University of
Natural Ressources and Life Sciences

Institute of Applied Microbiology

Insect cells as an engineering and production platform for antibodies and antibody derived products

Doctoral thesis of

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Abstract

In recent years there has been an increase in both availability and demand for new therapeutic antibody products. Thus, novel engineering and screening platforms as well as efficient and suitable expression schemes are required. In order to evaluate the specificities of newly designed antibody derived fragments we established an efficient selection method. Baculovirus vectors were designed to express and display an IgG_1 Fc-fragment fused the transmembrane region of the *Influenza* A virus neuraminidase on the surface of insect and mammalian cells. Cellular expression was confirmed by FACS analysis; proper folding and dimer formation was tested by binding to *Staphylococcus aureus* Protein A (SpA) and human FcγRI respectively. Further, an efficient insect cell based expression system was evaluated and established to produce human-like antibodies. We expressed the human anti-gp41 antibody 3D6 in different insect cells and compared product yield, specificity and glycosylation patterns to a 3D6 antibody expressed in Chinese hamster ovary cells. Using “High Five” cells we achieved amounts of secreted antibody comparable to those resulting from transient expression in mammalian cells. We determined the N-linked oligosaccharide structures present on asparagine-297 in IgG_1 heavy chains and tested the functionality in terms of antigen binding and the ability to elicit effector functions. “High Five” cells showed a glycosylation pattern that might lead to a reduced biological activity. We therefore established a glycosylation module giving the possibility to flexibly modify the glycosylation capability of different insect cell lines.

Zusammenfassung

In den letzten Jahren ist es zu einer deutlich gestiegenen Nachfrage an neuen therapeutischen Antikörper-Produkten gekommen. Die Entwicklung neuer Design- und Selektionsplatformen, sowie geeigneter Produktionssysteme ist somit von großer Bedeutung. Um die Eigenschaften dieser neu gestalteten Fragmente im Detail bewerten zu können haben wir ein effizientes Selektionsverfahren entwickelt. Hierfür wurden Baculovirus-Vektoren generiert die es ermöglichen ein IgG_1 Fc-Fragment, welches an die Transmembran-Region der Influenza A Neuraminidase fusioniert wurde, um auf der Oberfläche von Insekten- und Säugerzellen zu präsentieren. Zelluläre Expression wurde mittels Durchflusszytometrie bestätigt, eine korrekte Faltung und Dimerbildung wurde durch Bindung an Staphylococcus aureus Protein A (SpA) und den humanen FcγRI getestet. Weiters wurde ein auf Inseltenzellen basierendes System für die Produktion von human-ähnlichen Antikörpern entwickelt. Wir haben den humanen anti-gp41 Antikörper 3D6 in verschiedenen Insektenzellen exprimiert und in Bezug auf Produktausbeute, Spezifität und Glykosylierungsmuster mit einem in CHO exprimierten 3D6 Antikörper verglichen. "High Five" Zellen zeigten eine Ausbeute vergleichbar mit jener aus transienten Expressionen in Säugerzellen. Wir bestimmten die an Asparagin-297 gebundenen Oligosaccharid Strukturen der schweren Kette und testeten deren Funktionalität in Bezug auf Antigen-Bindung und ihre Fähigkeit Effektor-Funktionen auszulösen. "High Five" Zellen zeigten hierbei ein Glykosylierungsmuster das auf eine reduzierte biologische Aktivität vermuten lässt. Deshalb entwickelten wir ein Glykosylierung-Modul welches eine flexible Modifizierung der Glykosylierungskapazität diverser Insektenzellinien ermöglicht.

1. Introduction

1.1 The antibody molecule

Immunoglobulines, more generally called antibodies, are Y shaped molecules with an antigen-binding site located on each tip of the Y. The basic structural unit of an antibody consists of four polypeptide chains, two identical light chains (LC) (each containing about 220 amino acids) and two identical heavy chains (HC) (each usually containing about 440 amino acids), all held together by a combination of non-covalent and covalent, disulfide, bonds.

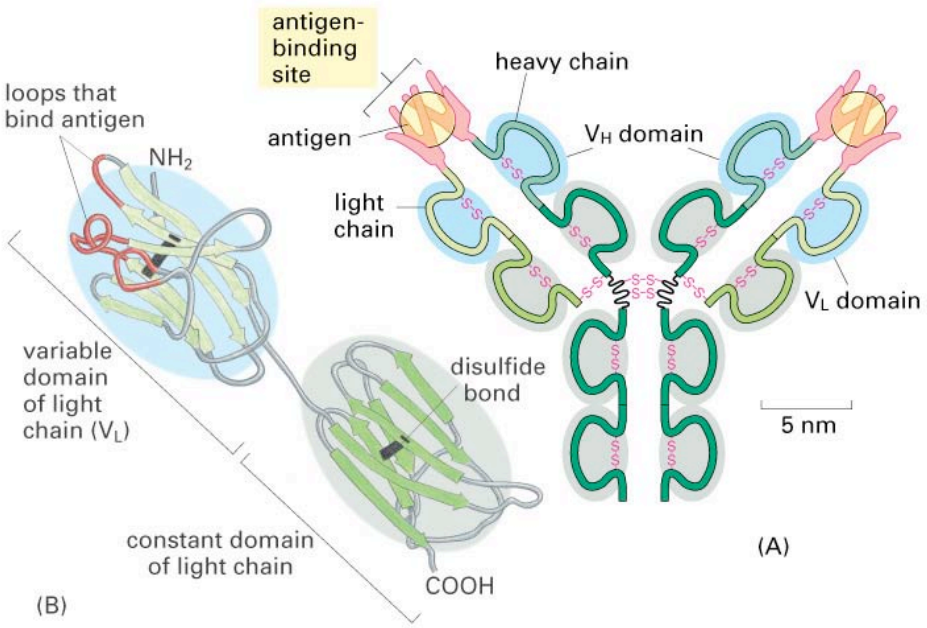

Figure 1.1 Schematic representation of antibody structure (adapted from Alberts, B., „Essential Cell Biology, 2nd edition“, Fig. 4.32, Garland Science textbooks, 2004).

Light and heavy chains themselves are composed of repeating, independently folded domains forming compact functional units. The light chain consists of one variable (VL) and one constant (CL) domain; the heavy chain of one variable (VH) and 3 - 4 constant (CH) domains (depending on antibody subtyp). VL and VH together form the antigen binding site while constant domains of the heavy chain determine the other biological functions of the antibody (Alberts et al., 2002).

In mammals, antibodies are present in 5 different variants also known as isotypes: IgA, IgD, IgE, IgG and IgM, each composed of an α, δ, ε, γ, or μ heavy chain and lambda (λ) or kappa (κ) light chain.

IgA is usually found in mucosal areas and is transported through secretory epithelial cells from the extracellular fluid into the gut, respiratory tract, saliva, tears and milk by a receptor specific pathway. Whereas IgA is present as a monomer in the blood, in secretions it is present as a dimer (Underdown and Schiff, 1986).

IgD mainly functions as an antigen receptor on B cells that have not been exposed to antigens right now and acts as a stimulator of basophils and mast cells to secrete immunoactivating, proinflammatory and antimicrobial mediators (Chen and Cerutti, 2011).

IgE binds to allergens and is responsible for a mast cell derived histamine secretion after binding to specific receptors (Lowell, 2011).

IgG is the major class of immunoglobulin in the blood. It is a monomer produced in large quantities and provides the majority of antibody-based immunity against invading pathogens. IgG further is the only antibody isotyp able to cross the placenta and give passive immunity to the foetus.

IgM is the first class of antibody that is produced by an immature B cell after activation by an antigen. In its secreted form, IgM is a pentamer composed of five antibody monomers hold together by a joining, „J“, polypeptide chain giving it a total of 10 antigen-binding sites (Tchoudakova et al., 2009).

1.2 Antibodies – a major part of our immune system

Antibody molecules play a major part in the adaptive immune system. Initially, during B cell development in the bone marrow, the antibody molecules are displayed on their plasma membrane, where they serve as receptors for antigen. An antigen-binding event to these receptors in peripheral lymphoid organs, together with co-stimulatory signals provided by helper T cells, activates the B cells to proliferate and differentiate into either memory- or antibody-secreting plasma cells. These plasma cells secret millions of copies of the specific antibody, that then circulate in the blood stream (Alberts et al., 2002). After a secreted antibody bound an antigen two kinds of effector functions are possible:

Antibody dependent cellular cytotoxicity (ADCC) starts with the binding of monoclonal antibodies Fc-fragment to Fc-receptors found on monocytes, macrophages, and natural killer cells. These cells then engulf the antibody-antigen immune-complex and dispose it (Weiner et al., 2009). Natural killer cells further secrete cytokines leading to cell death, and also recruit new B cells.

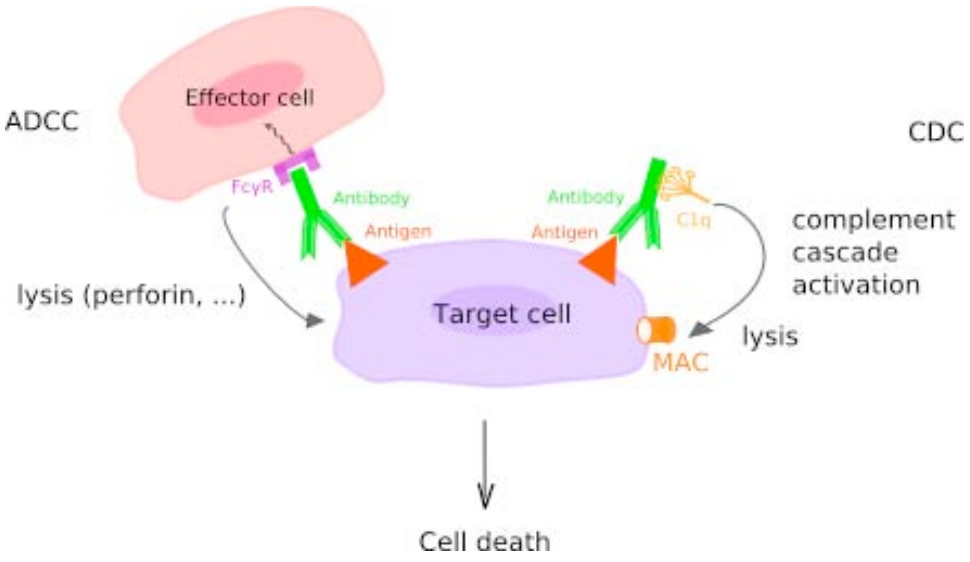

Figure 1.2 Schematic representation of IgG triggered effector functions (from IMGT lexique: http://www.imgt.org/textes/IMGTeducation/IMGTlexique/A/ADCC_and_CDC.html).

Another mechanism is the so-called Complement dependent cytotoxicity (CDC). CDC is a cytolytic cascade mediated by a series of 20 complement proteins abundantly present in serum. The cascade starts with the binding of C1q to antibody-antigen complexes and finally leads to the formation of a membrane attack complex that makes a whole in the cell membrane, further causing cell lysis and death (Natsume et al., 2009).

1.3 Antibody derived products

The dual functionality of an antibody, facilitating antigen binding and effector functions, is ideal for therapeutic treatments, but the Fc mediated effects are some times undesirable. A long serum half life for example minimizes the use of antibodies for in vivo diagnostics by medical imaging, resulting in poor contrasts. A further very important disadvantage is the decreased ability of large, full-sized antibodies to penetrate tissue. To overcome these problems scientists started to remove the Fc-region by proteolytic digestions using papain or pepsin (Holliger and Hudson, 2005). Genetically engineering further increased the possibilities for antibody-fragment design as shown in Figure 1.3.

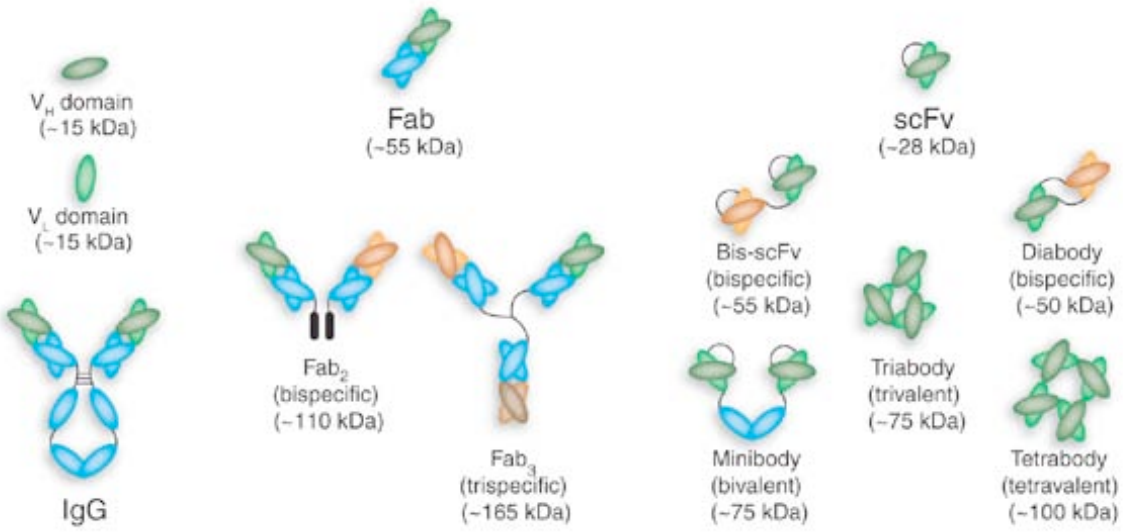

Figure 1.3 Schematic representation of different antibody fragment designs (adapted form Holliger and Hudson (2005).

The most commonly used designs are Fab and scFv fragments. A Fab consists of the variable and the first constant domain of heavy and light chains hold together by their natural disulphide linkage. A scFv, in contrast, is only composed of VH and VL held together by a flexible polypeptide linker. These fragments show a retained monovalent antigen binding ability and an increased tissue penetration (Kim et al., 2005). Besides the formats mentioned and in order to further decrease fragment size single domain fragments consisting of either VH or VL could

potentially target inaccessible epitopes. Although promising, these fragments suffered from a high tendency for aggregation (Ward et al., 1989).
Due to the fact that Fab and scFv possess only a monovalent antigen binding, fast dissociation rates and short antigen retention times have been observed. To increase the avidity of antigen binding, multivalent fragments composed of naturally or chemically linked scFv's or Fab's have been generated. Combining fragments specific for different targets, further leads to the generation of multi-specific formats (Casey et al., 2002; Wittel et al., 2005).

In recent years many attempts have been made to modify the abilities of an antibody fragment, by e.g. genetically engineering the structural loops of various domains. (Wozniak-Knopp et al., 2010) engineered an antigen-binding site in the CH3 domain of an Fc-fragment, resulting in an Fcab (Fc with antigen-binding) (Figure 1.4). This design enables antigen binding and the ability to elicit effector functions from a fragment of only 55 kDa in size, with the predominant advantages of enhanced serum half-life and increased tissue penetration.

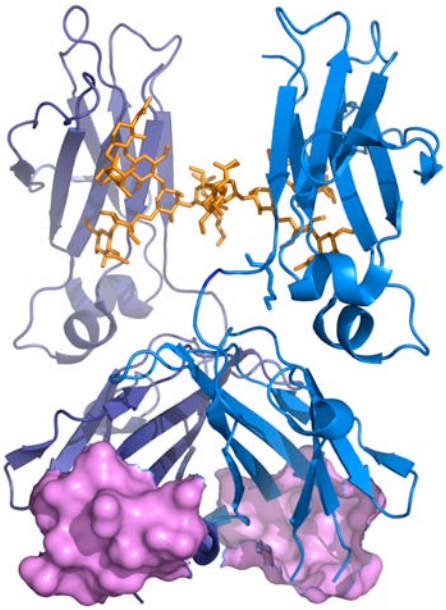

Figure 1.4 Schematic representation of an Fcab molecule. The engineered binding site is indicated as purple surface.

1.4 Screening systems

Since many different antibody variants have been developed there is a subsequent need for assays in order to discriminate specific binders. A screening system must give the possibility to link the phenotyp of high affinity binders to their corresponding genotype. The display of protein libraries on the surface of bacteriophages, viruses or cells has evolved to be ideal for that purpose.

Phage display is currently the predominat system and it is based on the display of antibody fragments fused to coatprotein pIII of filamentous bacteriophage M13. Using that system it is easily possible to generate libraries of high diversity, but displaying large fragments is tricky (Smith, 1985). Although phage display is a well established system, the display of antibody libraries on the surface of bacteria, yeast or mammalian cells has shown to give advantages like reduced limit in fragment size and the possibility to perform the selection using flow-cytometry.

Yeast surface display utilizes the fusion of antibody fragments to a native agglutinin receptor (aga2p) so that it is possible to generate libraries of around 10^7 different clones on the surface of e.g. *Saccharomyces cerevisiae* (Boder and Wittrup, 1997). Due to the reduced diversity, yeast display has most successfully been applied for affinity maturation approaches. *E. coli* surface display, that is based on the fusion of antibody fragments to a natural surface protein, on the other hand allows the generation of libraries with high diversity, but problems dealing with the translocation of large fusion proteins through the periplasm to the outer cell membrane have to be kept in mind (Daugherty et al., 1998).

The systems mentioned so far possess either no or different post-translational modification machineries as compared to mammalian cells and are not feasible to display full-length IgGs. Scientists therefore evaluated the use of mammalian cells for the display of full-length properly processed antibodies, using different membrane proteins for anchorage (Akamatsu et al., 2007)

Another alternative is *baculovirus surface/insect cell display* which combines several advantages of the ystems described. Offering large insertion capacities and post translational modifications, baculoviral particles and infected insect cells are both suitable for display of foreign peptides and proteins. Different membrane anchoring strategies are available offering a spectrum of possibilities to display large, complex proteins as well as small peptides at high avidity. Baculoviruses are non-pathogenic to mammals and thus, are an ideal carrier for antigenic epitopes or proteins intended to induce neutralizing immune response when administered as vaccines. As compared to mammalian cell lines, insect cells such as *Sf*9 and T. *ni* cells may be considered as a more robust and efficient display scaffold, since handling is less laborious, less costly and target proteins are incorporated more efficiently and homogeneously into the cellular membrane. Selection of specific clones by FACS is a fast and attractive method and does not require the cells to stay intact, but instead baculoviruses can be recovered by filtration and be used for re-infection. Overall, the procedures for producing recombinant baculoviruses with desired display properties and the analytic methods as well as selection schemes are almost as simple and fast as microbial surface platforms.

1.5 Expression systems and their capability for the production of glycosylated, full-sized antibodies

After engineered and screening of antibody fragments and whole antibodies, the next step is production thereof, at sufficient amounts and in required quality. Several expression systems are available for the production of antibodies and antibody fragments including bacteria, yeast, plants, insect and mammalian cells as well as transgenic animals (Jefferis, 2009; Kipriyanov and Little, 1999).

1.5.1 History

(Köhler and Milstein, 1975) established the first method for production of mouse monoclonal antibodies, by fusing an antibody expressing B cell with a myeloma cell, but the use of these mouse antibodies for treating humans was not successful because of their immunogenicity. In following years many attempts have been made to overcome this problem. Starting 1984 the first chimeric antibodies, composed of human and mouse sequences were generated (Morrison et al., 1984). The ratio mouse:human has further been directed to human sequences using techniques like CDR grafting (Jones et al., 1986) and transgenic mice (Green et al., 1994) finally resulting fully human antibodies.

1.5.2 Bacteria

The idea of expressing full-length antibodies using *E.coli* sounds reasonable when thinking about costs, because it grows in simple inexpensive media, has very short cultivation times and an easy handling. *E*.coli on the other hand lacks the post-translational and glycosylation machinery resulting in non-processed, non-glycosylated antibodies. There have been some attempts to express fully assembled IgG, but yields were very low, mainly due to very poor intracellular assembling (Simmons et al., 2002). These antibodies have been shown to give proper antigen binding, but totally missed the ability to elicit effector functions.

Antibody fragments possessing only an antigen-binding site, like Fab, Fv and scFv, where effector functions and further biological activities are not required, can readily be produced by bacterial fermentation (Boss et al., 1984). *E.coli* gives three major routes for protein expression: Although the cytoplasm would be favourable in terms of possible yield, the reducing conditions do not allow the production of fully assembled antibodies, requiring disulphide bonds. Therefore, secretion into the periplasm, where the oxidative environment is ideal for the formation of disulphide bonds is the method of choice.

Despite *E.coli*, the gram-positive *Bacillus* species like *Bacillus brevis* (Inoue et al., 1997), *Bacullus subtilis* (Wu et al., 1998) and *Bacillus megaterium* (Jordan et al., 2007) have already been successfully used for the production of antibody fragments; they lack an outer cell membrane allowing direct secretion of the protein in the growth medium.

1.5.3 Yeast

Yeasts have gained significant interest for the production of recombinant antibodies. *Pichia pastoris,* is the preferred host among yeasts, because of high cell densities, strong promoters and glycosylation patterns similar to that observed in human systems. Although promising, the production of monoclonal antibodies and / or antibody fragments in yeast is not always trivial. The host cell may on the one hand be stressed by environmental conditions, but even more by the over-expression of the heterologous protein itself. Especially overloading the secretory pathway often results in the inability of proper HC-LC dimer formation, which may be a major hindrance. High intracellular concentrations of misfolded antibodies further lead to stress reactions and direct loss of product (Gasser and Mattanovich, 2007). To overcome this problem many attempts have been made to co-express several chaperons or foldases leading to an improved protein folding (Damasceno et al., 2007). Therefore a lot of attempts have been made to design glycoengineered *Pichia pastoris* strains giving the possibility to produce human like glycosylation patterns (Choi et al., 2003; Li et al., 2006).

1.5.4 Mammalian cells

Mammalian cells, especially Chinese hamster ovary (CHO) and mouse myeloma (NS0) cells are currently the state of the art platform used for the production of antibodies for clinical applications, because of high expression rates, their capacity for proper protein folding and assembly and their posttranslational modification machinery. Using this benefits there are no limits for the production of therapeutically active antibody products (Kelley, 2009). There are two principle techniques for heterologous protein production in mammalian cells:

The most widely used system is the stable transfection of mammalian cells with an expression cassette for antibody heavy and light chains and selecting for high expresing clones by antibiotic pressure or more frequently by using like dihydrofolate reductase (DHFR). CHO cells lacking DHFR activity can only grow in supplemented medium. When these cells are transfected with DHFR-containing constructs, they are able to grow in non supplementded media. In the presence of methotrextrate (MTX), which is a DHFR inhibitor, cells can compensate for this effect by increasing the DHFR copy number and if the antibody expression cassette is located at the same site on the chromosome it gets amplified as well (Kaufman et al., 1985). Typical production processes using this technique will run in a fed-batch format in vessels from 5000 L to 25 000 L and yield up to 4 g antibody/L (Wurm, 2004).

Another method is the transient expression of antibodies mainly in CHO cells. Here the plasmid coding for antibody heavy and light chains is transfected in the cell using transfection agents like Polyethylenimine (PEI). (Muller et al., 2007) have reported a scable transient antibody expression with yields up to 22 mg antibody/L. A great advantage of that system is time, because the whole top-clone selection procedure necessary for the generation of highly expressing recombinant CHO clones can be avoided.

Despite all advantages, one always has to keep in mind, although inferior in terms of quality and biological activity, that hypersensitive reactions to mouse cell derived antibodies, e.g. Cetuximab have been reported (Chung et al., 2008) and that the costs for a mammalian cell culture process is drastically higher.

1.5.5 Plants

Plants allow a very cost effective production of full-sized antibodies and antibody fragments on an agricultural scale, while eliminating the risk of contaminations with endotoxins or mammalian pathogens (De Muynck et al., 2010). Different expression strategies have successfully been used, including whole-plant or plant-cell systems.

