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New generation recombinant antigenic peptides for vaccine and diagnostic antibody production

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Summary

INTRODUCTION

1. The immune system

The immune system is a system of biological structures and processes within an organism that protects it against external agents.

Two notable features that characterize the vertebrate immune system are its ability to distinguish between closely related substances (specificity) and its ability to "remember" previously exposures to extraneous substances (memory).

The features of the immune system are exploited not only therapeutically (antibodies are used in treatment of various health conditions) but also the molecules that comprise the adaptive immune system, antibodies in particular, are indispensable tools for the modern molecular biologist. Antibodies allow the visualization and isolation of the molecules they recognize, and can do so with extremely high precision.

The immune system comprises three layers:

- mechanical/chemical defenses
- innate immunity
- adaptive immunity

Mechanical and chemical defenses operate endlessly without solicitation. Innate immune responses, which involve cells and molecules always present in the host, are rapidly activated (minutes to hours). Adaptive immune responses take several days to develop fully and are highly specific: they can discriminate between closely related pathogens based on very small molecular structural differences.

The enormous diversity of antigens (any molecule that can induce an immune response) recognized by the immune system finds its explanation in unique rearrangements of the genetic material in B and T lymphocytes (B-cells and T cells) which are the white blood cells that carry out antigen-specific recognition. Even if the mechanisms that generates antigen-specific receptors on B and T cells are very similar, the way in which these receptors identify antigens is very different. The receptors on B-cells can directly interact with intact antigens, the receptors on T cells recognize processed (cleaved) forms of the antigen, presented on the surface of target cells by glycoproteins encoded by the major histocompatibility complex (MHC).

MHC-encoded glycoproteins are also involved in deciding the development of T cells so that an organism's own cells and tissues (self-antigens) normally do not induce an immune response.

Knowledge of antigen processing and presentation thus affects both vaccine design to protect against infectious disease and the generation of tools essential for research.

1.1. Mechanical and chemical defenses

Mechanical and chemical defenses constitute the first line of host defense against pathogens. Mechanical defenses include the skin, epithelia, and arthropod exoskeleton, which are barriers that can be opened only by mechanical damage or through specific chemo-enzymatic attacks. Chemical defenses include not only the low pH found in gastric secretions but also enzymes such as lysozyme, found in tear fluid, which can attack microbes directly. Mechanical defenses operate continuously without any pathogen provocation.
Viruses and bacteria have evolved strategies to breach the integrity of these physical barriers.

1.2. Innate immunity

The innate immune system is activated once the mechanical and chemical defenses have proven unsuccessful and the presence of an external agent is sensed. The innate immune system comprises cells and molecules that are immediately available for responding to pathogens.
Animals that lack an adaptive immune system, such as insects, exclusively depend on innate defenses to contrast infections.

1.2.a Phagocytes

Phagocytes are cells that can ingest and destroy pathogens normally. They are spread throughout various tissues and can be recruited to infection sites. Several soluble proteins present constitutively in the blood, or produced in response to infection or inflammation, also contribute to innate defense. The innate immune system phagocytes include macrophages, neutrophils, and dendritic cells. All of these cells express with Toll-like receptors (TLRs). Members of this family of cell surface proteins detect wide-ranging patterns of pathogen-specific markers and thus being key sensors for sensing the presence of viral or bacterial aggressors. Engagement of Toll-like receptors is important in eliciting effector molecules, including antimicrobial peptides. Dendritic cells and macrophages whose Toll-like receptors have detected pathogens also function as antigen-presenting cells (APCs) by exposing processed (cleaved) external agents to antigen-specific T cells.

1.2.b The complement system

Another main element of the innate immune system is the complement: an assortment of serum proteins that can bind directly to microbial or fungal surfaces. This binding activates a proteolytic cascade that end

in the expression of pore-forming proteins constituting the membrane attack complex (MAC) , which is capable of permeabilizing the pathogen's protective membrane.

At least three distinct pathways can activate complement.

The classical pathway requires the presence of antibodies produced in the course of an adaptive response and bound to the surface of the micro-organism. Many microbial surfaces have physico-chemical properties that result in activation of complement via the alternative pathway. Finally, pathogens that contain mannose rich cell walls activate complement through the mannose-binding lectin pathway.

The three pathways converge at the activation of complement protein C3 resulting in a covalent bond between C3 and a nearby protein or carbohydrate. This ensures that C3 will be covalently deposited only on antigen–antibody complexes in close proximity. Surfaces properly decorated with mannose-binding lectin or that received C3 deposits via the alternative pathway are similarly targeted. Activated C3 unleashes the terminal components of the complement cascade, C5 through C9, culminating in formation of the membrane attack complex, which inserts itself into most biological membranes and renders them permeable. The resulting loss of electrolytes and small solutes leads to lysis and death of the target cell.

All three complement activation pathways also generate the C3a and C5a cleavage fragments, which bind to G protein–coupled receptors and function as chemo-attractants for neutrophils and other cells involved in inflammation. All three pathways also result in the covalent tagging of the structures targeted by complement activation with fragments of C3. Phagocytic cells make use of these C3-derived tags to recognize, ingest, and destroy the labeled particles (opsonization). The complement cascade thus accomplishes multiple roles in host defense: it can destroy the membranes that envelope a pathogen, it covalently labels the targeted pathogen so that it may be promptly ingested by phagocytes and it induce the release of chemotactic signals to attract cells of the innate and adaptive immune system to the site of infection.

1.2.c Natural killer (NK) Cells

Natural killer (NK) cells are cytotoxic lymphocytes that provide rapid responses against virus-infected cells and tumor formation. Recognition by NK cells involves several classes of receptor, however they recognize stressed cells in the absence of antibodies and MHC.

For example, many virus-infected cells produce type I interferons, which, among others, activate NK cells. The interferons are classified as cytokines: small, secreted proteins that help regulate immune responses in various ways. Once activated, NK cells not only directly kill infected cells but also secrete interferon γ (IFN-γ), which is essential for orchestrating many aspects of the immune response.

1.3. Adaptive immunity

Adaptive or acquired immunity consist in a highly specific recognition of foreign substances, the full elaboration of which requires days or weeks after occurrence of the initial exposure to fully develop. Lymphocytes bearing antigen-specific receptors (lymphocytes B and T) are the key cells responsible for adaptive immunity.

Adaptive immunity is started in vertebrates when a pathogen eludes the innate immune system. The main function of the acquired immune system is the recognition of specific exogenous antigens during the process of antigen presentation. This process cause the development of responses that are designed to efficiently eliminate specific pathogens or pathogen-infected cells.

The last feature of this long term response is the generation of immunological memory, in which each different pathogen is remembered and thus recognized by highly specific antibodies and/or T cell receptors. These memory cells can be quickly recruited to contrast any subsequent infection by a known pathogen.

2. Antibodies

An early indication of the specific nature of adaptive responses came with the discovery of antibodies, key effector molecules of adaptive immunity, by von Behring and Kitasato in 1905. Von Behring hypnotized the existence of corpuscles ("Antikörper"), or antibodies (Abs), as a transferable factor responsible for protection.

We now know that von Behring's antibodies are serum proteins referred to as immunoglobulins (Ig) which carry out pathogen destruction.

Immunoglobulins can neutralize not only bacterial toxins but also harmful agents such as viruses (by binding directly to them, Abs prevent the virus from attaching to host cells) or venoms (Abs bind to the venom neutralizing it by keeping it from binding to its targets in the host). This is called passive immunization.

The region on an antigen where it makes contact with the corresponding antibody is called epitope. An antigen usually contains multiple epitopes, however each antibody, derived from a clonal population of B-cells, recognizes a single molecularly defined epitope on the corresponding antigen.

2.1. Immunoglobulins: Structure and Function

Immunoglobulins are abundant serum proteins that can be classified by their structural and functional properties. Fractionation of antisera (the blood plasma containing Abs), based on their functional activity, led to the identification of the immunoglobulins as the class of serum proteins responsible for antibody activity. Immunoglobulins are composed of two identical heavy (H) chains, covalently attached to two identical light (L) chains. The classic immunoglobulin thus has a twofold-symmetrical structure, described as H_2L_2 . An exception to this basic H_2L_2 architecture occurs in the camelids (camels, llamas, vicunas).

Proteolytic enzymes were used to fragment immunoglobulins to categorize the regions directly involved in antigen binding. The protease papain yields monovalent fragments, called F(ab) (antigen binding fragment), that can bind a single antigen molecule. The protease pepsin yields bivalent fragments, referred to as F(ab')2 capable of antigen cross-linking. The portion released upon papain digestion and incapable of antigen binding is called Fc (crystallizable fragment).

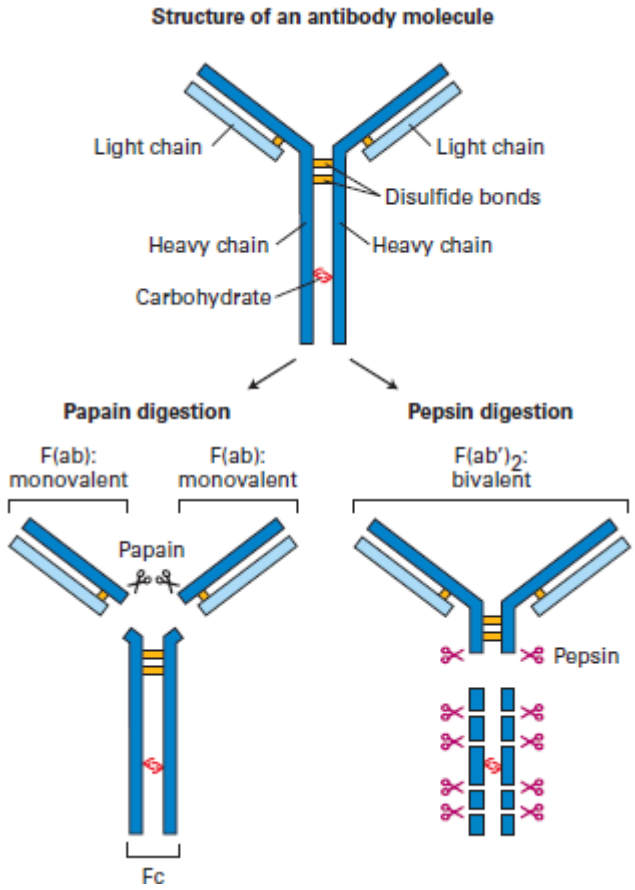

Figure 1 – Antibody structure

Antibodies/immunoglobulins are twofold-symmetrical structures composed of two identical heavy chains and two identical light chains. Fragmentation of antibodies with proteases yields fragments that retain antigen-binding capacity. The protease papain yields monovalent F(ab) fragments, and the protease pepsin yields bivalent F(ab')2 fragments. The Fc fragment is unable to bind antigen, but this portion of the intact molecule has other functional properties.

From Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company*.

Antibodies attached to a virus or microbial surface can be directly recognized by cells that expose receptors specific for the Ig Fc portion. These Fc receptors (FcRs), which are specific for individual classes and subclasses of immunoglobulins, show substantial structural and functional heterogeneity. By means of FcR-dependent events, specialized phagocytic cells such as dendritic cells and macrophages can engage antibody-decorated particles, then ingest and destroy them in the process of opsonization. FcR-dependent events also allow some immune-system cells (e.g., monocytes and natural killer cells) to directly engage target cells that display viral or other antigens to which antibodies are attached. This engagement may induce the immune-system cells to release toxic small molecules (e.g., oxygen radicals) or the contents of cytotoxic granules, including perforins and granzymes. This process, called antibody-dependent cell mediated cytotoxicity, illustrates how cells of the innate immune system interact with, and benefit from, the products of the adaptive immune response.

2.2. Immunoglobulins: Isotypes

Based on their biochemical properties, immunoglobulins are divided into different classes, or isotypes. There are two light-chain isotypes: lambda (λ) and kappa (κ). The heavy chains show more variation: in mammals, the major heavy-chain isotypes α, δ, ε, γ, and μ. These heavy chains can associate with either λ or κ light chains.

The fully assembled Ig gets its name from the heavy chain: α chains, IgA; δ chains, IgD; ε chains, IgE; γ chains, IgG; μ chains, IgM.

Each of the different Ig isotypes carries out specific functions.

2.2.a IgA

The IgA molecule interacts with an additional chain, the J chain, forming a dimeric structure. Dimeric IgA can bind to the polymeric IgA receptor on the basolateral side of epithelial cells, where it causes receptor-mediated endocytosis. Subsequently, the IgA receptor is cleaved and the dimeric IgA with the proteolytic receptor fragment (secretory portion) still attached is released from the apical side of the epithelial cell. This process, called transcytosis, is an effective means of delivering immunoglobulins from the basolateral side of an epithelium to the apical side. Tear fluid and other secretions are rich in IgA and so provide defense against environmental pathogens.

2.2.a IgD

IgDs are expressed as monomers and their function is still unclear. Their almost ubiquitous presence in species with an adaptive immune system suggests that IgD has important immunological functions.
In B lymphocytes, IgD function is to signal the B-cells to be activated.
During B-cell differentiation, IgM is the exclusive isotype expressed by immature B-cells. IgD starts to be expressed when the B-cell leaves the bone marrow to occupy peripheral lymphoid tissues. When a B-cell reaches its mature state, it co-expresses both IgMs and IgDs.
IgDs may also have some function in allergic reactions.

2.2.a IgE

IgEs main function is immunity to parasites.
IgEs also have a crucial role in allergic reactions provoked by exposure to a specific type of antigen (allergen). This reaction is called type 1 hypersensitivity and its symptoms vary from mild irritation to rapid death from anaphylactic shock.
IgE is expressed as a monomer and it is normally the least abundant isotype, immediately followed by IgD.

2.2.a IgG

Representing approximately 75% of serum immunoglobulins in humans, IgG is the most abundant antibody isotype. In humans 4 IgG subclasses can be distinguished by their abundance in serum (IgG1, 2, 3, and 4).
IgGs are essential for neutralization of pathogenic agents and in preparing particulate antigens for acquisition by cells equipped with receptors specific for the Fc portion of IgG molecules.
IgG is secreted as a small allowing it to perfuse tissues. It is the only isotype that has receptors to facilitate passage through the human placenta: transcytosis of IgG antibodies (mediated by the FcRn receptor) from the maternal circulation across the placenta delivers maternal antibodies to the fetus. These maternal antibodies will protect the newborn until its own immune system is sufficiently mature to produce antibodies. In adults, FcRn is also expressed on endothelial cells and helps control the turnover of IgG in the circulation.
IgGs are responsible of pathogen opsonization and toxin neutralization.
Depending on the immunoglobulin isotype, antigen antibody (immune) complexes can initiate the classical pathway of complement activation (see Figure 23-4). All IgG subclasses can activate complement, whereas IgA and IgE are unable to do so.

2.2.a IgM

The IgM molecule is secreted as a pentamer, stabilized by disulfide bonds and the J chain. In its pentameric form, IgM possesses 10 identical antigen-binding sites, which allow high-avidity interactions with the corresponding antigen. Avidity is defined as the sum total of the strength of interactions (affinity) of the available individual binding sites and the number of such binding sites. Upon deposition of IgM onto a surface that carries the antigen, the pentameric IgM molecule assumes a conformation that is highly contributing to the activation of the complement cascade.

As IgDs, IgMs are expressed as membrane-bound B-cell receptors on newly generated B-cells. Here the μ chains have an important role in B-cell development and activation.

IgMs (and IgGs) are particularly effective in activating the complement cascade.

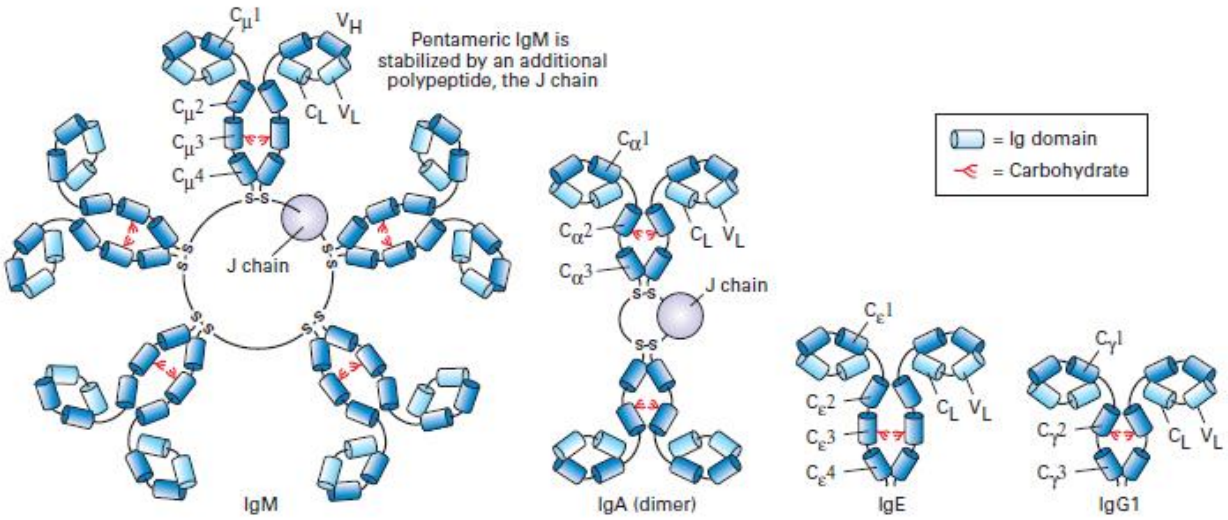

Figure 2 - Immunoglobulin isotypes
The different classes of immunoglobulins, called isotypes, may be distinguished biochemically and by immunological techniques.

From Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company*.

2.3. The clonal selection theory

The clonal selection theory states that each lymphocyte carries an antigen-binding receptor of exclusive specificity. When a lymphocyte encounters its specific antigen, clonal expansion (rapid cellular division) occurs and so allows an intensification of the response, concluding in the antigen clearance.

In a typical immune response, the antigen that elicits the response is of complex composition: even the simplest virus contains several distinct proteins. Many individual lymphocytes respond to a given antigen

and expand in response to it, each producing its own antigen receptor of unique structure and therefore with different affinity.

Because each lymphocyte is endowed with a unique receptor and clonally expands in response to antigen, this response is called polyclonal.

2.4. Variable and constant regions of immunoglobulins

The light chains of immunoglobulins produced by different B-cells have not identical biochemical properties, suggesting that they all have unique sequences.

The differences in amino acid sequence that distinguish one light chain from another are not randomly distributed but occur clustered in a domain referred to as the variable region of the light chain (V_L). This domain comprises the N-terminal 110 amino acids.

The remainder of the sequence is identical for the different light chains (provided they derive from an identical isotype) and is therefore referred to as the constant region (C_L).

Sequencing of the heavy chains revealed that the variable residues that distinguish one heavy chain from another were again concentrated in a well demarcated domain, referred to as the variable region of the heavy chain (V_H).

An alignment of sequences of different light chains showed a non-casual pattern of regions of variability, revealing three hypervariable regions *(*HV1, HV2, and HV3) which are sandwiched between what are called framework regions*.*

In the properly folded three-dimensional structure of immunoglobulins, these hypervariable regions are in close proximity and make contact with antigen. Thus that portion of an Ig molecule containing the hypervariable regions constitutes the antigen-binding site.

For this reason, hypervariable regions are also referred to as complementarity determining regions (CDRs).

3. Generation of antibody diversity and B-cell development

Pathogens have short replication times, are quite diverse in their genetic makeup, and evolve quickly, generating even more antigenic variation. An adequate defense must thus be capable of mounting an equally diverse response. Antibodies fulfill this role. The timing of the antibody response and its necessary adjustment to changes in the antigenic makeup of the pathogen in question pose unique demands on the organization and regulation of the adaptive immune system. The coding capacity of the typical vertebrate genome could not possibly encode the large number of antibodies required for adequate protection against the diversity of microbes to which the host is exposed. A unique mechanism evolved to allow not only sheer limitless variability of the antibody repertoire, but also to enable rapid adjustment of the quality of

the antibodies produced, to meet the demands posed by an unfolding viral or bacterial infection. Because optimal antibody production requires assistance in the form of T-cell help, we shall see that molecular mechanisms underlying receptor diversity are fundamentally similar for B and T cells. B-cells, which are responsible for antibody production, make use of a unique mechanism by which the genetic information required for synthesis of immunoglobulin heavy and light chains is stitched together from separate DNA sequence elements, or Ig gene segments, to create a functional transcriptional unit.

The act of recombination that combines Ig gene segments itself dramatically expands the variability in sequence precisely where these genetic elements are joined together. This mechanism of generating a diverse array of antibodies is fundamentally different from meiotic recombination, which occurs only in germ cells, and from alternative splicing of exons.

Because this recombination mechanism occurs in somatic cells but not in germ cells, it is known as somatic gene rearrangement or somatic recombination. This unusual recombination mechanism, unique to antigen receptors on B and T lymphocytes, makes it possible to specify an enormously diverse set of receptors with minimal expenditure of DNA coding space. The ability to combine at will discrete genetic elements (combinatorial diversity), in addition to the generation of yet more sequence diversity in the encoded receptors by the underlying recombination mechanisms themselves, allows adaptive immune responses against a virtually limitless array of antigens, including molecules encoded by the host. Thus there are mechanisms at work that not only create this enormous diversity, but there are also processes that impose tolerance to curtail unwanted reactivity against “self” components; the result of such reactivity is autoimmunity.

3.1. Light chains development: V and J gene segments

Immunoglobulin genes encoding intact immunoglobulins do not exist already assembled in the genome, ready for expression. Instead, the required gene segments are brought together and assembled in the course of B-cell development.

The immunoglobulin light chains are encoded by clusters of V gene segments, followed downstream by a single C segment. Each V gene segment carries its own promoter sequence and encodes the bulk of the light chain variable region, although a small piece of the nucleotide sequence encoding the light chain variable region is missing from the V gene segment. This missing portion is provided by one of the multiple J segments located between the V segments and the single C segment in the un-rearranged κ light-chain locus. This J segment is a genetic element, not to be confused with the J chain subunit.

In the course of B-cell development, the random commitment to a particular V gene segment results in its combination with one of the J segments, again a random event, thus forming an exon encoding the entire light chain variable region. The act of recombination not only generates an intact and functional light-chain

gene, it also places the promoter sequence of the rearranged gene within controlling distance of enhancer elements (located downstream of the light chain constant region exon) required for its transcription. Only a rearranged light chain gene can be transcribed.

3.2. Heavy chains development: V, D, and J gene segments

The organization of the heavy chain locus is more complex than that of the κ light-chain locus. The heavy chain locus contains not only a large tandem array of V gene segments (each equipped with its own promoter) and multiple J elements, but also multiple D (diversity) segments. Somatic recombination of a V, D, and J segment generates a rearranged sequence encoding the heavy chain variable region. Heavy chains rearrangements proceed via the same way of the light chains rearrangements. In the course of B-cell development, the heavy chain locus always rearranges first, starting with D-J rearrangements. D-J rearrangement is followed by V-DJ rearrangement. Only one in three rearrangements yields the proper reading frame for the rearranged VDJ sequence.

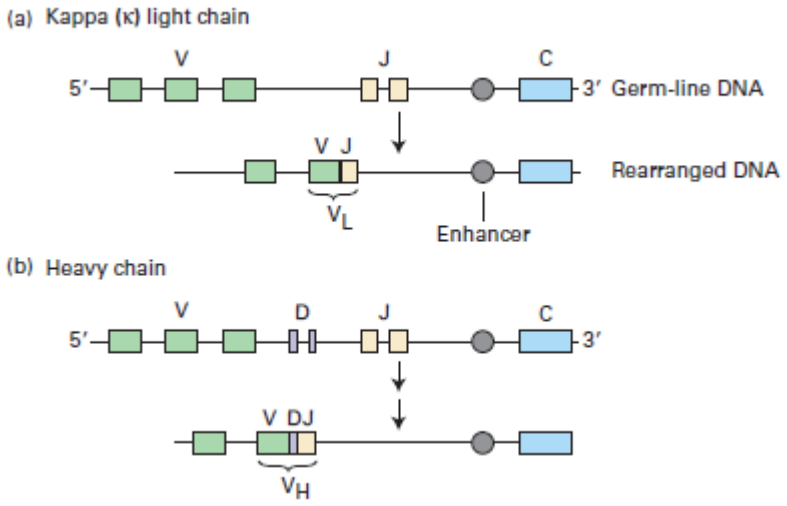

Figure 3 - Overview of somatic gene rearrangement in immunoglobulin DNA
During development of a B cell, somatic recombination of these gene segments yields functional light-chain genes (a) and heavy-chain genes (b).

From Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company*.

3.3. Somatic recombination and somatic hypermutation

Detailed sequence analysis of the light and heavy chain loci revealed a conserved sequence element at the 3' end of each V gene segment.

This conserved element, called a recombination signal sequence (RSS), is composed of heptamer and nonamer sequences separated by a 23 bp spacer. At the 5' end of each J element,
there is a similarly conserved RSS that contains a 12 bp spacer. Somatic recombination is catalyzed by the RAG1 and RAG2 recombinases, which are expressed only in lymphocytes.
The combination of the two gene segments to be joined is stabilized by the RAG1/RAG2 complex.
Defects in the synthesis of RAG proteins completely deny the possibility of somatic gene rearrangements.
In addition to the diversity created by random somatic recombination, antigen activated B cells can undergo somatic hypermutation: upon receipt of proper additional signals, most of which are provided by T cells, expression of activation induced deaminases (AID) is turned on. This enzymes deaminate cytosine residues to uracil so when a B cell that carries this mutation replicates, it may place an adenine on the complementary strand, thus generating a G to A transition. Alternatively, the uracil may be excised by DNA glycosylases to produce an abasic site. These abasic sites, when replicated, give rise to possible transitions as well as transversions. Mutations accumulate with every successive round of B cell division, yielding numerous variations in the rearranged VJ and VDJ segments. Even if many of these mutations are deleterious, the final result is the generation of a B cell population whose antibodies show a higher affinity for the antigen. In the course of an immune response or upon repeated immunization, the antibody response exhibits affinity maturation: an increased affinity of antibodies for antigen, as the result of somatic hypermutation.

3.4. B cell development

The B cell genetic rearrangements occur in a carefully ordered sequence during development of a B cell, starting with heavy chain rearrangements. The rearranged heavy chain is immediately used to build a membrane-bound receptor responsible for a cell fate decision in order to drive further B cell development and antibody synthesis. Only a productive rearrangement that produces an in-frame VDJ combination can generate a complete μ heavy chain, the production of which serves as a signal to indicate to the B cell that it has successfully accomplished rearrangement, and that no further rearrangements of the heavy chain locus (on the remaining allele) are required. B cells at this stage of development are called pre-B cells, as they have not yet completed assembly of a functional light chain gene and consequently cannot participate in antigen recognition.
The μ chain made at this stage of B cell development is a membrane-bound version. In pre-B cells, newly made μ chains form a complex with two surrogate, not functional light chains (λ5 and VpreB). The μ chain itself possesses no cytoplasmic tail and is therefore incapable of recruiting cytoplasmic components for the purpose of signal transduction. Instead, early B cells express two supporting transmembrane proteins, called Igα and Igβ, each of which carries in its cytoplasmic tail an immunoreceptor tyrosine-based activation

motif (ITAM). The entire complex constitutes the pre-B cell receptor (pre-BCR). Engagement of this receptor by appropriate signals results in the recruitment and activation of a tyrosine kinase, which phosphorylates the ITAMs. Once phosphorylated, ITAMs recruit other molecules essential for signal transduction.

