From the Department of Medicine
Karolinska Institutet, Stockholm, Sweden

Expression and Interaction Studies of Recombinant Human Monoclonal Antibodies

Daniel X. Johansson

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ABSTRACT

The highly specific binding characteristics of monoclonal antibodies (mAbs) have made them one of the most useful tools in the fields of research and biotechnology where they provide the basis for a large number of diagnostic and technical assays. In recent years, with the advent of techniques to generate fully human antibodies, mAbs have also emerged as a new important class of therapeutics.
Accordingly, improved technologies for faster isolation and characterization of mAbs are of great demand. The studies included in this thesis are all related to monoclonal antibody technologies.

In order for a new mAb to be characterized, it has to be produced in large quantities. This has been problematic since established production systems for recombinant antibodies either express at too low levels, are time consuming or are unreliable only allowing successful expression of certain clones.
We explored the *Drosophila* S2 cell line for high-level expression of mAbs in the form of human IgG1. A series of vectors were designed to allow easy transfer of isolated mAb genes from hybridomas and commonly used phage-display vectors. The S2 cells showed a number of advantages including rapid establishment of stable cellines that express at high levels, easy culturing and easy production scale-up.
The S2 produced mAbs had indistinguishable binding properties compared to mAbs produced in mammalian CHO cells as shown by antigen binding in ELISA, immunofluorescent staining of cells and virus neutralization capacity.

Next, we used the S2 system to produce three previously isolated mAbs targeting the envelope E2 of the highly sequence variable Hepatitis C virus (HCV). The subsequent characterization of the mAbs showed that two of them bound and neutralized a broad range of HCV isolates. The epitopes of both mAbs were mapped to a highly conserved region of E2 strongly indicated to be involved in interaction with CD81, a cell receptor important for HCV cell entry.

In a following study, a new mAb targeting the adhesion protein BabA of the human pathogen *Helicobacter pylori* was isolated from an immune combinatorial scFv antibody library. BabA is involved in *H. pylori* adherence to fucosylated blood sugars such as Lewis b (Leb) on the gastric mucosa. Again, the S2 system was used for high level production of mAb in IgG1 format allowing a detailed characterization of the antibody specificity profile. The mAb bound BabA derived from a wide range of *H pylori* strains.

Finally, we investigated the possibilities to develop a system for high throughput screening of mAb-antigen interactions in living cells. Such a system would be of great use since the antigen is displayed in its natural environment. This allows screening of antibodies targeting antigens not suitable for protein purification such as membrane proteins. Our system was based on Förster Resonance Energy Transfer (FRET). To circumvent background signal problems associated with established FRET-pairs, we investigated a novel pair of fluorescent proteins and subsequently showed its potential in library screenings by FACS sorting. However, proteins bound to the ER-membrane were suboptimal for analysis in this system, as false-positive FRET signals were observed when investigating interactions between antigen-antibody anchored to the ER.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. Daniel X. Johansson, Katarina Drakenberg, Kathrin H. Hopmann, Alexej Schmidt, Fayezeh Yari, Jorma Hinkula and Mats A. A. Persson. Efficient expression of recombinant human monoclonal antibodies in Drosophila S2 cells.
Journal of Immunological Methods **318**, 37-46 (2007).

II. Daniel X. Johansson, Cécile Voisset, Alexander W. Tarr, Mie Aung, Jonathan K. Ball, Jean Dubuisson, and Mats A. A. Persson.
Broadly neutralizing human monoclonal antibodies mapped to a CD81 binding region of the Hepatitis C Virus glycoprotein E2
Manuscript

III. Alexej Schmidt, Daniel X. Johansson, Rolf Sjöström, Kjell Hultenby, Åsa Holmner-Rocklöv, Anna Hultberg, Mats A A Persson, Thomas Borén and Lennart Hammarström.
A recombinant human monoclonal antibody against the Helicobacter pylori adhesion BabA with an ability to compete with the natural receptor Lewis b.
Manuscript

IV. Daniel X. Johansson, Hjalmar Brismar, Mats A. A. Persson.
Fluorescent protein pair emit intracellular FRET signal suitable for FACS screening.
Biochemical and Biophysical Research Communications **352**, 449-455 (2007)

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LIST OF ABBREVIATIONS

aa	Amino acid
ADCC	antibody-dependent cell-mediated cytotoxic activity
CD81-LEL	Large extracellular loop of the protein CD81
C_H	Constant domain of antibody heavy chain
C_L	Constant domain of antibody light chain
ER	Endoplasmatic reticulum
Fab	Fragment antigen binding (antibody V_LC_L + V_HC_H1)
FACS	Fluorescence-activated cell sorter
Fc	Fragment crystallizable, effector domain of antibody (C_H2-C_H3…)
Fd	Fragment diversity, the heavy chain portion of Fab (V_HC_H1)
FRET	Förster (Flourescence) resonance energy transfer
HAMA	Human Anti-Mouse Antibodies
HCV	Hepatitis C virus
HCVcc	HCV grown in cell culture
HCVpp	HCV pseudo particles
HDL	High density lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Leb	Lewis b
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PCA	Protein Complementation Assay
V_H	Variable domain of antibody heavy chain
V_L	Variable domain of antibody light chain
scFv	Single chain fragment variable (VH fused to VL via a linker)
scFv-κ	scFv where the full LC is included (κ-format)
SR-B1	Scavenger receptor class-B type-1
TcR	T-cell receptor
TLR	Toll-like receptor

AIMS OF THE PROJECT

The studies included in this thesis are aimed at:

- establishing a technological platform for mid-scale production of cloned antibodies allowing their subsequent characterization.
- establishing methods to investigate and screen for antibody-antigen interactions in living cells.

INTRODUCTION

The Immune System

The principal task of the immune system is to identify and eliminate pathogens, both foreign, such as bacteria and virus, and corrupt self tissue, such as cancer cells. Two basic systems, the innate immune system and the adaptive immune system, cooperate in this task.

The **innate immune system** provides an immediate, but non-specific response[1]. Pathogens are identified in a generic way via receptors such as the toll-like-receptors (TLR)[2] recognizing foreign molecular patterns normally not present in our body, but common in microbes. Examples of these pathogen-associated molecular patterns (PAMPs) are double stranded RNA (dsRNA) that is found in some viruses and lipopolysaccharides (LPS) that is part of the membrane of gram negative bacteria.
PAMP-ligands binding TLR may trigger several responses including secretion of cytokines that attract other cells from the immune system to the site of pathogen encounter, and an up-regulation of the general state of alert in surrounding cells e.g. via interferon (IFN)-α [3].
The plasma proteins known as the complement system is an important part of the innate immune system. The complement system triggers recruitment of inflammatory cells and destroys or marks pathogens for destruction, sometimes in cooperation with antibodies of the adaptive immune system.
Another important function carried out by innate immune cells is antigen presentation, i.e. engulfing and digestion of pathogens followed by presentation of the resulting peptide fragments to the adaptive immune system. This is performed by antigen presenting cells (APC) such as dendritic cells and macrophages, but also by B-cells belonging to the adaptive immune system[4].

The **adaptive immune system** reacts slower to pathogens, but instead mounts a specific response that can be improved and strengthened over time. Contrary to the innate immune response, the adaptive immune system is retaining a memory of the pathogen even after it has been eliminated. This immunological memory will allow the adaptive immune system to mount a faster and stronger response should the pathogen be encountered again.
The adaptive immune response is directed and executed by T- and B-cells. Antigens are recognized via cell surface receptors: T-cells carry T-cell receptors (TcR)[5] and B-cells carry surface bound antibodies as receptors.
Each cell clone has a receptor specific for one epitope of a specific antigen, though the total repertoire of these receptors recognizes virtually all possible non-self antigens.

The TcR only recognizes processed antigen peptides presented by another cell via the major histocompatibility complex (MHC) whilst surface bound antibodies recognize the full unprocessed antigen.
MHC class I (MHC-I) is expressed by all nucleated cells in the body and continuously presents peptides derived from proteins synthesized inside the cell[4]. Peptides presented via MHC-I are only recognized by CD8+ T-cells (a.k.a. cytotoxic T lymphocytes). CD8+ T-cells kill any cell presenting a non-self peptide via its MHC-I since this indicates that the cell is infected.
MHC class II (MHC-II) is expressed by APCs and presents peptides derived from phagocytosed antigens such as bacteria, virus or dead cells. Peptides presented via MHC-II are recognized by CD4+ T-cells (a.k.a. T-helper cells or T_h cells) that play a central role in directing the adaptive immune response. Should a CD4+ T-cell be presented its specific antigen by an APC, it differentiates towards either a T_h1 or a T_h2 CD4+ T-cell depending on simultaneous cytokine stimuli[4].

The **T_h1 response** is generally mounted upon encountering intracellular pathogens such as viruses. By secreting certain cytokines such as IFN-γ, the T_h1 cells activate CD8+ T-cells and cells of the innate immune system such as natural killer cells (NK) and macrophages. In addition, a T_h1 response up-regulates antigen presentation in surrounding cells by increasing the expression of MHC molecules. CD8+ T-cells play an important role in elimination of virus infected cells by perforin/granzyme induced lysis or Fas/FasL induced apoptosis[6, 7]. The cytotoxic activity of CD8+ T-cells sometimes harms non-infected bystander cells, as in the case of chronic hepatitis C [8].
IFN-γ and human IL-10 (hIL-10) stimulate B-cells to produce antibodies of class IgG1 and IgG3[9-12]. These are the most common antibody isotypes produced in response to viral infection[13]. IL-10 is secreted by T_h2-cells in mice, but in humans both T_h1 and T_h2 cells secrete this cytokine[14]. hIL-10 is reported to suppress proliferation and cytokine production of all T-cells, but stimulate plasma cells and antibody production[15].

The **T_h2 response** is generally associated with toxin exposure or extracellular pathogens such as bacteria and parasites. This response is mediated by antibodies targeting the pathogens. T_h2 cells secrete cytokines as IL-4, IL-5 and IL-13 that stimulate antigen-activated B-cells to proliferate and differentiate into antibody-producing plasma cells. Antibodies are mainly of class IgG4 and IgE[16-18]. In addition, the T_h2 response activates mast cells and eosinophils.
In humans, exclusive T_h1 or T_h2 responses are usually not seen, but rather an activation of both responses with a polarization towards one of them[19-21]. It is probably more accurate to view the classification of the response as T_h1 and T_h2 as extremes in a continuous spectrum.
In addition to differentiating into effector cells of T_h1 or T_h2 type, CD4+ T-cells may also differentiate into memory T_h cells and T_{reg} cells[4]. T_{reg} cells are important

for maintaining the immunological tolerance by suppressing auto-reactive T-cells. T_{reg} cells also downregulate T-cell mediated immunity towards the end of an immune reaction.

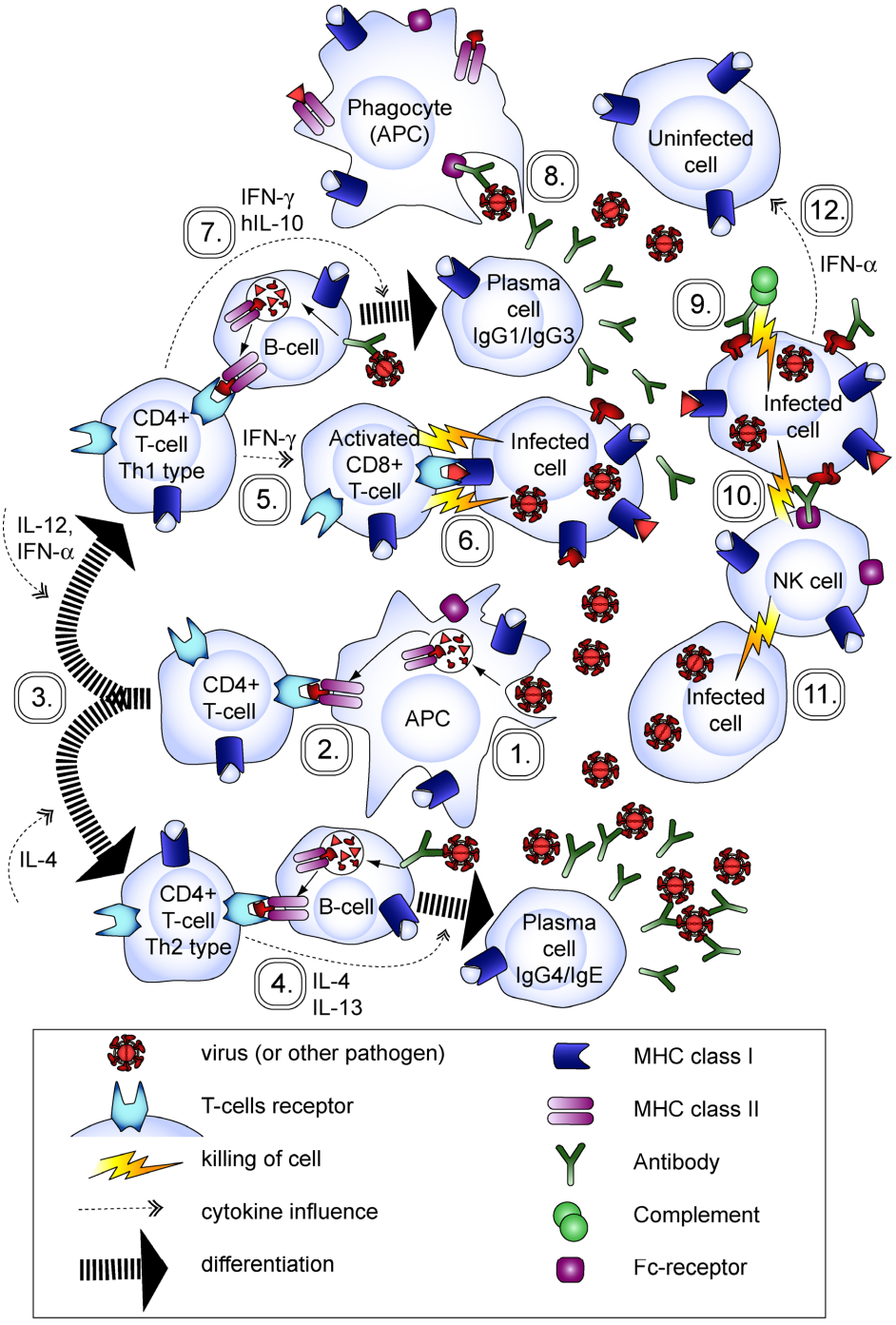

Phagocyte (APC)
8.
Uninfected cell
12.
IFN-γ hIL-10
7.
IFN-α
Plasma cell IgG1/IgG3
9.
B-cell
Infected cell
CD4+ T-cell Th1 type
IFN-γ
Activated CD8+ T-cell
Infected cell
5.
6.
10.
IL-12, IFN-α
NK cell
3.
CD4+ T-cell
APC
Infected cell
11.
2.
1.
IL-4
CD4+ T-cell Th2 type
B-cell
Plasma cell IgG4/IgE
4.
IL-4
IL-13
virus (or other pathogen)
T-cells receptor
killing of cell
cytokine influence
differentiation
MHC class I
MHC class II
Antibody
Complement
Fc-receptor

Figure 1:

1. A virus is phagocytosed by a professional APC. The virus is processed and presented on the cell surface via MHC-II complexes.
2. CD4+ T-cells having a TcR recognizing the presented antigen become primed.
3. Depending on the surrounding cytokine milieu, primed CD4+ T-cells shift into either a T_h1 or T_h2 type cell. A high level of IFN-α (produced by virus infected cells) and IL-12 (produced by APCs) is associated with intracellular pathogens and shift the response towards T_h1[22], while IL-4 is associated with extracellular microorganisms and parasites and shift the response towards T_h2.
4. T_h2 cells secrete IL-4 and IL-13 that stimulate differentiation of B-cells into IgG4 or IgE producing plasma cells. These antibody isotypes are effective against parasites and other extracellular pathogens including toxins.
5. T_h1 cells secrete IFN-γ that activates primed CD8+ T-cells.
6. MHC-I is expressed by all cells and continuously present samples of what is produced inside the cell. Here, an activated CD8+ T-cell is presented viral material by a cell indicating that it is infected. The CD8+ T-cell releases toxic compounds to destroy the infected cell.
7. hIL-10 stimulates the production of IgG1 and IgG3, both potent mediators of antibody-dependent cell-mediated cytotoxic activity (ADCC) and complement activation.
8. An antibody has bound to a virus and thereby opsonized it, i.e. made it more susceptible to phagocytosis. The phagocyte (e.g. a macrophage or a neutrophile) engulf and digest the virus and present the resulting peptides on MHC-II.
9. An antibody has bound a viral protein displayed on the surface of an infected cell. The antibody recruits components of the complement system that destroy the cell.
10. Antibodies binding cell surface displayed viral proteins may also induce ADCC by binding to the Fc receptor of Natural Killer (NK) cells.
11. Some viruses have evolved stealth techniques to avoid detection by the immune system. One such feature is to downregulate MHC-I to make it impossible for CD8+ T-cells to see if the cell is infected or not. However, the NK-cells constantly scan the surface of all cells and should a cell for some reason not express MHC-I on its surface, the NK cell destroys it[4].
12. Cells infected with virus produce IFN-α to inhibit viral replication, but it also warns surrounding cells and upregulates their antiviral defense mechanisms.