Most of plant derived antibodiy products have been produced by a *Agrobacterium tumefaciens mediated* nucelar transformation of the expression cassette that stably integrates in the plant genome. The gene transfer itself is very fast, but the regeneration of transgenic plants a very time consuming process and therefore not applicable for an easy testing of new antibody variants. The production of antibodies using this system has been shown in a variety of plant species including arabidopsis (Eeckhout et al., 2000), soybean (Zeitlin et al., 1998), tobaco (Hiatt et al., 1989) and rice (Torres et al., 1999).
Transient expression that is usually used for rapid testing the activity of a desired product can be achieved by agroinfiltration, where *Agrobacterium tumefaciens* carrying th expression cassette is infiltrated in plant leaves, or by the use of plant viruses as expression vectors.

Although whole-plant systems are a very promising for the large scale production of recombinant antibodies there are still a number of drawbacks. The glycosylation of an antibody is crucial for it’s biological function, but plants differ in their glycosylation machinery, giving need for the generation of glycoengineered hosts to remove immunogenic α 1,3-fucose and β 1,2-xylose (Koprivova et al., 2004). Subcellular targeting is another important factor. Full-sized immunoglobulines need to be addressed to the secretory pathway, for proper assembly and N-glycan processing. Although it is possible to guide antibodies to special cellular compartments, partial mistargeting always happens, resulting in antibody structural heterogeneity. Proteolytic degradation is a further drawback that has to be eliminated. These hindrances can be overcome by the use of plant cell cultures, where yields of 30 mg antidody/L have been reported, but the advantage of low costs is minimized.

1.5.6 Insect cells

Insect cells are a very powerful tool for the expression of large, highly processed proteins such as monoclonal antibodies because of their ability to perform signal-peptide cleavage, N- and O-linked glycosylation and efficient extracellular secretion. By using baculoviruses it is possible to transiently express large DNA fragments under the control of very strong viral promoters (Jarvis, 2009; Kost et al., 2005; Luckow, 1993). Again, the glycosylation patterns (Figure 1.5) were shown to differ somewhat as compared to mammalian cells (Betenbaugh et al., 2004; Rendic et al., 2007); thus, several glycoengineering strategies were developed (Jarvis et al., 1998).

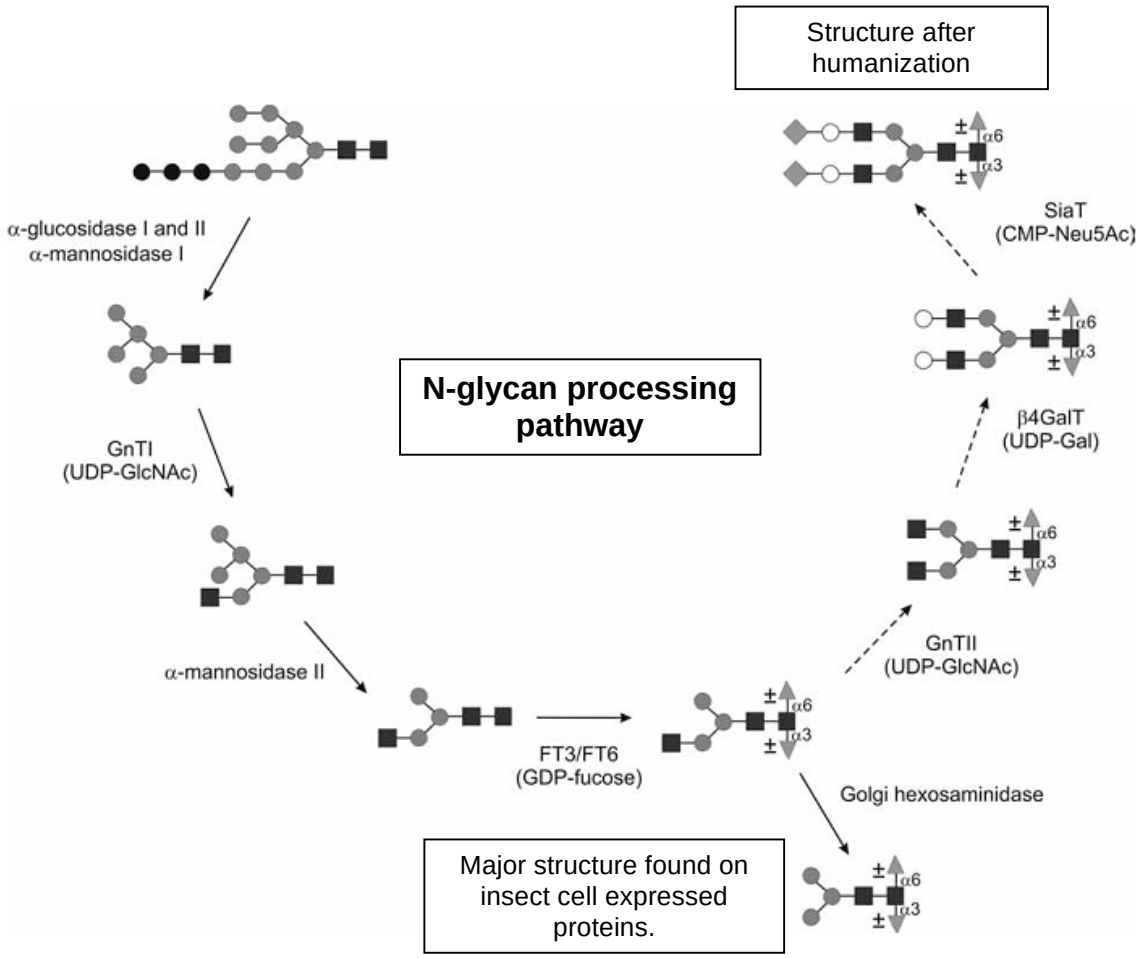

Figure 1.5 Schematic representation of the N-glycan processing pathway of insect cells. Possible humanization is indicated with dotted arrows (adapted form Rendic et al. (2007).

So far, insect cells have never been use for the production of monoclonal antibodies for the use in clinic, but the baculovirus-insect cell system may be advantageous as compared to stably transfected CHO cells or transient expressions in mammalian cells. Especially when considering a need for fast and easy production systems which may be increasingly important in the future due to novel, “personalized” medical treatments or when time consuming production schemes are not acceptable.

1.6 Aims of this work

In recent years there has been an increase in both availability and demand for new therapeutic antibody products. We therefore evaluated the use of the baculoviral insect cell system for the screening of new antibody variants, as well as the expression of therapeutically active antibody products.

The main aims were:

- The Establishment of a system enabling the efficient display of large antibody fragment libraries on the surface of insect and mammalian cells.

- The expression of the monoclonal antibody 3D6 in different insect cell lines and the characterisation regarding biological activity.

- The Establishment of a system allowing flexible modification of insect cell glycosylation capabilities.

2. Materials and Methods

All DNA manipulations were carried out essentially as summarized by Sambrook *et al.* (Sambrook, 1989). Kits for DNA purification were purchased form Machery-Nagel (Düren, Germany). Restriction enzymes, T4 DNA ligase and Calf Intestinal Alkaline phosphatase were purchased form New England Biolabs (Ipswich, USA). DNA polymerase was purchased from Novagen (Darmstadt, Germany). All enzymes were used according to manufacturer's recommendation. All primers and oligos were synthesized by Sigma-Aldrich (St. Louis, USA). All cell culture materials were purchased form Nunc (USA) and Corning (NY, USA).

2.1 Equipment

Table 3.1 Special equipment used in this work

device	supplier
Thermocyclers	Biometra (Germany)
Agarose gels unit	Biorad (USA)
Electroporator	BTX (USA)
Gel documentation system	Biorad (USA)
SDS-PAGE electrophoresis units	Biorad (USA)
Chemiluminescence imager	Peqlab (Germany)
Centrifuges	Heraeus (Germany) Eppendorf (Germany)
Incubators	Heraeus (Germany)
Class II laminar flow	Heraeus (Germany)
FACS Calibur	Becton Dickinson (USA)
FACS Canto	Becton Dickinson (USA)
MALDI-TOF MS	Burker-Flex (USA)

2.2 Plasmids

2.2.1 Map of pBacPAK8 (Novagen)

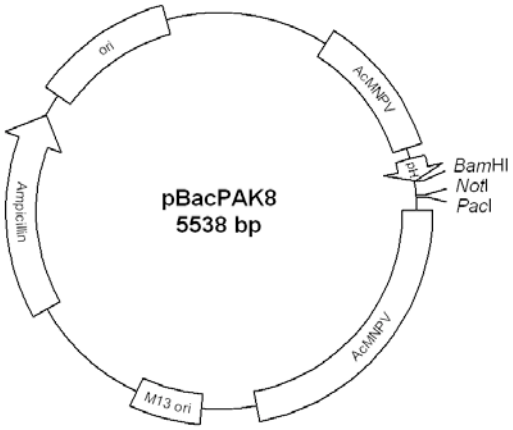

Figure 2.1 Map of pBacPAK8 vector. Important restriction sites are indicated. The vector provides a polyhedrin promoter for baculoviral gene expression, an antibiotic resistance gene and origins of replication. Flanking AcMNPV sequences allow recombination to transfer the expression cassette to the polyhedrin locus of the viral DNA

2.2.2 Map of pFastBac Dual (Invitrogen)

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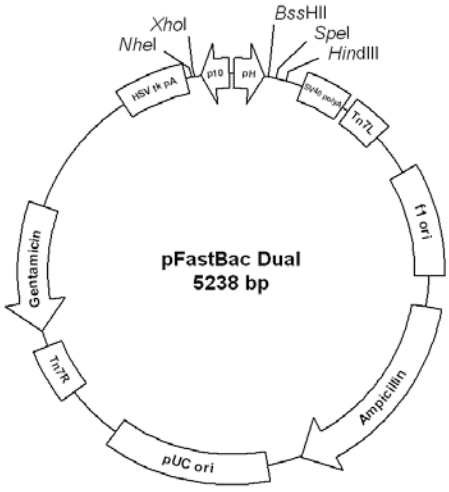

Figure 2.2 Map of pFastBac Dual vector. Important restriction sites are indicated. The vector provides an expression cassette containing two viral promoters (polyhedrin and p10) as well as two polyadenylation sites (HSV tk pA and SV40 polyA), two antibiotic resistance genes and an origin of replication. The left and the right arm of a Tn7 site enable the integration of the expreesion cassette in the viral genome.

2.2.3 Map of pDEST8 (Invitrogen)

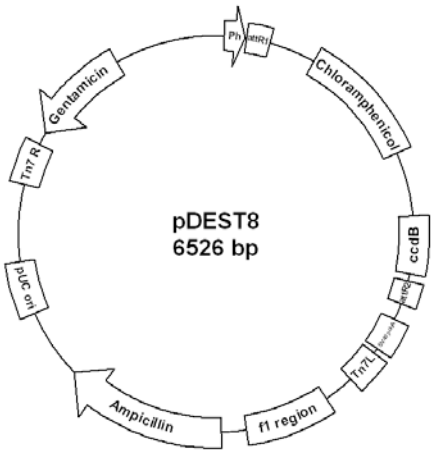

Figure 2.3 Map of pDEST8 vector. The vector provides an expression cassette containg the viral polyhedrin promoter (Ph) as well as a SV40 polyadenylation site (SV40 polyA), two antibiotic resistance genes, an origin of replication and a death gene (ccdB). The left and the right arm of a Tn7 site enable the integration of the expreesion cassette in the viral genome. The left and the right arm of the attR site enable homologous recombination with Entry vectors (pENTR 1A) carrying attL sites.

2.2.4 Map of pENTR 1A (Invitrogen)

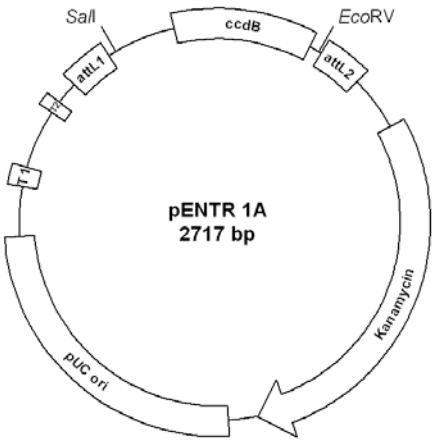

Figure 2.4 Map of pENTR 1A vector. Important restriction sites are indicated. The vector provides the left and the right arm of the attL site enable homologous recombination with Donor vectors (pDEST 8) carrying attR sites, an antibiotic resistance gene, an origin of replication and a death gene (ccdB).

2.2.5 Map of pUCDM (EMBL-Grenoble)

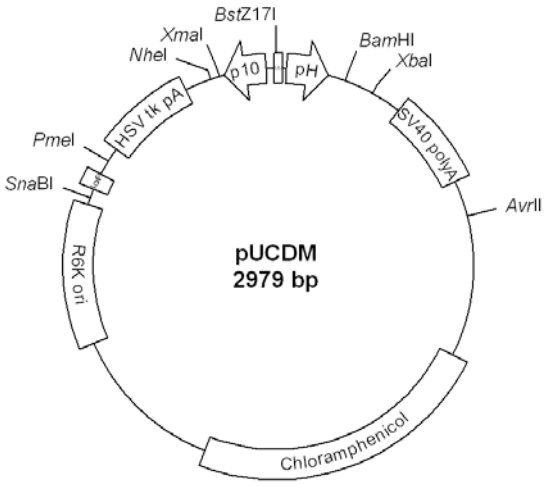

Figure 2.5 Map of pUCDM donor vector. Important restriction sites are indicated. This vector provides an expression cassette containing two viral promoters (polyhedrin and p10) as well as two polyadenylation sites (HSV tk pA and SV40 polyA), an antibiotic resistance gene, an origin of replication and a multiplication module. The loxP site enables a cre-mediated fusion with acceptor vectors (pKL).

2.2.6 Map of pKL (EMBL-Grenoble)

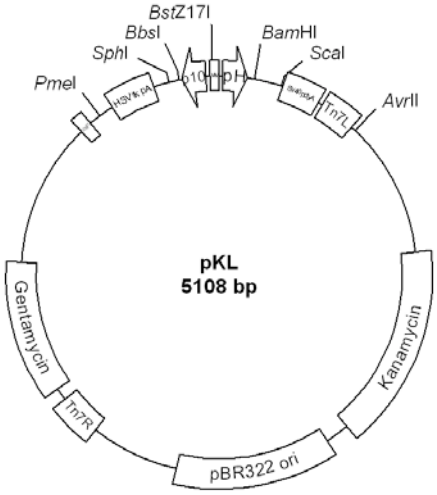

Figure 2.6 Map of pKL acceptor vector. Important restriction sites are indicated. This vector provides an expression cassette containing polyhedron and p10 promoters, two polyA sites (HSV tk pA and SV40 polyA), two antibiotic resistance genes, an origin of replication and a multiplication module. The loxP site enables a cre-mediated fusion with donor vectors (pUCDM). The left and the right arm of a Tn7 site enable the integration of the expression cassette in the viral genome.

2.3 Microorganisms

Escherichia coli (*E. coli*) strains JM109 and DH10B were used for standard cloning procedures. DH5α cells giving a pir^+ phenotyp were used for the propagation of pUCDM vectors. DH10Bac cells were used for the generation of recombinant baculovirus genome.

2.4 Cells and viruses

*Spodoptera frugiperda Sf*9 cells (ATCC CRL-1711) (Summers and Smith, 1987), *Trichoplusia ni* BTI-TN5B1-4 “High Five”, cells (ATCC CRL-10859) (Wickham and Nemerow, 1993), S*f*SWT-1 Mimic™ insect cells (Invitrogen, Carlsbad, USA) (Hollister et al., 2002) and *Ascalapha odorate Ao*38 cells (Hashimoto et al., 2010) were grown in IPL-41 medium (SAFC Biosciences, St. Louis, USA) containing yeast extract, a lipid mixture supplemented with either 0%, 3% or 10% fetal calf serum (FCS) at 27°C using T-flasks.

African green monkey, Vero, cells (ATCC CCL-81) were grown in Dulbecco`s modified Eagles medium/Ham’s F12 (Biochrom AG, Berlin, Germany) containing sojapepton and 4 mM glutamine.

Human U937 leukemic monocyte lymphoma cells (ATCC CRL-1593.2) were cultivated in RPMI1640 medium (PAA, Pasching, Austria) containing 10% FCS and 4mM glutamine at 37°C using T-flasks.

Recombinant *Autographa californica* nucleopolyhedrovirus was isolated and plaque purified by standard procedures.

2.5 Display Library

2.5.1 Establishing an optimal display system

2.5.1.1 Construction of transfer plasmid and generation of recombinant baculovirus

The human IgG_1-Fc fragment was PCR amplified using primers Fc-EcoRI-back and Fc-NotI-for / Fc-PstI-for. The products were EcoRI / PstI digested. The Cytomegalovirus promoter/enhancer (CMV) was PCR amplified using primers CMV-BamHI-back / CMV-BssHII-back and CMV-XbaI-for. The products were XbaI digested. The transmembrane domain of influenza A strain A/PR8/34 (NA_{tm}), the gp64 signal domaine (SD) and membrane anchorage sequence (mars) were generated by annealing of phosphorylated oligos: NA_{tm}-1 and NA_{tm}-2; SD-1 and SD-2; mars-1, mars-2, mars-3 and mars-4. CMV was ligated to SD (CMV-SD), PCR amplified using primers CMV-BssHII-back and SD-EcoRI-for and further EcoRI digested. Fc was ligated to NA_{tm} (NA_{tm}-Fc) and mars (Fc-mars), the ligation products were PCR amplified using primers NA_{tm}-XbaI-back / Fc-EcoRI-back and Fc-NotI-for / Fc-PstI-for and further XbaI (NA_{tm}-Fc) and EcoRI (Fc-mars) digested. NA_{tm}-Fc was ligated to CMV (CMV-NA_{tm}-Fc), PCR amplified using primers CMV-BamHI-back and Fc-NotI-for and digested with BamHI and NotI. The insert was further cloned into a BamHI / NotI digested dephosphorylated pBacPAK8 vector (Clonetech, CA, USA) resulting in pBac8-NA_{tm}. CMV-SD was ligated to Fc-mars, PCR amplified using primers CMV-BssHII-back and mars-HindIII-for and digested with BssHII and HindIII. The insert was further cloned into a BssHII / HindIII digested, dephosphorylated pFastBac Dual vector (Invitrogen, Carlsbad, USA) resulting in pFBD-mars.

The Her2 binding Fcab clone 317-SA was PCR amplified using primers Fc-EcoRI-back / Fc-NotI-for and cut with EcoRI / NotI. The insert was ligated into a EcoRI / NotI digested pBac8-NA_{tm} resulting in pBac8-317.

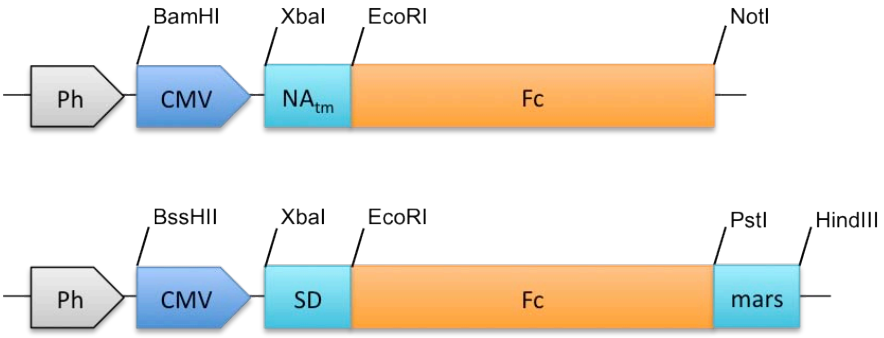

Figure 2.7 Schematic representation of surface display constructs. The expression cassetts are under control of a dual promotor system composed of polyhedrin promoter (Ph) coming form the vector backbone and a cytomegalovirus promotor (CMV). The IgG_1 Fc-fragment (Fc) is either fused to influenza A neuraminidase transmembrane domain (NA_{tm}) or fused to the signal domain (SD) and membrane anchorage sequence (mars) of the baculoviral gp64.

Recombinant baculovirus was generated using Bac-to-Bac (pFBD-mars) (Invitrogen, Carlsbad, USA) resulting in Ac-mars and BaculoGold system (pBac8-NA_{tm}, pBac8-317) (Clonetech, CA, USA) resulting in Ac-NA and Ac-317, according to manufacturer's recommendations.

Table 2.2 Primers used for the construction of displyay constucts

designation	Sequence 5’ → 3’
Fc-EcoRI-back	GAT GAT GAA TTC ACG TGT CCC CCA TGT CCC G
Fc-NotI-for	GAT GAT GCG GCC GCT CAT TTA CCC GGA GAC AGG G
Fc-PstI-for	GAT GAT CTG CAG TCA TTT ACC CGG AGA CAG GG
CMV-BamHI-back	GAT GAT GGA TCC TCA ATA TTG GCC ATT AGC CAT AT
CMV-BssHII-back	GAT GAT GCG CGC TCA ATA TTG GCC ATT AGC CAT AT
CMV-XbaI-for	GAT GAT TCT AGA AGA TCT GAC GGT TCA CTA AAC G
NA_{tm}-1	ATG AAT CCA AAT CAG AAA ATA ATA ACC ATT GGA TCA ATC TGT CTG GTA GTC GGA CTA ATT AGC CTA A
NA_{tm}-2	GAT GAT *GAA TTC* GCT AAT CCA TAT TGA GAT TAT ATT TCC TAT CTG CAA TAT TAG GCT AAT TAG TCC GAC TAC CAG
NA_{tm}-XbaI-back	GAT GAT TCT AGA ATG AAT CCA AAT CAG AAA ATA ATA AC
SD-1	CTA GAA TGG TAA GCG CTA TTG TTT TAT ATG TGC TTT TGG CGG CGG CGG CGC ATT CTG CCT TTG CGG AT
SD-2	ATC CGC AAA GGC AGA ATG CGC CGC CGC CGC CAA AAG CAC ATA TAA AAC AAT AGC GCT TAC CAT T
mars-1	GGC TGA AGG CGA ATT GGC CGC TAA ATT GAC TTC GTT CAT GTT TGG TCA TGT AGT TAA CTT TGT AAT TAT ATT AAT TG
mars-2	TGA TTT TAT TTT TGT ACT GTA TGA TTA GAA ACC GTA ATA GAC AAT ATT AA
mars-3	CTA CAT GAC CAA ACA TGA ACG AAG TCA ATT TAG CGG CCA ATT CGC CTT CAG CCT GCA
mars-4	TTA ATA TTG TCT ATT ACG GTT TCT AAT CAT ACA GTA CAA AAA TAA AAT CAC AAT TAA TAT AAT TACA AAG TTA A
HindIII-for	GAT GAT AAG CTT TTA ATA TTG TCT ATT ACG GTT TCT AA

2.5.1.2 Establishing optimal surface display parameters

To confirm the correct size of fusion proteins *Sf*9 cells were seeded in cell culture flasks with a density of 1 x 10^5 cells / cm^2 and infected with Ac-mars and Ac-NA at a multiplicity of infection (MOI) of 10 and harvested 72 hours post infection (hpi). The cells were immediately subjected to SDS-PAGE and western blotting as described in section 2.5.3.1.

For determination of optimal display parameters *Sf*9 cells were again seeded in cell culture flasks with a density of 1 x 10^5 cells / cm^2 and infected with Ac-mars and Ac-NA at a MOI of 1, 10 and 20. 24, 48 and 72 hpi cells were harvested and washed with phosphate buffered saline (PBS). Subsequently 1x10^6 cells were incubated with Anti-Human IgG (γ-chain specific) R-Phycoerythrin Conjugate (Sigma-Aldrich, P8047) diluted in PBS containing 10% Fetal Calf Serum (FCS) for 1 h. After a washing step with PBS cells were analysed on a FACS-Calibur (Becton Dickinson, San Jose, CA).

