Mucin-like Fusion Proteins Produced in *Pichia pastoris* as Enhancers of Immunogenicity of Recombinant Vaccines

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Abstract

Targeting antigens to certain mannose specific receptors of the immune system is a potentially efficient way to enhance the immunogenicity of recombinant vaccines. The present thesis demonstrates the fundamental steps in the development and production of the mucin like fusion protein PSGL-1/mIgG 2b, capable of carrying 106 O-glycans and 6 N-glycans, as a vaccine adjuvant, using the yeast *P. pastoris* as the production platform. The main areas covered are the production of the recombinant protein in lab-scale (1-3L) stirred tank bioreactors, characterisation of the expressed recombinant protein with emphasis on its glycosylation and its binding to the mannose specific receptors MR, MBL, and DC-SIGN known to have immune response modulating properties. The ability of *P. pastoris* produced PSGL-1/mIgG 2b to elicit improved ovalbumin, OVA, specific immune responses in mouse models is also covered.

PSGL-1/mIgG 2b was stably expressed and secreted to media concentrations of 92±12 mg L⁻¹ (p=0.05) at pH 3.5.

LC-MS analysis of the O-glycans released from PSGL-1/mIgG 2b revealed Hex₂₋₉ structures, with indication of phosphorylation. Enzymatic digestion of the O-glycans revealed α₁,₂ bonds and another α₁ glycosidic bond not being α₁,6. NMR analysis could not detect beta 1,2- or α₁,4 or 1,6 bonds leading to the conclusion that α₁,3 linkages may also be present in some of the O-glycans. LC-MS analysis of N-glycans of the fusion protein revealed structures consistent with high mannose Man₅₋₁₅GlcNAc₂ for expression at pH 6.0 and Man₅₋₁₂GlcNAc₂ for expression at pH 3.5 suggesting that low expression pH influences golgi processing of the N-glycans.

Surface plasmon resonance analysis demonstrated that *P. pastoris* produced PSGL-1/mIgG 2b had the ability to bind the three mannose specific receptors (MR, DC-SIGN, MBL) with apparent binding affinities in the nM range. Removing the N-glycans of PSGL-1/mIgG 2b did not reduce its binding affinity to the receptors demonstrating, for the first time, that the O-glycans alone are capable of mediating high affinity binding to all receptors.

The in vivo activity of *P. pastoris* produced PSGL-1/mIgG 2b was investigated by immunizing C57BL/6 mice with OVA or OVA conjugated to or mixed with PSGL-1/mIgG 2b produced in *P. pastoris* or CHO cells in combination with the iscomatrix™ adjuvant (AbiSCO®-100).
OVA-specific immune responses were investigated. When OVA was conjugated to *P. pastoris* produced PSGL-1/mIgG2b, a more rapid and stronger immune response was observed compared to when OVA was conjugated to CHO produced PSGL-1/mIgG2b or given alone with AbiSCO®-100. The influence of a T\(_{\text{H1}}\) response was particularly pronounced in the group receiving OVA conjugated to *P. pastoris* produced PSGL-1/mIgG2b, indicated by increased IgG\(_{2a}\) antibody titers, a strong OVA-specific CTL response and increased numbers of IFN-\(\gamma\) and IL-2 producing splenocytes compared to controls. These results show that the mannose structures of the *P. pastoris* produced fusion protein play a decisive role for inducing a broad and strong OVA-specific immune response.

An attempt to identify potential limitations to secretion of PSGL-1/mIgG2b was made to aid in process development. PSGL-1/mIgG2b was genetically fused with the green fluorescent protein (GFP) and expressed using different exponential methanol feeding models. The 310 kDa PSGL-1/mIgG2b/GFP was secreted up to 18 mg L\(^{-1}\) but progressively accumulated as insoluble fluorescent protein inside the cells, ultimately leading to a stop in recombinant protein production and secretion. Western blot analysis of intracellular insoluble PSGL-1/mIgG2b/GFP suggested a bottle-neck in the golgi apparatus, where incomplete processing of the alfa secretion signal sequence appeared to be the main reason for intracellular aggregation. Postponing intracellular accumulation by reducing the specific methanol up-take rates prolonged the secretion phase which improved total productivities from 9 to 18 mg.

In summary, this work demonstrates that *P. pastoris* offers a potent production platform for the production of PSGL-1/mIgG2b, which also was demonstrated to act as a vaccine adjuvant in a mouse model system, possibly by targeting mannose specific receptors including MR, DC-SIGN and MBL.
Acknowledgements

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List of papers

I. Secretion dynamics of a GFP-tagged mucin-like protein in high cell density bioreactor cultures of *Pichia pastoris*
Magnus Sjöblom, Linda Lindberg, Jan Holgersson, Kris A. Berglund and Ulrika Rova
(Manuscript)

II. Influences of pH on cellular growth and secretion and glycosylation of the mucin like protein P-selectin glycoprotein ligand 1/mouse immunoglobulin G2b in high cell density cultures of *Pichia pastoris*
Magnus Sjöblom, Diarmuid Kenny, Niclas Karlsson, Anki Gustafsson, Tomas Johansson, Linda Lindberg, Jan Holgersson and Ulrika Rova
(Manuscript)

III. *Pichia pastoris*-produced mucin-type fusion proteins with multivalent O-glycan substitution as targeting molecules for mannose-specific receptors of the immune system
Anki Gustafsson, Magnus Sjöblom, Lena Strindelius, Tomas Johansson, Linda Lindberg, Ulrika Rova and Jan Holgersson
Glycobiology vol. 21 no. 8 pp. 1071-1086, 2011

IV. Mannosylated mucin-type fusion proteins enhance antigen-specific antibody and T lymphocyte responses
Gustaf Ahlén, Lena Strindelius, Tomas Johansson, Anki Gustafsson, Nathalie Chatzissavidou, Magnus Sjöblom, Ulrika Rova, and Jan Holgersson
(Manuscript)
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AGP-1/mIgG&lt;sub&gt;2b&lt;/sub&gt;</td>
<td>alpha 1-acid glycoprotein/mouse immunoglobulin G&lt;sub&gt;2b&lt;/sub&gt;</td>
</tr>
<tr>
<td>AOX</td>
<td>alcohol oxidase</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate binding domain</td>
</tr>
<tr>
<td>CTL</td>
<td>cytolytic T lymphocyte</td>
</tr>
<tr>
<td>CTLD</td>
<td>C-type lectin like domain</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>dendritic cell specific intracellular adhesion molecule 3 grabbing non integrin</td>
</tr>
<tr>
<td>DCW</td>
<td>dry cell weight</td>
</tr>
<tr>
<td>Dol</td>
<td>dolichol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fuc</td>
<td>fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose binding lectin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLFB</td>
<td>methanol limited fed-batch</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PMT</td>
<td>protein O-mannosyl transferase</td>
</tr>
<tr>
<td>PPM</td>
<td><em>Pichia pastoris</em> produced PSGL-1/mIgG&lt;sub&gt;2b&lt;/sub&gt;</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>UDP</td>
<td>uracil diphosphate</td>
</tr>
</tbody>
</table>
Symbolic representations of monosaccharides used in this thesis

- ● = Mannose (Man)
- □ = N-acetylglactosamine (GalNAc)
- ■ = N-acetylglucosamine (GlcNAc)
- ▲ = Fucose (Fuc)
- ● = Glucose (Glc)
- ○ = Galactose (Gal)
- ◆ = Sialic acid (Sia)
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Introduction

The body is constantly subjected to numerous immunological challenges including viruses, bacteria, fungi and cancer. To protect from these threats the body relies on a highly interactive and cooperative system composed of innate and adaptive immunity [1]. Innate immunity acts in a direct and non-specific manner to prevent antigen entry into the body and to eliminate pathogens invading the tissues. In contrast, the adaptive immunity is characterized by a delayed but highly antigen specific immune response and immunological memory. Immunological memory ensures that a second challenge with the same antigen results in a faster and often more efficient antigen specific immune response [2]. The goal of a vaccine is to generate protective immunity and long term immunological memory so that the host is protected from the pathogen in the future [2, 3]. To combat different types of pathogens the body needs to generate different types of immune responses. Vaccines need to elicit the adequate branch of the immune system to be effective against a particular immunological challenge.

Pathogenic microorganisms which have been processed to retain their ability for transient growth but not to cause significant disease are generally the most efficient stimulators of the immune system, inducing both the humoral- and cell-mediated branch of the immune system with generation of memory cells [2, 4, 5]. However, the inherent risk of these so called attenuated whole-organism vaccines to revert back to their virulent form, or to cause severe disease in immune-suppressed individuals and the potential presence of contaminating viruses often excludes them from public use today [4, 6]. Modern vaccines are therefore generally based on recombinant protein antigens which are safer and more defined on the molecular level [6, 7]. There are also well established microbial platforms for large scale production of recombinant proteins [8].

On the other hand, recombinant antigens are frequently poor immunogens and may not instruct adaptive immunity to generate the appropriate response. Because of this vaccines based on recombinant antigens are often used in conjunction with adjuvants which serve to enhance the immunogenicity of the antigen [9, 10]. Today there are very few vaccine adjuvants approved for prophylactic use in humans and those in use may not efficiently enhance the induction of the desired immune response [9].
Consequently there is a need to investigate novel immunological signalling pathways which may lead to the identification of new adjuvants. Certain receptors of the innate immunity may instruct the adaptive immune system on how to respond to antigenic challenge and are therefore attractive targets for recombinant vaccines [1]. Some of these receptors specifically recognize mannose and mannose containing glycoconjugates of microbial origin [11-13]. In this respect, yeast which naturally mannosylate recombinant glycoproteins, presents an opportunity to produce efficient targeting molecules to these types of receptors. The methylotrophic yeast *Pichia pastoris* is a robust microorganisms for industrial cultivation and may provide a cost effective way of producing mannosylated recombinant proteins as novel adjuvants to subunit vaccines [14].
PART I

Receptor targeting for improved immunogenicity of recombinant vaccines

This part describes the basic aspects of how pattern recognition receptors of the innate immunity may be exploited to control adaptive immunity. It also describes the background for utilizing P. pastoris produced mucin-like proteins as enhancers of the immunogenicity of recombinant antigens.

1.1 Innate immunity and pattern recognition receptors

Microbial challenge may activate several innate mechanisms which serve to clear the threat from our system and influence the direction of adaptive immunity [1, 15]. These mechanisms include the complement, the inflammatory response, but also phagocytosis and degradation of antigens by a variety of immune cells. The complement is an intricate system of serum proteins which are capable of eliminating pathogens either by generating cytolytic membrane-attack complexes on the surfaces of microorganisms, or by promoting phagocytosis of the antigen through molecular marking of the antigen – opsonisation [16]. Molecular tagging of the antigen by the complement has also shown to lower the activation threshold of B-cells which is important for the development of the humoral part of adaptive immunity with the generation of antigen specific antibodies [17]. Other functions of the complement entail induction of the inflammatory response [17]. The acute inflammatory response is an important reaction to invading pathogens resulting in the release of various inflammatory mediators that modify the properties of local endothelial cells [18]. As a consequence, different types of immune cells and plasma proteins which normally are restricted to the blood vessels are recruited to the site of infection where they may interact with the pathogen [18]. Phagocytic cells are for example capable of internalize and degrade microorganisms. In addition, specialized antigen presenting cells (APCs), most notably dendritic cells, are able to process antigen and present antigen peptides to cells of the adaptive immunity in
combination with co-stimulation which potentiates development of antigen specific immune responses and immunological memory [2, 19]. The ability of the innate immune system to detect antigens and subsequently instruct adaptive immunity reside in special pattern recognition receptors (PRRs) capable of recognising conserved microbial structures termed, pathogen associated molecular patterns (PAMPs) [1]. Pathogens come in a variety of shapes and sizes and express different types of PAMPs and may invade our tissues from outside the cells or from the inside. Because the PRRs recognize different types of PAMPs and are found both as soluble plasma proteins as well as transmembrane- and cytosolic receptors, different pathogens are likely to engage different combinations of PRRs. The combined signals emanating from such distinct combinations of PRRs are hence likely to direct a pathogen specific response [15]. In general, ligand binding by the PRRs activates different signal transduction pathways leading to different expression profiles of genes encoding proteins involved in immunological events [20]. The cytokines are an important group of these proteins which mediate several complex interactions between immune cells, which for example may lead to the generation of immunological memory [21, 22]. Consequently, the PRRs function as links between innate and adaptive immunity [1]. Evidence has shown that physical association between an antigen and PAMPs often are necessary for efficient development of an antigen specific response [23, 24]. Hence, using PAMPs for targeting antigens to PRRs of innate immunity may be an efficient way to enhance and shape the antigen specific immune response.

1.2 Adaptive immunity
The adaptive immunity can be divided into the humoral and cellular branches which act in concert to eliminate and control chronic microbial infections and tumor growth [2, 25]. The humoral immune response relies on B-cells secreting antigen-specific antibodies which may prevent infection or neutralize toxins by binding to critical parts of the surfaces of viruses, microorganisms or foreign proteins [3]. Antibodies may also target microorganisms for destruction by the complement system [16, 17]. The cell-mediated immune response is comprised of two major subpopulations of T cells, generally referred to as T helper (T_{H}) and T cytotoxic (T_{C}) cells, and can be identified
Based on the presence of specific glycoproteins termed CD4 or CD8 on their cell membranes [2]. T<sub>H</sub> and T<sub>C</sub> cells are therefore often referred to as CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively. Whereas the T<sub>H</sub> cells support and direct an immune response, activated T<sub>C</sub> cells, referred to as cytotoxic T lymphocytes (CTL) can target infected or altered self-cells and lyse them [6]. CTL-responses are important in eliminating and controlling altered self-cells including virus infected cells and tumours [25-27].

On a basic level (Figure 1) activation of T<sub>H</sub> and T<sub>C</sub> cells both require that protein antigens are processed to peptides and presented on the surface of antigen presenting cells in combination with major histocompatibility complexes (MHCs) and co-stimulation [6]. Co-stimulation are signals provided by the interaction between special co-stimulatory molecules of the APC and co-stimulatory receptors of the T cells [28]. However, T<sub>H</sub> and T<sub>C</sub> cells require different MHCs to be activated. Whereas T<sub>H</sub> cells require that antigen peptides are presented by MHC II molecules, T<sub>C</sub> cells require antigen peptide presentation by MHC I molecules [6]. Whether the protein antigen is processed to be presented by MHC I or II molecules is therefore important for the immune response elicited by the antigen and consequently also for the effects of vaccination. Exogenously administered antigens tend to be processed through the endocytic pathway leading to MHC II presentation, and endogenously produced antigens enter the endogenous pathway for MHC I presentation [6, 29]. However, depending on the nature of the antigen and the APC, exogenously administered antigens may also be presented by MHC I presentation, which is referred to as cross-presentation [30-32]. For vaccine design it is therefore important to be able to control which processing pathway the antigen enters.
Figure 1. Important steps during development of adaptive immunity. 1. Antigen capture by pattern recognition receptor of antigen presenting cell, APC (in this case dendritic cell). The antigen is processed and antigen peptides presented on MHC I and II molecules to naïve T<sub>11</sub> and T<sub>C</sub> cells in combination with co-stimulation which leads to their activation. By the influence of various cytokines activated T<sub>11</sub> cells may differentiate into antigen specific T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub> cells which support different lines of immune responses. T<sub>C</sub> cells may differentiate into antigen specific CTLs by cytokines secreted by T<sub>11</sub> cells. 3. Following activation B-cells interact with activated T-cells to generate antibody secreting plasma cells and memory B-cells.
TH cells are central for the development of adaptive immunity and support differentiation and proliferation of for example B cells and CTLs cells [2, 17]. Following activation, B and T cells may, under optimal stimulation, differentiate and proliferate into effector cells and memory cells capable of defending our body against immediate and future microbial assault. There are several subtypes of TH cells, including TH1, TH2 and TH17 cells, which support various lines of immune responses effective against different types of pathogens [33, 34]. To instruct the differentiation of naïve TH cells, APCs utilizes a variety of cytokines. For example, IL-12 promotes the development of TH1 cells, IL-4 TH2 cells and IL-23 TH17 cells [33, 34]. Vaccines need to be able to direct all these aspects of activation and differentiation stages if they are to establish efficient and long lasting protection against a particular class of infection.

1.3 Control of adaptive immunity by PRRs
Professional antigen presenting cells including dendritic cells (DCs) and macrophages are among the most potent immune modulating cells [19, 35, 36]. They carry several types of surface and intracellular PRRs enabling them to sense, capture and internalize antigen and present antigen peptides with MHC II molecules in combination with co-stimulation, which is crucial for activating CD4+ T cells [19, 37, 38]. DCs are also the main cross-presenting cells \textit{in vivo}, allowing them to present antigen peptides from exogenously acquired antigens with MHC I molecules, which is crucial for the activation of TC cells [30]. When DCs interact with antigen through their PRRs they may undergo a maturation program which improves its T cell stimulation capability [19]. In addition, ligand binding by PRRs also triggers up-regulation of co-stimulatory molecules, T cell adhesion molecules and chemokine receptors which allow DCs to migrate to secondary lymphoid organs including local lymph nodes and the spleen [19, 39]. These are highly organized structures, largely occupied by DCs, macrophages and lymphocytes and provide the perfect microenvironment for the development of adaptive immunity [39]. Professional antigen presenting cells, especially DCs, are therefore likely to relay a significant part of the instructive signals from PRRs to
adaptive immunity. Hence, targeting PRRs of professional antigen presenting cells may be particularly efficient for improving the antigen specific immune responses.

1.4 Mannose specific receptors
Several of the transmembrane PRRs expressed by professional APCs and soluble plasma PRRs selectively bind terminal mannoses or epitopes of high mannose N-glycans common on yeast derived glycoproteins and other glycoconjugates of microbial origin (Table 1) [13, 37, 40]. These mannose specific receptors are known to have several important functions in the innate immune responses and in linking the innate- with the adaptive immune responses including antigen capture and internalization for improved T-cell presentation [41, 42], modulation of cytokine expression by DCs [43], activation of the complement and direct opsonisation of antigen for phagocytosis [13, 44, 45]. The mannose specific receptors are therefore interesting for the development of novel vaccine adjuvants [11, 46].

1.4.1 Ligand binding of mannose specific receptors
The majority of the mannose specific receptors identified today belong to a large family of carbohydrate binding proteins termed the C-type lectins [13]. The C-type lectins bind their ligands in a calcium dependent way. The principal ligand binding activity of the mannose specific receptors of the C-type lectin superfamily resides in special domains termed, C-type lectin like domains (CTLDs) [40]. In general the mannose specific CTLDs also display specific binding to L-fucose and N-acetyl-D-glucoseamine which shares the stereochemistry of the 3- and 4-OH groups of D-mannose [12, 13]. These hydroxyl groups adopt equatorial positions which appear to be the fundamental binding determinant.
Table 1. Selected trans-membrane and soluble C-type lectins with mannose binding activity

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Selected ligands</th>
<th>Possible functions</th>
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<tr>
<td><strong>Trans-membrane receptors</strong></td>
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<td>Mannose receptor</td>
<td>Man, Fuc, GlcNAc</td>
<td>Antigen up-take</td>
<td>[47]</td>
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<td>Endo180</td>
<td>Man, Fuc, GlcNAc</td>
<td>Antigen up-take?</td>
<td>[48]</td>
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<td>Man, Fuc, Glc</td>
<td>Antigen up-take</td>
<td>[11, 12, 49]</td>
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<td></td>
<td>Man,GlcNAc₂</td>
<td>DC-migration</td>
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<td>T-cell interaction</td>
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<td>DC-SIGNR</td>
<td>Man, Fuc, Glc</td>
<td>Antigen capture</td>
<td>[12, 50]</td>
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<td></td>
<td>Man,GlcNAc₂</td>
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<td>Dectin-1*</td>
<td>Beta-glucans</td>
<td>Antigen up-take</td>
<td>[51, 52]</td>
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<td></td>
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<td>T-cell interaction</td>
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<td>Man,GlcNAc₂</td>
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<td>[53]</td>
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<td>Man, Fuc, GlcNAc</td>
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<td>Opsonisation</td>
<td>[55]</td>
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<tr>
<td></td>
<td>ManNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-D</td>
<td>Glc, Man, maltose, inositol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Dectin-1 has not shown mannose binding activity but because it recognizes yeast derived oligosaccharides and has an established role in the development of adaptive immunity, which is relevant to this work, it has been included.

Binding of monosaccharides tend to be weak with dissociation constants in the 1 mM range [13]. However, *in-vivo* these lectins are involved in recognising larger oligosaccharides of glycoproteins, glycolipids and cell surfaces. Binding to
oligosaccharides, in particular when the oligosaccharides are bound to a protein, often generates much higher affinities, reaching dissociation constants in the \( \mu M \) and nM ranges, suggesting that interactions outside the principle carbohydrate binding domain are of major importance for high affinity binding [49]. The CTLD of the mannose specific receptor DC-SIGN has for example shown to have ten-fold higher affinity for a branched oligomannoside compared to mannose [49]. When this oligomannoside was presented on a protein scaffold an additional 15-20 fold enhancement was observed [49]. Multivalent binding, whereby a lectin oligomer interacts with glycoproteins bearing multiple oligosaccharides have also been shown to be an additional contributing factor to the high affinity binding observed between lectins and their ligands [54]. The particular set of carbohydrate ligands that a receptor recognizes is largely influenced by the fine specificity of the CTLDs, number and organisation of the CTLDs as well as oligomerisation of the receptor polypeptides (Figure 2) [13, 56]. Members of the mannose receptor family for example, can carry eight or ten CTLDs within a single receptor molecule, although all may not participate in binding, whereas DC-SIGN and MBL carry a single CTLD [12, 57]. In-vivo however, DC-SIGN and MBL oligomerizes which may determine their fine specificities [12, 13]. Moreover, it has been found that DC-SIGN is organised in distinct molecular clusters on the surface of immature DCs which is believed to enhance binding to bacteria and virus particles by multivalent binding [58, 59]. In general, highly mannosylated glycoproteins or neoglycoconjugates have been shown to be good ligands for mannose specific receptors such as MBL, DC-SIGN and MR [12, 13, 47, 54].

Several of the mannose specific transmembrane receptors carry special motifs on their cytoplasmic tails which may be involved in their ability to influence events following ligand binding. The mannose receptor and DC-SIGN for example carry tyrosine based endocytic sorting signals which promote internalization in clathrin coated vesicles [60, 61]. The ability of DC-SIGN to transduce signals to the interior of the cell is thought to require a di-leucin motif and a tyrosine residue in its cytoplasmic tail [62]. In contrast to the other C-lectins, Dectin-1 has a functional tyrosine based activation motif in its cytoplasmic tail which is a common motif in antigen receptors and functions as a signal transducer [51].
Subtle differences of the ligands used to target mannose specific receptors may have profound influences on the immunological outcome. For example, it was shown that the oxidised form of mannan conjugated to a synthetic peptide of mammary mucin (MUC1) resulted mainly in the induction of the cellular immune response contrary to the reduced form of mannan which mainly induced a humoral response [64]. However, the relationships between the structures of the ligands of these receptors and the immunological outcome are poorly understood. The overlapping ligand specificities of the mannose specific receptors and also the complex interaction between several other types of receptors makes the exact contribution of a certain ligand to the immunological outcome very hard to predict.
1.4.2 Yeast glycosylation provides natural ligands for the mannose specific receptors  
Glycosylation is a common co- and post-translational modification of endogenous and heterologous proteins [65]. It involves the sequential addition of monosaccharides to certain glycosylation sites on the protein which is catalyzed by a family of enzymes termed glycosyltransferases [65]. The oligosaccharides generated are categorized based on their linkage to the protein which most often is by a N-glycosidic linkage to an asparagine residue (generating N-glycans) or via an O-glycosidic linkage to a serine or threonine residue (generating O-glycans) of the protein. The characteristic feature of yeast glycosylation is the high mannose content in both N- and O-linked oligosaccharides [65]. Hence yeast glycosylation provides natural ligands for the mannose specific receptors. In yeast, such as *Saccharomyces cerevisiae*, glycoproteins are typically hyperglycosylated with N-glycans of Man$_{2-6}$GlcNAc$_2$ in size [65, 66]. In these N–glycans the mannoses are commonly linked by α1,2, α1,3, α1,6 linkages and can carry phosphate groups. The N-glycans of *P. pastoris* derived glycoproteins are frequently smaller in size compared to those derived from *S. cerevisiae* typically with N-glycans Man$_{1-4}$GlcNAc$_2$ in size, although hyper glycosylation have been reported for *P. pastoris* as well (Figure 3) [65, 67, 68].

**Figure 3.** Representative structures of N- and O-linked glycans in *P. pastoris*. The N-glycan is (Man$_3$GlcNAc$_2$-Asn) O-glycan is (Man$_4$-Ser/Thr). High mannose content is typical of yeast glycosylation.

O-glycans are generally much smaller than N-glycans with straight polymers with up to about 6 hexoses which may be phosphorylated [69, 70]. Of the *P. pastoris* derived glycoproteins studied, the majority of the O-glycans are Man$_{2-6}$ structures linked through α1,2 glycosidic linkages with indication of phosphorylation [69, 70]. Penta-
and hexasaccharides (mannosides) where the terminal and subterminal mannoses are bound by β1,2 glycosidic linkages have also been found with indications of phosphorylation [69]. β1,2 glycosidic linkages in glycans of glycoproteins derived from S. cerevisiae have not been observed. Interestingly however, β1,2-linked oligomannosides have been observed in cell wall components of the pathogenic yeast Candida albicans [71]. Recently the genes responsible for β-mannosyltransferase activity in P. pastoris were identified [71]. The ability of yeast to mannosylate recombinant glycoproteins presents the opportunity to produce targeting molecules for mannose specific receptors which could be used as vaccine adjuvants for recombinant vaccines.

1.4.3 Mucin-like fusion proteins produced in Pichia pastoris as enhancers of immunogenicity of recombinant vaccines

Mucins are highly O-glycosylated proteins and constitute the major component of the protective mucous membranes of the respiratory, digestive and urogenital tracts [72]. Mucins are generally long, extended molecules where the carbohydrate content can constitute up to 90% of their molecular weight [72]. Expressed in P. pastoris mucins would be highly mannosylated glycoproteins. The high O-glycan substitution in combination with the linear shape of mucins may be particularly efficient to bind mannose specific receptors of the immune system by multivalent binding – the generation of strong bonds by the combination of many weak ones [73]. Physical linkage between an antigen and a PAMP has been shown to be important for coupling the immune response towards the antigen [23, 24]. Physical linkage between a P. pastoris produced recombinant mucin and an antigen of choice could therefore serve to enhance the antigen-specific immune response, possibly by targeting mannose specific receptors of professional APCs which improves T-cell presentation [41, 42]. The homodimeric fusion protein consisting of the extracellular part of P-selectin glycoprotein ligand 1 (PSGL-1) and the Fc part of mouse immunoglobulin G2b (mIgG2b) has been suggested as a versatile scaffold molecule for presentation of biologically active carbohydrates [73]. Dimeric PSGL-1/mIgG2b has the theoretical capacity to carry 106 O-glycans and six N-glycans and mannosylated by P. pastoris it should be well apt to target mannose specific receptors in vivo [73]. By utilizing
existing cross-linking chemistry, the antigen could be conjugated to the *P. pastoris* produced PSGL-1/mIgG₂₅, creating a significantly more immunogenic species compared to the antigen alone (Figure 4).

![Figure 4](image.png)

**Figure 4.** Schematic representation of how the *P. pastoris* produced mucin-like fusion protein PSGL-1/mIgG₂₅ could be used to enhance the immunogenicity of recombinant antigens.

### 1.5 Glycan biosynthesis in yeast

Glycosylation takes place in the endoplasmic reticulum (ER) and Golgi apparatus and the carbohydrate-protein conjugate formed is referred to as a glycoprotein [65]. N-glycans are attached to the amide nitrogen of an asparagine (Asn) residue through an N-glycosidic linkage within the consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline. O-glycans are attached to the –OH of a Ser or Thr with its anomeric carbon through a glycosidic linkage. There are no consensus sequences for O-linked glycans known.

The initial steps of N-glycan biosynthesis is an evolutionary conserved sequence of events between yeast and higher eukaryotes resulting in the covalent attachment of a Glc₃Man₆GlcNAc₂ precursor oligosaccharide to a suitable asparagine residue on the protein being translated [65]. The Glc₃Man₆GlcNAc₂-Asn is then trimmed by certain glucosidases and mannosidases to remove the glucose and a specific α1,2-linked
mannose giving a Man₆GlcNAc-Asn structure which is released to the Golgi for further processing. This oligosaccharide is common for both yeast and higher eukaryotes and from this point yeast and mammalian cells take different glycosylation routes. Mammalian cells generally continue the trimming process in the Golgi to give Man₃GlcNAc₂-Asn or Man₃GlcNAc₂-Asn structures which are precursors for the generation of hybrid and complex types of N-glycans (Figure 5).

![Figure 5. The three N-glycan subtypes.](image)

Both *S. cerevisiae* and *P. pastoris* have an α1,6-mannosyltransferase which adds a critical mannose to the Man₆GlcNAc-Asn structure [74-76]. This feature commits the N-glycan to be a substrate for additional α1,2-, α1,3, α1,6-mannosyltransferases as well as phosphor mannosyltransferases resulting in a highly mannosylated structures typical for yeast derived glycoproteins. However, in contrast to *S. cerevisiae*, no terminal α1,3-linked mannosylation seem to occur in *P. pastoris* [14]. Diversification and elongation of the core structure then occurs through cis-, medial and trans-Golgi compartments with the more distal parts of the N-glycan being formed in the later parts of the golgi. Therefore, enzymes responsible for the synthesis of the terminal regions of the oligosaccharide usually reside in the trans-Golgi compartments [76, 77].

In contrast to N-glycan biosynthesis, O-glycan biosynthesis between yeast and lower eukaryotes and vertebrates is different from the initial glycosylation step. In yeast, O-glycosylation is initiated in the ER by the addition of a single mannose from a dolichol linked mannose donor by a seven-membered family of protein O-mannosyl transferases (PMT1-PMT7) [77]. The protein is subsequently transported to the Golgi where
further glycosylation takes place by other types of mannosyltransferases where the nucleotide sugar GDP-Man act as sugar donor [77]. In mammalian cells, O-glycan biosynthesis starts in the Golgi usually by the addition of GalNAc from a nucleotide sugar donor.