Immunoglobulins

Immunoglobulins (Ig) are a class of homologous multidomain glycoproteins making up an important part of our immune defense. Igs are often referred to as antibodies in the literature even though this is a functional definition of an Ig capable of binding a specific antigen. Antibodies are produced by vertebrate B-lymphocytes in response to antigen exposure (e.g. bacteria, virus, toxin or other molecules regarded as non-self). The immune system can generate highly specific antibody clones that bind virtually any antigen, but each clone exclusively binds a specific epitope of a specific antigen.

Upon antibody binding, pathogen activity may be neutralized and the effects of toxins blocked [23, 24]. In addition to the direct sterical effects of binding, antibodies may exert a number of effector functions to help eliminate the antigen such as ADCC and mediating opsonization.
The highly specific binding characteristics of antibodies have made them one of the most useful tools in the fields of research and biotechnology. Antibodies are used in affinity purification, diagnostic tools, detection and labeling of antigens, biocatalysis (catalytic antibodies) [25, 26] and more recently monoclonal antibodies have taken a promising step into therapeutic medicine [27-30].

Antibody structure and isotypes

The monomeric antibody molecule is made up of two identical polypeptide light chains (LC) and two identical heavy chains (HC) all linked by disulfide bridges (see figure 2). LC consists of two structural domains: the N-terminal variable domain (V_L), that is part of the antigen binding site, and the C-terminal constant domain (C_L), that is either of the kappa (κ) or lambda (λ) class. In a similar way HC has an antigen binding variable domain (V_H), while its constant part consists of 3-4 domains (C_H) depending on class. There are five major classes of H-chains in mammals: μ, γ, α, ε and δ. These are further divided into subclasses. The class of H-chain included in an antibody determines the antibody isotype (IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgE and IgD in humans) and hence also its effector functions. Each isotype is evolved to handle a different kind of antigen or to be active at certain stages of the immunological response. IgM functions as the first wave of defense upon an immunological challenge, IgG make up the majority of antibody-based immunity, IgA defend mucosal areas, IgE is involved in defense against parasites and binding to allergens (triggering allergic reactions) and IgD functions as a B-cell receptor. All antibody isotypes are monomeric except IgA (dimeric) and IgM (pentameric).

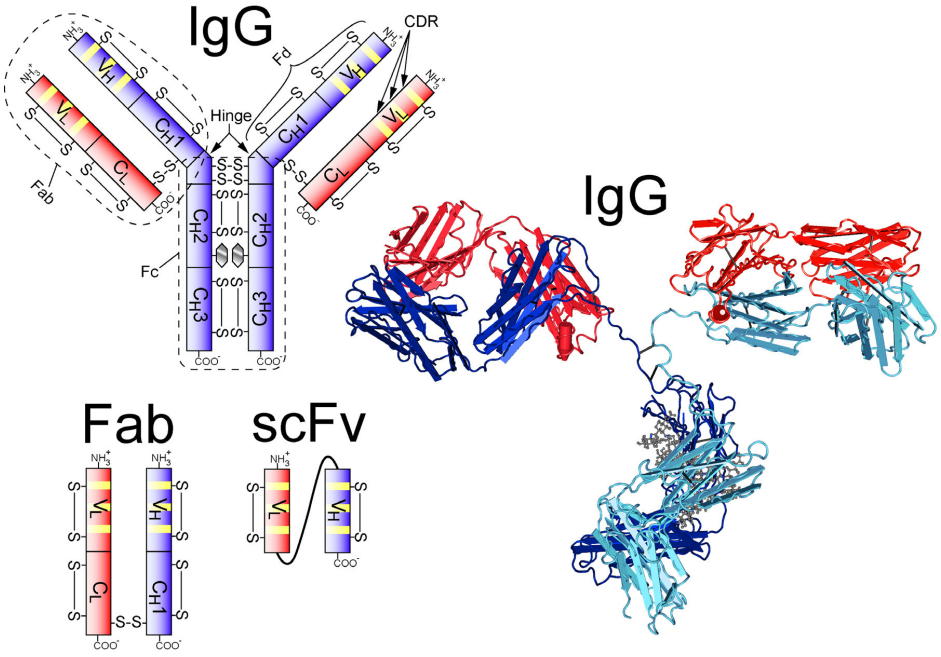


Figure 2: Full length antibody of isotype IgG and antibody fragments Fab and scFv. HC is shown in blue and LC in red. Many inter- and intramolecular disulfide bridges are required to stabilize the structure and the glycosylation on the C_H2 domain (grey) are necessary for mediating effector functions. A molecular structure of IgG is shown on the right hand side.

Functionally, each Ig monomer can be divided into two antigen binding structures (Fabs) and one effector domain (Fc) (see figure 2)
Each **Fab** consists of a light chain together with $V_H + C_H1$ (Fd-fragment) of the heavy chain. The specific antigen recognition of the variable domains is mediated by three hypervariable regions called the complementary determining regions (CDR) each typically 6 to 15 amino acids long. Between the CDRs are the more conserved framework regions (FR) that jointly play an important structural role.

The **Fc** fragment consists of the remaining constant domains of the heavy chain. The C_H2 domain is glycosylated which has been shown important for mediating the effector functions including stimulation of phagocytosis of the antigen [31-33], activation of ADCC[34], activation of mast cells (including allergic responses)[35] and binding to complement that further activates the immune system[33].

Using genetic engineering, antibodies have been modified in a number of ways. These include the expression of the Fab-fragment only or as single-chain Fv

(scFv) consisting of V_H and V_L fused via a polypeptide linker. These smaller fragments are suitable for many biotechnological applications where full Ig is not needed since they are easier and cheaper to produce as they can be produced in prokaryotic hosts. Antibody fragments could also be interesting in certain therapeutic settings because of their lower retention in non-target tissue, more rapid blood clearance and better tumor penetration. However, antibody fragments do not have any effector domain (which is sometimes desirable) and they are commonly expressed as monovalent molecules.

Technologies used to generate monoclonal antibodies

When an antigen is encountered, the humoral response will generate a multitude of different antibody clones, a **polyclonal antibody** response. Serum from immunized subjects thus contains a mixture of antibodies which can be purified and used for binding the antigen. Polyclonal antibodies have been used for decades as research tools, but also in therapy such as venom anti-serum purified from venom immunized animals.
However, there are several limitations with polyclonal antibodies, e.g. production which relies on animal immunization rendering both quantity and quality differences in between batches.

It was not until the advent of the **hybridoma technology**[36] that one single antibody clone, a **monoclonal antibody (mAb)**, could be selected and produced in an adequate manner. The technique is based on immortalizing single antibody producing mouse B-cells by fusing them to myeloma (tumor) cells. Each of the resulting mouse hybridoma cells produces one antibody clone which is screened for antigen binding characteristics. Hybridomas producing interesting mAbs are expanded in culture allowing large scale production *in vitro* with good batch consistency. Contrary to the case with polyclonal sera, the specificity and affinity can be clearly defined for mAbs.

Even though the hybridoma technology was a revolutionary technique, it still suffers from several limitations. Establishing and selecting hybridomas are labor and cost intensive and the fusion step is not very efficient, resulting in poor representation of the immunological repertoire. It is also difficult to control the resulting mAb's isotype and hence its effector function which is a disadvantage in a therapeutic setting. In addition, effector functions of murine Ig administered to humans are usually insufficiently activated, serum half life is short and repeatedly treated patients frequently develop human anti-mouse antibodies (HAMA) which severely reduces the desired effect[37].

Attempts to avoid HAMA have been made by genetically modifying selected antibodies. Among the attempted strategies are the "chimera" consisting of the

originally selected murine V-domains, while the constant domains have been exchanged by corresponding human domains[38].
A more thorough approach is "CDR-grafting" or "humanization", where the entire antibody is replaced by human sequences except for the murine CDRs[39]. Using any of these techniques will result in an antibody having a human Fc-domain which allows full effector functions.

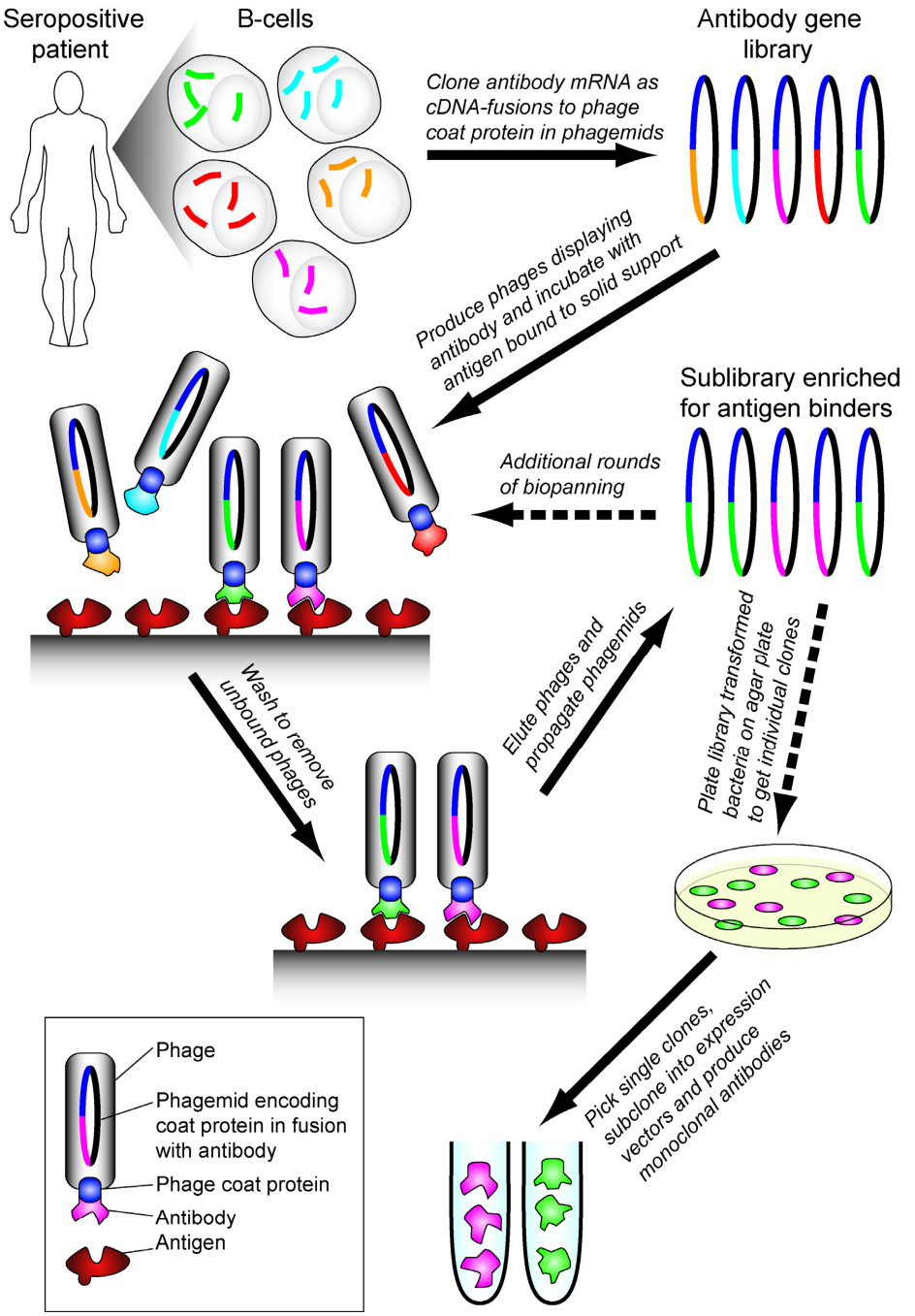
Selecting mAbs from human hybridoma would of course generate 100% human antibodies with null or very small immunogenicity. Unfortunately, the hybridoma technology has shown to function poorly in the human case, mainly due to the lack of a suitable human myeloma cell line. In cases where immunization is needed to stimulate B-cell differentiation, the ethical considerations are more complicated when working with humans than with mice. Another limitation is that the human B-cell repertoire generally does not contain antibodies targeting "self" proteins which represent a large part of the targets for human antibody therapeutics. Approaches to circumvent this fact have been made by moving the human immune system to mice where it matures and regards mouse proteins as "self". Examples of these are the transgenic Xenomouse®, HuMab^{TM} Mouse and the Transchromo Mouse[40].

Another great leap in the field of antibody engineering occurred when it was shown that functional antibody fragments such as scFv and Fab could be produced in *Escherichia coli* (*E.coli*) [41, 42]. This opened up possibilities to express vast cloned **combinatorial antibody libraries** representing a large part of the immune repertoire of an individual and from it isolate single mAbs binding to a certain antigen. The system most widely used to isolate desired antibodies from combinatorial libraries is **phage display** [43-46] (see figure 3). These libraries may contain over 10^{10} individual antibody clones[46]. Each bacteriophage carries genes encoding one of the library antibody members and at the same time displays the translated antibody on its surface as a fusion protein, i.e. genotype and phenotype are connected on the same particle. During the actual screening procedure (e.g. via biopanning) bacteriophages are mixed with the antigen which is coated on a solid support. Phages displaying a relevant antibody clone bind the antigen and stick to the solid phase, whilst non-binding phages are washed away. The rescued phages are usually amplified and subjected to additional rounds of biopanning. Washing stringency is often increased in subsequent rounds to select successively stronger binders from the enriched sublibrary members.
The antibody genes from the resulting isolated phages may then be cloned into expression vectors for production and characterization of the mAb.

Many other techniques for displaying and selecting mAbs from antibody libraries have been reported including display on bacteria[47], yeast[48, 49], baculovirus[50] and cell-free ribosome display technologies[51].