The pre-B-cell receptor has several important functions. First, it shuts off expression of the RAG recombinases, so rearrangement of the other heavy chain locus cannot progress. This allelic exclusion, ensures that only one of the two available copies of the heavy chain locus will be rearranged and thus expressed as a complete μ chain. Secondly, because of the association of the pre-B-cell receptor with Igα and Igβ, the receptor becomes a functional signal transduction unit. Signals that emanate from the pre-BCR initiate pre-B cell proliferation to increase the numbers of those B cells that have undergone productive D-J and V-DJ recombination. In the course of this development, expression of the surrogate light chain subunits decreases. The progressive dilution of VpreB and λ5 with every successive cell division allows the resume of expression of the RAG enzymes, which now target the κ or λ light chain locus for recombination.

A productive light-chain V-J rearrangement also yields allelic exclusion. Upon completion of a successful V-J light-chain rearrangement, the B-cell can make both μ heavy chains and κ or λ light chains, and assemble them into a functional B-cell receptor (BCR), which can recognize antigen.

Once a B cell expresses a complete BCR on its cell surface, it can recognize antigen, and all subsequent steps in B cell activation and differentiation involve antigen specific engagement by the antigen for which that BCR is specific. The BCR not only plays a role in driving B-cell proliferation upon successful encounter with antigen, it also functions as a device for capturing and ingesting antigen, an essential step that allows the B cell to process the acquired antigen and convert it into a signal that sends out a call for assistance by T lymphocytes.

3.4. B cells switching from membrane-bound to secreted Igs production

The proper functioning of immunoglobulins, such as neutralization of antigen or killing of bacteria, requires that these products be released by the B cell. The choice between the synthesis of membrane-bound or secreted immunoglobulin is made during processing of the heavy chain primary transcript.

The μ locus contains two exons (TM1 and TM2) that together encode a C-terminal domain that anchors IgMs on the plasma membrane. One polyadenylation site is found upstream of these exons while a second site is present downstream. If the downstream poly(A) site is chosen, then further processing yields an mRNA that encodes the membrane-bound form of μ while if the upstream poly(A) site is chosen the secreted version of the μ chain is produced. Similar arrangements are found for the other Ig constant region gene segments, each of which can specify either a membrane-bound or a secreted heavy chain. The ability to switch between the membrane-anchored and the secreted form of Ig heavy chains by alternative

use of poly(A) sites (not by alternative splicing) is peculiar of this family of gene products. The capacity to switch from the synthesis of exclusively membrane Igs to the assembly of secreted Igs is acquired by B cells in the course of their differentiation: terminally differentiated B cells (plasma cells) are almost exclusively dedicated to the production of secreted antibodies.

3.5. Immunoglobulin class-switch recombination

In the immunoglobulin heavy-chain locus, the exons that encode the μ chain lie immediately downstream of the rearranged VDJ exon. This is followed by exons that specify the δ chain. Transcription of a newly rearranged immunoglobulin heavy chain locus yields a single primary transcript that includes the μ and δ constant regions. Splicing of this large transcript determines whether a μ chain or a δ chain will be produced. Downstream of the μ/δ combination are the exons that together encode all of the other heavy chain isotypes. Upstream of each isotype encoding exon cluster (with the exception of the δ locus) there are repetitive sequences (switch sites) that are recombination prone. Because each B cell necessarily starts out with surface IgM, recombination involving these sites results in a class switch from IgM to one of the other isotypes. The light chain is not affected by this process nor is the rearranged VDJ segment with which the B cell began its pathway. Class-switch recombination thus generates antibodies with different constant regions but identical antigenic specificity. Class-switch recombination is dependent on activation-induced deaminase (AID), the presence of antigen and T lymphocytes. Somatic hypermutation and class-switch recombination occur concurrently, and their combined effect allows fine regulation of the adaptive immune response against a particular antigen.

4. MHC and T lymphocytes

Antibodies can recognize antigen without the participation of any third-party elements: the presence of the antigen and the antibody is sufficient for their interaction. In the course of their differentiation, B cells receive support from T cells. This assistance is antigen specific, and the T cells responsible for providing it are called helper T cells (or T helper cells). Even though antibodies contribute to the elimination of pathogens, it is often required to destroy also the infected cells that function as a source of new virus particles. This operation is carried out by T cells with cytotoxic activity. These cytotoxic T cells and helper T cells utilize antigen-specific receptors whose genes are arranged by mechanisms similar to those used by B cells to generate immunoglobulin genes. However, T cells engage in antigen recognition in a differently from B cells. The antigen specific receptors on T cells recognize short fragments of protein antigens, presented by membrane glycoproteins encoded by the major histocompatibility complex (MHC). Many antigen presenting cells, in the course of their normal activity, digest pathogen and self-proteins exposing

the resulting small peptides to their cell surface thus promoting a physical complex with an MHC protein. T cells can distinguish these complexes, and if they sense a pathogen derived peptide they can take appropriate action.

4.1. MHC classes

The MHC encodes two types of glycoproteins essential for immune recognition, commonly called class I and class II MHC molecules. The class I and class II MHC products are both involved in presenting antigen to T cells, but they serve two broadly distinct functions. Class I MHC products mostly serve to alert cytotoxic T cells of the presence of intracellular intruders and allows the cytotoxic T cell to destroy the infected cell. Class II MHC products are found on antigen presenting cells: when the class II molecules on such cells present the antigen to helper T cells, this is commonly interpreted by the immune system as an immediate need for an adaptive immune response that requires B cell involvement, cytokine production and assistance to cytotoxic T cells.

In addition to the class I and class II MHC molecules, the MHC encodes essential components of the antigen processing and presentation machinery and also key components of the complement cascade. Class I and class II MHC molecules are recognized by different populations of immune system cells and therefore serve different functions.

Both class I and class II MHC molecules are highly polymorphic: many allelic variants exist among individuals of the same species, however both classes of MHC molecules also are structurally similar in many aspects, as are their interactions with peptides and the T cell receptor.

4.1.a Class I MHC cells

These T cells mostly use class I MHC molecules as their restriction elements, and also are characterized by the presence of the CD8 glycoprotein marker on their surface. Most, if not all, nucleated cells constitutively express class I MHC molecules and can support replication of viruses. Cytotoxic T cells recognize and kill the infected targets via the expressed class I MHC molecules that display virus-derived antigen.

The structure of class I MHC molecules reveals two membrane-proximal Ig-like domains. These domains support an eight-stranded β sheet topped by two α helices. Jointly the β sheet and the helices create a cleft, closed at both ends, in which a peptide can bind. The mode of peptide binding by a class I MHC molecule requires a peptide of rather fixed length, usually 8 to 10 amino acids, so that the ends of the peptide can be inserted into pockets that accommodate the charged amino and carboxyl groups at the termini.

The peptide is anchored into the peptide binding cleft by means of a small number of amino acid side chains, each of which is accommodated by a pocket in the MHC molecule that precisely fits that particular amino acid residue. The polymorphic residues that distinguish one allelic MHC molecule from another are mostly located in and around the peptide-binding cleft. These residues therefore define the architecture of the peptide-binding pocket and hence the specificity of peptide binding affecting the surface of the MHC. A T cell receptor designed to interact with one particular class I MHC allele will therefore not interact with unrelated MHC molecules because of their different surface architectures (MHC restriction). The CD8 marker functions as a co-receptor, binding to conserved portions of the class I MHC molecules thus giving restriction specificity of any mature T cell that bears it.

4.1.b Class II MHC cells

B cells do not undergo final differentiation into antibody secreting plasma cells without assistance from another subset of T cells, the T helper cells. Those cells express on their surface the CD4 glycoprotein marker and use class II MHC molecules as restriction elements. The constitutive expression of class II MHC molecules is confined to so called professional antigen presenting cells, including B cells, dendritic cells, and macrophages.

The two subunits (α and β) of class II MHC molecules are both type I membrane glycoproteins and belong to the Ig superfamily. Like the large subunit of class I molecules, both the α and β subunits of class II molecules show genetic polymorphism. The basic three-dimensional design of class II MHC molecules resembles that of class I MHC molecules: two membrane proximal Ig-like domains support a peptide-binding portion composed of an eight-stranded β sheet and two α helices. For class II MHC molecules, the α and β subunits contribute equally to the construction of the peptide binding cleft. This cleft is open at both ends and thus supports the binding of longer peptides that protrude from it. The mode of peptide binding involves pockets that accommodate specific peptide side chains, as well as contacts between side chains of the MHC molecule with main-chain atoms of the bound peptide. As for class I MHC, class II MHC polymorphisms mainly affect residues in and around the peptide-binding cleft, so that the peptide-binding specificity will usually differ among different allelic products. A T cell receptor that interacts with a particular class II MHC molecule will not interact with a different allelic molecule, not only because of the difference in the peptide-binding specificity of the allelic molecules, but also because of the polymorphisms that affect the contact residues with the T-cell receptor: as for class I MHC, this is the basis for class II MHC restricted recognition of antigen. The CD4 co-receptor recognizes conserved features on class II MHC molecules. Any mature T cell that bears the CD4 co-receptor uses class II MHC molecules for antigen recognition.

Multiple types of CD4 T cells have been identified based on the cytokines they produce and their functional properties. Whereas all activated T cells can produce IL-2, other cytokines are produced by particular CD4 T-cell subsets, these CD4 T cells are classified as T_{H1} cells, characterized by the production of interferon γ and TNF, and T_{H2} cells, characterized by the production of IL-4 and IL-10.

The Th1 response is characterized by the activation of the bactericidal activities of macrophages, and induces B cells to make opsonizing and complement-fixing antibodies (IgG1≈IgG2b>>IgG2a), and leads to "cell-mediated immunity". The Th2 response is characterized by the stimulation of B cells to produce neutralizing non-cytolytic antibodies (IgG1≈IgG2a≈IgG2b≈IgG3), leading to "humoral immunity".

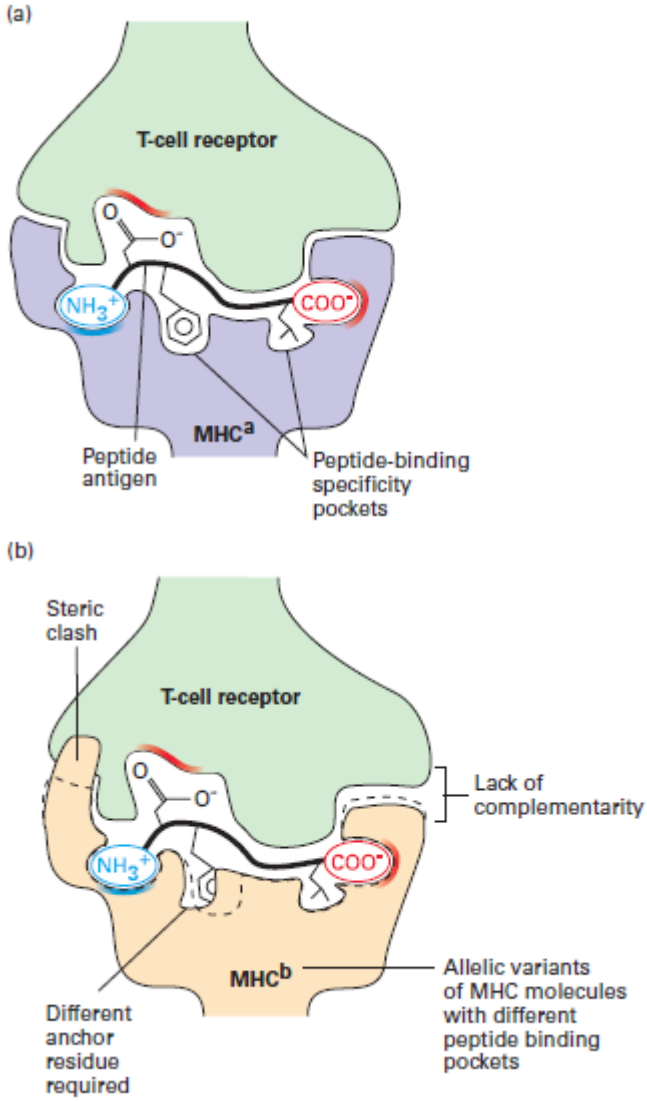

Figure 4 - Peptide binding and MHC restriction

a) Peptides that bind to class I molecules are on average 8–10 residues in length, require proper accommodation of the termini, and include two or three residues that are conserved (anchor residues). Positions in class I molecules that distinguish one allele from another (polymorphic residues) occur in and around the peptide-binding cleft. The polymorphic residues in the MHC affect both the specificity of peptide binding and the interactions with T-cell receptors. Successful "recognition" of a peptide antigen–MHC complex by a T-cell receptor requires a good fit among the receptor, peptide, and MHC molecule. (b) Steric clash and a lack of complementarity between anchor residues and the MHC molecule prevent proper binding. T-cell receptors are thus restricted to specific peptide-MHC products.

From Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company*.

4.2. Antigen processing and presentation

The process by which extraneous molecules enter the immune system is the crucial stage that determines the eventual outcome of a response. A successful adaptive immune response, which includes the production of antibodies and the generation of helper and cytotoxic T cells, cannot unfold without the involvement of professional antigen-presenting cells. Professional antigen-presenting cells include B cells, dendritic cells and macrophages. It is these cells that acquire the antigen, process it, and then display it in a form that can be recognized by T cells. The pathway by which antigen is converted into a form suitable for T cell recognition is commonly called antigen processing and presentation. The class I MHC pathway focuses predominantly on presentation of proteins synthesized by the cell itself while the class II MHC pathway is centered on materials acquired from outside the antigen-presenting cell. Antigen processing and presentation in both the class I and II pathways may be divided into six phases:

1. acquisition of antigen
2. tagging the antigen for destruction
3. proteolysis
4. delivery of peptides to MHC molecules
5. binding of peptide to a MHC molecule
6. display of the peptide loaded MHC molecule on the cell surface

4.3. T cells

T lymphocytes recognize antigen through MHC molecules. These antigen specific receptors structurally related to immunoglobulins, to generate these antigen wich T cells rearrange the genes encoding the T cell receptor (TCR) subunits by mechanisms of somatic recombination matching those used by B cells (to rearrange immunoglobulin genes). The development of T cells is strictly dependent on successful completion of the somatic gene rearrangements that yield the TCR subunits. T cells use their T cell receptors as B cells utilize the BCRs to recognize antigens and then transduce signals that lead to their clonal expansion. T cells that have been activated through engagement of these antigen specific receptors proliferate and acquire the capacity to kill antigen-bearing target cells or to secrete cytokines that will assist B-cells in their differentiation. The T-cell receptor for antigen recognizes MHC molecules complexed with appropriate peptides. T cells recognize antigens only together with the polymorphic MHC molecules present in that host. In the course of T cell development, T cells must learn to identify these self MHC molecules and receive instructions about which MHC-peptide combinations to ignore, so as to avoid reactivity of newly generated T cells with the host's own tissues (autoimmunity).

4.3.a Structure of the T cell receptor

The T cell receptor for antigen recognizes MHC molecules complexed with appropriate peptides. The TCR is composed of two glycoprotein subunits, each of which is encoded by a somatically rearranged gene. The receptors are composed of either an α-β pair or a γ-δ pair. The structure of these subunits shows structural similarity to the F(ab) portion of an immunoglobulin: at the N-terminal end there is a variable domain, followed by a constant-region domain and a transmembrane segment. The cytoplasmic tails of the TCR subunits are too short to allow recruitment of cytosolic factors that assist in signal transduction. Instead, the TCR associates with the CD3 complex, a set of membrane proteins. The external domain of the CD3 subunits is homologous to immunoglobulin domains, and the cytoplasmic domain in each contains an ITAM domain, through which adapter molecules may be recruited.

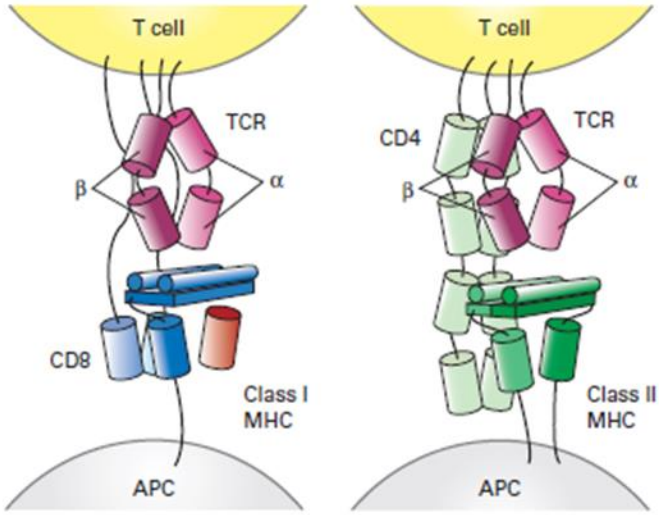

Figure 5 - Structure of the T-cell receptor and its co-receptors
The T-cell receptor (TCR) for antigen is composed of two chains, the α and β subunits, which are produced by V-J and V-D-J recombination, respectively. The αβ subunits must associate with another complex (CD3) to allow the transduction of signals. The formation of a full TCRαβ–CD3 complex is required for surface expression. The T-cell receptor further associates with the co-receptors CD8 (light blue) or CD4 (light green), which allow interaction with conserved features of class I MHC and class II MHC molecules, respectively, on antigen-presenting cells.

From Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company*.

5. Interactions between B and T Cells

To produce high affinity antibodies B cells necessitate support from T cells. B cell activation requires an antigen source to engage the B cell receptor and the presence of activated antigen-specific T cells. Soluble antigen reaches the lymph node through the afferent lymphatics. Bacterial growth is accompanied by the release of microbial products that can serve as antigens. If the infection is goes along with by local tissue destruction, activation of the complement cascade results in bacterial killing and the associated release of

bacterial proteins, which are also delivered via the lymphatics to the draining lymph node. Complement-modified antigens are superior in the activation of B cells by engaging complement receptors on B cells, which serve as co-receptors for the BCR. B cells that acquire antigen via their B cell receptors internalize the immune complex and process it for presentation via the class II MHC pathway. Antigen-experienced B cells consequently convert the BCR-acquired antigen into a request for T cell help in the form of a class II MHC–peptide complex. The epitope recognized by the B cell receptor may be quite different from the peptide ultimately displayed on the surface associated with a class II MHC molecule. As long as the B cell epitope and the class II-presented peptide (a T cell epitope) are physically linked, successful B cell differentiation can be initiated. The concept of linked recognition explains why there is a minimum size for molecules that can successfully stimulate a high affinity antibody response. Such molecules must satisfy several criteria: they must contain the epitope perceived by the B cell receptor, undergo endocytosis and proteolysis, and a proteolyzed fragment of the molecule must bind to the allelic class II MHC molecules available in order to be presented as a class II MHC–peptide complex (which functions as a demand for T cell help). For this reason, synthetic peptides used to elicit antibody production are conjugated to carrier proteins to improve their immunogenicity, where the carrier proteins serve as the source of peptides for presentation via class II MHC products. Only through recognition of this class II MHC–peptide complex via its TCR can T cells provide the help necessary for the B cell to run its complete course of differentiation. This concept applies equally to B cells capable of recognizing particular modifications on proteins or peptides. Antibodies that recognize the phosphorylated form of a protein are commonly generated by immunization of experimental animals (e.g., mice, rabbits, goats) with the phosphorylated peptide in question, conjugated to a carrier protein. An appropriately specific B cell recognizes the phosphorylated site on the peptide of interest, internalizes the phosphorylated peptide (plus its carrier), and generates a complex set of peptides by endosomal proteolysis of the carrier protein. Among these peptides there should be at least one that can bind to the class II MHC molecules carried by that B cell for a successful response to arise. If properly displayed at the surface of the B cell, this class II MHC–peptide complex becomes the call for T cell help, provided by CD4 T cells equipped with receptors capable of recognizing the complex of class II MHC molecule and carrier-derived peptide. The T cell identifies, via its TCR, an antigen-experienced B cell by means of the class II MHC–peptide complexes displayed by the B cell. The B cell also displays co-stimulatory molecules and receptors for cytokines produced by the activated T cell (e.g., IL-4). These B cells then proliferate. Some of them differentiate into plasma cells, others are set aside and become memory B cells. The first wave of antibodies produced is always IgM. Class switching to other isotypes and somatic hypermutation requires the persistence of the antigen or repeated exposure to it. In addition to cytokines, B cells require cell-to-cell contacts to initiate somatic hypermutation and class switch recombination. These contacts involve two members of the TNF/TNF-receptor family: CD40 on B cells and CD40L on T cells.

6. Biotechnological applications of immunological principles

Antibodies have proven to be an essential tool in scientific research. Purified antibodies have proven to be highly specific, stable and easy to manipulate consequently they are currently used in many molecular biology techniques and applications. Antibodies can be conjugated to other proteins (e.g., HRP peroxidase, alkaline phosphatase), various molecules (e.g., fluorophores, chromophores, biotin) or even small drugs. Their most common use is in protein identification and/or quantification. Antibodies are used in Western blot, in ELISA and in DOTBLOT/ELISPOT assays to analyze proteins . They are also used in immune-precipitation/co-immune-precipitation to isolate proteins (and their interactors) and in immunohistochemistry/immunofluorescence to examine protein expression levels or to locate proteins within cells (using a microscope). In flow cytometry the use of immunoglobulins consents protein expression studies in order to distinguish cell types. Antibodies can also detect protein modifications (phosphorylation, nitrosylation, methylation, acetylation, etc.), complex carbohydrates, (glyco)lipids, nucleic acids and their modifications, having therefore found extensive use not only in research techniques but in diagnosis procedures as well.

6.1 Polyclonal antibodies

Antibodies for research use are obtained by inoculation of a suitable animal (e.g., mouse, rabbit, goat, chicken) with the target antigen. The objective is to stimulate antibody production and obtain high titer, high affinity antisera for research or diagnostic procedures. The antisera consist in a pool of anti-antigen generated antibodies extracted from the blood of the immunized animal.
Due to the polyclonal B cell response these antibodies are called polyclonal antibodies. The major drawback with a polyclonal serum is that due to multiple epitope recognition (non-specific binding) other molecules could been recognized other than the target antigen thus generating a background signal. To overcome this issue the antisera is often affinity purified increasing the specificity but with the risk of losing detection capability.

6.2 Monoclonal antibodies

Monoclonal antibodies (mAbs) are mono-specific antibodies re made by clones of a unique parent B cell. Differently from polyclonal antibodies which are generated from different immune cells, mAbs have monovalent affinity: they all react with the same epitope. These antibodies are produced forming hybrid cell lines (hybridomas) by fusing a specific antibody-producing B cell with a myeloma (malignant monoclonal expansions of immunoglobulin-secreting B cells) cell. The creation of immortalized cell

guarantees the yield of unlimited quantities of highly specific antibodies much more sensitive than a polyclonal antiserum. The extraordinary specificity of mAbs reduce background noise and cross-reactivity, helps provide reproducible results and ensures efficiency in affinity purification. Polyclonal antibodies, however, have a more robust detection (due to multiple epitopes) and require much less time/money to be prepared.

6.3 Immunotherapies

The increasing knowledge of the mechanism that regulates the immune system has opened the way to new approaches in disease treatment. Inducing, enhancing, or suppressing an immune response is the base of immunotherapy.

6.3.a Cell based immunotherapy

Cell based immunotherapy involves immune cells such as NK cells Cytotoxic T lymphocytes and dendritic cells which are either activated in vivo by administering certain cytokines or they are isolated, enriched and transfused to the patient (adoptive cell transfer). In the latest decade adoptive cell transfer began to use genetically engineered T cells produced by infecting human cells with a virus (or a retrovirus) encoding a TCR gene specialized to recognize a particular antigen.
The use of T cells is one of the current major researched approach to treat cancer (Morgan et al., 2006; Ankri et al., 2013).

6.3.b Therapeutic antibodies

Monoclonal antibodies are currently use as a very powerful therapeutic tool. The clinical uses of these antibodies are almost endless and they are currently employed against cancer, auto-immune disorders, transplant rejection and cardiovascular diseases.
During the development of therapeutic mAbs four types have been developed: murine, chimeric, humanized and human.
The murine mAbs are analogues of the human Abs, produced by hybridoma technology. Due to the divergence between murine and human immune systems, murine mAbs had little benefits (reduced stimulation of cytotoxicity and complement cascade) and serious drawbacks (mild to strong allergic reactions).
To overcome these problems chimeric Abs (composed of murine variable regions attached to human constant regions) and humanized Abs (produced by attaching murine hypervariable regions on human antibodies) were developed. These two types of antibodies lack the collateral effects of murine mAbs,

however, due to their mixed nature, they sometimes show inferior antigen affinity. With the development of antibody engineering techniques, it became possible to introducing alterations into the complementarity CDR thus increasing mAb antigen binding strength.

The latest entry in the therapeutic Abs family are human monoclonal antibodies. These types of Abs are produced by replacing murine immunoglobulin genes with human homologous genes, thus creating transgenic mice. The inoculation with the target antigen, yields fully human antibodies which can be used to generate human mAbs.

Monoclonal antibodies can be further engineered to be a drug delivery system. Antibody drug conjugates (ADCs) are a complex of an antibody (a whole mAb or a fragment) linked to a biological active cytotoxic drug. As of 2013, the majority of the ADCs are employed against cancer exploiting the mAb ability to target the cancer cells while not reacting with normal cells.

6.3.c Vaccines

Perhaps the most significant application of immunological knowledge are vaccines.

Vaccines are compounds intended to be harmless but that can stimulate an immune response against a particular pathogen thus granting protection against it.

Vaccines may be composed of live attenuated variants of more virulent pathogens. The attenuated version of the pathogen can cause a mild form of the disease or no symptoms at all. However, by recruiting all the components of the adaptive immune system, vaccines can elicit protecting levels of antibodies. These levels may fade with progressing age, and repeated immunizations (booster injections) are often required to preserve a full protection.

Another major type of vaccine is called subunit vaccine: rather than using live attenuated pathogens, only one of its molecular components (e.g., proteins or membranes) is used to provoke an immune response. In certain cases this is sufficient to afford lasting protection against a challenge with the live, virulent source of the antigen used for vaccination.

Thanks to recombinant DNA technology, recombinant vaccines are composed by short antigenic fragments of the molecular components (or the whole components) used in subunit vaccines which can be expressed in bacteria or yeast. This allows to better aim the immune response towards a specific molecular target. Recombinant vaccines eliminate the need of the live pathogen or the extraction of one of its components thus reducing production costs.

From a public health perspective, cheaply produced and widely distributed vaccines are formidable tools in eradicating infectious diseases. Current efforts are directed at producing vaccines against diseases for which no other suitable therapies are available (e.g., Alzheimer's disease, HIV/AIDS) or where socio-economic conditions have made distribution of drugs (or conventional vaccines) problematic (e.g., HPV).

Through a more detailed understanding of how the immune system functions, it should be possible to improve the design of existing vaccines and extend these principles to diseases for which currently no successful treatments are available. One of the most challenging vaccine design of the latest decade has been the one against Alzheimer's disease.

7. Alzheimer's disease

Alzheimer's disease (AD) is a degenerative disease of the central nervous system and is the most common form of dementia.

Most often, AD is diagnosed in people over 65 years of age(affecting more than 5% of the population) even though the less-prevalent early-onset Alzheimer's can occur much earlier.

The disease, which worsens as it progresses, deprives people of their human defining qualities (reason, memory, recognition, language, etc.) and eventually leads to death.

Initially underestimated and classified as a form of presenile dementia, AD is now considered one of the major health threats in developed countries. To date there is not a proper cure and more than 25 million of individuals suffer from Alzheimer's which is predicted to affect 1 in 85 people globally by 2050.