1.5.1 Cultivation parameters may influence glycosylation
In the development of glycosylated protein therapeutics it is important to develop bioprocesses for large scale production in parallel with in vitro and in vivo functional studies. Several reports have demonstrated how the glycosylation profile of the same recombinant protein was altered due to changes in cultivation parameters such as temperature, various growth factors, nitrogen sources, specific growth rate, etc [78-80]. The inherit complexity of the glycosylation processes makes it very difficult to predict how a certain parameter will change the glycosylation profile of a glycoprotein and it has to be investigated for each cell type and recombinant protein. The US Food and Drug Administration (FDA) requires detailed carbohydrate characterisation of glycoproteins with therapeutic use in humans [81] and changes in glycosylation due to changes in bioprocess parameters can, besides changes in therapeutic potency of the drug, also impart costly problems in the entire chain of approval.
Pichia pastoris biotechnology

In addition to provide recombinant proteins with a suitable glycosylation for targeting mannose specific receptors of the immune system, P. pastoris is also a robust microorganisms for the industrial production of recombinant proteins [14]. This part will cover important aspects of heterologous protein production associated with P. pastoris. Recently P. pastoris was classified into a new genus, Komagataella which in turn was split into three species, K. pastoris, K. phaffii and K.pseudopastoris [82]. Because the majority of the literature uses P. pastoris, this name will also be used in this thesis.

2.1 Historical background of Pichia pastoris

P. pastoris is a methylotrophic budding yeast first described by Koichi Ogata in 1969 and was initially intended for single cell protein production by Phillips Petroleum Company due to low cost of methanol at the time [14, 83]. However, because of unfavourable production economics this application was never realized, and in the early 1980’s Salk Institute Biotechnology/Industrial Associates (SIBIA), Inc was contracted to develop P. pastoris as an expression platform for heterologous protein production. The interest for using this yeast for production of recombinant proteins stems to a large extent from its ability to grow to very high cell densities in simple mineral media together with unique enzymes expressed in large amounts only when utilizing methanol. One of these enzymes, alcohol oxidase 1 (AOX1), was found to be expressed in particularly high amounts when growing on methanol, but barely present when growing on repressing carbon sources such as glycerol or glucose [84]. The strong and tightly regulated promoter associated with the AOX1 gene (P_{AOX1}) was identified and isolated by SIBIA in the 1980’s concomitantly with the development of strains, vectors and methods for molecular genetic manipulation [14, 85]. Today, the P.
pastoris expression system has been used to express hundreds of recombinant proteins and several new promoters for recombinant protein production have been isolated [14, 86]. Glycoengineering of P. pastoris has also demonstrated that glycoproteins such as erythropoietin and human antibodies can be expressed with human like glycosylation and high product homogeneity [75, 87]. Hence, certain glycoproteins traditionally produced by the comparably complex and expensive mammalian cell lines, could be expressed by P. Pastoris in the future [88]. Several recombinant therapeutic proteins are under development for the market with the P. pastoris expression system (Table 2) [74].

Table 2. Therapeutical proteins under development with the P. pastoris expression system 2004 [74]

<table>
<thead>
<tr>
<th>Protein</th>
<th>Therapeutical target</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiostatin</td>
<td>Antiangiogenic factor</td>
<td>EntreMed</td>
</tr>
<tr>
<td>Elastase inhibitor</td>
<td>Cystic fibrosis</td>
<td>Dyax</td>
</tr>
<tr>
<td>Endostatin</td>
<td>Antiangiogenic factor</td>
<td>EntreMed</td>
</tr>
<tr>
<td>Epidermal growth factor analog</td>
<td>Diabetes</td>
<td>Transition Therapeutics</td>
</tr>
<tr>
<td>Insulin-like growth factor-1</td>
<td>Insulin-like growth factor-1</td>
<td>Cephalon</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>Stabilizing blood volume in burns/shock</td>
<td>Mitsubishi Pharma (formerly Welfide)</td>
</tr>
<tr>
<td>Kallikrein inhibitor</td>
<td>Hereditary angiodema</td>
<td>Dyax</td>
</tr>
</tbody>
</table>
2.2 Advantages with *P. pastoris* as an expression platform

Yeasts may be a good alternative when prokaryotic production platforms cannot provide the correct folding environment or perform the appropriate co- and posttranslational modifications for the recombinant protein. Yeasts combine the abilities to perform several post-translational modifications of mammalian cell lines with the attractive cultivation features and ease of genetic manipulation characteristic for prokaryotic cells [89]. Although *S. cerevisiae* is the most characterized yeasts today it tend to hyperglycosylate recombinant glycoproteins and generally excrete lower amounts of recombinant proteins as compared to *P. pastoris* [90]. Furthermore, because of its low maintenance demand and preference for respiratory growth *P. pastoris* can efficiently grow to very high cell densities (>150 g dry cellular weight per liter) [14, 91]. In contrast, *S. cerevisiae* which prefers fermentative growth even under aerobic conditions, may quickly accumulate ethanol to toxic levels which limits growth and heterologous protein production [14, 92]. The combination of the strong and tightly regulated *P._AOX1* with an efficient secretion system and the capability of *P. pastoris* to grow to exceptionally high cell densities in bioreactor cultivations have resulted in several expressions with intra- and extracellular recombinant protein titres in the g per liter range [14, 91]. Moreover, *P. pastoris* can grow within a wide pH range (pH 3-7) without any major changes in specific growth which presents the opportunity to counteract competing microorganisms and proteolytic activity in the cultivation medium [93].

2.3 The *P. pastoris* expression system

The *P. pastoris* expression system comprises several expression strains and vectors for introducing the recombinant gene into the chromosome of the host cells concomitantly with control elements and markers, table 3. All strains derive from the NRRL-Y 11430 (Northern Regional Research Laboratory, Peoria, IL) strain and commonly have one or several auxotrophic mutations to allow for selection of transformants during the process of molecular cloning [14]. There are also strains where the genes for certain proteases have been disrupted in order to reduce proteolytic degradation of the product in the media; a problem frequently hampering high cell density cultivations.
Recombinant genes are generally introduced to the *P. pastoris* genome through *Escherichia coli/P. pastoris* shuttle vectors enabling integration into the chromosomes by homologous recombination yielding stable transformants [14]. By multiple integration events multicopy strains may be generated. Multiple copies of the recombinant gene, the gene copy number, may be important for the productivity [14, 94, 95]. There are several vectors currently available for integrating recombinant genes in the genome of *P. pastoris*, providing various promoters, secretion signals, selection markers and other features which may be required in a particular case (Table 3). They also contain essential elements for maintenance in *E. coli* such as origin of replication and markers.

Table 3. Examples of commercially available vectors compatible with the *P. pastoris* expression system (Invitrogen).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter</th>
<th>Induction</th>
<th>Secretion signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPICZ A, B, C</td>
<td>AOX1</td>
<td>Methanol</td>
<td>None</td>
</tr>
<tr>
<td>pPICZα A, B, C</td>
<td>AOX1</td>
<td>Methanol</td>
<td>α-MF</td>
</tr>
<tr>
<td>pGAPZ A, B, C</td>
<td>GAP</td>
<td>Constitutive*</td>
<td>None</td>
</tr>
<tr>
<td>pGAPZα A, B, C</td>
<td>GAP</td>
<td>Constitutive*</td>
<td>α-MF</td>
</tr>
<tr>
<td>pFLD1</td>
<td>FLD1</td>
<td>Methylamine/methanol</td>
<td>α-MF</td>
</tr>
<tr>
<td>pHIL-S1</td>
<td>AOX1</td>
<td>Methanol</td>
<td>PHO1</td>
</tr>
</tbody>
</table>

* Strong constitutive on glucose. 2/3 and 1/3 of glucose induction on glycerol and methanol respectively

2.3.1 Secretion

Most vectors available to the *P. pastoris* expression system have the option to generate in-frame fusions between the recombinant protein and a secretion signal to direct the recombinant protein to the secretory pathway [14]. Secretion of the recombinant protein is highly advantageous from a bioprocess engineering point of view since it simplifies separation of the recombinant protein from other cellular matter [96]. Moreover, *P. pastoris* has been found to secrete low amounts of endogenous proteins which in combination with simple mineral salts media may result in the recombinant product being the major constituent in the culture supernatant [90]. However, the option of secretion is generally limited to proteins which are intrinsically secreted by their native host. The most commonly utilized secretion signals are those of the
heterologous *S. cerevisiae* alfa-mating factor (alfa-MF) and of the endogenous *P. pastoris* acid phosphatase (PHO1) [14]. Genome sequence studies have revealed a multitude of “new” endogenous signals that potentially can be used to improve secretion compared to expressions where the alfa-MF signal or PHO1 signal are used [97]. In addition to a secretion signal peptide which directs translocation to ER and progression through golgi, there may also be cell wall targeting signals and signals facilitating permeation through the cell wall to the culture media [98]. These additional signals could possibly be incorporated into vectors to improve excretion to the cultivation media.

### 2.3.2 Promoters

Most of the promoters isolated for controlling recombinant protein production with *P. pastoris* are associated with methanol metabolism. The reason for this is that they typically are highly repressed when the cells grow on carbon sources such as glucose and glycerol, whereas they are greatly induced when grown on methanol or other substrates involved in methanol metabolism. Today several promoters, both constitutive and inducible, have been isolated although relatively few are used [86, 90]. The most commonly used is the PAOX1, but alternative promoters, including the glyceraldehyde-3-phosphate dehydrogenase promoter (PGA) and formate dehydrogenase promoter (PF1D1), are gaining increasingly more interest largely because they offer methanol independent expressions.

#### 2.3.2.1 The alcohol oxidase 1 promoter

The most commonly used promoter is the methanol inducible PAOX1 due to its strength, tight regulation and possibly also for the highly developed technology around this promoter [90]. Tight regulation is particularly important when expressing recombinant proteins that are toxic to the cells, as it presents the option to grow a high biomass before the production phase. In this way the total productivity can be high although the cell-viability would be compromised upon induction. There are actually two alcohol oxidase genes and promoters in wild-type *P. pastoris*, AOX1 and AOX2, PAOX1 and PAOX2, but it is the AOX1 gene product which is most abundant in wild type, methanol grown cells [99]. The reason for this is primarily due to the much
stronger AOX1 promoter. Functional studies where the AOX1 gene was exchanged by the coding region of the AOX2 gene demonstrated that AOX2 could functionally substitute AOX1 when put under P_{AOX1} [99]. Steady state levels of AOX1 message have also been shown to be considerably higher than for AOX2 [85, 99]. The protein coding regions of AOX1 and AOX2 genes share 92% and 97% homology at the nucleotide and amino acid sequence levels, respectively, but exhibit no homology in the promoter regions [100].

Studies on the regulation of several methanol inducible genes including the AOX1 and AOX2 genes have indicated a repression/derepression and induction mechanism operating on these genes [99, 101-103]. For example, AOX1 protein and mRNA are undetectable during growth on glucose but during growth on methanol AOX1 protein can reach over 30% of total soluble protein and mRNA levels up to 5% of poly(A)+ mRNA [84, 99]. Growth limiting amounts of glucose or glycerol leads to derepression of the P_{AOX1} with some transcriptional activity. Fully, methanol induced cells exhibit about a 1000 fold increase in transcriptional activity compared to completely repressed cells whereas derepressed cells has about 20 times higher transcriptional activity than repressed cells [103]. Observations have also indicated the P_{AOX1} is subjected to catabolite repression, particularly when cells are grown on methanol at non-growth-limiting rate [96]. This repression is reflected by a sharp increase in alcohol oxidase activity during the initial phase of the induction whereas the alcohol oxidase activity subsequently decreases four to five times for the remainder of the cultivation [96]. In contrast, when the cells are grown on methanol at growth-rate limiting rate catabolite repression is less pronounced, especially at lower cultivation temperatures [104]. Although the molecular details remain to be elucidated, a growing amount of data continuously reveals more about the repression/derepression induction mechanism of the AOX1 promoter.

### 2.3.2.2 Methanol utilization phenotypes

The P_{AOX1} can be used in different contexts which are reflected through different methanol utilization phenotypes. When the AOX1 and AOX2 genes are intact, the
cells are able to grow on methanol with wild-type growth rate and their phenotype is termed methanol utilization positive or Mut+. When the AOX1 gene is disrupted, the cells rely on the much weaker $P_{AOX2}$ for generation of the alcohol oxidase required for methanol metabolism [105]. This disruption results in a lower growth rate on methanol and the phenotype is termed methanol utilization slow or Mut-. Mut' strains can either be obtained premade, as with the KM71 strain or generated by using special vectors, which disrupt the AOX1 gene during integration of the recombinant gene into the AOX1 locus of the chromosome. In the case when both AOX1 and AOX2 genes have been disrupted, as in the MC100-3 strain, the cells are unable to utilize methanol at all and the phenotype is termed methanol utilization negative or Mut-.

Generally, the Mut+ phenotype is the most commonly used phenotype because of the higher productivities associated with its higher growth rates on methanol [105]. However, the Mut+ phenotype is more sensitive to residual methanol concentrations in the cultivation media compared to Mut' or Mut- which puts higher demands on process control [94, 105]. Moreover, for large scale production the high methanol- and oxygen consumption rates in combination with large heat removal requirements of the bioreactors associated with the Mut+ phenotype are disadvantages [80, 106]. The Mut' is not as sensitive to residual methanol and may be better suited for larger scale expressions because of less strict requirement on methanol concentration in the media [94]. On the other hand, the expression levels with the Mut' phenotype are generally lower compared to the Mut+ phenotype, and large scale cultivations still need large amounts of methanol, although some proteins are expressed better with Mut' [105]. The Mut- phenotype requires much less methanol compared to Mut+ and Mut', but requires a carbon source which support growth and does not repress the $P_{AOX1}$. Due to the repression/derepression induction mechanism of the $P_{AOX1}$, growth limiting levels of glycerol results in some recombinant protein production. By exploiting this fact, Mut- cultivations with growth limiting feeds of glycerol have been shown to have the potential to give higher product yields than corresponding Mut+ phenotype with about 34 times less consumed methanol [105]. The specific productivity with glycerol feeds decreases, however, because of partial repression of the $P_{AOX1}$ by even small amounts of
residual glycerol [105, 107]. The requirement to keep the residual glycerol concentration within a narrow limit during cultivation, places high demands on process control. Certain carbon sources do not repress the $P_{\text{AOX1}}$ as much as glycerol and accordingly may be used without rigorous control of their media concentration. These so-called non-repressing carbon sources, including alanine, sorbitol, mannitol or trehalose have been found to work well with the Mut$^-$ phenotype in combination with 0.5% methanol to support induction [108]. By using these carbon sources in Mut$^-$ shake-flask cultivations similar product yields or higher compared to the Mut$^+$ have been achieved [108]. These non-repressing carbon sources can also be used with the Mut$^+$ and Mut$^+$ phenotypes to support growth and reduce the requirement for methanol as carbon source. Sorbitol is one of the most extensively studied [109, 110]. Mixed sorbitol/methanol feeds have been shown to improve production of avidin due to higher biomass yields concomitantly with a reduction of heat production and oxygen consumption rate by 38% compared to when methanol was used as the sole carbon source for a Mut$^+$ strain [110].

### 2.3.2.3 Alternative promoters

Methanol is generally not desired for the production of food grade or therapeutic proteins which may favour other promoters than the $P_{\text{AOX1}}$. In the case in which the recombinant protein is not toxic to the cells the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter ($P_{\text{GAP}}$) may be an alternative to the $P_{\text{AOX1}}$ [111]. For some recombinant proteins $P_{\text{GAP}}$ has been shown to generate similar or higher expression levels as $P_{\text{AOX1}}$ and importantly presents the opportunity to use less toxic and flammable carbon sources than methanol, including glucose and glycerol [111]. Moreover, compared to cells grown on methanol, cells grown on glucose or glycerol display higher viability, which in addition to higher yields also results in less release of product degrading proteases to the culture medium [82]. Another promoter which offers methanol-independent induction is the formaldehyde dehydrogenase promoter ($P_{\text{FLD1}}$) [112]. $P_{\text{FLD1}}$ is similar in strength to $P_{\text{AOX1}}$ and is tightly regulated but offers methanol-independent induction, although methanol can be used as inducer as well [113]. The major advantage of $P_{\text{FLD1}}$ is that glucose or glycerol can be used as the
carbon source when methylamine is used as the inducer and sole nitrogen source, which circumvents the problems associated with methanol usage [112]. Although there is a growing amount of data on alternative promoters to P_{AOX1}, the highly developed technology around the P_{AOX1} both for small scale and large scale expressions is likely to secure its role as one of the most important promoters for the _P. pastoris_ expression system in a foreseeable future.

### 2.4 Methanol metabolism

Methanol metabolism is intimately linked to the production of recombinant proteins with _P. pastoris_ and therefore some in depth discussion of this topic will be presented here.

#### 2.4.1 Methylo trophic metabolism in microorganisms

Microorganisms which can utilize C1 compounds at a more reduced state than CO₂ as a sole carbon and energy source are termed methylo trophs. Naturally occurring C1 compounds such as methane, methanol, formaldehyde, methylated amines, methylated sulphur compounds and methyl halogenates are principle substrates for methylo trophs [114, 115]. Whereas prokaryotic methylo trophs are able to use all these substrates as sole carbon and energy sources, methylo trophic yeasts are generally confined to use methanol [114]. The general feature of the metabolism of reduced C1 compounds is the initial oxidation to formaldehyde. Part of the formaldehyde generated from methanol oxidation is subsequently further oxidized to CO₂ with the coupled generation of reducing equivalents (Figure 6). Another part is assimilated by various cyclic pathways to generate cell constituents. Three pathways are known to assimilate formaldehyde in microorganisms; the xylulose monophosphate pathway, the ribulose monophosphate pathway and the serine pathway. In methylo trophic yeast however, only the xylulose monophosphate pathway is known to operate [114]. The specific reactions are dependent on the C1 substrate and the microbial species [115].
2.4.2 Methylo trophic yeasts
Facultative methylo trophic yeasts respond dramatically upon growth on methanol with biosynthesis of specialized enzymatic systems which are partly compartmentalized into peroxisomes [101]. Facultative methylo trophic yeasts are found in at least four genera; *Pichia, Torulopsis, Hansenula* and *Candida*. The natural habitats of these yeasts are probably decaying plant material where methanol can be found as a byproduct of pectin hydrolysis [116]. For example, *P. pastoris* was isolated from exudates of oak and *Hansenula polymorpha* was isolated from spoiled orange juice.

2.4.3 The initial steps of methanol oxidation
In the first step of methanol metabolism, methanol and molecular oxygen diffuses to the peroxisome where methanol is oxidized to formaldehyde and hydrogen peroxide by alcohol oxidase 1 (and alcohol oxidase 2) (Figure 7). Molecular oxygen functions as the electron acceptor and FAD acts as a non-covalently bound co-factor for AOX [114]. The high oxygen consumption rate of methanol grown *P. pastoris* (Mut⁺) cultures is linked to this first oxidation step [91]. The hydrogen peroxide may subsequently be decomposed to water and oxygen by catalase (Cat).

![Figure 6](image.png)

**Figure 6.** Principal outline of metabolism of C1 compounds by methylo trophic. Methylo trophic yeasts are generally confined to use methanol as the substrate and the xylulose monophosphate pathway for assimilation (Marked in yellow).
Figure 7. Schematic overview of the methanol metabolism and the glutathione redox system of P. pastoris. Aox (Alcohol oxidase), Das (Dihydroxy acetone synthase), Cat (catalase), Fld (Formaldehyde dehydrogenase), Fgh (S-formylglutathione hydrolase), Fdh (Formate dehydrogenase), GSH (glutathione, reduced form), GSSG (Glutathione, oxidized form), GS-CH$_2$OH (S-hydroxymethylglutathione), GS-CHO (S-formylglutathione), GAP (Glyceraldehyde-3-phosphate), DHA (Dihydroxyacetone phosphate), Xu5P (Xylulose-5-phosphate), FBP (Fructose-1,6-bisphosphate), F6P (Fructose-6-phosphate), Pnp20 (peroxisomal glutathione peroxidase), ROOH (alkylhydroperoxide where R denotes a hydrogen or an aliphatic or aromatic organic group), dashed arrow (indicates the generation of alkylhydroperoxides resulting from reaction between H$_2$O$_2$ and the peroxisomal membrane, TCA (tricarboxylic acid cycle) Illustration is a modified version of figure 1 [117].
Hydrogen peroxide is highly reactive and may also generate other reactive oxygen species (ROS) which have been found to be scavenged by another important, methanol inducible, peroxisomal antioxidant enzyme, Pmp20 (peroxisomal glutathione peroxidase), which is a part of the glutathione redox system [117]. The glutathione redox system has shown to play a critical role to prevent accumulation of ROS and formaldehyde during methanol metabolism in \textit{P. pastoris}, figure 2 ([117]. The formaldehyde generated from the oxidation of methanol can be diverted into two metabolic pathways; the dissimilatory- and assimilatory [114].

\section*{2.4.4 Dissimilatory pathway}
In the dissimilatory pathway formaldehyde is ultimately oxidized to CO$_2$ with concomitant generation of two reducing equivalents in the form of NADH. Firstly, formaldehyde spontaneously reacts with glutathione in the peroxisome to form S-hydroxymethylglutathione (S-HMG). S-HMG may subsequently be transported to the cytosol where it is converted to S-formylglutathione (S-FG) by the NAD$^+$-dependant formaldehyde dehydrogenase (Fld) [117]. S-FG is hydrolysed by S-formylglutathione hydrolase (Fgh) to formic acid and glutathione. Finally, formic acid is oxidized to CO$_2$ by the NAD$^+$-dependant formate dehydrogenase (Fdh). The two NADH generated per dissimilated formaldehyde could via mitochondrial oxidative phosphorylation yield four ATP in total which means that 4 moles of ATP could be generated per mole of dissimilated methanol (Table 4). There is some debate whether the dissimilatory pathway is essential for growth on methanol as the sole carbon and energy source for methylotrophic yeast [118]. Deletion strains of \textit{Candida boidinii} in which each gene in the oxidative pathway of formaldehyde to CO$_2$ was disrupted showed that formaldehyde dehydrogenase was essential for growth [115]. On the other hand S-FG hydrolase and formate dehydrogenase was not essential for growth although the growth yield of the S-FG hydrolase- and formate dehydrogenase disruptants were 10\% and 25\%, respectively, compared to wild type strain [115].
Table 4. Number of moles of NADH and ATP formed per mole of dissimilated formaldehyde following the formaldehyde oxidation pathway. fld1Δ, fgh1Δ and fdh1Δ refers to the strains where formaldehyde dehydrogenase (fld), S-formylglutathione hydrolase (fgh) and formate dehydrogenase (fdh) have been disrupted [115].

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative cell yields</th>
<th>NADH</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>fld1Δ</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>fgh1Δ</td>
<td>10</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>fdh1Δ</td>
<td>25</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

On the other hand, strains of *H. polymorpha* in which both NADH generating enzymes were disrupted, still had the same growth yield as the wild type strain [118]. In these mutants it was suggested that the tricarboxylic acid cycle (TCA) was essential for generating energy during methylotrophic growth of yeast and so the dissimilatory pathway would not be crucial for growth but rather have a pure detoxification role [118]. Calculations based on ATP generation from TCA and oxidative phosphorylation suggest that 5.3 moles of ATP can be generated per mole of dissimilated methanol, which is higher than from direct pathway of methanol dissimilation (Table 5) [115]. Common to all disruptants was a higher sensitivity to formaldehyde which supports that the oxidative pathway does have a detoxification role.

Table 5. Number of moles of ATP per dissimilated methanol for direct methanol dissimilation or by the TCA [118].

<table>
<thead>
<tr>
<th>Metabolic pathway</th>
<th>nATP per dissimilated CH₃OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct methanol dissimilation</td>
<td>4</td>
</tr>
<tr>
<td>TCA and oxidative phosphorylation</td>
<td>5.3</td>
</tr>
</tbody>
</table>
2.4.4 The assimilatory pathway

Part of the formaldehyde generated from the methanol oxidation is assimilated by the xylulose monophosphate (Xu5P) cycle to provide precursors for cell material. Xu5P is transferred into the peroxisome, possibly by an active transport, where the peroxisomal enzyme dihydroxyacetone synthase (Das) catalyzes its reaction with formaldehyde to produce glyceraldehydes-3-phosphate (GAP), and dihydroxyacetone, (DHA) in a transketolase reaction [114]. GAP and DHA subsequently enters the cytosol where DHA is phosphorylated by dihydroxyacetone kinase to give dihydroxyacetone phosphate, DHAP. DHAP then reacts with GAP to produce fructose-1,6-bisphosphate in an aldolase reaction which is dephosphorylated to fructose-6-phosphate by a phosphatase. In a series of rearrangement reactions Xu5P is regenerated. It is estimated that one third of the GAP formed is utilized for biomass generation [114]. As mentioned previously, part of the GAP has also been proposed to generate ATP through the TCA and oxidative phosphorylation and that this would be essential energy source for methylotrophic growth of yeast [118].

2.4.5 Function of peroxisomes

The ability of methylotrophic yeasts to grow on methanol as the sole carbon and energy source is dependent on the localization of AOX, Cat and Das to peroxisomes [114]. All other enzymes in the methanol utilization pathways are found in the cytoplasm. There are several reasons for this. First, AOX of *P. pastoris* fails to oligomerize in the cytoplasm [119]. It is interesting to note that the alcohol oxidase from *H. polymorpha* can oligomerize in the cytoplasm indicating that there may be drastic differences between different strains of methylotrophic yeast [119]. Compartamentalization of AOX and Das to peroxisomes is also required to direct the formaldehyde generated from oxidation of methanol over the dissimilatory and assimilatory pathways [114]. The assimilation of formaldehyde requires certain amounts of Xu5P in the peroxisome. However, the formation of Xu5P in the cytoplasm and its transport into the peroxisome are energy requiring reactions (Klei2006). Consequently, when the energy status of the cells is increased, the flux of Xu5P into the peroxisome may increase and a larger fraction of formaldehyde may enter the assimilatory pathway.
Conversely, when the energy status of the cells is low, less Xu5P will be available in the peroxisome and a larger fraction of the formaldehyde may react with GSH and enter the dissimilatory pathway. The energy status of the cells is hence sufficient to regulate the flux of formaldehyde over the assimilatory and dissimilatory pathways [114].

Formaldehyde and H₂O₂ are highly toxic compounds because of their unspecific reactivity towards nucleic acids and proteins; therefore, compartmentalization may have been developed to protect the rest of the cells from such reactions. Compartmentalization of CAT is also required because otherwise hydrogen peroxide would most likely be metabolized by peroxidase activities of the mitochondria, which are energy requiring reactions. For example, cytochrome c peroxidase can metabolize H₂O₂ via an energy dependant process and also has a considerably higher affinity for H₂O₂ than Cat [120]. In addition, H₂O₂ will spontaneously oxidize GSH which in turn might limit the energy generation through the dissimilatory pathway by limiting the formation of S-hydroxymethylglutathione.

2.5 Bioreactor cultivation strategies using the Pₐox₁
To exploit the full potential of the P. pastoris expression system and the Pₐox₁, high cell density cultivation is essential. Although the specific productivity may be low for several proteins, the exceptionally high cell densities obtainable with P. pastoris (>150 g cry cell weight L⁻¹) may compensate for that and often generates high total productivities [14, 96]. However, to sustain aerobic processes (DOT>20% air saturation) with high cell density P. pastoris cultures, particularly with methanol grown Mut⁺ strains, bioreactors with high oxygen transfer capabilities and efficient cooling systems are crucial [80, 121]. Bioreactor controlled cultivation is also required to maintain the methanol concentration at a specified level [96]. This cultivation is normally done as fed-batch, whereby the methanol feed rate is controlled not to exceed the maximum methanol consumption rate of the cells. Initially the methanol feed rate may be increased exponentially to maintain a certain specific growth rate, but because of the increasing oxygen consumption rate of the growing biomass, the methanol feed rate must eventually be restricted to prevent oxygen limitation. By supplying a constant
feed of methanol, restricted to keep the dissolved oxygen at a desired value, biomass can be generated without oxygen limitation [91].

2.5.1 Standard fed-batch cultivation

Because of the repression/derepression and induction mechanism of the P_{AOX1} promoter a three stage operation strategy is commonly used for expression of recombinant proteins with the \textit{P. pastoris} system (Figure 8). These steps consist of a glycerol batch phase to generate biomass but no recombinant protein, a glycerol fed-batch phase to generate more biomass and derepress the P_{AOX1} and finally a methanol fed-batch phase to induce recombinant protein production. The methanol fed-batch phase is the most critical to achieve high yields of recombinant protein and several operational strategies have been studied [93, 96]. The methanol feeding strategy implemented for the production phase depend on several factors such as operational conditions including temperature, pH, cultivation medium etc, but also on the strain used and the particular recombinant protein expressed [93]. The standard methanol fed-batch cultivation is generally performed at a temperature of 30 °C, a pH of 5-6 and a DOT > 20 % air saturation [93, 96]. Cultivation at temperatures of 32 °C or above is detrimental to recombinant protein production [93].

Figure 8. The three main steps of standard \textit{P. pastoris} high cell density fed batch technique for recombinant protein production. (1) Biomass generation by batch growth on glycerol for about 24 h. (2) Derepression of the P_{AOX1} and biomass generation by growth on limited glycerol for about 4 hours. (3) Induction by growth on pure methanol for about 72 hours.
2.5.1.1 The glycerol batch phase
During the glycerol batch phase biomass is generated by growth on specified amount of glycerol which represses the \( P_{AOX1} \) and consequently no recombinant protein is produced. Repression of the \( P_{AOX1} \) ensures that a high cell density is reached before the onset of any adverse effects caused by recombinant protein expression and the selection for potential mutants defective in recombinant expression may be minimized. Most media formulations use a glycerol concentration of 40 g L\(^{-1}\) for the batch phase because there are indications that higher levels of glycerol may inhibit growth [93]. Maximum specific growth rate on glycerol for \( P. pastoris \) (Mut\(^+\)) has been reported to be 0.26 h\(^{-1}\) which can be compared to 0.14 h\(^{-1}\) for methanol [91, 122]. In addition, the cell viability is higher for cells grown on glycerol compared to those grown on methanol which have the additional benefit of reducing release of product degrading proteases in the cultivation medium. Collectively, these facts reflect the main purposes with the glycerol batch phase, which are to quickly generate a high viability biomass with low selection of mutants defective in recombinant protein expression. Exhaustion of the glycerol is commonly indicated by a sharp increase in the dissolved oxygen tension which marks the end of the glycerol batch phase.