Display systems have a number of advantages over the hybridoma selection technique. First, much greater library sizes are possible with phage display. Theoretically, if the library is large enough it should be possible to isolate an antibody clone recognizing any antigen including self-antigens [52, 53]. Second, the selection conditions and buffer in which antibody-antigen interaction takes place can be controlled, including additions of competitors or blocking of certain epitopes. Finally, affinity maturation *in vivo* reaches a plateau[54, 55] while *in vitro* techniques such as phage display do not suffer from these limitations. This means that higher affinity binders can be isolated by *in vitro* techniques. In addition, the isolated antibody genes can be subjected to affinity maturation by error-prone PCR[56], chain shuffling[57] or other genetic modifications. The resulting antibody mutants are subjected to new rounds of biopanning, isolating even higher affinity binders.

Figure 3: Phage display as performed in paper III: Antibody genes are cloned from B-cells to construct a phagemid library. Phages are produced in *E.coli*. Each phage displays one antibody library member on its surface and carries the genetic material encoding the same antibody. Phages are incubated with antigen bound to a solid phase. Washing removes all phages not binding the antigen. Phages are often eluted by changing pH and the phagemid is propagated. The resulting sublibrary is enriched for antibody genes encoding antigen binding antibodies. The sublibrary may be subjected to new rounds of biopanning to select stronger binders. Single antibody clones are isolated by transforming the sublibrary into bacteria subsequently plated on agar. Each of the resulting bacterial colonies will contain one library member allowing cloning of individual antibody genes.


Seropositive patient
B-cells
Antibody gene library
Clone antibody mRNA as cDNA-fusions to phage coat protein in phagemids
Produce phages displaying antibody and incubate with antigen bound to solid support
Sublibrary enriched for antigen binders
Additional rounds of biopanning
Elute phages and propagate phagemids
Wash to remove unbound phages
Plate library transformed bacteria on agar plate to get individual clones
Pick single clones, subclone into expression vectors and produce monoclonal antibodies
Phage
Phagemid encoding coat protein in fusion with antibody
Phage coat protein
Antibody
Antigen

Alternative systems for screening libraries

Even though the use of phage display has been very successful for isolating specific binders from combinatorial libraries, it still suffers from drawbacks. These include the fact that some antibody clones aggregate or fold incorrectly, thereby excluding them from selection. Normally, the antigen used in biopanning has to be expressed and purified which is not always easily accomplished. Furthermore, the purified antigen has to retain its correct folding during biopanning – something especially troublesome for membrane proteins. Another drawback is the need for subcloning of the isolated mAb genes into eukaryotic expression vectors to allow full length Ig production. Normally, the binding properties of the produced full length mAbs have to be verified again.

It is of great interest to develop a system where both antibody and antigen are expressed in their natural environment – the cell – and still allow tens or hundreds of thousands of library members to be screened in a high-throughput fashion. Such a system would allow full length expression of Ig and correct folding and post-translational modifications of the antigen. As mentioned before, this would be especially beneficial when selecting mAbs against membrane proteins - a group of proteins containing many potentially interesting targets as they fill functions as cell receptors, surface markers for cancer and viral envelope proteins.

The most widely used system for screening libraries for protein-protein interactions *in vivo* is the **yeast-2-hybrid system**[58]. However, this system is not suitable for screening antibody interactions since the actual interaction takes place in the reducing environment of the nuclei. Another limitation is that interactions are limited to soluble proteins, i.e. not membrane bound ones.
To get around these limitations, a set of assays collectively known as **protein fragment complementation assays** (PCAs) has been developed. These are based on the genetic splitting of a reporter protein where one half is fused to a library and the other half fused to the target. Upon [library member] – [target protein] interaction, the two halves of the split reporter are brought in close proximity and reconstituted. This restores reporter functionality which could be fluorescence[59], essential enzymatic activity[60, 61] etc. that enables isolating or selecting for positive interacting clones.

Another interesting technique that has been used for real-time monitoring of protein interactions in living cells is based on the physical phenomenon known as **Förster (fluorescence) resonance energy transfer** (FRET). FRET is a radiation-less transfer of energy from an excited donor fluorophore to an acceptor fluorophore. Upon interaction with an external electromagnetic field, such as light, fluorophores form an oscillating dipole. The donor dipole may potentially influence the acceptor dipoles to start oscillating in synchrony, i.e. energy transfer may occur.

The efficiency of FRET is affected by the relative orientation of fluorophore dipoles and the spectral overlap of donor emission spectrum and acceptor excitation spectrum. The transfer efficiency drops with the sixth power of the distance between the donor and acceptor fluorophores and FRET will only occur at distances typically less than 10nm[62].

A popular set of fluorophores used in FRET is the enhanced cyano fluorescent protein (ECFP) as donor and the enhanced yellow fluorescent protein (EYFP) as acceptor [63, 64], both having a molecular diameter of approximately 3nm. For FRET to occur there must be less than two molecular diameters in between these fluorophores, which is ordinarily only achieved during protein-protein interaction. Positive FRET can be identified by measuring the emission from the acceptor fluorophore after exclusive excitation of the donor. Another way to measure FRET is to compare the emission of the donor before and after photobleaching of the acceptor. An intensified donor emission post-bleaching indicates that it previously was quenched by the acceptor, i.e. FRET occurred (see figure 4).

FRET allows the studying of interactions in any cell compartment, even though studies of antibodies are best performed in the secretory pathway and on the plasma membrane. Interactions in between membrane proteins have been successfully studied using FRET[65].

By genetically fusing a library to the donor fluorophore and fusing the target to the acceptor fluorophore, FRET only occurs if the expressed library member and target interact thereby bringing donor and acceptor together.
Cells expressing large libraries could potentially be screened for FRET using fluorescent-activated cell sorting (FACS). Indeed, FACS has been used to detect FRET for determining whether two specific proteins interact or not[64, 66-68], but inherent spectral problems with the ECFP/EYFP pair has limited their use for library screening applications. The main problems are their overlapping excitation spectra, making it difficult to exclusively excite ECFP, and overlapping emission spectra which make it difficult to exclusively detect EYFP emission. These spectral overlaps result in high background signals.

A new pair of fluorescent protein allowing FRET with low background signal in FACS applications is therefore desired.
Recently, a new FRET pair called CyPET and YPet was developed that showed favorable spectral properties[69]. This pair has since been used to screen a peptide library for specific interactions to a target protein in a prokaryotic system[70]. CyPET/YPet has great potential for future library screenings, including antibody-based libraries in mammalian cells.

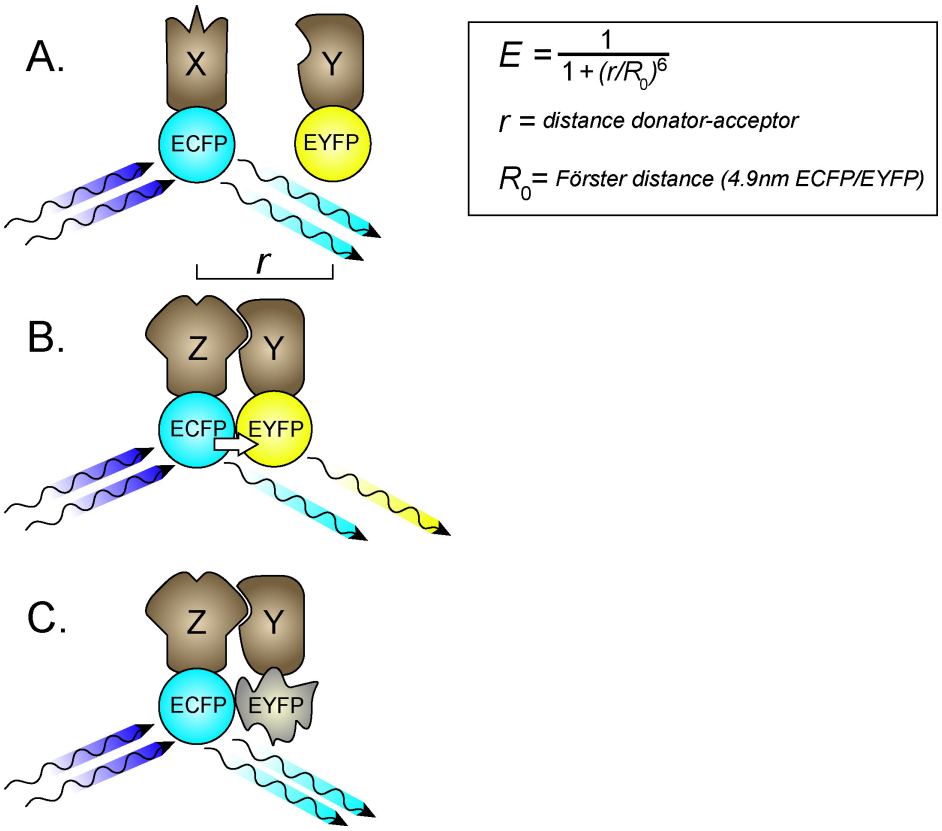


Figure 4: The donor ECFP is specifically excited by a laser and emits light (A). If the acceptor EYFP is close enough, FRET is possible. Fusion partners can mediate fluorophore proximity if they interact as Z and Y do (B). FRET can be detected either by measuring emission from the acceptor (B) or by comparing donor emission before (B) and after photobleaching (C). The Förster radius R_0 is defined as the distance where FRET efficiency is 50%. FRET efficiency rapidly drop with distance and when r = 2xR_0 , it is only 1.5%. R_0 for the pair ECFP/EYFP has been determined to 4.9nm[71]. As a comparison, the diameter of ECFP is about 3 nm.

Expression of recombinant antibodies

As seen in figure 2, a correctly assembled antibody including its post-translational modifications is a very complex molecule. Antibodies are naturally translated by ribosomes residing on the membrane of the endoplasmatic reticulum (ER). Inside the ER-lumen, the H- and L-polypeptides fold and assemble with the help of

chaperones, the many disulphide bonds are formed in the oxidizing luminal milieu assisted by disulphide isomerases and finally the antibody is glycosylated.

After isolating a desired antibody gene, the next step is to produce the mAb in larger quantities to allow further characterization of the protein. This is performed by subcloning the antibody genes (from hybridoma or the isolated combinatorial library member) into a suitable expression vector. Using standard genetic engineering techniques, the mAb gene can easily be manipulated at this stage, changing mAb format or isotype or adding a fusion partner such as a toxin or enzyme to add extra functionality [72, 73].

Several expression systems are currently available for production of mAbs including mammalian cells, bacteria, yeast, insect cells, baculovirus, plants and transgenic animals. The choice of expression system depends on the mAb application (therapeutic, diagnostic, experimental tool), mAb format (whole Ig, Fab, scFv) and required quantity and quality.

Prokaryotic expression systems such as the commonly used *E.coli* have the advantage of high protein expression levels, low cost of production and easy scale-up. However, these systems are best suited for production of antibody fragments rather than full Ig, since polymeric polypeptide assembly is not well supported. One strategy is to express scFv as cytosolic inclusion bodies[74]. These insoluble aggregates are denatured followed by renaturing of the scFv. However, not all antibody clones tolerate this treatment without loosing their binding properties. Another strategy is to add bacterial signal sequences to the mAb genes which will make the proteins end up in the periplasmic space. There, chaperones and the oxidizing milieu assist in forming the many disulphide bridges necessary for mAb folding and functionality. However, the high expression levels often result in the formation of insoluble aggregates[75].
Another disadvantage is the lack of glycosylation machinery in *E.coli* rendering all products unglycosylated which may affect stability, solubility, half-life and, in the case of full length Ig, also effector functions.
Prokaryote production of mAbs is also somewhat unreliable. Even though many mAb clones express successfully in prokaryotes, others missfold or aggregates thereby losing its function. The sequence of the V-regions appear to determine whether prokaryote production of the mAb is successful or not[76].

Yeast such as *Pichia pastoris* is a simple eukaryote capable of glycosylation and secretion. Culturing is fast, cheap and easily scaled up. Yeast grows on chemically defined media and several yeast produced proteins have been validated for therapeutic applications[77]. Antibody fragments as well as full length Ig have been produced in yeast[78, 79], but assembly of LC and HC is often incomplete[78]. In

addition, full length Ig shows altered effector functionality due to dissimilar glycosylation pattern than that of humans.

Mammalian cell expression gives a high quality, correctly glycosylated product. However, culturing and especially scale-up is very expensive. Production levels are relatively low and it is time consuming to generate a high producing stable cell line. Still, mammalian cell culturing has emerged as the method of choice for the production of most commercialized mAbs[80]. Popular cell lines used are Chinese hamster ovary (CHO) cells, mouse myelomas (e.g. NSO) and human embryonic kidney cells (HEK-293). Transient expression in mammalian cells has been used for rapid production of smaller amounts of mAbs[81]. Great progress has been made in this system during recent years and it has become a popular alternative for producing smaller batches. However, transient expression is labor intensive, costly and difficult to scale up. In addition, it is per definition not stable and producing a second batch is as demanding as the first one.

Insect cells have mainly been used as hosts for **baculovirus** expression of mAbs[82]. These cells harbor a more advanced glycosylation machinery than yeast, giving almost the same carbohydrate structures as mammalian cells. Baculovirus driven expression is very high, however a major disadvantage stems from the fact that baculovirus lyses its host insect cell upon infection, narrowing the window for optimal production to 3-5 days and making scale up difficult. In addition, the infection process may have negative effects on post-translational processing[83].
To take advantage of the good expression and processing properties of **insect cells,** attempts to express mAbs in other ways than by baculovirus have been made. However, expression levels have been generally low, even in the case of stably transfected cell lines[84].
From a therapeutic point of view, insect cells as well as yeast do not harbor any viruses that potentially could be transmitted to patients.

Hepatitis C virus

More than 3% of the world population - 170 million persons - are infected with Hepatitis C virus (HCV)[85]. About 70-90% of acute infections become persistent and the resulting chronic hepatitis is associated with an increased risk for liver cirrhosis and hepatocellular carcinoma[86]. Chronic HCV infection is the most common cause for liver transplantation in the world today and will remain so for many years to come.

HCV is primarily transmitted via blood[87] and before diagnostic tests were developed, infection was passed in between patients via blood products, haemodialysis, organ transplantation and sharing of non-sterilized medical equipment such as needles. This is still a problem in regions of the world were

access to, use of or quality of diagnostics are poor while in the developed world, HCV mainly spreads among intravenous drug users. In total, 2-5 million people become newly infected by HCV every year[88, 89].
Since the discovery of HCV in 1989[90] diagnostic methods have been successfully developed and implemented, considerably reducing the incidence of new infections. However, attempts to develop satisfactory vaccines and fully efficient treatments have failed. The available therapy today is a combination of pegylated interferon-α2a (peg-IFN-α2a) and ribavirin[91]. This therapy gives sustained response in only about 50% of the chronic cases depending on the infecting HCV genotype[92-94] and it is associated with substantial toxicity[95] excluding patients who cannot tolerate this treatment[96].

Research on HCV has long been hampered by the absence of a non-primate animal model and the lack of an *in vitro* culturing system. Hopefully, the recent introduction of the HCVcc cell culture system[97-99] that allows *in vitro* culturing of infectious HCV particles, will assist in rapid development and evaluation of new more effective therapeutic drugs and vaccines.