8. History of Alzheimer's disease

Initially considered as a dark form of presenile dementia, Alzheimer's disease was described for the first time in 1906 by the German neuropathologist Alois Alzheimer (1863-1915). During the Tubingen psychiatric Convention, in 1907, Dr. Alzheimer presented the case of Auguste Deter, a 51 year old woman suffering from a strange form of dementia whom he followed from 1901 to her death in 1906. In the clinical description of the patient, Alzheimer pointed out those symptoms that today characterize the syndrome: the progressive loss of memory and cognitive skills, such as language, orientation and visual-spatial skills. Following autopsy, the doctor noticed strange and peculiar traits in the brain tissue of the patient and highlighted, by silver staining, the presence of two anomalies at the level of the cerebral cortex: the agglomerates, then defined amyloid plaques (already observed in elderly people) and tangled bundles of fibers whom he called neurofibrillary tangles.

Those tangles were not described until then and in doing that Alzheimer understood that the disease was as well not characterized.

The amyloid deposits were formerly thought to be starch-like aggregates (hence the name).

Alzheimer's disease got its official name in 1910 when Emil Kraepelin, the most famous German psychiatrist at the time, republished his article "Psychiatry", in which he defined a new form of dementia from Alzheimer's discovery, calling it after the doctor.

It was Divry, in 1930, who first visualized the senile plaques, using Congo red staining, focusing on the presence of the amyloid deposits at the center of the plaque.

Roughly 30 years later Kidd and Terry described the senile plaques and the neurofibrillary tangles using electronic microscopy.

In 1970 the scientific research was focused on cerebral cellular neurotransmitters. In Alzheimer's disease patients a misregulation of cerebral acetylcholine levels can be observed due to the reduction or inactivity of acetylcholine modifying enzymes. The researchers discovered quickly that a therapeutic approach on those enzymes gave little results as the regulation pathway of neurotransmitters is too multifaceted and heterogeneous.

The scientific attention then shifted on the neurofibrillary tangles and the neuritic/senile plaques in order to define their exact molecular composition and their development pathways.

A major breakthrough came in the 80s when Glenner and Wong identified "a novel cerebrovascular amyloid protein", known as beta-amyloid and when Wisniewski and Brion discovered that the Tau protein is a key component of tangles. These two protein quickly became the pathological hallmark of Alzheimer's disease and the prime suspects in nerve cell damage.

Since then many bio-pathological, biochemical and genetic studies were carried out.

In the 1990-2000 period a transgenic mouse, model of the pathology, was created along with a vaccine prototype and various drugs.

Over the first decade of the 21st century, scientists have significantly progressed in understanding potential environmental, genetic and other risk factors for AD, some of the processes leading to development of plaques and tangles, and the brain areas that are affected. Specific genes related to both the early-onset and late-onset forms of AD have been identified and the role of beta-amyloid has been one of the focal points of nowadays research. As of 2012, more than 1000 clinical trials have been or are being conducted to test various compounds in the hope to counteract the pathology. However, AD is still incurable.

9. Characterization of Alzheimer's Disease

9.1. Clinical symptoms

Although Alzheimer's disease develops differently for every individual, there are many common symptoms: patients with AD most generally present insidiously progressive memory loss, to which other spheres of cognitive impairment are added over time (Selkoe and Schenk, 2003). In the early phase of the disease, the most common and known symptom is difficulty in remembering recent events, often incorrectly supposed to be age-related or displays of high levels of stress. As the disease advances, symptoms can include confusion, irritability, aggression, mood swings and long-term memory loss. After memory loss occurs,

patients may also experience language disorders and deficiency in their executive functions and visuospatial cognition.

At the latest stage of the pathology, bodily functions are progressively, lost, ultimately leading to death.

Alzheimer's disease develops for an unknown and variable amount of time before becoming entirely apparent, and it can evolve undiagnosed for years.

The rapidity of the development of AD and the intensification of its symptoms differs between patients from months to several years.

As the sufferers conditions worsen they often estrange themselves from family and society.

Due to the incurable and degenerative nature of Alzheimer's disease, the sufferer relies on others for assistance placing a great burden on caregivers.

9.2 Neuropathological phenotype and diagnosis

The clinical diagnosis of possible or probable AD is done following some internationally recognized neuropsychological criteria (NINCDS-ADRDA, DSM-IV-TR) aided by modern medical apparatuses such as PET or SPECT. Although these methods are able to identify Alzheimer's disease, the absolute certainty of the pathology can only be obtained *post mortem* by an histological exam of the brain.

The current validated markers of AD are the senile plaques and the neurofibrillary tangles (Fig. 6).

In the last decade several new bio-markers, from genes to proteins, have been proposed. A major research focus is now pointed at the role of small soluble intracellular oligomers of Aβ40 and Aβ42 (the protein responsible for the plaques and the fibrils). These oligomers are both intracellular that circulating and their detection by biopsy could be accurate as the brain histological exam though not requiring the death of the patient.

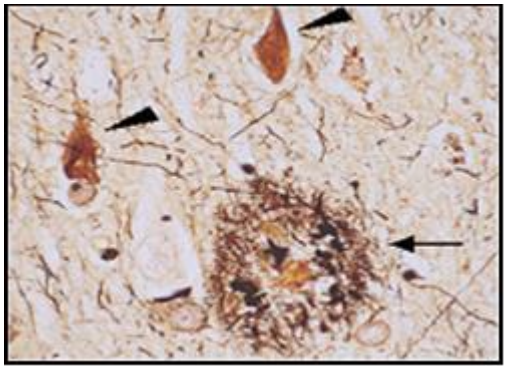

Figure 6 - Senile plaques and the neurofibrillary tangles
***Post mortem* histological exam of brain tissue from an AD patient. The arrow indicates the senile plaque while the black triangles indicates the neurofibrillary tangles**

9.2.a The neuritic/senile plaques

Neuritic plaques are extracellular deposits of beta amyloid in the gray matter (limbic system and cerebral cortex) of the brain. They have a spherical appearance and they are surrounded by dystrophic neurites (axons and dendrites), activated microglial cells and reactive astrocytes. The amyloid (Aβ) peptide derives from the proteolytic cleavage of the Amyloid precursor protein (APP) that produce Aβ fragments of 39-42 amino acids. The common fragment, in the amyloid aggregates, is Aβ42 which being the highly hydrophobic variant mostly promotes the polymerization of the fragments (seeding). Aβ40 is the most expressed cellular peptide, its much less hydrophobic than Aβ42 but nonetheless is involved in the formation of the fibrils/plaques.

Other than the "Aβ40/42 mixed" plaques, in the same cerebral areas, Aβ42-only plaques have been spotted. These plaques are widely distributed and they precede the formation of the former neuritic plaque (pre-amyloid plaques). These plaques seems to have a non-fibrillar (amorphous) conformation and they do not seem to be directly involved with typical AD symptoms. The discussion about the role and the risk of the pre-amyloid plaques is still open.

The β-amyloid proteins can amass, as fibrils, on the walls of the intra-parenchymal arteries and arterioles and, ad as deposits on the cerebral capillaries.

Amyloid deposition predisposes these blood vessels to failure, increasing the danger of a hemorrhagic stroke.

This Cerebral amyloid angiopathy (CAA) is more common in people who suffer from Alzheimer's but can also occur in those who have no sign of dementia. CAA it's not strictly related to the number of cerebral amyloid plaques and it is still unclear if CAA can lead to dementia.

9.2b Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are intersected perinuclear fibers that amass and deposit on the apical dendrites of AD patients brains. NFTs derive from the hyper-phosphorylation of a cytosolic microtubule-associated protein known as tau.

This protein normally binds to microtubules and has a crucial role in their formation and stabilization. However when tau is hyper-phosphorylated, it becomes incapable of binding the microtubules which become unstable and begin collapsing. The unbound tau clusters in an insoluble helix structure formed by couples of filaments (paired helical filaments, PHF) which then aggregates forming the NFTs. As tau is abundant in neurons of the central nervous system, the tangles are involved with many neurodegenerative diseases called tauopathies. Both tau phosphorylation pathway and its role in Alzheimer's Disease have been controversial even if the understanding of tauopathies have greatly increased in the last years.

Tau pathobiology involves an extensive assortment of other cellular abnormalities including an interference in autophagy, vesicle trafficking mechanisms, axoplasmic transport, neuronal polarity, and even the secretion of tau itself into the extracellular space.
It is commonly presumed that NFTs are central mediators of AD pathogenesis.
Nevertheless some novel studies propose a model in which tau phosphorylation and aggregation in AD might essentially be a defensive (antioxidant) response that functions as a mechanism to save threatened neurons. As of 2013 tau and its related kinases remains one of the major therapeutic target.

9.3 Neurotransmitters alterations

In AD affected patients a decrease of both the number and the activity of acetylcholine synthetizing and degrading enzymes can be observed. Low levels of acetylcholine are responsible for the typical AD cognitive collapse. Some attempts to find a possible cure for AD involved the inhibition of the acetylcholinesterase enzyme in order to augment and/or stabilize the neurotransmitter levels in the synaptic junctions. The treatment gave no considerable clinical benefits due to the fact that the pathway of acetylcholine synthesis/regulation is incredibly complex involving several diversified neurotransmitters (e.g., GABA, glutamate, corticotrophin) (Selkoe and Schenk, 2003).

9.4 Origin of the Aβ peptides: the Amyloid Precursor Protein pathways and the secretases

9.4a The Amyloid Precursor Protein

Amyloid precursor protein (APP) is a type-1 membrane glycoprotein expressed in several tissues and concentrated in the synapses of neurons. Its function is still unclear but it has a confirmed role in synapse formation, neural plasticity and iron export.
There are many APP variants due to various alternative splicing isoforms.
In human, the three principal isoforms are composed by 695, 751 and 770 amino acids and they undergo different posttranslational modification (N- and O-glycosylation, phosphorylation and tyrosine sulfation).
Isoform 695 is particularly abundant in neurons while 751 and 770 are widely expressed in non-neuronal cells.
The main difference between these three isoforms is that isoform 695 lacks a 56 amino acids sequence very similar to the typical Kunitz-type motif of serine proteases.
A number of distinct, largely independently-folding structural domains have been identified in the APP sequence. The extracellular region (N-terminus), much larger than the intracellular region (C-terminus), is

divided into two domains (E1 and E2), connected by an acidic domain (AcD). The E1 domain contains two subdomains including a growth factor-like domain (GFLD) and a copper-binding domain (CuBD). The E2 domain contains a coiled coil dimerization motif. A serine protease inhibitor domain, absent in the 695 isoform is found between the AcD and E2 domains.

9.4b APP proteolysis

APP is co-translational translocated in the endoplasmic reticulum by a signal peptide and then secreted. During this phase many post-translational modification occur and the APP undergoes several proteolytic cuts by aspartate proteases called secretases.

Aβ peptides are the resulting fragments of the APP proteolysis: they are initially released in secretory vesicles and ultimately in the extracellular space. The most common pathway is the non-amyloidogenic one. The first cut is made by an α-secretase, 12 amino acids from the transmembrane domain N-terminus. This first cleavage causes the release of a large soluble domain (α-APPs) in extracellular space while a 83 amino acids peptide (CTF) is retained in the cellular membrane. The subsequent action of a γ-secretase generates another extracellular released peptide (p3) and a C-terminus fragment further processed into the soluble intracellular APP internal domain (AICD).

In the amyloidogenic pathway the first cleavage of the APP is made by a β-secretase which cuts 12 amino acids from the transmembrane domain N-terminus thus generating a smaller extracellular soluble domain (β-APPs) and a 99 CTF peptide. γ-secretase then cut CTF producing the AICD and the extracellular Aβ peptide.

Aβ peptides are produced in healthy subjects thus suggesting that the amyloid peptides production is a normal metabolic event.

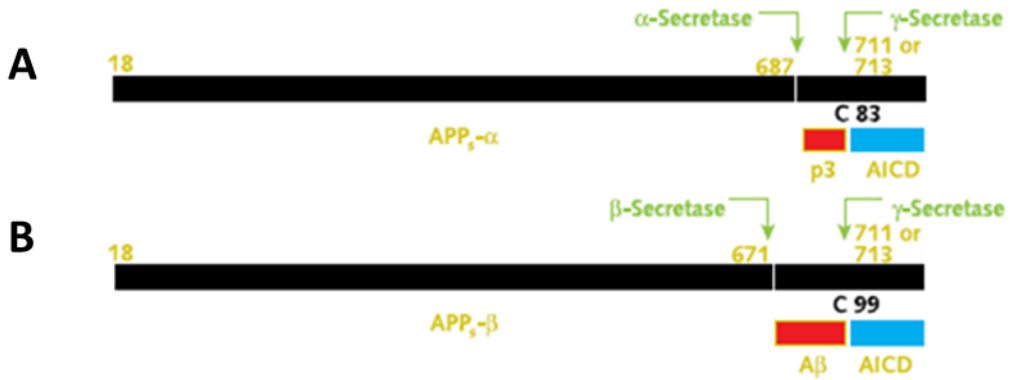

Figure 7 – Schematization of the APP processing
A - α-secretase cut, non-amyloidogenic pathway
B - β-secretase cut, amyloidogenic pathway

9.4c Function of APP and its byproducts

Functional consequences of APP proteolysis are still unclear.

Putative functions of APP and proteolysis byproducts include:

- Serine protease inhibition (APP isoforms containing the Kunitz protease inhibitor domain)
- Cellular adhesion to other cells or substrates
- Transcriptional activator (AICD fragment)
- Neuroprotection (α-APPs fragment)
- Axonal vesicular transport
- Iron transport
- Hormonal regulation

The main focus is, however, the main *in vivo* role of the APP. It is widely assumed that APP plays a fundamental part in synaptic formation and repair. Experiments with APP knockout mice showed relatively minor phenotypic effects including impaired long-term potentiation and memory loss without general neuron loss. On the other hand, transgenic mice with APP overexpression have been reported to show impaired long-term potentiation.

9.4d α-secretases

α-secretases are membrane metalloproteases of the ADAM family that cut APP in the amyloid region on the KLVFF sequence thus preventing neurotoxic Aβ42 peptide formation. The α-secretase cleavage is typical of the non-amyloidogenic pathway and the resulting α-APPs fragment has demonstrated neuro-protective functions.

9.4e β-secretases

β-secretases or β-site APP cleaving enzymes (BACE) are type I transmembrane proteases of the aspartate protease family. They are composed by 501 amino acids divided in:

- signal peptide (amino acids 1-21)
- pre-protein domain (amino acids 22-45)
- catalytic domain (amino acids 45-459) with two highly conserved aspartate protease active sites (DTGS, amino acids 93-96; DSGT, amino acids 289-292)
- C-terminal extension region comprising a transmembrane domain (amino acids 460-477) and a cytosolic domain (amino acids 477-501)

β-secretases are responsible for the generation of the toxic Aβ peptides. Since α -secretases cleave APP closer to the cell membrane than β-secretases, it removes a fragment of the amyloid-β peptide. The cleavage of APP by BACE cause the generation of a longer peptide (C99) precursor of the toxic amyloid peptides.

9.4f γ-secretases

γ-secretases is an integral membrane multi-subunit protease complex, composed by four major components: presenilin 1 (PS1), nicastrin, APH-1 (anterior pharynx-defective 1) and PEN-2 (presenilin enhancer 2).

Presenilin 1, the catalytic core of the complex, is composed by:

- 8 transmembrane domain
- an extended cytosolic loop (between the 6th and the 7th domain)
- cytosolic C- and N- terminus

Presenilins (PS) are subject to endo-proteolysis in the cytosolic loop thus generating two fragments (NTF and CTF, N- and C-terminal fragment respectively). NTF and CTF interacts to form a stable and functional heterodimer. Domains 6 and 7 each contain aspartate protease motifs critical for substrate (α -secretase C83 or β-secretase C99 generated fragments) recruitment.

The endo-proteolysis is necessary for the correct activation of the presenilins by the exposition of the substrate interacting hydrophobic pocket.

The CTF of the PS1s is in connection with nicastrins (type I transmembrane glycoproteins) which allow their correct translocation from the endoplasmic reticulum (processing site) to the surface of the cytoplasmic membrane or to the Golgi network vesicles. Moreover nicastrins stabilize the CTF-NTF heterodimer and act as a connection with the substrate (Edbauer et al., 2002).

PEN-2 plays a role in the PS endo-proteolysis. It takes a reverse hairpin form with both its extremities towards the vesicles lumen or the exterior of the plasma membrane.

APH-1 is a protein composed by seven transmembrane domains , it undergoes endo-proteolysis and its C-terminus stabilizes the unaltered prensinilins.

γ–secretases have low specificity considering they can generate various peptides different in the C-terminus. In the amyloidogenic pathway, Aβ40 is the most common peptide, followed by Aβ42 and Aβ43. Other less frequent amyloid peptides are Aβ38, Aβ39 and Aβ34 (Wolfe et al., 2001).

Another target site of the γ–secretase complex is the ε site of the C99 fragment. This region is downstream of the Aβ C-terminus generating site (between Aβ amino acids 49 and 50) of the APP.

The cut on the ε site produces the cytoplasmic AICD fragment.

APP is not the only target of γ–secretases activity, other substrates are: ErbB4, CD44, syndecan3, E-cadherin, N-cadherin and the Notch receptor. The cleavage of Notch in its proteolytic pathway by the γ–secretase complex cause the release of the transcriptional regulator NICD (Notch intracellular domain) (Kopan, 2002).

9.5 Aβ peptides aggregation and fibrillogenesis

Aβ peptides are, under physiological conditions, 39-42 amino acids long (4 KDa ca.).
The NMR analysis of their structure shows five different regions:

1. a non-structured, polar region (amino acids 1-14) solvated by water solutions
2. a first amphipathic helix (amino acids 15-24), probably implicated in the interaction with cellular surfaces
3. a hinge region (amino acids 25-27)
4. a second hydrophobic helix (amino acids 28-36)
5. the C-terminal region (amino acids 37-42), highly responsible for the polymerization (Coles et al., 1998)

40 and Aβ42 peptides can form fibrils without the need of other proteins suggesting that the fibril assembly process is intrinsic in the Aβ peptide structure.
Replacing the hydrophobic 17-21 region with hydrophilic amino acids inhibits fibrillogenesis showing that this event is driven by hydrophobic interactions.
In order to form fibrils, Aβ peptides undergo a conformational change from a random coil / α-helix structure to a antiparallel β-sheets one. This transition is assisted by various factors, among them: metal ions, chaperon proteins (Apolipoprotein E, amyloid P component), pH changes, oxidative stress and Aβ concentration increments.
When in random coil / α-helix conformation Aβ peptides aggregate slowly however when in antiparallel β-sheets structure the aggregation is very rapid. It is very plausible that an hydrophobic environment aids monomers interaction to generate oligomers and then fibrils.
The suggested conformational change mechanism involves side chain de-protonation of residues Asp7, Glu11, Glu22 and Asp23 when at pH greater than 4 and protonation of His 7, 13 and 14 thus destabilizing the α-helical structures (Serpell, 2000). The fibril core is the 10-42 region while the 1-9 sequence remains exposed and leads to interactions between fibrils.
Antiparallel β-sheets form tetramers which aggregates into protofilaments. Fibrils are then formed by the (lateral) interaction of 3-5 protofilaments.

9.6 Genetic of the Alzheimer's disease

Alzheimer's disease can be classified in two groups: sporadic AD and familiar AD (FAD). The former represent the vast majority of the cases (90% ca.) and has unknown origins. FAD covers the remaining 10% of the cases and is phenotypically identical to the sporadic variant the only difference being only the earlier age when AD symptoms appear. Four human key genes have been identified as related to AD. They respectively code for proteins APP, ApoE4, PS1 and PS2.
Other gene are being investigated as risk factors or active players in the disease mechanism.

Chromosome	Genotype	Phenotype
21	APP mutation	Augmented production of Aβ42 and/or other β-amyloid peptides
19	Apolipoprotein E4 (ApoE4) polymorphism	Increase of amyloid deposit and plaque density
14	PS1 mutation	Augmented production of Aβ42
1	PS2 mutation	Augmented production of Aβ42

Table 1 – Resume of the principal AD related mutations

9.6a APP mutation

Missense mutation in the APP gene are responsible for less than 0.5% of AD cases. These mutation are however invaluable to understand of the pathology and its mechanisms. Transgenic mice over-expressing mutated forms of APP are the actual standard animal model of AD. Being the APP gene on the 21th chromosome it is well know that there are many correlation between AD and Down syndrome (Selkoe, 2004).

9.6b ApoE4 mutation

Apolipoprotein E (ApoE) is an important transport factor of cholesterol, mutations in its gene or its promoter are considered risk elements for AD predisposition. The ApoE locus has three alleles: E2, E3 and E4. The responsible for Aβ deposit and accumulation is E4 and E2 seems to contrast these events (Xing and Higuchi, 2002). One or multiple copies of the E4 allele are considered the main genetic cause of AD (25% of the cases).

9.6c PS1 and PS2 mutation

Mutations in the presenilins genes alter the γ-secretases APP cleavage causing the most aggressive form of AD. More than 100 mutations have been identified on the PS1 gene and more than 10 on the PS2 gene. These mutations modify the transmembrane domains of the presenilins.
The phenotype is an early manifestation of the disease due to the over-accumulation of amyloid products. Presenilins play a fundamental role in correct development and reproduction (Notch proteolysis) and also interacts with the GSK3 kinase, one of the enzyme involved in tau phosphorylation. Mutated PS are responsible for tau hyper-phosphorylation and the formation of neurofibrillary tangles (Selkoe, 2001-2004)

9.7 Inflammation and immune response

Various AD related alterations reveal the activation of inflammatory mechanisms: accumulation of complement components, deposit of acute phase proteins and cytokines, cyclooxygenase 2 up-regulation and microglia activation.
A large number of the complement system components are up-regulated in the brain of AD patients and the membrane attack complex (MAC) has been found in cerebral areas with neuritic plaques. The presence of the MAC suggests a major role of the complement in neuronal loss. The exact role is however still unclear: in this case also some of the components (e.g., C5a) could be function as neuro-protectors.
Around neuritic plaques, in AD patients, an elevated amount of active microglia cells (exposing MHC-II antigens) an integrins can be found. Microglia has many putative roles in AD pathogenesis:

- cytokine, acute phase proteins and complement proteins synthesis
- nitric oxide, superoxide and other radicals generation
- conversion of soluble amyloid peptides into fibrils

Microglia cells are capable of releasing neurotoxic components suggesting an active role in cerebral neuronal death. There are, however, some evidences that these cells could possibly have the function of limiting brain damage in Alzheimer's disease. The overall role of these immune response components is, as often happens is the study of AD, still unclear. Some of the acute phase response elements (IL-1, IL-6 and S100-b) have a demonstrated neuro-degeneration accelerating effect, others (TGF-α and TGF-β) could protect brain cells from neuro-toxic and oxidative stress. The role and the specificity of the inflammation process in correlation with AD and other neuro-degenerative diseases (e.g., Parkinson, Pick, Huntington) is one of the many unclear aspect of this ailments.

9.8 Oxidative stress

Cerebral metabolism requires high energetic levels and it is strictly dependent from aerobic conditions. The brain is enriched in easily oxidizable polyunsaturated fats and radical generating transition metals. These factors along a reduced quantity of anti-oxidizing compounds make the brain an organ particularly susceptible to oxidative damage. An excess of oxidizing activities or the failure of defensive anti-oxidizing systems, both related to AD, could be responsible for an aggravated and irrecoverable neuronal damage. Another plausible theory is that an age dependent alteration of the normal cerebral metabolism can make the brain more vulnerable to neuro-toxic amyloid peptides or fibrils. Although it is not possible to assess the exact role of every single free radical source, it is known that Aβ peptides can oxidize membranes, mitochondria and DNA compromising their biological functions.

9.9 The amyloid cascade hypothesis

The ultimate cause of Alzheimer's disease is a combination of age, genetic predisposition and environmental stress. These factors lead to neuro-biological changes (e.g., Aβ deposits, inflammation reactions, oxidative stress and vascular defects) that manifest themselves at various stages of the pathology. The exact timeline of these AD dependent anomalies is still unknown and varies depending on the considered assumption. The current most accredited theory is the amyloid cascade hypothesis, presented for the first time in 1991 (Selkoe, 1991; Hardy and Higgins, 1992). The core of this model is that Aβ deposition is the initial pathological event in AD, leading to the formation of senile plaques and then to NFTs, neuronal cell damage/death, and eventually dementia. The over production of Aβ42 is observed in cerebral fluids and in neurons. Aβ monomers are released in the extracellular space by endosomes fusion to the plasma membrane. Aβ42 aggregates quickly and forms amyloid deposit to which Aβ40 is added. The accumulation of Aβ peptides is the begin of the cascade, then the plaques trigger an inflammatory response characterized by astrogliosis, microglia activation and an abnormal release of interleukins and cytokines. The next step is an overproduction of free radicals that damage proteins, lipids and nucleic acids. The metabolic effects (e.g., alteration of Ca^{2+} homeostasis) continue the cascade starting other harmful effects such as uncontrolled kinases activation. The alteration of Ca^{2+} levels triggers the hyper-phosphorylation of protein tau which aggregates into PHFs and ultimately into NFTs. The final stage of the cascade is the neuronal death that impairs the normal brain functions .

Since 1992 the amyloid cascade hypothesis has been the scientific reference for the AD although, during the years, new knowledge has been acquired and some points have been updated or modified. Overall, there is extensive evidence (genetic studies, AD patients and Tg mice evaluations, studies on the formation of Aβ and tau, clinical trials targeting Aβ and tau) supporting this hypothesis. However, as this theory does not fully explain the pathogenesis of AD there are other schemes that partially or totally contrast the amyloid cascade hypothesis.

The main observation of these theories is that a major part of the population has plaques without any sign of dementia and that the role of Aβ should not be considered univocally harmful. As of 2013 the debate is still open (Reitz, 2012).

9.10 Amyloid-β derived diffusible ligands (ADDLs)

The amyloid cascade hypothesis has dominated research and subsequent therapeutic drug development for over two decades (Karran et al., 2011). More recent evidence, however, suggests that the presence or absence of plaque is insufficient to entirely account for the Aβ damaging role in AD. These studies support an alternate interpretation of the cascade hypothesis focused on Aβ soluble aggregated particles. These amyloid monomers interact in a non-covalent way to generate intermediate soluble Aβ oligomers (from trimers to 12mers) hence called amyloid-β derived diffusible ligands (ADDLs).

ADDLs can accumulate and cause functional deficits prior to proper plaque deposition. These theory provide the basis for a different view of the amyloid cascade hypothesis and acts as the "missing link" explaining the low correlation between plaque deposition and cognitive status (Catalano et al., 2006; Krafft et al., 2010).

Researchers discovered massive quantities of ADDLs in post-mortem brains of AD patient and relatively low levels of ADDLs have been found in the brains of healthy people. Further support to comes from experiments with transgenic mice in where it was observed that the neurons function is restored when ADDLs are removed. Involvement of soluble Aβ oligomers in synaptic failure and Alzheimer's disease associated memory deficiency is now widely accepted, however a divergence concerning which particular oligomers are toxic remains. ADDLs are now regarded as one of the major target for AD understanding, diagnosis and targeted drug discovery.

10. Therapeutical anti β-amyloid approaches

As 2012, there are no drug treatments that can deliver a remedy for Alzheimer's disease. However, medicines that can temporarily slow down the pathology advancement have been developed. There are two main categories of drug used to treat AD: cholinesterase inhibitors (donepezil hydrochloride, rivastigmine and galantamine) and N-methyl-D-aspartate (NMDA) receptor antagonists (memantine). Although these drugs don't work on every patient, they are recommended for patients with mild-to-moderate Alzheimer's disease symptoms.