2.5.1.2 The glycerol fed-batch phase
After the initial batch glycerol is depleted the cells are provided with a growth limiting feed of glycerol to generate more biomass without oxygen limitation, and for Mut\(^+\) strains, also to derepress the \( P_{AOX1} \) [91]. When the \( P_{AOX1} \) is derepressed in Mut\(^+\) strains, some alcohol oxidase (and recombinant protein) is produced which makes the cells less sensitive to a shift of carbon sources to methanol and allows them tolerate higher initial feed rates of methanol in the methanol fed-batch phase, which is important to reduce the lag-phase [96]. Both constant and exponential feed rates have been used. Constant feeds are simpler from a practical point of view but are less efficient in terms of generating biomass compared to when exponential feed rates are used. The exponential feed profile may be estimated from
\[ F(t) = \frac{\mu X_0 V_0}{G} e^{\mu x} \]  

Where \( F(t) \) (L h\(^{-1}\)) is the glycerol feed rate at time \( t \) (h) after the start of the feed, \( \mu \) (h\(^{-1}\)) is the specific growth rate, \( X_0 \) is the biomass concentration in gram dry cell weight (gDCW) L\(^{-1}\) and \( V_0 \) culture volume (L) at the start of the feed, \( G \) (g L\(^{-1}\)) is the glycerol concentration in the feed and \( Y_{x/s} \) is the growth yield coefficient on glycerol [96]. Maintaining a specific growth rate of 0.18 h\(^{-1}\) has been reported to be sufficient for the derepression of the P\(_{AOX1}\) [96].

### 2.5.1.3 The methanol fed-batch phase

After the glycerol fed-batch phase has ended, methanol is used to induce expression of the recombinant protein. The standard methanol fed-batch phase is typically started with very low feed rates of methanol (often around 100 \( \mu \)L g DCW\(^{-1}\) h\(^{-1}\)) the first 2-3 hours to let the cells fully adapt to methanol metabolism [91]. During the adaptation period the cells generate peroxisomes and the adequate enzymes required for methanol metabolism [114]. Subsequently, the feed rate may be increased exponentially to maintain constant specific growth rate until the oxygen transfer, or other mechanism, ultimately limits further increase and the methanol feed rate has to remain constant or even decreased [96]. To improve oxygen transfer pure oxygen can be supplemented to the air feed or a higher total pressure can be used in the reactor [91, 123]. Furthermore, because \( P. \) \textit{pastoris} is an obligate aerobe when growing on methanol, oxygen limiting conditions can also be used to increase the driving force for oxygen transfer without the generation of growth inhibiting fermentation products [124].

### 2.5.2 Methanol concentration

Careful control of the methanol concentration in \( P. \) \textit{pastoris} bioreactor cultivations is crucial for process development and to ensure stable and reproducible production of a recombinant protein based on the P\(_{AOX1}\) promoter. Too high concentrations of methanol will be toxic to the cells, and too low levels of methanol may not be enough
to induce AOX transcription [93, 125]. There are several studies on how the residual methanol concentration influences the specific growth rate and productivity. For example, in a Mut+ strain expressing the heavy-chain fragment C of botulinum neurotoxin, serotype A intracellularly, the maximum specific growth rate, $\mu_{\text{max}}$, was found to be 0.071 h$^{-1}$ at a methanol concentration of 3.65 g L$^{-1}$, which also led to the highest specific productivity of 0.082 mg g wet cell weight$^{-1}$ h$^{-1}$ [126]. In another study, where a Mut+ strain secreted human $\beta_2$-glycoprotein I domain V, $\mu_{\text{max}}$ declined from about 0.012 h$^{-1}$ to 0.01 h$^{-1}$ when the methanol concentrations were increased from 1.5 g L$^{-1}$ to 17 g L$^{-1}$ and to 0.009 h$^{-1}$ at a methanol concentration of 31 g L$^{-1}$ [125]. However, the specific productivity was increased from about 0.05 to 0.12 mg gDCW$^{-1}$ h$^{-1}$ as the methanol concentration increased to 31 g L$^{-1}$ [125]. In a third study where a Mut+ strain secreted avidin, the $\mu_{\text{max}}$ was found to be about 0.14 h$^{-1}$ at methanol concentrations between 1-6 g L$^{-1}$ and decreased to about 0.10 h$^{-1}$ at 15 g L$^{-1}$ [122]. In the same study the specific productivity was relatively constant at about 0.025 mg g DCW$^{-1}$ h$^{-1}$ for all methanol concentrations, including a control run using a predefined exponential feeding with methanol concentration at 0.05 g L$^{-1}$ [122]. Collectively, these results demonstrate that the particular induction strategy often has to be tailored for a particular recombinant protein.

As a consequence of the high oxygen demands of high cell density $P.\ pastoris$ Mut+ cultures, high residual methanol cannot be maintained for aerobic processes and cultivations must eventually progress with very low methanol concentrations (< 25 mg L$^{-1}$) [124]. On the other hand, under oxygen- or temperature limited cultivation higher residual methanol can be maintained even in high cell density Mut+ cultures [104, 124]. The possibility to maintain higher residual methanol concentrations in oxygen limited fed-batch cultivation compared to methanol limited fed-batch cultivation has been shown to have positive effects on the $P_{\text{AOX1}}$ promoter activity, particularly at the later part of the methanol fed-batch phase [124]. By using Mut- phenotypes, which do not utilize methanol as a carbon source, and a limited feed of a non-repressive carbon source such as sorbitol, the residual methanol concentration may also be maintained at high levels without oxygen limitation [108]. However, the requirement to control an additional nutrient at a specified level makes process control more complex, and hence less attractive.
2.5.2.1 Control of the methanol concentration

On-line control of the methanol concentration is highly advantageous for the development of *P. pastoris* bioprocesses where the P_{AX} is used as the promoter, as it allows induction at specified methanol concentrations and prevents overfeeding to toxic levels of methanol. Overfeeding may also present safety hazards, particularly at large scale cultivation. Several on-line techniques have been developed to monitor the methanol concentration in the cultivation media, including mid-IR spectroscopy [122], automated sequential injection analysis [127], a silicon tubing sensor [128] and gas analyzers monitoring the exhaust gas of the bioreactor [104, 129]. The simplest way to monitor the methanol concentration is perhaps through the exhaust gas of the bioreactor by the use of commercially available gas analyzers [104, 129]. In contrast to the other techniques, this technique doesn’t require any direct interaction between the sensing device and the cultivation medium and has a simpler more direct set up. One disadvantage may be its sensitivity to ammonium hydroxide, which is often used as a nitrogen source for *P. pastoris* cultivations [129]. Methanol detection systems can be connected to a variety of controllers that regulate the methanol feed rate in response to the detected methanol concentrations. One major drawback of these systems is that their sensitivities are not high enough for control of methanol-limited processes [96].

Methanol limitation can be monitored indirectly by stopping the methanol feed briefly and observing/measuring how the DOT responds. If there is no or very little residual methanol DOT will respond quickly (<15 seconds for 1L bioreactors) and the methanol feed rate can be increased [96]. Conversely, when the response time is increased it may indicate residual methanol and the methanol feed can be reduced or stopped. This procedure can be repeated until oxygen transfer limitation prevents further increase in methanol feed rate. DOT can also be used as a set point for the bioreactor so that the methanol feed pump is regulated to maintain the specified [124].

2.5.3 Cultivation media
The high cell densities obtainable with *P. pastoris* require a cultivation medium which provides a rich supply of the appropriate nutrients. Work in the early 1980s determined ranges of basic media components suitable for high cell density fermentation with yeast, table 6 [130]. High cell density *P. pastoris* cultivations are generally performed in the basal salts medium (BSM) described in the *Pichia* fermentation guidelines provided by Invitrogen [131].

The basal salts medium consists of mineral salts and glycerol as the sole carbon source. In addition the BSM is supplemented by a trace element solution referred to as PTM\(_i\) trace salts which also includes biotin. The BSM media, including PTM\(_i\) solution has a composition in the high range suggested by table 6 [93]. The PTM\(_i\) trace salt solution is also included in the glycerol- and methanol feeds during glycerol and methanol fed-batch phases. The only nitrogen source is ammonium hydroxide which is added as the pH is regulated. BSM has some drawbacks including precipitation of media components and high ionic strength which, in turn, may cause problems with cell density determinations, cell-lysis and downstream processing [96, 132]. Other media including FM22 has been developed for high cell density cultivation and generally has lower amounts of salts compared to the BSM, except potassium which is higher. It has been estimated that under high cell density cultivation (>50 gDCW L\(^{-1}\)) in BSM and FM22, the cells may suffer from nitrogen starvation which in turn may increase the proteolytic activity in the medium [93]. Optimization studies have indicated that inclusion of ammonium sulphate to about 7.5 g L\(^{-1}\) in the medium can remedy this problem [132]. Compared to mineral salts media, rich media such as the BMGY media described in EasySelect\textsuperscript{TM} *Pichia* Expression Kit (Invitrogen) which include 1% yeast extract, 2% peptone and about 10 g L\(^{-1}\) ammonium sulphate may result in substantially higher yields and less product degradation (>10 fold increase – unpublished results).

Table 6. Suggested composition of basic elements for high cell density cultivation of yeast (Wegner1983)

<table>
<thead>
<tr>
<th>Element</th>
<th>Range (L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>2.9-20 g</td>
</tr>
<tr>
<td>K</td>
<td>1.0-20 g</td>
</tr>
<tr>
<td>Mg</td>
<td>0.15-3 g</td>
</tr>
<tr>
<td>Ca</td>
<td>0.06-1.6 g</td>
</tr>
<tr>
<td>S</td>
<td>0.1-8 g</td>
</tr>
<tr>
<td>Fe</td>
<td>6-140 mg</td>
</tr>
<tr>
<td>Zn</td>
<td>2-100 mg</td>
</tr>
<tr>
<td>Cu</td>
<td>0.6-16 mg</td>
</tr>
<tr>
<td>Mn</td>
<td>0.6-20 mg</td>
</tr>
</tbody>
</table>
2.6. Factors limiting recombinant protein production with the *P. pastoris* system

There are several factors that potentially may limit the final productivities of secreted recombinant proteins. These factors may be associated with technical aspects of the bioreactor system, the recombinant protein expression and secretion processes, and the stability of the product in the cultivation media.

2.6.1 Technical limitations

Utilization of methanol as a carbon source puts particularly high demands on the oxygen- and heat transfer capabilities of the bioreactor system and are major concerns during scale-up [121]. The high oxygen consumption rates of methanol grown cells are related to the first oxidation step of methanol to formaldehyde by alcohol oxidase which requires substantial amounts of oxygen [91]. Moreover, compared to other common substrates used for *P. pastoris* cultivation including glycerol and sorbitol, methanol has the highest heat of combustion on a carbon mole basis which contributes to the high heat generation [110].

The acceptable specific growth rate in a bioreactor with a certain cooling power can be estimated from the following equation which is derived from an enthalpy balance on the bioreactor [80]:

\[
\mu \leq \frac{Q_{\text{cool}}Y_{X/S}}{XY_{Q/S}}
\]  

(2)

where $Q_{\text{cool}}$ is the cooling power of the bioreactor in $W \ L^{-1}$, $Y_{X/S}$ is the biomass to substrate yield (C-mol C-mol$^{-1}$ or g g$^{-1}$), $Y_{Q/S}$ is the heat to substrate yield (J C-mol$^{-1}$ or J g$^{-1}$) and $X$ is the biomass concentration in g DCW L$^{-1}$. If representative data are inserted in (2) with the cooling power of the bioreactor being 20 $W \ L^{-1}$, $Y_{X/S}$ on methanol 0.45 C-mol C-mol$^{-1}$, $Y_{Q/S}$ on methanol 510 KJ C-mol$^{-1}$, the C-molar weight of the biomass 25 g C-mol$^{-1}$ and the biomass concentration 100 g L$^{-1}$, the specific
growth must not exceed about 0.02 h⁻¹ [80]. The maximum growth rate on methanol is 0.14 h⁻¹; therefore the cooling capacity of a standard bioreactor places serious limitations to the interval of specific growth rates which can be used. Consequently, the cells may not be allowed to grow at a specific growth rate which may be optimal for recombinant protein production.

Similarly, the oxygen transfer capacity of the reactor is limited and a constant methanol feed rate has to be maintained when the maximum oxygen transfer capacity of the bioreactor has been reached [91]. Because of the increase in total biomass the specific growth rate under constant feeds will decline until it reaches the dilution rate which is the point where the maximum biomass concentration has been reached [91]. The specific oxygen consumption rate during constant methanol fed-batch is often in the range of 40-50 mg g DCW⁻¹, when no oxygen supplementation or overpressure is used and the highest possible methanol feed rate is applied (around 6 mL h⁻¹ L⁻¹) [91]. To ensure an equal oxygen supply in the larger bioreactor during scale-up, a common strategy is to maintain the same power input per volume ratio (P/V), where P refers to the power input from the impeller, and V is the liquid volume [121]. Stirred tank reactors larger than 1500 L are generally limited to (P/V) of about 8 kW m⁻³ because of mechanical reasons, which in turn limits the oxygen transfer rate (OTR) of the reactor to around 4 g L⁻¹ h⁻¹ using only air and no over pressure in the reactor [121]. A rough estimation suggests that under standard methanol fed-batch processes at 1500 L scale a biomass concentration of around 80-100 gDCW L⁻¹ could be sustained. Although pure oxygen improves the OTR it is generally regarded unsafe at larger scales and alternative techniques, including increased reactor pressure or oxygen limited techniques, may present more attractive operational strategies [121, 123, 124]. Mixed feeds which utilize carbon sources resulting in reduced heat generation and oxygen consumption rates, including sorbitol, are also interesting although such systems often results in more complex set-ups and process control.
2.6.2 Limiting factors during expression and secretion

Before larger scale processes are developed around an expression strain, it is important to ensure that potential factors limiting expression and secretion on the cellular level are minimized. The factors associated with the expression and secretion steps involve transcription, stability of mRNA, translation, translocation, folding, co- and post-translational modifications, vesicle trafficking, exocytosis and cell wall permeation, (Figure 9).

![Diagram of protein expression and secretion in yeast.](image)

**Figure 9. Critical points during expression and secretion of recombinant proteins in yeast.**
Although the cultivation strategy inevitably influences several of these steps, this section will focus on how improvement of expression and secretion can be achieved by genetic design of the expression strain.

Several efforts have been made to improve secretion to the cultivation media by optimization of gene copy number and promoter strength [95], optimization of secretion signals [98, 133, 134], overexpression of genes associated with the secretory pathway [135], overexpression of chaperones and foldases [136], manipulation of cell wall structure and permeability by disruption of a gene involved in cell wall biogenesis [137]. Because of the different natures of recombinant proteins the particular steps required to improve secretion is varied and generally has to be tailored for a particular recombinant protein.

2.6.2.1 Transcription
The amount of specific transcripts is primarily influenced by the promoter but is also influenced by the gene copy number and stability of the mRNA [89, 95]. When the \text{P}_{\text{Aox1}} \text{ and P}_{\text{Gap}} \text{ were compared at different gene copy numbers no difference in expression levels of human trypsinogen 1 could be seen for the P}_{\text{Gap}} \text{ whereas a gene copy number of two resulted in the highest expression levels for P}_{\text{Aox1}} [95]. Other studies have also indicated how the gene copy number has to be optimized for a particular recombinant protein and the promoter used [138, 139]. Limitation to the gene dosage effect is generally believed to occur because other processes in the expression pathway becomes limiting at a particular gene copy number. When a certain amount of transcript has been generated the translation machinery may become the limiting factor due to depletion of precursor molecules and energy [140].

2.6.2.2 Translocation and folding
Low folding rate is probably one of the major factors leading to limited secretion rates of recombinant proteins in yeast [141]. If the folding rate cannot meet the translational rate, accumulation of misfolded proteins may induce stress responses and interfere with other processes essential for the viability of the cells ultimately reducing recombinant protein secretion [142]. To improve the folding rate a general strategy is to improve the
folding environment for the recombinant protein. This change has been done by overexpression of various chaperones and foldases involved in protein folding [136]. For example, the secretion of recombinant human serum albumin, which has a high content of disulfide bridges, is thought to be limited because of low productive folding during translocation [136]. By duplication of the genes encoding protein disulfide isomerase (PDI) and polyubiquitin, the secretion could be increased more than 15- and 8.8-fold respectively, compared to the parental strain [136]. On the other hand, the same gene duplications did not influence the secretion of human interleukin-1beta, pointing out the fact that different strategies have to be evaluated for every recombinant protein.

It has also been shown that the secretion can be improved by stimulation of the unfolded protein response [143]. The UPR is a stress response to the accumulation of misfolded proteins in the lumen of ER and generally serves to provide a better folding environment in the ER by up-regulation of a multitude of genes including those encoding chaperones and foldases [144]. Hence, stimulation of the UPR could have beneficial effects for the expression of recombinant proteins which undergo folding in the ER. However, the UPR may also act on a more global level by suppression of protein synthesis and degradation of persistently misfolded species by a mechanism termed ER associated degradation [144-146]. Briefly, increased levels of misfolded proteins activates a transmembrane protein termed Ire1p, which has both kinase and ribonuclease activity. The ribonuclease activity of activated Ire1p potentiates splicing of a precursor mRNA, which in its mature form encodes a transcription factor termed Hac1p. Hac1p may subsequently induce transcription of UPR associated genes which have a special UPR element in their promoter regions [147, 148].

By overexpressing this transcription factor a constitutive expression of the UPR could be achieved which was demonstrated to improve secretion for certain recombinant proteins in S. cerevisae [143]. Controlled stimulation of the UPR by, for example, a regulated overexpression of the UPR transcription factor could therefore be a strategy to improve secretion.
2.6.2.3 Secretion signal processing
The most successful secretion signal used with the *P. pastoris* expression system so far is the *S. cerevisiae* α-factor (α-MF) prepro signal [14]. However, variability in the amino terminus of recombinant proteins secreted by *P. pastoris* utilizing the α-MF prepro signal is still commonly seen, reflecting incomplete signal sequence processing [14]. The α-MF basically consists of three parts which are sequentially removed as the recombinant protein progresses through the secretory pathway [89]. The first part, or pre-sequence, is a 19-amino acid residue which directs the protein to the ER where it subsequently is removed by signal peptidase. The second part, or the pro-sequence, is a 66-amino acid residue which is removed in the late golgi by the Kex2 endopeptidase. The pro-sequence carries three consensus sequences for N-glycosylation. Finally there is a Glu-Ala repeat which is removed by the Ste13 exopeptidase exposing the N-terminal of the recombinant protein. The pre-region can function on its own to direct secretion although the pro region has been found to be essential in some cases [89].

2.6.2.3 Cell wall permeation
From a heterologous protein expression point of view the cell wall is highly interesting as it presents a physical barrier to secretion of recombinant proteins. It is a dynamic barrier which can change its properties as a response to various stimuli [149]. Cell wall structure and porosity is also dependant on strain and the physiological state of the cells and hence cultivation conditions might influence the efficiency of secretion [150]. To efficiently secrete larger recombinant proteins it is important that the cell wall has a high permeability for the protein of interest or the cell wall permeation may present a bottle neck for secretion. This in turn could cause aggregation of the recombinant protein in the periplasmic space and perhaps also intracellularly which ultimately may reduce productivity of the process. Studies made on a killer toxin secreted by *Pichia anomala* have indicated that the protein accumulates in the cell wall which may be a result of a slower rate of diffusion through the cell wall than transport through the ER-Golgi system [149].
The cell wall may act as a molecular sieve letting smaller proteins pass through easier than larger ones [151]. It may also function as an ion-exchanger as charged groups of the secreted protein may interact with cell wall components [152]. Interactions between the cell wall and the protein to be secreted can thus be quite complex and the outcome can be difficult to predict. For example, a highly glycosylated 400 kDa form of the Epstein-Barr virus envelope glycoprotein was excreted by *S. cerevisiae* whereas smaller and less glycosylated forms were not [150]. These findings indicate that the sieving effect is less compared to other factors which might include interactions between charged groups of the cell wall and the glycoprotein.

In order to increase excretion of proteins across the cell wall, manipulation of cell wall cross-linking enzyme glycoprophospholipid-anchored surface protein (Gas1p) has been studied in *S. cerevisiae* and *P. pastoris*. Deletion of the gene coding for Gas1p in *S. cerevisiae*, GAS1, increased excretion of human insulin-like growth factor 1 seven-fold [153]. In *P. pastoris* disruption of its GAS1 homologue did not have any effect on excretion of trypsinogen or albumin but the amount of excreted lipase was doubled [137]. This cross linking enzyme influences the structure and permeability of the cell wall and an absence of its activity could possibly cause a higher porosity making it easier for proteins to be excreted. In addition to manipulation of the cell wall structure and permeability by disruption of genes involved in the cell wall biogenesis, there are indications that a short amino acid sequence of the secreted protein may promote excretion to the growth medium. Residues 1-8 of mature glucoamylase was shown to enhance excretion of glucoamylase with the *S.cerevisiae* system but also beta-galactosidase when genetically fused to this enzyme [98]. Transfer of a protein from the periplasmic space across the cell wall to the growth medium may hence be promoted by growth conditions loosening the cell wall structure, manipulation of genes resulting in the loosening of the cell wall structure or addition of certain amino acids sequences to the recombinant protein to be excreted.
2.6.3 Proteolytic degradation

High cell density cultivations are often hampered by proteolytic degradation of secreted recombinant proteins which can result from activities of intracellular-, cell-bound or secreted proteases [96]. The proteolytic activities in the cultivation media are mainly thought to originate from intracellular proteases of dead lysed cells [96]. In *P. pastoris* bioreactor cultivations where extremely high cell densities are achieved leakage of intracellular proteases from lysed cells may be particularly prominent and present a serious limitation for achieving high protein titres [154]. Several strategies have been developed to minimize proteolytic degradation of recombinant proteins. These strategies may include the use of protease deficient strains, modification of the recombinant protein to resist proteolytic attack [155], changing operational conditions like pH and temperature to reduce activity of proteases and reduce cell lysis which may be a source for proteases in the media [104, 156] and supplementation of peptone, casaminoacids and/or protease inhibitors to the cultivation media [157]. Peptones and casamino acids may function as competitive substrates for the proteases which limits the degradation of the recombinant protein.

2.6.3.1 Proteolytic systems in *P. pastoris*

The proteases in *P. pastoris* belong to three basic groups; the cytosolic proteosomes, vacuolar proteases and proteases associated with the secretory pathway [158, 159]. The cytosolic proteosomes are multicatalytic protease complexes which degrade proteins and peptides in an ATP dependent manner. They have important functions in the proteolytic pathways of the eukaryotic cell including stress mediated protein degradation and proteolysis of short lived proteins [160]. Since degradation by the proteasomes require ATP and often ubiquitinylation of the protein to be degraded, they are probably not responsible for the major proteolytic activities in the cultivation media of high cell density cultures of *P. pastoris* [96]. Vacuolar proteases play a major part in the total proteolytic activities in yeast which involve maturation of precursors, activation of zymogens, and in general degradation of proteins which may include peptides supplied in the growth media, etc. General degradation of proteins may be enhanced during nutritional stress. Nitrogen starvation has been shown to induce
proteolytic activity in the cultivation media [161]. There are seven known vacuolar proteases in yeast of which endoproteinase A and B, carboxypeptidase Y and S, aminopeptidase I and yscCo act in an autocatalytic manner with low substrate specificity [158]. This feature allows them to be active after leaking out to the media from lysed cells and potentially target secreted recombinant proteins [158]. For this reason cell viability is an important factor to reduce proteolytic activities in the cultivation media [104].
Part III

Present Study

This part summarizes the background of the present study, study objectives and results of the present study.

3.1 Background summary

Modern vaccines are often based on recombinant proteins derived from pathogens. However, recombinant vaccines are generally poor immunogens and are therefore used in conjunction with adjuvants to enhance and direct the antigen specific immune response. Because of the low availability of approved vaccine adjuvants for human use there is a need to investigate novel strategies for adjuvant design. One strategy is to target recombinant vaccines to special pattern recognition receptors (PRRs) of the immune system which have the ability enhance and direct the antigen specific immune responses.

The mannose specific receptors are a group of PRRs which specifically bind to highly mannosylated carbohydrates of microbial glycoconjugates. The mannose receptor (MR), the dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and the mannose binding lectin (MBL) are three mannose specific receptors with documented functions within the immune system [13, 40, 43, 55]. MR and DC-SIGN are transmembrane endocytic receptors found on professional antigen presenting cells, and have shown to improve antigen up-take and T-cell presentation [41, 42, 162]. MBL is a soluble plasma protein which is involved in activation of the complement system and opsonophagocytic activites which in turn may influence adaptive immunity [16, 44]. Targeting these receptors is therefore interesting in the development of novel vaccine adjuvants. However, structural and functional characterisation of oligosaccharides involved in targeting mannosylated antigens to mannose specific, immunoregulatory receptors like MR, DC-SIGN and MBL is generally poor.
P-selectin glycoprotein ligand 1/mouse immunoglobulin G2b (PSGL-1/mIgG2b) is a homodimeric mucin-like fusion protein which, as a dimer, has 106 theoretical sites for O-glycans and six sites for N-glycans. Expressed and secreted by yeasts PSGL-1/mIgG2b would be extensively mannosylated and could therefore have the potential to bind mannose specific receptors particularly well through multivalent binding. By linking antigens to PSGL-1/mIgG2b, the antigen could be targeted to mannose specific receptors of the immune system which in turn could generate enhanced antigen specific immune responses. Compared to N-glycans, little is known about O-glycans as ligands for mannose specific receptors. PSGL-1/mIgG2b presents the opportunity to investigate the ability of yeast produced O-glycans to mediate high affinity binding to mannose specific receptors.

The methylotrophic yeast *P. pastoris* is well documented for efficient secretion, is suitable for large scale cultivation and intrinsically mannosylate recombinant glycoproteins with low incidence of hyperglycosylation. *P. pastoris* may therefore provide a suitable, cost effective platform for the production of vaccine adjuvants based on targeting mannose specific receptors of the immune system.

### 3.2 Study objectives

The general study objective of the work presented in this thesis was to explore the *P. pastoris* produced recombinant mucin-like fusion protein PSGL-1/mIgG2b as a vaccine adjuvant for sub-unit vaccines and to evaluate *P. pastoris* as a platform for production of PSGL-1/mIgG2b. The specific study objectives were:

- **Paper I and II.** To identify important factors for expression and secretion of PSGL-1/mIgG2b in *P. pastoris* high cell density bioreactor cultures. Secretory bottle-necks and influence of pH on cellular growth, productivity and glycosylation were investigated to assess the potential of the *P. pastoris* system to produce PSGL-1/mIgG2b.

- **Paper III.** To characterize the O-glycans of *P. pastoris* produced PSGL-1/mIgG2b and demonstrate that they are capable of mediating high affinity
binding to recombinant versions of representative mannose specific receptors of the immune system. These results are important for showing that \textit{P. pastoris} produced PSGL-1/mIgG\textsubscript{2b} could function as an efficient targeting molecule for mannose specific receptors and that the O-glycans play an important role for efficient binding.

**Paper IV.** To demonstrate that \textit{P. pastoris} produced PSGL-1/mIgG\textsubscript{2b} has the ability to enhance antigen-specific immune responses in mice systems, to demonstrate that the glycans of \textit{P. pastoris} produced PSGL-1/mIgG\textsubscript{2b} are decisive for the enhanced immune responses, and also to characterize the type of immune responses elicited. These results are important for demonstrating the \textit{in vivo} functionality of PSGL-1/mIgG\textsubscript{2b} as an adjuvant.

### 3.3 Summary of papers

#### 3.3.1 Paper I

Preliminary expression studies of PSGL-1/mIgG\textsubscript{2b} showed that increased methanol feed rates stopped secretion at increasingly earlier time points. Because the methanol feed rates were not excessive it was assumed that there was a secretory bottle-neck which caused higher intracellular aggregation under higher transcriptional activity of the \textit{P\textsubscript{AOX1}}, ultimately limiting secretion. In this study the green fluorescent protein was genetically linked to the C-terminal end of PSGL-1/mIgG\textsubscript{2b} in order to identify potential bottle-necks to secretion within the secretory pathway. For quick evaluation of fusion protein expression, an in-house measuring device was constructed based on a commercially available fluorometer (Figure 10).
Figure 10. Schematic layout of a system for on-line monitoring of fluorescent protein expression. The cell suspension can continuously be pumped through a flow cell in a loop arrangement. Excitation light is passed from the light source by fiber-optic cables to the flow cell where the fluorescent protein is excited and emits light. The emission is subsequently passed back through another set of fiber-optic cables, to the spectrometer and data acquisition.

With this system the cellular fluorescence, which was a measure of intracellular retained fusion protein, could be observed either in real-time by directly measuring the fluorescence of the cell suspension in the reactor, or by measuring the fluorescence of cell pellets directly in standard micro centrifuge tubes. To exclude the effects of increasing cell density in the cultivation broth, fluorescence was measured on cell pellets in standard microcentrifuge tubes. ELISA was used to verify that the relative changes in cellular fluorescence also reflected changes in intracellular fusion protein concentrations. The fusion protein was expressed using three predefined exponential methanol feeding models which were evaluated based on intracellular retained- and secreted fusion protein.

It was found that part of the fusion protein aggregated intracellularly as insoluble protein and that higher methanol feed rates increased the rate of aggregation. At a certain intracellular fusion protein concentration recombinant protein stopped. This point also appeared to coincide with an increasingly lower rate of secretion. This was consistent with the preliminary trials where the secretion of PSGL-1/MLgG2a without
GFP was found to decrease with increasing methanol feed rates. It appeared as if the intracellular fusion protein concentration was decisive for the ability of the cells to secrete. By using lower methanol feed rates, aggregation could be postponed and secretion maintained for longer time which resulted in higher productivities. It was concluded that the high transcriptional activity of $P_{aox1}$ imparted a translational rate which was too high and ultimately resulted in the aggregation. The fact that the intracellularly aggregated protein was fluorescent and had its full-length molecular weight suggested that it was correctly folded and extensively N- and O-glycosylated. The high level of glycosylation can only occur in the golgi which in turn implied that the rate limiting step for secretion was located post ER. Confocal microscopy showed that the fusion protein was gradually sorted to large single spots close to the cell wall. Western blot analysis revealed that the aggregated insoluble fusion protein had a slightly higher molecular weight compared to the intracellular soluble fraction of the fusion protein, suggesting that incomplete signal sequence processing could be the cause of the aggregation of insoluble fusion protein.