HCV genome, proteome and virion structure

HCV belongs to the genus Hepacivirus of the Flaviviridae family. The genome is a 9.6-kilobase uncapped linear positively stranded RNA (ssRNA(+)). It contains one open reading frame (ORF) flanked by highly structured untranslated regions important for controlling translation and replication[100]. The ORF encode one single polyprotein of about 3010 amino acids which is processed into structural (core, E1 and E2) and non-structural (NS2, NS3, NS4A, NS5A and NS5B) proteins co- and post-translationally by host and viral proteases[101, 102]. The classification of p7 is still debated and its function not yet elucidated, but it has been reported to form ion channels[103]. In addition, a small protein called F (frame shift) is encoded in an alternative reading frame within the core gene[104].

The **non-structural proteins** are involved in the intracellular life cycle of HCV. NS2 is a metallo-/thiol protease responsible for the NS2-NS3 autocleavage[105-107], NS3 is a NTPase/helicase and serine protease responsible for processing of NS3-5B[108-110], NS4A functions as a cofactor to NS3[108, 111], NS4B induces membrane alterations necessary for replication[112, 113] and NS5A is a RNA-binding phosphoprotein[114, 115] closely associated with NS5B which is a RNA dependent RNA polymerase[116].

The **structural proteins** are processed by host signal peptidase cleavage and are integrated into the virions. The core protein forms the nucleocapsid encapsulating the HCV genome[117]. The highly *N*-glycosylated envelope proteins E1 and E2 undergo a complex folding pathway and contain many intramolecular disulphide bridges[118]. Once processed, E1 and E2 are integrated in the virion's surrounding

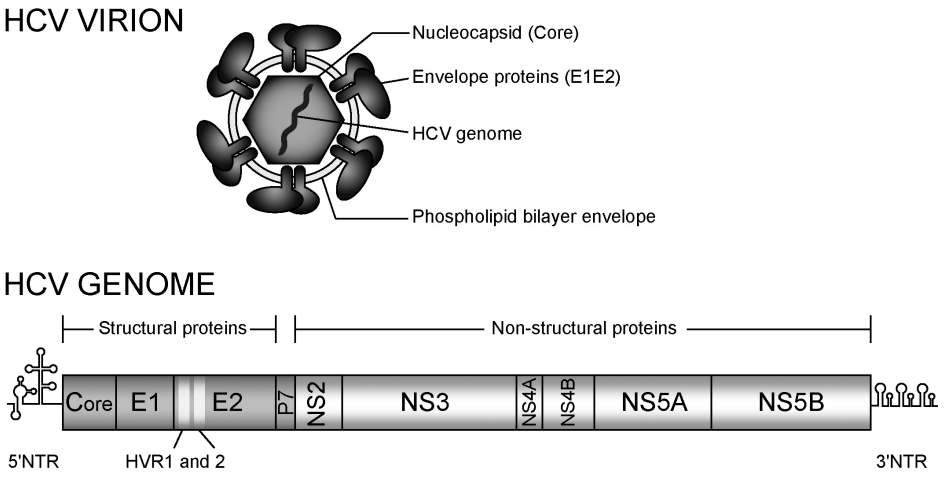


Figure 5: The HCV virion has a diameter of about 50nm and carries the genome packed in a nucleocapsid surrounded by a lipid bilayer envelope. The HCV genome is about 9.6kb and contains one ORF encoding all viral proteins.

lipid bilayer as non-covalent heterodimers, filling important functions for viral interaction with cell receptors and cell entry. An interesting feature of E2 is that it shows a large sequence variation among HCV genotypes, especially in two regions close to its amino terminus called the hypervariable region 1 and 2 (HVR1 and HVR2).

HCV genotypes

As with all RNA viruses, the HCV polymerase lacks a proof-reading function. The resulting high mutation rate has created a considerable genetic heterogeneity of the virus population[119]. Based on nucleotide diversity HCV have been divided into six main genotypes (1-6) with numerous subtypes (a, b etc.). The different genotypes are associated with specific geographic distribution, disease progression and response to therapy.

HCV model systems

The fact that HCV only propagates in human or chimpanzee hosts have made studies of infection and immune response difficult. Small animal models are desired, but so far the few mouse models developed require extensive and laborious procedures including transplantation of human liver tissue [120, 121]. In addition, the immune response and course of infection differ between man and mouse, rendering these models suboptimal.

Some processes of the HCV lifecycle can be studied *in vitro*. Unfortunately, the propagation of HCV in both established cell lines and in primary hepatocytes turned out to be very inefficient. In the absence of a fully propagating culturing system, Lohmann and colleagues created a **subgenomic HCV replicon** by replacing the structural genes with a neomycin resistance gene[122]. Transfected Huh-7, a human hepatoma cell line, did replicate the HCV RNA subgenome under selective neomycin pressure. This allowed detailed molecular studies of basic HCV replication and how this machinery is affected by different therapeutic agents[123, 124].

To elucidate the mechanisms involved in virion receptor interactions, cell entry and virion formation, other surrogate systems has been developed. Among the more thoroughly investigated systems are formation of virus-like particles in insect cells[125], pseudotyped retroviruses (HCVpp)[126, 127] and finally the recently developed HCV cell culturing system (HCVcc) that allow production of infectious viral particles[97-99]. Of these systems, HCVpp and HCVcc are worth a closer look since they have produced a wealth of data.

Bartosch and colleagues developed the **HCVpp** system[126] which relies on the fact that retroviruses frequently and non-specifically incorporate cell surface proteins into the viral membrane as they bud from the plasma membrane. By co-expressing E1 and E2 with a retrovirus lacking its own envelope proteins, the budded viral particles will incorporate E1E2 and has to rely on the envelope proteins of HCV for subsequent cell entry. HCVpp carries a reporter gene, usually luciferase or the green fluorescent protein (GFP)[128], which is used to measure infectivity. This system mimics the early steps of the HCV life cycle and has been used for studies of virus entry and antibody neutralization.

In 2005, three independent groups simultaneously reported the successful production of infectious HCV particles in cell culture (**HCVcc**)[97-99]. They had all used JHF-1, a genotype 2a clone isolated from a patient with fulminant hepatitis C[129]. For some unknown reason, this particular HCV isolate had the ability to propagate in cell culture. For the first time, the entire life cycle of HCV could be studied in one system. The HCVcc system is an important breakthrough for HCV research and opens up for established genetic and biochemical techniques used in virology. Studies exploiting the HCVcc system will most likely result in a plentitude of interesting data over the coming years.

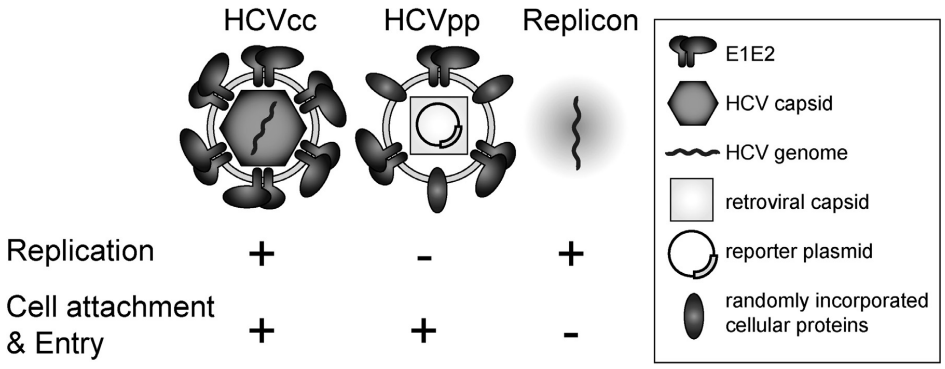


Figure 6: The different *in vitro* systems used to study HCV. HCVcc allows all stages of the cell cycle to be studied while HCVpp only allows studies of cell receptor interaction and entry as well as antibody neutralization. The subgenomic replicon allows studies of HCV genome replication and its effects on the host cell and how replication is affected by drugs.

HCV entry and following life cycle

The viral life cycle is initiated when the HCV virion attaches to a host cell via one or several receptors. HCV uses hepatocytes as its main host for replication, but other cells including dendritic cells and B-cells have been shown to contain negative strand RNA indicating extrahepatic replication[130, 131].

The envelope proteins E1 and E2 are exposed on HCV virions and are thought to mediate attachment to cells by specifically interacting with cellular surface receptors. One of the more characterized interactions is that of E2 and the large extracellular loop of CD81 (CD81-LEL)[132]. CD81 is a tetraspanin known to be involved in membrane fusion processes[133]. It has been shown in several studies that HCVpp only infect human hepatic cells expressing CD81[126, 127, 134-136]. HepG2, a human hepatoma cell line not expressing CD81, is not susceptible to infection by HCVpp or HCVcc. However, by recombinantly expressing CD81 in HepG2, the cells become permissive to infection[98, 134-137] supporting the notion of CD81 involvement in HCV entry. Further evidence have been given by the inhibition of HCVpp entry by knocking down CD81 expression in Huh-7 cells by small interfering RNA[136] and by the inhibition of entry of HCVpp and HCVcc by addition of anti-CD81 mAbs or recombinant soluble CD81-LEL[97-99, 126, 127, 135, 137]. Even though CD81 seems to be crucial for mediating cell entry, this does not explain the hepatotropism of HCV since CD81 is expressed in a wide variety of non-hepatic cell types that is not infected. Most likely, a unique combination of CD81 together with one or several other co-receptors found on hepatocytes and other permissive cells are required for entry.

Among the other suggested candidate receptors are scavenger receptor class-B type-1 (SR-B1) which has been shown to specifically interact with a truncated soluble form of E2 (sE2)[138, 139]. Interestingly, studies have not been able to show interaction between purified E1E2 heterodimer and SR-B1. However, studies with HCVpp support the notion that SR-B1 has a role in cell attachment and/or entry. By pre-incubating the target cells with anti-SR-B1 antibodies prior to infection, HCVpp infectivity is reduced[134].
SR-B1 is a multiligand receptor that among other molecules bind low-density lipoprotein (LDL) and high-density lipoprotein (HDL)[140, 141]. Interestingly, HDL has been shown to enhance HCVpp infectivity in a SR-B1 dependent manner[139, 142, 143]. This is fascinating since both HDL and HCVpp bind SR-B1 while no interaction between HCVpp and HDL has been shown. Indications are that HDL in some manner activates SR-B1 to promote HCVpp entry. Some studies suggest that HCV exploits the physiological lipid-transfer activity of SR-B1 to enter the cell[139, 143].
As CD81, SR-B1 is expressed in a large variety of cell types even though its expression is particularily high in hepatocytes[141]. However, ectopic expression of both CD81 and SR-B1 in non-permissive cells is not enough to allow HCVpp entry[134] indicating that yet more cellular molecules are involved.
Among other proposed receptors are low-density lipoprotein receptor[144] and the C-type lectins L-sign and DC-sign[145-147]. Interestingly, DC-sign is not expressed in hepatocytes, but well on dendritic cells reported to possibly support HCV replication[130].

These cellular receptors may have different functions including initial attachment of HCV to the host cell, internalization of the viral particle into endosomes and finally fusion of the viral envelope with the endosomal membrane. The fusion of the viral and cellular membranes are thought to be triggered by the lowered pH in endosomes as seen in related Flavivirus[127, 148-151]. The capsid enters the cytoplasm and releases the HCV genome. Initially, the genome functions as a messenger RNA (mRNA) for translation of the viral polyprotein. When the viral replication complex has been translated and processed, the viral genome also functions as a template for replication. The subsequent process of virion assembly and budding is largely unknown. Somehow, the viral genome is packed into nucleocapsids composed by the core protein and thought to form virions by budding into the lumen of the ER. Assembled HCV virions have been found close to the ER membrane[152] indicating that budding occurs in compartments before the nucleocapsids reach the plasma membrane. The E1E2 carrying virions are then thought to be released from the cell via the secretory pathway, albeit this has yet to be elucidated.

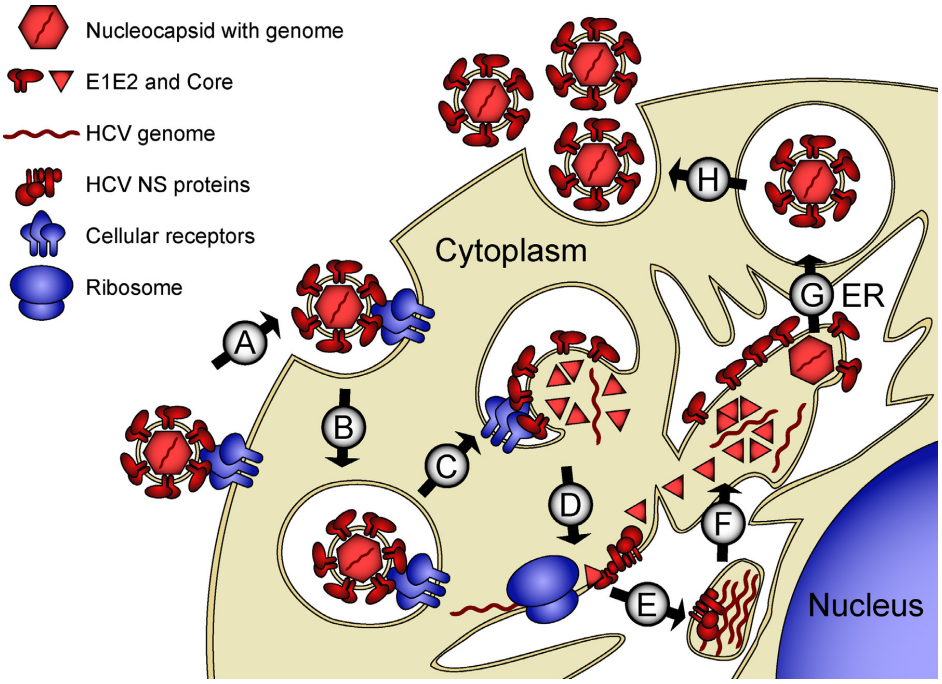


Figure 7: Schematic overview of the putative HCV life cycle. Virus attaches to specific cellular receptors (A) and enters the cell by endocytosis (B). Low pH triggers viral envelope fusing with endosome membrane (C) and the RNA genome is uncoated and translated on ribosomes (D). The translated transcription machinery replicates the viral genome (E) which is packed into core nucleocapsids (F). Nucleocapsids are enveloped by budding (G) and exit the cell via exocytosis (H).

Immunological response to HCV infection and viral evasion strategies

It is generally considered that HCV is noncytopathic and does not kill its host cells[153-155]. However, an immune-mediated inflammatory response (hepatitis) is triggered as the virus multiply and spread. Depending on the efficiency of the induced antiviral immune response during the acute infection phase, HCV infection is either rapidly cleared or progress towards chronicity including a slow destruction of the hepatic tissue due to a long-lasting inflammatory milieu.
The quantitative and qualitative composition of immune responses needed to clear HCV infection are complex and their relative importance have not been fully dissected. Similarly, the role of viral immunosupression, viral escape mutants and impact of host genetic factors are yet to be elucidated. However, it seems like the critical period determining the outcome of the infection is during the early acute phase.