Vero cells were seeded in 6-well plates and transduced with an MOI of 200 by incubating the cells with the desired volume of virus for 5 h while shaking. The virus was removed and cells were further incubated with DMEM / Ham's F12 media.

2.5.1.3 Cellular localization – Immunofluorescence

*Sf*9 and vero cells were infected / transduced with Ac-NA at MOI 10 and 200 respectively and harvested 72 hpt / 48 hpi. Cells were allowed to attach to adhesion slides (Biorad, Hercules, USA) for 3 h and washed with PBS. Cells were fixed with paraformaldehyd for 10 min and after one round of washing swollen for 10 min using Triton x-100 (1% in PBS). Unspecific binding sites were blocked by incubation with PBS containing 20 % FCS for 30 min. Cells were stained with an Anti-Human IgG (γ-chain specific) R-Phycoerythrin Conjugate (Sigma-Aldrich, P8047) antibody for 45 min, washed carefully and imaged using a scanning confocal microscope (Leica, Wetzlar, Germany).

2.5.2 Establishing an optimal library system

2.5.2.1 Construction of transfer plasmids and generation of recombinant baculovirus

Our recombination system is based on the Gateway® technology (Invitrogen, Carlsbad, USA). The part of pDEST 8 vector carrying all necessary fragments for Gateway recombination was PCR amplified using primers attR1-XbaI-back / attR2-PacI-for. The products were XbaI / PacI digested and ligated to a pBac8-NA$_{tm}$ digested with the same enzymes. For being able to linearize the finally resulting baculovirus genome, a unique Bsu36I restriction site was introduced into the ccdB gene by QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) using primers Ins-Bsu36-AS / Ins-Bsu36 according to manufacturer's recommendations. Recombinant baculovirus was generated using BaculoGold system, according to manufacturer's recommendations, resulting in Ac-attR (Destionation clone).

Table 2.3 Primers used for the amplification of recombination module.

designation	Sequence 5' → 3'
attR1-XbaI-back	GAT GAT TCT AGA CCA TCG GGC GCG GAT CAT C
attR2-PacI-for	GAT GAT CTA GTA CTT CTC GAC AAG CTA TC
Ins-Bsu36-AS	AAA CCT TAA ACT GCC CTT AGG TTT CAC CAG TCC CTG TTC TCG
Ins-Bsu36	CGA GAA CAG GGA CTG GTG AAA TCC TAA GGG CAG TTT AAG GTT

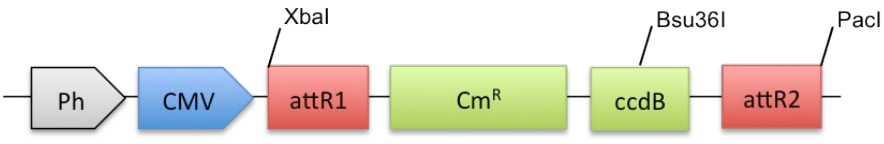

Figure 2.8 Schematic representation of Destination clone (Ac-attR).

The IgG_1-Fc fused to influenza A neuraminidase transmembrane domain was PCR amplified using primers NAtm-XbaI-back / Fc-EcoRV-for. The product was cut with EcoRV / XbaI and ligated to a pENTR 1A cut with the same enzymes resulting in pENTR-NA-Fc (Entry clone).

Table 2.4 Primers used for the generation of Entry vector.

designation	Sequence 5' → 3'
NAtm-XbaI-back	GAT GAT TCT AGA ATG AAT CCA AAT CAG AAA ATA ATA AC
Fc-EcoRV-for	GAT GAT GAT ATC TCA TTT ACC CGG AGA CAG GG

Figure 2.9 Schematic representation of Entry clone pENTR-NA-Fc.

2.5.2.2 Preparation of recombinant baculovirus genome

*Sf*9 cells were infected with Ac-attR, Ac-NA and a baculovirus expressing GFPmut7.1 (Ac-GFP) under control of the polyhedrin promoter at MOI 5 and harvested 72 hpi. Cells were washed with phosphate buffered saline (PBS), resuspended in TE buffer (10 mM Tris-HCL pH 8, 1 mM EDTA) and lysed by adding cell lysis buffer (50 mM Tris-HCL pH 8, 5% 2-mercaptoethanol, 0.4% SDS, 10 mM EDTA). For getting rid of disturbing RNA and proteins, the preparation was incubated with proteinase K (10 mg/mL in TE buffer) and RNase A (10 mg/mL) and incubated at 37°C for 30 min. DNA extraction was done by incubating the preparation 5 min with Phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase was removed and the DNA was precipitated with 3 M NaAc and 96% EtOH. The DNA was pelleted by centrifugation at 16 000 x g for 15 min, the pellet was washed twice with 70% EtOH and finally resuspended in TE buffer. For reducing the possible non-recombinant baculoviral background the Ac-attR genomic DNA was further cut with Bsu36I.

2.5.2.3 In vitro Gateway recombination and re-transfection

The recombination reaction, enabling direct in-vitro gene transfer in the baculovirus genome was done by mixing 300 ng of Bsu36I cut / non cut Baculovirus DNA (Destination clone) and 300 ng of pENTR-NA-Fc (Entry clone) with Gateway® LR Clonase™ II Enzyme Mix for BaculoDirect™ Kits (Invitrogen, Carlsbad, USA). The reaction was incubated at room temperature for 20 h, split and subsequently transfected in *Sf*9 insect cells using Cellfectin II (Invitrogen, Carlsbad, USA) according to manufacturer's recommendations. As a negative control a non-cut, non-recombined baculovirus genome was re-transfected as well.

2.5.2.4 Analysing recombination reactions – FACS, Plaque Assay, PCR

*Sf*9 cells were harvested 6 days post transfection and the presence of Fc-fragments on the cellular surface was measured by FACS as described in section 2.5.1.2. The virus containing supernatants were further subjected to a plaque assay for separating viral plaques and titer determination according to standard techniques. After 5 days viral plaques were visualised by incubation with Thiazolyl Blue Tetrazolium Bromide and random plaques were further amplified using *Sf*9 cells. 5 dpi cells were harvested and the baculovirus genome was isolated using alkaline lysis according to standard techniques. For confirming positive plaques samples were screened by PCR using primers CMV-Screen-back / 1660-for.

Table 2.5 Primers used for screening baculovirus genome.

designation	Sequence 5' → 3'
CMV-Screen-back	ACT TTC CAA AAT GTC GTA ATA AC
1660-for	CAA CGC ACA GAA TCT AGC GC

2.5.2.5 Screening for clones in large libraries – FACS

Isolated genomes of Ac-NA and Ac-GFP were mixed in ratios of 1:10 to 1:1000000 (1 µg of DNA / reaction) with Ac-GFP in the minor amount in order to transfect *Sf*9 insect cells using Cellfectin II according to manufacturer's recommendations. Cells were harvested 72 hpi and the presence of GFP expressing cells was evaluated using FACS.

2.5.3 Quality of displayed Fc-fragments

2.5.3.1 Fc dimer formation – SDS-PAGE and western blotting

*Sf*9 cells were infected with Ac-NA and prepared for SDS-PAGE 72 hpi by adding 2 x electrophoresis buffer containing 50 mM Tris/HCl, pH 6,8, 10% glycerol, 2% SDS and 0,1% bromphenol blue for the non-reducing PAGE and in addition 5% 2-mercaptoethanol for the reducing PAGE. Proteins were separated using 10% Tris-Glycin gels and electro-blotted to a PVDF membrane (Roth, Karlsruhe, Germany). Detection was performed using an Anti-Human IgG Alkaline phosphatase conjugate antibody (Sigma-Aldrich, A3187) in PBS containing 1% skim milk powder. The blot was developed using CDP-Star (New England Biolabs, USA).

2.5.3.2 Binding of marker proteins - FACS

*Sf*9 and Vero cells were infected and transduced with Ac-NA and Ac-317 at an MOI of 10 and MOI 200 respectively. 72 hpi and 48 hpt, respectively, cells were harvested and washed with PBS. Subsequently $1x10^6$ cells were incubated with marker proteins diluted in PBS containing 10% FCS for 1 h as shown in table 2.6

Table 2.6 Marker proteins used in FACS analysis.

	1st step	2nd step
AntiFc	Anti-Human IgG R-Phycoerythrin Conjugate (Sigma-Aldrich, P8047).	
Protein A	Protein A, Biotin Conjugate (Calbiochem, 203195)	Strepavidin-R-PE Conjugate (Qiagen, 922721)
CD64	CD64 - His (R&D Systems, 1257-Fc)	Penta-His Alexa Fluor 488 Conjugate (Qiagen, 35310)
Her2	Her2, Biotin Conjugate	Strepavidin-R-PE Conjugate (Qiagen, 922721)

After staining, cells were washed with PBS, resuspended in 300 µL PBS and analysed on a FACS-Calibur (Becton Dickinson, San Jose, CA).

2.6 Secreted 3D6 antibody

2.6.1 Construction of transfer plasmid and generation of recombinant baculovirus

The human anti-gp41 antibody 3D6 light chain was PCR amplified using primers LC-SpeI-back and LC-HindIII-for. The product was digested with SpeI / HindIII and ligated into pFastBac Dual vector linearized with the same enzymes, resulting in pFBD-LC. dsDNA encoding for 3D6 heavy chain was PCR amplified using primers HC-XhoI-back and HC-NheI-for. The product was XhoI / NheI digested and ligated into XhoI / NheI digested pFBD-LC, resulting in pFBD-3D6.

Recombinant baculovirus was generated using the Bac-to-Bac system, resulting in Ac-3D6.

Table 2.7 Primers used for the amplification of 3D6 light and heavy chains.

designation	Sequence 5' → 3'
LC-SpeI-back	AGT AGT AGT ACT AGT ATG GAC ATG AGG GTC CCC G
LC-HindIII-for	AGT AGT AGT AAG CTT CTA ACA CTC TCC CCT GTT GAA GC
HC-XhoI-back	AGT AGT AGT CTC GAG ATG GAG TTG GGA CTG AGC TG
HC-NheI-for	AGT AGT AGT GCT AGC TCA TTT ACC CGG AGA CAG GG

2.6.2 Expression of 3D6 antibody

For the expression of soluble 3D6 antibody *Sf*9, "High Five" and Mimic cells were transferred to shaker flasks with supplemented IP-L41 medium without FCS two days prior infection. For infection 1 x 10^8 cells were spun down at 900 x g for 10 min and resuspended in the minimum volume of virus needed for infecting with a multiplicity of infection (MOI) of 5. After 1 h of shaking, to the volume was adjusted to 100 mL with supplemented IP-L41 medium without FCS. Samples were taken 24, 48 and 72 hours post infection (hpi) and the supernatant was harvested 96 hpi by centrifugation at 2000g for 10 min. Concentration of 3D6 antibody was measured by Ali Assadian (Polymun Scientific, Vienna, Austira) using ELISA.

2.6.3 Purification of 3D6 antibody – Affinity chromatography

The cellular supernatant was filtered through a 0.22 µM filter cartridge (Millipore, Billerica, USA) and applied on a 1 mL Bio-Scale™ Mini UNOsphere SUPrA™ Cartridge (BioRad, Hercules, USA) using an Äkta purifier (GEHealthcare) at an flow rate of 1 mL / min. Elution was performed with 100 mM glycine buffer pH 3 at a flow rate of 0,5 mL / min. The eluted samples were adjusted to pH 7 using 100 mM Tris-HCl (pH 9).

2.6.4 Size determination of purified antibodies - SDS-PAGE and western blotting

For determining the size of purified heavy and light chains and of fully assembled antibody, samples were mixed with 2 x electrophoresis buffer containing 50 mM Tris/HCl, pH 6,8, 10% glycerol, 2% SDS and 0,1% bromphenol blue for the non-reducing PAGE and in addition 5% 2-mercaptoethanol for the reducing PAGE. Proteins were separated by SDS-Page using NuPAGE® Novex 12% Bis-Tris gels, stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, USA) or electroblotted to a PVDF membrane (Roth, Karlsruhe, Germany). Detection was performed using an anti-human IgG alkaline phosphatase conjugated antibody (Sigma-Aldrich, A3187) in PBS containing 1% skimmed milk powder. The blot was developed using alkaline phosphatase Conjugate Substrate Kit (BioRad, Hercules, USA).

2.6.5 N-glycan analysis – MALDI-TOF MS

N-glycan analysis of insect cell expressed antibodies were essentially performed as described Rendic et al., (Rendić et al., 2007). Briefly, after the heavy and light chains of purified IgG_1 were separated on an SDS-PAGE, coomassie stained bands corresponding to heavy chains were excised. After washing the bands, they were treated with dithiothreitol and iodoacetamide solutions to modify cysteine residues. The gel bands were washed again and then subject to trypsin digestion at 37 ^{o}C over night. The peptides extracted with AcN:H_2O:TFA solution (666:330:1) were then dried, resuspended in 20 µl of 50 mM NH_4Ac (pH 5) buffer and subject to PNGase A treatment at 37 ^{o}C over night. The released N-glycans were purified using Spec PT C18 (Varian, USA) single-use SPE pipette tips (containing 4 mg of a C18 RP gel) overlaid additionally with, first, 25 µL of packed Dowex 50 W X8 (100–200 mesh) and, second, 25 µL of packed AG 3-X4A (200–400 mesh) before equilibrating with 2% acetic acid. After application of the sample to the column, the column was washed with 2% acetic acid and the flow-through that contains purified N-glycans was collected and dried in a SpeedVac. The drying procedure was repeated twice after resuspending the N-glycans in 150 µL of deionized water. Dried N-glycans were finally resuspended in 3 µL of deionized water and used for MALDI-TOF-MS analysis (on a Bruker Ultraflex Maldi-TOF/TOF in positive reflectron mode using 2% 2,5-dihydroxybenzoic acid (DHB) as matrix).

2.6.6 Target and receptor binding - FACS

For testing the specific binding ability of insect cell expressed antibodies to their target*, Sf*9 cells were infected with a recombinant baculovirus expressing the 3D6 epitope (SGKLICTTAVPWNAS) on the cellular surface with MOI 10 and harvested 72 hpi. After brief washing with phosphate buffered saline (PBS), $1x10^6$ cells were incubated with 100 µL of each insect cell expressed 3D6 antibody (1 µg/mL in PBS containing 10 % FCS) for 1 h at room temperature while gently shaking. Cells were again washed with PBS and incubated with an anti-human IgG (γ-chain specific) R-phycoerythrin conjugate (Sigma-Aldrich, P8047) in PBS containing

10% FCS for 1 h at room temperature. After a final wash with PBS, cells were resuspended in 400 µL PBS and analysed on a FACS-CantoII (Becton Dickinson, San Jose, CA).

In order to test the ability of insect cell expressed antibodies to bind receptors important for the protective function of the immune system, $1x10^6$ U937 cells were washed with PBS and incubated with 100 µL of each insect cell expressed 3D6 antibody (1 µg/mL in PBS) for 30 min on ice. After a brief washing, cells were incubated with an anti-human κ light chain FITC conjugate (Sigma Aldrich, F3761). After washing with PBS, cells were resuspended in 400 µL PBS and analysed on a FACS-CantoII.

2.6.7 Receptor binding - Enzyme-linked Immunosorbent Assay

Coating of human FcγRI/CD64 to 96 well Maxisorp plates (Nunc, Rochester, USA) was performed by incubating 1 µg/mL recombinant human FcγRI/CD64 (R&D Systems, Minneapolis, USA) in 100 µL coating buffer (PBS, 1% BSA) at 4°C over night. Plates were washed with TPBS (PBS, 0.1% Tween20) and 50 µL serially diluted samples (in dilution buffer: TPBS, 1% BSA) were applied to the wells. After one hour incubation on a rocking platform, the plate was washed again with TPBS. Detection was performed with 50 µL/well of an anti-human k light chain peroxidase conjugate (Sigma Aldrich, A7164) 1:1000 diluted in dilution buffer for 1 h while shaking. Staining of the ELISA plate was performed by adding 100 µL/well staining buffer (citrate buffer, pH5) containing 1 mg/mL OPD (1,2-o-phenylenedihydrochloride) and 1 µL/mL H_2O_2 (35%). The reaction was stopped with 100 µL H_2SO_4 and plates were analysed on an Infinite® M1000 reader (Tecan Group, Männedorf, Switzerland).

2.7 Glyco-module

2.7.1 Construction of transfer plasmid and generation of recombinant baculovirus

The *Caenorhabditis elegans* N-acetylglucosaminyltransferase II was PCR amplified from a cDNA template using primers CeGntII-back and CeGntII-for. The product was digested with NheI / XmaI and ligated into pUCDM vector cut with the same enzymes, resulting in pUCDM-G. The pUCDM vector contains a R6γ ori, which requires pir^+ competent *E*.coli for transformation. The bovine β4–galactosyltransferase was PCR amplified from a cDNA template using primers b4GalT-back and b4GalT-for. The product was BamHI / XbaI digested and ligated into BamHI / XbaI digested pUCDM-G, resulting in pUCDM-GG. For being able to directly integrate the glycosyltransferases in the baculovirus genome, the glycomodule consisting of expression cassettes for GntII and GalT were subcloned in a pKL vector, carrying Tn7 sites. This was achieved by cutting pUCDM-GG with PmeI and AvrII and ligating the glycol-module into a pKL vector cut with the same enzymes resulting in pKL-GG. Recombinant baculovirus was generated using Bac-To-Bac system resulting in Ac-GG.

Table 2.8 Primers used for the amplification of glycosyltransferases

designation	Sequence 5' → 3'
CeGntII-back	GACTTGATCACCCGGG ATGATGGTCTATCGACGGATG
CeGntII-for	TGCATCAGCTGCTAGCTTAAGAAGTTGTAGATGTGATTGT
b4GalT-back	CATCGGGCGCGGATCCATGAAGTTTCGGGAGCGGCT
b4GalT-for	GACTGCAGGCTCTAGACTAGCTCGGCGTCCCGATG

2.7.2 Expression and purification of glyco-modified 3D6 antibody

For the expression of glyco-modified, soluble, 3D6 antibody the new *Ao*38 cell line and Mimic cells were transferred to shaker flasks with supplemented IP-L41 medium without FCS two days prior infection. For infection 1 x 10^8 cells were spun down at 900 x g for 10 min and resuspended in the minimum volume of virus needed for infecting the cells: Mimic insect cells with Ac-3D6 at an MOI of 5, *Ao*38 with Ac-3D6 at an MOI of 5 and one flask in addition with Ac-GG at an MOI of 5. After 1 h of shaking the cells were filled up to 100 mL with supplemented IP-L41 medium without FCS and supernatants were harvested 96 hpi by centrifugation at 2000 x g for 10 min. Purification was done as described in section 2.6.3.

2.7.3 N-glycan analysis – Lectin blotting

Purified antibodies were separated on a reducing SDS-PAGE and stained with SimplyBlue™ SafeStain, or electroblotted to a PVDF membrane as described in section 2.6.4. Detection was performed using biotinylated *Ricinus communis* agglutinin I (1:1000) in PBS containing 1% BSA and Strep-Tactin Alkaline Phosphatase conjugate (IBA, Göttingen, Germany). The blot was developed using Alkaline phosphatase Conjugate Substrate Kit (BioRad, Hercules, USA).

3. Results and Discussion

3.1 Display Library

3.1.1 The optimal display system

Over the last years many different strategies have been developed to display eukaryotic proteins on the surface of insect cells using *Autographa californica* nucleopolyhedrovirus (AcMNPV) (Grabherr et al., 2001; Mäkelä et al., 2010a; Mäkelä and Oker-Blom, 2008). Most of them are based on class I transmembrane proteins like the baculoviral gp64 major coat protein (Grabherr et al., 1997), where the signal peptide is located at the amino terminal end and the transmembrane region at the carboxy-terminal end. For many proteins this is not a problem and even advantageous, but when working with Fcabs this orientation would cause sterical hindrances and thus, alter the binding ability of engineered binding sites. By using the influenza A neuraminidase class II transmembrane domain, the problem could be overcome, because signal domain and membrane anchorage sequence are both located at the amino terminal end, creating a free C-terminus (Borg et al., 2004; Bos et al., 1984). In this study we have compared the use of both systems as demonstrated in Figure 3.1

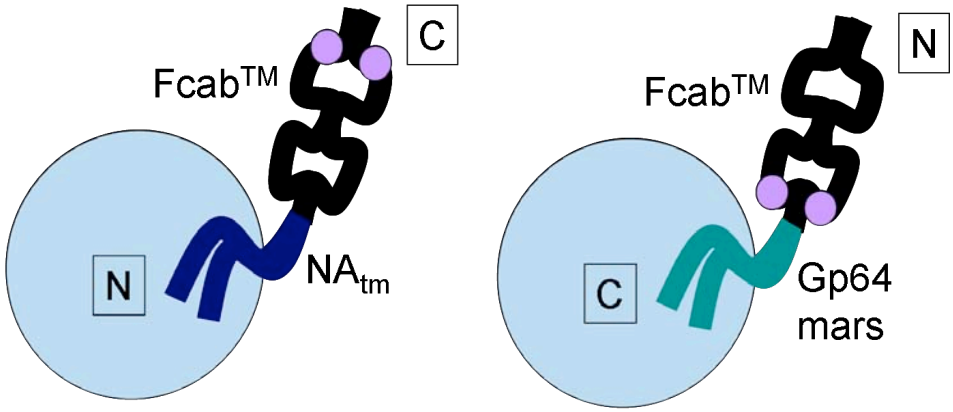

Figure 3.1 Schematic representation of the Fcab display.

In both cases, gene expression was driven by a dual promoter construct consisting of the baculoviral very late polyhedrin promoter, for insect cells, adjacent to the CMV promoter for mammalian cell expression. To confirm the expression of correct fusion proteins in insect cells, *Sf*9 cells were infected with Ac-NA and Ac-mars at an MOI of 10. Cellular proteins were separated by SDS-PAGE and analysed by western blotting 72 hpi, using an Anti-Human IgG antibody. Figure 3.2 shows strong bands at an estimated molecular weight of 29 kDa for the NA_{tm}-Fc fusion and 31 kDa for Fc-mars. This shows that the CMV promoter did not disturb the polyhedrin promoter driven gene expression.

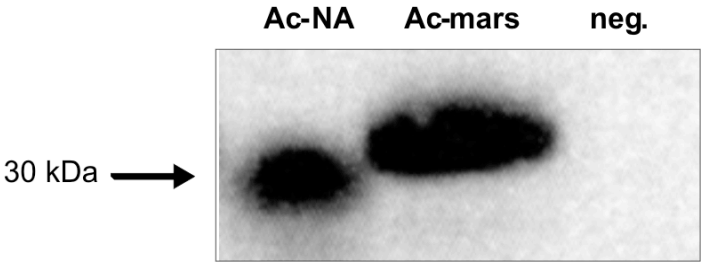

Figure 3.2 Western Blot analysis of Ac-NA and Ac-mars infected *Sf*9 cells (72 hpi) stained with an Anti-Human IgG (γ-chain specific) antibody. The fusion proteins are expressed in high amounts and the relation in matter of size is verified (NAtm-Fc: 29 kDa; Fc-mars: 32 kDa).