Even if there still are many unsolved question on Alzheimer's disease, the continuing understanding of AD pathogenesis allowed the identification of many possible targets and therapeutic strategies. In the last 15 years several strategies on different levels of the amyloidogenic pathway have been proposed:

- inhibition of the amyloid aggregation process and/or disaggregation of pre-existing senile plaques
- prevention of amyloid deposit formation

10.1. Inhibition of the amyloid aggregation process and/or disaggregation of pre-existing senile plaques

Two different strategies have been developed to contrast the aggregation process or to promote disaggregation of the senile plaques:

a. immunization
b. anti-aggregation agents

10.1a Immunization

Prophylactic vaccines have been successfully employed against a large number of pathogens micro-organisms. An efficient vaccine should promote strong immunity and induce a long term immunological memory. While prophylactic vaccines are effective with healthy patient in order to prevent the disease, therapeutic vaccines could be given to patients affected by chronic illnesses such as cancer, cardio-vascular pathologies and neuro-degenerative syndromes. Therapeutic vaccines are directed to those pathologies for which there is a lack of efficient or alternative treatments.
The immunization against AD has been one of the most promising approach in this decade. It takes advantage of the antibodies (Abs) capability of clearing many brain areas from the amyloid peptides. Alzheimer's disease affected patients show a reduced immunotiter against Aβ species along with a low T cells (Aβ induced) proliferation rate.
Passive immunization, in which antibodies against a particular infectious agent are given directly to the patient, involves the use of monoclonal antibodies or antibody fragments directed against Aβ epitopes.
The prototype for active immunization is considered to be the Élan vaccine which came in 1999.

The first evidence of the possible therapeutic use of immune-globulins against AD came in 1997 when Solomon's laboratory demonstrated the inhibition of Aβ42 *in vitro* fibrillogenesis using antibodies. In 1999, Schenk et al. showed the positive effects of the immunization of AD model transgenic mice (overexpressing the human APP770) with human Aβ42. The amyloid peptide generates antibodies capable of inducing plaque disaggregation by microglia phagocytosis. The final result of the study was the prevention of AD arise in young model mice while in old, already ill, mice the immunization lowered the number of senile plaques, dystrophic neurons and gliosis thus lessening symptoms of Alzheimer's disease. In a short time, many other laboratories independently confirmed the efficacy of the immunization approach and, by the

end of 1999, Élan Corporation. Called AN-1792, the vaccine is composed by the synthetic human Aβ42 and, as adjuvant, the purified plant saponin QS-21. In the first phase of the experimentation the vaccine gave no collateral effects in patients, however in the second phase roughly the 5% of the treated patients showed inflammation reactions which worsened into aseptic meningo-encephalitis. The immunization with AN-1792 was blocked in 2002. Élan Corporation and many other independent groups began to research the reasons of the vaccine failure. The inflammatory response had presumably been caused by multiple factors: pro-inflammatory cytokines, complement system triggering by the antibodies Fc, Th1 cells activation and an Aβ42 auto-immune response. In mice this auto-immune reaction didn't happen because the little although significant difference between the murine Aβ42 and the injected human Aβ42. In 2003 it was demonstrated that some of the phase 2 AN-1792 immunization developed an active anti amyloid immune-response and maintained stable functional and behavioral functions. The anti-AD vaccine project didn't met its demise but rather began a main worldwide research focus.

Since 1999 many laboratories over the world begin to develop various vaccine in order to find a cure to AD or to better understand its pathogenesis.

Over the last 10 years the mechanism by which Aβ immunization inhibits o reduce plaques formation has been thoroughly inspected and it is currently explained by two major hypothesis:

1. the vaccine induce Aβ species phagocytosis by microglia monocytes
2. the anti-Aβ serum takes away free Aβ peptides altering their equilibrium between the central nervous system and the plasma

Valid evidences for both theories exist and there's the possibility that both hypothesis take place.

The higher observed immune-response against Aβ is directed to the N-terminus of the amyloid peptide, in the 1-16 region (the smallest active epitope is the 4-10 fragment).

Despite the scientific attention is shifting from senile plaques to ADDLs, immunotherapy is still regarded as one of the most effective approach to treat or prevent AD (Schnabel, 2011).

10.1b Anti-aggregation agents: chemical compounds, β-sheet breaker peptides and proteins

Chemical compounds

Various little molecules, not necessarily correlated, can inhibit fibrillogenesis or Aβ toxicity *in vitro*. By chemical compounds libraries screening the anti-aggregation and/or disaggregating function of many chemicals was found. Among them the more acknowledged are Congo Red, the antibiotics rifampicin and anthracyclines (e.g., IDOX), Hexadecyl-N-methylpiperidinium (HMP), melatonin, nicotine, benzofurans, β-cyclodextrin and estrogens. Their mechanism of action consist in the stabilization of Aβ monomeric forms and/or the solubilization of Aβ aggregated forms thus reducing their toxicity. The usage of this compound is

however limited due to their dimension, non-specificity, side effects and sometimes to their unclear mechanism of action.
The research and development of new generation anti-aggregation drugs (e.g., chaperonins bound chemicals) is one, if not the main, pharmaceutical scientific target.

β-sheet breaker peptides

β-sheet breakers (BSB) are short synthetic peptides capable of binding soluble Aβ but unable to become part of a β-sheet structure hence precluding the amyloidogenic conformation and amyloid aggregation. The paradigm for BSB are two peptides synthetized by Soto (LPFFD peptide) and Tjernberg (KLVFF peptide) (Soto et al., 1999; Tjernberg et al., 1999). Soto's peptide binds to Aβ17-21 region while Tjernberg's one match the 16-20 region. In Soto's BSB the Aβ valine is replaced by a proline, this modification is essential to the anti-aggregation action because proline is a β-sheet conformation inhibitor.
Over the years these two peptides have been modified in order to optimize their β-sheet prevention action. Some of the modification involved adding a "destructive" polar element fused to the original peptide, fusing a large chemical group to one end of the BSB thus preventing β-sheet by steric hindrance, enriching the BSB in prolines or using n-methyl or n-acetyl amino acids.

Proteins

Various studies suggested that Aβ could show its toxicity before the extra-cellular aggregation. Experiments on neuronal cells demonstrated that Aβ can form dimers or small oligomers at an intra-cellular level. If this intra-cellular Aβ contributes to AD it is essential to find its interaction proteins. So far the vast majority of these proteins seems to be chaperonins (e.g., heat shock proteins). Many other proteins could be part of the Aβ pathway, promoting or limiting its harmful effects. The general idea is that among the cause of the disease could be mutated proteins unable to perform their anti-aggregation or Aβ homeostasis control functions.

10.2. Prevention of amyloid deposit formation

10.2a Reduction of APP expression

Using a modern targeted gene therapy approach (e.g., iRNA, antisense nucleotides) it is possible to reduce or to shut down APP expression. The desired effect is a reduction in Aβ levels however the possible side effect of a direct intervention on APP is not advisable since its biological role is still not completely clear.

10.2b α–secretase activation

The activation of α–secretase could bring a double benefit due to its cleavage in the amyloid region of APP: the prevention of toxic Aβ peptides formation and an augmented release of the neuro-protective α–APPs fragment.

The activity of α–secretases is regulated by protein kinase C (PKC) mediated phosphorylation. The stimulation of PKC related neuro-transmitters (e.g., muscarinic acetylcholine receptor M1 and M3) indirectly but efficiently up-regulate α–secretases cleavage thus releasing more α–APPs fragment and lowering Aβ concentrations. However APP is not the only substrate of α–secretases which up-regulation can lead to undesired biological side effect

10.2c β–secretase inhibition

Being the direct responsible for Aβ peptides production, β–secretases represent an attractive target for the therapeutic treatment of AD. Knock-out BACE1 deficient mice don't produce any of the Aβ forms however it seems that β–secretases are necessary for the proper function of muscle spindles. These results raise the possibility that BACE inhibitors currently being investigated are not specific for the treatment of Alzheimer's disease but inhibits other proteases giving major side effects. Many BACE inhibiting drugs have been designed, tested and modified during the last 10 years (e.g., OM99-2, MK-8931, LY2886721). As of 2013 advanced clinical trials (phase 2/3) on BACE inhibitors are still going on.

10.2d γ–secretase inhibition

γ–secretases are pivotal enzymes involved both in the amyloidogenic and non-amyloidogenic APP processing pathway. These proteases also have a fundamental role in other cellular pathways processing many other substrates (e.g., Notch, ErbB4, CD44, syndecan3, E-cadherin, N-cadherin). Studies in both transgenic and non-transgenic animal models of AD have indicated that γ-secretase inhibitors are able to lower brain Aβ levels but there currently are little insights on amyloid deposition after prolonged administration. γ-Secretase inhibitors may cause various side effects (abnormalities in the gastrointestinal tract, thymus, spleen, skin, and decrease in lymphocytes), associated with the inhibition of the cleavage of Notch. Some non-steroidal anti-inflammatory drugs (NSAIDs) and other small organic molecules have been found to modulate γ-secretase activity, shifting its cleavage action from longer to shorter non-toxic Aβ species without affecting Notch cleavage. Long-term histopathological and behavioral animal studies are available with these NSAIDs and new drugs are being researched. As for BACE, γ–secretases inhibitors are a

major pharmaceutical interest and many compounds have reached advanced clinical trial (Imbimbo et al, 2011).

10.2e Membrane cholesterol depletion

Membrane cholesterol levels have a role in non amyloidogenic APP soluble derivatives formation. Specifically augmented levels of cholesterol compromise the release of soluble APP forms. Researchers have found that, by the opposite mechanism, a depletion of membrane cholesterol by statins administration reduce the production of amyloid peptides. Considering the adverse effects of using statins and the fact that this is not an AD specific treatment, the use of this drug is not recommended.

RATIONALE AND SCOPE OF STUDY

Since the end of 1700, traditional vaccines are composed by dead /inactivated micro-organisms or purified products derived from them. Recombinant DNA technology and artificial DNA/protein synthesis play a major role in the development of new generation vaccines more efficient and safer than traditional ones. Antigenic peptides are the base of these new vaccines thus eliminating the need of the original micro-organism.

The recombinant/synthetic approach can be used not only to produce vaccine but also to produce diagnostic antibodies for medical or scientific purposes.

The presented study will be divided in two projects:

- the use of recombinant antigenic peptides to generate diagnostic antibodies against “problematic” antigens
- the development of a recombinant vaccine against Alzheimer's disease

1. Recombinant antigenic peptides for diagnostic antibody production

The purpose of this project is an original approach to the production of diagnostic immunoglobulins against “problematic” antigens to be used as a powerful molecular biology tool. A “problematic” antigen cannot usually be isolated from the natural source (or prepared by recombinant DNA technology) and/or does not guarantee the production of “good quality” antibodies (e.g., elevated titles and high specificity). To overcome these problems haptens are used. An hapten is a small molecule (in this case a short fragment of the whole antigen) which cannot elicit an immune response due to its small molecular dimension and thus has to be attached to a larger carrier (e.g., proteins). Keyhole limpet hemocyanin (KLH) is the most commonly employed carrier protein for this purpose.

KLH is an effective carrier protein for its large size (390 kDa) and numerous epitopes that generate a substantial immune response. Because KLH is derived from a gastropod, it is phylogenetically distant from mammalian proteins, thus minimizing false positives with immunologically-based research techniques in mammalian model organisms and humans.

Due to its exceptional size and unusual glycosylation, KLH cannot be easily synthesized, and is more efficiently and cost-effectively prepared by purification from the hemolymph of the source animal (*Megathura crenulata*).

Haptens/epitopes are cross-linked to the KHL as recombinants approach are restricted by the difficult expression/production of this protein in bacteria or yeast.

While quite straightforward, this methodology not always provides ideal immunogenicity, particularly in the case of weak epitopes, and does not preserve the structure of the parent antigen region from which the peptide is extracted.

The project presented in this thesis use the so-called TDMI technology (Thioredoxin-Displayed Multipeptide Immunogens) previously developed in the Department of Biosciences of the University of Parma. This approach takes advantage of the *E.coli* thioredoxin A protein (EcTRX), a small, nontoxic, soluble protein using it as an antigen-presenting thermostable and highly constrained scaffold.
By recombinant DNA technologies, it is possible to insert within EcTRX, in a well exposed loop, peptides as long as the TRX (100 aa ca.).
This thioredoxin loop has been effectively employed for the intracellular as well as cell surface exposure of antigenic peptides (Rubio et al., 2009).
Chosen the ("problematic") antigen, with an *in silico* analysis through several bioinformatic programs, epitopes able to stimulate the production of antibodies (B cell epitopes) are predicted.
Synthetic genes encoding these epitopes are than cloned into EcTRX gene and expressed as a fusion protein used to immunize a suitable animal.
The object of the study is to demonstrate the effectiveness of the TDMI approach and, eventually, optimize it.

2. Development of a recombinant vaccine against Alzheimer's disease

Starting from a previous study focused on the amyloid Aβ42 peptide and on the identification of one or more epitopes capable of inducing the production of antibodies against said peptide (Moretto et al., 2007), the second project of this study is the development of a vaccine against Alzheimer's disease.
The chosen epitope is composed by the first seven amino acids of Aβ42 (Aβ1-7) repeated nine times an inserted in the EcTRX.
The project is composed by two phases.
The first is the evaluation of the efficacy as "enhancer" of: i) different immune adjuvants approved for human use (alum and Montanide), ii) flagellin from Salmonella enterica (Fli) fused to Trx (Fli-EcTrx), produced in recombinant form, and iii) supramolecular constructs obtained from the aggregation of the antigen with the cellular walls of *Lattococcus lactis* (BLP-EcTrx). In the latter formulation, by acid treatment of *L. lactis* cells, protein and nucleic acids are removed, leaving an empty peptidoglycan shell. Multiple copies of the antigen can be attached to the surface of these bacterial-like particles (BLPs) when expressed as fusion constructs with a peptidoglycan binding domain (PNG-BD). For this vaccine the LysM domain of the *L .lactis* autolysin (AcmA) has been chosen as PNG-BD thus creating the EcTRX-LysM scaffold to be attached to the *L. lactis* BLPs.
The vaccination experiment is conducted on BALB/c mice.
Immuno-response data from the first phase is used to develop a prototype vaccine to be tested, in a 14 month study, on Tg2576 transgenic mice (overexpressing the Swedish variant, APPSwe, of the human APP

protein) that develop Alzheimer-like symptoms from the 9th month of life, and BALB/c mice as "normal" controls.

The primary objective of this phase is to evaluate the longterm efficacy of the vaccine in preventing the cognitive deficits associated with the progression of the mice Alzheimer-like pathology, as well as the development of histopathological symptoms of the disease such as amyloid plaques and the accumulation/overproduction of Aβ oligomeric structures following vaccination.

MATERIALS AND METHODS

1. Bacteria

1.1. *E. coli* BL21-CodonPlus(DE3)-RIL strain

BL21-CodonPlus(DE3)-RIL (BL21C+)competent cells are derived from the high-performance Stratagene BL21-Gold competent cell line. These cells enable efficient high-level expression of heterologous proteins in *Escherichia coli*.

BL21C+ cells contain extra copies of the *argU*, *ileY*, and *leuW* tRNA genes. These genes encode tRNAs that recognize the arginine codons AGA and AGG, the isoleucine codon AUA, and the leucine codon CUA, respectively. The CodonPlus-RIL strains have available the tRNAs that most frequently restrict translation of heterologous proteins from organisms that have AT-rich genomes.

For full specification see Agilent Technologies product catalog.

1.1. *L. lactis*

Lactococcus lactis (IL1403 strain) was kindly provided by Dr. Marco Ventura (Department of Life Sciences, Laboratory of Probiogenomics, Università di Parma)

2. Plasmids

2.1. pET28

The used pET28 plasmid is a modified version of the pET28 plasmid (Novagen). A unique NdeI restriction site allows in-frame cloning in-between the two 6xHisTAG sequences.

For full specification see Novagen product catalog.

2.2. pET28-EcTRX

pET28-EcTRX was obtained by cloning the *E. coli* thioredoxin A gene in the pET28 NdeI site.

2.3. pET28-PfTRX

pET28-PfTRX was obtained by cloning the *P. furiosus* thioredoxin gene in the pET28 NdeI site.

2.4. pET28-LysM

pET28-LysM was obtained by cloning the *L. lactis* AcmA LysM domain gene in the pET28 plasmid using the unique NdeI/HindIII sites.

3. Growth mediums

Luria-Bertani (LB) medium was used to grow *E. coli* cells. See Molecular cloning : a laboratory manual (Sambrook and Russel) for the recipe and specifications.

Sigma-Aldrich M17 broth (56156) was used to grow *L. lactis* cells.

4. Plasmid/DNA extraction

Plasmid extraction was performed following the Molecular cloning : a laboratory manual (Sambrook and Russel), using QIAGEN Plasmid Midi Kit (QIAGEN) and using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.

5. Restriction and ligation reactions

Restriction reactions were performed using Takara enzymes according to the manufacturer's instructions. Ligation reaction were performed using Takara T4 ligase according to the manufacturer's instructions.

6. Bacterial transformation

Transformation of bacteria was done by electroporation. See Molecular cloning : a laboratory manual (Sambrook and Russel) for the full protocol.

7. Bacterial colony PCR

Bacterial colony PCR was done using GoTAQ polymerase (Promega) according to the manufacturer's instructions. Bacterial colonies was directly added to the reaction mix immediately before the start of the reaction.

8. Recombinant protein expression

Recombinant protein expression was induced by growing BL21C+ cells to an OD(λ_{600nm}) value of 0.6 then adding 1mM isopropyl-β-Dthiogalactopyranoside (IPTG). Cells were then cultured for 3 hours at 37°C or for 16 hours at RT.

9. Cell lysis

After recombinant protein expression, BL21C+ cells were harvested by centrifugation. Cellular pellet was resuspended in Tris-HCl 25mM pH7.4, NaCl 300mM, PMSF 0.5 mM, Benzamidine 0.5 mM, Leupeptin 1 µM, Pepstatin 1 µM and was then subjected to 20 minutes of ice-cold sonication using 3' bursts at 20–30 W with a 6' cooling period in ice between each burst.

10. Protein purification

Following cell lysis, 6xHis-tagged polypeptides were bound to a metal-affinity resin (Talon, Clontech), purified as per the manufacturer‟s instructions and extensively dialyzed against phosphate-buffered saline (Sigma-Aldrich PBS, P3813).

11. Protein purification from inclusion bodies

Following cell lysis insoluble recombinant were solubilized from inclusion bodies with 8 M urea, 150 mM NaCl, 10 mM 2-mercaptoethanol, 20 mM Tris-HCl pH 8.0. and two rounds of 20 minutes ice-cold sonication (immediately followed by centrifugation). Renaturation was performed using a renaturing buffer solution (1M guanidine-Hcl, 50 mM Tris–HCl, pH 8.0) and one last round of 20 minutes ice-cold sonication.

12. BLPs assembly

12.1. *L. lactis* acid treatment

L. lactis cells were growth o/n at 30°C, harvested by centrifugation, washed with sterile water and then re-centrifuged. Cells were resuspended in 0.1M HCl, boiled for 30', centrifuged and washed 3 times with sterile PBS. All centrifugation were performed at 4°C.

12.2. Binding of LysM fusion constructs to BLPs

Acid treated BLPs were resuspended in 1M guanidine-Hcl, 50 mM Tris–HCl, pH 8.0 then centrifuged. BLP pellet was resuspended with an appropriate quantity of purified LysM fusion protein (in 1M guanidine-Hcl, 50 mM Tris–HCl, pH 8.0) followed by rocking /rotating incubation for 1 hour at RT.
BLPs underwent 3 steps of centrifugation and wash with sterile PBS. All centrifugation were performed at 4°C.

13. LPS removal

Removal of endotoxin from recombinant protein and BLP preparations was performed as described by Liu et al. (Liu et al., 1997)

14. Western Blot

Proteins were separated on a SDS-polyacrylamide gel and then transferred onto a 0.2 µm pore size nitrocellulose membrane (Bio-rad), using a wet/tank blotting apparatus (Bio-Rad Criterion Blotter). The membrane was successively blocked overnight at 4°C in Tris Buffered Saline (TBS) supplemented with 0.05% Tween 20 (TBST) containing 5% skim milk powder (blocking solution). Membranes were washed three times with TBST for 15 min and then incubated with the primary antibody (properly diluted in TBST) for 2 hours at RT. Immunoblots were then washed three times for 15 min with TBST followed by incubation with LiCor IRDye 680 Goat anti-rabbit or anti-mouse (properly diluted in TBST) for 1 hour at RT. The immunodetection was done with the Odyssey Infrared Imager (LiCor).

15. ELISA test

Wells of a 96-well Nunc Immunoplate were coated with 100µl of 5µg/ml antigen at 4°C o/n. Wells were washed 3 times with PBS, Tween20 0.1% (PBST) and then blocked with 200µl PBST, 5% Skim milk. Blocking was performed by incubation for at 37 °C for 1 hour.
Wells were washed 3 times with PBST and incubated with 100µl of the primary Ab/serum (properly diluted in PBST) for 1 hour at 37 °C.
Wells were again washed 3 times with PBST and incubated with 100µl of an anti-mouse/anti-rabbit HRP conjugated Ab (properly diluted in PBST) for 1 hour at 37 °C.

Wells were washed 3 times with PBST and then incubated with 100µl of ABTS Reagent (KPL, 50-66-01) for 30’ at 37°C. To stop the reaction, 100µl of 1% SDS were added to each well.

Absorbance values were detected at λ_{450nm} using an iMark Microplate Absorbance Reader (Bio-rad).

RESULTS

RECOMBINANT ANTIGENIC PEPTIDES FOR DIAGNOSTIC ANTIBODY PRODUCTION

In this particular project *Escherichia coli* thioredoxin A (EcTRX) has been exploited as a carrier scaffold for the presentation/stabilization of antigenic peptides. EcTRX is a small (109 residues), soluble and non-toxic protein that contains a surface exposed loop, corresponding to a unique CpoI restriction site in the nucleotide sequence that is suitable for directional in-frame cloning of peptide encoding oligonucleotides, stabilized at the base by a disulfide bond.
EcTRX has been used not only as scaffold but also to confer immunogenicity to peptide epitopes.

1. Antibody production: TDMI approach

Using the EcTRX unique CpoI restriction site, DNA sequences coding for tandemly repeated peptides were inserted in the EcTRX gene already cloned in an appropriate expression vector (pET28, Novagen).
This approach, developed in our research laboratory, is called Thioredoxin Displayed Multipeptide Immunogens (TDMI).
Prior to the start of this project *Escherichia coli* TRX coding sequence were chemically synthesized (Eurofins MWG Operon) and inserted into the NdeI site of the modified pET28 plasmid in frame with two 6xHis tag sequences. The resulting expression vector is called pET28-EcTRX.
Given a target protein against which an antisera is required, epitope peptides are *in silico* predicted and analyzed.
The codon-usage optimized (for expression in *E. coli*) DNA sequence coding for three tandemly repeated copies the desired epitopes were then chemically synthesized (Eurofins MWG Operon) and inserted into the CpoI site of the EcTRX gene of the previously described pET28-EcTRX plasmid.
The resulting pET28-EcTRX-(trimeric epitope) plasmids were used to transform *E. coli* BL21C+ cells.
Recombinant protein expression was induced by adding 1mM isopropyl-β-Dthiogalactopyranoside
(IPTG) to the various trasformants, which were then cultured for 3 hours at 37°C. Following cell lysis, 6xHis-tagged polypeptides were bound to a metal-affinity resin (Talon, Clontech).
The purified protein antigen constructs were sent to an antibody production facility (Preclinics GmbH) for rabbit immunization and consequently antiserum extraction.
The TDMI approach has been used, over the course of this thesis, to produce various antibodies against "problematic" antigens that cannot usually be isolated from the natural source (or prepared by recombinant DNA technology) and/or does not guarantee the production of "good quality" antibodies (eg, elevated titles and high specificity).
Some of them are hereafter presented as an example of the potential and adaptability of the TDMI approach.

1.1. Anti-Dok1

Docking protein 1 (Dok1) is a human protein constitutively (tyrosine) phosphorylated in hematopoietic progenitors isolated from chronic myelogenous leukemia (CML) patients. It may be a critical substrate for p210, a chimeric protein whose expression is associated with CML.

An antisera against Dok-1 was produced in the perspective of a collaboration with Dr. Tommasino (IARC, Lyon) research group. My work consisted in the production of the anti-Dok1 sera by TDMI technique while the quality testing of the antibodies was performed by Dr. Sylla (IARC, Lyon).

In a first experiment, a detectable molecular flag was attached to the recombinant Dok1 protein.

The flagged Dok1 protein (F-Dok1) and a negative control flagged protein (F-NC) underwent immunoprecipitation (IP) with the anti-Dok1 antiserum and then were detected in a western blot experiment using an anti-FLAG Ab (Fig.8 A).

In the second experiment, Human Embryonic Kidney 293 cells (HEK-293) were transfected with GFP or GFP-Dok1 fusion protein. An immunofluorescence (IF) test was performed to detect GFP-Dok1, using GFP fluorescence (Green) or anti-Dok1 antiserum (Red) (Fig.8 B).

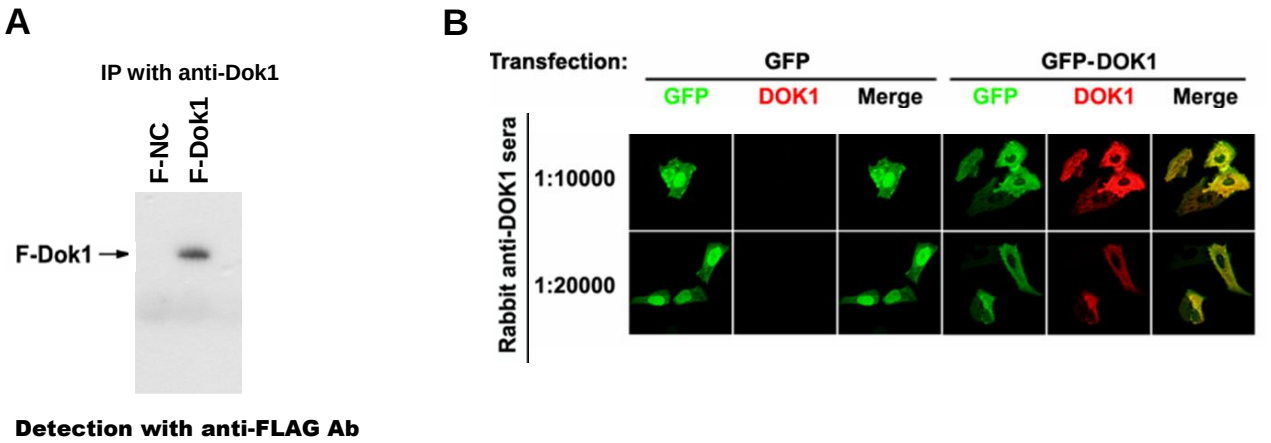

Figure 8 –Quality tests on anti-Dok1 antiserum
A – IP experiment
B – IF experiment

In figure 8 A, the western blot shows how the produced anti-Dok1 antisera is capable of selectively binding, and then precipitate, the target F-Dok1 but not the F-NC.

The specificity of the serum is also revealed in figure 8 B by the lack of signal on GFP alone and a very strong co-localization of green and the red signals to detect GFP-Dok1.