The first line of defense mounted upon viral infection is the **innate immune response** that includes a range of various non-specific components including IFN and NK-cells[156]. Even though HCV induces a strong type-1 INF (IFN-α/β) response, the virus somehow manages to evade the antiviral effects[157, 158]. Studies with chimpanzees have shown that type-1 IFN stimulated genes are expressed regardless of infection outcome suggesting viral resistance to downstream mechanisms in the signaling pathway[159, 160]. Studies using the replicon system have shown that NS3/4A is involved in blocking of the IFN-signaling pathway[161-163], but likely HCV have evolved several strategies to avoid the innate intracellular immune response. NS5A[164] and E2[165] have been suggested to be involved in downregulating IFN response.
Type-1 IFN has many functions including promoting memory T-cell proliferation, preventing T-cell apoptosis, activation of NK-cells, dendritic-cell maturation[166] and upregulation of MHC class I and II peptides[167].
MHC fills important functions in antigen processing and presentation and its HCV induced downregulation might lead to an inefficient activation of CD8+ T cells and an inability of the adaptive immune response to clear HCV-infected hepatocytes[160, 168]. This could be one explanation to why the defects in cellular immunity seem to be HCV specific. The ability of HCV to block IFN effects not only reduces the efficacy of IFN therapies, but may also explain why chronic infections can be established.

Even though the innate immune response most likely does play a role in the clinical outcome of HCV infection, it seems like the **adaptive immune response** is the most important factor. The clearance of acute infection is generally associated with a strong and polyclonal **cell mediated immune response,** while a weak and narrow response is associated with persistence[169-181]. An especially strong correlation exists between viral clearance and a CD4+ T-cell response to non-structural proteins of HCV[170, 172, 181-183]. The importance of CD4+ T-cells for resolving infection has also been shown by treating infected chimpanzees with anti-CD4 antibodies thereby depleting these cells which resulted in HCV persistence[175]. Also, the HCV specific CD4+ T cell response is generally weak, absent or defect in chronically infected individuals, further emphasizing its importance[171, 180, 183-185].
The role of cytotoxic activity of CD8+ T cells in clearing infection has also been examined. One study following the outcome of IFN-α + ribavirin treatment showed that responders had an increased number of HCV specific CD8+ T cells compared to non-responders suggesting that cell mediated cytotoxic activity is beneficial for successfully clearing infection[186]. Also, cell mediated cytotoxic activity has been shown to inversely correlate with viraemia in chronically infected patients, indicating that it limits the viral replication even though it cannot clear the infection[187-189]. It is possible that the lack of help from CD4+ T-cells

render the CD8+ T cell response functionally impaired[190-193]. Indeed, chronically infected individuals have been shown to harbor CD8+ T cells with impaired lytic activity[191] and defect IFN-γ release[177].

The role of the other side of the adaptive immune system, the **humoral response,** is not completely understood in HCV infection. It has been shown that antibodies can neutralize HCV infectivity *in vitro* before being administered to chimpanzees[194]. Other studies have shown a reduced peak viraemia in patients with neutralizing serum during the acute phase[195] and a sustained anti-E2 antibody response in chimpanzees has been shown to have the same effect[196].
In a recent study, chronically HCV infected patients treated with Rituximab (anti-CD20, for treatment of B-cell lymphomas) showed increased viral load upon B-cell depletion, suggesting that B-cells are involved in controlling viraemia[197].
An indirect indication of antibody impact on viral proliferation is the appearance of sequence changes in HVR-1 of E2 simultaneously with seroconversion[198]. This could be the appearance of virus escape mutants forced to evolve to evade antibody neutralization. HVR-1 is a main target for antibodies[199], but HCV escape mutants are readily produced[198, 200-202].

The studies above indicate a protective function of antibodies in HCV infection, but other studies have shown that resolving infection may occur in the absence of seroconversion in humans [203], in the absence of antibodies to E1 and E2 in chimpanzees[169] and even in agammaglobulinemic children[204-206]. Also, the observation that cross genotype neutralizing antibodies often are found in chronically infected individuals, but rarely in those who resolve infection[142, 207, 208] indicates that an antibody response is not necessary for resolving infection.
It appears that HCV also has mechanisms to modulate and impair the humoral response. Viral RNA can normally be detected in patient plasma 1-3 weeks post-infection, however antibodies do not appear until 7-10 weeks post-infection[209-214]. Interestingly, antibodies targeting E1 and E2 appear much later and of less magnitude than antibodies targeting the other HCV proteins[215, 216]. It appears that HCV has the capability to generally delay antibody response, in particular that to the envelope proteins. In addition, raised antibodies are almost exclusively of the IgG1 isotype indicating that the class-switch system is affected too[215, 216].
The strategy to delay the immune response may be especially successful for a highly mutating virus as HCV. Each newly generated HCV genome is expected to contain several nucleotide changes (10^{-3} per nucleotide per generation). In combination with its high replication rate of 10^{12} virions per day in established infections[217] a vast repertoire of genetically distinct quasispecies are produced. Among the many created HCV variants are plausible antibody and T-cell escape mutants that can evade the adaptive immune response[198, 218].
It is possible that once infection is established, the HCV replication exceeds the capacity of the immune system and a chronic situation arises.

In summary, HCV infection persists in the majority of individuals even in the presence of a mounted immune response. It appears that the cellular immunity is more important than the humoral response for determining the outcome of the infection even though the impact of these factors are not completely understood. HCV has evolved a number of mechanisms to evade the immune response including downregulating the effects of the innate immune system, corrupting cell mediated immunity, escaping adaptive responses via mutations and delaying antibody response to the envelope proteins.

Developing vaccines and new therapies for HCV

As mentioned above, no vaccine or completely efficient treatment for HCV infection is available today. There is a great need for a safe, efficient therapy from which the virus cannot escape via mutations.
Many small-molecule HCV enzyme inhibitors have been developed and tested such as NS3/4a serine protease inhibitors[219, 220], but escape mutants have evolved[221-223]. Likewise inhibitors of NS5B and HCV replication[224, 225] have given rise to escape mutants[225-227]. Many of these substances are in clinical trials.

There are studies showing that a vaccine approach is feasible. Previously HCV infected drug users who had resolved the infection were 12 times less likely to become persistently infected than those infected for the first time[228]. Studies have shown that patients can produce a broadly reactive antibody response also when infected by a single isolate[142, 208] and another study showed that raised immunity in chimpanzees could provide protection against different HCV genotypes[229]. These studies indicate that a broad immunological memory can be established and give protection against developing a chronic HCV infection.
However, studies in both humans and chimpanzees have shown that previously infected subjects often are unable to prevent reinfection[230-233].
This is not detrimental for a vaccine approach since the pathological effects of HCV are exerted during the chronic phase. Consequently, the primary goal of a vaccine should be set to avoid establishment of a chronic infection rather than giving sterilizing immunity.

What, then, gives protection against chronicity? In chimpanzees, a vigorous and proliferative CD4+ T-cell response to non-structural proteins has shown to give a long lasting protective immunity[196, 232]. In humans, CD8+ T-cells are maintained after a resolved infection and the number of HCV specific CD4+ cells (T_h1 type) was shown 10 times as many as in chronically infected patients[234].
Both CD4+ and CD8+ HCV specific T-cells are biomarkers for a previous HCV exposure and recovery. Conversely, the levels of anti-HCV antibodies in resolving patients are often undetectable[184].

Again, it seems like the cell mediated adaptive immune response is central in protection, at least in subjects previously infected with HCV. There is a possibility that protective immunity might be formed in a different way in the case of vaccination since the virus itself cannot tamper with the immune processes. Chimpanzees have been vaccinated with purified E1-E2 protein and the highest responders in terms of anti-E1-E2 antibody titers were completely protected against infection[235] indicating an efficient protection by the raised antibodies. Another study in chimpanzees has correlated sterilizing immunity to anti-E2 antibody titers[236].
Various E1-E2 vaccine formulations[237-239] together with different adjuvants have been studied of which many have reached clinical trials.

There is still much to learn before a successful vaccine can be developed. It is of great importance to identify conserved neutralizing HCV epitopes that the virus cannot mutate without loosing viability. These epitopes would constitute suitable components of a future vaccine.
Since antibodies seem to give protection if they are present at the time of infection[235, 236], administering immunoglobulins targeting conserved neutralizing epitopes might constitute an alternative for therapeutic passive protection as pre- or post-exposure prophylaxis. Another interesting application for anti-HCV antibodies would be the prevention of recurrent HCV infection after liver transplantation as is already in practice for hepatitis B virus (HBV) infected patients[240]. HCV reinfection is universal with a high level of viraemia due to immunosuppression of the transplant patient. Passively administered antibodies could suppress or eradicate HCV before it infects the liver allograft. Using monoclonal antibodies to prevent HCV reinfection of liver transplants have shown promising results in animal models[241].
An indirect evidence for antibody mediated suppression of HCV reinfection was shown in a group of liver transplant patients receiving blood containing anti-HBV antibodies (HBIG)[242]. These transplantations occurred before blood was screened for HCV and hence a large number of the donors having HBIG also had antibodies to HCV[243]. Chronically HCV infected patients receiving blood positive for HBIG after transplantation had a much lower incidence of reinfection than other patients (54% vs. 94%) in a one year follow-up. This indicated that the HBIG positive blood contained a protective substance, likely anti-HCV antibodies.

COMMENTS ON MATERIALS & METHODS

Cell lines

Human cell lines

The human hepatoma cell line **Huh-7** was used for expression of HCV E1E2 (paper I and II). Hepatocytes are the natural hosts for HCV and might contain unique features needed for correct E1E2 processing.
The human cervical cancer cell line **Hela-tat** was used for expression of HIV-1 gp160 and its fusions (paper I and IV). These cells have a stable integration of the HIV-1 tat-gene which boost transcription from the long terminal repeat (LTR) flanking the HIV-1 genome. The plasmids used for gp160 expression also contain the HIV-1 LTR, hence needing tat for efficient transcription.

The S2 cell line

The S2 cell line was originally isolated from a primary culture of late stage *Drosophila melanogaster* embryos[244]. It is believed that the cell line is derived from a macrophage-like lineage. S2 cells grow in suspension, at room temperature (optimal is 28°C) and in normal atmosphere. They can be grown in chemically defined media without any addition of serum.

All antibodies were expressed in S2 cells under the tight control of the inducible metallothionein (MT) promoter[245] which is induced by heavy metal ions such as Cu^{2+} and Cd^{2+} (paper I, II and III).
To direct the antibody chains to the secretory pathway, they were fused to the signal peptide of the *Drosophila* BiP homologue HSC72[246].

An interesting feature of S2 cells is their remarkable ability to integrate plasmids into their genome. Multicopy arrays of more than 500-1000 plasmid copies in a head to tail fashion are inserted in a single event.
Stable cell lines are obtained by co-transfection of the MT-driven expression plasmid together with pCoBlast (Invitrogen) conferring blasticidin resistance. Selection of stably transfected cells are made by growing cells in media containing 25µg/ml blasticidin.
The number of inserted gene copies can be manipulated by varying the ratio of expression and selection plasmids. We have used a 20:1 (w/w) ratio of expression to selection vector.

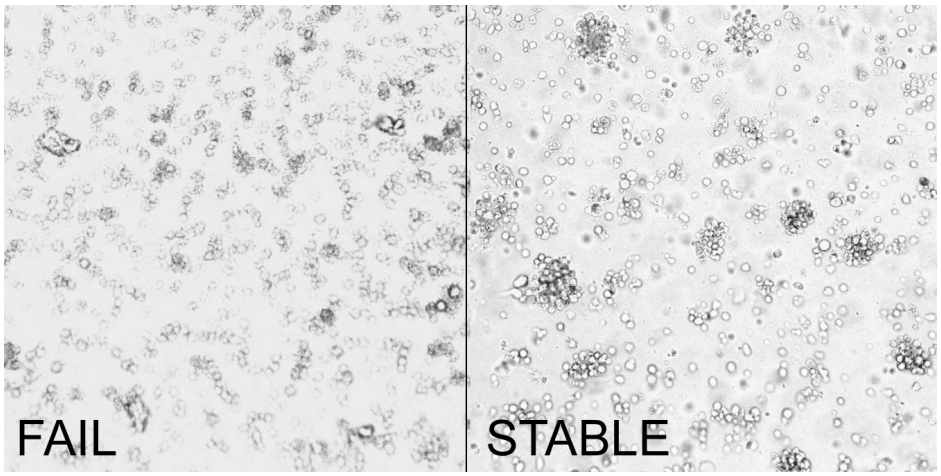


Figure 8: Selection of stable transformants. After 3-5 days, stable transformants form non-adherent aggregates containing 10-20 round cells. Cells not stably integrating the resistance gene are smaller, wrinkled and attach to the plate.

mAb expression in S2 cells

Stably transfected S2 cell cultures were grown in ordinary Ehrlenmayer flasks on a shaker at 90rpm. Expression was induced by addition of 500μM $CuSO_4$ once the cells reached a density $>10^7$ cells/ml. Media containing secreted antibody was generally harvested 10 days post induction. After sterile filtration, media was loaded on HiTrap protein A columns and the antibody purified.

Methods used for protein studies

Western Blot

The standard Western Blot (WB) is a powerful and robust method to detect a protein in a cell lysate or tissue extract. Normally the proteins are denatured by lithium or sodium dodecyl sulfate (LDS/SDS) and often treated with reducing agents to dissolve disulphide bridges. Proteins are then size separated on a polyacrylamide gel followed by transfer to a nitrocellulose membrane.
A primary antibody recognizing the protein of interest is incubated with the membrane followed by washing to remove unbound antibody. The secondary antibody recognizes the primary antibody and is linked to a reporter enzyme allowing detection of the protein of interest. This will result in an accumulation of reporter enzyme at the position of the protein of interest. The amount of accumulated reporter enzyme is dependent on the quantity of the protein of interest on the nitrocellulose membrane, but also on the affinity of the primary (and secondary) antibodies used for detection. Hence, the signal intensity of two

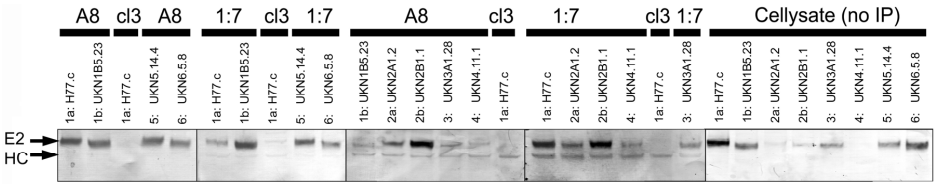


Figure 9: E1E2 from different genotypes were expressed in Huh-7 cells and subjected to immunoprecipitation using mAb cl3 (negative control), A8 or 1:7. Immunoprecipitates were run on WB and E2 detected with a mix of murine mAbs. To assess the murine mAb mixture's ability to recognize E2 of the different isolates, raw cellysates were examined directly on WB. As seen from the blot, genotype 2a, 2b and 4 are not detected efficiently (right blot). Yet, 2a and especially 2b are the strongest bands detected in IP showing that large quantities of E2 protein have been pulled down. This indicates that E2 of genotype 2b (isolate UKN2B1.1) is pulled down by both 1:7 and A8 better than other isolates. The HC of the antibody used in the IP is visible in some of the blots.

different proteins cannot be compared. One can only compare the detection of the same protein using the same antibody.
This is exemplified in paper II were E2 from different HCV isolates are detected with varying efficiency in a WB (see figure 9).
WB will not allow a primary antibody recognizing a strictly conformational epitope to detect its antigen since proteins are denatured. Milder denaturation and reduction protocols can be applied to preserve protein conformation to some extent. Such a protocol was applied in paper III, where the antibody Abba3 only recognized a mildly denatured protein.