In order to evaluate parameters optimal for surface display, *Sf*9 cells were infected with Ac-NA and Ac-mars at different MOIs and analysed at different time points after infection. Figure 3.3 (top) shows increased display efficiency and a more homogenous population using the amino terminal anchorage of the influenza A neuraminidase transmembrane region. Since a slight increase in display efficiency was observed when using an MOI of 10 (Figure 3.3 middle), all further infections were carried out at this MOI. However, for library screening the problem of cross-infections, resulting in multiple screening rounds, has to be kept in mind and the optimal MOI might have to be determined again for this purpose. The last critical point when it comes to baculoviral gene expression is the optimal time point for harvesting the cells. Cells were therefore infected with optimized parameters and analysed 24, 48 and 72 hpi. Figure 3.3 (bottom) shows a significant increase in amount of displayed Fc-fragments from 24 to 72 hours. We decided to carry out all further infections, with amino terminal anchored Fc-fragments at an MOI of 10 and to analyse the cells 72 hpi.

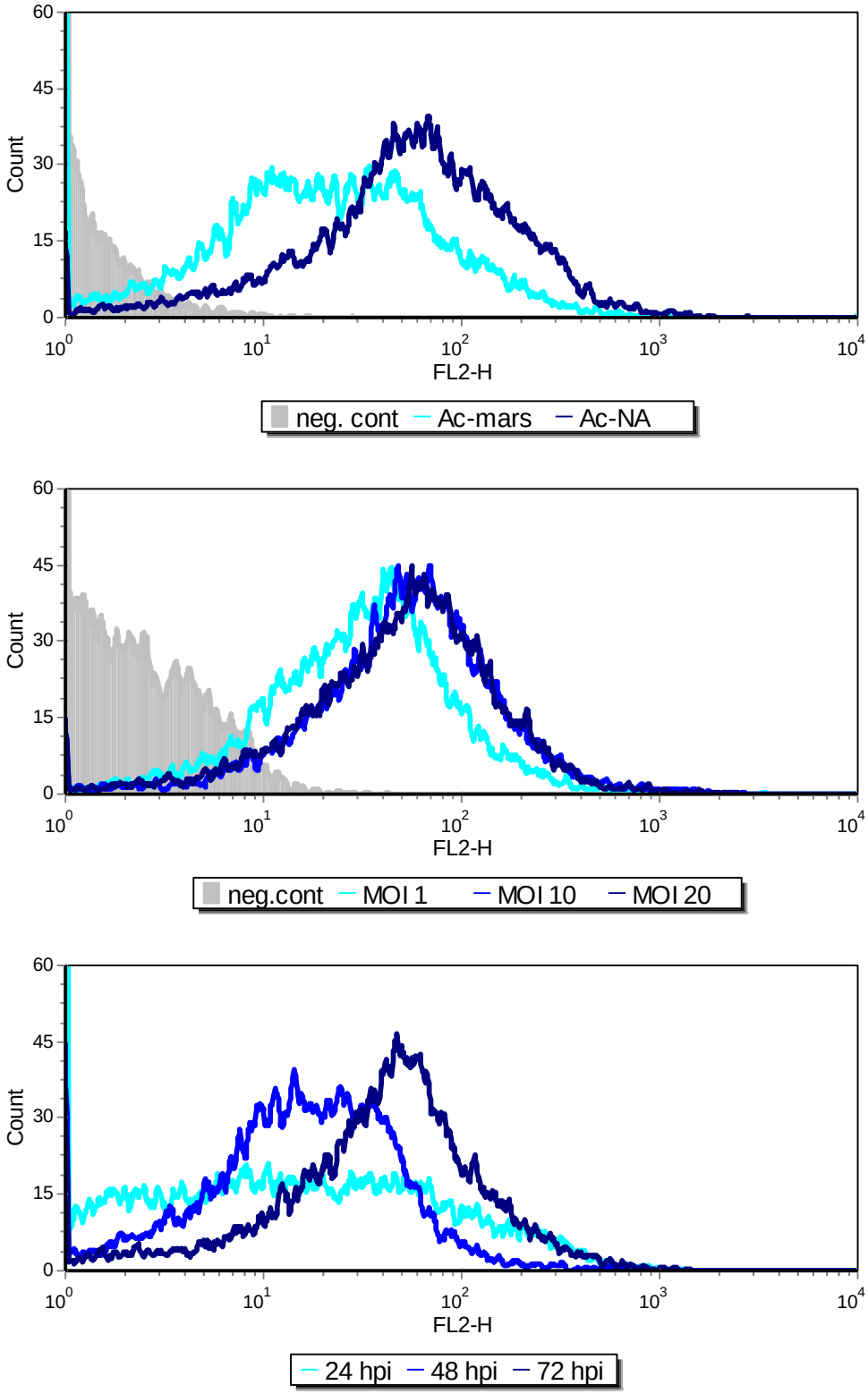

Figure 3.3 Evaluation of optimal display parameters using FACS. Using the aminoterminal anchorage of influenza A neuraminidase transmembrane domain gave highest expression rates (top). Infecting with an MOI of 10 (middle) and harvesting 72 hpi (bottom) turned out be ideal.

For testing the transduction efficiency and the full functionality of the dual promoter, Vero cells were transduced with Ac-NA at an MOI of 200 and subjected to FACS analysis 48 hpi. Figure 3.4 shows that the transduction efficiency is almost 90 %, giving no need for further optimization as done by Ernst et al. (Ernst et al., 2006).

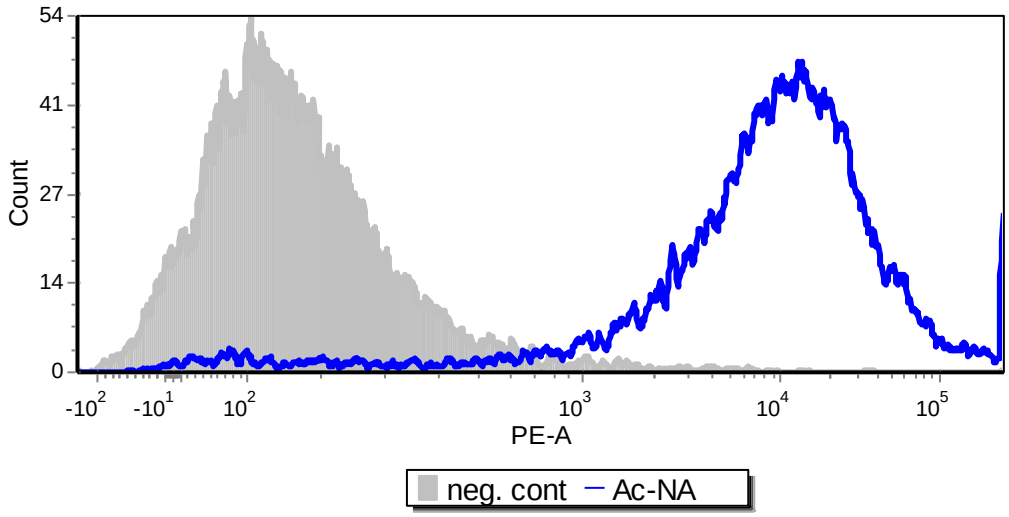

Figure 3.4 FACS analysis of Vero cells transduced with Ac-NA at an MOI of 200 48 hpi.

3.1.1.1 Cellular localisation

To get information about the cellular localization of NA_{tm}-Fc fusion proteins, insect and mammalian cells were infected and transduced, respectively, with Ac-NA and subjected to immunostaining, using a phycoerythrin labelled anti-human IgG antibody and confocal scanning microscopy 48 hpi and 36 hpt. There was only little to no protein detectable in cytoplasm or in the nucleus, but substantial amounts were detectable at almost all cellular membranes including the nuclear membrane and the endoplasmatic reticulum (Figure 3.5). Apparently, the N-terminal neuraminidase signal peptide was efficient in targeting the fusion protein through the secretory pathway to the cell surface plasma membrane where it was incorporated into the cell membrane and anchored by the neuraminidase transmembrane domain.

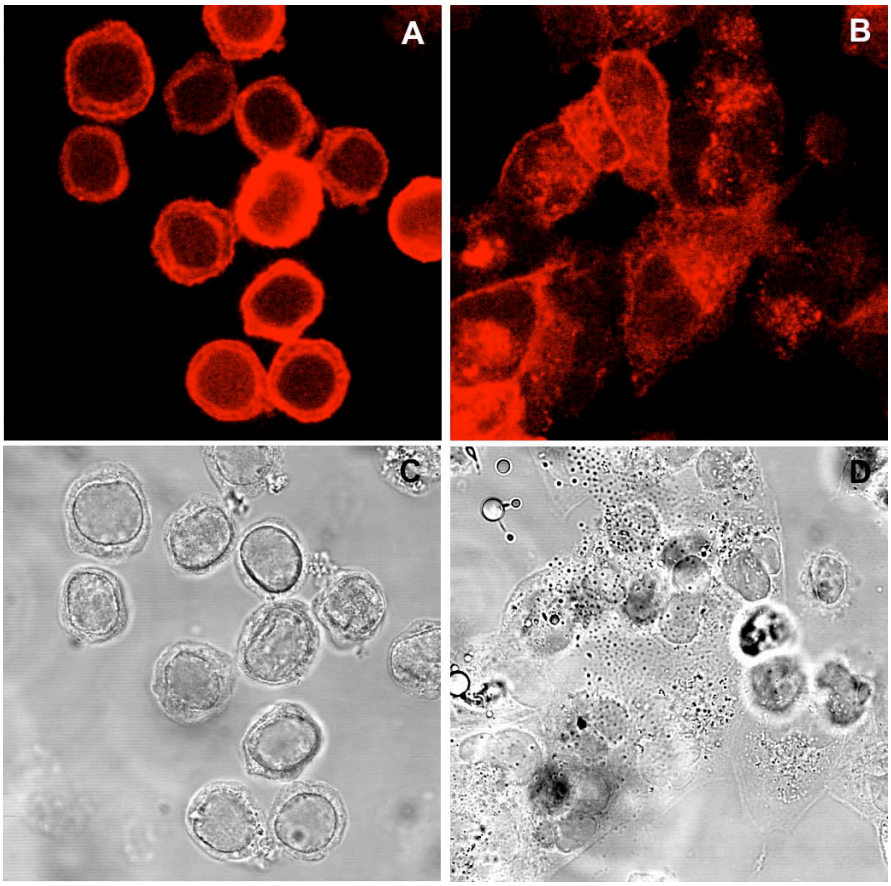

Figure 3.5 Pictures A – B show, with Ac-NA, infected *Sf*9- and transduced Vero-cells stained with a Phycoerythrin labeled Anti-Fc antibody. Pictures C – D show the corresponding cells in transmission microscopy.

3.1.2 The optimal Library system

Nowadays two major systems are used when it comes to the generation of surface display libraries: bacterial phage display and yeast surface display. Phage display allows the expression of exogenous (poly)peptides on the surface of filamentous phages (Hoogenboom, 2005; McCafferty et al., 1990; Smith, 1985). Thereby it becomes possible to create libraries in the range of 1 x 10^{10} different clones. However, a major drawback is the fact that *E*.coli is not able to glycosylate proteins. As an alternative, Yeast surface display systems provide eukaryotic posttranslational modifications (Kondo and Ueda, 2004). However, the yeast quality control system is unable to differentiate well-folded protein variants form collapsed ones, among those of high stability (Park et al., 2006) Eukaryotic surface display libraries based on AcMNPV, allow expression of proteins on the viral and cellular surface and selection of specific binding proteins while providing higher order post translational modifications (Ernst et al., 1998; Mäkelä et al., 2010b). A major drawback using baculovirus based surface display is the limited library size. Common methods based on in vivo homologous recombination produce approximately 10^3 individual clones, which would limit the number of mutants to be screened drastically. Therefore, our approach was to adapt a system that is based on direct in vitro recombination (Figure 3.6).

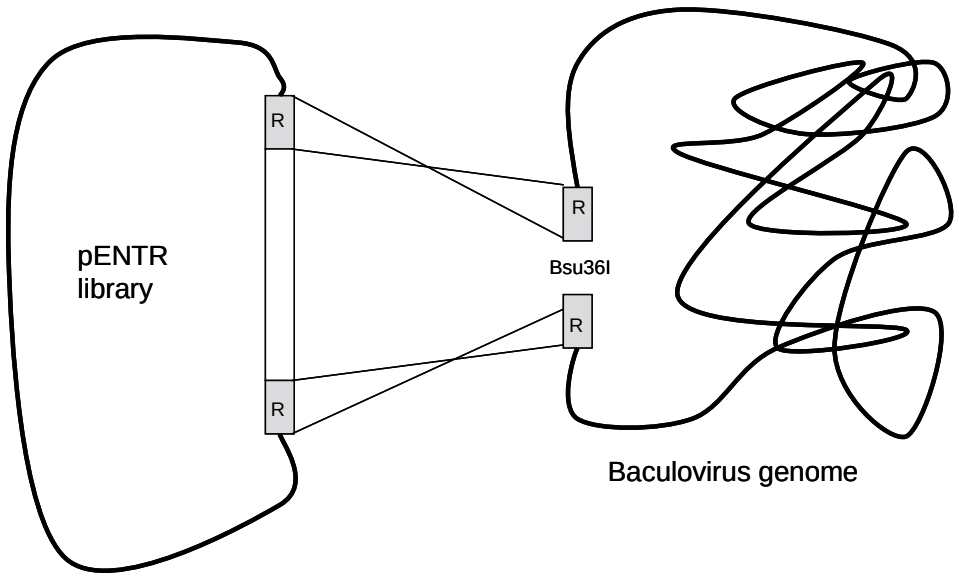

Figure 3.6 Schematic representation of in vitro recombination system.

A recombinant Acceptor baculovirus was generated carrying a dual promoter construct consisting of the baculoviral polyhedrin and the mammalian CMV promoter, adjacent to a recombination cassette based on Invitrogen's Gateway® technology. This cassette consists of the left and the right arm of an attR site spaced by "junk" DNA including a Bsu36I restriction. Bsu36I is one of the very few enzymes not cutting in the baculovirus genome de novo. Circular baculovirus DNA is infectious and in order to reduce non-recombinant background linearizing the genome with Bsu36I is expected to be favourable.

The Donor constucts consist of a plasmid containing the left and the right arm of an attL site homologous to attR sites present on the baculovirus genome. The desired insert is cloned in between those sites and recombination is performed using an LR Clonase enzyme mix in vitro.

To test the efficiency of our system, *Sf*9 cells were transfected with in vitro recombination reactions and analysed 6 days after transfection using FACS. Figure 3.7 shows that in vitro recombination is much more efficient when using Bsu36I cut baculovirus DNA as acceptor (94% of cells are displaying the desired protein). When using uncleaved viral DNA, the amount of non-recombinant baculoviral background is significantly higher (only 24% cells show transgene expression).

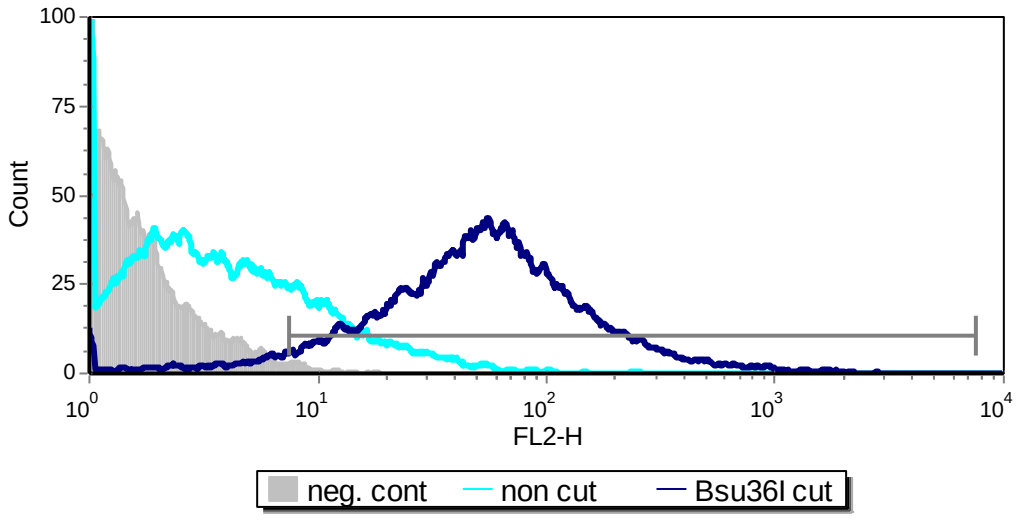

Figure 3.7 FACS analysis of in vitro recombinations.

In order to confirm the results obtained with FACS, virus generated from recombination with Bsu36I cut DNA was subjected to plaque purification and single plaques were analysed by PCR using primers flanking the recombination sites. Figure 3.8 shows that 17 out of 22 viral clones carry the desired insert (1000 bp), yielding a recombination efficiency of 77 %. A plaque containing a non-recombined virus gives a band of 2000 bp.

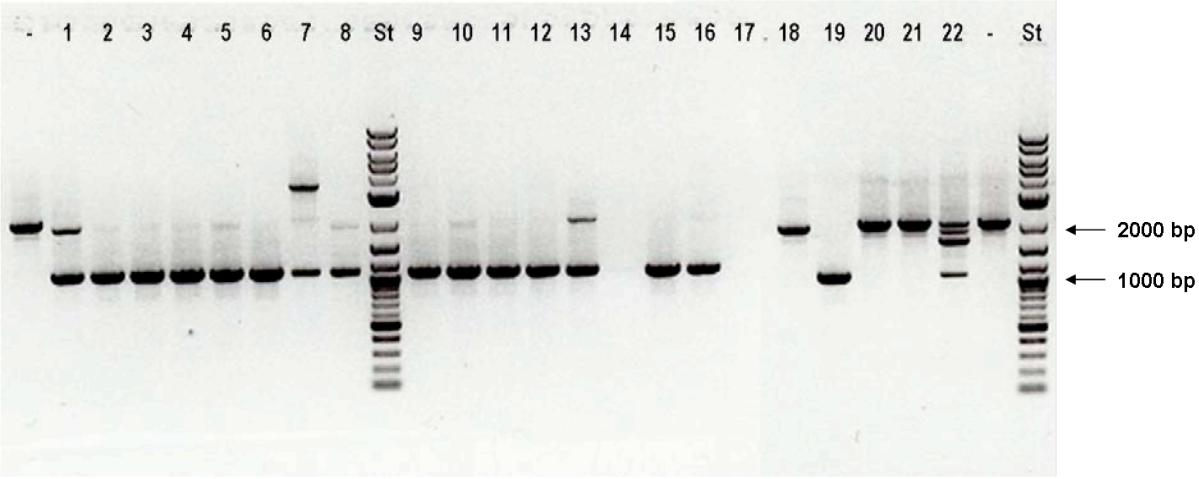

Figure 3.8 Agarose gel electrophoresis of PCR screened plaques.

The ability to select for single clones in large libraries is a key issue for screening systems. We therefore transfected *Sf*9 insect cells with baculovirus genomic DNA coding for GFP and Fcab mixed in different ratios. In order to set a negative control gate, cells were only transfected with genomic DNA coding for Fcab. Figure 3.9 shows that at a ratio of GFP to Fcab of $1{:}10^6$, there are still 21 GFP expressing cells detectable (0.21% of 10 000 cells measured). This supports the suggestion that our setting is useful for screening of large libraries.

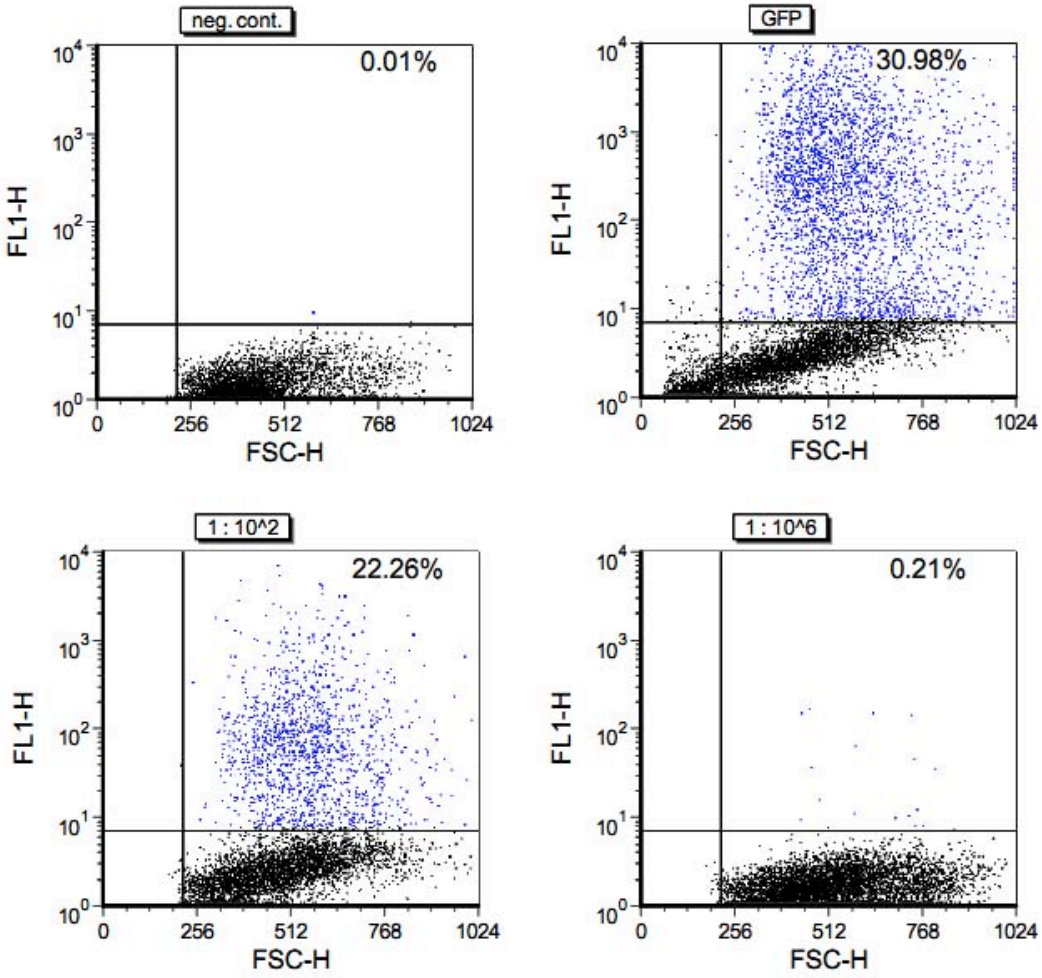

Figure 3.9 FCAS analysis of transfection experiments.

3.1.3 Quality of displayed Fc-fragments

3.1.3.1 Formation of Fc dimers

Dimer formation is one important factor for full functionality of IgG_1 Fc-fragments. To obtain information about dimer formation, insect and mammalian cells were infected and transduced as described above and subjected to reducing and non-reducing SDS-PAGE followed by western blot analysis. Under non-reducing conditions disulphide bonds linking two Fc's are expected to stay intact, thus a size shift of the band can be expected. The bands of *Sf*9 and vero cell derived samples show the estimated size of 29 kDa for a single-chain Fc on the reducing gel, and under non-reducing conditions the bands were shifted to 55 kDa and 50 kDa, respectively. This indicated that, although, expressed as single chains, Fc-fragments may form disulphide bonds during the secretory pathway and were displayed in the form of dimers on the cellular surface.