1.2. Anti-HPV16_E6/E7

Dr. Ferrari (Kurdistani Lab, UCLA) requested two antisera directed against HPV16 early proteins 6 (HPV16_E6) and 7 (HPV16_E7).

E6 and E7 are two of the eight early expressed proteins of *Papillomaviridae.* In cancerous human papilloma viruses sub-types (e.g., HPV16,HPV18,HPV38), E6 and E7 inactivate members of the pRb tumor suppressor proteins preventing apoptosis and promoting cell cycle progression, thus "instructing" the infected cell for replication of the viral DNA. E7 also seems to have a role in immortalization of HPV infected cells by triggering cellular telomerase hyperactivity.

I prepared (using TDMI and EcTRX) and purified the HPV16_E6 and HPV16_E7 antigen constructs.

The produced anti-HPV16_E6 and anti-HPV16_E7 antisera were tested, by Dr. Ferrari, in a western blot experiment against HPV16 E6 and E7 proteins (data not shown) and against HPV18 infected HeLa cells (Fig.9). The purpose of the second western blot was to see if the TDMI generated antisera showed cross-reactivity to a similar epitope (Fig. 10) without aspecific background noise.

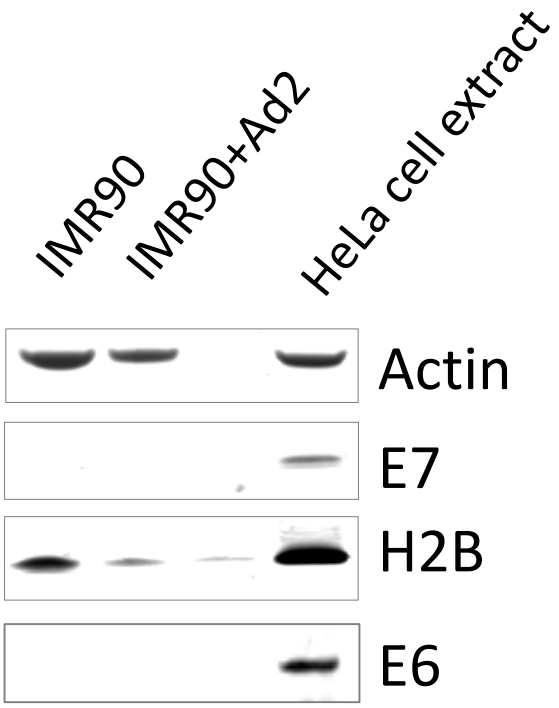

Figure 9 – Western blot against HPV18 infected HeLa cells, IMR90 cellsand Adenovirus infected IMR90 cells

Actin, E7, H2B and E6 indicates the antisera used as source of primary Abs

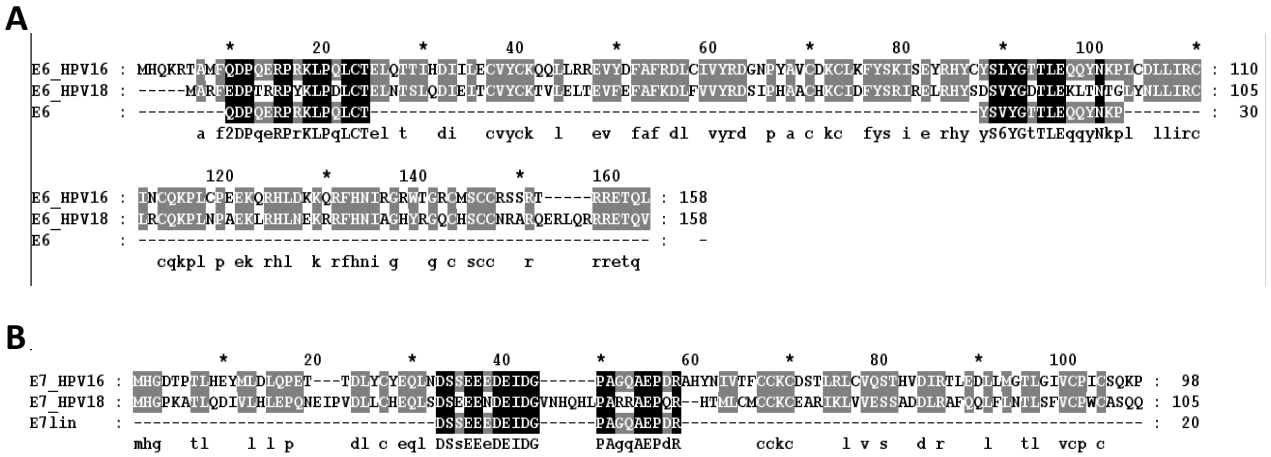

Figure 10 – Alignment between the HPV16_E6/E7 epitopes and their respective HPV16/18 full length proteins

As shown in figure 8, anti-HPV16_E6 and E7 specifically recognize HPV18 E6 and E7 in HeLa cells but not in HPV-non-infected human embryo fibroblasts (IMR90) and adenovirus infected IMR90 (IMR90+Ad2). Actin and histone protein 2b (H2B) are used as positive controls. Figure 10A is an alignment between the HPV16_E6 epitope (E6) and its respective HPV16/18 full length proteins. Figure 10B is the same alignment with the HPV16_E7 epitope (E7) and HPV16/18 full length E7. As shown, the epitopes are sufficiently conserved to generate antibody cross reactivity against HPV16 and HPV18.

1.3. Anti-EID1

EID1 protein (E1A-like inhibitor of differentiation 1) is an EP300 and Pcid2 interacting protein which is taught to be a stem cell pluripotency enhancer. Due to the lack of good quality commercial anti-EID1 antibodies, Dr. Ferrari (Kurdistani Lab, UCLA) requested an anti-EID1 Abs containing serum.

I prepared (using TDMI and EcTRX) and purified the EID1 antigen constructs.

Dr. Ferrari used the antisera for western blot (Fig. 11) and immunofluorescence experiments (Fig. 12).

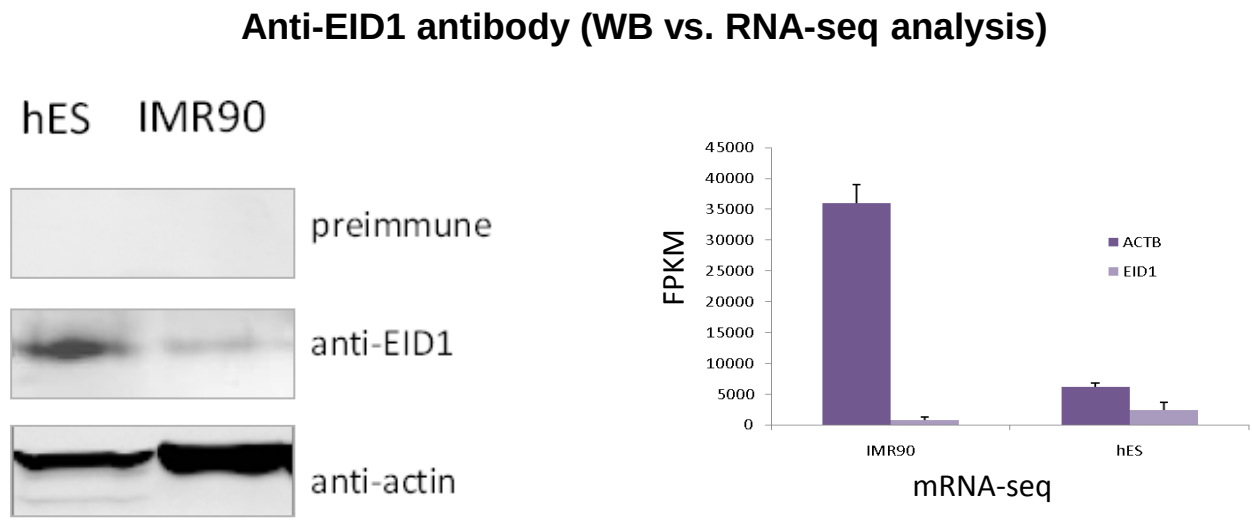

Figure 11 – EID1 western blot and RNA-seq analysis confrontation

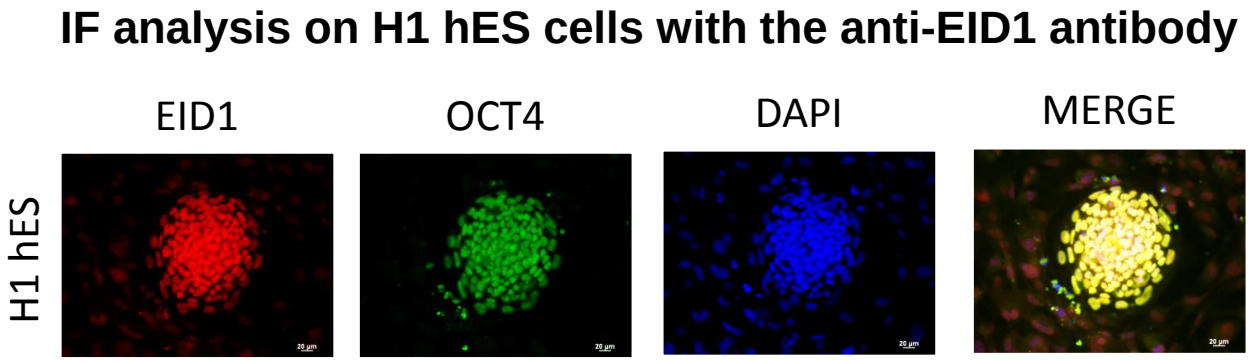

Figure 12 – EID1 IF assay

The western blot (Fig.11) shows how the anti-EID1 serum strongly recognize EID1 in human embryonic stem cells (hES) and not in IMR90 cells. RNA-seq data confirm the different level of expression of the EID1 protein confirming its role as a cell staminality marker. A commercial anti-actin Ab is used as positive control whereas the rabbit preimmune serum (derived from a blood sample of rabbit not yet immunized with the EcTRX-EID1 construct) acts as negative control. This experiments also show how a TDMI generated antibody serum can recognize even low levels of the target antigen as hES cells are not transfected or engineered to overexpress EID1.

Figure 12 is the representation of an IF experiment where the anti-EID1 Ab was confronted with an anti-OCT4 Ab and the fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) . Being OCT4 (octamer-binding transcription factor 4) critically involved in the self-renewal of undifferentiated embryonic stem cells it is often used as a marker for undifferentiated cells. DAPI was used to indifferently color hES cells and feeder cells which only serve as a substrate for the growth of hES cells. The EID1 signal is comparable to the OCT4

one, however a little aspecific recognition of feeder cells occurs even though not as elevated as with the DAPI coloration.

2. Improving antibody specificity

The major drawback of TDMI generated antisera is that, inevitably, antibodies against the TRX scaffold are as well generated.
The "aspecific" antibodies can give false detection results and, sometimes, an elevated background noise in molecular/diagnostic immune-techniques.
Even if the performance of the raw polyclonal TDMI produced antiserum is comparable to the vast majority of commercial polyclonal antibodies, the second part of the diagnostic antibody production project consisted in improving antibody specificity by minimizing the generation of anti-scaffold Abs.

2.1. Monoclonal Abs production

In order to generate better quality antibodies we used a TDMI generated antigenic peptide for an explorative experiment on monoclonal antibody production.
My work consisted in the production and purification of a TDMI based HPV38_E7 antigenic protein construct (EcTRX-HPV38_E7). Dr. Viarisio (DKFZ, Heidelberg) carried out mice immunization, hybridoma production and, ultimately, anti-HPV38_E7 mAb purification and testing.
Figure 13A shows a western blot experiment with a TDMI produced anti-HPV38_E7 rabbit polyclonal serum (produced prior to the start of the project presented in this thesis).
HEK293 cells were transfected in order to express the HPV38 E7 protein fused with a molecular flag (HPV38_E7-FLAG) and with a DNA sequence coding for an unrelated protein (NC) as negative control.
The experiment shows strong anti-HPV38 immune-reactivity but with an equally intense background noise.

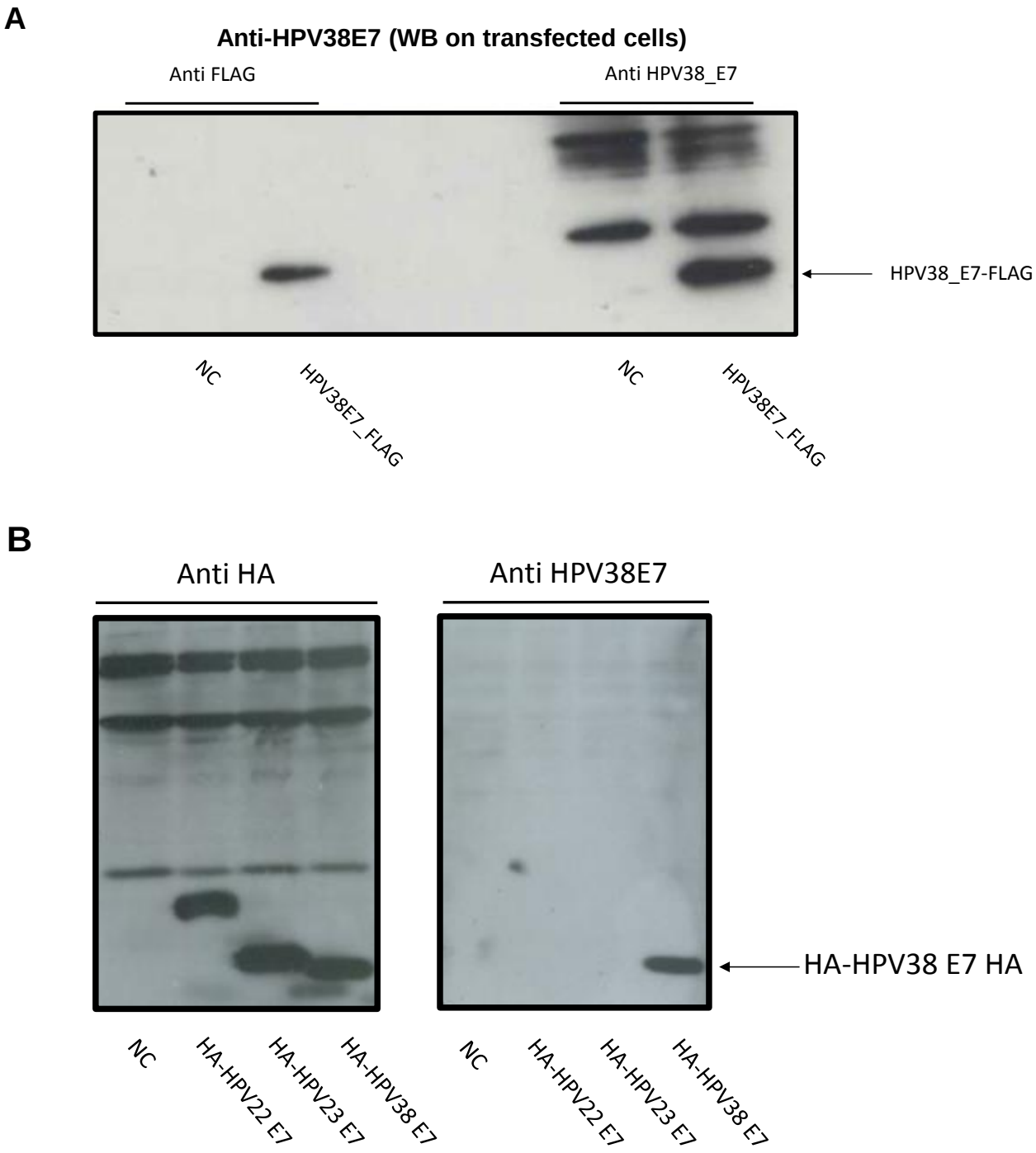

Figure 13 – TDMI generated polyclonal (A) and monoclonal (B) anti-HPV38_E7 Abs western blot experiments

Figure 13 shows the performance in western blot of the anti HPV38_E7 mAb.

HEK293 cells were transfected with the coding DNA of various E7 (from different HPV subtypes) and hemagglutinin (HA) fusion proteins. The negative control was the same as the one used in the previous western blot (Fig. 13A). Not only the mAb does not aspecifically binds other targets but it can also discriminate between unrelated HPV subtypes.

2.2. Homologous TRX based TDMI

In addition to conventional antibodies, *camelidae* possess an uncommon form of antibodies lacking the light chains. The variable domain of such a heavy-chain antibody (VHH) is fully capable of antigen binding even if it is not paired with the variable domain of the light chain like in all other antibodies.

Nanobodies (Ablynx) are small single-domain antibody fragments derived from camelid VHH through recombinant DNA technology. They retain the characteristics of the heavy-chain Abs but they feature small dimensions (110 aa) and the possibility to be produced in bacteria or in yeast.

A secondary antibody specifically targeting the VHH region could prove itself to be a very useful molecular tool and so it was requested by Preclincs GmBH company.

For the anti-VHH serum production we used TDMI exploiting not the EcTRX but the endogenous TRX of the rabbit (OcTRX), the chosen animal host for the immunization.

The general idea was that the rabbit immune system should detect the OcTRX as a self-protein thus not generating anti-scaffold antibodies but only VHH directed Abs.

Once obtained the serum was used in an ELISA test against the OcTRX scaffold and the protein construct used for immunization (OcTRX-VHH). Results are presented in figure 14.

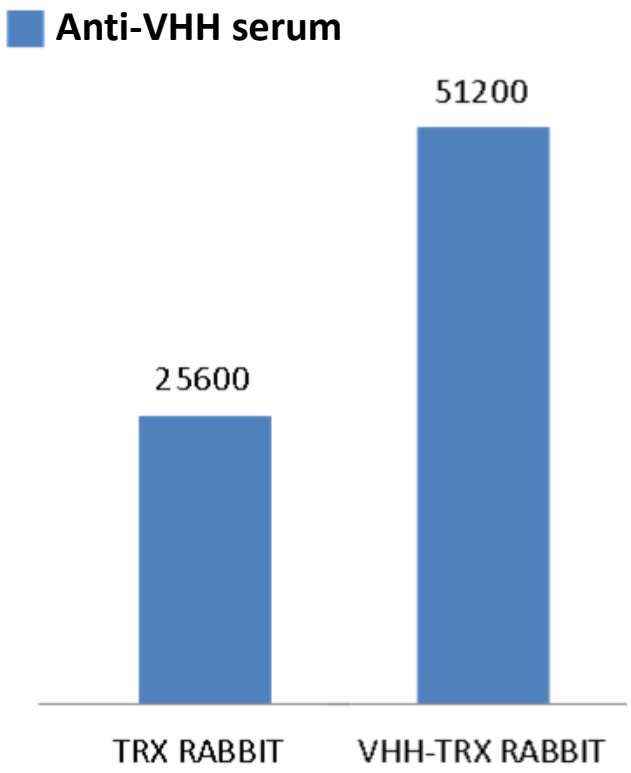

Figure 14 – ELISA test with the OcTRX-baseded anti-VHH serum
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

Although anti-VHH rabbit serum specifically recognized the antigen, the use of the homologous rabbit Trx scaffold does not prevent the formation of aspecific rabbit anti-OcTRX Abs, as half of the immunotiter (25600 of 51200) is directed against the rabbit thioredoxin.

2.3. Depletion of aspecific antibodies: the BLPtrap

Antibody purification consist of selective enrichment or precise isolation of Abs from serum (polyclonal antibodies), ascites fluid or cell culture supernatant of a hybridoma cell line (mAbs). Purification methods are various and comprise differential precipitation, size-exclusion chromatography and affinity purification.
Most antibodies are moderately resistant proteins that can tolerate purification conditions (e.g., low-pH, precipitation, gel filtration and dialysis) however it is not unusual the case in which antibody functionality was altered due to the purification procedure.
In order to maintain such functionality we designed and developed an alternate Ab purification procedure that targets aspecific antibodies leaving specific ones unharmed.
The technique, named “BLPtrap”, exploits supramolecular constructs obtained from the aggregation of the protein scaffold with the cellular walls of *Lattococcus lactis* (BLP-EcTrx).
By acid treatment of *L.lactis* cells protein and nucleic acids are removed, leaving an empty peptidoglycan shell. Multiple copies of a protein can be attached to the surface of these bacterial-like particles (BLPs) when expressed as fusion constructs with a peptidoglycan binding domain (PNG-BD): the LysM domain of the *L.lactis* autolysin (AcmA).
The production of EcTRX-LysM fusion protein allows us to prepare *L.lactis* BLPs displaying EcTRX scaffold.
The idea is that processing a (EcTRX) TDMI derived antiserum with EcTRX-covered BLPs the anti-scaffold Abs should bind the BLP based immuno-trap and be efficiently removed (Fig.15), leaving only specific anti-epitope Abs.

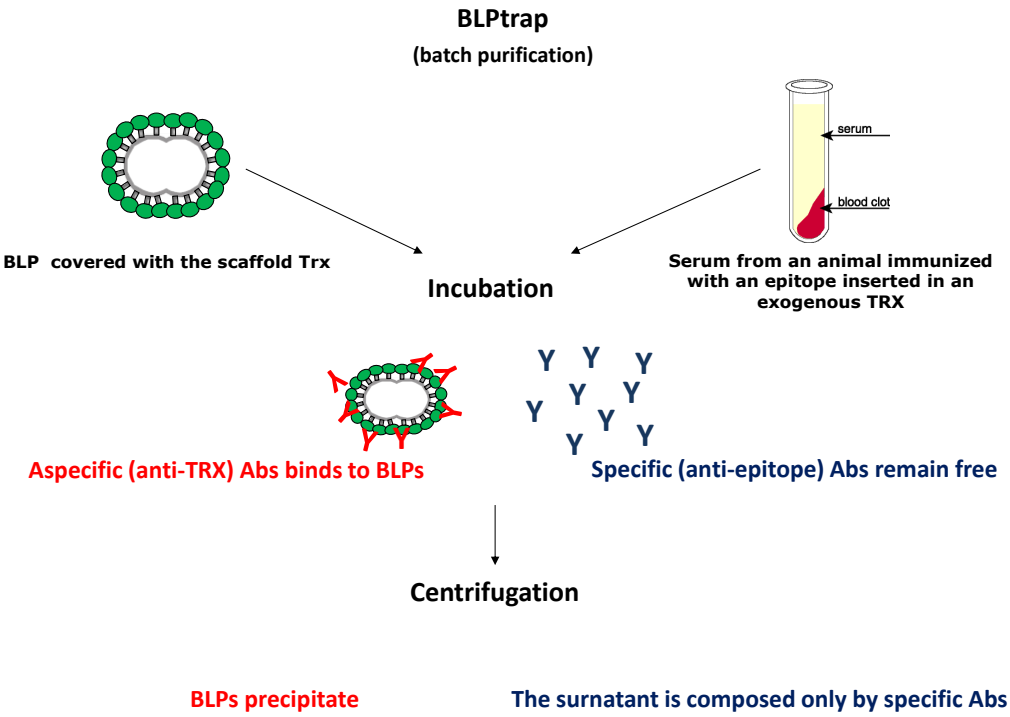

Figure 15 – Overview of the BLPtrap procedure

2.3.a BLP-EcTRX preparation

The EcTRX gene was extracted by digestion of the pET28-PfTRX plasmid with NdeI restriction enzyme (Takara) then cloned in the NdeI-digested pET28-LysM vector (already available in our research laboratory). Once obtained a confirmed clone containing and expressing the pET28-EcTRX-LysM construct, it was growth in 1l of LB medium and the expression of the protein was induced with IPTG.

The insoluble EcTRX-LysM was extracted and purified from inclusion bodies with urea and guanidine-HCl.

L. lactis (IL1403 strain) was growth o/n in 1l of M17 medium. Cells were pelleted and then treated with HCl to remove proteins and DNA. The EcTRX-LysM fusion protein was mixed to the treated BLPs in order to let the LysM domain bind the PNG.

2.3.b Serum treatment

The previously described anti-EID1 serum was used to test the efficacy of the BLPtrap approach. Anti-EID1 serum was mixed respectively with “empty” BLPs (50% of final volume) and with BLP-EcTRX (50% and 67% of final volume), then incubated under agitation for 1h at RT.

After precipitation by centrifugation of the BLPs the supernatant containing the still unbound Abs was collected.

The treated sera were used in an ELISA test against the epitope used for the immunization (EcTrx-EID1) and the scaffold protein (EcTRX)

Results are summarized in figure 16.

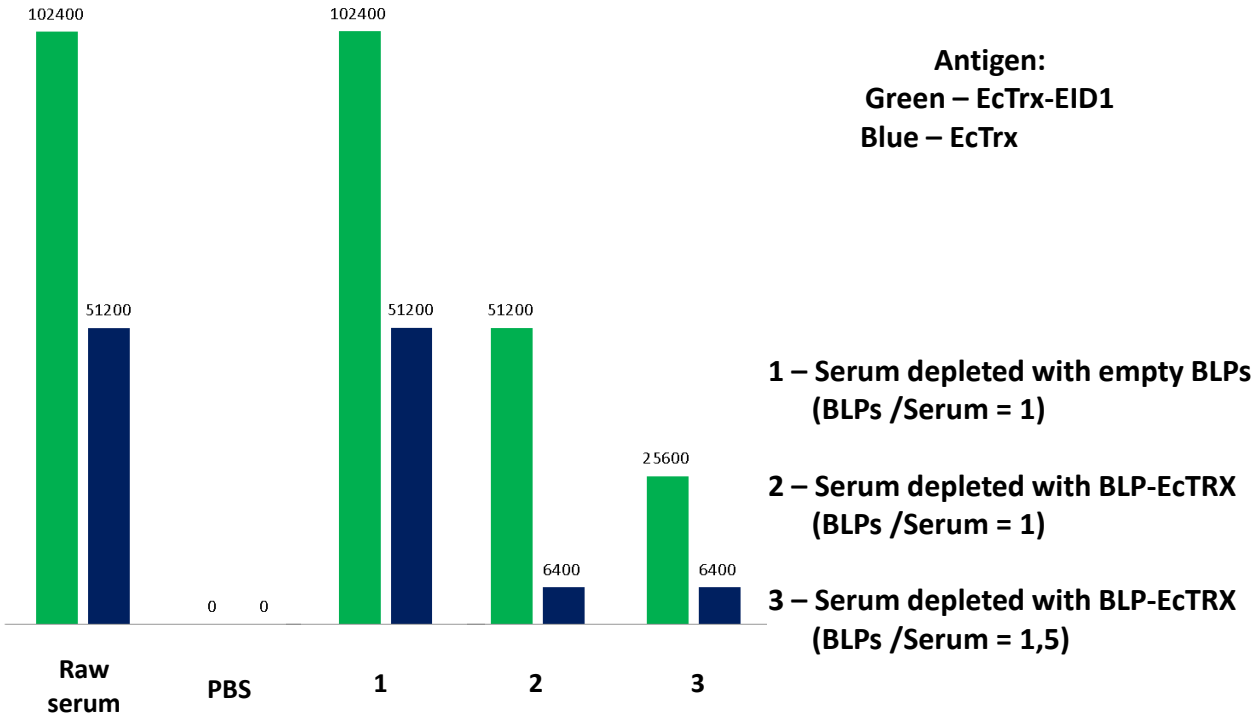

Figure 16 – ELISA testing of the BLPtrap treated anti-EID1 serum
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

The analysis of the antibody titer (Fig. 16) shows that in the raw (untreated) serum half of the signal (51200 of 102400) derives from Abs directed versus the scaffold. The BLPtrap treatment reduce the titer of specific anti-EID1 Abs but, on the other hand, substantially reduce the titer of aspecific scaffold-directed Abs. A BLPs-serum ratio of 1 seems to be optimal as increasing the quantity of BLPs not only does not affect the response against the scaffold but moreover reduce the anti-EID1 Ab titer. The serum treated with empty BLPs shows no difference with the untreated “raw” serum indicating that the depletion of the anti-scaffold Abs is not a BLP-dependent adsorption effect but, rather, a specific binding to the EcTRX protein. PBS was used as a negative control.