It was concluded that low methanol feed rates, which reduced intracellular aggregation, was beneficial for prolonged secretion but also that the $P_{aox1}$ ultimately was too strong for the GFP-fusion protein, due to a secretory bottleneck post ER. Strain development based on gene copy number, promoter and signal sequence would possibly tune secretion with protein synthesis rate so that continuous secretion could be maintained for extended periods of time.

### 3.3.2 Paper II

Cultivation pH is an important parameter which may be used to lower the proteolytic activity in the media and counteract competing microorganisms [156]. However, cultivation pH has also been shown to profoundly influence the glycosylation profile of some recombinant glycoproteins [79, 163]. Because the biological activity of PSGL-1/mIgG$_2b$ mainly resides in its glycans, its therapeutic effect could be influenced by changes in cultivation pH. Although this could have negative effects as it limits the cultivation conditions applicable for the production process if a single defined glycan
profile is desired, it could also present the opportunity to produce therapeutic glycoproteins with different therapeutic effects based on the same scaffold molecule.

In this study the influence of pH on productivity, degradation and glycosylation of PSGL-1/mIgG2b and cellular growth was investigated. Bioreactor cultivations were performed utilizing the same glycerol batch, fed-batch and methanol fed-batch procedures except that pH during the methanol fed-batch phase was either 3.5 or 6.0. For expressions at pH 3.5 the bioreactor set point was changed to 3.5 at the beginning of the glycerol fed-batch phase. The cultivation pH was subsequently lowered by cellular activity.

In general, product homogeneity was higher at pH 3.5 although growth and productivity was impaired. “Product homogeneity” refers to the number of bands observed on the western blot, where “high” means a lower number of bands. Induction pH did not have any influence on product degradation rate in cell free culture supernatant. The degradation did not alter the staining pattern observed on the western blot suggesting that the difference in product homogeneity is related to intracellular processes and not to degradation in the culture medium.

Western blot showed that the band of PSGL-1/mIgG2b expressed at pH 3.5 was weaker and more dispersed compared to that of PSGL-1/mIgG2b expressed at pH 6.0. Considering that anti-PSGL-1 antibodies were used and that PSGL-1 part of the fusion protein is the major glycan carrying part, the Western blot results suggested that induction pH influenced glycosylation.

Characterisation of the O-glycans of PSGL-1/mIgG2b expressed at the different pH values by LC-MS did not reveal any apparent differences. The O-glycans were Hex$_{2-9}$ structures where Hex$_{5-6}$ structures were most predominant. In addition, phosphorylated O-glycans were detected. However, when the N-glycans were removed from PSGL-1/mIgG2b, the bands on the western blot of PSGL-1/mIgG2b expressed under the two conditions became considerably more similar in size and strength suggesting that the pH primarily influenced N-glycosylation. LC-MS analysis of the released N-glycans revealed structures consistent with high mannose Man$_{5-12}$GlcNAc$_2$ for expression at pH 6.0 and Man$_{5-12}$GlcNAc$_2$ for expression at pH 3.5, indicating that the low induction pH adversely influenced Golgi processing of the N-glycans. This suggests that more robust mannose receptor targeting molecules could be designed by excluding the N-
glycosylation sites, as O-glycosylation appears less sensitive to pH changes in the medium.

In spite of the same methanol feed, the specific growth rate at pH 6.0 was constant at about 0.01 h\(^{-1}\), whereas at pH 3.5 it was about 0.005 h\(^{-1}\). This difference reflects the higher maintenance demand at the lower pH. The volumetric productivities were almost two times higher at pH 6.0 reaching 122±9 (p=0.05) mg L\(^{-1}\). However, because the fusion protein was truncated when produced at pH 6.0 the productivities might have been over estimated. Because of the higher product homogeneity, pH 3.5 was chosen for the production of PSGL-1/mIgG\(_{2b}\) to be used in the receptor binding- and immunological studies.

3.3.3 Paper III
In this study the ability of \(P.\) \textit{pastoris} produced PSGL-1/mIgG\(_{2b}\) to target the mannose specific receptors MR, DC-SIGN and MBL was investigated to assess its potential as a receptor targeting molecule for subunit vaccines.

The \(P.\) \textit{pastoris} produced PSGL-1/mIgG\(_{2b}\) was found to efficiently bind the mannose specific receptors MR, DC-SIGN and MBL displaying apparent dissociation constants in the nM region for all receptors. The binding of another \(P.\) \textit{pastoris} produced fusion protein (\(\alpha1\) acid glycoprotein mouse immune globulin G\(_{2b}\), AGP/mIgG\(_{2b}\), capable of carrying 12 N-glycans, was used as control and displayed similar binding affinities to the receptors as PSGL-1/mIgG\(_{2b}\). In contrast, PSGL-1/mIgG\(_{2b}\) produced in Chinese hamster ovary (CHO) cells, which have a completely different glycosylation, did not bind to the receptors, indicating that the mannose structures of \(P.\) \textit{pastoris} produced PSGL-1/mIgG\(_{2b}\) and AGP/mIgG\(_{2b}\) are crucial for efficient receptor binding. When the N-glycans of \(P.\) \textit{pastoris} produced PSGL-1/mIgG\(_{2b}\) were removed, the binding affinity to the receptors was retained demonstrating that the O-glycans could mediate strong binding to the receptors. Although MR and MBL have displayed specific binding to terminal mannoses in previous studies, DC-SIGN have been shown to preferentially bind a tri-mannose structure of high mannose N-glycans which are not present in the O-glycans [13, 56].
The ligands for DC-SIGN could hence be extended to include these types of O-glycans. The high O-glycan substitution of PSGL-1/mIgG_{2b} may also have promoted binding through multivalent binding mode which hence could be adopted as an efficient strategy to target mannose specific receptors.

Characterization of the O-glycans of \textit{P. pastoris} produced PSGL-1/mIgG_{2b} by $^1$H-nuclear magnetic resonance ($^1$H-NMR), electrospray ionization-ion trap mass spectrometry (ESI-MS) and enzymatic digestion, revealed Hex$_{2,9}$ with indications of mannoses linked by $\alpha$1,2- and $\alpha$1,3-glycosidic linkages. Inclusion of $\alpha$1,3-glycosidic linkages in \textit{P. pastoris} derived O-glycans have not been documented before and contribute to an increasingly more complex picture of O-glycans derived from \textit{P. pastoris} and yeasts in general.

3.3.4 Paper IV
In this study the results of Paper III was brought one step further by evaluating the ability of \textit{P. pastoris} produced PSGL-1/mIgG_{2b} (PPM) to improve antigen specific immune responses in mice system. Ovalbumin (OVA) was used as a model antigen due to its extensive use in immunological studies and hence the possibility to compare the results obtained in this study with the literature. To evaluate the function of PPM as an adjuvant, OVA-specific immune responses in mice immunized with OVA, OVA-PPM conjugates or mixtures, with or without an additional adjuvant in the form of Alum or AbiSCO®-100 were studied. Antibody responses, cytokine profiles of antigen induced splenocytes, CTL responses and antigen induced proliferation of T-lymphocytes were characterized, quantified and compared for the different vaccine compositions. To evaluate the effects of the mannose structures of PPM, PSGL-1/mIgG_{2b} carrying mono and disialylated core 1 structures produced in CHO cells was used as comparison.

When combining the OVA–PPM conjugate with AbiSCO®-100, a significantly more rapid and stronger antibody response was seen compared to when OVA was conjugated to PSGL-1/mIgG_{2b} with mono and disialylated core 1 structure or given alone with Abisco®-100. IgG\textsubscript{1} and IgG\textsubscript{2b} were the predominant IgG subclasses indicating a mixed $T_{H1}/T_{H2}$ type of immune response. IgG\textsubscript{2a} antibody titers were only detectable in the group that received OVA conjugated to PPM + AbiSCO®-100.
which would indicate a stronger influence of T<sub>h1</sub> response than the other groups. The T<sub>h1</sub> response is further evidenced by the generation of a strong OVA-specific CTL response and increased numbers of IFN-γ and IL-2 producing splenocytes seen in the group immunized with the OVA conjugated to PPM + AbiSCO®-100. These results show that the mannose structures in the fusion protein play a decisive role for inducing a broad immune response with a rapid and high antibody response including a strong CTL response. When comparing conjugated OVA with just mixing, conjugation of OVA to mannosylated PSGL-1/mIgG<sub>2b</sub> appear to give a more rapid, stronger and broader antibody responses than when OVA is mixed with mannosylated PSGL-1/mIgG<sub>2b</sub>. The fact that a physical link between OVA and PPM is required for inducing the strong and broad immune responses observed is consistent with the ability of PPM to target OVA to endocytic mannose specific receptors of professional antigen presenting cells. T cell presentation mediated by the mannose receptor has for example been shown to be a hundred fold more efficient compared to when the antigen was internalized by fluid phase pinocytosis [42, 162]. Other studies have also observed that physical association between a PAMP and an antigen is important for enhancing antigen specific immune responses, possibly because the PAMP can target the antigen directly to the activation signal of the APC in the periphery [24].

The results in this study demonstrate that PPM function as a vaccine adjuvant in mice, and appear to skew the immune responses toward T<sub>h1</sub> type. Considering the results in Paper III, it is likely that the adjuvantic effect is achieved by targeting the antigen to mannose specific receptors of the immune system.

3.4 Conclusions
The mannose structures of PPM were shown to be crucial for its high affinity binding to a wide selection of mannose specific receptors, and also for its ability to improve both humoral and cellular OVA-specific immune responses in mice. Physical linkage between OVA and PPM were found to be important for the enhanced immune responses observed. Collectively these results strongly suggest that the adjuvant effect mediated by PPM resides in its ability to deliver the antigen to mannose specific
receptors of APCs. *P. pastoris* produced mucin-like recombinant proteins hence comprise an interesting group of glycoproteins which potentially could be used with a wide repertoire of antigens to improve the potency of sub-unit vaccines in general.

Removing the N-glycans of PPM did not reduce its binding affinity to the receptors demonstrating that the O-glycans alone are capable of mediating high affinity binding to all receptors. In contrast to the N-glycans, the O-glycans of PPM did not appear to be significantly influenced by expression pH, suggesting that a more robust targeting molecule could be designed by excluding the sites for N-linked glycosylation in PPM.

PPM was stably expressed at pH 3.5 reaching volumetric productivities of around 90 mg L⁻¹ after 50 hours of induction which can be considered a good expression level, particularly when considering the large (250 kDa) and complex PPM protein. Based on the secretion dynamics of a GFP tagged PPM and comparison with expression of PSGL-1/mIgG₂b without GFP, the major limitation to secretion of PPM appeared to be a secretory bottle-neck post ER which caused increasing amounts of intracellular aggregation during induction. This result suggests that a significant amount of production capacity remains to be harnessed by strain development.

However, with the present strain the rate of protein synthesis had to be restricted to prevent aggregation. This restriction was implemented by controlling the methanol feed rates, which in turn extended expression and secretion times, and resulted in higher final protein titres. Methanol fed-batch with specific growth rates in the range of 0.005-0.01 h⁻¹ should allow expression and secretion for at least 40-50 hours generating volumetric productivities around 90 mg L⁻¹.
References


Paper I
Secretion and expression dynamics of a GFP-tagged mucin-like fusion protein in high cell density *P. pastoris* bioreactor cultivations.

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Abstract

The *Pichia pastoris* produced mucin-like fusion protein PSGL-1/mIgG2b is a potential vaccine adjuvant which motivates its larger scale production. Understanding secretory bottle-necks is important in process development and to study the secretion process of PSGL-1/mIgG2b, its C-terminal was fused to the green fluorescent protein and expressed in bioreactor cultures of *Pichia pastoris* (Mut*¹*). The fusion protein was partially found to accumulate intracellularly, where the major fraction was insoluble, fluorescent full-length protein. The high degree of glycosylation of the aggregated fusion protein indicated a secretory bottle-neck in the golgi-system. This result was consistent with low endoplasmic reticulum folding stress as quantified by RTqPCR of the unfolded protein response controlled KAR2 gene during cultivations. Reduction of intracellular recombinant protein synthesis rate by using lower feed rates of methanol enhanced productivity from 8 to 18 mg L⁻¹, demonstrating the importance of tuning the synthesis rate with secretory bottle-necks to maintain secretion.

**Keywords:** green fluorescent protein, GFP, GFP-fusion, KAR2, *Pichia pastoris*, secretion, yeast, unfolded protein response
Introduction

Considerable evidence supports that targeting mannose specific receptors of the immune system may improve both CD4\(^+\) and CD8\(^+\) T cell responses [1,2,3,4]. These receptors have shown to improve antigen up-take and T-cell presentation as well as influencing toll-like receptors signalling pathways to shape adaptive immunity [5,6,7,8]. Recombinant mucin-like proteins are interesting scaffold molecules for multivalent presentation of biologically active carbohydrates [9]. The homodimeric mucin-like fusion protein consisting of the extracellular part of P-selectin glycoprotein ligand-1 and the Fc part of mouse Immunoglobulin G\(_{2b}\) (PSGL-1/mIgG\(_{2b}\)) has the theoretical capacity to carry 106 O-glycans and 6 N-glycans [9]. Expressed and secreted by *Pichia pastoris*, it is a highly mannosylated, 250 kDa homodimer where the two monomers are linked by a disulfide bridge [9,10]. The mannose structures of *P. pastoris* produced PSGL-1/mIgG\(_{2b}\) are capable of mediating high affinity binding to the mannose binding lectin, mannose receptor( CD206) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN or CD209) [10]. Hence, *P. pastoris* produced PSGL-1/mIgG\(_{2b}\) could potentially be used to target antigens to mannose specific receptors of the immune system and have an important function as a vaccine adjuvant.

The methylotrophic yeast *Pichia pastoris* is a robust microorganism for industrial cultivation and has been used to express and secrete several recombinant proteins in the g per litre range [11]. For large scale production of recombinant proteins using the *P. pastoris* system it is important to understand the secretion dynamics in relation to the cultivation strategy as intracellular accumulation of recombinant proteins destined for secretion have been observed in several cases [12,13]. A clear tracking of the accumulation, sub-cellular distribution and secretion processes is often missing. Frequently, the major limitations to secretion in recombinant yeast are signal sequence processing, membrane translocation and folding within
ER [12]. Some proteins have also been shown to be retained in the cell wall [14,15]. Another factor is the proteolytic degradation of the product in the media, which often hampers high cell density cultivations and may reduce yields [16,17]. Various strategies have been employed to try to improve secretion including the use of different promoters [12], gene copy number [12,18], secretion signals [19,20], overexpression of proteins involved in the folding and secretion processes [21,22], manipulation of genes involved in cell-wall biogenesis [23] and different cultivation techniques [24,25]. Although secretion may be improved by one or several of these types of actions, there is no general method that ensures enhanced secretion of an arbitrary recombinant protein.

The most frequently used promoter used with the P. pastoris expression system is the methanol inducible alcohol oxidase 1 (AOX1) promoter due to its strength and tight regulation [11,24]. Because methanol functions both as carbon source and inducer of recombinant protein production, controlling the media methanol concentration and methanol up-take rate during the production phase are critical [25,26]. High residual methanol concentrations may be toxic to the cells whereas too low levels may be insufficient for transcription of the AOX1 promoter [27,28,29]. Controlling the transcriptional rate of the recombinant gene by the methanol feed rate may hence provide a way to tune the intracellular synthesis rate with secretion rate.

Fusion proteins with the green fluorescent protein, GFP, are interesting when studying the secretion process of recombinant proteins as it often allows monitoring of the secretion dynamics and sub-cellular localization of the recombinant protein by means of confocal microscopy and fluorescence measurements [30,31]. If GFP is linked to the carboxy terminal of the protein of interest, it can also be used as a folding reporter, where GFP fluorescence
would indicate productive folding for the entire fusion protein [32]. In addition, if the same conditions which favour secretion of the GFP-fusion protein also apply for the recombinant protein without GFP, process development time could be greatly reduced due to the convenient analytical techniques associated with GFP fluorescence [33,34]. Previous studies indicated that PSGL-1/mIgG2b/GFP quickly aggregated intracellularly which appeared to cause a premature stop in secretion. Therefore the aim of the present study was to identify the secretory bottle-neck and to investigate if secretion could be improved by tuning the intracellular synthesis rate with the secretion rate by controlling the methanol feed rate. In addition, confocal microscopy was used to evaluate intracellular distribution of the GFP tagged fusion protein during secretion. To assess potential folding stress in the ER during induction, RTqPCR analyses of the unfolded protein response controlled gene KAR2 was done before and after induction. Because a clear tracking of accumulation, sub-cellular distribution and secretion processes of the recombinant protein is often missing, the ambition was also to clearly display the expression dynamics by comparing the intracellular soluble, insoluble and secreted PSGL-1/mIgG2b/GFP during three predefined exponential methanol feeding models.

Materials and Methods

Strains and plasmids

The P. pastoris expression strain GS115 (Mut+), chemicals for transformation and plasmids for P. pastoris chromosome integration were acquired from the EasySelect™ Pichia Expression Kit version G (Invitrogen, Carlsbad, USA). For propagation of plasmids, E. coli XL-1 Blue (Agilent Technologies, Palo Alto, CA, USA) was used. The GFPuv cDNA
sequence was obtained from the GFPuv vector pGFPuv (Clontech, Palo Alto, CA, USA). All restriction endonucleases for molecular cloning were acquired from New England Biolabs.

**Chemicals**

Yeast nitrogen base without amino acids was from EasySelect™ Pichia Expression Kit version G (Invitrogen). Peptone (CAS: 91079-38-8) and yeast extract (CAS: 8013-01-2) were from (Merck, Darmstadt, Germany). All other chemicals were acquired from Sigma Aldrich unless otherwise stated.

**Construction of recombinant plasmids**

*pPICZα/PSGL-1/mIgG2b/GFP*. The cDNA encoding PSGL-1/mIgG2b was PCR amplified from the PSGL-1/mIgG2b expression plasmid [35] using 5'-CGC GGG AAT TCC AGC TGT GGG ACA CCT GGG -3’ and 5’-GCG GGG TAC CTT TAC CCG GAG ACC GGG AG-3’ as forward and reverse primers, respectively, and subcloned into the multiple cloning site of the pPICZα vector (Invitrogen) by EcoRI and Kpn1 digestion. A stop codon was excluded in the reverse primer. The cDNA encoding GFPuv was PCR amplified from the pGFPuv vector (Clontech) using 5’-GCG GGG TAC CCC AAC GCC GAC ACC AAC GCC GAC AGG AGA ATT CAT GAG TAA AGG AGA AGA ACT TTT CAC TGG AGT TG-3’ and 5’-CGC GGG CGG CTT ATT TGT AGA GCT CAT CCA TGC CAT GTG TAA TC-3’ as forward and reverse primers, respectively, and subcloned into the pPICZα/PSGL-1/mIgG2b (no stop codon) expression plasmid by Kpn1 and NotI digestion. A linker with the amino acid sequence 5’-GTPTPTPTPTGFT-3’ was included in the forward primer (underlined) [36]. The sequence was confirmed by DNA sequencing.

**Plasmid integration and selection of high producing clones**
The vector pPICZa/PSGL-1/mIgG2b/GFP was amplified in *E. coli* XL-1 Blue using 25 μg mL⁻¹ Zeocin™ as selective agent. Following purification, the vectors were linearized by *Pme*I and transformed into *P. pastoris* GS115 cells according to standard procedures (Easy Comp™, Invitrogen). Tranformants of the Mut⁺ phenotype were subsequently identified by growing nine clones from each transformation on MDH agar (1.34% yeast nitrogen base without amino acids, 4 x 10⁻³% histidine, 4 x 10⁻⁴% biotin, 2% glucose, 1.5% agar) and on MMH agar (same as MDH but with 0.5% methanol instead of glucose) using *P. pastoris* GS115/Albumin (Mut⁺) and *P. pastoris* GS115/pPICZ/lacZ (Mut⁺) from Invitrogen as negative and positive control, respectively. To screen for high expressing clones, seven transformants of each transformation exhibiting the Mut⁺ phenotype were selected and inoculated in BMGY (1% yeast extract, 2% peptone, 1% v/v glycerol, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) and grown for 24 hours at 29 °C. This was followed by a 72 hours induction period in BMMY (0.5% methanol, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) at 29 °C. Cell culture supernatants were then harvested by centrifuging at 10,000 x g, 10 minutes at 4 °C and the concentration of PSGL-1/mIgG2b and PSGL-1/mIgG2b/GFP in the supernatants was determined by ELISA using a goat anti-mouse IgG (Fc) antibody described below. The clone secreting most PSGL-1/mIgG2b/GFP was used to make glycerol stocks to be used in subsequent bioreactor cultivations.

**Inoculum preparation**

An inoculum was prepared by inoculating 50 mL BMGY media in a 500 ml shake flask with *P. pastoris* GS115 encoding PSGL-1/mIgG2b/GFP. The culture was incubated at 180 rpm, 29 °C until OD₆₀₀ was approximately five.
Bioreactor medium and set-up

Duplicate runs were made for all cultivation conditions. All bioreactor cultivations were started in modified BMGY media (same as BMGY but with 40 g L\(^{-1}\) glycerol and 1 g L\(^{-1}\) histidine). In addition, 500 µL of antifoam (Sigma A6426) was used per cultivation and added prior to inoculation. An additional supplementation of 5 mL of 3.5% histidine was added before induction to ensure histidine was never limiting. Preliminary experiments indicated that two and three times this amount of histidine did not result in higher or lower growth rates or productivities during the time interval studied. All cultivations were conducted at 29 ºC and pH 6.0 in 1 L bioreactors (Biobundle, Applikon, the Netherlands). The pH was maintained by automatic addition of 15% NH\(_4\)OH. Dissolved oxygen tension (DOT), measured by a pO\(_2\) electrode, was kept at 35% of air saturation by adjusting agitation from 700-900 rpm and feed rate of air and pure oxygen as required. The pO\(_2\) electrode was calibrated before inoculation with air saturation at 29 ºC, pH 6.0, one atmosphere absolute pressure, aeration of 0.75 L min\(^{-1}\) and an agitation of 700 rpm.

Glycerol batch and fed-batch phases

The glycerol batch phase was initiated by transferring 45 mL of the inoculum to 620 mL bioreactor medium. After the initial glycerol was consumed, indicated by a DOT value of 100%, the cells were fed 45 mL of a 50 % w/v glycerol solution including 12 mL PTM\(_1\) (0.6% CuSO\(_4\)x5H\(_2\)O, 8 x 10\(^{-3}\)% NaI, 0.3% MnSO\(_4\)xH\(_2\)O, 0.02% NaMoO\(_4\)x2H\(_2\)O, 2 x 10\(^{-3}\)% Boric Acid, 0.05% CoCl\(_2\), 2% ZnCl\(_2\), 6.5% FeSO\(_4\)x7H\(_2\)O, 0.02% Biotin, 0.5% v/v H\(_2\)SO\(_4\)) salts per litre glycerol at a constant rate of 14 mL h\(^{-1}\) giving a biomass concentration of approximately 48 g dry cell weight per litre cultivation volume.

Induction phase
The methanol feed rate was incrementally changed 4 times per hour according to three pre-defined feeding strategies with the equation:

\[ F(t) = F_0 e^{\mu t} \]  

(1)

where \( F \) is the feed rate in mL h\(^{-1}\), \( F_0 \) is the initial feed rate in mL h\(^{-1}\), \( t \) is the time after induction in hours (h) and \( \mu \) is the exponent in h\(^{-1}\). Preliminary experiments indicated that secretion was favoured under low initial feed rates and therefore the following conditions were investigated in this study: 1. \( F_0 = 1.4 \) mL h\(^{-1}\) L\(^{-1}\), \( \mu = 0.04 \) h\(^{-1}\); 2. \( F_0 = 1.4 \) mL h\(^{-1}\) L\(^{-1}\), \( \mu = 0.09 \) h\(^{-1}\); 3. \( F_0 = 3.5 \) mL h\(^{-1}\) L\(^{-1}\), \( \mu = 0.12 \) h\(^{-1}\). These feeding models are termed “Low”, “Medium” and “High” in the text. For the run with \( F_0 = 3.5 \) mL h\(^{-1}\) L\(^{-1}\), this feed rate was constant during the initial 2.5 hours of induction to let the cells adapt to methanol. The initial feed rate was increased to a maximum of 8 mL h\(^{-1}\) L\(^{-1}\), or until the cells maximum methanol consumption rate was reached, after which it was adjusted to the cells methanol consumption rate. To ensure that the methanol concentrations were growth limiting, the responsiveness of the cells to a complete stop in the methanol feed was measured. A 20% increase in DOT in less than 30 seconds was assumed to represent growth limiting concentrations of methanol. Off-line analysis of the supernatant with HPLC (described below) confirmed that residual methanol concentration was less than 100 mg L\(^{-1}\) during the time interval of the study, and was hence never toxic to the cells.

**Methanol concentration determinations**

An HPLC system equipped with a Series 200 refractive index (RI) detector and a BioRad Aminex HPX87-H column was used for analysis of methanol. The column was kept at 65°C and 5 mM H\(_2\)SO\(_4\) was used as the mobile phase. A flow rate of 0.6 mL min\(^{-1}\) was applied.
reference curve was prepared using methanol concentrations ranging from 100 to 10000 mg L\(^{-1}\). Bioreactor samples were centrifuged 10000 x g, 10 minute at 4 °C and sterile filtered using 0.22 μm PES filter prior to injection. Peak areas from the chromatograms were subsequently evaluated on the basis of the reference curve.

**Sample preparation for secreted and intracellular soluble and insoluble PSGL-1/mIgG2b/GFP**

To assay for secreted recombinant protein, cell culture supernatants were collected by centrifugation at 10,000 x g for 10 minutes at 4 °C. Cells and cell debris were removed by sterile filtration through 0.22 μm polyether sulfone (PES) filter (Nalgene) after which the supernatants were treated with 500 μL protease inhibitor cocktail (Sigma P8215) per litre supernatant and stored at -20 °C. To estimate the intracellular soluble PSGL-1/mIgG2b/GFP, expressed as micrograms per g dry cell weight (μg gDCW\(^{-1}\)), 250 μL bioreactor sample was centrifuged at 10000 x g for 10 minutes at 4 °C, the supernatant discarded and the pellet washed once with 1 mL of sterile, ice cold phosphate buffered saline (100 mM M phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). Subsequently the cell pellet was suspended in ice cold yeast breaking buffer (50 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5% v/v glycerol, pH 7.4) up to a volume of 500 μL. Acid washed glass beads (Sigma 8772) were added to a total volume of 1000 μL and the samples were vortexed 12 x 1 minute with 1 minute cooling on ice in-between. The samples were then centrifuged at 15000 x g for 30 minutes at 4 °C and the clear lysate was assayed for soluble PSGL-1/mIgG2b/GFP with ELISA described below. Estimation of specific soluble PSGL-1/mIgG2b/GFP concentration could then be made by dividing the total amount of extracted protein with the total dry biomass of the 250 μL bioreactor sample. To estimate the amount of specific insoluble PSGL-1/mIgG2b/GFP, expressed as μg gDCW\(^{-1}\), the remaining
cell debris was washed once by resuspending it in 1000 μL of YBB and transferring it to a fresh centrifuge tube and centrifuging at 10000 × g for 10 minutes at 4 °C. After washing, the pellet was resuspended in extraction buffer up to 1000 μL (YBB with 2% w/v of sodium dodecyl sulphate, SDS) and incubated in a water bath at 37 °C for 2 hours. Finally, the samples were centrifuged at 15000 × g for 30 minutes at 4 °C and the clear supernatant was assayed for insoluble PSGL-1/mIgG2b/GFP.

**Fluorescence measurements**

All fluorescence measurements were made directly on cell pellets in ordinary, transparent 1.5 mL microcentrifuge tubes of polypropylene (Plastibrand 780500). Hence the fluorescence data represent the relative specific fluorescence, i.e relative per cell fluorescence. Measurements were made on pellets from 1500 μL bioreactor samples, washed once in ice cold PBS and then centrifuged at 10000 × g for 10 minutes at 4 °C. To ensure that the microcentrifuge tubes were positioned exactly the same for each measurement an in-house positioning device was constructed. The fluorescence measurements were made with an USB2000-FLG spectrometer (Ocean Optics), a back-scattering probe R200-7-UV-VIS (Ocean Optics), and a PX-2 light source (Ocean Optics) in pulsed mode. An integration time of 1500 ms was used at all times. To shape the excitation wavelength with peak intensity at 395 nm, the linear variable filter LVH-HL (Ocean Optics) was used. Excitation light was always checked against a reference spectrum before sample measurements to ensure that fluorescence intensity differences were not due to different excitation. Entire spectra from the samples were collected and emission intensity at 508 nm was recorded (counts s⁻¹). The OOIBase32 operating software (Ocean Optics) was used to acquire data.

**ELISA**
The intra- and extracellular concentrations of recombinant fusion protein were determined by a two-antibody sandwich ELISA method as previously described Liu(2005)[37]. Briefly, 96-well ELISA plates (Corning) were coated with an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody (Sigma) at a concentration of 10 μg mL⁻¹. The plate was blocked with 1% BSA in PBS, which was also used for dilution of fusion protein as well as the second antibody (peroxidase-conjugated, anti-mouse IgG(Fc) antibody; Sigma). All incubations lasted for 2 hours. Between and after incubations the plates were washed with PBS containing 0.5% (v/v) Tween 20. Bound peroxidase-conjugated antibody was visualized with 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB, Sigma). The reaction was stopped by the addition of 2 M H₂SO₄ and the absorbance at 450 nm was recorded. The fusion protein concentration was estimated using a dilution series of purified mouse IgG₂b (Serotec) in blocking buffer as an internal standard.

**Western Blotting**

The recombinant proteins were analyzed by Western blotting. SDS-PAGE was run under non-reducing and reducing conditions using 4-12% gradient Bis-Tris gels and MES buffer (Invitrogen). For reducing conditions NuPAGE® Sample Reducing Agent was used according to manufacturer’s instructions (Invitrogen). Western blot membranes were probed with a mouse anti-human CD162 (BD Pharmingen) at a dilution of 1:500. The secondary antibody was a goat anti-mouse IgG-AP-conjugated antibody (Calbiochem) at a dilution of 1:5,000. Bound antibodies were visualized by colorimetric detection using His-Tag AP western reagents according to manufacturer’s instructions (Novagen).