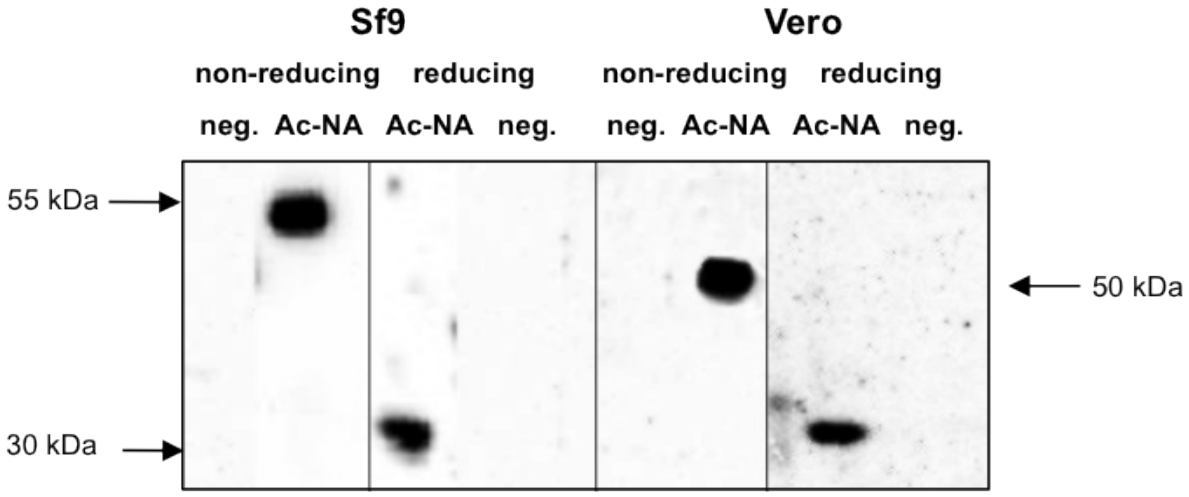

Figure 3.10 Western blot analysis from a non-reducing and reducing SDS-PAGE. Cells were stained with an Anti-human IgG antibody. Under non-reducing conditions, disulfide bonds, linking two Fc chains, stay intact, resulting in shift in size (55 / 50 kDa) compared to reducing conditions (30 kDa).

3.1.3.2 Binding of marker proteins

Structural integrity of an antibody Fc-region is a key issue for biological activities, such as Antibody dependent cellular cytotoxicity (ADCC) and Complement dependent cellular cytotoxicity (CDC). Special marker proteins serve to characterize the Fcab, since they only bind when certain structural features are present. *Staphylococcus aureus* protein A (SpA) binds to antibody Fc-fragments with high affinity only when correctly folded. FcγRI (CD64) is a cell surface protein, expressed constitutively on haemopoietic cells (e.g. macrophages, eosinophils, neutrophils, natural killer cells, lymphocytes) and other tissues and may up regulated, on each cell type when exposed to cytokines or other activating agents (Jefferis and Lund, 2002). The Human epidermal growth factor receptor 2 (Her2) is used for testing the engineered binding site of Fcab clone 317, specific for Her2.
Insect and mammalian cells have therefore been infected / transduced with Ac-NA and the Fcab clone Ac-317 and subjected to flow cytometric analysis 72 h post infection and 48 h post transduction respectively (Figure 3.11). Cells were stained with an Anti-human IgG_1 antibody (AntiFc) to confirm the overall expression of the Fc-fragments on the cellular surface. 95% of insect and 93% of mammalian cells displayed the wildtyp Fc on the outer cell membrane. The amount of displayed Fcab clone 317 is significantly lower (80% in insect and 40% in mammalian cells). This could be due to a reduced stability caused by the modifactions in CH3 domain. Staining with SpA gave, for wild type, 77 % positive insect and 79 % positive mammalian cells. Fcab clone 317, again, showed drastically lower amounts of bound SpA (28% and 8% respectively). In order to test the ability of the expressed Fc to exert effector immune functions, insect and mammalian cells were incubated with the human FcγRI (CD64). While only 18 % of the wild type expressing insect cell population showed FcγRI binding, 56 % of vero cells bound to human FcγRI. Fcab clone 317 showed almost no significant binding. We further tested the binding ability of our Fcab clone to its specific target, which is the human epidermal growth factor receptor 2 (HER2). However, while the corresponding yeast-clone (Saccharomyces cerevisiae displaying Her2 specific Fcab; kindly received from Gordana Wozniak) showed specific binding, for the insect cells no significant binding could be detected. This could be possibly due to the differences in the cellular quality control mechanisms.

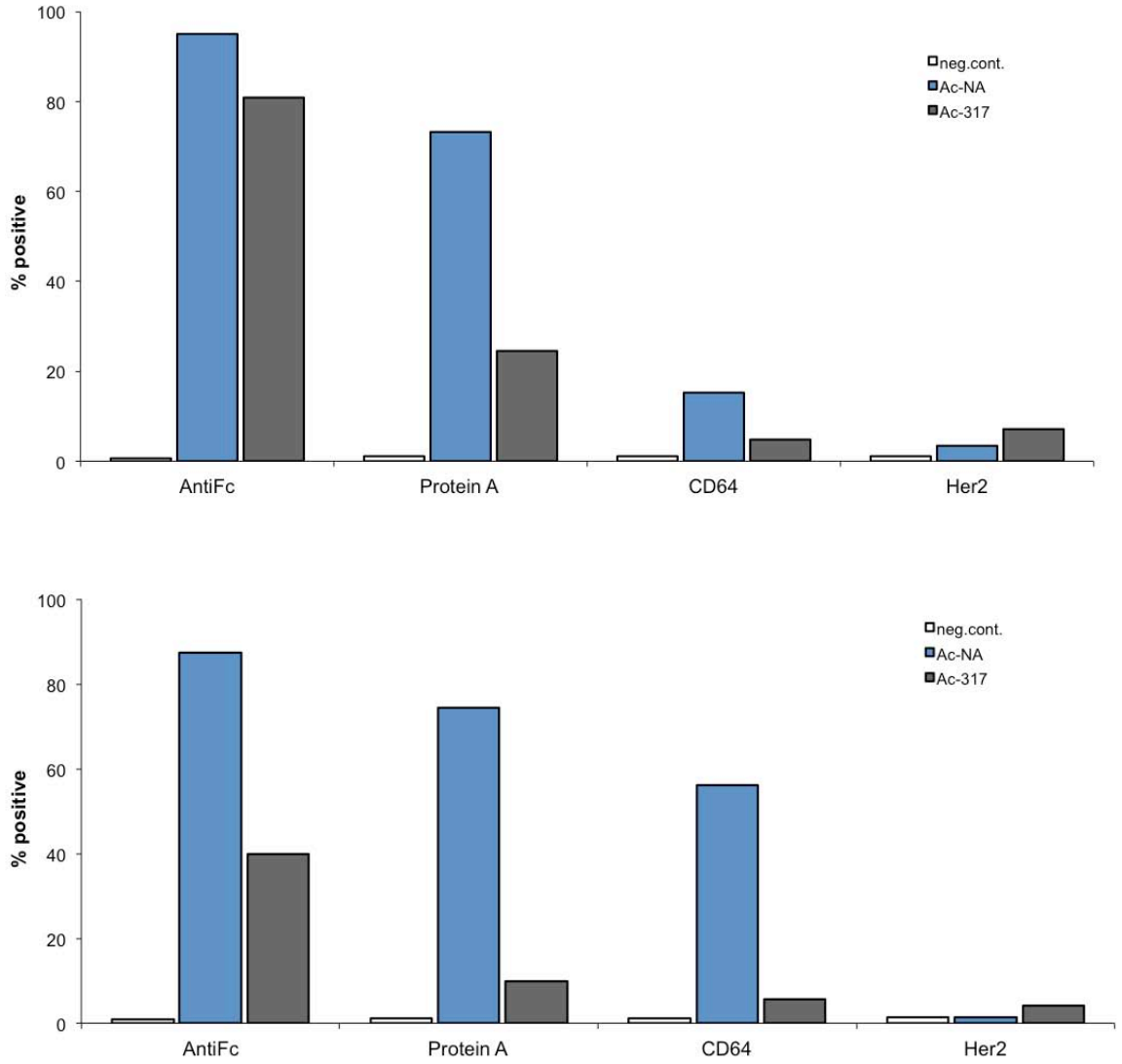

Figure 3.11 FACS analyses of *Sf*9 (top) and vero cells (bottom) displaying IgG1 Fc-fragment and Fcab clone 317, stained with an Anti-Fc antibody, SpA and CD64.

3.1.4 Conclusion I

It could be shown that display of human Fc-fragments, based on the transmembrane domain of influenza A neuraminidase was highly efficient on the surface of insect cells as well as on mammalian cells. For the generation of surface display libraries, an in vitro approach could be adapted to be highly suitable. Analyzing the quality of displayed Fc's expressed in described systems revealed a clear difference between the insect and mammalian expression system. Marker proteins, that are not dependent on the glycosylation pattern, showed almost no difference in their binding characteristics. However, the binding of highly glycosylation dependent FcγRI (Jefferis and Lund, 2002; Sibéril et al., 2006) was significantly different among the expression systems. Based on these findings, we concluded that differences in glycosylation pattern are another key factor in functionality of human Fc-fragments. Apparently the genetic differences of insect and mammalian cells are sufficient to strongly influence e.g. effector functions of expressed antibodies in vivo. To test the impact of glycosylation on the functionality of insect cell expressed antibodies we decided to produce a secreted antibody and characterize it in terms of biological activity.

3.2 Secreted 3D6 antibody

3.2.1 Expression of mAb 3D6 in different insect cell lines

In order to analyse the differences in glycosylation pattern of a whole antibody derived from different insect cell lines, we generated recombinant baculovirus clones expressing the heavy and light chain of the monoclonal HIV antibody 3D6 (Rüker et al., 1991). We evaluated the differences in expression yield and profile in *Spodoptera frugiperda* (*Sf*9), *Trichoplusia ni* "High Five" and glycoengineered *Sf*SWT-1 (Mimic^{TM}) insect cells. For monitoring the expression profile, samples were collected 24, 48, 72 and 96 hours post infection (hpi) and the mAb content was determined by ELISA (Figure 3.12). *Sf*9 and Mimic cells secreted 1–2 µg of antibody per mL of culture supernatant after 48 hours, rising to 3 µg/mL after 96 hours. Interestingly, "High Five" cells yielded significantly more secreted 3D6 antibody. At 48 hpi they produced an almost twofold higher amount of antibody, steadily increasing to more than 12 µg/mL after 96 hours.

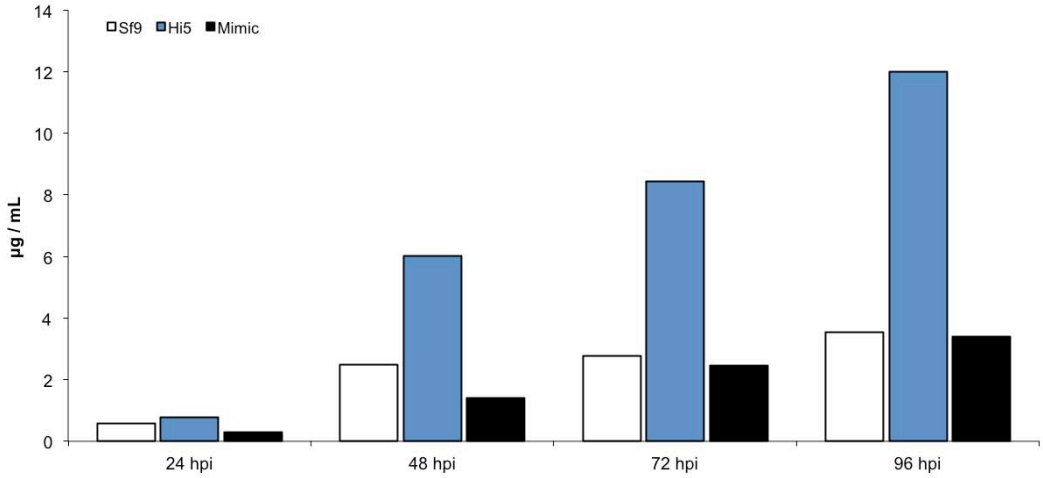

Figure 3.12 ELISA of 3D6 antibody secreted to insect cell culture supernatants. 24, 48, 72 and 96 hours post infection (hpi). (Experiments were carried out in triplicates)

Although somewhat higher expression rates can be reached by transient (22 µg/mL in culture supernatant) (Muller et al., 2007) and stable expression in CHO cells, (1 – 10 mg of antibody / mL culture supernatant in a fed batch process) (Kelley, 2009; (Huang et al., 2010), the fast production, ease of handling and lower costs of the insect cell system are definite advantages. Using this system, no time-consuming top-clone selection is required and stability of cell lines is not an issue.

The complexity of the downstream processing is a further important factor in recombinant protein production technologies. For purification, the crude culture supernatant was directly applied to a protein A column and eluted samples were subjected to a reducing as well as a non-reducing SDS-PAGE for purity and quality control. Figure 3.13 (A) shows that in all samples, the heavy chain (HC) and light chain (LC) bands were the most dominant proteins detected. Low amounts of additional proteins were visible and found to be present in the CHO expressed and purified 3D6 antibody preparation as well. HC and LC bands revealed a molecular weight of 55 and 27 kDa respectively. The CHO derived antibody showed the same size distribution. The non-reducing SDS-PAGE (Figure 3.13 B) indicates the presence of antibodies composed of two heavy and light chains each, since the molecular weight increased accordingly. All bands were further confirmed by Western blot analysis (data not shown). Taken together, insect cells especially “High Five” cells can be seen as being a potential tool for the production of sufficient quantifies of properly folded antibodies.

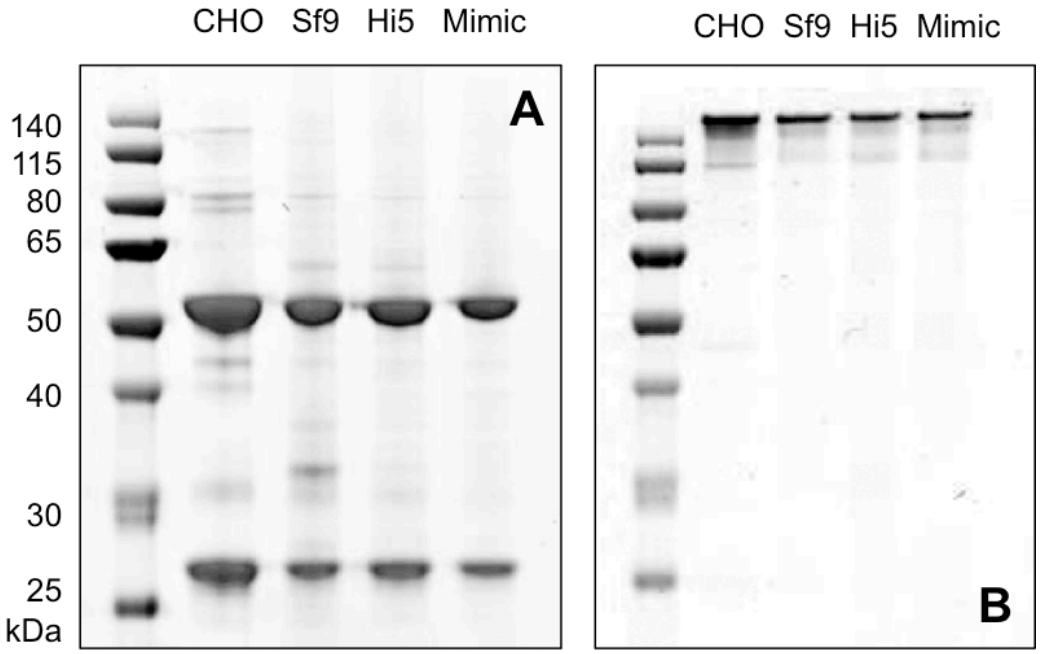

Figure 3.13 (A) Reducing SDS-PAGE shows heavy chain and light chain bands at 55 and 27 kDa respectively being equal among all expression systems. (B) Non-reducing SAS-PAGE indicates the presence of fully assembled antibodies composed of 2 HCs and 2 LCs at 160 kDa. The identity of all bands was further confirmed by Western blotting (data not shown).

3.2.2 Determination of N-glycan structures

The structure of the N-linked glycans in IgG_1 antibody heavy chains is very important for the ability to elicit different effector functions *in vivo* (Jefferis et al., 1998*)*. We, therefore, analyzed the N-glycosylation patterns of the different 3D6 antibodies by mass spectrometry. Figure 3.14 shows the MALDI / TOF-MS spectra of N-glycans from different insect cell lines and CHO cells after PNGase A digestion. Our N-glycan analyses were corroborated by the analyses of (glyco)peptides prior to PNGase A treatment (data not shown). The IgG_1 expressed in CHO cells exhibited fucosylated biantennary N-glycan structures carrying two terminal N-acetylglucosamine residues or one/two terminal galactose residues; based on our knowledge of the mammalian glycosylation machinery, the fucose is assumed to be α1,6-linked to the core. Smaller biantennary structures were identified on IgG_1 purified from *Sf*9 cells. These structures carry no terminal galactose residues, but are fucosylated; based on our mass spectrometric data traces of core α1,3-linked fucose can not be ruled out, but we presume a high degree of core α1,6-fucosylation. In the case of IgG_1 purified from “High Five” cells, the major structure carried two fucose residues; this indicates that a considerable portion of the N-glycans carry both core α1,3- and α1,6-linked fucose. For therapeutic applications of *Sf*9 and “High Five” cell derived antibodies, the core α-1,3-fucosylation is highly relevant as it is known to be immunogenic (Altmann, 2007). The structures identified in analyses of N-glycans from IgG_1 expressed in Mimic cells, closely resemble the ones found in IgG_1 expressed in CHO cells. Nevertheless, the amount of galactosylated IgG_1 in Mimic cells is, according to the estimated peak size (Figure 3.14), somewhat lower than in CHO cells (30% versus 50% as judged by the intensity).

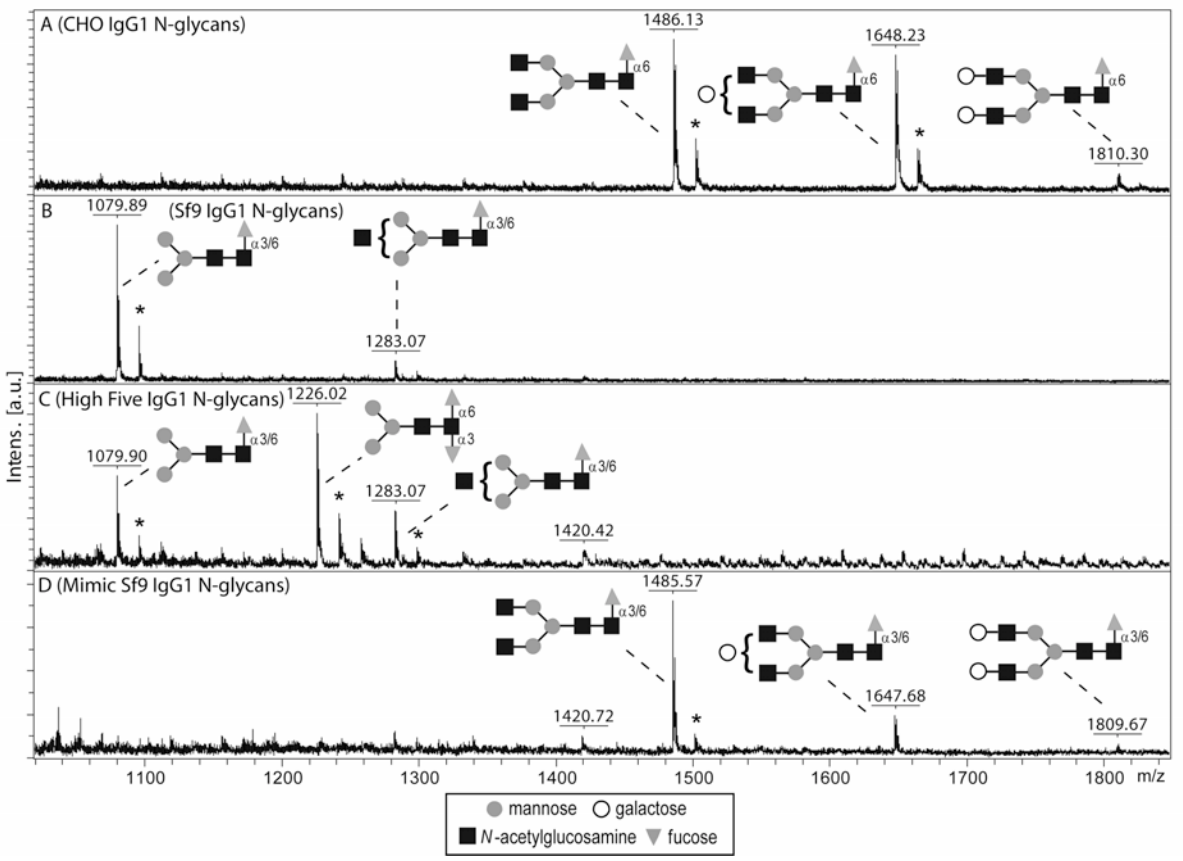

Figure 3.14 MALDI / TOF-MS spectra of IgG_1 N-glycan structures expressed in CHO, *Sf*9, "High Five" and Mimic *Sf*9 cell lines. Fucosylated biantennary N-glycan structures carrying two terminal N-acetylglucosamine residues or one or two terminal galactose residues were identified on IgG1 recombinantly expressed in and purified from CHO cells (A). Smaller biantennary structures were identified on IgG_1 purified from *Sf*9 cells; these fucosylated structures carry no terminal galactose residues (B). In addition to the structures found in *Sf*9 IgG_1, the major structure found in IgG_1 purified from "High Five" cells carries two fucose residues. This structure indicates that a considerable portion of the N-glycans carry the allergenic core α1,3-linked fucose (C). The structures identified in analysis of N-glycans from IgG_1 expressed in Mimic *Sf*9 cells, closely resemble the ones found in IgG_1 expressed in CHO cells. Nevertheless, the amount of galactosylated IgG_1 in Mimic *Sf*9 cells is considerably lower than in CHO cells. The major species $[M+Na]^+$ are annotated with *m/z* values of the structures; potassium adducts of identified major structures are labelled with an asterisk.

Of the insect lines tested, Mimic cells generally offer the most human-like glycosylation capacities. Although the detected N-glycan structures resembled mammalian complex N-linked glycans, the relative hypogalactosylation indicates that the full theoretical capacity was not realised (Figure 3.14). In terms of glycosylation, the Mimic cells would be the ideal choice for producing therapeutically active antibodies. However, due to the fact that cells from the *Spodoptera frugiperda* lineage are not able not secrete sufficient amounts (Figure 3.12), their biotechnological use is limited. “High Five” cells in contrast, are the ideal choice in terms of production yield, but have the disadvantages of non human glycosylation and furthermore, the synthesis of an immunogenic core α-1,3-linked fucose. These drawbacks could be overcome by genetically modifying “High Five” cells by introducing glycosyltransferases in a similar way to Mimic cells (Hollister et al., 2002) and/or by generating α1,3/α1,6-fucosyltransferase double knock-down or knock-out mutants. Furthermore, considering that ADCC activity of antibodies can be enhanced by changing their glycosylation from a typical core fucosylated complex type to a structure lacking this core fucosylation (Nechansky et al., 2007; Shinkawa et al., 2003), the knock-out of the relevant fucosyltransferase(s), e.g. using the Potelligent® technology (BioWa, Inc.) also seems reasonable (Beuger et al., 2009; Satoh et al., 2006), but has only been performed in mammalian cells. Indeed, realisation of a knock-out in “High Five” cells requires not just knowledge of the relevant gene sequence(s), but also development of the gene deletion technology in insect cells. The possible effects of these modifications on the production yield remain to be determined, but since the strategy was successfully employed in generating the Mimic cell line (see Figure 3.12) (Hollister et al., 2002), no changes in secretion performance are, *a priori*, to be expected.