DEVELOPMENT OF A RECOMBINANT VACCINE AGAINST ALZHEIMER'S DISEASE

1. Evaluation of the efficacy of different antigen formulations

The first phase of the project consisted in the evaluation of different antigens formulation.
The chosen antigens were two different fragments of the amyloid Aβ42 peptide: the first 15 amino acids of the peptide repeated four times (Aβ(1-15)4) and the first 7 amino acids repeated 9 times (Aβ(1-7)9). Aβ(1-15)4 derives from an already published work (Moretto et al., 2007) while Aβ(1-7)9 represent the attempt to minimize the Aβ42 antigen in order to avoid possible collateral effects due to the amyloid peptide intrinsic toxicity.
For both the antigens the scaffold was the *E. coli* thioredoxin (EcTRX)
Other than the epitopes, the evaluation regarded the effects of different immune adjuvants.
Two different human approved commercial adjuvants were chosen for this evaluation study: Montanide ISA720 (water-oil emulsion, Seppic) and alum (aluminium hydroxide).
A third adjuvant was *Salmonella thyphi* flagellin protein (Fli) used as a molecular TLR stimulant.
Fli was employed both as a fusion construct with EcTRX (Fli-EcTRX) and as protein bound together with EcTRX to *Lactococcus lactis* BLPs (BLP-EcTRX-Fli).
The selection of these adjuvants was made prior to the start of this study analyzing previous experiments (unpublished, not shown data).
Prior to the start of the projects 8 different formulations were prepared using the TDMI (Thioredoxin-displayed multipeptide immunogens) technique:

1. EcTRX-Aβ(1-7)9 + alum
2. EcTRX-Aβ(1-7)9 + Montanide ISA 720
3. EcTRX-Aβ(1-15)4 + Montanide ISA 720
4. Fli-EcTRX-Aβ(1-7)9 no adjuvant
5. Fli-EcTRX-Aβ(1-7)9 + Montanide ISA 720
6. BLP-EcTRX-Aβ(1-7)9 no adjuvant
7. BLP-EcTRX-Aβ(1-7)9 + Montanide ISA 720
8. BLP-EcTRX-Aβ(1-7)9-Fli

These 8 formulations were sub-cutaneously administrated to 60 BALB/c mice. The vaccination consisted in a first injection (priming) and three further immunizations (boost) at intervals of 15 days over a total time span of 3 months. At each injection 2 μg of antigen (EcTRX-Aβ) were administered.

BLPs based formulation gave origin to cutaneous wheals on the injection site of the mouse so, as precaution, the dose of the vaccine was halved.

After the sacrifice of the animals, serum was extracted from their blood and it was used for the ELISA analysis of the anti-Aβ antibody titer (Fig.17).

The titer is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values.

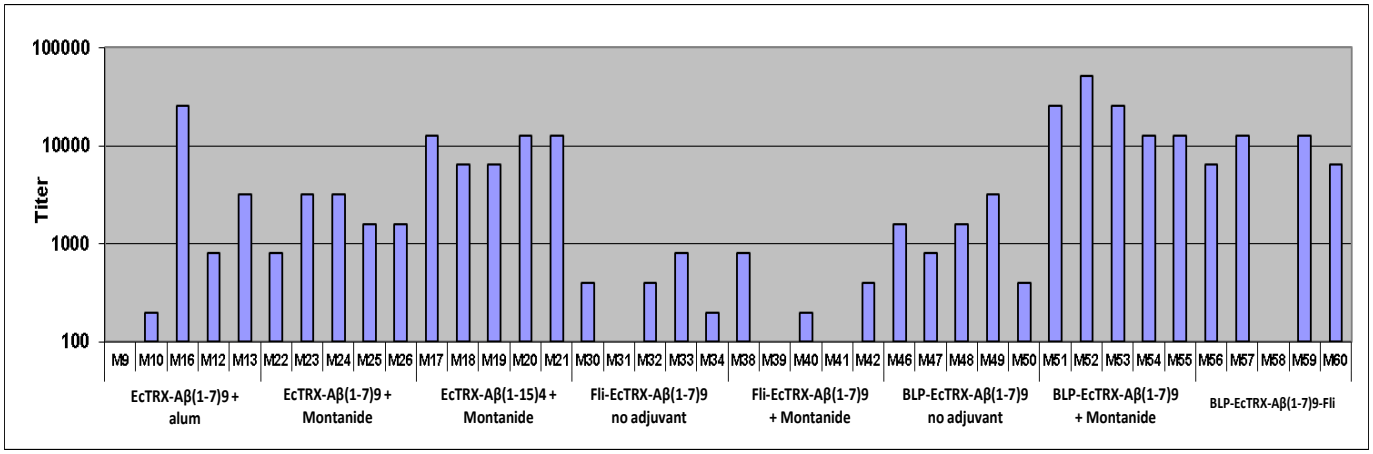

Figure 17 – ELISA analysis of the anti-Aβ antibody titer
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

Some consideration are made, after the analysis of the ELISA test results:

- Montanide seems to have superior and more constant immune-adjuvant properties than alum
- The Aβ(1-15)4 epitope has a 5-10 fold immunogenicity than the miniaturized Aβ(1-7)9 epitope
- The anchoring of EcTRX-Aβ(1-7)9 to BLPs produce a strong immune response albeit higher when administered with an adjuvant. This is most certainly due to the immune stimulatory effect of the peptidoglycan.
- Aβ(1-7)9 BLPs immunogenicity is comparable to the Aβ(1-15)4 reference, when supplemented with Montanide or when the epitope is co-anchored with Fli to the PNG shell.
- The Fli-EcTrx-Abeta(1-7)9, with or without adjuvant, does not seem to induce an effective immune response as documented by the low antibody titers. A possible explanation of this phenomenon is that when directly fused to the EcTRX scaffold, the flagellin “distract” the immune system being bigger and more antigenic than the scaffold containing the epitope.

From this first explorative phase we chose the formulations which gave the better antibody titer (4 or more animals showing a high antibody titer) for further testing. The chose formulations were:

- EcTRX-Aβ(1-7)9 + Montanide ISA 720
- EcTRX-Aβ(1-15)4 + Montanide ISA 720
- BLP-EcTRX-Aβ(1-7)9 + Montanide ISA 720
- BLP-EcTRX-Aβ(1-7)9-Fli

The sera derived from these formulation underwent an antibody isotyping ELISA test (Rapid ELISA Mouse mAb Isotyping Kit, Pierce) (Fig.18).

The result of this test clearly shows how the immune response generated by the soluble formulations (EcTRX-Aβ(1-7)9 / EcTRX-Aβ(1-15)4 + Montanide ISO 720) qualitatively differs from the one arose after the vaccination with the particulate BLP-based preparations.

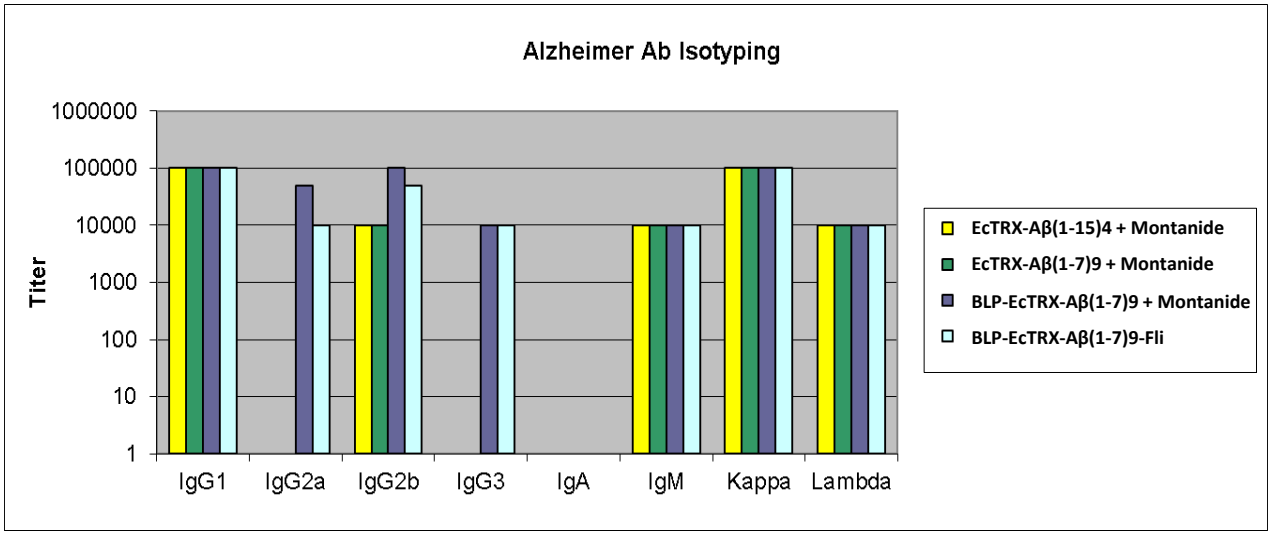

Figure 18 – Anti Aβ42 Ab isotyping ELISA analysis
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

The PNG-free soluble formulations origin a Th1 response (IgG1≈IgG2b>>IgG2a) oriented towards to an all-around humoral immunity. BLPs, on the other hand, give rise to a pro-inflammatory Th2 response (IgG1≈IgG2b>>IgG2a) due to the abundance of PNG in these formulation. This strong inflammatory response explains the wheals on the injected mice and the high antibody titer. Even if a pro-inflammatory response is not preferred in treating a delicate disease such as Alzheimer's, various studies on an Aβ42 vaccine showed a strong correlation between the efficacy of the vaccine and the production of IgG2a e IgG3, the classes intensely induced by the BLP formulations.

1.1. Evaluation of *Pyrococcus furiosus* thioredoxin as a scaffold

Parallel studies conducted in our research group (data in course of publication) demonstrated that *Pyrococcus furiosus* Trx (PfTRX) is better as a molecular scaffold for vaccination than the *E. coli* Trx. Valuable features of PfTRX are a higher thermal stability and protease resistance, a stronger solubilization capacity, and the complete lack of cross-reactivity of anti-PfTRX antibodies with other thioredoxins, including human, mouse and *E. coli* Trx.

2. Production of the new formulations

Considering the result of the preliminary vaccination and the tests on PfTRX, we decided to produce new formulations for the vaccine prototype to be used on Tg2576 transgenic mice (overexpressing the Swedish variant, APPSwe, of the human APP protein), one of the most used murin model of AD.
Two formulations, PfTrx-Aβ(1-7)9 + Montanide and BLP-PfTrx-Aβ(1-7)9-Fli plus their respective placebos PfTrx + Montanide and BLP-PfTrx-Fli (constructs containing the PfTrx without epitopes) were designed and produced, for a total of four new preparations. The scaffold was no longer EcTRX but PfTRX due to its conformational constrain features. Aβ(1-7)9 was chosen over Aβ(1-15)4 in order to avoid as much as possible amyloid toxicity and in light of the fairly comparable results in the ELISA test .

2.1. Production of PfTRX (placebo)

For the preparation of PfTRX we used a *E. coli* BL21C+ strain containing the pET28-PfTRX vector already available in our research laboratory prior to the start of this project.
Bacteria were grown in 1l of LB medium and the expression of the histidine tagged PfTRX was induced with IPTG. The protein was purified by cobalt affinity chromatography.
The purified PfTRX in the eluted fraction was verified by Bradford assay and SDS page (Fig.19).

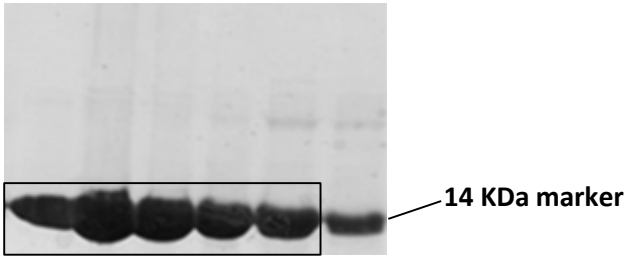

Figure 19 – SDS page verification of PfTRX eluted fractions

PfTRX containing fractions were unified then concentrated and dialyzed in PBS pH7.4 by ultrafiltration.
The protein has been detoxified with TritonX-114 then sterilized by filtration.
After quantification aliquots were made (at 20 µg/ml) and stored at -80°C.

2.2. Production of PfTrx-Aβ(1-7)9

The PfTrx-Aβ(1-7)9 antigen consist in the first 7 amino acids of Aβ42 repeated 9 times and inserted by TDMI in the PfTRX.
The production started from the pET28-EcTRX-Aβ(1-7)9 vector, already available and used in the first phase of the project for the expression of the EcTRX-Aβ(1-7)9 epitope construct.
The vector was extracted by plasmidic midi-preparation (QIAGEN Plasmid Midi Kit) then digested with CpoI (Takara) restriction enzyme. The CpoI-flanked Aβ(1-7)9 DNA digestion fragment was cloned in the CpoI digested pET28-PfTRX vector. The resulting pET28-PfTRX-Aβ(1-7)9 plasmid was used to transform *E. coli* BL21C+ cells. Correct clones were identified by bacterial colony PCR using T7 universal primers(Fig.20).

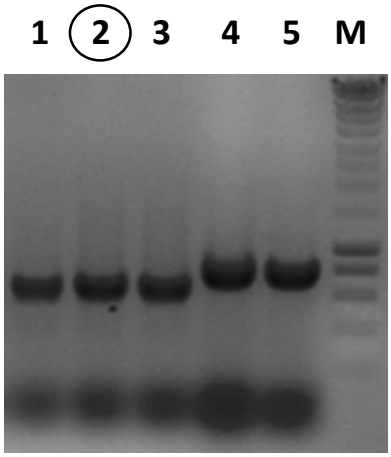

Figure 20 – Colony PCR on pET28-PfTRX-Aβ(1-7)9 transformed clones
1-5: clones
M: marker

Clone 2 circled, is a positive clone

Positive transformants were tested for epitope expression with IPTG induction and subsequently SDS PAGE analysis (Fig.21)

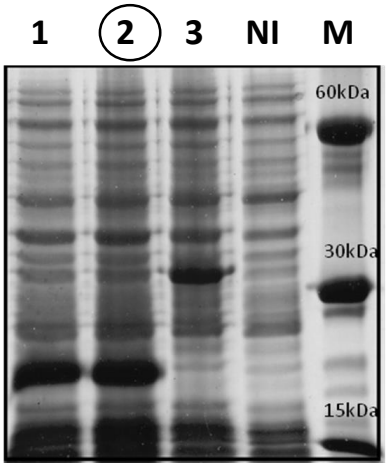

Figure 21 – SDS PAGE on induced pET28-PfTRX-Aβ(1-7)9 transformed clones
1-3: clones
NI: non induced clone 1
M: marker

Clone 2 circled, is a positive clone

From PfTRX- Aβ(1-7)9 inducing clones plasmidic DNA was extracted (mini-prep) and sequenced.
Once obtained a confirmed clone, containing and expressing the PfTRX-Aβ(1-7)9 construct, production and purification of the antigen was carried out like PfTRX (Fig.22).

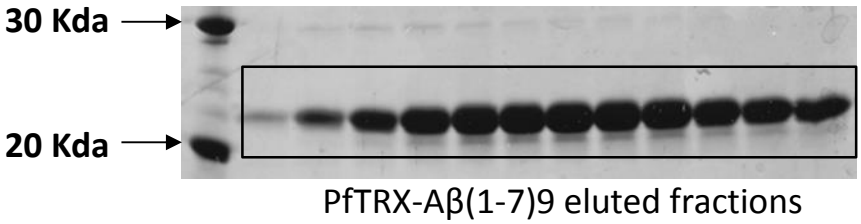

Figure 22 – SDS page verification of PfTRX-Aβ(1-7)9 eluted fractions

2.2. Production of BLP-PfTRX-Fli

In order to correctly prepare BLP formulation 3 molecular "characters" are needed:

1. The PNG empty shell
2. The scaffold-epitope construct fused to a PNG binding domain (LysM)
3. The flagellin (molecular adjuvant) fused to a PNG binding domain (LysM)

2.2.a BLPs preparation

L. lactis (IL1403 strain) was growth o/n in 1l of M17 medium. Cells were pelleted and then treated with HCl to remove proteins and DNA. BLPs were washed several times with sterile and LPS-free PBS buffer.

2.2.b PfTRX-LysM preparation

PfTRX gene was extracted by digestion of the pET28-PfTRX plasmid with NdeI restriction enzyme (Takara) then cloned in the NdeI-digested pET28-LysM vector (already available in our research laboratory).
The resulting pET28-PfTRX-LysM plasmid was used to transform *E. coli* BL21C+ cells. Afterwards correct clones were identified by bacterial colony PCR and IPTG induction test as described before.
Positive clones were verified by plasmid sequencing.
Once obtained a confirmed clone, containing and expressing the pET28-PfTRX-LysM construct, it was growth in 1l of LB medium and the expression of the protein was induced with IPTG.
The insoluble PfTRX-LysM was extracted and purified from inclusion bodies with urea and guanidine-HCl then verified and quantified by SDS-PAGE.
The construct was conserved at -80°C.

2.2.c Fli-LysM preparation

An expressing Fli-LysM bacterial clone was already available in our research lab at the beginning of this project.
Expression and purification of the Fli-LysM fusion protein was carried out like PfTRX-LysM.

2.2.d Assembly of the BLP-PfTRX-Fli formulation

BLPs, the Fli-LysM construct and the PfTRX-LysM construct were assembled together in a sterile environment. For each milliliter of BLPs, 500 µg of Fli-LysM and 250 µg PfTRX-LysM were used. The BLPs with the attached epitopes were pelleted and detoxified by TritonX-114. Verification and quantification of the antigen were done by SDS-PAGE (Fig.23).

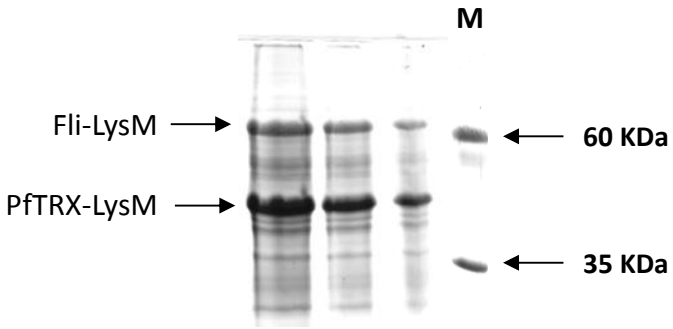

Figure 23 – SDS page verification of the BLP-PfTRX-Fli formulation
M: marker
The first three lanes (from the left) are different quantities of the BLP-PfTRX-Fli formulation

2.3. Production of BLP-PfTRX-Aβ(1-7)9-Fli

2.3a PfTRX-Aβ(1-7)9-LysM preparation

PfTRX-Aβ(1-7)9 DNA sequence was extracted by digestion of the pET28-PfTRX-Aβ(1-7)9 plasmid with NdeI restriction enzyme (Takara) then cloned in the NdeI-digested pET28-LysM vector.
The resulting pET28-PfTRX-LysM plasmid was used to transform *E. coli* BL21C+ cells afterwards correct clones were identified by bacterial colony PCR and IPTG induction test as described before.
Positive clones were verified by plasmid sequencing.
Once obtained a confirmed clone, containing and expressing the pET28- PfTRX-Aβ(1-7)9-LysM construct, it was growth in 1l of LB medium and the expression of the protein was induced with IPTG.
The insoluble PfTRX-Aβ(1-7)9-LysM was extracted and purified from inclusion bodies with urea and guanidine-HCl then verified and quantified by SDS-PAGE.
The protein construct was conserved at -80°C.

2.2.d Assembly of the BLP-PfTRX-Aβ(1-7)9-Fli formulation

BLPs, the Fli-LysM construct and the PfTRX-Aβ(1-7)9-LysM construct were assembled together in a sterile environment. For each milliliter of BLPs, 500 µg of Fli-LysM and 250 µg PfTRX-Aβ(1-7)9-LysM were used. The BLPs with the attached epitopes were pelleted and detoxified by TritonX-114. Verification and quantification of the antigen were done by SDS-PAGE (Fig.24).

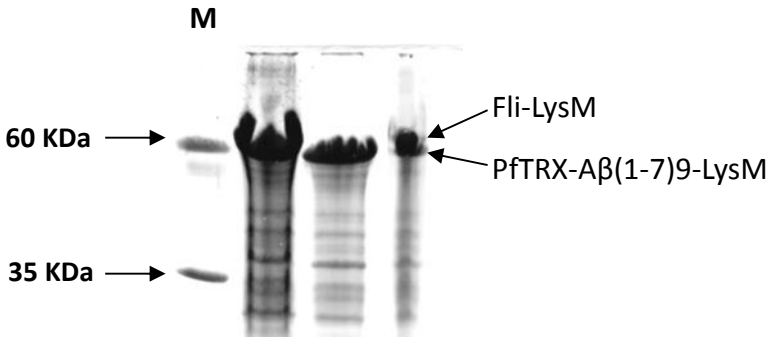

Figure 24 – SDS page verification of the BLP-PfTRX-Aβ(1-7)9-Fli formulation
M: marker
The first three lanes (from the left) are different quantities of the BLP-PfTRX-Aβ(1-7)9-Fli formulation

3. Immunization protocol

The two antigenic formulations and their respective were used in a 14 month study of 60 Tg2576 transgenic mice, overexpressing the Swedish variant (APPSwe) of the human APP protein. Due to this mutated (K670N/M671L) “hyper-aggregating” form of APP, these model mice develop Alzheimer-like symptoms from the 9th month of life.
The pathological phenotype consist in an augmentation of β-amyloid peptide levels and the development of amyloid plaques. A the 14-15 month the pathology reach its peak with mnemonic/behavioral deficits.
24 BALB/c mice were employed as "normal" controls to evaluate a potential toxicity of the scaffolds.

The immunization protocol consisted in the vaccination of 6 groups of mice:

Group 1
15 Tg2576 mice immunized each with 2 µg of PfTRX (100 µl) mixed with Montanide ISA 720 (50/50 mix)

Group 2
15 Tg2576 mice immunized each with 2 µg of PfTRX-Aβ(1-7)9 (100 µl) mixed with Montanide ISA 720 (50/50 mix)

Group 3
15 Tg2576 mice immunized each with 4 µg of BLP-PfTRX-Fli (2 µg of PfTRX) particulate formulation (100 µl)

Group 4
15 Tg2576 mice immunized each with 4 µg of BLP-PfTRX-Aβ(1-7)9-Fli (2 µg of PfTRX- Aβ(1-7)9) particulate formulation (100 µl)

Group 5
12 BALB/c mice immunized each with 2 µg of PfTRX (100 µl) mixed with Montanide ISA 720 (50/50 mix)

Group 6
12 BALB/c mice immunized each with 4 µg of BLP-PfTRX-Aβ(1-7)9-Fli (2 µg of PfTRX- Aβ(1-7)9) particulate formulation (100 µl)

All of these formulations were sub-cutaneously administrated. The vaccination consisted in a first injection (priming) and two further immunizations (boost) at intervals of 15 days. After the first three injections, nine other boost immunization were given once per month.
BLPs based formulation did not origin cutaneous wheals on the injection site.

Animal handling and keeping was performed by Professor Calzà's scientific equipe (Dipartimento di Scienze Mediche Veterinarie, University of Bologna)

3.1. Intermediate test

On the 5th month of treatment a sample of caudal blood from 12 of the 60 Tg2576 mice was taken (3 mice per group).

An indirect ELISA test on the sera extracted from the blood samples was done using: Aβ42 as the antigen fixed on the plate wells, the sera as the source of primary Abs and a HRP conjugated anti-mouse IgG as secondary/detection Ab. In figure 25 are represented the anti-Aβ42 Ab titer of the selected sera. The titer is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean value of the negative controls added to 4x the standard deviation of the same values.

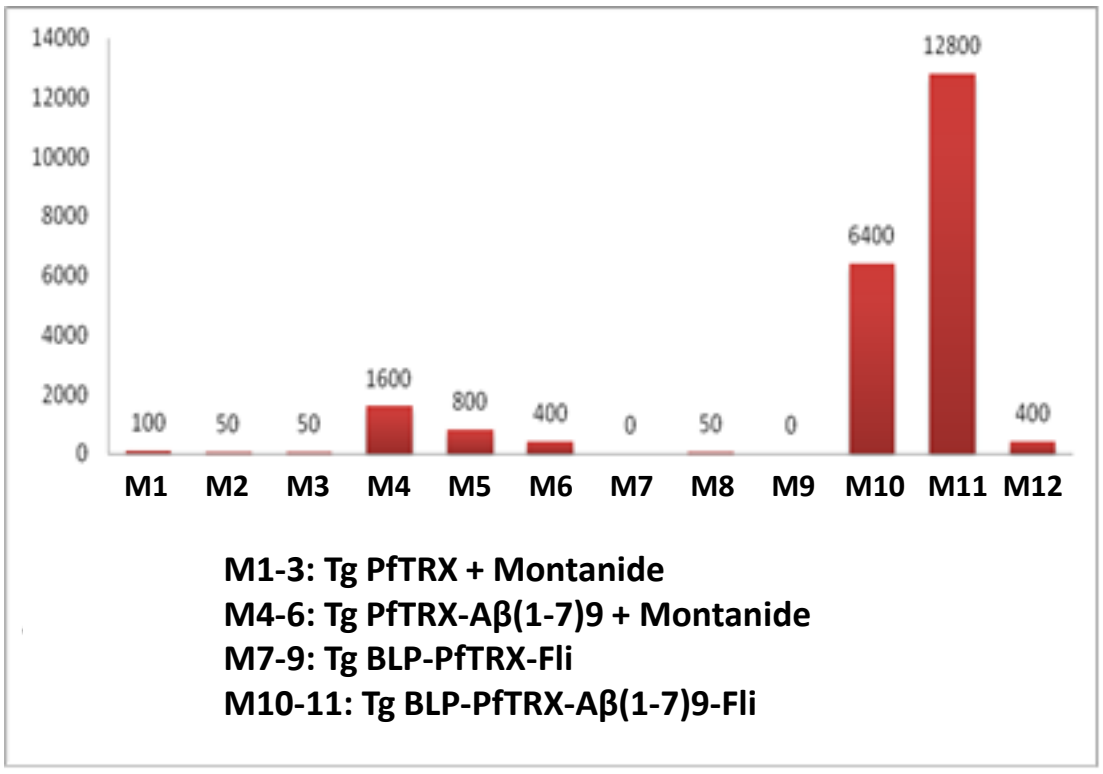

Figure 25 – ELISA analysis of the anti-Aβ antibody titer
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

The analysis of anti-Aβ42 antibody titer (Fig.25) revealed IgG levels far below the expected and we therefore decided to increase by about 10 times the dose of the formulation Trx PfTrx-Aβ(1-7)9 + Montanide and by 3 times the dose of the formulation BLP-PfTrx-Fli-Aβ(1-7)9.
The BLP formulations were modestly increased to avoid formation of wheals when injected.

4. Final tests

At the end of 14 months treatment were carried out two different behavioral tests (Y-Maze and Contextual Fear Conditioning) and another indirect ELISA test on the sera extracted from the blood samples (after the sacrifice of the animals).