**Biomass concentration**
Biomass concentration, $X$, expressed as gram dry cell weight (DCW) per litre ($g_{\text{DCW}} L^{-1}$), was determined by centrifuging 5 mL of cell suspension at 3,000 $x$ $g$ for 4 minutes at 4 ºC and then washing the cells with two volumes of distilled water. The cells were resuspended in distilled water, transferred to a pre-weighed aluminium dish and dried at 90 ºC until constant weight.

**Calculation of specific productivity**

The specific productivity was taken as:

$$ q_p = \frac{1}{X_{tot}} \frac{dP_{tot}}{dt}, $$

where $X_{tot}$ is the total dry biomass in g L$^{-1}$, $P_{tot}$ is the total PSGL-1/mIgG2b/GFP in the media in mg and $t$ is induction time in (h). Regression curves ($p=0.05$) were fitted to the secretion data points. To calculate the specific productivity at the sampling time points the derivatives of the regression models were used in combination with $X_{tot}$ for respective sample point.

**Confocal microscopy**

Glass slides, 76x26x1mm, (VWR) were prepared by etching two 9.5 mm circles with a diamond pen and then transferring 30 $\mu$L of 1% polyethyleneimine, PEI, (Sigma) to each circle, incubating 5 minutes and removing excess PEI by aspiration. Slides were then stored in a moist chamber until use. Cells taken from the bioreactor (equivalent to approximately 4 mg dry cell weight) at the different time points were immediately fixed in 4% formaldehyde for 15 minutes at 37 ºC, washed two times in 3 mL sterile filtered PBS and then adhered to the PEI areas of the glass slides. The cells were allowed to settle for 30 minutes after which the
slides were rinsed twice in PBS and allowed to air dry and put in a moist chamber. The cover glasses were subsequently mounted with ProLong Antifade Kit (Molecular Probes) according to manufacturer’s instructions. Epifluorescence images were taken using a Zeiss Axioimager Z1 system with AxioVision software, using a 63xplan-APOCHROMAT objective, at appropriate excitation and emission wavelengths. A *P. pastoris* GS115 strain not carrying the recombinant gene was used as control.

**Quantitative PCR**

RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Paisley, UK) and subjected to DNaseI (Ambion, Austin, Tx, USA) treatment to exclude genomic DNA contaminants. To generate cDNA from 1 mg RNA templates, the iScript cDNA synthesis kit (Bio-Rad laboratories, Hercules, CA, USA) was used. RNA was degraded by adding 2 μL 2.5M NaOH to each 20 μL reaction, followed by incubation at 42 °C for 10 min. Upon neutralization using 5 μL 1M HCl, the cDNA was diluted to 100 μL, diluted another 10 times and used for quantitative PCR using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Each 25 μL reaction mixture consisted of 5 μL template, 12.5 μL SYBR Green PCR master mix and 0.2 μM of each primer. For all samples, controls were made, where either template or superscript was omitted during cDNA synthesis. All reactions were performed in triplicate on independent RNA preparations from different time points taken from two bioreactor cultivations using the “high” methanol feeding model, with a 7900HT Sequence Detection System (Applied Biosystems) using the Sequence Detection System software and the program: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Samples were normalized against the *actin gene* [38] and compared to the KAR2 gene in *P. pastoris*. Results were analysed using the delta delta Ct method of analysis and converted to relative expression ratio (2^{-ΔΔCt}) for
statistical analysis [39]. Sample points taken from the glycerol batch phase, where no recombinant protein expression occurs, was used as a reference for calculating up- or down regulation of the KAR2 gene in response to induction. Primer sequences to the actin and KAR2 genes were designed according to [38].

### Results and Discussion

*Western blot confirms secretion of PSGL-1/mIgG2b/GFP but suggests incomplete prepro peptide processing and a secretion bottle-neck in the golgi*

Western blot analysis of the culture supernatants indicated that *P. pastoris* secreted two anti-PSGL-1-antibody reactive protein species, approximately 300-350 kDa in size under non-reducing conditions (Figure 1). Bands of lower molecular weight were also found and probably represent degradation products, monomers or differently glycosylated species. The double band at 300-350 kDa correlates with dimeric PSGL-1/mIgG2b/GFP which should have a theoretical molecular weight of approximately 310 kDa depending on glycosylation. We have previously shown that PSGL-1/mIgG2b is expressed as an extensively O-glycosylated 250 kDa dimer which also carries N-glycans [10]. Since there are two GFP molecules with a molecular weight of 27 kDa [40] on each dimeric PSGL-1/mIgG2b/GFP and two linkers, the total molecular weight of dimeric PSGL-1/mIgG2b/GFP would hence be around 310 kDa.
Figure 1. Representative Western blot analyses of supernatant and cell extract containing the soluble or insoluble fractions of PSGL-1/mIgG2b/GFP. Membranes were probed by anti-PSGL-1 antibodies.

The Western blot of intracellular protein revealed a difference in molecular weight between the soluble and insoluble fractions comparable with the molecular weight difference between the two bands of the double band observed for the secreted PSGL-1/mIgG2b/GFP (Figure 1). This suggests that the double band at 300-350 kDa observed for the secreted PSGL-1/mIgG2b/GFP is not a result of proteolytic clipping in the media, or different glycosylation, but rather concomitant secretion of PSGL-1/mIgG2b/GFP with differently processed signal peptide. The alpha factor secretion signal used to direct secretion of PSGL-1/mIgG2b/GFP consists of a 19 amino acid pre signal, a 66 amino acid pro signal and a 4 amino acid spacer (two Glu-Ala repeats) [11]. The pro signal also carries three sites for N-glycosylation. Incomplete processing of the pre pro peptide has been demonstrated for GFP-fusion proteins expressed by S. cerevisiae [41]. Under normal conditions the pre peptide is proteolytically removed during translocation by signal peptidase, whereas the pro peptide remains bound through the secretory pathway until it is eventually removed in the trans golgi by the endogenous Kex2p protease [11]. In addition, two Glu-Ala repeats following the Kex2p
cleavage site are removed by the Ste13 gene product exposing the native N-terminal end of the recombinant protein [11]. The difference in molecular weight between the intracellular insoluble and soluble PSGL-1/mIgG2b/GFP, and between the bands in the double band of the secreted PSGL-1/mIgG2b/GFP, is about 10-30 kDa. This result would be consistent with the theoretical molecular weight of 7.7 kDa for the propeptide plus two Glu-Ala repeats, assuming an average amino acid molecular weight of 110 Da, and no N-linked glycosylation. N-glycosylation of the propeptide would result in higher molecular weight. Too high protein synthesis rates might have saturated the Kex2p proteases resulting in incomplete processing of the propeptide leading to intracellular aggregation but also to some secretion of the incompletely processed form. Variation in the N-terminal is frequently observed with recombinant proteins secreted by P. pastoris [11]. Alternatively and/or in addition, the difference in molecular weight could be related to different glycosylation. Different glycosylation of the same protein core has been observed to result in distinct bands of different molecular weight [42].

Western blot analysis of intracellular soluble and insoluble PSGL-1/mIgG2b/GFP revealed two anti-PSGL-1-reactive protein species approximately 300-350 and 170 kDa in sizes under non-reducing conditions (Figure 1). The two bands correlate with the dimeric and monomeric form of PSGL-1/mIgG2b/GFP. The same band pattern was observed for the soluble and insoluble fractions indicating the insoluble form also consists of intact full length dimeric PSGL-1/mIgG2b/GFP or its corresponding monomer. The calculated molecular weight of non-glycosylated dimeric PSGL-1/mIgG2b/GFP is 174 kDa. SDS PAGE was run also under reducing conditions to break the disulfide bond linking the two monomers in the dimer to determine if the 170 kDa bands are the monomer and not an underglycosylated dimeric species. Subsequent western blot revealed a single 150 kDa species confirming that it is the
monomer (data not shown). The molecular weight of 300-350 kDa for the dimer or 170 kDa for the monomer is indicative of extensive O- and/or N-glycosylation, suggesting that PSGL-1/mIgG2b/GFP has progressed into golgi before aggregation. Hence the bottle-neck to secretion of PSGL-1/mIgG2b/GFP appears to be located within the golgi and not within the ER.

**RTqPCR of the KAR2 gene suggest low ER folding stress**

The high translational rates as imparted on the cell by the AOX1 promoter may overwhelm the folding capacity of the ER resulting in accumulation of missfolded proteins [11]. The unfolded protein response, UPR, serves to restore homeostasis in the ER following accumulation of missfolded proteins [43,44]. Briefly, increased levels of missfolded proteins in the ER induce transcription of genes encoding ER-resident chaperones, including KAR2, which improves the folding capacity in ER [44]. UPR may also reduce secretion by global suppression of protein synthesis and translocation into ER as well as enhanced degradation of missfolded proteins through a process termed ER associated degradation [44,45,46]. Accumulation of recombinant proteins in *P. pastoris* has been shown to induce the unfolded protein response in other studies [12]. Transcriptional activation of the KAR2 gene in response to induction may be analyzed by quantifying the KAR2 mRNA by RTqPCR [38]. RTqPCR analysis of the KAR2 gene was made for the cultivations with the highest methanol feed rates to determine if the UPR was differentially induced before and some hours after induction. Although the data suggests a slight up-regulation, there is no major up- or down regulations of the KAR2 gene following methanol induction (Figure 2). This would be in accordance with low folding problems in ER. Alternatively, it may also indicate that *P. pastoris* has a more constitutive active UPR, which have been noted in other studies as well [38].
Figure 2. Relative expression levels of the KAR2 gene. Data is based on duplicate runs but at different time points. Actin was used as control. The glycerol batch phase was used as a reference point when calculating up- or down regulation in response to induction as the AOX1 promoter should be fully repressed and hence no recombinant protein production should occur.
**Figure 3.** Methanol feeding, growth and expression dynamics.
Fluorescence of cell pellets shows a gradual accumulation of insoluble recombinant protein, suggesting a congestion of the secretory pathway

The fluorescence intensity at 508 nm of the cell pellets correlated approximately with the amount of specific insoluble PSGL-1/mIgG2b/GFP which indicates a high degree of productive folding despite aggregation, figure 3A and 3B. The specific amount of soluble PSGL-1/mIgG2b/GFP was consistently lower than the specific insoluble amounts, figure 3B and 3F. The increase in intracellular soluble PSGL-1/mIgG2b/GFP also occurred before increase in insoluble PSGL-1/mIgG2b/GFP, which is in accordance with a gradual congestion of the secretory pathway (compare figure 3B and 3F). Accumulation of insoluble PSGL-1/mIgG2b/GFP started at the time of induction whereas the soluble form accumulated already during the glycerol fed-batch phase which is consistent with the derepression of the AOX1 promoter during the fed-batch phase.

Intracellular fusion protein accumulation disturbs secretion and metabolic activity of the cells

It was generally observed that higher methanol feed rates, or methanol up-take rates, resulted in faster intracellular accumulation of both soluble and insoluble recombinant protein which is in accordance with a higher induction of the AOX1 promoter (Figure 3A, 3B, 3E and 3F). This confirms that the protein synthesis rate can be controlled to some extent by the methanol feed rates. In contrast, the total amount of secreted fusion protein decreased with higher methanol up-take rates (compare Figure 3C and 3E). Although the specific rate of secretion was initially higher under higher methanol up-take rates it declined faster and reached zero already after 12 hours induction (Figure 3G). At a certain time, indicated both by a peak in intracellular GFP fluorescence and intracellular PSGL-1/mIgG2b/GFP concentration, intracellular accumulation stopped and declined (Figure 3A, 3B and 3E). This peak level also appeared to coincide with a decrease in secretion rate and methanol consumption rate of the
cells (compare Figure 3A, 3B, 3C and 3D). To ensure that the methanol concentration in the media was growth limiting and that the cells were consuming all methanol supplied by the feed, regular controls of cells’ responsiveness to a complete stop in the methanol feed were made [25]. The “responsiveness” was taken to be the time for the dissolved oxygen tension to increase 20% after a complete stop in the methanol feed. Under conditions before the GFP fluorescence peaked, the responsiveness was 5-10 seconds ensuring that the methanol concentrations were very low. Off-line analysis of the culture supernatant showed that the methanol concentrations under these conditions were outside the detection limit of the HPLC instrument implying methanol concentrations < 100 mg L\(^{-1}\). After the GFP fluorescence peaked, the response time gradually increased to more than a minute suggesting slight accumulation of methanol and the methanol feeds were reduced. Off-line analysis of the culture supernatant showed that the methanol concentrations still were below 500 mg L\(^{-1}\) during the remainder of the cultivation. Hence the methanol should never be toxic to the cells. Because of the decreasing productivities the cultivations were terminated at these time points (data not shown). In spite of the reduced methanol consumption rate and the high intracellular concentrations of the fusion protein, cell growth appeared not to be immediately influenced (Figure 3H). The fact that the reduction in methanol consumption rate and secretion were not correlated with the level of methanol feed rate or specific methanol up-take rate (data not shown) but rather with the peak levels of intracellular GFP fluorescence, suggests that the intracellular fusion protein concentrations were decisive for the reduced metabolic activity and secretion capabilities of the cells. According to the data the intracellular concentrations of insoluble fusion protein tolerable by the cells, ranged between 300 to 545 μg gDCW\(^{-1}\). It appeared as the cells tolerated lower concentrations of fusion protein when the rate of accumulation was higher. This might be related to the fact that the cells had a shorter time to adapt to the increasing amounts of recombinant protein. Control experiments where the P.
*pastoris* GS115 strain without the recombinant gene was grown on methanol according to the high methanol feeding regime never displayed any reduction in methanol consumption rate during 48 hours induction (Figure 3D). This would be consistent with the idea that it is the intracellular concentration of fusion protein which disturbs the metabolic activity and secretion capabilities of the cells.

Postponing accumulation of PSGL-1/mIgG2b/GFP by using lower methanol feed rates was favourable for maintaining secretion. Although the protein synthesis rate could be controlled to some degree by using lower methanol feed rates, the AOX1 promoter seemed too strong for the expression and secretion of PSGL-1/mIgG2b/GFP unless solving the suggested Golgi bottle-neck. If Kex2p processing of the secretion signal propeptide is limiting secretion, a possibility to resolve this would possibly be to overexpress Kex2p concomitantly with fusion protein expression. Despite the secretion bottle-necks the productivities were amongst the highest reported for GFP fusion proteins secreted by yeast reaching media concentrations up to 18 mg L⁻¹.

*Confocal microscopy shows that intracellular PSGL-1/mIgG2b/GFP fluorescence is gradually sorted to an isolated area close to the cell wall*

The distribution and intensity of GFP fluorescence inside the cells appeared to change with time (Figure 4). Because ELISA quantification of intracellular PSGL-1/mIgG2b/GFP correlated rather well with the observed fluorescence intensity and western blot of intracellular PSGL-1/mIgG2b/GFP indicated full length protein, the GFP fluorescence observed with confocal microscopy is likely to reflect the localisation of full length PSGL-1/mIgG2b/GFP. After 13 hours of induction, with the low methanol feed rate \( F_0 = 1.4 \text{ ml h}^{-1}\text{L}^{-1} \) and \( \mu = 0.04 \text{ h}^{-1} \), GFP fluorescence has generally lower specific intensity and is more evenly
distributed within the cells compared to 42 hours of induction (Figure 4). After 42 hours of induction the specific fluorescence is higher in intensity and polarized to one small isolated area close to the cell wall. Other studies with *P. pastoris* have shown that smaller elements of transitional ER and Golgi structures may fuse with one another to generate larger structures [47]. Hence, in this study it could be speculated that the concentration of fusion protein in elements from the secretory machinery, possibly the golgi, become progressively higher and that these elements fuse with each other with time to form the larger spot seen in the confocal image (Figure 4). In addition, not all cells appeared to express PSGL-1/mIgG2b/GFP. Genetic instability has been observed in *P. pastoris* and has been suggested to be caused by excess methanol [48]; however, the confocal pictures were taken with cells that had been cultivated with low methanol concentration (<100 mg L⁻¹) suggesting other mechanisms.

![Confocal microscopy pictures](image)

*Figure 4.* Confocal microscopy pictures of cells at different time points of induction using the low methanol feed rate model (Fᵢ=1.4 ml h⁻¹ L⁻¹ and μ=0.04 h⁻¹). GFP fluorescence is gradually sorted into large single spots located close to the cell wall which appears to have high concentration of fusion protein. Note that different exposure times have been used due the high GFP fluorescence at 42 hours induction.
Conclusion

In this study the secretion dynamics of a 310-350 kDa GFP tagged mucin-like protein, PSGL-1/mIgG2b/GFP, under three predefined exponential methanol feeding regimes in high cell density bioreactor cultivations of *P. pastoris* GS115 (Mut+) were investigated. It was shown that the protein synthesis rate could be controlled by the methanol feed rates and that secretion could be maintained for longer times when using lower methanol feed rates. Tuning expression rates with secretion rates is therefore important for improving productivity. Secretion of PSGL-1/mIgG2b/GFP was favoured using low methanol feed rates and would probably benefit from using even lower induction of the AOX1 promoter; however, lower methanol feed rates would lead to starvation of the cells and viability loss. Mixed sorbitol/methanol feeds could be used to supply complementary energy when using very low feed rates of methanol. Alternatively mixed glycerol/methanol feeds could be used to suppress induction of the AOX1 promoter sufficiently to prevent intracellular aggregation. Using weaker promoters, including the PEX8 or YPT1 [11] promoters would perhaps be more attractive, particularly from a bioprocess engineering point of view, as subsequent cultivation becomes simpler compared to using mixed feeds. GFP allowed for a fast analysis of intracellular aggregation and should be a valuable tool during process development for PSGL-1/mIgG2b/GFP and also for studying secretion processes in general. The difference in molecular sizes of intracellular soluble and insoluble fractions of the fusion protein suggested incomplete processing of the alfa factor prepro signal which may have promoted the aggregation. The high molecular weight of the intracellularly accumulated fusion protein implies extensive N- and O-glycosylation which indicates that the secretion bottle-neck was located in the Golgi. This would also be consistent with the low folding stress as measured by the relative expression of the UPR controlled KAR2 gene before and after induction. The productivities were among the highest reported for GFP fusion proteins expressed in yeast,
reaching media concentrations up to 18 mg L\(^{-1}\) which confirms the high secretion capacity of
\textit{P. pastoris}.

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References


Paper II
Influences of pH on cellular growth, secretion and glycosylation of the mucin-like protein P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b in high cell density cultures of *Pichia pastoris*

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Abstract

_Pichia pastoris_-produced P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b, PSGL-1/mIgG2b, carries mainly O-glycans and has been shown to be a promising mannose receptor-targeting molecule for recombinant vaccines. This study investigates how pH influences productivity and growth kinetics as well as glycosylation and degradation of PSGL-1/mIgG2b, in bioreactor cultivations of _P. pastoris_. PSGL-1/mIgG2b was expressed and secreted at pH 3.5 and 6.0 using the same methanol feeding scheme. Cultivation pH did not influence product degradation rate. However, expression at pH 3.5 resulted in a less truncated version of the fusion protein, as indicated by one major band on the Western blot in contrast to three bands observed for the fusion protein expressed at pH 6.0. The overall volumetric productivity was 1.5 times higher at pH 6.0, reaching 122±9 mg L⁻¹ (p=0.05) after 48 hours induction. The specific growth rate during induction was two times higher at pH 6.0. Gel shift assay with PNGase F indicated pH dependent differences in N-glycosylation whereas differences in O-glycosylation appeared less pronounced. LC-MS analysis of the released N-glycans revealed structures with masses consistent with Man₅₋₁₅GlcNAc₂ and Man₉₋₁₂GlcNAc₂ for the fusion protein expressed at pH 6.0 and 3.5 respectively, suggesting that the low pH adversely influences processing in the Golgi. Because a fraction of the fusion protein expressed at pH 3.5 was of higher molecular weight than that expressed at pH 6.0, expression at pH 3.5 may potentiate a higher N-glycosylation site occupancy. LC-MS analysis of the O-glycans revealed structures with masses consistent with Hex₂-₉ oligosaccharides, some of which appeared phosphorylated. In conclusion, induction at pH 6.0 favoured cellular growth and productivity at the expense of protein homogeneity.

**Keywords:** glycoprotein, glycosylation, N-glycans, O-glycans, _Pichia pastoris_, pH, secretion
Introduction

Antigens glycosylated by *P. pastoris* have shown to elicit improved antigen-specific immune responses compared to their non-glycosylated counterparts, possibly by targeting mannose specific receptors of the immune system which potentiates antigen up-take and T-cell presentation [1,2]. Mannosylation of antigens, or of conjugation partners to antigens, may therefore provide means to produce more efficient vaccines and vaccine adjuvants [3,4]. The mucin-like homodimeric fusion protein consisting of the extracellular part of P-selectin glycoprotein ligand-1 (PSGL-1) and the Fc part of mouse immunoglobulin G2b (mIgG2b), carries 6 sites for N-glycosylation and 106 sites for O-linked glycosylation [5]. We have previously shown that the O-glycans of *P. pastoris*-produced PSGL-1/mIgG2b are capable of mediating high affinity binding to certain mannose specific receptors of the immune system [6]. Conjugating PSGL-1/mIgG2b to recombinant antigens could hence be expected to improve the antigen specific immune response and generate more potent vaccine compositions. This motivates larger scale production of PSGL-1/mIgG2b using *P. pastoris*.

The development of industrial bioprocesses for the production of therapeutic glycoproteins can be a particular challenge because of the requirement for a defined glycoform. Several process parameters such as cultivation pH, temperature, nitrogen source, etcetera have been shown to influence the glycoform profile of recombinant glycoproteins and even their biological activity. Therefore, the regulatory requirements for approval of a therapeutic glycoprotein are complex and include structural characterization of its glycans [7,8,9].

High cell-density bioreactor cultivations of *P. pastoris* have been demonstrated to be an efficient platform for expression of several secreted recombinant proteins [10,11]. However, proteolysis of the recombinant product can be a severe problem hampering the yields
P. pastoris is capable of growing under a wide pH range, which may present the opportunity to counteract proteolytic activity in the medium and competing microorganisms. For example, by decreasing pH from 5.0 to 4.0 in P. pastoris high cell density bioreactor cultivations, the fraction full-length fusion protein consisting of the cellulose-binding domain of Neocallimastix patricarum and lipase B from Candida Antarctica was increased from 40 to 90% [14]. However, the influences of pH on glycosylation of recombinant glycoproteins secreted by P. pastoris have not been studied in great detail. Lowering of cultivation pH has shown to have great impact on glycosylation of a recombinant glycoprotein secreted by S. cerevisiae. When a mutant form of lysozyme carrying one site for N-linked glycosylation was expressed and secreted to the medium by S. cerevisiae, a large polymannose (Man310GlcNAc2) was predominantly formed at pH 4 while a smaller Man14GlcNAc2 chain was found at pH 3 [9]. For the production of mannosylated vaccines designed to target mannose specific receptors such differences could have a profound impact on the biological activity of the vaccine. For the development of larger scale production of PSGL-1/mIgG2b it is hence important to evaluate the glycosylation profile under different cultivation parameters.

Compared to N-glycans, there are few studies done on O-glycans of glycoproteins derived from P. pastoris, both in terms of their structures and how different cultivation conditions influences their synthesis [16,17]. Although P. pastoris derived O-glycans generally are described as short (Man4-6) 1,2-linked mannose polymers, recent findings reveal greater diversity with O-glycans containing up to nine hexoses, including also α1,3- and β1,2 linkages, and some of the hexoses being phosphorylated [6,17]. Removal of the N-glycans of P. pastoris-produced PSGL-1/mIgG2b do not influence its high affinity binding to certain mannose specific receptors of the immune system, indicating that the O-glycans are important...
for mediating high affinity binding to the receptors [6]. Because preliminary observations indicated that PSGL-1/mIgG2b was less fragmented when expressed at pH lower than 6.0, the aim of this study was to investigate if the glycosylation of PSGL-1/mIgG2b was influenced by the cultivation pH. PSGL-1/mIgG2b was expressed at pH 3.5 and pH 6.0. Specific growth rate, productivity and proteolytic degradation were also investigated to evaluate potential benefits at either pH.
Materials and Methods

Generation of PSGL-1/mIgG2b producing clones

Isolation of PSGL-1/mIgG2b-expressing GS115 (Mut+) clones was done as previously described [6].

Bioreactor cultivation

Three bioreactor cultivations at pH 3.5 and three at pH 6.0 were done in random order. An inoculum was prepared by inoculating 100 mL BMGY media, pH 6.0, in a 1 L shake flask with P. pastoris GS115 Mut+ encoding PSGL-1/mIgG2b. The culture was incubated at 180 rpm, 29 ºC until OD600 was approximately four. The bioreactor was inoculated with 90 mL of the prepared inoculum. The bioreactor cultivations were conducted according to a methanol limited fed-batch strategy (MLFB) (Pichia Fermentation Process Guidelines Version B 053002, Invitrogen) at 29 ºC in 3 L bioreactors (Biobundle, Applikon, the Netherlands) with an initial volume of 2.0 L BMGY supplemented with 4% w/v glycerol and 1 g L⁻¹ histidine. The glycerol batch phase was conducted at pH 6.0 for all runs whereas the pH set point was changed to 3.5 during the glycerol fed batch phase for those runs induced at pH 3.5. The pH was maintained by automatic addition of 15% NH₄OH. During the glycerol batch phase the dissolved oxygen tension (DOT), measured by a pO₂ electrode, was kept at 30% oxygen saturation by keeping the agitation fixed at 835 rpm and varying the air and supply of pure oxygen as needed. The pO₂ electrode was calibrated before inoculation with air saturation at 29 ºC, pH 6.0, one atmosphere absolute pressure, air flow rate of 2.0 L min⁻¹ and an agitation of 835 rpm. After the initial glycerol was consumed, indicated by a DOT value of 100%, the cells were fed 150 g 50 % w/w glycerol at a rate of 35 g h⁻¹. The DOT was maintained at 30%. Following a 10 minutes starvation period a 100% methanol feed with 12 mL PTM₁ salts
(0.6% CuSO₄·5H₂O, 8 x 10⁻³% NaI, 0.3% MnSO₄·H₂O, 0.02% NaMoO₄·2H₂O, 2 x 10⁻³% Boric Acid, 0.05% CoCl₂, 2% ZnCl₂, 6.5% FeSO₄·7H₂O, 0.02% Biotin, 0.5% v/v H₂SO₄) per litre methanol was initiated at a rate of 0.9 mL h⁻¹ L⁻¹. The methanol feed was subsequently linearly increased according to F(t) = 0.425t+0.9 for 4 h and left constant at 2.6 mL h⁻¹ L⁻¹ for 14 h after which the feed was linearly increased according to F(t) = 0.13t+2.6 for 8 hours and left at 3.65 mL h⁻¹ L⁻¹ for the remainder of the cultivations. F(t) = methanol feed rate (mL h⁻¹ L⁻¹), t = induction time (h). Before induction, 5 mL L⁻¹ of a 3.5 % L-histidinemonohydrochloride (Merck, KGaA) solution was injected to the bioreactor to ensure that histidine never was limiting.

**Calculation of specific productivity**

The specific rate of product formation, qₚ, was calculated according to (Eq. 1)

\[
q_p = \frac{1}{X^T} \frac{dP^T}{dt}
\]

where \(X^T\) is the total dry cell weight in gram (gDCW) calculated as \(X^*V\) where \(X\) is the biomass concentration (gDCW L⁻¹) and \(V\) is culture volume (L), \(t\) is induction time in hours (h) and \(P^T\) is total amount of fusion protein in the culture supernatant in milligrams (mg) calculated as \(P^*V_{sup}\), where \(V_{sup}\) is the culture supernatant volume (L) and \(P\) is the fusion protein concentration in the culture supernatant (mg L⁻¹). The volume of the culture supernatant was calculated according to (Eq. 2) [18].

\[
V_{sup} = V - aVX
\]

where \(a\) is the cell volume per gram dry cell weight (0.0032 L gDCW⁻¹) [18]. The bioreactor cultivations were done in triplicates for each pH setting. Quadratic regression models
(p=0.05) were fitted to the total fusion protein data points. To calculate the specific rate of secretion at the sampling time points, expressed as mg h\(^{-1}\) gDCW\(^{-1}\), the derivative of the regression models were used in combination with the total dry biomass and time for respective sampling point.

**Calculation of volumetric productivity**

Volumetric productivity, \(q_v\) (mg L\(^{-1}\)) was calculated according to (Eq. 3).

\[
q_v = \frac{P}{V}
\]  

(3)

**Calculation of specific growth rate**

The specific growth rate, \(\mu\) (h\(^{-1}\)), was calculated according to (Eq. 4).

\[
\mu = \frac{1}{X^2} \frac{dX}{dt}  
\]  

(4)

To determine the specific growth rate, (Eq. 4) was integrated and the slope of the resulting lines was taken as the specific growth rates. Because the cells adapt to methanol during the 2-4 hours of induction, specific growth rates were calculated after the adaptation period.

**Calculation of apparent yield coefficients**

The apparent growth yield coefficients on methanol were calculated after the methanol adaptation period (4 hours), whereas the apparent product yield coefficients were calculated from the start of induction as the fusion protein expression does not require an adaptation period. The apparent yield coefficients were calculated according to

\[
Y_{ji} = \frac{X_j}{X_i} 
\]
where i refers to biomass or product formed, and j refers to the amount of substrate required for the particular amount of biomass or product formed.

**Sample preparation**

To assay for secreted recombinant protein, cell culture supernatants were collected by centrifugation at 10,000g for 10 min at 4 °C. Cells and cell debris were removed by sterile filtration through 0.22 μm polyether sulfone (PES) filter (Nalgene) after which the supernatants were treated with 500 μL protease inhibitor cocktail (Sigma-Aldrich, P8215) per litre supernatant and stored at -20 °C until assay.

**ELISA**

The media concentration of recombinant fusion protein was determined by a two-antibody sandwich ELISA method as previously described [19]. Briefly, 96-well ELISA plates (Corning Inc.) were coated with an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody (Sigma) at a concentration of 10 μg mL⁻¹. The plate was blocked with 1% BSA in PBS which was also used for dilution of fusion protein as well as the second antibody (peroxidise conjugated, anti-mouse IgG(Fc) antibody; Sigma-Aldrich, 1:1000. All incubations lasted for 2 hours. Between and after incubations the plates were washed with PBS containing 0.5% (v/v) Tween 20. Bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB, Sigma-Aldrich). The reaction was stopped by the addition of 2 M H₂SO₄ and the absorbance at 450 nm was recorded. The fusion protein concentration was estimated using a dilution series of purified mouse IgG₂b (AbD Serotec, Oxford, UK) in blocking buffer as an internal standard.