3.2.3 Functionality of insect cell expressed antibodies

So far we could show that the baculovirus insect cell system is suitable for the expression of fully assembled antibodies carrying complex N-linked glycan structures similar to those found in humans, in particular when using Mimic cells. Whenever antibodies are intended for therapeutical use they have to exert full functionality regarding antigen binding and the ability to elicit effector functions. For testing the target binding ability, *Sf*9 cells were infected with a recombinant baculovirus expressing the HIV gp41 3D6 epitope on the cellular surface. The epitope presenting cells were incubated with insect and CHO expressed 3D6 antibody and target binding was measured by FACS. Figure 3.15 shows that no difference in binding abilities among antibodies expressed in different cell lines could be observed. The broadening of the peaks could be explained by a non uniform distribution of the 3D6 epitope on the cellular surface.

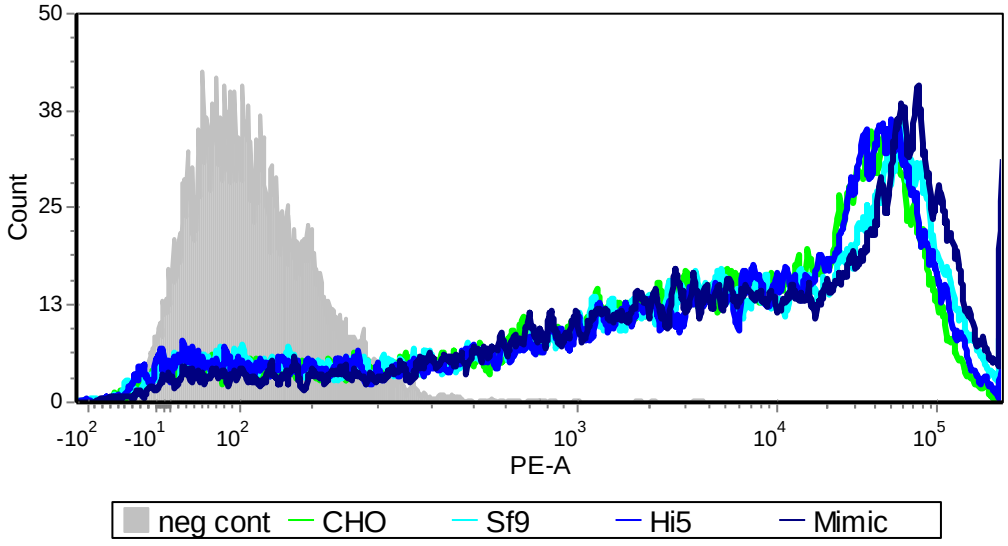

Fig 3.15 Binding of insect and CHO cell expressed antibodies to their target. *Sf*9 cells presenting the 3D6 epitope were incubated with purified antibodies and target binding was measured using FACS. Antibodies expressed in insect and CHO cells show a highly specific binding. The broadening of the peak in lower fluorescent intensity regions can be explained by different amounts of 3D6 epitope displayed on *Sf*9 cells.

Besides target binding, the effector functions of the Fc region, such as ADCC and CDC are essential for the immune system to trigger potent responses against a variety of pathogens. The binding of insect cell expressed 3D6 antibody to FcγRs present on leukocytes was measured by incubating human Caucasian histiocytic lymphoma (U937) cells with insect cell expressed antibodies. (Dai et al., 2009) have shown that U937 express, in absence of interferon-γ, suitable amounts of human CD64 and CD32, but not CD16 on the cellular surface, with intention of making use of interaction between IgG_1 with CD64 and CD32. Figure 3.16 shows that U937 cells treated with 3D6 displayed a similar amount of bound antibody for all samples tested.

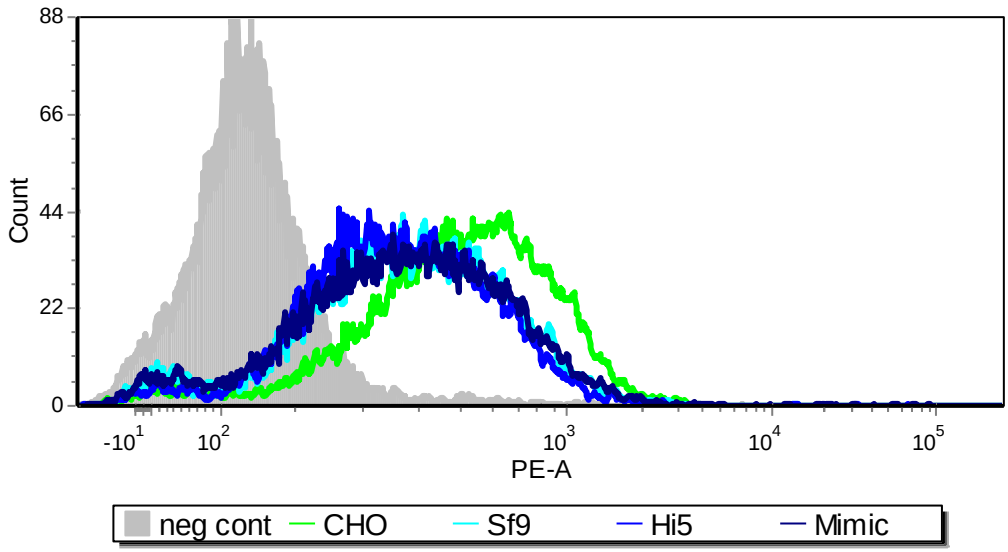

Figure 3.16 Binding of insect and CHO cell expressed 3D6 antibody to FcγRs. FACS analysis of U937 cells incubated with 3D6 antibody. Insect and CHO expressed antibodies bound to FcγRs present on cellular surface. The peak for the CHO sample is shifted to the right indicating a slightly increased binding capacity.

The peak of cells treated with the CHO expressed antibody was slightly shifted towards higher fluorescent intensities indicating somewhat increased antibody binding. This difference may be the result of the more authentic glycosylation capacity of CHO cells as compared to insect cells. The difference between the N-glycans found in CHO and Mimic cell expressed 3D6 is mainly the amount of galactose, suggesting that the presence of this sugar residue is important for FcγR binding (Kumpel et al., 1994). In order to confirm that the binding on the cellular

surface was specific for FcγRs, we tested the antibodies in terms of binding to the high affinity receptor CD64 by ELISA (Figure 3.17). All insect cell expressed antibodies showed similar binding properties, which were comparable to CHO expressed samples.

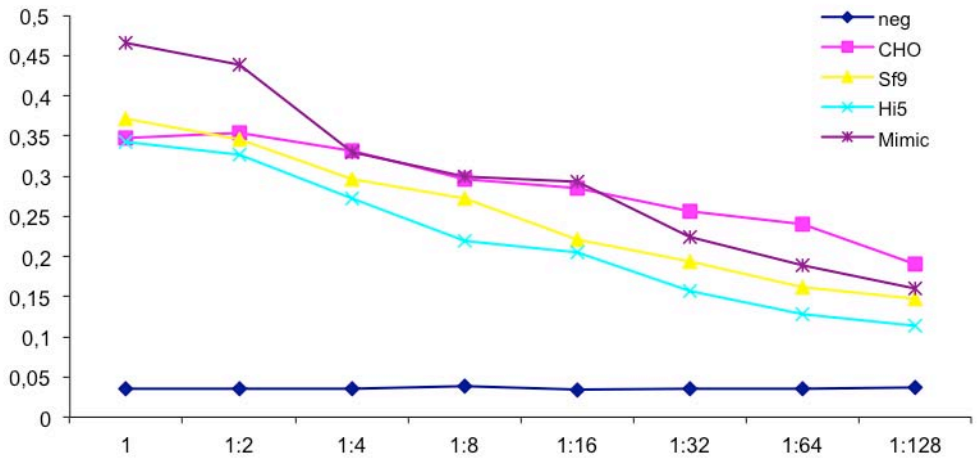

Figure 3.17 Binding of insect and CHO cell expressed 3D6 antibody to FcγRs. ELISA determining the binding of 3D6 antibody to the specific FcγRI (CD64).

Apart from the parameters tested in this study, the half-life of recombinantly produced antibodies is another important factor. While galactosylation (Huang et al., 2006) and fucosylation (Kanda et al., 2007) were shown to have little or no effect in terms of half-life, IgGs carrying high-mannose type N-glycans (*e.g.*, Man_5, $Man_5GlcNAc_2$) were found to clear faster from serum in mice. It was furhter shown that the absence of sialic acid greatly influences the survival of glycoproteins in the blood stream (Morell et al., 1971). However, IgGs carrying hybrid structures (*e.g.*, Man5GnF, $Man_5GlcNAc_3F$) showed higher stability when compared to IgGs carrying Man_5 (Kanda et al., 2007). Half-life and clearance behaviour of IgG carrying Man_3 ($Man_3GlcNAc_2$) and GnM/MGn ($Man_3GlcNAc_3$) has yet to be determined.

3.2.4 Conclusion II

We evaluated the feasibility of the baculovirus insect cell system for the transient expression of monoclonal antibodies suitable for therapeutic use. Insect cells, especially *Trichoplusia ni* “High Five” cells could be proven to be a very powerful tool for the generation of complex, secreted, glycosylated proteins, as they express high amounts of antibody, comparable to those found in transient mammalian cell systems. Although stable CHO expression schemes offer even higher product yields, cost and time consuming generation and characterisation of stable integrations is not required. Functionality studies of insect cell derived antibodies showed a comparable antigen binding ability and a slightly reduced binding to human FcγRs. These results emphasize the power of insect cells for the production of therapeutically active antibodies. Our results are highly encouraging to modify the glycosylation pathways of “High Five” cells, as done for *Sf*9 cells by (Breitbach and Jarvis, 2001), and thereby, anticipating the establishment of insect cells as a fast, economic and powerful system for antibody production. Consequently, we started to create a flexible glycomodifying-platform in insect cells.

3.3 Glyco-Module

The differences in N-glycan processing between insect and mammalian cells are mainly due to insignificant expression of glycosyltransferases responsible for generating complex-type structures. Glycoengineering in insect cells is mainly done by stably integrating enzymes into the cellular genome (Hollister et al., 2002). This, however, is very time consuming process and the flexible choice of best expressing cell lines is lost. In order to provide a more flexible system that can be used for a wide range of insect cell lines, we generated a recombinant baculovirus expressing the *C.elegans* N-acetylglucosaminyltransferase II and the bovine β4-galactosyltransferase together with the 3D6 antibody. “High Five” cells and the new insect cell line *Ao*38 (Hashimoto et al., 2010) were co-infected with Ac-3D6 and Ac-GG, the supernatant was harvested 96 hpi and the antibodies were purified using a protein A column. To quickly check the functionality of glycosyltransferases and the resulting glycan structures present on 3D6 antibody, samples were subjected to SDS-PAGE and western blotting. The detection was performed using *Riccinus communi*s agglutinin I (RCA I) which specifically binds galactosylated structures (Iskratsch et al., 2009). A CHO, a Mimic and a native “High Five” / *Ao*38 expressed 3D6 were included as controls.

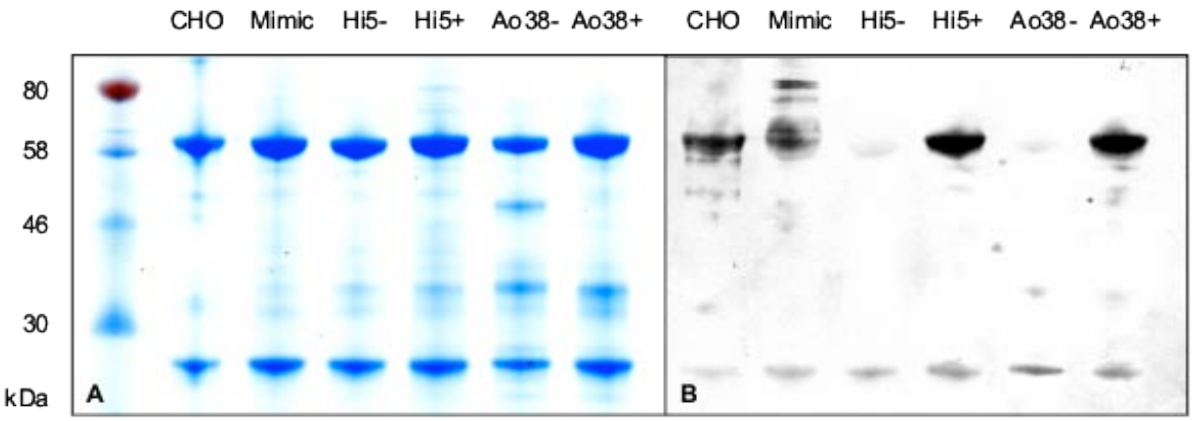

Figure 3.18 Coomassie stained gel (A) and lectin blot using *Ricinus comminus* agglutinin I (B) of glycomodified IgG_1 antibody expressed in different cell lines (“-“ without the presence of glycomodule; “+” coexpressed with the glyco module).

Figure 3.18 shows that the CHO expressed 3D6 antibody specifically bound to RCA I, confirming the presence of the expected galactosylated structures, whereas Mimic cells gave more unspecific signal, which is in accordance with the results obtained by mass spectometry. Only a little portion of Mimic cells expressed antibodies carry terminal galactose, which is somewhat in contrast to the predicted potential of this cell line (Hollister et al., 2002). When 3D6 was coexpressed with glycosyltransferases in the “High Five” (Hi5+) and *Ao*38 cells (Ao38+) a very strong signal was detected, indicating the functionality of glycosyltransferases and the presence of terminal galactose.

3.3.1 Conclusin III

Using our glycosylation module we have shown that it is possible to flexibly modify the glycosylation capacity of different insect cell lines. It is now possible to first evaluate an optimal cell line for the expression of specific proteins and if glycosylation is crucial for proper function, to easily add the sugar residues needed.

4. Abbreviations

Ac-3D6	Baculovirus expressing 3D6 heavy and light chain.
Ac-317	Baculovirus expressing Fcab clone 317 fused influenza A neuraminidase transmembrane domain.
Ac-attR	Baculovirus containing in vitro recombination cassette.
Ac-GG	Baculovirus expressing N-acetylglucosaminyltransferase II and β4-galactosyltransferase
Ac-GFP	Baculovirus expressing GFP.
Ac-mars	Baculovirus expressing IgG_1 Fc-fragment fused to membrane anchorage sequence of baculoviral gp64.
Ac-NA	Baculovirus expressing IgG_1 Fc-fragment fused to influenza A neuraminidase transmembrane domain.
AcMNPV	*Autographa californica* nucleopolyhedrovirus
ADCC	Antibody dependent cellular cytotoxicity
BSA	Bovine Serum Albumin
CDC	Complement dependent cytotoxicity
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluoresence Avtivated Cell Sorting
FCS	Fetal Calf Serum
hpi	hours post infection
hpt	hours post transduction
MOI	Multiplicity of Infection
PBS	Phosphate Buffered Saline

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6. Literature

Akamatsu, Y., Pakabunto, K., Xu, Z., Zhang, Y., Tsurushita, N., 2007. Whole IgG surface display on mammalian cells: Application to isolation of neutralizing chicken monoclonal anti-IL-12 antibodies. J Immunol Methods 327, 40-52.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell. Garland Science, New York.

Altmann, F., 2007. The role of protein glycosylation in allergy. Int Arch Allergy Immunol 142, 99-115.

Betenbaugh, M.J., Tomiya, N., Narang, S., Hsu, J.T., Lee, Y.C., 2004. Biosynthesis of human-type N-glycans in heterologous systems. Curr Opin Struct Biol 14, 601-606.

Beuger, V., Kunkele, K.P., Koll, H., Gartner, A., Bahner, M., Burtscher, H., Klein, C., 2009. Short-hairpin-RNA-mediated silencing of fucosyltransferase 8 in Chinese-hamster ovary cells for the production of antibodies with enhanced antibody immune effector function. Biotechnol Appl Biochem 53, 31-37.

Boder, E.T., Wittrup, K.D., 1997. Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15, 553-557.

Borg, J., Nevsten, P., Wallenberg, R., Stenstrom, M., Cardell, S., Falkenberg, C., Holm, C., 2004. Amino-terminal anchored surface display in insect cells and budded baculovirus using the amino-terminal end of neuraminidase. J Biotechnol 114, 21-30.

Bos, T., Davis, A., Nayak, D., 1984. NH2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. Proc Natl Acad Sci U S A 81, 2327-2331.

Boss, M., Kenten, J., Wood, C., Emtage, J., 1984. Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in E. coli. Nucleic Acids Res 12, 3791-3806.

Breitbach, K., Jarvis, D.L., 2001. Improved glycosylation of a foreign protein by Tn-5B1-4 cells engineered to express mammalian glycosyltransferases. Biotechnol Bioeng 74, 230-239.

Casey, J.L., Napier, M.P., King, D.J., Pedley, R.B., Chaplin, L.C., Weir, N., Skelton, L., Green, A.J., Hope-Stone, L.D., Yarranton, G.T., Begent, R.H., 2002. Tumour targeting of humanised cross-linked divalent-Fab' antibody fragments: a clinical phase I/II study. Br J Cancer 86, 1401-1410.

Chen, K., Cerutti, A., 2011. The function and regulation of immunoglobulin D. Curr Opin Immunol.

Choi, B., Bobrowicz, P., Davidson, R., Hamilton, S., Kung, D., Li, H., Miele, R., Nett, J., Wildt, S., Gerngross, T., 2003. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris. Proc Natl Acad Sci U S A 100, 5022-5027.

Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A., 2008. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N Engl J Med 358, 1109-1117.

Dai, X., Jayapal, M., Tay, H., Reghunathan, R., Lin, G., Too, C., Lim, Y., Chan, S., Kemeny, D., Floto, R., Smith, K., Melendez, A., MacAry, P., 2009. Differential signal transduction, membrane trafficking, and immune effector functions mediated by FcgammaRI versus FcgammaRIIa. Blood 114, 318-327.

Damasceno, L.M., Anderson, K.A., Ritter, G., Cregg, J.M., Old, L.J., Batt, C.A., 2007. Cooverexpression of chaperones for enhanced secretion of a single-chain antibody fragment in Pichia pastoris. Appl Microbiol Biotechnol 74, 381-389.

Daugherty, P.S., Chen, G., Olsen, M.J., Iverson, B.L., Georgiou, G., 1998. Antibody affinity maturation using bacterial surface display. Protein Eng 11, 825-832.

De Muynck, B., Navarre, C., Boutry, M., 2010. Production of antibodies in plants: status after twenty years. Plant Biotechnol J 8, 529-563.

Eeckhout, D., Fiers, E., Sienaert, R., Snoeck, V., Depicker, A., De Jaeger, G., 2000. Isolation and characterization of recombinant antibody fragments against CDC2a from Arabidopsis thaliana. Eur J Biochem 267, 6775-6783.

Ernst, W., Grabherr, R., Wegner, D., Borth, N., Grassauer, A., Katinger, H., 1998. Baculovirus surface display: construction and screening of a eukaryotic epitope library. Nucleic Acids Res 26, 1718-1723.

Ernst, W., Schinko, T., Spenger, A., Oker-Blom, C., Grabherr, R., 2006. Improving baculovirus transduction of mammalian cells by surface display of a RGD-motif. J Biotechnol 126, 237-240.

Gasser, B., Mattanovich, D., 2007. Antibody production with yeasts and filamentous fungi: on the road to large scale? Biotechnol Lett 29, 201-212.

Grabherr, R., Ernst, W., Doblhoff-Dier, O., Sara, M., Katinger, H., 1997. Expression of foreign proteins on the surface of Autographa californica nuclear polyhedrosis virus. Biotechniques 22, 730-735.

Grabherr, R., Ernst, W., Oker-Blom, C., Jones, I., 2001. Developments in the use of baculoviruses for the surface display of complex eukaryotic proteins. Trends Biotechnol 19, 231-236.

Green, L.L., Hardy, M.C., Maynard-Currie, C.E., Tsuda, H., Louie, D.M., Mendez, M.J., Abderrahim, H., Noguchi, M., Smith, D.H., Zeng, Y., 1994. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. Nat Genet 7, 13-21.

Hashimoto, Y., Zhang, S., Blissard, G.W., 2010. Ao38, a new cell line from eggs of the black witch moth, Ascalapha odorata (Lepidoptera: Noctuidae), is permissive for AcMNPV infection and produces high levels of recombinant proteins. BMC Biotechnol 10, 50.

Hiatt, A., Cafferkey, R., Bowdish, K., 1989. Production of antibodies in transgenic plants. Nature 342, 76-78.

Holliger, P., Hudson, P.J., 2005. Engineered antibody fragments and the rise of single domains. Nat Biotechnol 23, 1126-1136.

Hollister, J., Grabenhorst, E., Nimtz, M., Conradt, H., Jarvis, D.L., 2002. Engineering the protein N-glycosylation pathway in insect cells for production of biantennary, complex N-glycans. Biochemistry 41, 15093-15104.

Hoogenboom, H.R., 2005. Selecting and screening recombinant antibody libraries. Nat Biotechnol 23, 1105-1116.

Huang, L., Biolsi, S., Bales, K.R., Kuchibhotla, U., 2006. Impact of variable domain glycosylation on antibody clearance: an LC/MS characterization. Anal Biochem 349, 197-207.

Huang, Y.M., Hu, W., Rustandi, E., Chang, K., Yusuf-Makagiansar, H., Ryll, T., 2010. Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. Biotechnol Prog 26, 1400-1410.

Inoue, Y., Ohta, T., Tada, H., Iwasa, S., Udaka, S., Yamagata, H., 1997. Efficient production of a functional mouse/human chimeric Fab' against human urokinase-type plasminogen activator by Bacillus brevis. Appl Microbiol Biotechnol 48, 487-492.

Iskratsch, T., Braun, A., Paschinger, K., Wilson, I.B., 2009. Specificity analysis of lectins and antibodies using remodeled glycoproteins. Anal Biochem 386, 133-146.

Jarvis, D.L., 2009. Baculovirus-insect cell expression systems. Methods Enzymol 463, 191-222.

Jarvis, D.L., Kawar, Z.S., Hollister, J.R., 1998. Engineering N-glycosylation pathways in the baculovirus-insect cell system. Curr Opin Biotechnol 9, 528-533.

Jefferis, R., 2009. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sci 30, 356-362.

Jefferis, R., Lund, J., 2002. Interaction sites on human IgG-Fc for FcgammaR: current models. Immunol Lett 82, 57-65.

Jefferis, R., Lund, J., Pound, J., 1998. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol Rev 163, 59-76.

Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G., 1986. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321, 522-525.

Jordan, E., Hust, M., Roth, A., Biedendieck, R., Schirrmann, T., Jahn, D., Dübel, S., 2007. Production of recombinant antibody fragments in Bacillus megaterium. Microb Cell Fact 6, 2.

Kanda, Y., Yamada, T., Mori, K., Okazaki, A., Inoue, M., Kitajima-Miyama, K., Kuni-Kamochi, R., Nakano, R., Yano, K., Kakita, S., Shitara, K., Satoh, M., 2007. Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. Glycobiology 17, 104-118.

Kaufman, R.J., Wasley, L.C., Spiliotes, A.J., Gossels, S.D., Latt, S.A., Larsen, G.R., Kay, R.M., 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol Cell Biol 5, 1750-1759.

Kelley, B., 2009. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. MAbs 1, 443-452.

Kim, S.J., Park, Y., Hong, H.J., 2005. Antibody engineering for the development of therapeutic antibodies. Mol Cells 20, 17-29.

Kipriyanov, S., Little, M., 1999. Generation of recombinant antibodies. Mol Biotechnol 12, 173-201.

Kondo, A., Ueda, M., 2004. Yeast cell-surface display--applications of molecular display. Appl Microbiol Biotechnol 64, 28-40.

Koprivova, A., Stemmer, C., Altmann, F., Hoffmann, A., Kopriva, S., Gorr, G., Reski, R., Decker, E.L., 2004. Targeted knockouts of Physcomitrella lacking plant-specific immunogenic N-glycans. Plant Biotechnol J 2, 517-523.