GNA capture ELISA of HCV E1E2

Galanthus nivalis agglutinin (GNA) is a lectin with selective specificity for mannosidic type glycans found on HCV E1E2.
E1E2 from a wide range of genotypes were expressed in Huh-7 cells.
Cellysates were incubated in GNA coated ELISA wells resulting in binding of the heavily glycosylated HCV envelope proteins to GNA.
Since different HCV isolates may express E1E2 at different levels, GNA coating was titrated to be the limiting factor for E1E2 binding to the well. This way, the ELISA wells were saturated with E1E2 for all HCV isolates.

mAb epitope determination using alanine substitution mutants

The term "Alanine scanning" refers to protein-protein interaction studies where one of the proteins carries an alanine-substitution mutation at a certain amino-acid position[247]. The investigated protein-protein interaction could be an antibody and

an antigen. By comparing relative binding between the antibody and a collection of differently alanine-substituted antigens, it is possible to draw conclusions of what aminoacid positions are important for the interaction.
Libraries of linear peptides displayed on phages can be used to map linear epitopes[248], but for mapping conformational epitopes, alanine scanning is the preferred method.
However, the introduced substitution mutations in the antigen might induce changes in the overall structure of the protein thereby possibly altering the epitope. A structurally disturbed epitope could also abolish antibody interaction to a certain mutant even though its primary sequence is optimal for binding. Hence, it is of great importance to confirm the overall structure of the alanine-mutants.
To try to map mAb binding epitopes on HCV E2, a panel of H77c derived E1E2 alanine-mutants were used. Each member of the panel contains one alanine substitution and is named according to the identification and position of the wildtype amino acid in H77c, e.g. clone G530A has its glycine in position 530 mutated to alanine. This panel had previously been used to map E2 interaction with its putative receptor CD81[249].
The panel of mutant E1E2 was expressed and GNA captured in an ELISA as described above.
mAbs were then tested for binding to the different mutants. A mutant giving a modified binding signal compared to the wildtype could be an indication of that particular amino acids importance for mAb interaction with E1E2.
The introduced mutations could possibly alter the overall conformation, interaction with GNA or expression levels. To assure that none of these factors influenced the resulting binding signal in the mAb epitope mapping assay, all mutants had previously been checked by a panel of conformation dependent murine antibodies[249]. This verified that they were all correctly folded, with the possible exception of mutants in the 540-550 region. The binding of several antibodies were affected by mutations in this region, even though the antibody's epitope was located elsewhere. This indicates that the region 540-550 might be important for E2 overall conformation.

Neutralization studies

Neutralization of virus, i.e. blocking its infectivity, seems to be accomplished in two ways. For some virus it seems like neutralization is obtained if a critical number of antibodies bind the virion thereby sterically blocking the virus from interacting with its target cell[23]. In this case, neutralization capacity of antibodies binding virions is proportional to antibody affinity.
For other viruses, it seems like neutralization can be obtained if certain epitopes are blocked such as epitopes responsible for virus attachment, interaction with receptors and co-receptors, and initiation of viral envelope fusion with the cellular membrane[250]. In this case, antibodies binding the virion are not necessarily neutralizing virus infection. A low affine antibody may just as well be better at

neutralization than a high affine one depending on what epitope they target[251]. Hence, it is not enough to know that an antibody binds the virion to determine its neutralization properties.

The capacity of antibodies to neutralize virus infectivity was examined in both paper I and paper II.
Neutralization of virus that can be cultivated *in vitro* is normally measured by quantification of total virus a fixed time post-infection, often by quantifying a certain viral protein. The neutralization efficiency is calculated by comparing the amount of viral protein in the absence (set to 100% infectivity) or presence of an antibody.
In paper I the amount of HIV-1 p24 was used as a benchmark for viral propagation, while HCVcc E2 envelope protein was used in paper II.
However, paper II mainly looked at neutralization of the surrogate model HCVpp since only one genotype was available for HCVcc at the time. HCVpp relies on the non-specific incorporation of cellular proteins into the viral envelope as retroviral Gag-Pol buds from the plasma membrane. The retroviral envelope proteins are deleted in the HCVpp system and instead HCV E1E2 are co-expressed. The formed virion will incorporate HCV E1E2 and rely on these envelope proteins for subsequent cell entry. The pseudoparticles carry a vector encoding luciferase that will be expressed upon infection. By measuring luciferase activity, infection efficiency can be determined.
It is known that the formed HCV pseudo particles carry varying numbers of correctly folded and aggregated E1E2[252], rendering some pseudoparticles noninfectious. The ratio between noninfectious and infectious enveloped particles may also vary in between isolates.
This makes it hard to compare neutralization efficiency both in between genotypes, but especially in between HCVpp and HCVcc. However, neutralization data of HCVcc is probably more reliable than of HCVpp. Most likely, the quality and homogeneity of inserted E1E2 is better in HCVcc than in HCVpp.

Detection of antibody interactions *in vivo*

We wanted to develop a system to measure the interaction between antibodies in the scFv-κ format connected to a transmembrane domain (TM) and the HIV-1 envelope protein gp160. Initially, we tried to adapt the yeast split-ubiquitin system to work in our mammalian cell system. This system had previously been used in *Saccharomyces cerevisiae* to study membrane protein interactions[60]. It is based on the interaction dependent reconstitution of a split ubiquitin fused to the interaction partners. Upon reconstitution, a specific protease recognizes the full ubiquitin domain and cleaves off a reporter molecule. We tried two different reporters. The first was the recombinase Cre which upon release is translocated to the nucleus and there recombine a reporter construct leading to expression of fluorescent

ECFP. Unfortunately, we got release/activity of Cre also when singly transfecting gp160 fused to half-ubiquitin-Cre (see figure 10). This indicated that the release was not dependent on the reconstitution of ubiquitin. Mammalian cells possibly contain proteases with different specificity than yeast.

In parallel we tried Arginine-GFP (RGFP) as a reporter using the two constructs gp160-UbC-RGFP and scFv-κ-TM-UbN. RGFP is rapidly targeted for degradation upon release since it contains an N-terminal Arginine[253]. A positive interaction would result in loss of fluorescence. Unfortunately, no difference in fluorescence intensity could be detected in between singly transfecting gp160-UbC-RGFP compared to co-transfection of both constructs.

Since we had problems getting the split-ubiquitin system to work in mammalian cells, we tried another system based on complementation of the enzyme dehydrofolate reductase (dhfr)[254]. This system is based on the interaction dependent reconstitution of dhfr, thereby restoring its enzymatic activity. By using a cell line with a knocked out dhfr gene, reconstitution of the split dhfr was necessary for survival when growing cells in selective media.

Unfortunately, cells did not survive even when co-transfecting both interaction partners carrying the two halves of dhfr. However, transfection of a positive control consisting of one of the interaction partners fused to the full dhfr led to survival of transfected cells.

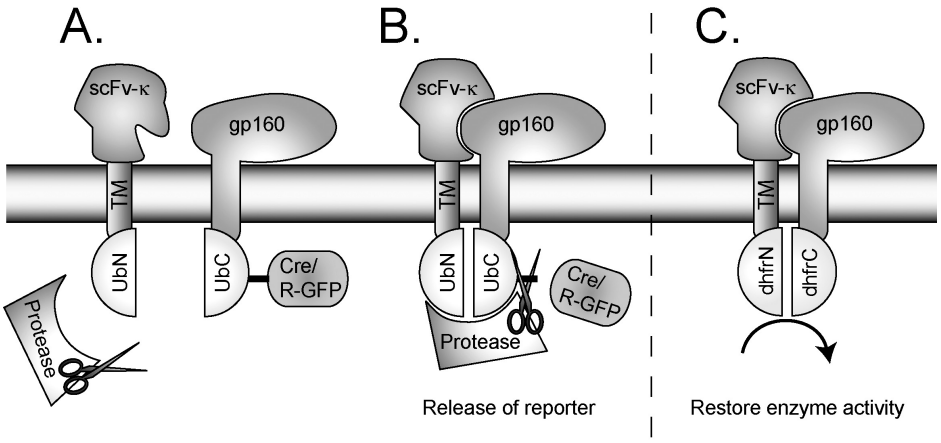


Figure 10: The way the split ubiquitin system (A, B) and dhfr complementation system (C) were supposed to work. Upon interaction between scFv-κ and HIV-1 gp160, the split ubiquitin domain is reconstituted and recognized by a specific protease that releases the reporter (B). In the case of split dhfr, enzymatic activity is restored upon interaction (c).

Our third attempt was based on FRET. To allow high-throughput screening of libraries, the fluorescent signal resulting from interaction needed to permit detection by FACS.
The most commonly used protein fluorophore pair used for FRET is ECFP and EYFP. These proteins allow efficient FRET, but have a significant spectral overlap both for excitation and more importantly emission. These properties result in a significant portion of false positives which is suboptimal in a library screening situation where only a minor fraction are true positives.
Our goal was to find a new pair of fluorescent proteins with the following properties:

- allows efficient FRET in cell environment
- excitation of the donor should be possible without any background excitation of the acceptor
- detection of emission from the acceptor should possible without any background detection of donor emission.

Based on their spectrograms and other fluorescent properties, the fluorescent proteins ECFP and HcRed[255] were chosen (see figure 11).

A Becton-Dickinson FACSDiVa Sorter was used to measure FRET in FACS. ECFP was excited by a 457nm laser and emission from HcRed was detected in FL4 (figure 16).
Photobleaching FRET (pbFRET) was quantified in a Leica TCS SP inverted confocal scanning laser microscope using a 40x/1.4 NA objective. A region of interest in a fixed cell was chosen and fluorescence intensity of ECFP and HcRed was individually quantified by excitation with 457nm and 543nm respectively. HcRed was then irreversibly photobleached by continuous excitation for 30-90s with 543 and 633nm lasers. The fluorescence intensity of ECFP and HcRed was then individually quantified again. The FRET efficiency was determined by comparing the intensity of ECFP before and after bleaching of HcRed. Calculating a valid FRET efficiency from pbFRET relies on the complete bleaching of the acceptor without bleaching the donor, something that could be difficult to obtain.

FRET is a sensitive method giving very low background if the fluorophores are chosen carefully. The energy transfer only occurs if the fluorophores are in very close proximity. However, a positive FRET signal is not necessary a *specific* interaction. There could be other reasons why the fluorophores are in close proximity such as protein aggregation.

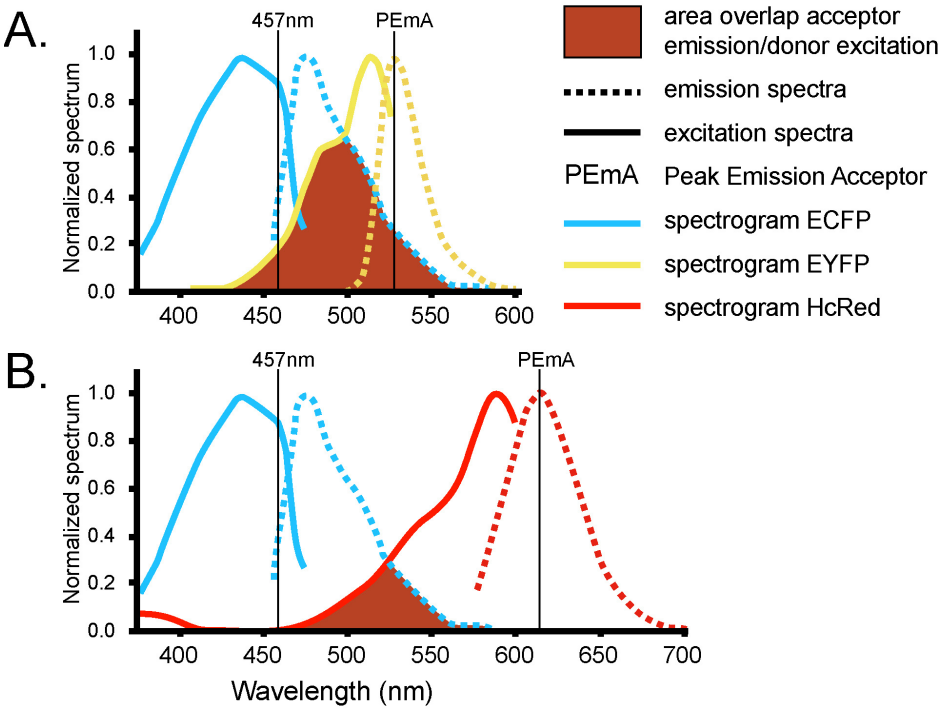


Figure 11: Spectrogram of FRET pairs ECFP/EYFP (A) and ECFP/HcRed (B). As seen from the top spectrogram, excitation of ECFP using a 457nm laser will also excite EYFP to some extent. When measuring EYFP emission, ECFP emission is overlapping. These overlaps will cause false positives. On the contrary, excitation using 457nm laser will not excite HcRed, and emission from HcRed is clearly distinguished from ECFP. ECFP-HcRed gives a clear FRET signal, but is less efficient at transferring energy.

RESULTS AND DISCUSSION

Exploiting *Drosophila* S2 cells for mid-scale mAb production

The high specificity of antibodies has made them one of the most important tools in biological research for decades. Today they are also used routinely in diagnostics and are emerging as an important new class of therapeutics. Nothing indicates that the demand for antibodies will cool off for a foreseeable future. On the contrary an increasing number of antibodies are isolated from combinatorial libraries.

These newly isolated mAbs need to be produced to allow characterization including determination of affinity and specificity, mapping of epitope and to studying behavior and interactions in a biological context.
Production in prokaryotes and transient expression in mammalian cells are both alternatives for rapid mid-scale production of mAbs, but both have limitations. Neither one make up a really good alternative for routine production of experimental amounts of mAbs especially when a larger number of antibody clones are to be characterized as is often the case when screening antibody libraries.
In Paper I we investigated the possibilities of using stably transfected *Drosophila* S2 cells for mAb expression.

S2 cells grow in suspension, in room temperature, in normal atmosphere and can grow on chemically defined media which are considerable advantages.
The S2-expression vectors were designed to allow easy transfer of isolated mAb genes. pMThIgG1 allows transfer of Fab genes from phagemid vectors such as pComb3 and pComb3H[256-258] while pMThIgG1-V allows transfer of V-genes cloned from hybridomas or from isolated scFv. Expression in both vectors is tightly regulated by the strong inducible MT-promoter.

In our hands, the system performed over expectations with mAb expression levels of 5-35μg/ml from stable cell lines. The high expression level could possibly be explained by the extreme multicopy gene insertions taking place in these cells during selection. Another reason explaining the efficient secretion and high quality of produced mAbs could be the presence of HSC72, the *Drosophila* homologue of the immunoglobulin binding chaperone protein (BiP). This chaperone has been shown to bind expressed Ig H-chain in S2 cells[246] and likely assist its folding.

Time is also an important factor for an expression system aimed at rapid characterization of isolated candidate mAbs. The S2 cells turned out to perform well also in this respect. Stable cell lines were obtained 2-3 weeks after transfection with no need for further gene amplification rounds as is often necessary for mammalian CHO cells which is a process that may require months

to complete. Milligram quantities of mAb could be obtained within a month post initial transfection.
A series of experiments were conducted to assure that the binding properties were identical for S2 produced mAbs to that of CHO produced ones. Matching performance of a mAb expressed in the two systems was shown in ELISA antigen binding, immunofluorescence staining of fixed antigen expressing cells and neutralization of virus infectivity.

We have not made any attempts to optimize expression levels and the S2 cultures were grown in ordinary Ehrlenmayer flasks on a shaker at room temperature. Most likely, expression levels could be increased significantly by optimizing cell culture density, oxygenation, temperature, induction conditions, shaking and/or stirring. As seen from the expression curves, mAb is accumulating in the media even at the time of harvest (see figure 12). This indicates that the starting culture could be denser or time of expression longer for maximal production.