**Western Blotting**
The recombinant protein was analyzed by Western blotting. SDS-PAGE was run under non-reducing conditions using 4-12% Bis-Tris gradient gels and MES buffer (Invitrogen). Precision protein standards (Hi-Mark, Invitrogen) were applied as reference for protein molecular weight determination. Western blot membranes were probed with a mouse anti-human CD162 (BD Pharmingen) at a dilution of 1:500. The secondary antibody was a goat anti-mouse IgG-AP-conjugated antibody (Calbiochem) at a dilution of 1:5000. Bound antibodies were visualized by colorimetric detection using His-Tag AP Western reagents according to manufacturer’s instructions (Novagen).

**Cell concentration determinations**

Dry cellular weight (DCW), was determined by centrifuging 5 mL of cell suspension at 3,000g for 4 minutes at 4 °C and then washing the cells with two volumes of distilled water. The cells were finally resuspended in distilled water, transferred to a preweighed aluminium dish and dried at 90 °C until constant weight.

**Fusion protein degradation analysis**

A two-level, three factor factorial design was used to analyse the fusion protein degradation in cell free culture supernatant after 48 hours of induction. The three factors were induction pH (3.5 or 6.0), temperature (4 °C or 29 °C) and addition of protease inhibitor cocktail (“with” or “without”). At 48 hours of induction, a sample of the culture supernatant from each induction condition was prepared as described in “Sample preparation” except that this sample was not supplemented with protease inhibitor cocktail. From this sample, five 100 μL aliquots were transferred to 1 mL microcentrifuge tubes. All samples were initially stored at – 20 °C for about 96 hours before the analysis commenced. Subsequently, four aliquots were thawed whereof two aliquots were supplemented with protease inhibitor cocktail (50 μL mL⁻¹)
(Sigma). One aliquot with and without protease inhibitor cocktail were incubated at 4 °C, and one aliquot with and without protease inhibitor cocktail were incubated at 29 °C, for 93 hours respectively. One aliquot was used as control and left frozen until screening. After incubation all samples were put on ice and the samples without protease inhibitor cocktail were supplemented with 50 μL mL⁻¹ of the cocktail. The control was thawed, supplemented with protease inhibitor cocktail (50 μL mL⁻¹) and ELISA was performed to determine the fusion protein concentrations. One replicate of the screen was conducted and the results were analysed with the statistical software MODDE 9.0 (Umetrics), at a 5% significance level. Significant factors were displayed by a coefficient diagram.

Purification of PSGL-1/mIgG₂b

Culture supernatants were centrifuged 10,000g, 10 minutes at 4 °C and sterile filtered with 0.22μm polyether sulfone (PES) filter (Nalgene) before loading onto a pre-equilibrated protein A sepharose column (Biovision, 6501-25). The column was washed with 10 column volumes (CV) of washing solution (0.05 M Sodium Borate, 0.15 M Sodium Chloride, pH 8.0) and elution of recombinant fusion protein was achieved using 5 CV of 0.1 M sodium citrate, pH 3.0. After elution, selected fractions were pooled, neutralized with 300 μL per mL of 1 M Tris-HCl, pH 9.0 and then dialyzed extensively (12-14 kD cut-off, Spectra/Por 132700) against MilliQ water at 4 °C. After dialysis, the samples were frozen, lyophilized and stored at -80 °C before further purification.

Lyophilized samples were dissolved to approximately 10 mg mL⁻¹ in gel filtration buffer (0.1 M sodium phosphate pH 7.2, 0.5 M sodium chloride). Gel filtration of the PSGL-1/mIgG₂b was carried out on a pre-equilibrated HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). Typically, 1 mL sample was applied to the gel filtration column and eluted with a flow rate of 0.5 mL min⁻¹. Eluted fractions were kept at 4 °C until pooling was done on
the basis of Western blot analysis. Pooled fractions were then dialyzed as above, frozen, lyophilized and stored at -80 °C.

**N-glycan release for gel-shift assay**

Approximately 200 μg purified lyophilized PSGL-1/mIgG2b expressed at either pH was dissolved in 300 μL phosphate buffered saline, pH 7.5. Subsequently, 45 μL of the protein solution was incubated with 2.5 μL 2% SDS solution at 70 °C for 10 minutes. Following brief cooling on ice, 2.5 μL 15 v/v % Triton X-100 was added. After mixing, 2.0 μL of PNG:ase F solution (Sigma-Aldrich G5166) was added and the mixture incubated at 37 °C for 16 hours in a water bath. Release was evaluated with Western blotting as described above.

**Release of N-glycans for LC-MS analysis**

The fusion proteins were blotted to PVDF membrane and stained by staining solution (0.8% (w/v) Direct blue 71 (DB71), 10% acetic acid and 40% ethanol in MilliQ water) for 20 minutes with shaking. The blots were subsequently destained by destaining solution (10% acetic acid, 40% ethanol in MilliQ water) for 5 x 2 minutes with shaking followed by drying in air. Spots visualized by DB71 were cut out of the PVDF membrane and transferred to separate microtitre plate wells with 100 μL of 1% (w/v) PVP solution (Polyvinyl Pyrolidone 40 Solution; 1 % (w/v) in 50% methanol) in 50% (v/v) methanol to wet and block the membrane. Samples were incubated for 5 minutes with shaking. Spots were subsequently washed 3 x 5 minutes with MilliQ water and incubated for 15 minutes with 5 μL PNG:ase F (0.5 U μL−1) (Roche, Mannheim, Germany) followed by addition of 10 μL MilliQ water and incubation overnight at 37 °C. The blots were sonicated for 5 minutes and the liquid containing the released oligosaccharides transferred to a new microtitre plate. This was repeated once with 20 μL MilliQ water and the liquids containing the released
oligosaccharides were combined. Any remaining N-glycans were subsequently released by \(\beta\)-elimination on the PVDF membranes as described below.

**Chemical release of O-linked glycans from purified PSGL-1/mIgG\(_2\)\(_b\)**

The O-glycans were released by reductive \(\beta\)-elimination as described (Carlstedt1993). Briefly, lyophilized PSGL-1/mIgG\(_2\)\(_b\) proteins were dissolved in water to 1 mg mL\(^{-1}\) concentration. The solution was mixed with an equal volume of 1M NaBH\(_4\), 0.1 M KOH and incubated over night at 60° C. The reaction was stopped with addition of acetic acid. The released glycans were desalted with AG50WX8 cation exchange beads (Bio-Rad) packed on top of a C18 Zip-tip (Millipore, Bedford, MA). Samples were eluted with water and dried in a speed-vac centrifuge. Borate complexes were removed with 1% acetic acid in methanol and subsequent vacuum centrifugation (5x). The dried glycans were dissolved in water for analysis on LC-MS.

**Mass spectrometry**

Liquid chromatography-mass spectrometry (LC-MS) was performed with a graphitized carbon column coupled to an LTQ LC/MS\(^2\) ion trap instrument (Thermo Fischer Scientific). The column was packed with 5\(\mu\)m Hypercarb particles (Thermo, Hypersil-Keystone, Runcorn, UK). The glycans were released with a gradient from 0-80% acetonitrile in 8mM NH\(_4\)HCO\(_3\) buffer and detected in negative mode by full scan (\(m/z\) 380-2000) followed by MS\(^2\) scans of the most intense ions.
Results and Discussion

The fusion protein expressed at pH 3.5 is less truncated than the protein expressed at pH 6.0. Western blot analysis of the culture supernatant from all cultivations revealed an approximately 260 kDa anti-PSGL-1-reactive protein under non-reducing conditions which is in accordance with its dimeric form [19] (Figure 1). Bands of lower molecular weight were also observed and probably represent degradation products, differently processed forms and the monomeric form of PSGL-1/mIgG2b. Expression at pH 3.5 was clearly more stable resulting in only one major band on the Western blot compared to at least three major bands at pH 6.0 (Figure 1). This staining pattern observed on the Western blots was the same from 2 hours of induction until harvest at 48 hours induction, suggesting different intracellular processing of the fusion protein rather than different proteolytic degradation or fragmentation in the cultivation medium.

The degradation rate of the fusion protein at 29 °C and 4 °C was assessed in cell free culture supernatant from 48 hours of induction, with and without a protease inhibitor cocktail designed to reduce the activity of several types of proteases including serine-, cysteine-, aspartic- and metalloproteases. It was clear that the fusion protein was subjected to degradation in the culture supernatant. However, neither the cultivation pH nor the protease inhibitor cocktail significantly (p=0.05) influenced fusion protein degradation rate. The only significant factor was temperature (Figure 2). At 4 °C 84±22 % (p=0.05) of the initial fusion protein amount was left after 96 hours incubation in culture supernatant, whereas at 29 °C 54±2 % of the initial amount of the fusion protein remained after the same time. Hence, temperature-limited processes [20] might be particularly suitable to improve yields of PSGL-1/mIgG2b.
Induction pH appear to influence glycosylation

When run on the same gel and when using anti-PSGL-1 antibodies to probe the membrane, the band of the fusion protein expressed at pH 3.5 were consistently weaker and more dispersed than the bands of the species expressed at pH 6.0 even though the same amounts were applied (Figure 3, controls). It should be noted that the PSGL-1 part of the fusion protein is the major glycan carrying part of the fusion protein, with 106 sites for O-glycosylation and 6 sites for N-glycosylation for the dimer [5,21]. The different appearances of the Western blot bands suggest that there is a difference in the glycosylation of PSGL-1/mIgG2b depending on expression pH. The more dispersed bands observed for the fusion protein expressed at pH 3.5 could be a result of a more heterogeneous glycosylation, both in terms of glycosylation site occupancy and the particular N- and O-linked oligosaccharides being expressed, compared to the fusion protein produced at pH 6.0. Hence at pH 6.0, the individual molecular species of the fusion protein may have a more homogenous glycosylation profile and exhibit a more focused migration pattern on the SDS-PAGE gel. In contrast, at pH 3.5 the individual fusion protein species may not have been uniformly glycosylated and as a result exhibit a more dispersed migration pattern with weaker bands on the Western blot.

In contrast to the anti-PSGL-1-antibodies, anti-mIgG2b-antibodies did not give different band strengths on Western blots (data not shown). This would be consistent with a change of the glycans of the PSGL-1 part which may interfere with antibody binding. Such interference could be expected from higher glycosylation site occupancy, charged groups or larger glycans.

In summary, expression at pH 6.0 appears to give a more homogenous glycosylation profile but a more truncated version of the fusion protein compared to expression at pH 3.5.
Low pH appear to adversely influence N-glycosylation

Purified PSGL-1/mIgG2b expressed at pH 3.5 and 6.0 were subjected to PNGase:F treatment to remove the N-glycans (Figure 3). Western blot of PSGL-1/mIgG2b with released N-glycans revealed major bands at approximately 160 kDa which are likely to correspond to the dimer. The bands at approximately 110 kDa may correspond to the monomeric form of PSGL-1/mIgG2b or degradation products still carrying the PSGL-1 epitope. The molecular weight shift from 260 kDa to approximately 160 kDa for the dimer suggests that the N-glycans comprised a large part of the molecular weight of PSGL-1/mIgG2b regardless of which pH they were expressed in. The theoretical molecular weight of the fusion protein dimer without glycosylation is about 117 kDa suggesting that there is a substantial amount of O-glycans present as well. When the N-glycans were removed from PSGL-1/mIgG2b expressed at pH 3.5 and 6.0, the bands on the Western blot became considerably more similar (Figure 3). Notably, the weak and dispersed band of the fusion protein expressed at pH 3.5 became stronger and less dispersed after PNG:ase F treatment. Therefore, it appears as if the N-glycosylation of PSGL-1/mIgG2b expressed at pH 3.5 is different compared to that of PSGL-1/mIgG2b expressed at pH 6.0.

When the N-glycans of the fusion proteins were investigated by LC-MS it was found that the fusion protein expressed at pH 6.0 carried N-glycans with higher mass, compared to the fusion protein expressed at pH 3.5, (Table 1). Whereas the fusion protein expressed at pH 6.0 carried structures consistent with N-linked high mannose oligosaccharides with 5 up to 15 mannose residues (Man5,15GlcNAc2), the fusion protein expressed at pH 3.5 carried structures consistent with N-linked oligosaccharides with up to only 12 mannose residues (Man12GlcNAc2). Structures consistent with Man12,8GlcNAc2 oligosaccharides were not detected on
the fusion protein expressed at either pH. Briefly, N-glycosylation in yeast, like in mammalian cell lines, starts in the ER by the en bloc addition of a \( \text{Glc}_3\text{Man}_9\text{GlcNAc}_2 \) oligosaccharide to the nascent polypeptide [22,23]. This oligosaccharide is subsequently trimmed down to \( \text{Man}_9\text{GlcNAc}_2 \) before the glycoprotein is released to the Golgi where further processing of the oligosaccharide occurs, typically by the addition of more mannose residues by various mannosyl transferases [22,23]. Because the observed differences in N-glycosylation of the fusion protein expressed at pH 3.5 and pH 6.0 were outside the \( \text{Man}_9\text{GlcNAc}_2 \) core, it appears as if low cultivation pH adversely influences processing in the Golgi. This would be in accordance with other studies on N-glycosylation in \( S. \text{cerevisiae} \), which reported that low cultivation pH influenced processing of N-glycans in the Golgi, generating shorter N-glycans compared to expression at higher pH [9]. Adverse effects of pH on processing in Golgi could also contribute to a more heterogeneous glycosylation which possibly would be reflected as more dispersed Western blot bands.

However, the major structures under both expression conditions had masses consistent with \( \text{Man}_{10,11}\text{GlcNAc}_2 \) oligosaccharides. This is consistent with \( P. \text{pastoris} \) N-glycosylation in general which typically comprises high mannose \( \text{Man}_{8,14}\text{GlcNAc}_2 \) oligosaccharides [16]. No phosphorylation of the N-glycans was detected at either pH. Western blot of the purified fusion protein (Figure 3) indicated that a fraction of the fusion protein expressed at pH 3.5 is of higher molecular weight compared to that expressed at pH 6.0. Because LC-MS analysis indicated that the fusion protein expressed at pH 6.0 carried N-glycans with higher molecular weight compared to the fusion protein expressed at pH 3.5, expression at pH 3.5 might therefore generate species with a higher N-glycosylation site occupancy. This would also imply that the low pH might influence the processes involved in the addition of the \( \text{Glc}_3\text{Man}_9\text{GlcNAc}_2 \) oligosaccharide in the ER.
As mentioned previously, N-glycosylation in yeasts starts in the ER with the \textit{en bloc} addition of Glc$_1$Man$_9$GlcNAc$_2$ which subsequently is trimmed down to Man$_8$GlcNAc$_2$ before leaving ER. The Man$_8$GlcNAc$_2$ then becomes the substrate for a \( \alpha 1,6 \) mannosyl transferase (Och1p) which commits the glycans to be substrates for further mannose additions throughout the Golgi [22,23,24]. It is therefore somewhat surprising to see structures consistent with masses of Man$_5$-6GlcNAc$_2$ oligosaccharides. This suggests that some of the Man$_8$GlcNAc$_2$ oligosaccharides of the fusion protein released from the ER may not be accessible for the mannosyltransferases in the Golgi which transfer mannoses outside the Man$_8$GlcNAc$_2$ core, and that these oligosaccharides subsequently might be subjected to mannosidase activity in the Golgi. However, yeasts have not been reported to remove more mannoses than to the Man$_8$GlcNAc$_2$ structure [23].

An additional factor that may contribute to dispersion of the Western blot bands is incomplete processing of the secretion signal. The \textit{S. cerevisiae} alpha mating factor prepro signal was used in this study to direct the fusion protein through the secretory pathway. Generally the pre region is cleaved off in the ER whereas the pro region, which carries three sites for N-glycosylation, is cleaved off in the Golgi by the KEX2 gene product [10,25]. There is also a Glu-Ala repeat (GluAlaGluAla) closest to the N-terminal of the recombinant protein which is removed by the STE13 gene product in the Golgi. Secreted recombinant proteins using the alpha prepro secretion signal do often display heterogeneous N-termini [10]. However this is mainly because of incomplete processing of the Glu-Ala repeat and hence there should not be any additional N-glycosylation sites from the pro region remaining [10,25]. On the other hand there are implications that some recombinant proteins may be secreted with a small fraction still carrying the pro region [26]. If the properties of the Golgi are distorted because of the low
pH, the secretion signal processing might be incomplete leaving some recombinant molecules with the pro region and the chance of six additional N-glycans per PSGL-1/mIgG2b dimer. However, in general full processing of the prepro leader is required for secretion [10,26].

*Induction pH does not appear to influence O-glycosylation*

LC-MS analysis of the O-glycans released from PSGL1/mIgG2b expressed at pH 6.0 and 3.5 did not reveal any apparent differences as demonstrated by the base peak chromatograms (Figure 4). The individual mass spectra did not reveal any apparent differences either. This would be consistent with the idea that the major differences observed on the Western blots are related to differences in the N-glycosylation. However, different O-glycosylation site occupancy cannot be ruled out based on the LC-MS data. If the relative abundance of the different O-glycans is the same regardless of induction pH, but the O-glycan site occupancy is higher on the fusion protein expressed at pH 3.5, the same LC-MS data would result. On the other hand, considering the similar molecular size of the fusion protein expressed at either pH after N-glycan release, and that the same types of O-glycans were detected on the fusion proteins, it appears as if the O-glycosylation site occupancy is similar regardless of expression pH. All spectra, each summed from 2.5 to 16 minutes of the chromatogram, displayed eight peaks corresponding to fragments with masses explained by the alditols of Hex2,3 structures, with Hex5,6 being most predominant (Figure 5). These findings correspond well with our previous analyses of O-glycans of PSGL-1/mIgG2b expressed at pH 3.5 [6]. In addition, some peaks corresponded to fragments with masses explained by phosphorylated alditols, with phosphorylated Hex2,3 (P-Hex2,3) being the most common (m/z 423.08 and 585.17) (Figure 5). This is in accordance with O-glycans derived from glycoproteins expressed in *P. pastoris* in other studies [17]. Phosphorylated structures up to Hex7 could be detected although at very
low abundance (data not shown). MS\(^2\) also indicated that both the non-reducing and reducing end of the Hex\(_2\) and Hex\(_3\) structures might have been phosphorylated (data not shown).

*Low pH impairs growth*

The same methanol feeding protocol was used for all cultivations so that the influence of pH could be clearly observed. Because the cells need to adapt to methanol during 2-4 hours the growth data was calculated based on data after 4 hours of induction (Table 2). Although the same amount of methanol was consumed in all cultivations (~237g after about 48 hours induction) the biomass generation was lower at pH 3.5 compared to at pH 6.0 during the interval studied (Figure 6A). The apparent growth yields on methanol after 48 hours of induction were approximately 0.08 g g\(^{-1}\) at pH 3.5 and 0.2 g g\(^{-1}\) at pH 6.0, which is consistent with a higher maintenance requirement at pH 3.5 than at pH 6.0. At pH 3.5, there was an approximately 10 hour lag period during induction at pH 3.5 where the biomass decreased somewhat. After this the cells displayed a constant specific growth rate at about 0.005 h\(^{-1}\) for the remainder of the cultivation (Figure 6B). At pH 6.0 the specific growth rate was approximately constant at 0.01 h\(^{-1}\).

*Induction pH does not impart major influences on specific productivities*

Because the fusion protein was truncated at pH 6.0, where one of the species may be the monomer, the volumetric productivities at pH 6.0 may be overestimated. For this reason “overall” volumetric productivities was used. By using reducing conditions the dimers could have been split into their monomeric forms and a more accurate comparison between the expressions could have been done. The overall volumetric productivities at pH 3.5 and 6.0 were approximately 80 and 122 mg per litre cultivation volume respectively, after about 48 hours induction (Table 1, Figure 6C). The apparent product yields on methanol after 48 hours
of induction at pH 3.5 and pH 6.0 were 0.7 mg g\(^{-1}\) and 1.1 mg g\(^{-1}\) respectively. The specific productivities decreased from the start of the induction (Figure 6D). Although the specific productivities were different between expression at pH 3.5 and 6.0, the difference was quite small. Hence, the higher total productivity at pH 6.0 appear to mainly results from a higher biomass generation, stressing the importance of growing a high biomass before pH change and induction. The specific productivities were also shown to decrease at a similar rate for both pH values. We have previously shown that intracellular aggregation of a GFP-tagged PSGL-1/mIgG\(_{2b}\) within the secretory pathway is likely to interfere with secretion (Manuscript under preparation). Gradual intracellular aggregation of PSGL-1/mIgG\(_{2b}\) could hence result in the gradual decrease in specific secretion. On the other hand, there are indications that the alcohol oxidase 1 promoter (P\(_{AOX1}\)) is subjected to catabolite repression which potentially could lead to decreasing transcriptional activity of the P\(_{AOX1}\) and hence decreasing rate of fusion protein secretion [11]. Reduced transcriptional activity of the P\(_{AOX1}\) could also be due to the decreasing specific methanol up-take rates due to increased biomass during constant feed of methanol.

Although growth and productivity data strongly favours expression at pH 6.0, there were also some disadvantages. After affinity chromatography, dialysis and lyophilisation of the fusion protein expressed at pH 3.5, SDS-PAGE and coomassie staining revealed one major band at the expected molecular weight. In contrast, the fusion protein expressed at pH 6.0 revealed four major bands after affinity chromatography. As a result a larger fraction of the fusion protein expressed at pH 6.0 had to be discarded during the gel filtration step, compared to the fusion protein expressed at pH 3.5.
Conclusion

The mucin-like fusion protein PSGL-1/mIgG2b was expressed and secreted in high cell density bioreactor cultivation of *P. pastoris* under pH 3.5 and 6.0, primarily to investigate the influence of pH on glycosylation but also on cellular growth, productivities and degradation of the fusion protein. It was clear that the induction pH influenced the character of the fusion protein. In general, expression at pH 6.0 appeared to give a more homogenous glycosylation profile but a more truncated version of the fusion protein compared to expression at pH 3.5. Gel shift assay with PNG:ase F suggested that N-glycosylation was different depending on the expression pH. LC-MS analysis of the released N-glycans revealed structures with masses consistent with high mannose Man$_{5,15}$GlcNAc$_2$ from the fusion protein expressed at pH 6.0 and Man$_{5,12}$GlcNAc$_2$ at pH 3.5, which indicated different processing in the Golgi. However, because a fraction of the fusion protein expressed at pH 3.5 was of higher molecular weight compared to that expressed at pH 6.0, it appears as if the N-glycosylation site occupancy might generally have been higher for expression at pH 3.5. The initial steps of N-glycosylation might therefore also have been influenced by the induction pH.

No apparent differences in the types of O-glycans, or their relative abundance on the fusion protein, under the different cultivation conditions were observed. Structures consistent with Man$_{2,9}$ oligosaccharides were detected where Man$_{5,6}$ were most predominant. There was also indication of phosphorylation, most notably phosphorylated Hex$_{2,3}$ structures. We have previously shown that the O-glycans alone are able to mediate high affinity to certain mannose specific receptors of the immune system. Removing the N-glycosylation sites of PSGL-1/mIgG2b could hence generate a simpler and more robust targeting molecule for mannose-specific receptors, as O-glycosylation in contrast to N-glycosylation does not appear to be influenced by expression pH between 3.5 and 6.0 as N-glycosylation.
Compared to expression at pH 3.5, the overall volumetric productivity was about 1.5 times higher at pH 6.0 and the specific growth was about two times higher. The specific productivities on the other hand were similar. Collectively these facts strongly favour production at pH 6.0. However, because of the truncation of the fusion protein expressed at pH 6.0 the productivities might have been overestimated. Moreover, cultivation pH did not influence fusion protein degradation rate. Finally, the potential influence of induction pH on the actual *in vivo* activity of PSGL-1/mIgG2b as a vaccine adjuvant would be decisive for the choice of expression pH.

**Acknowledgements**

This work was supported by the Research Council of Norrbotten, Innovationsbron and Längmanska företagarfonden. J.H. was supported by the Swedish Research Council (no. K2011-65X-3031-01-6) and the County Council of Västra Götaland (ALF).
References


Figure Legends

Figure 1.
Western blot of culture supernatant from expression at pH 3.5 and 6.0. Induction times are indicated. Membrane was probed with anti-human PSGL-1 antibodies.

Figure 2.
Coefficient plot of factors tested to be significant for degradation of the fusion protein in cell free culture supernatant. The factors are considered significant when the confidence intervals do not cross zero. Temp = temperature, Inh = protease inhibitor cocktail.

Figure 3.
Western blot of purified PSGL-1/mIgG2b expressed at pH 3.5 and 6.0, treated with PNGase F to remove the N-glycans. Membrane was probed with anti-PSGL-1-antibodies. Approximately 2 μg protein was loaded per well.

Figure 4.
Base peak chromatogram of the O-glycans released from PSGL-1/mIgG2b expressed at pH 3.5 and 6.0.

Figure 5.
Representative mass spectrogram of O-glycans released from PSGL-1/mIgG2b expressed at pH 3.5 and pH 6.0.
Figure 6A.
Biomass concentration verses induction time for growth on methanol at pH 3.5 and pH 6.0

Figure 6B.
Specific growth verses induction time for growth on methanol at pH 3.5 and pH 6.0.

Figure 6C.
Volumetric productivity verses induction time for expression at pH 3.5 and 6.0.

Figure 6D.
Specific productivity verses induction time for pH 3.5 and 6.0.
Figure 1

**pH 6.0**
- Control
- 48 h induction
- 30 h induction
- 20 h induction
- 10 h induction
- 2 h induction

**pH 3.5**
- Control
- 48 h induction
- 30 h induction
- 20 h induction
- 10 h induction
- 2 h induction

**Protein Bands**
- 220 kDa
- 97 kDa
- 66 kDa
- 45 kDa
Figure 3

260 kDa
160 kDa
110 kDa
80 kDa
60 kDa
50 kDa

Control pH 3.5
PNGase F pH 3.5
PNGase F pH 6.0
Control pH 6.0
<table>
<thead>
<tr>
<th>M/Z (charge)</th>
<th>Retention times (minutes)</th>
<th>Assigned High Mannose structure</th>
<th>Relative intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
<td>pH 3.5</td>
<td>pH 6.0</td>
</tr>
<tr>
<td>1233(^1)-</td>
<td>12.51</td>
<td>14.46</td>
<td>Man(_5)GlcNAc(_2)</td>
</tr>
<tr>
<td>1395(^1)-</td>
<td>13.25</td>
<td>15.17</td>
<td>Man(_5)GlcNAc(_2)</td>
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<td>1557(^1)-</td>
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<td>Man(_7)GlcNAc(_2)</td>
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<td>1719(^1)-</td>
<td>-</td>
<td>-</td>
<td>Man(_3)GlcNAc(_2)</td>
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<tr>
<td>1881(^1)-</td>
<td>17.88</td>
<td>17.85</td>
<td>Man(_3)GlcNAc(_2)</td>
</tr>
<tr>
<td>1021(^2)-</td>
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<td>17.69-18.85 (3 peaks)</td>
<td>Man(_1)GlcNAc(_2)</td>
</tr>
<tr>
<td>1102(^2)-</td>
<td>17.55-19.64 (4 peaks)</td>
<td>17.69-19.25 (3 peaks)</td>
<td>Man(_1)GlcNAc(_2)</td>
</tr>
<tr>
<td>1183(^2)-</td>
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<td>17.21-17.83 (2 peaks)</td>
<td>Man(_1)GlcNAc(_2)</td>
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<td>1264(^2)-</td>
<td>18.54</td>
<td>-</td>
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</tr>
<tr>
<td>1346(^2)-</td>
<td>18.33</td>
<td>-</td>
<td>Man(_1)GlcNAc(_2)</td>
</tr>
<tr>
<td>1425(^2)-</td>
<td>18.6</td>
<td>-</td>
<td>Man(_1)GlcNAc(_2)</td>
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Table 1
Table 1. Growth and productivity data. Growth data were based on induction time points after 4 hours until 48 hours of induction. The apparent growth- and product yields are based on methanol.

<table>
<thead>
<tr>
<th>pH</th>
<th>Specific growth rate (h(^{-1}))</th>
<th>Volumetric productivity(^a) (mg L(^{-1}))</th>
<th>Growth yield (gDCW g(^{-1}))</th>
<th>Product yield (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>0.01</td>
<td>122±9(^b)</td>
<td>0.19±0.03(^b)</td>
<td>1.12±0.07(^b)</td>
</tr>
<tr>
<td>3.5</td>
<td>0.005</td>
<td>80±7(^b)</td>
<td>0.08±0.03(^b)</td>
<td>0.71±0.05(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Volumetric productivity is the amount of fusion protein in culture supernatant per culture volume at 48 hours induction

\(^b\) Shows 95% confidence interval
Figure 6A

Total biomass (gDCW) vs. Induction time (h)

- pH 6.0
- pH 3.5
Figure 6B

- pH 6.0
- pH 3.5

Equation: 

- $y = 0.0004x - 0.061$ 
  - $R^2 = 0.973$
- $y = 0.0009x - 0.163$ 
  - $R^2 = 0.965$

Induction time (h)

$\ln$
Figure 6C

- pH 6.0
- pH 3.5
Paper III
Pichia pastoris-produced mucin-type fusion proteins with multivalent O-glycan substitution as targeting molecules for mannose-specific receptors of the immune system

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Mannose-binding proteins like the macrophage mannose receptor (MR), the dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and mannose-binding lectin (MBL) play crucial roles in both innate and adaptive immune responses. Attempts to elucidate their detailed ligand specificity have intensified in recent years because of their potential use as targeting receptors for vaccines (Keler et al. 2004) and other therapeutics (Irache et al. 2008).

The mannose receptor (MR or CD206, previously known as the macrophage MR, MMR), a C-type lectin abundantly expressed on macrophages and dendritic cells (DCs), binds glycoconjugates with high affinity and promotes endocytosis through several carbohydrate recognition domains (CRDs; Taylor et al. 1990, 1992). Different CRDs in conjunction with an extended conformation enable it to distinguish between endogenous and exogenous glycosylated molecules (Taylor and Drickamer 1993; Napper et al. 2001), thus participating in as diverse functions as immune responses to pathogens such as Mycobacterium tuberculosis (Schlesinger 1993) and the regulation of endogenous protein levels (e.g. tissue plasminogen activator; Otter et al. 1991). The MR is a type 1 transmembrane protein. The extracellular part consists of a cysteine-rich domain, a fibronectin type II repeat and eight CRDs (Taylor et al. 1990). Binding studies have shown that CRD4 is the only one of the eight CRDs capable of binding mannose on its own, albeit with low affinity. The affinity is considerably improved upon the involvement of CRD5 in the binding, while high-affinity binding of natural ligands requires the involvement of CRD4–8 (Taylor et al. 1992; Taylor and Drickamer 1993).