4.1. Behavioral test: Y Maze

Y Maze is a behavioral test for measuring the willingness of mice to explore new environments. Testing occurs in a Y-shaped maze with three arms at a 120° angle from each other. After introduction to the center of the maze, the mouse is allowed to freely explore the three arms. Mice typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited.
Over the course of multiple arm entrances, the animal subject should display a tendency to enter a less recently visited arm. The number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation.
This test is commonly used to quantify cognitive deficits.
The main considered parameter was the percentage of alternating entries (Fig.26).

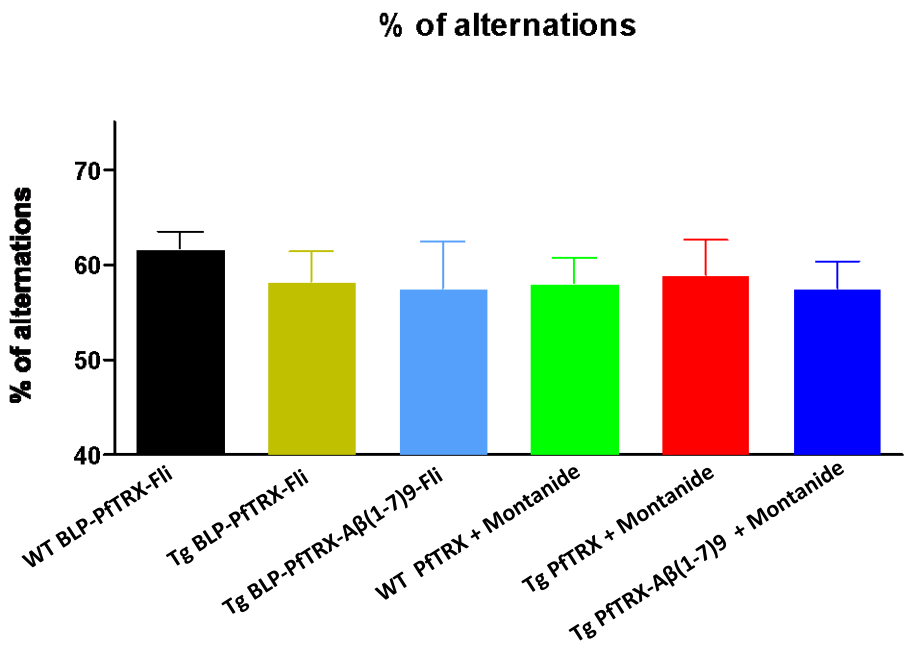

Figure 26 – Schematization of Y-maze test Bars represent the mean of the analyzed mice immunization group

Other parameters (total arm entries and distance), were considered less relevant and in any case did not gave innovative informations (Fig. 27).

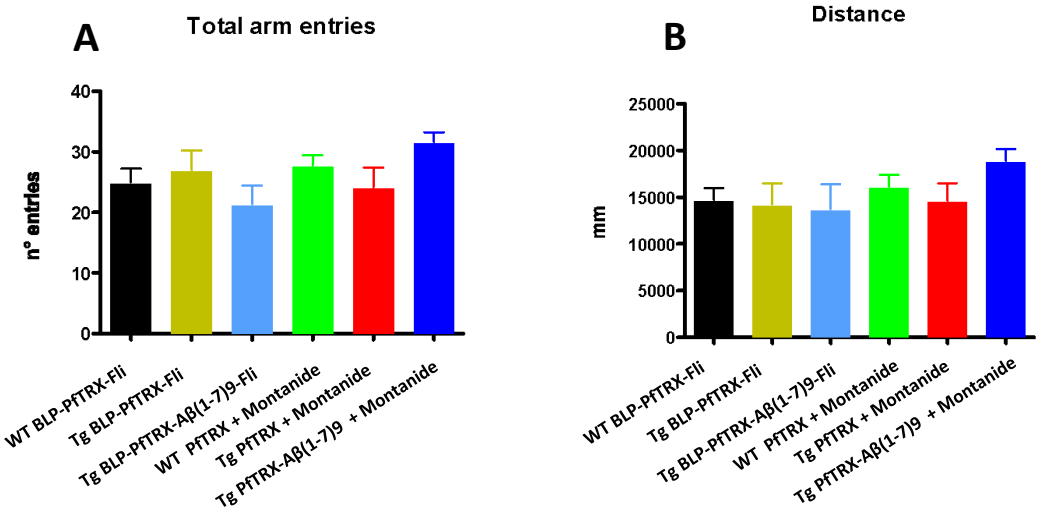

Figure 27 – Schematization of Y-maze test result (parameters not considered)
Bars represent the mean of the analyzed mice immunization group
A: number of arm entries
B: total distance run

The test shows how placebo treated WT mice behavior effectively differs from placebo treated Tg mice.

None of the vaccine formulations seem to have had a positive effect on the treated mice. The “odd-data” in the Y-maze is the PfTRX+Montanide treated Tg mice showing a “healthier” behavior than WT mice treated with the same placebo.

This is very likely an artifact of the Y-maze test but could suggest a possible not conformity of the employed animals.

4.2. Behavioral test: Contextual Fear Conditioning

Contextual fear conditioning (CFC) is the one of the most basic conditioning procedures. It involves taking an animal and placing it in a unique environment, providing a painful stimulus, and then removing it. When the animal is returned to the same environment, it commonly demonstrates a “fear response” if it remembers and associates that environment with the painful stimulus. Freezing is the mice response to fear, which has been defined as “total absence of movement (except for respiration).”

The basic idea is that an healthy mouse should remember and correlate the environment with the painful stimulus showing the freezing response. A mouse with cognitive deficit should, on the other hand, not freeze as it is not capable of making the environment-pain association.

Results of the test are represented in figure 28.

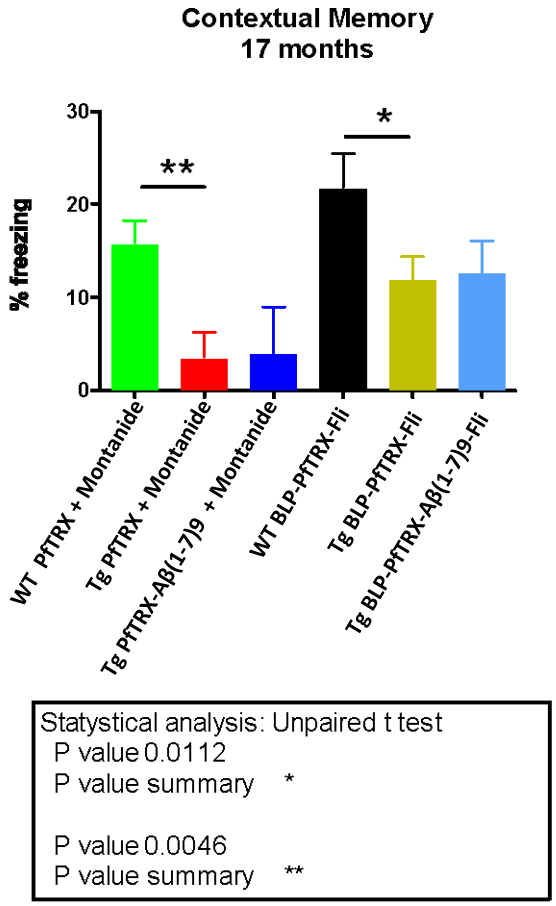

Figure 28 – Schematization of CFC test result
Bars represent the mean of the analyzed mice immunization group

As Y-maze, CFC showed the non-effectiveness of the vaccine in preventing mnemonic/behavioural deficits in treated mice. CFC results are clearer than Y-maze ones due to the overall better accuracy of the former test. Without any doubt there is statistical difference in the response of placebo treated WT mice from the Tgs. Both the vaccines graphically seems to have had a minor effect albeit not statistically relevant and much inferior to the values of WT mice.

4.2. ELISA test

Another indirect ELISA test, identical to the intermediate one, was done on the sera extracted from the blood samples of all immunized mice.

Aβ42 was used as the antigen fixed on the plat wells, the sera as the source of primary Abs and a HRP conjugated anti-mouse IgG as secondary/detection Ab.

Results (Fig.29) are again shown as anti- Aβ42 Ab titers of the various extracted antisera.

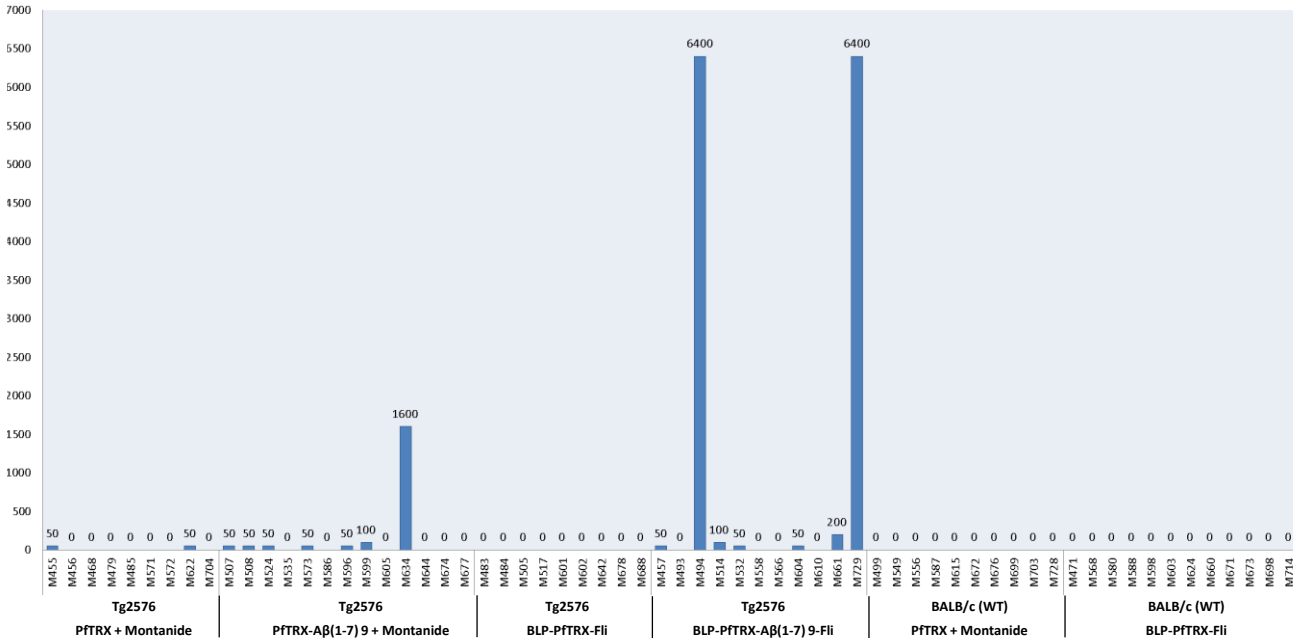

Figure 29 – ELISA analysis of the anti-Aβ antibody titer
Values indicate Ab immunotiter.
Immunotiter is intended as the reciprocal of the highest dilution showing an absorbance (λ=405nm) higher to the mean of the negative controls added to 4x the standard deviation of the same values

Anti- Aβ42 IgGs levels are extremely low and way inferior to the expected ones. This ELISA experiment gives "molecular confirm" the Y-maze and CFC tests results. The behavioral CFC data of the only respondent mice (M634, M494, M729) was singularly re-analyzed (Fig.30)

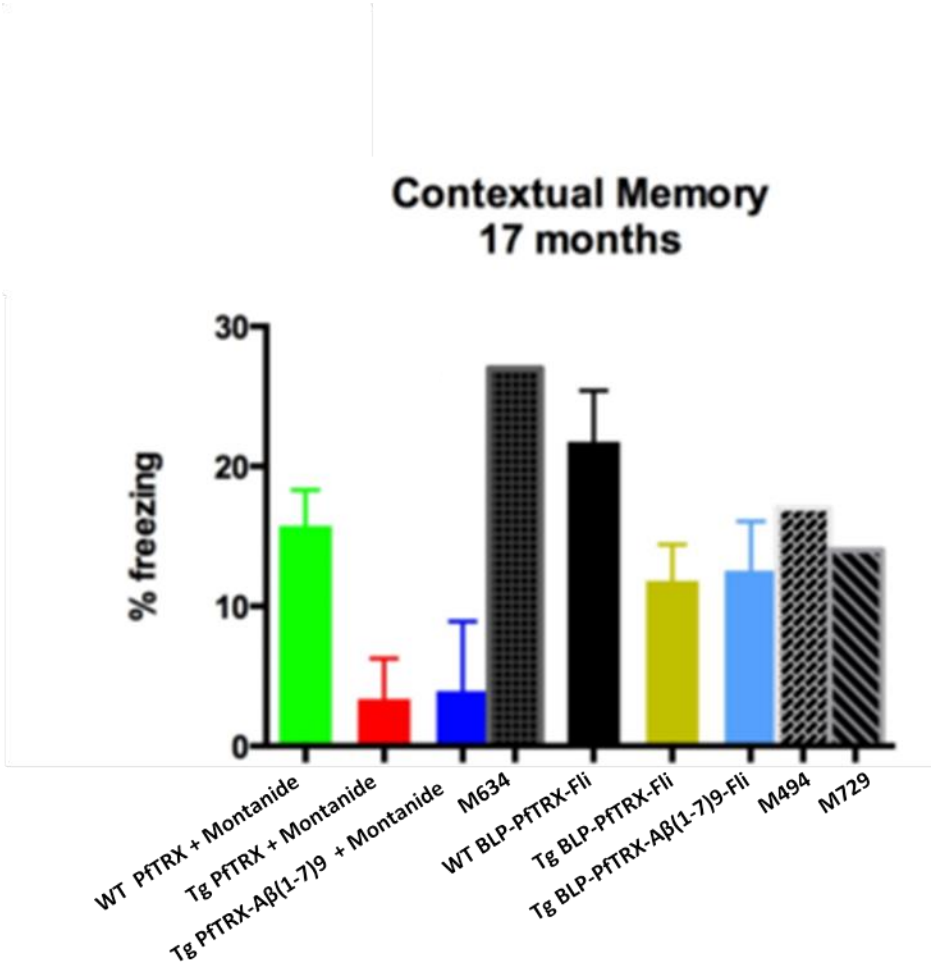

Figure 30 – Schematization of CFC test result for M634, M494 and M729
Except for M634, M494 and M729, bars represent the mean of the analyzed mice immunization group

M494 and M729 showed a very little increase over the mean of their group (Group 4 - BLP-PfTRX-Aβ(1-7)9-Fli) while M634 showed a behavior better than the mean of WT values. These data, however, have no statistic reliability and does not change the overall "failure" of the vaccine prototype.

There are some possible explanations:

- the epitope length could be too small to stimulate an adequate immuno-response;
- the same antigen may have been administered at an insufficient dose;
- the onset of a tolerance against the amyloid peptide due to the nature of the Tg2576 mice;
- the possible intervention of disease chronicity factors in some way related to the extremely long duration of the study (one of the longest ever conducted so far on this mouse model).

CONCLUSIONS AND FUTURE PERSPECTIVES

RECOMBINANT ANTIGENIC PEPTIDES FOR DIAGNOSTIC ANTIBODY PRODUCTION

Through the presented experiments we have proven the flexibility and the efficacy of antibody production by the TDMI approach: "good quality" polyclonal Abs are generated against a specific antigen and can be used in routine laboratory applications (immunoprecipitation, immunofluorescence, western blot, ELISA).

1. Optimizing the TDMI approach

Even if the immunoglobulins generated by TDMI approach are abundant (high titer) and normally show high specificity against their molecular target, some issues remains and the whole methodology could benefit an upgrading/optimization.

1.1. Technical difficulties

The feasibility of the TDMI technique is strongly dependent from the epitope solubility: if the *in silico* predicted epitope cannot be expressed as TRX-fusion protein (in *E. coli* cells) or shows solubility complications, the entire Abs production process cannot proceed.
Insolubility of the scaffold-antigen fusion protein is, in this approach, a minor yet obnoxious issue: insoluble antigenic constructs can be purified by inclusion bodies denaturation; however it is possible that this procedure compromise the antigen original tridimensional conformation lowering the specificity of generated Abs.

1.2. Replacing EcTRX with PfTRX

In a parallel study, our research group, demonstrated the superiority of the *P. furiosus* TRX over the *E. coli* TRX as an immunization scaffold/carrier (data in course of publication). Exploiting the stronger epitope solubilization capacity and intrinsic higher conformational constrain of PfTRX, could be an option for resolving the antigen solubility issue.
Other PfTRX features such as a higher thermal stability and protease resistance could be used for an improved and more efficient TRX-antigen purification procedure thus removing the need to use an His-tagged scaffold and avoiding anti-HisTAG Abs generation.
The lack of cross-reactivity of anti-PfTRX antibodies with other thioredoxins (especially eukaryotic TRXs) could reveal itself invaluable for the production of a polyclonal antiserum to be used with mammalian cells, tissues or samples (Fig. 31)

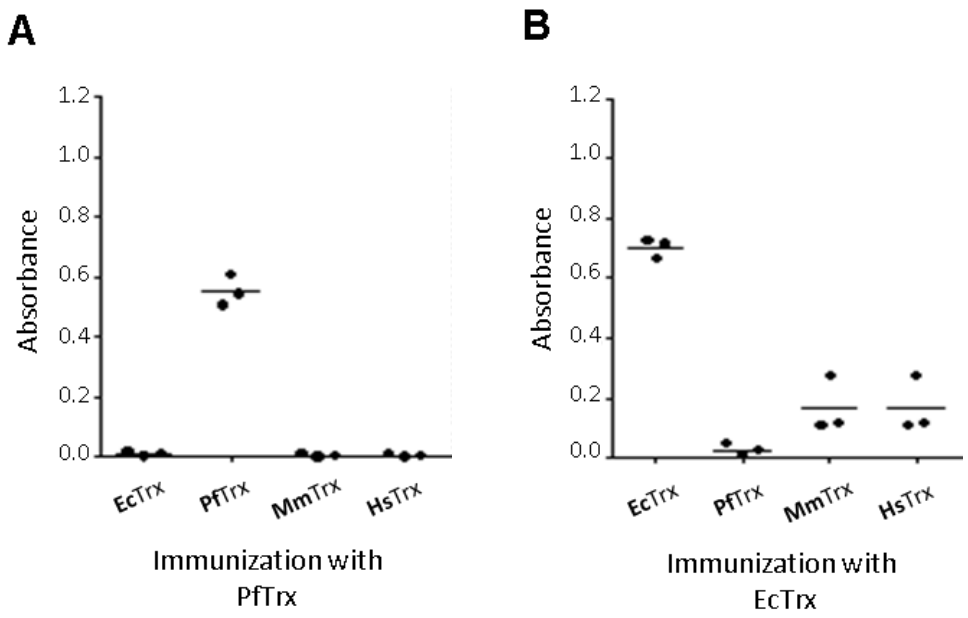

Figure 31 – Immune cross-reactivity profiles of anti-PfTrx and anti-EcTrx antibodies. Sera from mice immunized with empty PfTrx and EcTrx and MmTrx were analyzed by ELISA using the corresponding GST-fusion proteins plus murine and human thioredoxin (GST-MmTrx and GST-HsTrx) as capture antigens. Each dot represents an individual mouse serum.

1.2. Improving Abs specificity

As shown in the presented experiments, in this thesis I analyzed three different ways to achieve higher immunoglobulin specificity.

1.2.a Homologous TRX based TDMI

One of the approach, the use of an endogenous TRX (OcTRX in rabbit), revealed itself to be unsuccessful as nearly half of the Abs reactivity was directed against the scaffold. A possible explanation of this "failure" could be that, even if the host animal immune system tolerates and recognizes as self the vast majority of the endogenous TRX sequence/structure, there are some "junctional epitopes" located in-between the region where the TRX exposed loop ends and the epitope region begins. These non-self junctional epitopes generate Abs directed against both the scaffold and the antigen which might compromise the specificity of serum against the latter (Fig.32).

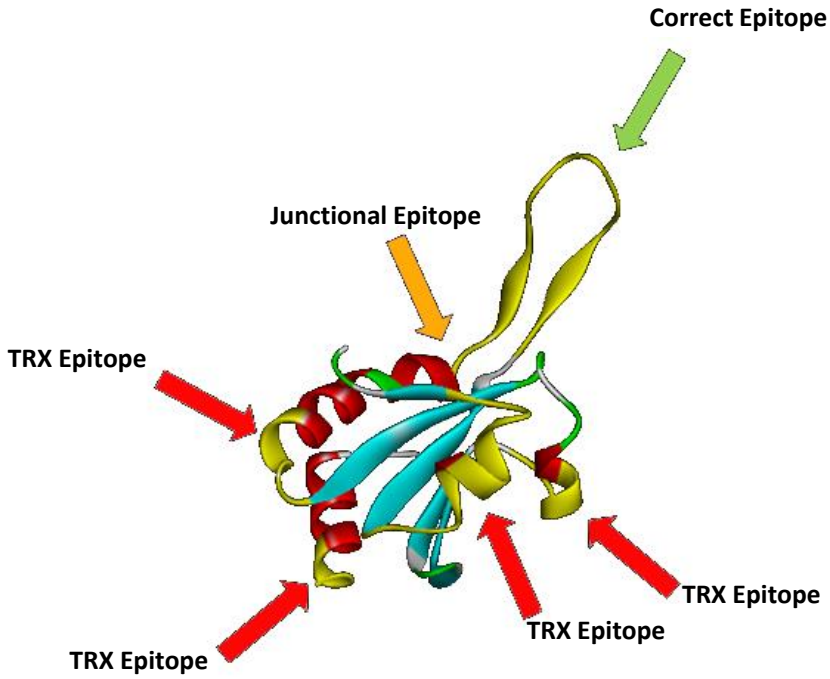

Figure 32 – Predicted structure of EcTRX containing an antigenic peptide (yellow loop). Colored arrows represent B-cell epitopes.

1.2.b BLPtrap

The immuno-sequestration of anti-scaffold Abs by BLPtrap yielded positive results. The procedure is fast, inexpensive and requires little practical work. The trial was although an explorative one and the procedure needs to be optimized. The serum/BLP-TRX ratio seems to be a crucial point as, for instance, the presented data show how by increasing the serum/BLP-EcTRX ratio from 1 to 1,5, the serum becomes depleted of antigen specific Abs .

1.2.c Monoclonal Abs production

The better results came, with little surprise, from the production of mAbs with TDMI generated antigens.
Even if mAb production has not moderated costs, need to be performed by qualified personnel and requires months of work, the overall quality of the Ab and the possibility to obtain a productive immortal cell line justify the effort. Due to the high specificity of the mAbs one perspective is to generate TDMI derived immunoglobulins that can specifically recognize and bind peptide epitope bearing a post-translational modification, like phosphorylation, ignoring the same non-modified peptide. In order to achieve with this methodology mAbs capable to distinguish a phosphorylated form of a protein, phosphomimetic epitopes are in production. Phosphomimetics are amino acid that mimic a phosphorylated

residue of a different amino acid: for example aspartic acid is chemically similar to phospho-serine and could mimic it. Consequently by replacing in an peptide epitope a serine with an aspartic acid the mAbs generated against said peptide should recognize only its phosphorylated form.

1.2. Improving the *in silico* epitope prediction

In our classical strategy, once antigen has been chosen we proceed with an *in silico* analysis through several programs for the prediction of epitopes (linear and conformational) that might be able to stimulate the production of antibodies (B cell epitopes).

These programs are BepiPred (http://www.cbs.dtu.dk/services/BepiPred/), ABCPred (http://www.imtech.res.in/raghava/abcpred/), BCEPred (http://www.imtech.res.in/raghava/bcepred/) and the BCPreds suite (BCPred, FBCPred, AAP, http://ailab.cs.iastate.edu/bcpreds/predict.html).

The results of these bio-informatic programs has to be combined by an operator, creating a prediction overall score value which allows us to choose the best B-cell epitope.

Recently it has been developed and made available a new prediction platform called BEST (B-cell Epitope prediction using Support Vector Machine Tool) which is a significant improvement compared to traditional programs for B -cell epitopes prediction (Gao et al., 2012).

I put this platform in use and proceeded with its *in silico* validation through the systematic comparison with epitopes that we had already produced and tested (Table 2).

Epitope	Molecular target	Scaffold	Ab type	Verified laboratory application	BEST prediction
EID1	human EID1 protein	EcTRX	polyclonal	WB, IF	excellent
Dok1	human Dok1 protein	EcTRX	polyclonal	WB, IF, IP	good
HPV16_E6	E6 protein of HPV16	EcTRX	polyclonal	WB, IP	average
HPV16_E7	E7 protein of HPV16	EcTRX	polyclonal	WB, IP	excellent
HPV38_E7	E7 protein of HPV38	EcTRX	monoclonal	WB, IP	not performed
DeltaNp73	human DeltaNp73 protein	EcTRX	polyclonal	WB	not performed
Phosphomimetic DeltaNp73 (S422D)	Phosphorylated DeltaNp73 protein	EcTRX	monoclonal	not yet tested	not performed
VHH	camelid IgG VHH region	OcTRX	polyclonal	WB, ELISA	good
PI3K delta	human PI3K delta protein	EcTRX	polyclonal	none (bad quality)	bad
PI3K gamma	human PI3K gamma protein	EcTRX	polyclonal	none (bad quality)	bad
SHOX1	human SHOX1 protein	EcTRX	polyclonal	WB	excellent
HPV16_E6	E6 protein of HPV16	PfTRX	polyclonal	not yet tested	average
HPV16_E7	E7 protein of HPV16	PfTRX	polyclonal	not yet tested	excellent

Table 2 – Resume of produced antibodies using the TDMI approach during the progress of the presented (thesis) project

A noteworthy point is the case of the PI3K epitopes against which our TDMI approach did not give production of good quality antibodies. In this specific case the BEST prediction did not match ours and indicates alternative epitopes to be used to stimulate B-cell Ab production.

Except for this unfortunate case, overall BEST prediction and our past prediction method seem to match and the former could be used to standardize epitope prediction for future projects.

DEVELOPMENT OF A RECOMBINANT VACCINE AGAINST ALZHEIMER'S DISEASE

The primary objective of this study was to evaluate the long-term efficacy of the vaccine in preventing the cognitive deficits associated with the progression of the mice Alzheimer-like pathology, in addition to the development of histopathological symptoms of the disease such as amyloid plaques and the accumulation / overproduction of Aβ oligomeric structures.

At the end of treatment, we carried out two different behavioral tests (Y-Maze and Contextual Fear Conditioning), which revealed no significant differences between the treated and untreated mice.

We wanted to verify whether, in spite of the negative behavioral outcome, the treated mice had a different antibody titer compared to the untreated ones. Even in this case there were no significant differences between placebo and vaccine treated mice, except in few individuals.

Some conceivable explanations come to mind.

The main hypothesis is that, due to their nature (overexpressing APP and thus "acquainted" with high levels of amyloid peptides) the Tg2576 mice developed a tolerance against the Aβ peptide. This can also explain the abundant anti- Aβ42 Ab immunotiter originated in BALB/c mice after immunization in the first phase of the project, which had the aim to evaluate the efficacy of different antigen formulations.

The second hypothesis regards the suitability of the prototype vaccine.

A plausible idea is that the antigen was administered at a sufficient dose to stimulate a response in BALB/c mice but not in Tg2576. Although the injected amount has been increased during the experiment on transgenic mice, it may have been below the effective threshold.

A parallel/complementary assumption is that the miniaturization of the antigen from 15 residues (Aβ1-15) to 7 (Aβ1-7), in order to reduce toxic effects of the amyloid peptide, was too extreme to produce an adequate immune-response in Tg2576 mice.