Kost, T.A., Condreay, J.P., Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23, 567-575.

Kumpel, B.M., Rademacher, T.W., Rook, G.A., Williams, P.J., Wilson, I.B., 1994. Galactosylation of human IgG monoclonal anti-D produced by EBV-transformed B-lymphoblastoid cell lines is dependent on culture method and affects Fc receptor-mediated functional activity. Hum Antibodies Hybridomas 5, 143-151.

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

Li, H., Sethuraman, N., Stadheim, T.A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B.K., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y., Kim, N., Mansfield, R., Nett, J.H., Rios, S., Strawbridge, R., Wildt, S., Gerngross, T.U., 2006. Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol 24, 210-215.

Lowell, C.A., 2011. Neutrophils give us a shock. J Clin Invest 121, 1260-1263.

Luckow, V.A., 1993. Baculovirus systems for the expression of human gene products. Curr Opin Biotechnol 4, 564-572.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552-554.

Morell, A.G., Gregoriadis, G., Scheinberg, I.H., Hickman, J., Ashwell, G., 1971. The role of sialic acid in determining the survival of glycoproteins in the circulation. J Biol Chem 246, 1461-1467.

Morrison, S.L., Johnson, M.J., Herzenberg, L.A., Oi, V.T., 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A 81, 6851-6855.

Muller, N., Derouazi, M., Van Tilborgh, F., Wulhfard, S., Hacker, D.L., Jordan, M., Wurm, F.M., 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol Lett 29, 703-711.

Mäkelä, A.R., Ernst, W., Grabherr, R., Oker-Blom, C., 2010a. Baculovirus-based display and gene delivery systems. Cold Spring Harb Protoc 2010, pdb.top72.

Mäkelä, A.R., Ernst, W., Grabherr, R., Oker-Blom, C., 2010b. Creation of baculovirus display libraries. Cold Spring Harb Protoc 2010, pdb.prot5393.

Mäkelä, A.R., Oker-Blom, C., 2008. The baculovirus display technology--an evolving instrument for molecular screening and drug delivery. Comb Chem High Throughput Screen 11, 86-98.

Natsume, A., Niwa, R., Satoh, M., 2009. Improving effector functions of antibodies for cancer treatment: Enhancing ADCC and CDC. Drug Des Devel Ther 3, 7-16.

Nechansky, A., Schuster, M., Jost, W., Siegl, P., Wiederkum, S., Gorr, G., Kircheis, R., 2007. Compensation of endogenous IgG mediated inhibition of antibody-dependent cellular cytotoxicity by glyco-engineering of therapeutic antibodies. Mol Immunol 44, 1815-1817.

Park, S., Xu, Y., Stowell, X.F., Gai, F., Saven, J.G., Boder, E.T., 2006. Limitations of yeast surface display in engineering proteins of high thermostability. Protein Eng Des Sel 19, 211-217.

Rendic, D., Wilson, I.B.H., Paschinger, K., 2007. The glycosylation capacity of insect cells.

Rendić, D., Wilson, I.B., Lubec, G., Gutternigg, M., Altmann, F., Léonard, R., 2007. Adaptation of the "in-gel release method" to N-glycome analysis of low-milligram amounts of material. Electrophoresis 28, 4484-4492.

Rüker, F., Ebert, V., Kohl, J., Steindl, F., Riegler, H., Katinger, H., 1991. Expression of a human monoclonal anti-HIV-1 antibody in CHO cells. Ann N Y Acad Sci 646, 212-219.

Sambrook, J., Fritsch, E.F. & Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual. New York.

Satoh, M., Iida, S., Shitara, K., 2006. Non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies. Expert Opin Biol Ther 6, 1161-1173.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., Shitara, K., 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 278, 3466-3473.

Sibéril, S., Dutertre, C., Boix, C., Bonnin, E., Ménez, R., Stura, E., Jorieux, S., Fridman, W., Teillaud, J., 2006. Molecular aspects of human FcgammaR interactions with IgG: functional and therapeutic consequences. Immunol Lett 106, 111-118.

Simmons, L.C., Reilly, D., Klimowski, L., Raju, T.S., Meng, G., Sims, P., Hong, K., Shields, R.L., Damico, L.A., Rancatore, P., Yansura, D.G., 2002. Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. J Immunol Methods 263, 133-147.

Smith, G.P., 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228, 1315-1317.

Summers, M.D., Smith, G.E., 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin* 1555.

Tchoudakova, A., Hensel, F., Murillo, A., Eng, B., Foley, M., Smith, L., Schoenen, F., Hildebrand, A., Kelter, A.R., Ilag, L.L., Vollmers, H.P., Brandlein, S., McIninch, J., Chon, J., Lee, G., Cacciuttolo, M., 2009. High level expression of functional human IgMs in human PER.C6 cells. MAbs 1, 163-171.

Torres, E., Vaquero, C., Nicholson, L., Sack, M., Stöger, E., Drossard, J., Christou, P., Fischer, R., Perrin, Y., 1999. Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. Transgenic Res 8, 441-449.

Underdown, B.J., Schiff, J.M., 1986. Immunoglobulin A: strategic defense initiative at the mucosal surface. Annu Rev Immunol 4, 389-417.

Ward, E.S., Güssow, D., Griffiths, A.D., Jones, P.T., Winter, G., 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. Nature 341, 544-546.

Weiner, L.M., Dhodapkar, M.V., Ferrone, S., 2009. Monoclonal antibodies for cancer immunotherapy. Lancet 373, 1033-1040.

Wickham, T.J., Nemerow, G.R., 1993. Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. Biotechnol Prog 9, 25-30.

Wittel, U.A., Jain, M., Goel, A., Chauhan, S.C., Colcher, D., Batra, S.K., 2005. The in vivo characteristics of genetically engineered divalent and tetravalent single-chain antibody constructs. Nucl Med Biol 32, 157-164.

Wozniak-Knopp, G., Bartl, S., Bauer, A., Mostageer, M., Woisetschläger, M., Antes, B., Ettl, K., Kainer, M., Weberhofer, G., Wiederkum, S., Himmler, G., Mudde, G.C., Rüker, F., 2010. Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng Des Sel 23, 289-297.

Wu, S.C., Ye, R., Wu, X.C., Ng, S.C., Wong, S.L., 1998. Enhanced secretory production of a single-chain antibody fragment from Bacillus subtilis by coproduction of molecular chaperones. J Bacteriol 180, 2830-2835.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22, 1393-1398.

Zeitlin, L., Olmsted, S.S., Moench, T.R., Co, M.S., Martinell, B.J., Paradkar, V.M., Russell, D.R., Queen, C., Cone, R.A., Whaley, K.J., 1998. A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nat Biotechnol 16, 1361-1364.

7. Appendix

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Insect cells for antibody production: Evaluation of an efficient alternative

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ABSTRACT

In recent years there has been an increase in both availability and demand for therapeutic monoclonal antibodies. Currently, most of these antibodies are produced by stably transfected mammalian cells. In this study we evaluated the use of different baculoviral insect cell systems as an alternative for commonly used production schemes. We expressed the human anti-gp41 antibody 3D6 in *Spodoptera frugiperda Sf*9, *Trichoplusia ni* BTI-TN5B1-4 "High Five", and *Spodoptera frugiperda Sf*SWT-1 "Mimic™" insect cells and compared product yield, specificity and glycosylation patterns with a 3D6 antibody expressed in Chinese hamster ovary cells. Using "High Five" cells we achieved amounts of secreted antibody comparable to those resulting from transient expression in mammalian cells. We determined the N-linked oligosaccharide structures present on asparagine-297 in IgG_1 heavy chains and tested the functionality in terms of antigen binding and the ability to elicit effector functions. Antibodies expressed in all insect cell lines displayed highly specific antigen binding. In general, the insect-produced antibodies carried, as the CHO-produced form, fucosylated N-glycans, including, in the case of "High Five" cells, high levels of core α1,3-fucose. This indicates that in all systems glycoengineering may be required in order to produce optimal glycoforms of this antibody.

1. Introduction

Monoclonal antibodies have attracted great attention from the pharmaceutical industry based on their potential to treat diseases ranging from autoimmune disorders through to cancer and viral or bacterial infections. Antibodies can exert different potent biological functions such as blocking interactions between receptors and ligands or triggering apoptosis; they can also act as adaptor molecules linking the humoral and cellular immune mechanisms. Binding of an antibody to human FcγRs, a group of surface glycoproteins belonging to the Ig superfamily expressed on leucocytes, results in a variety of effector functions. For example, antibody dependent cellular cytotoxicity (ADCC) and complement dependent cellular cytotoxicity (CDCC) are highly important for clinical efficacy. FcγRs can be divided into three classes named FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), each exhibiting different affinities for different IgG subtypes. Crucial for receptor binding in IgG_1 heavy chains is the residue asparagine-297 in the N-linked glycosylation motif $-_{297}$Asn-Ser-Thr$_{299}$-. Replacement of the motif results in non-glycosylated antibodies with drastically reduced FcγR binding abilities (Jefferis and Lund, 2002; Sibéril et al., 2006). Thus, there are multiple requirements for a production system for therapeutically active antibodies including yield, protein folding, glycosylation, costs and speed.

Several expression systems are available for the production of antibodies and antibody fragments including bacteria, yeast, plants, insect and mammalian cells as well as transgenic animals (Jefferis, 2009; Kipriyanov and Little, 1999). While bacteria are not able to assemble complete antibodies and lack the cellular machinery to glycosylate proteins, antibody fragments possessing only the antigen binding site can readily be produced by bacterial fermentation (Boss et al., 1984). When using yeast it becomes possible to produce secreted, fully sized antibodies; however, these are defective in effector functions, other than those expressed in glycoengineered *Pichia pastoris* strains (Choi et al., 2003; Gasser and Mattanovich, 2007; Li et al., 2006). Mammalian cells, especially Chinese hamster ovary (CHO) and mouse myeloma (NS0) cells are currently most commonly used to produce therapeutic antibodies because of high expression rates and their human-like posttranslational modification machinery (Birch and Racher, 2006; Kelley, 2009). Nevetherless, hypersensitive reactions to mouse cell derived antibodies, *e.g.* Cetuximab have been reported (Chung et al., 2008). Using plant cells as an expression systems eliminates the risk of endogenous mammalian pathogens; the plant cells have the machinery for complex N-glycosylation, but there are still problems regarding expression rates, sub-cellular localization, proteolytic degradation and immunogenic α1,3-fucosylation and β1,2-xylosylation (Koprivova et al., 2004; Stoger et al., 2002). Insect

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cells are a very powerful tool for the expression of large, highly processed proteins such as monoclonal antibodies because of their ability to perform signal-peptide cleavage, N- and O-linked glycosylation and efficient extracellular secretion. By using baculoviruses it is possible to transiently express large DNA fragments under the control of very strong viral promoters (Jarvis, 2009; Kost et al., 2005; Luckow, 1993). Again, the glycosylation patterns were shown to differ somewhat as compared to mammalian cells (Betenbaugh et al., 2004; Rendic et al., 2008); thus, glycoengineering strategies were developed (Jarvis et al., 1998). The baculovirus-insect cell system may have the advantage over stably transfected CHO cells, when considering a need for fast and easy production systems which may be increasingly important in the future due to novel, "personalized" medical treatments or when time consuming production schemes are not acceptable. Therefore, in this study we evaluated different insect cell lines for their ability to express properly folded antibodies exhibiting antigen binding and the ability to trigger effector functions.

2. Materials and methods

All DNA manipulations were carried out essentially as summarized by Sambrook and Russel (2001). Restriction enzymes, T4 DNA ligase and Calf Intestinal Alkaline phosphatase were purchased form New England Biolabs (Ipswich, USA). DNA polymerase was purchased from Novagen (Darmstadt, Germany). All enzymes were used according to manufacturer's recommendation. All primers and oligos were synthesized by Sigma–Aldrich (St. Louis, USA).

2.1. Cells and viruses

*Spodoptera frugiperda Sf*9 cells (ATCC CRL-1711) (Summers and Smith, 1987), *Trichoplusia ni* BTI-TN5B1-4 "High Five", cells (ATCC CRL-10859) (Wickham and Nemerow, 1993) and *Sf*SWT-1 Mimic™ insect cells (Invitrogen, Carlsbad, USA) (Hollister et al., 2002) were grown in IPL-41 medium (SAFC Biosciences, St. Louis, USA) containing yeast extract, a lipid mixture supplemented with either 0%, 3% or 10% fetal calf serum (FCS) at 27 °C using T-flasks.

Human U937 leukemic monocyte lymphoma cells (ATCC CRL-1593.2) were cultivated in RPMI1640 medium (PAA, Pasching, Austria) containing 10% FCS and 4 mM glutamine at 37 °C using T-flasks. Recombinant *Autographa californica* nucleopolyhedroviruses were isolated and plaque purified by standard procedures.

2.2. Construction of transfer plasmid and generation of recombinant baculovirus

The human anti-gp41 antibody 3D6 light chain was PCR amplified using primers LC-SpeI-back (5′- AGTAGTAGTACTAGTATGGACATGAGGGTCCCCG -3′) and LC-HindIII-for (5′- AGTAGTAGTAAGCTTCTAACACTCTCCCCTGTTGAAGC -3′). The product was digested with SpeI/HindIII and ligated into pFastBac Dual (Invitrogen, Carlabed, USA) vector cut with same enzymes, resulting in pFBD-LC. The dsDNA encoding for 3D6 heavy chain was PCR amplified using primers HC-XhoI-back (5′- AGTAGTAGTCTCGAGATGGAGTTGGGACTGAGCTG -3′) and HC-NheI-for (5′- AGTAGTAGTGCTAGCTCATTTACCCGGAGACAGGG -3′). The product was XhoI/NheI digested and ligated into XhoI/NheI digested pFBD-LC, resulting in pFBD-3D6. Using the pFBD-3D6 plasmid, the recombinant baculovirus was generated using Bac-to-Bac system (Invitrogen, Carlsbad, USA) according to manufacturer's recommendation.

2.3. Expression and purification of mAb 3D6

*Sf*9, "High Five" and Mimic cells were transferred to shaker flasks with supplemented IP-L41 medium without FCS two days prior infection. For infection 1×10^8 cells were spun down at $900 \times g$ for 10 min and resuspended in the minimum volume of virus needed for infecting with a multiplicity of infection (MOI) of 5. After 1 h of shaking the cells were filled up to 100 mL with supplemented IP-L41 medium without FCS. Samples were taken 24, 48 and 72 h post infection (hpi) and the supernatant was harvested 96 hpi by centrifugation at $2000 \times g$ for 10 min.

The cellular supernatant was filtered through a 0.22 μM filter cartridge (Millipore, Billerica, USA) and applied on a 1 mL Bio-Scale™ Mini UNOsphere SUPrA™ Cartridge (BioRad, Hercules, USA) using an Äkta purifier (GEHealthcare). Elution was performed with 100 mM glycine buffer pH 3. The eluted samples were adjusted to pH 7 using 100 mM Tris–HCl (pH 9).

2.4. SDS-page and Western blotting

Samples were mixed with 2× electrophoresis buffer containing 50 mM Tris/HCl, pH 6,8, 10% glycerol, 2% SDS and 0.1% bromphenol blue for the non-reducing PAGE and in addition 5% 2-mercaptoethanol for the reducing PAGE. Proteins were separated by SDS-Page using NuPAGE® Novex 12% Bis–Tris gels, stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, USA) or electroblotted to a PVDF membrane (Roth, Karlsruhe, Germany). Detection was performed using an anti-human IgG alkaline phosphatase conjugated antibody (Sigma–Aldrich, A3187) in PBS containing 1% skimmed milk powder. The blot was developed using alkaline phosphatase Conjugate Substrate Kit (BioRad, Hercules, USA).

2.5. N-Glycan analysis

N-Glycan analyses were essentially performed as described in Rendić et al. (2007). Briefly, after the heavy and light chains of purified IgG_1 were separated on an SDS-PAGE, bands corresponding to heavy chains were excised. After washing the bands, they were treated with dithiothreitol and iodoacetamide solutions to modify cysteine residues. The gel bands were washed again and then subject to trypsin digestion at 37 °C over night. The peptides extracted with AcN:H_2O:TFA solution (666:330:1) were then dried, resuspended in 20 μL of 50 mM NH_4Ac (pH 5) buffer and subject to PNGase A treatment at 37 °C over night. The released N-glycans were purified using Spec PT C18 (Varian, USA) single-use SPE pipette tips (containing 4 mg of a C18 RP gel) overlaid additionally with, first, 25 μL of packed Dowex 50 W X8 (100–200 mesh) and, second, 25 μL of packed AG 3-X4A (200–400 mesh) before equilibrating with 2% acetic acid. After application of the sample to the column, the column was washed with 2% acetic acid and the flow-through that contains purified N-glycans was collected and dried in a SpeedVac. The drying procedure was repeated twice after resuspending the N-glycans in 150 μL of deionized water. Dried N-glycans were finally resuspended in 3 μL of deionized water and used for MALDI-TOF-MS analysis (on a Bruker Ultraflex Maldi-TOF/TOF in positive reflectron mode using 2% 2,5-dihydroxybenzoic acid (DHB) as matrix).

2.6. Flow cytometric analysis

*Sf*9 cells were infected with a recombinant baculovirus expressing the 3D6 epitope (SGKLICTTAVPWNAS) on the cellular surface with MOI 10 and harvested 72 hpi. After brief washing with phosphate buffered saline (PBS), 1×10^6 cells were incubated with 100 μL of each insect cell expressed 3D6 antibody (1 μg/mL in PBS containing 10% FCS) for 1 h at room temperature while gen-

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tly shaking. Cells were again washed with PBS and incubated with an anti-human IgG (γ-chain specific) R-phycoerythrin conjugate (Sigma–Aldrich, P8047) in PBS containing 10% FCS for 1 h at room temperature. After a final wash with PBS, cells were resuspended in 400 μL PBS and analysed on a FACS-CantoII (Becton Dickinson, San Jose, CA).

U937 cells were washed with PBS and incubated with 100 μL of each insect cell expressed 3D6 antibody (1 μg/mL in PBS) for 30 min on ice. After a brief washing, cells were incubated with an anti-human κ light chain FITC conjugate (Sigma Aldrich, F3761). After washing with PBS, cells were resuspended in 400 μL PBS and analysed on a FACS-CantoII.

2.7. Enzyme-linked immunosorbent assay

Coating of human FcγRI/CD64 to 96 well Maxisorp plates (Nunc, Rochester, USA) was performed by incubating 1 μg/mL recombinant human FcγRI/CD64 (R&D Systems, Minneapolis, USA) in 100 μL coating buffer (PBS, 1% BSA) at 4 °C over night. Plates were washed with TPBS (PBS, 0.1% Tween20) and 50 μL serially diluted samples (in dilution buffer: TPBS, 1% BSA) were applied to the wells. After one hour incubation on a rocking platform, the plate was washed again with TPBS. Detection was performed with 50 μL/well of an anti-human κ light chain peroxidase conjugate (Sigma Aldrich, A7164) 1:1000 diluted in dilution buffer for 1 h while rocking. Staining of the ELISA plate was performed by adding 100 μL/well staining buffer (citrate buffer, pH5) containing 1 mg/mL OPD (1,2-o-phenylenedihydrochloride) and 1 μL/mL H_2O_2 (35%). The reaction was stopped with 100 μL H_2SO_4 and plates were analysed on an Infinite® M1000 reader (Tecan Group, Männedorf, Switzerland).

3. Results and discussion

3.1. Expression of mAb 3D6 in different insect cell lines

The baculovirus insect cell system is a very powerful tool for the routine production of recombinant proteins. In our study we evaluated the expression of the monoclonal anti-gp41 antibody 3D6 in *Spodoptera frugiperda* (*Sf*9), *Trichoplusia ni* "High Five" and glyco-engineered *Sf*SWT-1 (Mimic™) insect cell by infecting them with a recombinant baculovirus. For monitoring the expression profile, samples were taken 24, 48, 72 and 96 h post infection (hpi) and the mAb content was determined by ELISA (Fig. 1). *Sf*9 and Mimic cells secreted 1–2 μg of antibody per mL of culture supernatant after 48 h, rising to 3 μg/mL after 96 h. Interestingly, "High Five" cells yielded more secreted 3D6 antibody. At 48 hpi they produced an almost two-fold higher amount of antibody, steadily increasing to more than 12 μg/mL after 96 h. Although somewhat higher expression rates can be reached by transient (22 μg/mL in culture supernatant) (Muller et al., 2007) and stable expression in CHO cells (1–10 mg of antibody/mL culture supernatant in a fed batch process) (Kelley, 2009; Huang et al., 2010), the fast production, ease of handling and lower costs of the insect cell system are definite advantages. Using this system, no time consuming top-clone selection is required and stability of cell lines is not an issue.

The complexity of the downstream processing is a further important factor in recombinant protein production technologies. For purification, the crude culture supernatant was directly applied to a protein A column and eluted samples were subjected to a reducing as well as a non-reducing SDS-PAGE for purity and quality control. Fig. 2A shows that in all samples, the heavy chain (HC) and light chain (LC) bands were the most dominant. Low amounts of additional proteins were visible and found to be present in the CHO expressed and purified 3D6 antibody preparation as well. HC and LC bands revealed a molecular weight of 55 and 27 kDa, respectively. The CHO derived antibody showed the same size distribution. The non-reducing SDS-PAGE (Fig. 2B) indicates the presence of antibodies composed of two heavy and light chains each, since the molecular weight increased accordingly. All bands were further confirmed by Western blot analysis (data not shown). Taken together, insect cells especially "High Five" cells can be seen as being a potential tool for the production of sufficient quantifies of properly folded antibodies.

3.2. Determination of N-glycan structures

The structure of the N-linked glycans in IgG_1 antibody heavy chains is very important for the ability to elicit different effector functions *in vivo*. We, therefore, analysed the N-glycosylation patterns of the different 3D6 antibodies by mass spectrometry. Fig. 3 shows the MALDI/TOF-MS spectra of N-glycans from different insect cell lines and CHO cells after PNGase A digestion. Our N-glycan analyses were corroborated by the analyses of (glyco)peptides prior to PNGase A treatment (data not shown). The IgG_1 expressed in CHO cells exhibited fucosylated biantennary N-glycan structures carrying two terminal N-acetylglucosamine residues or one/two terminal galactose residues; based on our knowledge of the mammalian glycosylation machinery, the fucose is assumed to be α1,6-linked to the core. Smaller biantennary structures were identified on IgG_1 purified from *Sf*9 cells. These structures carry no terminal galactose residues, but are fucosylated; based on our mass spectrometric data traces of core α1,3-linked fucose cannot be ruled out, but we presume a high degree of core α1,6-fucosylation. In the case of IgG_1 purified from "High Five" cells, the major structure carried two fucose residues; this indicates that a considerable portion of the N-glycans carry both core α1,3- and α1,6-linked fucose. For therapeutic applications of Sf9 and "High Five" cell derived antibodies, the core α-1,3-fucosylation is highly relevant as it is known to be immunogenic (Altmann, 2007). The structures identified in analyses of N-glycans from IgG_1 expressed in Mimic cells, closely resemble the ones found in IgG_1 expressed in CHO cells. Nevertheless, the amount of galactosylated IgG_1 in Mimic cells is, according to the estimated peak size (Fig. 3), somewhat lower than in CHO cells (30% versus 50% as judged by the intensity).