Another mannose-binding receptor is DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN).
whether the binding of these receptors is restricted to high-mannose structures like fungi that carry mannosylated glycans on their surfaces, have evolved to protect us against the invasion of pathogens. For example, on a bacterial or viral surface. Expression pattern than the MR as it is mainly expressed on immature and mature DCs with crucial functions in DC trafficking, T-cell interactions and pathogen recognition (Geijtenbeek et al. 2002). It is a type II membrane protein consisting of an extracellular part including a CRD and a neck domain, a transmembrane and a cytoplasmic region. The latter region contains several internalization motifs, allowing DC-SIGN to recycle between the cell surface and endosomes. DC-SIGN has been shown to preferentially bind to high-mannose structures (Feinberg et al. 2001; Mitchell et al. 2001). Binding of DC-SIGN to the internal pentasaccharide GlcNAcβ1,2Manα1,3(GlcNAcβ1,2Manα1,6)Man revealed that two monomers, cross-linked by the oligosaccharide, are involved in binding. The trimannose part of this pentasaccharide is present in both high-mannose and complex N-glycan structures but binding to the trimannose core in the latter is prevented by steric interference caused by different aminoceric linkages. On the cell surface, it appears that high-affinity binding of high-mannose structures is achieved by homotetramer formation via the neck region (Mitchell et al. 2001; Bernhard et al. 2004). Despite the seemingly high affinity for high-mannose structures, it has been shown that in the tetramer formation also multivalent monomeric mannose, as in, for example, Manα1,2-glycan-binding proteins (BSA), is bound with high affinity by DC-SIGN (Mitchell et al. 2001).

The mannose-binding lectin (MBL) is yet another C-type lectin which selectively binds mannose-containing oligosaccharides. MBL belongs to the collectins, a group of proteins comprised of a collagenous region, a neck region and a CRD (Geijtenbeek et al. 2002). It is a type II membrane protein (P. pastoris) carrying mainly N-acetylgalactosamine-based glycoconjugates. The tetramer structure of MBL has been shown to be a high-affinity binding to mannosylated glycans, which is likely to play a significant role in opsonophagocytic processes, in modulation of inflammation as well as in promotion of apoptosis (Donnet et al. 2006). As for DC-SIGN, the oligomerization of MBL seems important for its carbohydrate-binding properties. Most common are tri- and tetrameric homo-oligomers, referred to as MBL-I and MBL-II, where it has been shown that the tri- and tetramer structures have similar affinities for both mannose- and N-acetylgalactosamine-based glycoconjugates. The tetramer shows a considerably higher binding capacity and a lower dissociation rate when compared with the trimeric structure. On the other hand, no differences were seen between the two oligomers with regard to the complex formation with the MBL-associated serine protease-1, -2 and -3 (Teillet et al. 2005). Conformational studies have shown that the CRDs exhibit substantial flexibility in their orientation (Jensenius et al. 2009), which is likely to play a significant role for recognition of different ligands in a multivalent fashion, for example, on a bacterial or viral surface.

Even though the mannose-specific receptors to some extent have evolved to protect us against the invasion of pathogens like fungi that carry mannosylated glycans on their surfaces, their fine specificity toward more complex mannolyslated structures is incompletely characterized. It is also not clear whether the binding of these receptors is restricted to high-mannose type N-glycans or whether O-glycans carrying oligomannose structures can also be bound. To address this question, a P-selectin glycoprotein ligand-1 immunoglobulin fusion protein (PSGL-1/mIgG2b) carrying mainly O-glycans was expressed in P. pastoris and characterized with regards to its binding to the MR, DC-SIGN and MBL. An α1-acid glycoprotein immunoglobulin fusion protein (AGP/mIgG2b) was characterized by exoglycosidase cleavage, Western blotting, mass spectrometry (MS) and 1H-nuclear magnetic resonance (1H-NMR) spectroscopy.

**Results**

**PSGL-1/mIgG2b and AGP/mIgG2b expression in Pichia pastoris**

The biomass concentration (expressed as g dry cell weight per liter cultivation volume) was after glycerol batch and fed-batch phases 29.1 ± 0.8 g/L and 44.2 ± 1.0 g/L and grew to ~55 g/L for both strains after 40–60 h of induction (Figure 1). Although there was no significant difference (P = 0.05) in biomass generation between the PSGL-1/mIgG2b and AGP/mIgG2b expressing strains during the glycerol batch and glycerol fed-batch phases, there appeared to be a slight difference during the methanol fed-batch phases. According to the regression models fitted to the data with a 95% confidence interval, fusion protein concentration in the culture supernatant reached 92 ± 12 mg/L for PSGL-1/mIgG2b and 13.4 ± 2.5 mg/L for AGP/mIgG2b after 48 h induction (Figure 1). Fusion protein accumulation in the culture supernatant stopped after ~50 h of induction for both strains.

![Fig. 1. Bioreactor cultivation of P. pastoris clones. Variations in fusion protein concentration in culture supernatant and dry cellular mass during the methanol fed-batch (induction) phase. PSGL-1/mIgG2b concentration (crosses); AGP/mIgG2b concentration (filled squares); dry cellular mass for PSGL-1/mIgG2b expression (filled triangles); dry cellular mass for AGP/mIgG2b expression (filled diamonds). Quadratic regression models were fitted to the data using a significance level of 0.05. Statistical analysis was based on least duplicate cultivations.](image-url)
PSGL-1/mIgG2b and AGP/mIgG2b produced in P. pastoris are both expressed as dimers and are heavily mannosylated. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses of purified PSGL-1/mIgG2b produced in Chinese hamster ovary (CHO)-K1 (lane 1) and P. pastoris (lane 2) cells revealed a protein of 200–350 kDa under non-reducing conditions (Figure 2A–D). The apparent MW of AGP/mIgG2b was ∼120 kDa under non-reducing conditions, which is likely to represent the dimeric form of AGP/mIgG2b (Figure 2A–C and E, lane 3). All three proteins (lanes 1–3) bound anti-mouse IgG (Figure 2C), while as expected only the PSGL-1 fusion proteins bound anti-PSGL-1 (Figure 2D). The anti-PSGL-1 antibody bound considerably weaker to P. pastoris- (lane 2) when compared with CHO-produced (lane 1) PSGL-1/mIgG2b (Figure 2D). The AGP fusion protein was reactive with the anti-AGP antibody (Figure 2E, lane 3). Pichia pastoris-produced PSGL-1/mIgG2b (lane 2), AGP/mIgG2b (lane 3) and the bovine thyroglobulin control (lane 4; positive control for Con A) bound strongly to the concanavalin A (Con A) lectin (Figure 2F). Also PSGL-1/mIgG2b expressed in CHO-K1 cells reacted with the Con A lectin (Figure 2F, lane 1), albeit considerably weaker than the P. pastoris-produced mucin-type fusion protein. This binding is due to the presence of internal mannoses in the N-glycan pentasaccharide core as verified by abolished binding upon PNGase F cleavage (see section “PSGL-1/mIgG2b produced in P. pastoris carries mannosose-containing O-glycans”). In addition to bands corresponding to the major glycoforms of the PSGL-1 and AGP Ig fusion proteins, bands of lower and higher molecular weight were found.

PSGL-1/mIgG2b produced in P. pastoris carries mannosose-containing O-glycans

The binding of Con A to CHO-produced PSGL-1/mIgG2b was abolished by PNGase F-treatment (Figure 3A; compare lane 1 with lane 2), showing that CHO-produced PSGL-1/mIgG2b carries mannose residues only as part of its N-glycans. In contrast, Con A binding to P. pastoris-produced PSGL-1/mIgG2b resisted PNGase F-treatment (Figure 3B; compare lane 3 with lane 4) indicating that the carbohydrate determinants recognized by the Con A lectin were carried also on O-glycans. The complete shift in molecular size without any appearance of intermediary sized forms of PSGL-1/mIgG2b upon PNGase F-treatment suggests that the enzymatic release was complete (Figure 3C and D, compare lane 1 with lane 2, and lane 3 with lane 4). As noted above, anti-PSGL-1 binds very weakly to P. pastoris-produced PSGL-1/mIgG2b (Figure 3D, lane 3). However, binding is increased upon PNGase F-treatment (Figure 3D, lane 4).

![Fig. 2. SDS-PAGE and Western blot analysis of PSGL-1/mIgG2b and AGP/mIgG2b produced in P. pastoris cells. PSGL-1/mIgG2b purified from CHO cells (lane 1) or P. pastoris cells (lane 2), AGP/mIgG2b purified from P. pastoris (lane 3) and bovine thyroglobulin (lane 4; positive control for Con A). Five micrograms of protein were loaded per well and glycosylated proteins detected by Pro Q Emerald (A) followed by a detection of all proteins by Ruby (B). For Western blot analyses, 500 ng of protein were loaded per well and membranes probed with anti-mIgG(Fc) (C), anti-PSGL-1 (D), anti-AGP (E) and Con A (F).](image-url)
PNGase F-treated PSGL-1/mIgG2b (cf. lane 3 with lane 2, αfianalyzed by western blot in order to elucidate the linkage configuration of its oligomannose O-glycans. Treatment with an α1,2-mannosidase created a shift in the molecular weight of PNGase F-treated and purified PSGL-1/mIgG2b produced in P. pastoris was exposed to different mannosidases and then analyzed by western blot in order to elucidate the linkage configuration of its oligomannose O-glycans. Treatment with an α1,2-mannosidase created a shift in the molecular weight of PNGase F-treated PSGL-1/mIgG2b (cf. lane 3 with lane 2, Figure 4A). In contrast, α1,6-mannosidase treatment did not cause a shift in the PSGL-1/mIgG2b molecular weight (cf. lanes 2 and 4). Further, incubation with both an α1,2- and α1,6-mannosidase resulted in a molecular weight shift comparable with that seen following α1,2-mannosidase treatment alone (cf. lanes 3 and 5). This suggests that the O-glycans of P. pastoris-produced PSGL-1/mIgG2b do not contain α1,6-linked mannoses either terminally or subterminally. Enzymatic cleavage with a non-linkage-specific α-mannosidase resulted in a considerably larger molecular weight shift of the fusion protein compared with that when only an α1,2-mannosidase was used (Figure 4A, lane 6). This implies that a fraction of O-glycans on PSGL-1/mIgG2b produced in P. pastoris contains α-linked mannosides that are not α1,2- or α1,6-linked. PNGase F-treated PSGL-1/mIgG2b was also incubated with a β-mannosidase for different time periods and at different temperatures. Overnight digestion at 37°C resulted in total degradation of the fusion protein demonstrated by the lack of anti-PSGL-1 binding (not shown). Incubation at 50°C for 2 h resulted in some remaining anti-PSGL-1 binding (not shown), but most of the fusion protein was still degraded suggesting the presence of proteases in the β-mannosidase solution provided by the manufacturer. Protein degradation was avoided by addition of a protease inhibitor cocktail to the β-mannosidase incubation at 50°C for 2 h (Figure 4A, lane 7). No molecular weight shift was seen, indicating that terminal β-linked mannose is not present in the O-glycans of P. pastoris-produced PSGL-1/mIgG2b provided that the β-mannosidase was not also inhibited by the protease inhibitor cocktail (cf. lanes 2 and 7). Mannosidase-treated PSGL-1/mIgG2b was analyzed with regard to its binding to Con A (Figure 4B); a binding that verified the results seen in Figure 4A. However, additional bands of lower molecular weight and not detected by anti-PSGL-1 were seen (lanes 2–7). They most likely represent fusion protein break-down products devoid of the anti-PSGL-1 epitope.

indicating that the N-glycans are partly responsible for the weak recognition by the anti-PSGL-1 antibody.

O-glycans carried by P. pastoris-produced PSGL-1/mIgG2b contain mainly α-linked mannose residues

PNGase F-treated and purified PSGL-1/mIgG2b produced in P. pastoris was exposed to different mannosidases and then analyzed by western blot in order to elucidate the linkage configuration of its oligomannose O-glycans. Treatment with an α1,2-mannosidase created a shift in the molecular weight of PNGase F-treated PSGL-1/mIgG2b (cf. lane 3 with lane 2, Figure 4A). In contrast, α1,6-mannosidase treatment did not cause a shift in the PSGL-1/mIgG2b molecular weight (cf. lanes 2 and 4). Further, incubation with both an α1,2- and α1,6-mannosidase resulted in a molecular weight shift comparable with that seen following α1,2-mannosidase treatment alone (cf. lanes 3 and 5). This suggests that the O-glycans of P. pastoris-produced PSGL-1/mIgG2b do not contain α1,6-linked mannoses either terminally or subterminally. Enzymatic cleavage with a non-linkage-specific α-mannosidase resulted in a considerably larger molecular weight shift of the fusion protein compared with that when only an α1,2-mannosidase was used (Figure 4A, lane 6). This implies that a fraction of O-glycans on PSGL-1/mIgG2b produced in P. pastoris contains α-linked mannosides that are not α1,2- or α1,6-linked. PNGase F-treated PSGL-1/mIgG2b was also incubated with a β-mannosidase for different time periods and at different temperatures. Overnight digestion at 37°C resulted in total degradation of the fusion protein demonstrated by the lack of anti-PSGL-1 binding (not shown). Incubation at 50°C for 2 h resulted in some remaining anti-PSGL-1 binding (not shown), but most of the fusion protein was still degraded suggesting the presence of proteases in the β-mannosidase solution provided by the manufacturer. Protein degradation was avoided by addition of a protease inhibitor cocktail to the β-mannosidase incubation at 50°C for 2 h (Figure 4A, lane 7). No molecular weight shift was seen, indicating that terminal β-linked mannose is not present in the O-glycans of P. pastoris-produced PSGL-1/mIgG2b provided that the β-mannosidase was not also inhibited by the protease inhibitor cocktail (cf. lanes 2 and 7). Mannosidase-treated PSGL-1/mIgG2b was analyzed with regard to its binding to Con A (Figure 4B); a binding that verified the results seen in Figure 4A. However, additional bands of lower molecular weight and not detected by anti-PSGL-1 were seen (lanes 2–7). They most likely represent fusion protein break-down products devoid of the anti-PSGL-1 epitope.

PSGL-1/mIgG2b and AGP/mIgG2b produced in P. pastoris are highly mannosylated and show completely different lectin-binding patterns compared with CHO-produced PSGL-1/mIgG2b.

A lectin array was used in order to establish a glycan profile for PSGL-1/mIgG2b produced in P. pastoris and CHO as well as for AGP/mIgG2b produced in P. pastoris (Figure 5). In summary, P. pastoris-produced fusion proteins reacted strongly with MBLs such as Con A, GNA, HHL, LCA, NPA, PSA and UDA (for lectin specificity, Table I). However, the signal of Calsepa, which also recognizes mannose, was weak indicating that this lectin recognizes a mannosylated structure that is not present on either AGP/mIgG2b or PSGL-1/mIgG2b. Pichia pastoris-produced fusion proteins were further characterized by weak expression of galactose (BPL, ECA, PHA-E and RCA120), fucose (AAL, AOL, TJA-II and UEA-I). CHO-produced PSGL-1/mIgG2b on the other hand showed binding specific for complex-type N-glycans (ACG, DSA, ECA, PHA-E and RCA120), fucose (AAL, AOL, TJA-II and TxLClI) and the determinants carried by O-glycans, Tn, T, sialyl-T and disialyl-T (ABA, ACA, BPL, Jacalin, MAH and MPA).

The total binding intensity of AGP/mIgG2b was much weaker than that of PSGL-1/mIgG2b, indicating that the overall glycan expression level is considerably lower for AGP/mIgG2b. This is consistent with the fact that AGP is a globular protein with N-linked glycans only, while PSGL-1 is a heavily O-glycosylated mucin-type protein with 53 potential O-glycosylation sites.

![Western blot analysis of PNGase F treated CHO- and P. pastoris-produced PSGL-1/mIgG2b. CHO-produced PSGL-1/mIgG2b (lane 1, non-treated; lane 2, PNGase F-treated) and P. pastoris-produced PSGL-1/mIgG2b (lane 3, non-treated; lane 4, PNGase F-treated). Proteins were analyzed under reducing conditions and 1 μg of protein was loaded per well. Membranes were probed with Con A (A and B), anti-mIgG(Fc) (C) and anti-PSGL-1 (D). Due to weaker staining with Con A of CHO-produced PSGL-1/mIgG2b, this membrane (A) was incubated with the film for a longer time (3 min) as opposed to the membrane containing P. pastoris-produced PSGL-1/mIgG2b (B, 20 s).](image)
MS of permethylated oligosaccharides released from purified, recombinant PSGL-1/1mlgG2b, produced in P. pastoris

O-glycans released by reductive β-elimination from P. pastoris-produced PSGL-1/1mlgG2b were permethylated and characterized using electrospray ionization MS (ESI-MS). Five peaks corresponding to fragments with masses explained by the sodiated molecular ions of permethylated Hex2-6 structures were seen (Figure 6A). These findings correspond well with previous analyses of O-glycans from P. pastoris-derived...
glycoproteins (Trimble et al. 2004). In addition, three peaks corresponding to Hex7-9 structures were seen. The Hex7-9 structures were confirmed by MS analyses (see representative daughter ion spectra; Figure 6B–E), while the peak most likely corresponding to Hex6 was too small for MS analysis.

NMR analyses confirm the presence of α- as not β-linked mannose residues in O-glycans from recombinant PSGL-1/mlgG2a produced in P. pastoris. Figure 7 shows the anomeric region of the one-dimensional and two-dimensional double quantum-filtered correlated spectroscopy (DQF COSY) spectra of reduced O-glycans released from PSGL-1/mlgG2a, revealing a number of both α- and β-signals as well as several broader resonances of most likely protein origin. The β-signals appearing at 4.725 ppm (GlcNAcβ1) and 4.461 ppm (GlcNAcβ1,4) and an α-signal at 5.245 ppm (GlcNAcβ) probably represent the first two residues of N-linked glycan core structures (GlcNAcβ1, 4GlcNAcβ; van Halbeek et al. 1980, 1981; Jars et al. 1995). A third β-signal can be seen at 4.655 ppm whose origin cannot be determined at present. The remaining α-signals are all due to mannoses. Literature values for O-linked mannose sequences from P. pastoris are lacking but closely related structures from Schizosaccharomyces pombe and S. cerevisiae have been characterized by MS and/or NMR. In the case of S. pombe, linear structures up to three mannoses (Man1,2Man,1,2Man)–1 have been found (Gemmill and Trimble 1999). From the COSY spectrum in Figure 7, the H1/H2 connectivities corresponding to Man1,2Man-ol (5.01/4.01 ppm) and Man1,2Man,1,2Man-ol (5.07/4.09 ppm and 5.26/4.03 ppm) are found. These values are precisely in accord with those found for the S. pombe structures (Gemmill and Trimble 1999). It is to be noted that no NMR evidence was found in P. pastoris for the presence of the Gal1,2Gal1,3Man1,2 hybrid structures up to the pentasaccharide level as in S. pombe. It is clear, however, that structures longer than three sugar residues are present since the H1/H2 connectivities at 5.42/4.08 and 5.22/3.89 ppm remain to be assigned. Considering that the MS data above indicated linear oligosaccharide sequences (Figure 6A–C) and that only the Manα1,2 glycosidase trimmed the non-reducing end(s) and that the Manα1,6 glycosidase was ineffective also at the penultimate level suggests that Manα1,3 residues are blocking further trimming. In fact, literature values for internal Manα3 residues found in N-glycans vary from 5.33/4.10 to 5.41/4.09 ppm when followed by one or two Manα1,2 residues (Dorland et al. 1981). Thus, the H1/H2 connectivity at 5.42/4.08 ppm in Figure 7 is consistent with the presence of a penultimate Manα1,3 residue which minimally would result in a Manα1,2Man,1,2Man-ol sequence. Furthermore, the H1/H2 connectivity at 5.22/3.89 ppm may then be rationalized as stemming from the Manα1,2 residue preceding Manα1,3. Inspection of both the one-dimensional and COSY spectra reveals, moreover, that most peaks consist of overlapping components as well as minor cross peaks at 5.25/4.06, 5.19/3.94 and 5.01/4.05 ppm (not seen at the gain used in Figure 7) in close proximity of the major ones suggesting the presence of even longer sequences.

Multivalent expression of oligosaccharide on P. pastoris-produced PSGL-1/mlgG2a and AGP/mlgG2a confers high-affinity binding to recombinant MR, DC-SIGN and MBL as revealed by surface plasmon resonance

The apparent equilibrium dissociation constants, Kd, for P. pastoris- and AGP-produced AGP/mlgG2a, and PSGL-1/mlgG2a, were in the nanomolar range for all recombinant receptors, indicating specific, high-affinity binding in all cases. The dissociation constants for each combination are listed in Table II. In contrast, no binding was observed for α-mannose, and poor

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**Table 1. Specificities of lectins in the lectin array**

<table>
<thead>
<tr>
<th>Lectin no.</th>
<th>Lectin</th>
<th>Reported specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LTL</td>
<td>Fuc(α1-3)Galβ1-4GlcNAc, Fuc(α1-2)Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>2</td>
<td>PSA</td>
<td>Fuc(α1-3)Galβ1-4GlcNAc, α-D-Glc, α-D-Man</td>
</tr>
<tr>
<td>3</td>
<td>LCA</td>
<td>Fuc(α1-6)GlcNAc, α-D-Glc, α-D-Man</td>
</tr>
<tr>
<td>4</td>
<td>UE-A</td>
<td>Fuc(α1-2)Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>5</td>
<td>AOL</td>
<td>Fuc(α1-2)Galβ1-4GlcNAc (core fucose)</td>
</tr>
<tr>
<td>6</td>
<td>AAL</td>
<td>Fuc(α1-2)Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>7</td>
<td>SNA</td>
<td>Sia(β2-3)Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>8</td>
<td>SAA</td>
<td>Sia(β2-6)Galβ1-4GlcNAc</td>
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<tr>
<td>9</td>
<td>TJA-I</td>
<td>Sia(β2-6)Galβ1-4GlcNAc</td>
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<tr>
<td>10</td>
<td>PHA-I</td>
<td>Tri-/tetra-antennary complex-type N-glycan</td>
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<tr>
<td>11</td>
<td>ECA</td>
<td>Gaβ1-4GlcNAc</td>
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<tr>
<td>12</td>
<td>RCA120</td>
<td>Gaβ1-4GlcNAc</td>
</tr>
<tr>
<td>13</td>
<td>PHA-II</td>
<td>Bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNAc</td>
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<tr>
<td>14</td>
<td>DSA</td>
<td>Gaβ1-4GlcNAc, Gaβ1-6GlcNAc</td>
</tr>
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<td>15</td>
<td>GSL-II</td>
<td>Agalactosylated tri-/tetra-antennary glycans, GlcNAc</td>
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<td>16</td>
<td>NPA</td>
<td>High-mannose, Manα1,3Man</td>
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<td>17</td>
<td>Cun</td>
<td>High-mannose, Manα1,6Manα1,3Man</td>
</tr>
<tr>
<td>18</td>
<td>GNA</td>
<td>High-mannose, Manα1,3Man</td>
</tr>
<tr>
<td>19</td>
<td>HHL</td>
<td>High-mannose, Manα1,3Man, Manα1,6Man</td>
</tr>
<tr>
<td>20</td>
<td>ACG</td>
<td>Sia(β2-3)Galβ1-4GlcNAc</td>
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<td>21</td>
<td>TxlCI</td>
<td>Manα1,3(Manα1,6)Man, bi- and tri-antennary complex-type N-glycan, Gaβ1-4GlcNAc</td>
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<tr>
<td>22</td>
<td>BPL</td>
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<tr>
<td>23</td>
<td>TJA-II</td>
<td>Fuc(α1-2)Galβ1-3→ or Gaβ1-4GlcNAcβ1,4→ groups at their non-reducing terminals</td>
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<td>24</td>
<td>EEL</td>
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<tr>
<td>25</td>
<td>ABA</td>
<td>Gaβ1-3Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>26</td>
<td>LEL</td>
<td>GlcNAc trimers/tetramers</td>
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<tr>
<td>27</td>
<td>STL</td>
<td>GlcNAc oligomers, oligosaccharide containing GlcNAc and Murα1-4GlcNAc</td>
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<tr>
<td>28</td>
<td>UDA</td>
<td>Gaβ1-3Galβ1-4GlcNAc, mixture of Man5 to Man9</td>
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<tr>
<td>29</td>
<td>PWM</td>
<td>(GlcNAcβ1,4Man)</td>
</tr>
<tr>
<td>30</td>
<td>Jacalin</td>
<td>Gaβ1-3Galβ1-4GlcNAc, Gaβ1-4GlcNAc</td>
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<tr>
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<td>PNA</td>
<td>Gaβ1-3Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>32</td>
<td>WPA</td>
<td>Gaβ1-3Galβ1-4GlcNAc, Gaβ1-3Galβ1-6Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>33</td>
<td>ACA</td>
<td>Gaβ1-3Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>34</td>
<td>MPA</td>
<td>Gaβ1-3Galβ1-4GlcNAc, Gaβ1-4GlcNAc</td>
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<tr>
<td>35</td>
<td>HPA</td>
<td>o-linked terminal Gaβ1-4GlcNAc</td>
</tr>
<tr>
<td>36</td>
<td>VNA</td>
<td>o-linked terminal Gaβ1-4GlcNAc, Gaβ1-3Galβ1-4GlcNAc</td>
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<td>Blood group A antigen, Gaβ1-3GlcNAc, Gaβ1-4GlcNAc</td>
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<tr>
<td>38</td>
<td>SBA</td>
<td>o- or β-linked terminal Gaβ1-4GlcNAc, Gaβ1-3GlcNAc, Gaβ1-4GlcNAc</td>
</tr>
<tr>
<td>39</td>
<td>Calsep</td>
<td>Mannose, maltose</td>
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<tr>
<td>40</td>
<td>PTL-I</td>
<td>o-linked terminal Gaβ1-4GlcNAc</td>
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<tr>
<td>41</td>
<td>MAH</td>
<td>Sia(β2-3)Galβ1-3Sia(β2→6)Galβ1-4GlcNAc</td>
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<tr>
<td>42</td>
<td>WGA</td>
<td>Chitin oligomers, Sia</td>
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<tr>
<td>43</td>
<td>GSL-I A4</td>
<td>o-linked GalNAc</td>
</tr>
<tr>
<td>44</td>
<td>GSL-I B4</td>
<td>o-linked Gal</td>
</tr>
</tbody>
</table>

These data were collected from lectin vendors and reports found by internet searches. Copied from (http://www.gebio.jp) with permission from GP Biosciences.
binding was observed for oligomannose-9 (data not shown) and CHO-produced PSGL-1/mIgG2b. Therefore, no affinity constants could be calculated for these compounds.

MR, DC-SIGN and MBL bind with high affinity to both N- and O-glycan oligomannose structures

The apparent equilibrium dissociation constants ($K_d$) for untreated $P.\ pastoris$-produced PSGL-1/mIgG2b ranged from 16 to 307 nM for all recombinant receptors. PNGase F cleavage of the N-glycans did not significantly affect the binding to MR or DC-SIGN, suggesting that the O-glycans are very important for binding of the mannosylated mucin-type fusion protein to these receptors (Table II). Representative curves for the binding of PNGase F-treated PSGL-1/mIgG2b to the MR, DC-SIGN and MBL are shown in Figure 8.

Discussion

$Pichia\ pastoris$-produced PSGL-1/mIgG2b and AGP/mIgG2b displayed specific, high-affinity binding to the MR, DC-SIGN and MBL, while CHO-produced PSGL-1/mIgG2b, which carries complex-type N-glycans and O-glycans without terminal mannoses, displayed poor binding. These findings are generally consistent with previous studies which have indicated that the CRDs of the MR and MBL specifically interact with single terminal mannose residues and that the CRD of DC-SIGN binds selectively to high-mannose type N-glycans (Taylor et al. 1992; Weis et al. 1992; Feinberg et al. 2001). Upon the removal of the high-mannose type N-glycans from $P.\ pastoris$-produced PSGL-1/mIgG2b by PNGase F-treatment, no major differences in binding affinities to the receptors were observed. The results show that mannosylated O-glycan structures are capable of mediating high-affinity binding to the MR, MBL and DC-SIGN similar to what has been reported before for high-mannose type N-glycans. Although it may be expected that the terminal mannoses of O-glycans on the $P.\ pastoris$-produced PSGL-1/mIgG2b can mediate binding to the MR and MBL, previous studies on the selective binding of DC-SIGN to high-mannose type N-glycans have not indicated that it can bind mannosylated O-glycan structures (Feinberg et al. 2001).

The results suggest that the O-glycans of $P.\ pastoris$-derived PSGL-1/mIgG2b were mainly composed of Man$_{2,3}$ oligosaccharides in which the individual mannose residues were $\alpha$1,2- and $\alpha$1,3-linked, but not $\alpha$1,6-linked. These findings are generally consistent with previous studies. The Man$_{2,3}$ O-glycans of both endogenous cell wall proteins and the kringle 1-4 domain of human plasminogen expressed in $P.\ pastoris$ had the mannose residues $\alpha$1,2-linked (Duman et al. 1998). The O-glycans of human bile salt-stimulated lipase secreted by $P.\ pastoris$ were mainly comprised of $\alpha$1,2-linked Man$_{2,4}$ oligosaccharides (Trimble et al. 2004). Some Man$_{3,5}$ structures were also identified, in which the terminal mannose of

![Fig. 6.](image_url)

Mannose-specific receptors recognize $P.\ pastoris$-produced O-glycans

![Mannose-specific receptors recognize $P.\ pastoris$-produced O-glycans](image_url)
Man₅ and the terminal and subterminal mannoses of Man₆ were β₁,₂-linked (Trimble et al. 2004). This is in contrast to the O-glycans found on PSGL-1/mIgG₂b which appeared not to contain any β-linkages. When *Toxoplasma gondii* surface antigen 1 (SAG1) and gelatinase B were expressed and secreted by *P. pastoris*, O-glycans were also shown to include α₁,₃- and α₁,₆-linkages besides α₁,₂ (Van den Steen et al. 1998; Letourneur et al. 2001). In contrast to O-glycans in 1078
S. cerevisiae, terminal α1,3-linked mannoses have, to the best of the authors’ knowledge, not been found in P. pastoris-derived O-glycans (Duman et al. 1998; Brethauer and Castellino 1999; Romero et al. 1999; Trimble et al. 2004). Our results are consistent with this, as α1,3-linkages were only identified as penultimate mannose residues.