Once the mAb has been characterized using S2 mid-scale production, the step to further scale up production would be easy since the stable cell line is already established. This is contrary to transient mammalian mid-scale production which must be succeeded by the time-consuming process of establishing high-expressing stably expressing CHO cells for large scale production. Possibly, the S2 system could also be used for large scale or industrial scale production of mAbs.

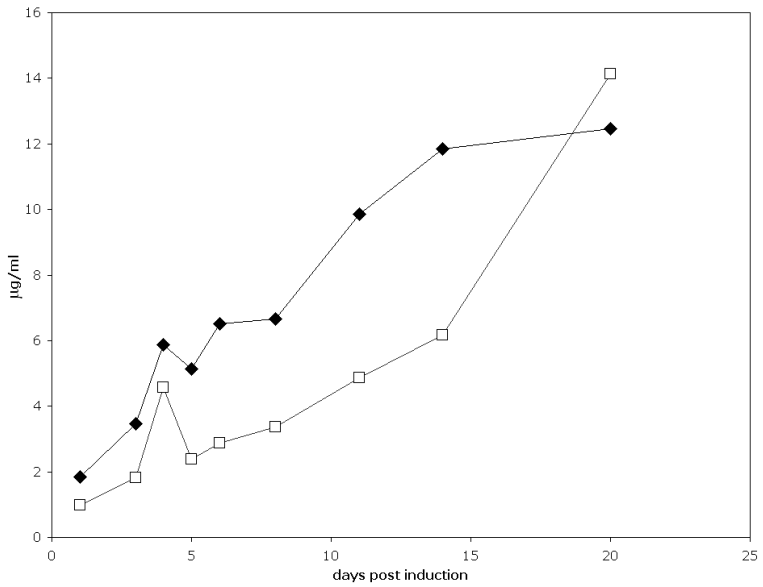


Figure 12: The accumulation of two different mAbs in culturing media over time. Even after 20 days, production is still high.

We have not investigated the behavior of S2 produced mAbs in an *in vivo* context. Glycosylation differs between insect cells and mammalian cells with *N*-glycans generally more trimmed in the former (reviewed in ref [259]) which affects the *in vivo* properties. It is known that lack of/heterologous glycosylation of antibodies lowers serum half-life since the H-chain glycosylation is essential for interaction with the so-called neonatal Fc receptor (FcRn)[260] also known as the Brambell receptor. This interaction is necessary for antibody recirculation since it rescues antibodies from proteolysis in the endothelium. In addition, glycosylation differences might affect effector functions[261] and trigger allergic reactions[262-264]. However, insect cell produced antibodies have been shown to retain intact effector functions such as ADCC and complement activation[265].

Human Ig *N*-linked glycosylation is of the complex biantennary type and exhibits heterogeneity with respect to the terminal sugars attached (see figure 13). Analysis of erythropoietin (EPO) expressed in S2 cells showed that oligo-saccharides mainly consisted of paucimannose or higher mannose structures [266]. It has been shown that the presence of certain terminal carbohydrate moieties may trigger lectin mediated clearance in humans. Glycoproteins lacking terminal sialic acids are cleared via the asialoglycoprotein receptor[267, 268] and terminal acetylglycosamine (GlcNAc) or mannose is cleared via several receptors in the reticulo-endothelial system[269].
To obtain long serum half-life of recombinant glycoproteins, the ambition should be to produce them fully sialylated.

Lepidopteran cells mainly used with the baculovirus expression system have been genetically engineered to produce complex sialylated proteins[270]. It is thought that these cells naturally lack the enzymes and substrates needed for sialylation[271-274]. However, *Drosophila* has been shown to contain both sialic acids[275] and a functional α2-6-sialyltransferase[276] contrary to the more commonly used lepidopteran cells. This indicates that *Drosophila* have all the necessary constituents to sialylate *N*-glycans even though it seems like this only occurs during certain developmental stages[276].
It would be interesting to try to genetically engineer S2 cells to activate or complement the endogenous pathways to allow sialylation.

It should be noted that the most commonly used system for expression of therapeutic mAbs, CHO cells, do not produce fully human homologous glycosylation. Addition of *N*-glycolylneuraminic acid[277, 278] might lead to rapid clearance by anti *N*-glycolylneuraminic acid antibodies since this type of glycan is not present in humans[279]. CHO produced mAbs are generally of the G_0, G_1 or G_2 type, i.e. not sialylated[280] (see figure 13).

The number of recombinant antibody-based products in clinical trials and entering the market is steadily increasing and is predicted to do so for many years to come[281].The mAb production capacity of the industry does not satisfy current demand and there is a great need for development of new systems allowing more efficient and cheaper production of mAbs.
Testing S2 produced mAbs in preclinical (animal models) and eventually in a clinical setting would demonstrate the S2-expression systems potential for production of therapeutic antibodies.

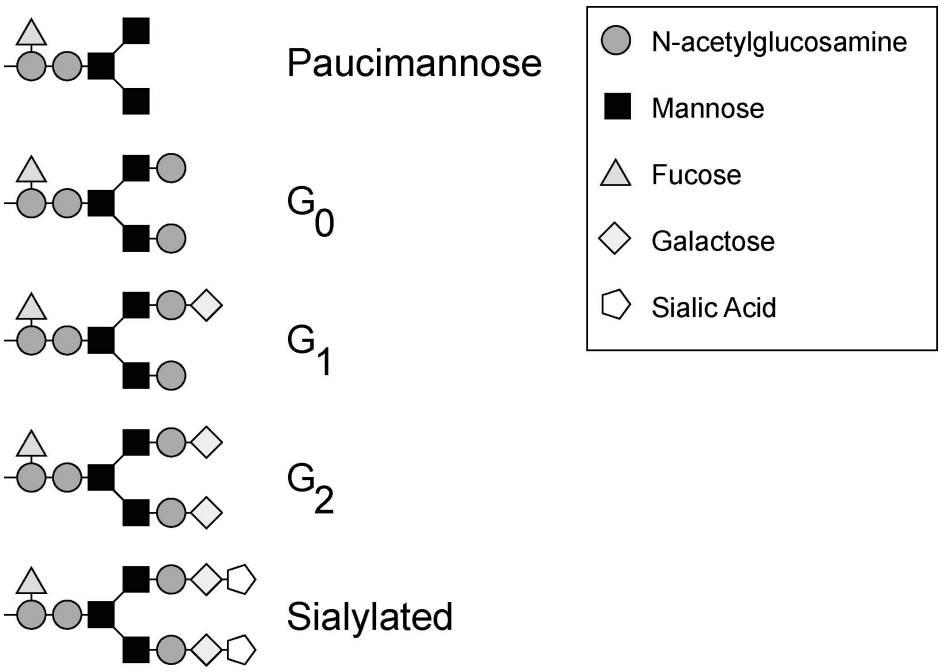


Figure 13: different types of *N*-glycosylation. Insect cells are thought to mainly form paucimannose. Human Ig glycosylation is generally of G_0-, G_1-, G_2-type or sialylated. Human glycosylation machinery sometimes adds extra oligosaccharide chains forming tri- or tetra-antennary complexes. CHO cells mainly glycosylate Ig to the G_0, G_1 or G_2 forms.

Characterization of broadly HCV neutralizing antibodies

The HCV envelope proteins are extremely variable in their amino acid sequence[282], but some residues critical for cell interaction and entry seem conserved across the different genotypes.
These conserved regions comprise interesting targets for HCV entry inhibitors and make up suitable vaccine candidates. The reason for this is twofold: first, the same therapy should be able to treat infections of all different genotypes of HCV and second, HCV escape mutants are not easily generated.
In Paper II, we looked closer at three antibodies known to bind HCV E2 called 1:7, A8 and L1. These had previously been isolated from an antibody library constructed from a patient infected with HCV gt2b, but biopanned against purified gt1a E2 protein[283]. This approach aimed at isolating antibodies targeting residues conserved in between the two HCV isolates. Interestingly, all isolated mAbs using this approach were shown to inhibit E2 interaction with CD81, a cellular receptor needed for HCV cell entry[283].
The aim of paper II was to characterize the binding of these mAbs including determination of affinity, mapping of binding epitope, genotype binding range and to investigate whether they neutralized HCV infection using HCVpp and HCVcc model systems.

To produce the amount needed for these studies, the three mAbs were expressed and purified as IgG1 using the S2 expression system described in paper I.
GNA capture ELISA revealed that all three mAbs bound E1E2 from the gt1a isolate H77c with Kd values in the nanomolar range.
Next, the genotype binding range was investigated in another GNA capture ELISA where mAb 1:7 and A8 bound broadly over all genotypes.
Huh-7 cells expressing E1E2 from various genotypes were immunofluorescently stained (IF) or lysed and subjected to immunoprecipitation (IP) using either 1:7 or A8. These experiments confirmed the broad genotype binding range of these mAbs.
The signals from IF staining and IP were not easily quantifiable, but the IP showed that both 1:7 and A8 clearly precipitated gt2a (UKN2A1.2) and gt2b (UNK2B1.1) better than the other gts (see figure 9).
The described experiments showed that 1:7 and A8 had a broad genotype binding range in binding E1E2 protein. Indeed, the mAb epitopes seemed markedly conserved and thereby possibly important for HCV functionality.

Binding is necessarily not the same as blocking infectivity[250] and the next question was whether the mAbs neutralized HCV infectivity when binding these epitopes.
A series of neutralization experiments were performed to answer this question.
Since HCVpp representing all genotypes were available, this system was chosen to study the cross-genotype neutralization of the mAbs.

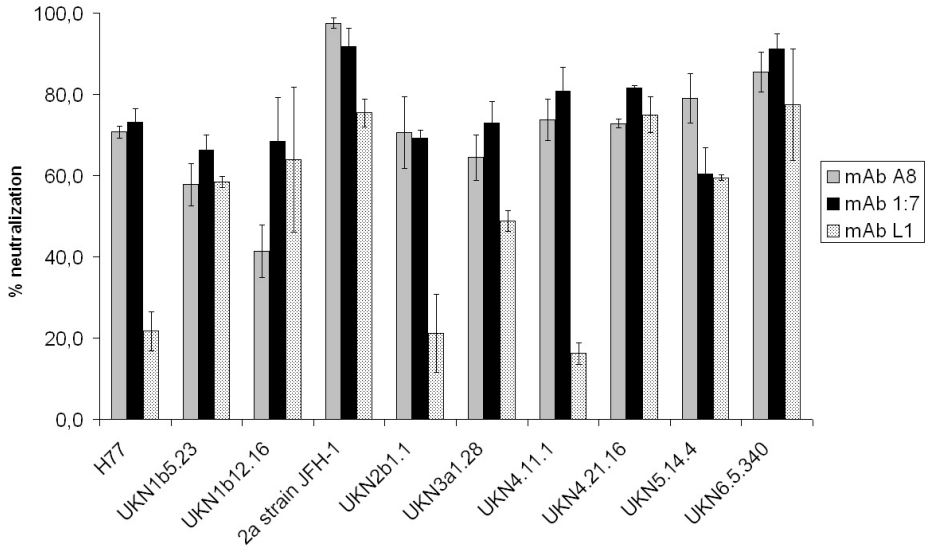


Figure 14: neutralization of HCVpp. mAb 1:7 and A8 showed good neutralization capacity over a broad range of isolates while L1 had a more limited range.

Again A8 and 1:7 showed a remarkably broad reactivity, while the range of L1 was more limited (see figure 14).
Next, the mAb ability to neutralize HCVcc was investigated. This model is more relevant than HCVpp since it is closer to the real *in vivo* process, but at the time we only had access to one single isolate of HCVcc (gt2a JFH-1).
A8 and 1:7 both efficiently neutralized HCVcc infection (IC_{50} 560ng/ml and 60ng/ml respectively), while L1 failed at all concentrations tested.

The neutralization capacity of L1 is interesting since it neutralizes the gt2a isolate JFH-1 in the HCVpp assay (75% - see figure 14), but not in the HCVcc assay (0%) at the concentrations tested. Explaining this is not straightforward. One possible explanation could be that much fewer E1E2 are present on each HCVpp particle than in HCVcc. This is not far-fetched as HCVpp randomly integrate proteins residing in the plasma membrane, while HCVcc most likely specifically incorporate E1E2. The difference in neutralization capacity would be many times magnified the more envelope proteins were incorporated, since it is likely that the majority of E1E2 need to be blocked on a particle to stop cell entry. Hence, two antibodies with only a small difference in affinity might look similar in HCVpp, but very different in HCVcc.
The difference could also be explained by the differing virion assembly process of HCVpp and HCVcc, where the epitope recognized by L1 might be hidden in

HCVcc. Again, this could be due to a more tightly E1E2 packed envelope of HCVcc.
Another explanation could be that L1 only recognizes infectious virions while 1:7 and A8 recognizes also non-infectious virions. If most HCVpp particles are noninfectious due to missfolded E1E2[252], the neutralizing effect of 1:7 and A8 will be underestimated compared to L1 in the HCVpp assay.
In the following HCVcc assay 1:7 and A8 will perform much better relative L1 since the quality of these virions are better and relatively more of them infectious. Possibly, L1 will neutralize HCVcc, but at higher concentrations.

It is hard to compare the absolute IC_{50} figures in between HCVpp and HCVcc due different characteristics of the particles both quantitatively and qualitatively.
It would be interesting to test the mAbs in neutralization experiments of HCVcc of other genotypes. Hybrid-genotype HCVcc has recently been created by replacing the structural genes in the original genome with that of other genotypes[284]. These hybrids make up obvious tools for future neutralization studies since HCVcc most likely is closer to the real virus than HCVpp.

High-density lipoproteins (HDL) have been suggested to reduce the neutralization effect of antibodies blocking E2-CD81 interaction [285], which both 1:7 and A8 do[283]. Neutralization of HCVpp by 1:7 and A8 in the presence of HDL has been performed and preliminary data show a 10-20% reduced neutralization capacity in the presence of HDL.

The data from our GNA capture and HCVpp neutralization implied that both A8 and 1:7 bound cross-genotype conserved neutralizing epitopes. Such epitopes make up interesting targets for both entry inhibitors and for use in vaccination. The epitopes were mapped using a panel of E1E2 single alanine-substitution mutants based on the gt1a H77c isolate. Interestingly, many of the residues critical for 1:7 and A8 binding to E2 had previously been shown critical for E2 interaction with CD81. The epitopes of both 1:7 and A8 were mapped to the amino acid region 523-535 with G523A, W529A, G530A and D535A entirely destroying the interaction. These residues were all conserved over a wide range of isolates.
The alanine-mutant panel was based upon the gt1a isolate H77c which L1 bound poorly as seen in GNA capture ELISA. Hence, the epitope of L1 could not be mapped using this panel.

mAb 1:7 and A8 both neutralized HCV infection over a wide range of isolates. It would be interesting to add these antibodies in sub-neutralization concentrations to HCVcc cultures and see if escape mutants emerges. This would show if the antibodies target an epitope requiring conservation for HCV functionality. If this is the case, not only are the mAbs interesting for therapeutic applications, but their epitopes make up possible vaccine constituents.