Of the insect lines tested, Mimic cells generally offer the most human-like glycosylation capacities. Although the detected N-glycan structures resembled mammalian complex N-linked glycans, the relative hypogalactosylation indicates that the full theoretical capacity was not realised (Fig. 3). In terms of glycosylation, the Mimic cells would be the ideal choice for producing therapeutically active antibodies. However, due to the fact that cells from the *Spodoptera frugiperda* lineage are not able not secrete sufficient amounts (Fig. 1), their biotechnological use is limited. "High Five" cells in contrast, are the ideal choice in terms of production yield, but have the disadvantages of non human glycosylation and furthermore, the synthesis of an immunogenic core α-1,3-linked fucose. These drawbacks could be overcome by genetically modifying "High Five" cells by introducing glycosyltransferases in a similar way to Mimic cells (Hollister et al., 2002) and/or by generating α1,3/α1,6-fucosyltransferase double knock-down or knock-out mutants. Furthermore, considering that ADCC activity of antibodies can be enhanced by changing their glycosylation from a typical core fucosylated complex type to a structure lacking this core fucosylation (Nechansky et al., 2007; Shinkawa et al., 2003), the knock-out of the relevant fucosyltransferase(s), *e.g.* using the Potelligent® technology (BioWa, Inc.) also seems reasonable (Beuger et al., 2009; Satoh et al., 2006), but has only been performed in mammalian cells. Indeed, realisation of a knock-out in "High Five" cells requires not just knowledge of the relevant gene sequence(s), but also develop-

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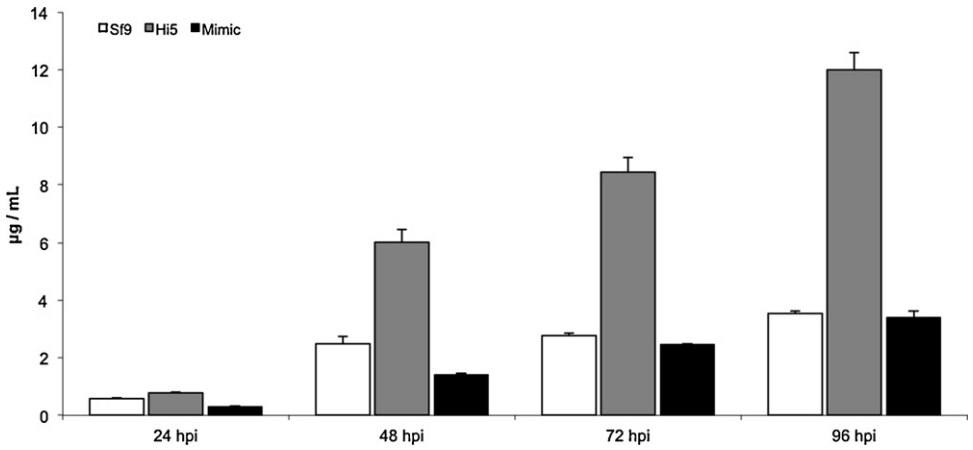

Fig. 1. ELISA of 3D6 antibody secreted to insect cell culture supernatants. 24, 48, 72 and 96 h post infection (hpi). (experiments were carried out in triplicates).

ment of the gene deletion technology in insect cells. The possible effects of these modifications on the production yield remain to be determined, but since the strategy was successfully employed in generating the Mimic cell line (see Fig. 1) (Hollister et al., 2002), no changes in secretion performance are, *a priori*, to be expected.

3.3. Functionality of insect cell expressed antibodies

So far we have shown that the baculovirus insect cell system is suitable for the expression of fully assembled antibodies carrying complex N-linked glycan structures similar to those found in humans, in particular when using Mimic cells. Whenever antibodies are intended for therapeutical use they have to exert full functionality regarding antigen binding and the ability to elicit effector functions. For testing the target binding ability, *Sf*9 cells were infected with a recombinant baculovirus expressing the HIV gp41 3D6 epitope on the cellular surface. The epitope presenting cells were incubated with insect and CHO expressed 3D6 antibody and target binding was measured by FACS. Fig. 4 shows that no difference in binding abilities among antibodies expressed in different cell lines could be observed. The broadening of the peaks could be explained by a non uniform distribution of the 3D6 epitope on the cellular surface.

Besides target binding, the effector functions of the Fc region, such as ADCC and CDCC are essential for the immune system to trigger potent responses against a variety of pathogens. The binding of insect cell expressed 3D6 antibody to FcγRs present on leukocytes was measured by incubating human Caucasian histiocytic lymphoma (U937) cells with insect cell expressed antibodies. Dai et al. (2009) have shown that U937 express, in absence of interferon-γ, suitable amounts of human CD64 and CD32, but not CD16 on the cellular surface, with intention of making use of interaction between IgG_1 with CD64 and CD32. Fig. 5A shows that U937 cells treated with 3D6 displayed a similar amount of bound antibody for all samples tested. The peak of cells treated with the CHO expressed antibody was slightly shifted towards higher fluorescent intensities indicating somewhat increased antibody binding. This difference may be the result of the more authentic glycosylation capacity of CHO cells as compared to insect cells. The difference between the N-glycans found in CHO and Mimic cell expressed 3D6 is mainly the amount of galactose, suggesting that the presence of this sugar residue is important for FcγR binding. In order to confirm that the

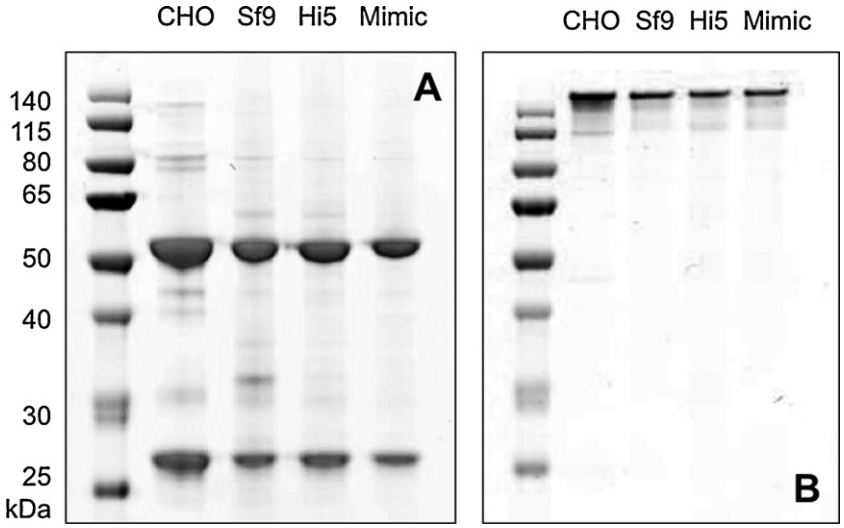

Fig. 2. (A) Reducing SDS-PAGE shows heavy chain and light chain bands at 55 and 27 kDa, respectively being equal among all expression systems. (B) Non-reducing SDS-PAGE indicates the presence of fully assembled antibodies composed of 2 HCs and 2 LCs at 160 kDa. The identity of all bands was further confirmed by Western blotting (data not shown).

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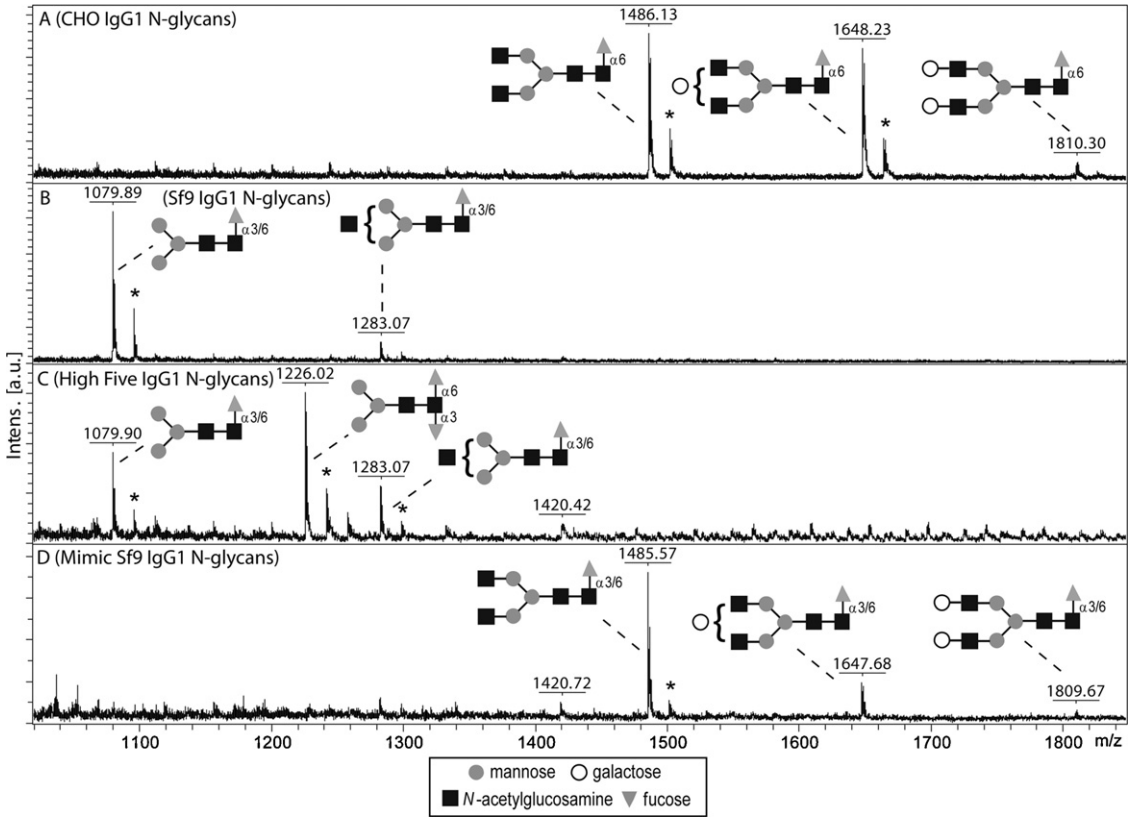

Fig. 3. MALDI/TOF-MS spectra of IgG_1 N-glycan structures expressed in CHO, Sf9, "High Five" and Mimic Sf9 cell lines. Fucosylated biantennary N-glycan structures carrying two terminal N-acetylglucosamine residues or one or two terminal galactose residues were identified on IgG1 recombinantly expressed in and purified from CHO cells (A). Smaller biantennary structures were identified on IgG_1 purified from Sf9 cells; these fucosylated structures carry no terminal galactose residues (B). In addition to the structures found in Sf9 IgG_1, the major structure found in IgG_1 purified from "High Five" cells carries two fucose residues. This structure indicates that a considerable portion of the N-glycans carry the allergenic core α1,3-linked fucose (C). The structures identified in analysis of N-glycans from IgG_1 expressed in Mimic Sf9 cells, closely resemble the ones found in IgG_1 expressed in CHO cells. Nevertheless, the amount of galactosylated IgG_1 in Mimic Sf9 cells is considerably lower than in CHO cells. The major species $[M+Na]^+$ are annotated with *m/z* values of the structures; potassium adducts of identified major structures are labelled with an asterisk.

binding on the cellular surface was specific for FcγRs, we tested the antibodies in terms of binding to the high affinity receptor CD64 by ELISA (Fig. 5B). All insect cell expressed antibodies showed similar binding properties which were comparable to CHO expressed samples.

Apart from the parameters tested in this study, the half-life of recombinantly produced antibodies is another important factor. While galactosylation, sialylation (Huang et al., 2006) and fucosylation (Kanda et al., 2007) were shown to have little or no effect in terms of half-life, IgGs carrying high-mannose type

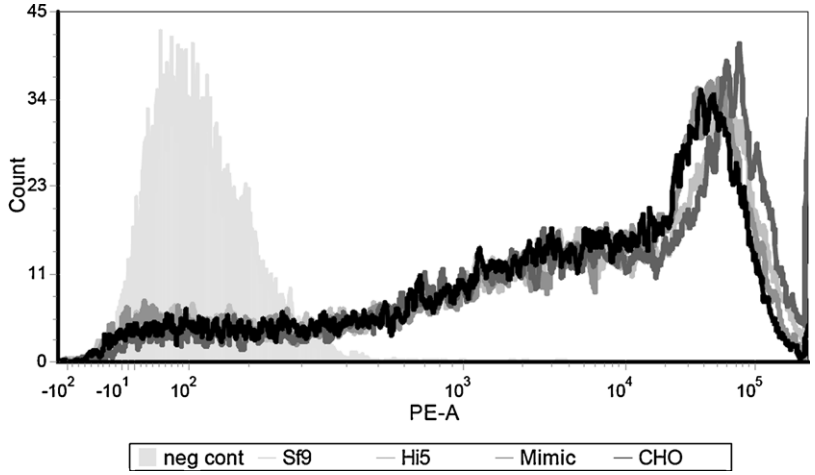

Fig. 4. Binding of insect and CHO cell expressed antibodies to their target. Sf9 cells presenting the 3D6 epitope were incubated with purified antibodies and target binding was measured using FACS. Antibodies expressed in insect and CHO cells show a highly specific binding. The broadening of the peak in lower fluorescent intensity regions can be explained by different amounts of 3D6 epitope displayed on Sf9 cells.

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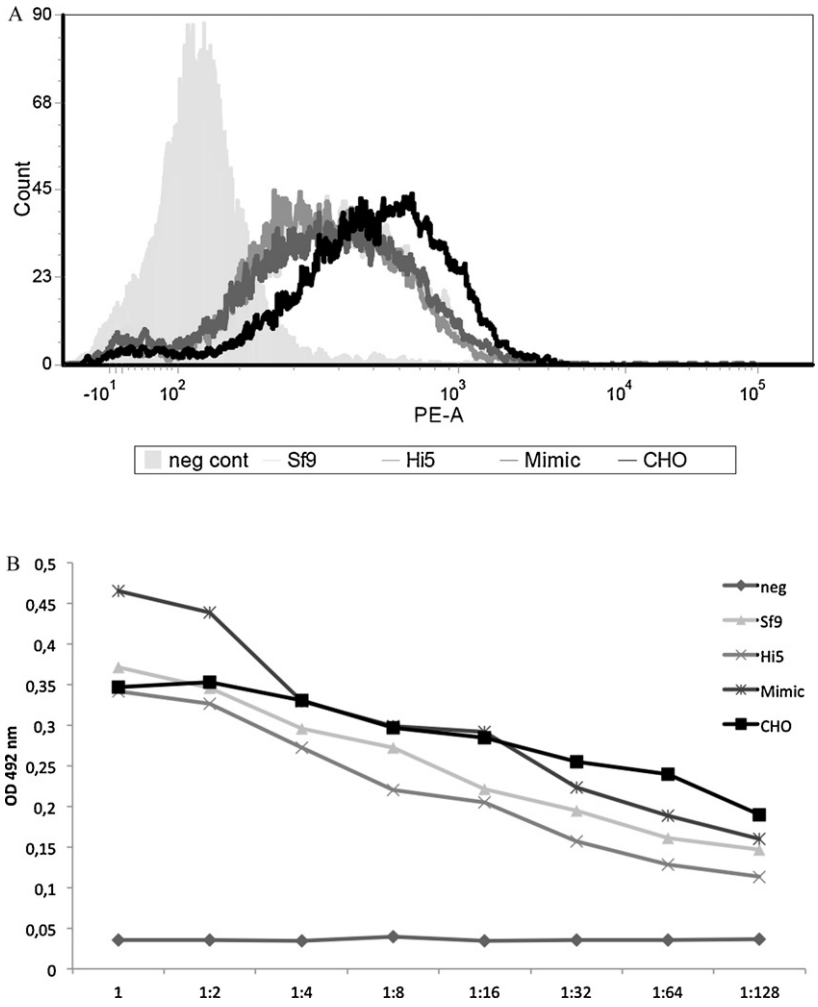

Fig. 5. Binding of insect and CHO cell expressed 3D6 antibody to FcγRs. (A) FACS analysis of U937 cells incubated with 3D6 antibody. Insect and CHO expressed antibodies bound to FcγRs present on cellular surface. The peak for the CHO sample is shifted to the right indicating a slightly increased binding capacity. (B) ELISA determining the binding of 3D6 antibody to the specific FcγRI (CD64).

N-glycans (*e.g.*, Man_5, $Man_5GlcNAc_2$) were found to clear faster from serum in mice. However, IgGs carrying hybrid structures (*e.g.*, Man5GnF, $Man_5GlcNAc_3F$) showed higher stability when compared to IgGs carrying Man_5 (Kanda et al., 2007). Half-life and clearance behaviour of IgG carrying Man_3 ($Man_3GlcNAc_2$) and GnM/MGn ($Man_3GlcNAc_3$) has yet to be determined.

4. Conclusion

In this study we evaluated the feasibility of the baculovirus insect cell system for the transient expression of monoclonal antibodies suitable for therapeutic use. Insect cells, especially *Trichoplusia ni* "High Five" cells could be proven to be a very powerful tool for the generation of complex, secreted, glycosylated proteins, as they express high amounts of antibody, comparable to those found in transient mammalian cell systems. Although stable CHO expression schemes offer even higher product yields, cost and time consuming generation and characterisation of stable integration is not required. Functionality studies of insect cell derived antibodies showed a comparable antigen binding ability and a slightly reduced binding to human FcγRs. These results emphasize the power of insect cells for the production of therapeutically active antibodies. Modifying the glycosylation pathways in "High Five" cells, as done by Breitbach and Jarvis (2001), might serve to solve the stated problems, anticipating the establishment of insect cells as a fast, economic and powerful system for antibody production.

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References

Altmann, F., 2007. The role of protein glycosylation in allergy. Int. Arch. Allergy Immunol. 142, 99–115.
Betenbaugh, M.J., Tomiya, N., Narang, S., Hsu, J.T., Lee, Y.C., 2004. Biosynthesis of human-type N-glycans in heterologous systems. Curr. Opin. Struct. Biol. 14, 601–606.
Beuger, V., Kunkele, K.P., Koll, H., Gartner, A., Bahner, M., Burtscher, H., Klein, C., 2009. Short-hairpin-RNA-mediated silencing of fucosyltransferase 8 in Chinese-hamster ovary cells for the production of antibodies with enhanced antibody immune effector function. Biotechnol. Appl. Biochem. 53, 31–37.
Birch, J.R., Racher, A.J., 2006. Antibody production. Adv. Drug Deliv. Rev. 58, 671–685.
Boss, M., Kenten, J., Wood, C., Emtage, J., 1984. Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli*. Nucleic Acids Res. 12, 3791–3806.

Breitbach, K., Jarvis, D.L., 2001. Improved glycosylation of a foreign protein by Tn-5B1-4 cells engineered to express mammalian glycosyltransferases. Biotechnol. Bioeng. 74, 230–239.
Choi, B., Bobrowicz, P., Davidson, R., Hamilton, S., Kung, D., Li, H., Miele, R., Nett, J., Wildt, S., Gerngross, T., 2003. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris. Proc. Natl. Acad. Sci. U. S. A. 100, 5022–5027.
Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A., 2008. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N. Engl. J. Med. 358, 1109–1117.
Dai, X., Jayapal, M., Tay, H., Reghunathan, R., Lin, G., Too, C., Lim, Y., Chan, S., Kemeny, D., Floto, R., Smith, K., Melendez, A., MacAry, P., 2009. Differential signal transduction, membrane trafficking, and immune effector functions mediated by FcgammaRI versus FcgammaRIIa. Blood 114, 318–327.
Gasser, B., Mattanovich, D., 2007. Antibody production with yeasts and filamentous fungi: on the road to large scale? Biotechnol. Lett. 29, 201–212.
Hollister, J., Grabenhorst, E., Nimtz, M., Conradt, H., Jarvis, D.L., 2002. Engineering the protein N-glycosylation pathway in insect cells for production of biantennary, complex N-glycans. Biochemistry 41, 15093–15104.
Huang, L., Biolsi, S., Bales, K.R., Kuchibhotla, U., 2006. Impact of variable domain glycosylation on antibody clearance: an LC/MS characterization. Anal. Biochem. 349, 197–207.
Huang, Y.M., Hu, W., Rustandi, E., Chang, K., Yusuf-Makagiansar, H., Ryll, T., 2010. Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. Biotechnol. Prog. 26, 1400–1410.
Jarvis, D.L., 2009. Baculovirus-insect cell expression systems. Methods Enzymol. 463, 191–222.
Jarvis, D.L., Kawar, Z.S., Hollister, J.R., 1998. Engineering N-glycosylation pathways in the baculovirus-insect cell system. Curr. Opin. Biotechnol. 9, 528–533.
Jefferis, R., 2009. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol. Sci. 30, 356–362.
Jefferis, R., Lund, J., 2002. Interaction sites on human IgG-Fc for FcgammaR: current models. Immunol. Lett. 82, 57–65.
Kanda, Y., Yamada, T., Mori, K., Okazaki, A., Inoue, M., Kitajima-Miyama, K., Kuni-Kamochi, R., Nakano, R., Yano, K., Kakita, S., Shitara, K., Satoh, M., 2007. Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. Glycobiology 17, 104–118.
Kelley, B., 2009. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. MAbs 1, 443–452.
Kipriyanov, S., Little, M., 1999. Generation of recombinant antibodies. Mol. Biotechnol. 12, 173–201.
Koprivova, A., Stemmer, C., Altmann, F., Hoffmann, A., Kopriva, S., Gorr, G., Reski, R., Decker, E.L., 2004. Targeted knockouts of Physcomitrella lacking plant-specific immunogenic N-glycans. Plant Biotechnol. J. 2, 517–523.
Kost, T.A., Condreay, J.P., Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. 23, 567–575.
Li, H., Sethuraman, N., Stadheim, T.A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B.K., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y., Kim, N., Mansfield, R., Nett, J.H., Rios, S., Strawbridge, R., Wildt, S., Gerngross, T.U., 2006. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. Nat. Biotechnol. 24, 210–215.
Luckow, V.A., 1993. Baculovirus systems for the expression of human gene products. Curr. Opin. Biotechnol. 4, 564–572.
Muller, N., Derouazi, M., Van Tilborgh, F., Wulhfard, S., Hacker, D.L., Jordan, M., Wurm, F.M., 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol. Lett. 29, 703–711.
Nechansky, A., Schuster, M., Jost, W., Siegl, P., Wiederkum, S., Gorr, G., Kircheis, R., 2007. Compensation of endogenous IgG mediated inhibition of antibody-dependent cellular cytotoxicity by glyco-engineering of therapeutic antibodies. Mol. Immunol. 44, 1815–1817.
Rendic, D., Wilson, I.B., Paschinger, K., 2008. The glycosylation capacity of insect cells. Croatia Chimica Acta 81, 7–21.
Rendić, D., Wilson, I.B., Lubec, G., Gutternigg, M., Altmann, F., Léonard, R., 2007. Adaptation of the "in-gel release method" to N-glycome analysis of low-milligram amounts of material. Electrophoresis 28, 4484–4492.
Sambrook, J., Russel, D.W., 2001. Molecular Cloning, Third edition. Cold Spring Harbor Laboratory Press, New York, USA.
Satoh, M., Iida, S., Shitara, K., 2006. Non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies. Expert Opin. Biol. Ther. 6, 1161–1173.
Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., Shitara, K., 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. 278, 3466–3473.
Sibéril, S., Dutertre, C., Boix, C., Bonnin, E., Ménez, R., Stura, E., Jorieux, S., Fridman, W., Teillaud, J., 2006. Molecular aspects of human FcgammaR interactions with IgG: functional and therapeutic consequences. Immunol. Lett. 106, 111–118.
Stoger, E., Sack, M., Fischer, R., Christou, P., 2002. Plantibodies: applications, advantages and bottlenecks. Curr. Opin. Biotechnol. 13, 161–166.
Summers, M.D., Smith, G.E., 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Station Bull., 1555.
Wickham, T.J., Nemerow, G.R., 1993. Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. Biotechnol. Prog. 9, 25–30.

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