One last hypothesis regards the possible intervention of disease chronicity factors in the immunized animals. It is possible that due to the exceptionally long duration of the study the mice developed a chronic and irreversible AD-like behavior, which could not be removable by a vaccine with prophylactic activity.

1. Future developments

Even if the prototype vaccine failed in preventing AD cognitive deficits and in inducing a strong immune response, further test will be performed to check if the immunization gave other "molecular effects".

The experience and knowledge gain with this project opened up a collaboration for the development of another prototype of AD vaccine.

1.1. Upcoming experiments

It will be considered to analyze, in the same two groups of animals (treated and placebo), intra-cerebral levels of Aβ42 oligomers, the species currently considered the primarily responsible for the amyloid neurotoxicity. For this purpose, I have optimized and validated an up-to-date methodology for the determination of the Aβ oligomers, based on an ELISA assay and a particular monoclonal antibody (a low affinity/high avidity IgM; OMAB, Agrisera) able to selectively capture the oligomeric forms of the Aβ peptide.

The higher selectivity/reliability of this assay makes it suitable not only for analyzing brain extracts, but also for other biological samples of diagnostic interest such as the cerebro-spinal fluid (CSF) and the peripheral blood of Alzheimer's human patients.

Histopathological examination and amyloid plaque quantitation of the tested animals brain hemisphere will be performed in order to establish if the vaccine has or has not prevented the formation of senile plaques.

1.2. Parallel projects

In parallel with the aforementioned studies, I participated in the development of a different anti-Aβ vaccine designed and produced by the research group coordinated by Dr. Antonella Prisco (Institute of Genetics and Biophysics ABT, CNR, Naples).

In this case the vaccine consists of the first 11 amino acids of the peptide Aβ42 (Aβ1-11) fused to the N terminus of the E2 subunit of the *Bacillus stearothermophilus* pyruvate dehydrogenase (Mantile et al., 2011). These E2-based constructs have the capacity to self-assemble into "virus-like" particles (VLP) consisting of 60 elements (monomers) (Fig.33).

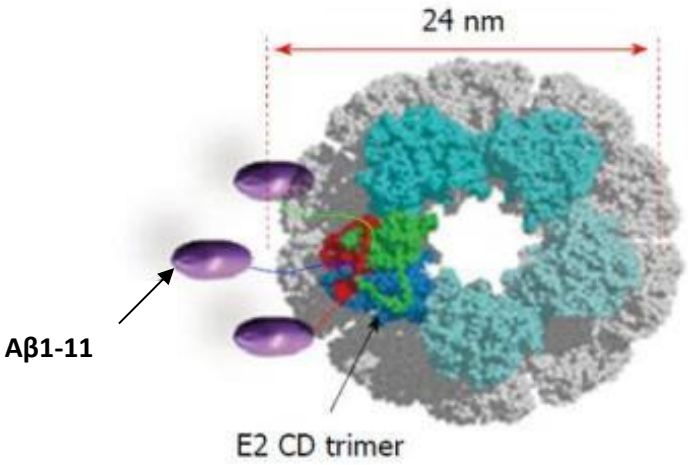

Figure 33 – Predicted structure of a VLP-Aβ

The immunization of BALB/c mice with these VLP-Aβ generates very high and long-lasting antibody titer.

The goal of this parallel project was to evaluate the immunogenicity of (1-11)E2 formulated in alum (Alhydrogel 2%), or in a squalene oil-in-water emulsion (AddaVax), or without adjuvant.

I am now dealing with the evaluation of the capacity of recognition of different forms of Ab (monomer, oligomer or fibril) by antibodies produced in response to immunization with different formulations of antigen VLP-Aβ (paper in review).

REFERENCES

Akira, S., K. Kiyoshi Takeda, and T. Kaisho. (2001) Toll-likereceptors: critical proteins linking innate and acquired immunity.*Nature Immunol.* **2:** 675–680.

Amzel, L. M., and R. J. Poljak. (1979) Three-dimensional structure of immunoglobulins. *Ann. Rev. Biochem.* **48:** 961–997.

Ankri C, Shamalov K, Horovitz-Fried M, Mauer S, Cohen CJ. (2013). Human T cells engineered to express a programmed death 1/28 costimulatory retargeting molecule display enhanced antitumor activity. *J Immunol*. **191**:4121-9.

Banchereau, J. (2002) The long arm of the immune system. *Sci.Am.* **287:** 52–59.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D. and Yednock, T. (2000) Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* **6**: 916-919.

Bergman et al. (2003) App intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations. *Experimental Cell Research* **287**: 1-9.

Bertini R et al. (1999) Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells. *J. Exp. Med.* **189**: 1783-1789.

Bjorkman, P. J., et al. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329:** 506–512.

Blass, J.P. (2002) Alzheimer's disease and Alzheimer's dementia: distinct but overlapping entities. *Neurobiol. Aging* **23**:1077-1084; *author reply* 1101-1075.

Brown, J. H., et al. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature***364:** 33–39.

Capurso, A. (2001) The promise of statins. *Ital. Heart J. Suppl.* Mar;**2**(3): 224-9.

Catalano SM, Dodson EC, Henze DA, Joyce JG, Krafft GA, Kinney GG. (2006). The role of amyloid-beta derived diffusible ligands (ADDLs) in Alzheimer's disease. *Curr Top Med Chem*. **6**:597-608. Review.

Chaney, O., Webster, D.S., Yu-Min Kuo and Roher, E.A. (1998) Molecular modeling of the Aβ1-42 peptide from Alzheimer's disease. *Protein Engineering* **11**: 761-767.

Cheret C, Willem M, Fricker FR, Wende H, Wulf-Goldenberg A, Tahirovic S, Nave KA, Saftig P, Haass C, Garratt AN, Bennett DL, Birchmeier C. (2013). Bace1 and Neuregulin-1 cooperate to control formation and maintenance of muscle spindles. *EMBO J.* **32**:2015-28.

Coles, M., Bicknell, W., Watson, A.A., Fairlie, D.P., Craik, D.J. (1998) Solution structure of amyloid beta-peptide(1-40) in a water-micelle environment. Is the membrane-spanning domain where we think it is? *Biochemistry* **37**: 11064-11077.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Dodart, J.C., Paul, S.M., Holtzman, D.M. (2001) Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U S A* **98**:8850-8855.

Dodart ,J.C., Bales, K.R, Paul, S.M., (2003) Immunotherapy for Alzheimer's disease: will vaccination work? *Trends Mol. Med.* **9**:85-87.

Dumery, L., Bourdel, F., Soussan, Y., Fialkowsky, A., Viale, S., Nicolas, P., Reboud-Ravaux (2001) β-amyloid protein aggregation: its implication in the physiopathology of Alzheimer's disease. *Pathol. Biolog.* **49**: 72-85.

Edbauer, D., Winkler, E., Haass, C. And Steiner, H. (2002) Presenilin and nicastrin regulate each other and determine amyloid β-peptide production via complex formation. *Proc. Natl. Acad. Sci. USA* **99**: 8666-71.

Evin, G., Weidemann, A. (2002). Biogenesis and metabolism of Alzheimer's disease Aβ amyloid peptides. *Peptides* **23**: 1285-1297.

Frenkel, D., Dewachter, I., Van Leuven, F., Solomon, B. (2003) Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. *Vaccine* **21**: 1060-1065.

Ferreira, T.S., Defelice, F. (2002) Physiopathological modulators of amyloid aggregation and novel pharmacological approaches in Alzheimer's disease. *Annals of the Brazilian Academy of Sciences* **74**: 265-284.

Findeis, M.A., Musso, G.M., Arico-Muendel, C.C., Benjamin, H.W., Hundal, A. M., Lee, J-J, Chin J., Kelley, M., Wakefield, J., Hayward, N. J., Molineaux, S. M. (1999) Modified-peptide inhibitors of amyloid β-peptide polymerization. *Bioch.* **38:** 6791-6800.

Fonte, V. et al. (2002) Interaction of intracellular b amyloid peptide with chaperone proteins. *PNAS* **99**: 9439-9444.

Gao CM, Yam AY, Wang X, Magdangal E, Salisbury C, Peretz D, Zuckermann RN, Connolly MD, Hansson O, Minthon L, Zetterberg H, Blennow K, Fedynyshyn JP, Allauzen S. (2010) Aβ40 oligomers identified as a potential biomarker for the diagnosis of Alzheimer's disease. *PLoS One*. **5**:e15725.

Gendreau KL, Hall GF. (2013). Tangles, Toxicity, and Tau Secretion in AD - New Approaches to a Vexing Problem. Front Neurol. **4**:160. Review.

Gestwicki, J. E., et al. (2004) Harnessing chaperones to generate small-molecule inhibitors of amyloid b aggregation. *Science* **306**: 865-869.

Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. and et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial alzheimer's disease. *Nature* **349**: 704-706.

Guzmán-Martinez L, Farías GA, Maccioni RB. (2013). Tau Oligomers as Potential Targets for Alzheimer's Diagnosis and Novel Drugs. Front Neurol. **4**:167. Review.

Jung, D., et al. (2006) Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Ann. Rev.Immunol.* **24:** 541–570.

Haass C, Selkoe DJ. (2007)Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol*. **8**:101-12. Review.

Hardy, J., Selkoe, D.J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**: 353-356.

Heyman, B. (2000) Regulation of antibody responses via antibodies, complement, and Fc receptors. *Ann. Rev. Immunol.***18:** 709–737.

Hinds, T.R., Kukull, W. A., Van Belle, G., Schellenberg, G. D., Villacres, E. C., and Larson, E. B. (1994) Relationship between serum α1-antichymotrypsin and Alzheimer's disesase. *Neurobiol. Aging* **15:** 21-7.

Hozumi, N., and S. Tonegawa. (1976) Evidence for somaticre arrangement of immunoglobulin genes coding for variable and constant regions. *Proc. Nat'l Acad. Sci. USA* **73:** 3628–3632.

Imbimbo, B.P. (2002) b-Amyloid immunization approaches for Alzheimer disease. *Drug Dev. Res* **56**: 150-162.

Imbimbo, B.P. (2004) The potential role of non-steroidal anti-inflammatory drugs in treating Alzheimer's desease. *Expert Opin. Investig. Drugs* **13**: 1469-1481.

Imbimbo BP, Giardina GA. (2011). γ-secretase inhibitors and modulators for the treatment of Alzheimer's disease: disappointments and hopes. *Curr Top Med Chem.* **11**:1555-70. Review.

Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N. and Ihara, Y. (1994). Visualization of Aβ 42 (43) and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ 42 (43). *Neuron* **13**: 45-53.

Janus, C. et al. (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* **408**: 979-982.

Kakimura, J. et al. (2002) Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins. *Faseb J*. **16**: 601-603.

Kang, J. Y., and J.-O. Lee. (2011) Structural biology of the Toll-like receptor family. *Ann. Rev. Biochem.* **80** :917–941.

Karran E, Mercken M, De Strooper B. (2011). The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov.* **10**:698-712.

Kenney, R.T., Regina Rabinovich, N., Pichyangkul, S., Price, V.L., Engers, H.D. (2002) 2nd meeting on novel adjuvants currently in/close to human clinical testing. World Health Organization-Organization Mondiale de la Sante Fondation Merieux, Annecy, France, 5-7 June 2000. *Vaccine* **20**: 2155-2163.

Kim, H., Kim, E., Lee, J., Park, H.C., Kim, S., Seo, J., Chang, K., Yu, E., Jeong, S., Chong, Y.H., Suh, Y. (2003) C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3β expression. *The FASEB Journal* **17**: 1951-53.

Kopan, R., (2002) Notch: a membrane-bound transcription factor. *Journal of Cell Science* **115:** 1095-1097.

Klein, W.L. (2002) Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem. Int.* **41**: 345-352.

Klein, W.L., Stine, Jr W.B., Teplow, D.B. (2004) Small assemblies of unmodified amyloid β-protein are the proximate neurotoxin in Alzheimer's disease. *Neuro. of aging* **25:** 569-580.

Krafft GA, Klein WL. (2010). ADDLs and the signaling web that leads to Alzheimer's disease. *Neuropharmacology*. **59**:230-42.

LaVallie, E.R, McCoy, J.M. (1995) Gene fusion expression systems in Escherichia coli. *Curr. Opin. Biotechnol.* **6**: 501-506.

Legleiter, J., Czilli, D.L., Gitter, B., DeMattos, R.B., Holtzman, D.M. and Kowalewski, T. (2004). Effect of different Anti-Ab Antibodies on Ab Fibrillogenesis as Assessed by Atomic Force Microscopy. *J. Mol. Biol.* **335**: 997-1006.

Lichtenthaler, S.F. and Haass, C. (2004) Amyloid at the cutting edge: activation of α-secretase prevents amyloidogenesis in an Alzheimer disease mouse model. *J. Clin. Invest.* **113**:1384–1387

Lopez OL, McDade E, Riverol M, Becker JT. (2011) Evolution of the diagnostic criteria for degenerative and cognitive disorders. *Curr Opin Neurol*. **24**:532-41.

Lowe, T.L., Strzelec, A., Kiessling, L.L., Murphy R.M. (2001) Structure-function relationships for inhibitors of β-amyloid toxicity containing the recognition sequence KLVFF. *Bioch.* **40**: 7882-7889.

Maccioni, R.B., Munoz, J.P., Barbeito, L. (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch. Med. Res.* **32**: 367-381.

Mason JM, Kokkoni N, Stott K, Doig AJ. (2003).Design strategies for anti-amyloid agents. *Curr Opin Struct Biol*. **13**:526-32. Review.

Mantile F, Basile C, Cicatiello V, De Falco D, Caivano A, De Berardinis P, Prisco A. (2011). A multimeric immunogen for the induction of immune memory to beta-amyloid. I*mmunol Cell Biol*. **89**:604-9.

McGeer, P. L. and McGeer, E. G. (2001). Inflammation, autotoxicity and Alzheimer disease. *Neurobiol Aging* **22**: 799-809.

Morgan, D. et al. (2000) A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* **408**: 982-985.

Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*.**314**:126-9.

Muramatsu, M., et al. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID),a potential RNA editing enzyme. Cell 102: 553–563.

Neefjes, J. J., et al. (1990) The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell***61:** 171–183.

Okuizumi, K., Onodera, O., Namba, Y., Ikeda, K., Yamamoto, T., Seki, K, Ueki, A., nanko, S., Tanaka, H., Takahashi, H. and et al. (1995). Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. *Nat. Genet.* **11**: 207-9.

Outeiro, T.F. and Muchowski, P.J. (2004) Molecular genetics approaches in yeast to study amyloid diseases. *J. Neurosci*. **23**: 49-59.

Permanne, B. et al. (2002) Reduction of amyloid load and cerebral damage in a transgenic mouse model of Alzheimer's disease by treatment with a beta-sheet breaker peptide. *Faseb J.*, 860-862.

Peters, P. J., et al. (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349:** 669–676.

Petrushina I, Tran M, Sadzikava N, Ghochikyan A, Vasilevko V, Agadjanyan MG,
Cribbs DH. (2003)Importance of IgG2c isotype in the immune response to beta-amyloid in amyloid precursor protein/transgenic mice. *Neurosci Lett. 338*:5-8.

Porayette P, Gallego MJ, Kaltcheva MM, Bowen RL, Vadakkadath Meethal S, Atwood CS. (2009). Differential processing of amyloid-beta precursor protein directs human embryonic stem cell proliferation and differentiation into neuronal precursor cells. *J Biol Chem.* 2009 **284**:23806-17

Reitz C. (2012) Alzheimer's disease and the amyloid cascade hypothesis: a critical review. *Int J Alzheimers Dis.* **2012**:369808.

Renbaum, P. and Levy-Lahad, E. (1998). Monogenic determinants of familial Alzheimer's disease: presenilin-2 mutations. *Cell Mol. Life Sci.* **54**: 910-9.

Rudolph, M. G., R. L. Stanfield, and I. A. Wilson. (2006) How TCRs bind MHCs, peptides, and coreceptors. *Ann. Rev. Immunol.***24:** 419–466.

Sambamurti, K., Kinsey, R., Maloney, B., Yuan-Wen Ge, and Lahiri, K. (2004) Gene structure and organization of the human β-secretase (BACE) promoter. *The FASEB Journal* **10**.1096-1378.

Saunders.Rajewsky, K., et al. (1969) The requirement of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **129:**1131–1143.

Sharpe, A. H., and A. K. Abbas. (2006) T-cell costimulation: biology, therapeutic potential, and challenges. *N. Engl. J. Med. **355:** 973–975.*

Schatz, D. G., M. A. Oettinger, and D. Baltimore. (1989) The V(D)J recombination activating gene, RAG-1. *Cell* **59:** 1035–1048.

Schmechel, A., Strauss, M., Schlicksupp, A., Pipkorn, R., Haass, C., Bayer, A. And Multhaup, G. (2004). Human BACE Forms Dimers and Colocalizes with *APP. J. Biol. Chem.* **279**: 39710-39717.

Schnabel J. (2011). Vaccines: chasing the dream. *Nature*. **475**:S18-9.

Schenk, D. et al. (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**: 173-177.

Selkoe, D. J. (1991). The molecular pathology of Alzheimer's disease. *Neuron* **6**: 487-98.

Selkoe, D.J. (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* **81**: 741-766.

Selkoe, D.J., Schenk. D. (2003) ALZHEIMER'S DISEASE: Molecular Understanding Predicts Amyloid-Based Therapeutics. *Annu. Rev. Pharmacol. Toxicol.* **43**: 545-584.

Selkoe, D.J. (2004) Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann. Intern. Med*. **140**: 627-638.

Serpell, L.C. (2000) Alzheimer's amyloid fibrils: structure and assembly. *Biochim. Biophys. Acta* **1502**: 16-30.

Sigurdsson, E.M., Wisniewski, T., Frangione, B. (2002) A safer vaccine for Alzheimer's disease? *Neurobiol. Aging* **23**: 1001-1008.

Smith, M.A., Casadesus, G., Joseph, J.A., Perry, G. (2002) Amyloid-beta and tau serve antioxidant functions in the aging and Alzheimer brain. *Free Radic. Biol. Med.* **33**: 1194-1199.

Solomon, B., Koppel, R., Frankel, D., Hanan-Aharon, E. (1997) Disaggregation of Alzheimer beta-amyloid by site-directed mAb. *Proc. Natl. Acad. Sci. U S A* **94**: 4109-4112.

Soto, C., Kindy, MS., Baumann, M., Frangione, B. (1996) Inhibition of Alzheimer's amyloidosis by peptides that prevent β-sheet conformation. *Bichem. Biophys. Res. Commun*. **226**: 672-680.

Soto, C. (1999) Alzheimer's and prion disease as disorders of protein conformation: implications for the design of novel therapeutic approaches. *J. Mol. Med.*, 412-418.

Steinman, R. M., and H. Hemmi. (2006) Dendritic cells: translating innate to adaptive immunity. *Curr. Top. Microbiol.Immunol.* **311:** 17–58.

Suzue, K., Zhou, X., Eisen, H.N., Young, R.A. (1997) Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. *Proc. Natl. Acad. Sci. U S A* **94**: 13146-13151.

Thal DR, Griffin WS, de Vos RA, Ghebremedhin E (2008). Cerebral amyloid angiopathy and its relationship to Alzheimer's disease. *Acta Neuropathol.* **115**: 599–609.

Tell V, Hilgeroth A. (2013). Recent developments of protein kinase inhibitors as potential AD therapeutics. Front Cell Neurosci. **7**:189. Review

Tjernberg, O., Callaway, J.E., Tjernberg, A., Hahne, S., Lilliehook, C., Terenius, L., Thyberg, J., and Nordsted, C. (1999) A molecular model of Alzheimer amyloid β-peptide fibril formation. *J. Biol. Chem*. **274**: 12619-12625.

Tjibbe Bosma, Rolf Kanninga, Jolanda Neef, Sandrine A. L. Audouy, Maarten L. van Roosmalen, Anton Steen, Girbe Buist, Jan Kok, Oscar P. Kuipers, George Robillard, and Kees (2006) Novel Surface Display System for Proteins on Non-Genetically Modified Gram-Positive Bacteria. *Appl Environ Microbiol*. **72**:880–889.

Vagnucci, A.H. Jr., Li, W.W. (2003) Alzheimer's disease and angiogenesis. *Lancet* **361**: 605-608.

van Roosmalen ML, Kanninga R, El Khattabi M, Neef J, Audouy S, Bosma T, Kuipers A, Post E, Steen A, Kok J, Buist G, Kuipers OP, Robillard G, Leenhouts K. (2006). Mucosal vaccine delivery of antigens tightly bound to an adjuvant particle made from food-grade bacteria. *Methods*. **38**:144-9.

Vassar, R., Bennet, B. and Citron, M. (1999) B-secretase Cleavage of Azheimer's Amyloid Precursor Protein by the Transmembrane Aspartic Protease BACE. *Science **286***: 735-741.

Viet MH, Ngo ST, Lam NS, Li MS. (2011). Inhibition of aggregation of amyloid peptides by beta-sheet breaker peptides and their binding affinity. *J Phys Chem B*. **115**:7433-46.

Wurth, C., Guimard, N.K., Hecht, M.H. (2002) Mutations that reduce aggregation of the Alzheimer's Abeta42 peptide: an unbiased search for the sequence determinants of Abeta amyloidogenesis. *J. Mol. Biol.* **319**: 1279-1290.

Williams, A. F., and A. N. Barclay. (1988) The immunoglobulin superfamily—domains for cell-surface recognition. *Ann. Rev.Immunol.* **6:** 381–405.

Willingham, S., Outeiro, T.F., DeVit, M.J., Lindquist,S. L., Muchowski, P.J. (2003) Yeast genes that enhance the toxicity of a mutant Huntingtin fragment or α-synuclein. *Science* **302**: 1769-1772.

Wolfe, S.M., and Haas, C. (2001) The role of Presenilins in γ-secretase activity. *J. Biolog. Chem*. **276**: 5413-5416.

Xing, Y., Higuchi, K. (2002) Amyloid fibril proteins. *Mech. Ageing Dev.* **123:** 1625-1636.

Xinwei, C. and Sudhof, C.T. (2004) Dissection of Amyloid precursor protein-dependent trascriptional transactivation. *J. Biol. Chem*. **279**: 24601-24611.

Books

Lodish "Molecular Cell Biology", seventh edition, *W. H. Freeman and Company.*

Sambrook and Russel, "Molecular Cloning: A Laboratory Manual", third edition, *Cold Spring Harbor Laboratory Press*.

PUBLICATIONS

Results related to the presented thesis are in course of publication.

The following manuscript is under review for publication.

Mantile F, Trovato M, Santoni A, Barba P, Ottonello S, De Berardinis P, Prisco A (2014) Alum and squalene-oil-in-water emulsion enhance the titer and avidity of anti-Aβ antibodies induced by multimeric protein antigen (1-11)E2, preserving the IgG1-skewed isotype distribution. *PLoS One* (in review)

The following publication is not related to the presented thesis but it was achieved during the PhD studentship.

Turroni F, Serafini F, Foroni E, Duranti S, O'Connell Motherway M, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sánchez B, Santoni A, Gioiosa L, Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M. (2013) Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. *Proc Natl Acad Sci U S A* **110**(27) 11151-11156.

APPENDIX

Sequences

EcTRX

ATGGGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAGCGGACGGGGCGATCCTCGTCGA
TTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCCCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAAC
TGACCGTTGCAAAACTGAACATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTG
CTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAA
CCTGGCGTGA

OcTRX

ATGGTGAAACAGATTGAAAGCAAAAGCGCGTTTCAGGAAGTGCTGGATAGCGCGGGCGATAAACTGGTGGTGGTGGATTT
TAGCGCGACCTGGTGCGGTCCGTGCAAAATGATTAAACCGTTTTTTCATGCGCTGAGCGAAAAATTTAACAACGTGGTGT
TTATTGAAGTGGATGTGGATGATTGCAAAGATATTGCGGCGGAATGCGAAGTGAAATGCATGCCGACCTTTCAGTTTTTT
AAAAAAGGCCAGAAAGTGGGCGAATTTAGCGGCGCGAACAAAGAAAAACTGGAAGCGACCATTAACGAACTGCTGTGA

PfTRX

ATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCCGGGATGCGGTCC
GTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAATTGTCCATATCAATGCCGGCA
AATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGA
CGCCAAAACCTGATTCGTTCTAAAGAAGAGATTCTGAAAAAACTGAAAGAGCTGCAGGAGAAA

Dok1

CGGTCCGCCGGAGGGCCCGGCGTTCCCGGAGCCGGGCACTGCGACTGGCAGCTGCGGCGGTCCGCCGGAGGGCCCGGCGT
TCCCGGAGCCGGGCACTGCGACTGGCAGCTGCGGCGGTCCGCCGGAGGGCCCGGCGTTCCCGGAGCCGGGCACTGCGACT
GGCAGCTGCGGCGGTCCG

HPV38 E7

CGGTCCGCTGCCGGATCTGCCGGAGGATATTGAGGCGAGCGTGGTGGAGGAAGAGCCGGCGGGCGGTCCGCTGCCGGATC
TGCCGGAGGATATTGAGGCGAGCGTGGTGGAGGAAGAGCCGGCGGGCGGTCCGCTGCCGGATCTGCCGGAGGATATTGAG
GCGAGCGTGGTGGAGGAAGAGCCGGCGGGCGGTCCG

VHH

cGGtCCGGGtCCGGGCATGGGCTGGTTTCGTCAGGCGCCGGGCAAAGAACGTGAATTTGTGGCGGGGCCAGGGCCAGGGA
TGGGGTGGTTCCGCCAAGCACCAGGGAAGGAGCGCGAGTTCGTTGCAGGTCCTGGTCCTGGTATGGGTTGGTTTCGCCAG
GCCCCTGGTAAAGAGCGTGAGTTTGTCGCCGGCCCCGGTCCG

HPV16_E6

CGGTCCGGGCCCGGGCCAGGATCCGCAGGAACGTCCGCGTAAACTGCCGCAGCTGTGCACCGGGCCAGGGCCAGGGCAAG
ACCCACAAGAGCGCCCACGCAAGTTGCCACAATTGTGTACAGGTCCTGGTCCTGGTCAAGATCCTCAGGAGCGTCCTCGT
AAGCTCCCTCAGCTCTGCACGGGCCCGGGCCCGGGCTACAGCGTGTACGGCACCACCCTGGAACAGCAGTACAACAAACC

GGGGCCAGGGCCAGGGTATTCCGTTTATGGGACAACATTGGAGCAACAATATAATAAGCCAGGTCCTGGTCCTGGTTATT
CTGTCTACGGTACGACGCTCGAGCAGCAATACAATAAACCTGGTCCCGGTCCG

HPV16_E7

CGGTCCGGGCCCGGGCGATAGCAGCGAAGAAGAAGATGAAATTGATGGCCCGGCGGGCCAGGCGGAACCGGATCGTGGGC
CAGGGCCAGGGGACTCCTCCGAGGAGGAGGACGAGATCGACGGGCCAGCAGGGCAAGCAGAGCCAGACCGCGGTCCTGGT
CCTGGTGACTCTTCTGAAGAGGAAGACGAAATCGATGGTCCTGCCGGTCAAGCCGAGCCTGACCGTGGTCCCGGTCCG

EID1

CGGTCCGGGCCCGGGCATGCCGGGCGAAGGCGATCTGCCGCAGATGGAAGTGGGCAGCGGCAGCCGTGAAGGGCCAGGGC
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GGTGAAGGTGATCTCCCTCAAATGGAGGTCGGTTCTGGTTCTCGCGAAGGCCCGGGCCCGGGCAGCGGCGCGCAGCAGCT
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Aβ(1-7)9

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LysM

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