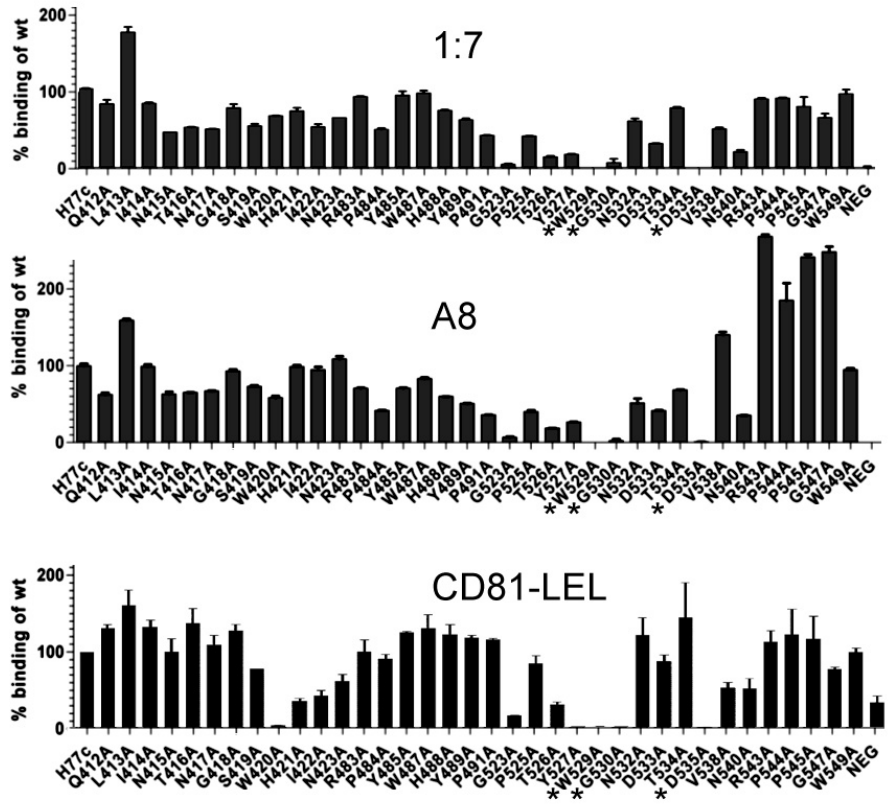


Figure 15: mAb 1:7 and A8 binding to a panel of E2 alanine substitution mutants. Residues G523, W529, G530, D535 are critical for E2 recognition by both mAbs indicating that they have overlapping epitopes. A previous study using the same panel of E2 mutants showed that W420, Y527, W529, G530 and D535 are critical for CD81-LEL interaction[249]. As seen, W529, G530 and D535 are critical residues for E2 interaction with both CD81 and 1:7/A8.

All three mAbs examined in this paper had previously been shown to recognize a conformational epitope [283], just like many other characterized HCV neutralizing mAbs[286-288]. In the vaccine setting, conformational epitopes might be suboptimal. To support the correct fold of the target epitope, portions of the rest of the protein have to be included. Vaccinations using this construct would result in many off-target antibodies raised against structurally supporting regions that might constitute non-neutralizing epitopes or non-conserved regions. This is especially true if immunodominant regions such as the HVR[238] are included.

On the other hand, if antibodies are raised to a conformational epitope only existent on infectious clones, the efficiency would be greatly increased. Much of the circulating HCV virions might be non-infectious as studies have shown that the ratio of HCV RNA : infectious titer is in the range of 10-100 in serum[233, 289].

However, conserved neutralizing linear epitopes might be more suitable for use in vaccination since their conformation is only dependent on the primary sequence of the peptide itself. Vaccination with a short, easy-to-manufacture peptide would raise an immune response only to the interesting epitope.
It would be interesting to try to select antibodies to conserved linear and neutralizing regions. This could possibly be done by using envelope proteins derived from different genotypes in the different biopanning rounds in combination with rounds against denatured protein.
Identifying these linear targets would be an important step in the development of cross-reactive vaccines. Indeed, broadly reactive mouse mAbs targeting linear epitopes have been isolated[290, 291].

Even though the conformational epitope targeted by 1:7 and A8 is not optimal for vaccination, the antibodies are still very interesting for passive immunization. As mentioned in the introduction, liver transplant allografts are often reinfected. Normally, patients are immunosuppressed to avoid rejection of the graft which takes the immunological pressure off HCV. The virus vividly propagates and reaches high level of viraemia. The result is often damage or loss of graft.
In this setting, administering neutralizing mAbs could potentially be very effective to battle reinfection. Since they are blocking viral infection without the need of an activated immune system, neutralizing mAbs would still be efficient even if the patient is immunosuppressed. The potential of anti-HCV antibodies administered after liver transplantation have been indirectly shown[242].

The most successful strategy would probably be to administer a cocktail of mAbs targeting several neutralizing epitopes on E2 in combination with HCV enzyme inhibiting drugs. Possibly, mAbs targeting E1 would be an interesting supplement as one study showed that all patient sera able to neutralize HCV contained demonstrable levels of antibodies to both E1 and E2[216]. Antibodies targeting E1 alone are capable of virus neutralization, further underscoring E1 as a suitable neutralization target[284].

Another aspect worth considering is what antibody isotype to use in therapy. HCV virion contains many envelope proteins and neutralization of infectivity probably only occurs if antibodies block the majority of E1E2. However, in the case of opsonization, one single antibody binding a virion might be enough to have it destroyed. It is interesting to note that antibody class switching seems blocked in HCV infection and that the majority of produced antibodies are IgG1[215, 216]. It has

been shown that IgG3 is more efficient than IgG1 in blocking HIV-1 infection[292], something possibly worth investigating also for HCV. However, the serum half-life of IgG3 is shorter than of IgG1 which is a non-desirable property in therapy.

Human monoclonal antibody targeting *Helicobacter pylori*

The aim for paper III was to select an antibody inhibiting the interaction between *Helicobacter pylori* protein BabA and fucosylated blood groupABO/Lewis b (Leb) antigens. This interaction is involved in *H. pylori* attachment to the gastric mucosa, where colonization is associated with peptic ulcers and gastric cancer[293].
A combinatorial antibody library in scFv-format was constructed based on the antibody repertoire of three BabA seropositive patients.
Using phage display, an antibody named Abba3 was selected by biopanning against purified BabA protein.

Abba3 was expressed as scFv in *E.coli* and tested for binding against *H. pylori* in an ELISA. scFv Abba3 bound *H. pylori* strain 17875/Leb which do express BabA, but not strain 17875/DM which has deleted *babA*-genes.
scFv Abba3 also bound purified BabA in a Western Blot, but only if BabA had been mildly treated. Full denaturation at 96°C and treatment with reducing agents destroyed recognition, indicating that ABBA3 is targeting a conformational epitope. The milder denaturation protocol of BabA is also a prerequisite for Leb binding to BabA in ImmunoBlots[294].
Abba3 was subcloned into the vector pMThIgG1 and expressed as human IgG1 in the S2 system described in paper I.

Different concentrations of Abba3 were incubated with a constant amount of radioactively labeled Leb to test for competitive binding against *H. pylori* strain 17875/Leb. Abba3 competed efficiently in both scFv and IgG format reducing the Leb binding to 50% at 247pM and 47pM respectively. As seen from this data, IgG was about 15 times more efficient/mol binding site in IgG format than as scFv. This could be explained by a better quality of produced IgG than of scFv, but also by IgG avidity effects by binding to the potential multimeric BabA.
Electron microscopy revealed Abba3 staining of *H. pylori* strain 17875/Leb, but no straining of strain 17875/DM which has its *babA* gene knocked out.

H. pylori exists as many different strains with varying expression and affinity of BabA to Leb. The strains are classified as “Generalists” if they have a broader binding phenotype against blood group ABO/Leb and as “Specialists” if they selectively bind blood group O.
To investigate the binding range of Abba3, different clinical isolates of *H. pylori* were examined in ELISA. Interestingly, Abba3 bound the majority of generalist strains (30/31), but only about half the specialist strains (11/21).

The approach to prevent or treat diseases transmitted through mucosal sites by direct administration of antibodies or via antibody fragment producing microorganisms[295] could be interesting in the case of *H. pylori* infections. Infection experiments in mice have shown that scFv targeting *H. pylori* reduced stomach colonization[296] indicating that this approach could be feasible also in humans.
Unfortunately, initial experiments where Abba3 was expressed as scFv in *Lactobacillus casei* dramatically reduced binding affinity of antibody to BabA (data not shown). Whether this is due to missfolding or reduced expression or secretion has to be investigated. The general feasibility of scFv expressing *Lactobacilli* reducing the load of pathogenic bacterial had been shown by Kruger and colleagues[295].
Another interesting study would be to map the seemingly fairly conserved ABBA-3 epitope (among generalists) to identify a BabA region suitable for use in vaccination trials.

Investigations of new *in vivo* antibody selection systems

The screening/binding step in most systems used for antibody library screening is carried out *in vitro*. As mentioned in the introduction, this is suboptimal in many situations, e.g. when screening for antibodies targeting membrane proteins.
The aim for paper IV was to investigate the possibilities to develop a screening system where the antigen-antibody interaction is taking place inside a living cell.

As a model system we chose to look at the interaction between HIV-1 envelope protein gp160 and three antibodies anchored to the membrane in scFv-κ format, i.e. VH linked to VL+CL (κ) fused to a transmembrane domain (TM) (as in figure 10, but with ECFP fused to scFv-κ-TM and HcRed fused to gp160).
Two of the investigated antibodies, scFv-κ-kl3 and scFv-κ-17, were used as positive controls since they were known to bind gp160[297]. scFv-κ-anti-TT specific for tetanus toxin were used as a negative antibody control[298].

We based our antigen-antibody detection system on FRET and used the fluorescent proteins ECFP and HcRed as donor and acceptor respectively.
To confirm that this pair did allow FRET, a fusion expressing construct consisting of the two fluorescent proteins was made. FRET signal from the cells expressing HcRed-ECFP fusion could be detected using FACS and could clearly be distinguished from cells co-expressing ECFP and HcRed (see figure 16).

To investigate whether cells positive for FRET could be enriched from a library, cells cytosolically expressing the fusion were mixed with cells co-expressing ECFP and HcRed.

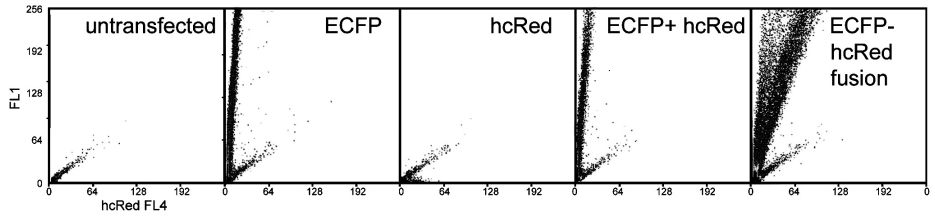


Figure 16: Cells expressing different combinations of fluorescent proteins analyzed by FACS. FRET is not occurring in cells co-expressing ECFP and HcRed, but it does in cells expressing the fusion protein as seen by an increase in HcRed emission (X-axis).

One round of FACS sorting enriched cells transfected with the fusion from originally 10% to 89% indicating that this FRET pair has suitable properties for library screenings.
Next, fusion proteins of scFv-κ-TM-CFP and gp160-HcRed were investigated for FRET. Unfortunately, co-expression levels were too low to analyze these constructs by FACS. Instead, photobleaching FRET (pbFRET)[299] was used which is not suitable for high-throughput screening since each cell takes several minutes to investigate. However, we wanted to confirm that the constructs worked. Unfortunately, all antibodies including the negative control gave high FRET signals. This could be the result of non-specific interactions due to aggregation or overloading of the ER membrane.

To try to elucidate the reason behind the false-positive FRET, two new constructs were made where the scFv-κ was exchanged with small affinity proteins called "affibodies"[300]. When co-transfected, the constructs affibody1-TM-ECFP and affibody2-TM-HcRed also gave high FRET signals even though no specific interaction were expected in between affibody1 and affibody2.
These results indicated that the problem is of a more general nature such as aggregation of the TM-domain or overloading of the ER membrane.
However, the method pbFRET worked as such, since cells expressing the fusion HcRed-ECFP were easily distinguished from cells co-expressing HcRed and ECFP in pbFRET (giving 14.8% and 2.3% FRET efficiency respectively).

As seen, our attempts to use this novel FRET pair for detection of membrane protein interactions were not successful. However, it seems like the problems is not related to the FRET-pair itself, but rather to the expression constructs we tried. It would be interesting to look at a less comlicated system such as ECFP and HcRed fused to leucine zippers that would facilitate interaction. These constructs could be compared for FRET effiency in the cytosol and the ER to investigate whether the ER compartment itself is part of the problem.

Concluding Remarks

Antibodies are truly fascinating proteins making up an important part of our immune system. Hundreds of millions of years of evolution have shaped this unique protein fold allowing high affine interaction with virtually any antigen. These properties have made antibodies extremely useful tools in biological research and lately also as a new class of therapeutics. The mAb therapeutic market has grown rapidly in recent years, reaching sales of $14bn in 2005, an increase of 36.5% from 2004 sales of $10.3bn[301].

Our lab has previously isolated several interesting mAbs from combinatorial libraries, but the lack of a suitable expression system has hampered their further characterization. This is exemplified by the anti-E2 mAbs investigated in this thesis (paper II) which were originally isolated over 7 years ago.
Hence, we were in great need of a system suitable for mid-scale production of mAbs.
The S2 system performed well in this aspect with great reliability, easy and rapid production and high expression levels. Still, we have not made any genuine attempts to optimize the expression levels. A colleague has reported a 150-fold increase in production of a protein in the S2 system after optimization of oxygenation, temperature, induction, shaking conditions and cell culture density (Fernando Arenzana, Institute Pasteur in Paris, personal communication).
The expression levels of mAbs in the S2 system are already high, but could they be increased further, the S2 system might constitute a good alternative for industrial scale production.

The versatility of the S2 system was shown in paper II and III where antibodies were produced in large amounts. In addition, about 15 other mAbs not reported in this thesis have been expressed and purified in our lab. They all expressed at high levels confirming the robustness of the S2 expression system.

The characterized mAbs 1:7 and A8 (targeting HCV E2) and ABBA-3 (targeting *H. pylori* BabA) make up possible therapeutic agents since they all target conserved regions of their corresponding antigens and inhibit pathogen interaction with its host. However, it should be noted that the most valuable information obtained might not be the mAbs themselves, but rather the identification of conserved regions of the antigens targeted by the mAbs. These regions make up possible vaccine candidates that could confer broad immunity.

A lot of time has been invested in our attempts to develop a system suitable for combinatorial antibody library screenings in living eukaryotic cells. This turned out to be very difficult, and in the end we did not reach our goals. However, we investigated a novel pair for FRET studies that might be suitable for library screening using FACS. More experiments are needed, but a functional system

would be very rewarding not only for screening antibody libraries but also for studying protein-protein interactions in general.
One central problem with library screenings in higher eukaryotic cells is the transfection procedure since each cell should optimally express only one library member. This criterion cannot be fulfilled by many of the established transfection methods such as lipofection and calcium precipitation. Lenti- and retroviral vectors are currently the most commonly used systems for expression of one single library member per cell in higher eukaryotes. However, the transfection efficiency of these viral systems is several orders of magnitude less than its bacterial counterparts. Hence, library sizes in higher eukaryotes are limited to somewhere in the range of 10^4-10^6. This size is generally not enough to find binders in naïve antibody libraries, but works for libraries derived from immune individuals or libraries pre-screened in prokaryotes.

To conclude, the future for monoclonal antibodies is indeed very bright both in the field of research and for therapy. The technologies reported in this thesis can be added to the collection of available tools making it easier to develop and characterize new monoclonal antibodies.

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