Reports have suggested that synthetic oligolysine-based dimannoside clusters with at least a trilysine core are good...
ligands for the MR (Frison et al. 2003). The anameric region of the one-dimensional and two-dimensional DQF COSY spectra of reduced O-glycans obtained at 600 MHz and 25°C. The residual water signal (HDO) was used as internal standard and set to 4.80 ppm. The numbers above the anameric glycan resonances represent approximate intensities (the resonances appearing at 5.22 ppm were arbitrarily set to a two-proton intensity). Di-s, tri-s and penta-s stand for di-, tri- and pentasaccharide, respectively. Cross peaks marked with an asterisk represent artifacts.

**Table II.** Apparent equilibrium dissociation constants ($K_D$) for binding of PSGL-1/mlgG2b and AGP/mlgG2b to MMR, DC-SIGN and MBL

<table>
<thead>
<tr>
<th>Analyte/receptor</th>
<th>MMR (nM)</th>
<th>DC-SIGN (nM)</th>
<th>MBL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pastoris-produced PSGL-1/mlgG2b</td>
<td>307</td>
<td>40.3</td>
<td>16</td>
</tr>
<tr>
<td>PNGase F-treated P. pastoris-produced PSGL-1/mlgG2b</td>
<td>126</td>
<td>56.2</td>
<td>67.7</td>
</tr>
<tr>
<td>P. Pastoris-produced AGP-1/mlgG2b</td>
<td>84.1</td>
<td>22.4</td>
<td>40.8</td>
</tr>
<tr>
<td>CHO-produced PSGL-1/mlgG2b</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The larger Man$_{a,6}$ O-glycans observed on PSGL-1/mlgG2b have not been confirmed in previous studies, although hyper-O-glycosylation has been suggested for $P$. pastoris-produced SAGI (Letourneur et al. 2001).

Dimeric AGP/mlgG3b has the potential to carry 12 N-linked glycans, which upon expression in $P$. pastoris are likely to be of the high-mannose type. The presence of different AGP/mlgG3b species of different molecular weight indicates variation in N-glycan size and site occupancy. Based on other studies, these N-glycans are likely to be Man$_{a,6}$GlCNAC$_2$ oligosaccharides, although the majority of them might be Man$_{a,6}$ GlCNAC$_2$ oligosaccharides (Trimble et al. 1991; Montesino et al. 1998; Blanchard et al. 2008). The poor binding of α-mannose and oligomannose-9 to the MR, MBL and DC-SIGN suggests that interactions between individual CRDs of the receptors and a single, $P$. pastoris-produced O-glycan would be weak and that other complementary interactions are important to generate the high binding affinity observed between PNGase F-treated PSGL-1/mlgG2b and the different receptors. Such complementary interactions could be multivalent binding and non-specific interactions between the protein part of PSGL-1/mlgG2b and the CRDs of the receptors (Lee et al. 1992; Coombs et al. 2010). The distinguishing feature of $P$. pastoris-produced PSGL-1/mlgG2b is its potential to present a large number, potentially 106, of O-glycans on an extended polypeptide core, as opposed to the
more scarcely placed N-glycans on a globular protein such as AGP/mIgG2b. It has been suggested that MBL is particularly well suited to bind repeating sugar structures of microbial origin (Weis and Drickamer 1994). The distinct conformation of PSGL-1/mIgG2b with the PSGL-1 part covered with O-glycans and being at least 200 Å long, resembles in a way a microbial surface and should therefore be well suited to present terminal mannosse residues to the three CRDs of MBL. 

Similar to MBL, the MR seems to rely on several active CRDs to generate strong interactions with an oligosaccharide ligand. However, in contrast to MBL which present multiple CRDs by oligomerization, the MR carries its eight CRDs within a single polypeptide (Taylor et al. 1992; Mullin et al. 1997). It has been suggested that the MR adopts an extended conformation possibly reaching ~380 Å from the cell surface where CRD4 and 5 may be close enough to interact with terminal mannososes on a single high-mannose type N-glycan (Mullin et al. 1997). Simultaneous interaction with other CRDs of the MR may require more widely spaced terminal mannososes. PSGL-1/mIgG2b with its extended conformation might be expected to provide terminal mannososes which could potentially interact with MR CRDs other than 4 and 5. In fact, it has been shown that CRD4-8 are required to achieve high-affinity binding to multivalent glycoconjugates which would imply that PSGL-1/mIgG2b and MR, MBL and DC-SIGN suggest that these receptors also in vivo. The MR and DC-SIGN are endo-cytotic receptors which have the potential to improve antigen internalization and subsequent T-cell presentation (East and Isacke 2002; Engering et al. 2002), whereas MBL is a soluble plasma protein which participates in the activation of the complement system (Turnier 1996). Conjugation of antigens to mannosylated PSGL-1/mIgG2b may provide an efficient way to target antigens to these receptors and thereby enhance antigen-specific immune responses. It has been shown that the type of mannosylation carried by the antigens is critical for the kind of response they elicit (Luong et al. 2007). For example, extensive O-linked mannosylation generated increased lymphoproliferative responses, while the presence of N-linked mannosylation was associated with decreased proliferative responses (Luong et al. 2007). The different conformations and carbohydrate substitutions of PSGL-1/mIgG2b may thus provide the means to modulate the immune responses toward conjugated antigens.

Materials and methods

Chemicals

Ammonium hydroxide, BSA, dithiothreitol (DTT), ethylene-diaminetetraacetic acid (EDTA), glucose, hydrochloric acid, sodium bicarbonate, sodium chloride, sodium citrate, sodium hydroxide, sodium phosphate, sulfuric acid, tris-base and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO). Calcium chloride, magnesium chloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy succinimide (EDC/NHS), ethanolamine, glycine and sodium acetate were purchased from Difeco. Glutaraldehyde, glutaric acid, hydrochloric acid, trypsin and Zeocin™ (Invitrogen) as selective drug. Membrane separation was performed using 10,000 MWCO membranes (Millipore, Bedford, MA) and, likewise, subcloned into the multiple cloning site of the pPICZα vector (Invitrogen) following EcoRI digestion. The cDNA-encoding AGP/mIgG2b, fragment was digested by EcoRI and Kpn1 (New England Biolabs, Ipswich, MA) and, likewise, subcloned into the multiple cloning site of the pPICZα vector (Invitrogen). The sequences were confirmed by DNA sequencing.

Plasmid integration and selection of high-producing P. pastoris clones

The vectors pPICZαA:PSGL-1/mIgG2b and pPICZαA:AGP/mIgG2b were amplified in Escherichia coli XL-1 Blue using 25 μg/mL Zeocin™ (Invitrogen) as selective drug. Following purification, the vectors were linearized by PmeI (New England Biolabs) and transformed into P. pastoris GS115 cells according to the standard procedures (Easy Comp™, Invitrogen). Transformants of the Mia’ phenotype were subsequently identified by growing nine clones from each transfection on MDH (minimal dextrose medium + histidine) agar (1.34% w/v yeast nitrogen base, 4 × 10^{-2} % w/v histidine, 1081
4 × 10^{-7} \text{w/v} \text{bixin, } 2\% \text{w/v yeast, } 1.5\% \text{w/v agar} \text{ and on MMH (minimal methanol } + \text{ histidine)} \text{ agar (MDH but with 0.5\% methanol instead of glucose) using } P. \text{ pastoris GS115/Mut}\text{ and } P. \text{ pastoris GS115/PICZuZac/Mut}\text{ as negative and positive controls, respectively. To screen for high-expressing clones, seven transformants of each transformation exhibiting the Mut\text{} phenotype were selected and inoculated in bufferued glycerol complex medium (BMYG; } 1\% \text{w/v yeast extract, 2\%w/v peptone, 1\%v/v glycerol, 1.34\%w/v yeast nitrogen base, 100 m\text{M potassium phosphate, pH } 6.0, \text{ 4} \times 10^{-7}\text{w/v bixin)} \text{ and grown for 24h at } 29\text{°C, at 180 rpm (Shake Incubator model 481, Thermo Fisher Scientific, Waltham, MA). This was followed by a 72-h induction period in bufferued methanol-complex medium (0.5\%v/v methanol, 1\%w/v yeast extract, 2\%w/v peptone, 1.34\%w/v yeast nitrogen base, 100 m\text{M potassium phosphate, pH } 6.0, \text{ 4} \times 10^{-7}\text{w/v bixin)} \text{ at } 29\text{°C, } 180 \text{ rpm. MDH agar, MMH agar and BMYG components were purchased from Sigma-Aldrich with the exception of peptone and yeast extract which were from Merek (Darmstadt, Germany). Cell culture supernatants were then harvested by centrifuging at } 10,000 \times \text{g for 10 min at } 4\text{°C, and the concentration of PSGL-1/mIgG2b, and AGP/mIgG2b, in the supernatants was assessed by enzyme-linked immunosorbent assay (ELISA) using a goat anti-mouse IgG (Fc) antibody (see section “Quantification of fusion protein using ELISA”).}

Construction of an expression plasmid for subsequent CHO transfection

The PSGL-1/mIgG2b expression plasmid (Liu et al. 1997) was modified to contain an enterokinase (EK) cleavage site (Asp Asp Asp Asp Lys) by PCR amplification using 5'-CGC GAT CCC CTT TAT CGT CAT CGT CCT TCA CAG ACA TGT GCT CGT G-3' and 5'-CGC GGG AAG CGT GTC TCG CTG GAC CAG CTC G3' as forward and reverse primers, respectively, and subsequent ligation of fusion protein using ELISA) using a goat anti-mouse IgG (Fc) antibody (see section “Quantification of fusion protein using ELISA”).

Transfection and clonal selection of CHO cells

CHO-K1 (ATCC®), Manassas; cat. no. CCL-61) cells, gradually adapted to serum-free medium, were seeded in Ex-cell 302 (JRH Bioscience, Lenexa, KS) medium in a Corning® (Corning Inc., NY) CellBind 75 cm² flask at a density of 1 × 10^5 cells/mL. The cells were transfected with the Avr2 New England BioLabs) linearized PSGL-1/EK/mIgG2b expression plasmid, using Lipofectamine 2000 CD (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours after transfection, the cells were incubated in selection medium containing 6 \mu{g}/mL puromycin (Sigma-Aldrich). The selection medium was changed every second to third day. After ~2 weeks, dead cells were removed using Dead Cell Removal MicroBeads (Miltenyi Biotech, Auburn, CA), according to the manufacturer’s instructions. Viable cells were single-cell cloned in 96-well plates and expanded in selection medium for ~2 weeks. To screen for high expressing clones, the concentration of PSGL-1/EK/mIgG2b in the supernatants was assessed by ELISA using a goat anti-mouse IgG(Fc) antibody (see section “Quantification of fusion protein using ELISA”). The clone C-P55 was selected for further expansion and a second single-cell cloning step was performed in ProCHO-4 (Lonza, Basel, Switzerland) medium to generate the clone C-P55.2.

Bioexpressor cultivation of P. pastoris clones

An inoculum was prepared by inoculating 50 mL BMGY media in a 500 mL shake flask with P. pastoris GS115 Mut+ encoding either PSGL-1/mIgG2b or AGP/mIgG2b. The culture was incubated at 180 rpm (Shake Incubator model 481, Thermo Electron Cooperation) at 29°C until OD_600 was ~2. The bioexpressor cultivations were conducted according to a methanol-limited fed-batch strategy (Invitrogen Pichia Fermentation Process Guidelines Version B 053002) in 1 L bioexpressors (Biobundle, Applikon, The Netherlands) with an initial volume of 750 mL BMGY supplemented with 4% w/v glycerol and 1 g/L histidine. The glycerol batch phase was conducted at 29°C, pH 6.0. To reduce pH to 3.5 for the methanol fed-batch phases (induction phase), the pH controller was set to 3.5 during the glycerol fed-batch phase and allowed to be lowered by the metabolic activity of the cells. The pH was maintained by automatic addition of 15% NH_4OH. During the glycerol batch phase, the dissolved oxygen (DO) concentration, measured by a pO_2 electrode, was kept at 30% of oxygen saturation by keeping the agitation fixed at 700 rpm and varying the aeration and supply of pure oxygen as needed. The pO_2 electrode was calibrated before inoculation with oxygen saturation at 29°C, pH 6.0, one atmosphere and aeration of 0.75 L/min and an agitation of 700 rpm. After the initial glycerol was consumed, indicated by a DO value of 100%, the cells were fed with 50 mL of a 50% w/v glycerol including 12 mL PTM_3 (0.6% CuSO_4 × 5H_2O, 8 × 10^{-7} \text{Na}, 0.3% MnSO_4 × H_2O, 0.02% NaMoO_4 × 2H_2O, 2 × 10^{-7} \text{boric acid}, 0.05% CoCl_2, 2% ZnCl_2, 6.5% FeSO_4 × 7H_2O, 0.02% bixin, 0.5% v/v H_2SO_4) salts per liter of glycerol at a rate of 12.5 mL/h. The DO was maintained at 30%. Following a 10 min starvation period, a 1.6 mL/h feed of pure methanol with 12 mL PTM_3 salts per liter of methanol was initiated. The feed was linearly increased to 5 mL/h over a 20 h period after which the feed was kept constant for the remainder of the cultivation. Aeration and pure oxygen feed were adjusted to maintain DO at 30%. Preliminary experiments indicated that histidine was never limiting when 5 mL of a 3.5% histidine solution was injected to the bioexpressor prior to induction. Cell culture supernatants were harvested by centrifuging at 10,000 x g for 10 min at 4°C and filtered through sterile, 0.2 μm pore size, polyether sulfone (PES) vacuum filters (TPP, Trasadingen, Switzerland). The supernatants were finally treated with 0.5 mL of protease inhibitor cocktail (Sigma-Aldrich) per liter supernatant and stored at 4°C.

Large-scale cultivation of CHO cells

The C-P55.2 cell line was cultured in serum-free ProCHO4 medium (Lonza) in repeated batch mode in a 20 L wave bioreactor (Wave System 20/50 EH, GE Healthcare). The bioreactor was inoculated at 0.79 × 10^5 viable cells/mL in a
volume of 5.2 L. At regular intervals, fresh cultivation medium with 2 mM glutamine was added as a bolus until the final volume in the bioreactor reached 10.3 L. The culture was harvested when the final cell density had reached 4.6 × 10^8 total cells/mL and the viability had dropped to 88%. The glucose, glutamine and pH levels were monitored daily and adjusted at optimal levels using sodium bicarbonate. Total cultivation time was 11 days. Cell culture supernatant was clarified by microfiltration using a 0.054 μm Millistak-POD C0H filter (Millipore, Billerica, MA). The clarified supernatant was concentrated 20× using a 0.11 m² Pellicon 3 case-sette (Millipore) connected to a Cogent M TFF system (Millipore), then further dialyzed against six volumes of phosphate-buffered saline (PBS). Finally, the product solution was treated with 1 mL/L protease inhibitor cocktail (Sigma-Aldrich) and 0.02% NaN₃ (Sigma-Aldrich) and stored at 4°C until purification.

Purification of PSGL-1/mIgG2b and AGP/mIgG2b fusion proteins

All chromatographic procedures were carried out on an ÄKTAExplorer 100 controlled by the Unicorn software (v. 5.11; GE Healthcare). The clarified supernatants were sterile filtered with 0.22 μm PES filter (TPP) before loading onto a MonoSelect SuRe column (GE Healthcare) pre-equilibrated with PBS. The column was washed with 10 column volumes (CVs) of PBS, and elution of recombinant fusion protein was achieved using 5 CVs of 0.1 M sodium citrate, pH 3.0. After elution, selected fractions were pooled, neutralized with 300 μL/mL of 1 M tris-HCl, pH 9.0, and then dialyzed extensively (12–14 kDa cutoff) against MilliQ water at 4°C. After dialysis, the samples were frozen, lyophilized and stored at −80°C before further purification.

Lyophilized samples were dissolved to 5 mg/mL in gel filtration buffer (0.1 M sodium phosphate, pH 7.2, 0.5 M sodium chloride). Gel filtration of the PSGL-1/mIgG2b was carried out on a pre-equilibrated HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare). Typically, 5 mL sample was applied to the gel filtration column and eluted with a flow rate of 1 mL/min. Eluted fractions were kept at 4°C until pooling were done on the basis of western blot analysis. Pooled fractions were then dialyzed as above, frozen, lyophilized and stored at −80°C.

Quantification of fusion protein using ELISA

The concentrations of recombinant fusion protein in supernatants and in purified fractions were determined by a two-antibody sandwich ELISA method. The 96-well ELISA plates (Corning Inc.) were coated with an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody (Sigma-Aldrich) at a concentration of 10 μg/mL at 4°C overnight. The plate was blocked with 1% BSA in PBS which was also used for dilution of fusion protein as well as the second, detecting anti-body (peroxidase-conjugated, anti-mouse IgG(Fc) antibody; PharMingen, San Diego, CA) diluted 1:10,000. All incubations lasted for 2 h. Between and after incubations, the plates were washed with PBS containing 0.5% (v/v) Tween 20. Bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (Sigma-Aldrich). The reaction was stopped by the addition of 2 M H₂SO₄ and the absorbance read at 450 nm. The fusion protein concentration was estimated using for calibration a dilution series of purified mouse IgG₂b (AbD Serotec, Oxford, UK) in blocking buffer as an internal standard.

PNGase F-treatment

After dialysis and lyophilization, purified PSGL-1/mIgG2b was dissolved in sodium phosphate buffer (0.1 M, pH 7.6) with 25 mM EDTA and 2% Triton X-100 and incubated at 100°C for 10 min. Samples were allowed to cool to room temperature, after which 60 U of PNgase F (Roche, Basel, Switzerland) per milligram protein were added and samples incubated at 37°C overnight. For control samples, an equal volume of buffer was added instead of PNgase F. PNgase F-treated PSGL-1/mIgG2b was re-purified on a HiPrep 26/60 Sephacryl S-300 HR column as described (see section “Purification of PSGL-1/mIgG2b and AGP/mIgG2b fusion proteins”).

Mannosidase treatment

PNGase F-treated and re-purified P. pastoris-produced PSGL-1/mIgG2b was treated with α1,2- and α1,6-mannosidases (Prozyme, San Leandro, CA) and non-linkage-specific α- and β-mannosidases (Sigma-Aldrich). α1,2- and α1,6-mannosidases were used at 15 and 40 U/g fusion protein, respectively. Incubations were performed in the reaction buffers provided by the manufacturer at 37°C for 20 h. α-mannosidase was used at 0.25 g/L and the incubation performed in 0.05 M citrate buffer, pH 4.6, at 37°C for 20 h. The β-mannosidase had a specific activity of 5–30 U/mL of which 25 μL was used per 100 μg of fusion protein. Incubations were performed in 0.01 M NaOAc, pH 4.5, at 37°C for 20 h or at 50°C for 2 h with or without protease inhibi-
tor cocktail (Sigma-Aldrich).

SDS-PAGE and western blotting

Recombinant proteins were analyzed by SDS-PAGE under reducing (5 mM DTT) and non-reducing conditions using 3–8% tris-acetate gradient gels and tris-acetate SDS running buffer (Invitrogen). Precision protein standards (Candycane, Molecular Probes, Leiden, the Netherlands, or Hi-Mark, Invitrogen) were applied as reference for protein molecular weight determination. Protein gels were stained using the Pro Q Emerald 300 Glycoprotein Detection Kit in combination with Ruby (Molecular Probes). These gels were visualized in a Fluor-S Max Multilager carrying a CCD camera (Bio-Rad, Hercules, CA). Separated proteins were also electrophoretically blotted using iBlot (Invitrogen) in combination with nitrocellulose membranes (Invitrogen). Western blot membranes were probed with biotinylated Con A (Vector, Burlingame, CA) 10 µg/mL, a mouse anti-PSGL-1 antibody (clone KPL-1, BD PharMingen, San Diego, CA) diluted 1:1000, and an anti-α-c globulin attacked (AGP) antibody (DakoCytomation, Denmark) diluted 1:500. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG F(ab)₂ (Sigma-Aldrich) diluted 1:50,000 and goat anti-rabbit IgG(H+L) (Sigma-Aldrich) diluted 1:10,000. Peroxidase-conjugated

Mannose-specific receptors recognize P. pastoris-produced O-glycans

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NeurAvidin (Pierce) 10 ng/mL was used to detect Con A binding. Bound lectins and antibodies were visualized by chemiluminescence using the ECL kit according to the manufacturer’s instructions (GE Healthcare). Bovine thyroglobulin (Sigma-Aldrich) was used as a Con A positive control.

**Glycan profiling of PSGL-1/mIgG2b and AGP/mlgG2b using lectin arrays**

Purified CHO- and *P. pastoris*-produced PSGL-1/mlgG2b and purified *P. pastoris*-produced AGP/mlgG2b were sent to GP Biosciences Ltd (Yokohama 225-0012, Japan; www.gpbio.jp) for glycan profiling analyses using their lectin arrays. Briefly, samples were labeled by Cy3 LecChip puriﬁcation arrays prior to detection. Deactivation of excess reactive groups was made with ethanolamine, 20 μL/min for 7 min. The immobilization level of the receptors were 11445 RU for MR, 8946 RU for DC-SIGN and 5040 RU for MBL. The analytes—PNGase F-treated and untreated PSGL-1/mlgG2b with mannose structures (P-PM) or with mono- and disialylated core 1 (C-P55.2) and AGP/mlgG2b with mannose structures (P-AM)—were dissolved in HBS-P buffer with 1 mM CaCl₂ and 1 mM MgCl₂ and injected on the CM5 sensor chip with a rate of 20 μL/min for 4 min with a 2.5 min waiting time. The analyte concentrations ranged from 0.64 nM to 10 μM. Regeneration of the surfaces was achieved by injection of glycine, pH 2.2, at 30 μL/min for 40 s. One channel on the CM5 sensor chip was immobilized only with buffer and was used as blank sensorgrams for subtraction of the bulk refractive index background. Data were calculated using BIAevaluation 4.1 software (Biacore, GE Healthcare) and the apparent equilibrium dissociation constants (K_D) were calculated by plotting steady-state binding levels against the analyte concentrations for several concentrations simultaneously.

The immobilized surface of the sensor chip was tested with mannan (Dextra Laboratories, Reading, UK) or mannosylated PSGL1/mIgG2b to verify that the surface was still active after the regeneration procedure. In addition, α-mannose (Dextra Laboratories) and oligomannose-9 (Dextra Laboratories) were also analyzed for possible binding to the receptors.

**Chemical release and permethylation of O-linked glycans from purified PSGL-1/mlgG2b**

Oligosaccharides were released by β-elimination as described (Carlstedt et al. 1993). The solution containing released oligosaccharides was evaporated under a stream of nitrogen at 45°C, and the oligosaccharides permethylated according to Ciucanu and Kerek (1984), with slight modifications as described (Hansson and Karlsson 1993).

**MS analyses**

ESI-MS in the positive-ion mode was performed using an LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Samples were dissolved in methanol/water (1:1) and introduced into the mass spectrometer at a flow rate of 5–10 μL/min. Nitrogen was used as sheath gas and the needle voltage set to 4.0 kV. The temperature of the heated capillary was set to 200°C.

**Proton NMR spectroscopy**

O-glycans were prepared as described (see section “Chemical release and permethylation of O-linked glycans from purified PSGL-1/mlgG2b”) yielding alditols at the reducing end of the O-glycans. The sample was subsequently lyophilized twice after having been dissolved in 100% D₂O and was thereafter dissolved in 0.5 mL of the same solvent. 1H-NMR spectra were acquired on a Varian 600 MHz spectrometer at 25°C. Two-dimensional DQF COSY spectra were recorded by the standard pulse sequence (Marion and Wuthrich 1983).

**Real-time surface plasmon resonance spectroscopy and data evaluation**

Analyses were performed using a Biacore 2000 instrument (Biacore, GE Healthcare). Recombinant human MMR (cat. no. 2534-MR/CF), DC-SIGN/Fc chimera (cat. no. 161-DC) and MBL (cat. no. 2307-MB/CF) were purchased from R&D Systems (Minneapolis, MN) and immobilized on a CM5 sensor chip using amine coupling chemistry according to the manufacturer’s instructions. Briefly, activation of the surface was made with EDC/NHS 1:1 at a flow rate of 10 μL/min for 7 min. The receptors were dissolved in sodium acetate buffer, pH 4.5, at concentrations of 20 μg/mL for MR, 20 μg/mL for DC-SIGN and 10 μg/mL for MBL, and were immobilized at 10 μL/min for 7 min. Deactivation of excess reactive groups was made with ethanolamine, 20 μL/min for 7 min. The immobilization level of the receptors were 11445 RU for MR, 8946 RU for DC-SIGN and 5040 RU for MBL. The analytes—PNGase F-treated and untreated PSGL-1/mlgG2b with mannose structures (P-PM) or with mono- and disialylated core 1 (C-P55.2) and AGP/mlgG2b with mannose structures (P-AM)—were dissolved in HBS-P buffer with 1 mM CaCl₂ and 1 mM MgCl₂ and injected on the CM5 sensor chip with a rate of 20 μL/min for 4 min with a 2.5 min waiting time. The analyte concentrations ranged from 0.64 nM to 10 μM. Regeneration of the surfaces was achieved by injection of glycine, pH 2.2, at 30 μL/min for 40 s. One channel on the CM5 sensor chip was immobilized only with buffer and was used as blank sensorgrams for subtraction of the bulk refractive index background. Data were calculated using BIAevaluation 4.1 software (Biacore, GE Healthcare) and the apparent equilibrium dissociation constants (K_D) were calculated by plotting steady-state binding levels against the analyte concentrations for several concentrations simultaneously. The immobilized surface of the sensor chip was tested with mannan (Dextra Laboratories, Reading, UK) or mannosylated PSGL1/mIgG2b to verify that the surface was still active after the regeneration procedure. In addition, α-mannose (Dextra Laboratories) and oligomannose-9 (Dextra Laboratories) were also analyzed for possible binding to the receptors.

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**Conflict of interest**

J.H. is acting CSO, shareholder and board member of Recopharma AB.

**Abbreviations**

AGP, α1-acid glycoprotein; BMGY, buffered glycerol complex medium; BSA, bovine serum albumin; CHO, Chinese hamster ovary; Con A, concanavalin A; CRD, carbohydrate recognition domain; CV, column volume; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin; DO, dissolved oxygen; DQF COSY, double quantum-filtered
correlated spectroscopy; DTT, dithiothreitol; EDC/NHS, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy succinimide; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; MBL, mannose-binding lectin; MDH, minimal dextrane medium + histidine; MMH, minimal methanol + histidine; MR, mannose receptor; MS, mass spectrometer; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PES, polyethylene sulfone; PGSP-1, P-selectin glycoprotein ligand-1; SAGI, Toxoplasma gondii surface antigen I; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References


Paper IV
Mannosylated mucin-type immunoglobulin fusion proteins enhance antigen-specific antibody and T lymphocyte responses

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Conflicts of interest: J.H. is a part time CSO, board member and shareholder of Recopharma AB.
Abstract

Targeting antigens to antigen-presenting cells (APC) improve their immunogenicity and capacity to induce Th1 responses and cytotoxic T lymphocytes (CTL). We have generated a mucin-type immunoglobulin fusion protein (PSGL-1/mIgG2b), which upon expression in the yeast *Pichia pastoris* became multivalently substituted with O-linked oligomannose structures and bound the macrophage mannose receptor (MMR) and dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) with high affinity *in vitro*. Here, its effects on the humoral and cellular anti-ovalbumin (OVA) responses in C57BL/6 mice are presented. OVA antibody class and subclass responses were determined by ELISA, the generation of anti-OVA CTLs was assessed in *in vitro*-stimulated immune spleen cells from the different groups of mice as effector cells and OVA peptide-fed RMA-S cells as targets, and evaluation of the type of Th cell response was done by IFN-γ, IL-2, IL-4 and IL-5 ELISpot assays. Immunizations with the OVA-mannosylated PSGL-1/mIgG2b conjugate, especially when combined with the AbiSCO®-100 adjuvant, lead to faster, stronger and broader (with regard to IgG subclass) OVA IgG responses, a stronger OVA-specific CTL response and stronger Th1 and Th2 responses than if OVA was used alone or together with AbiSCO®-100. Also non-covalent mixing of mannosylated PSGL-1/mIgG2b, OVA and AbiSCO®-100 lead to relatively stronger humoral and cellular responses. The O-glycan oligomannoses were necessary because PSGL-1/mIgG2b with mono- and disialyl core 1 structures did not have this effect. Mannosylated mucin-type fusion proteins can be used as versatile APC-targeting molecules for vaccines and as such enhance both humoral and cellular immune responses.

**Keywords:** mannos, glycoprotein, mucin, ovalbumin, ISCOM, vaccine, cytotoxic T-lymphocyte, ELISpot, cytokines, humoral immune response
Introduction

Targeting antigens to endocytic receptors on professional antigen-presenting cells represents an attractive strategy to enhance the efficacy of vaccines. Mannosylated antigens have been demonstrated to enhance MHC class I- and MHC class II-restricted antigen presentation, increase T-cell proliferation, and promote T cell effector responses [1-3] through mannose-mediated binding to endocytic receptors on dendritic cells and macrophages [4]. These receptors belong to the family of calcium-dependent C-type lectin receptors and include the mannose receptor (MR) and dendritic cell-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). They are particularly designed to sample antigen (self and non-self), much like pattern recognition receptors, and to integrate innate and adaptive immune responses [5-7]. DC-SIGN is mainly expressed on immature and mature dendritic cells (DCs) with crucial functions in DC trafficking and T-cell interactions as well as pathogen recognition [8]. MR is expressed primarily by tissue macrophages, and lymphatic and hepatic endothelia in humans and mice [9, 10]. MR is also expressed by subsets of dendritic cells (DC), primarily interstitial DCs, as well as on cultured DCs from human monocytes [11, 12].

Several studies have shown that polymeric mannose (mannan) improve antigen presentation and that oxidized and reduced mannan can induce antigen-specific Th1/CTL and Th2/humoral responses, respectively [13-16]. Tumor immunity was induced with the generation of both antigen-specific humoral and cellular immunity in hMR transgenic mice by antigenic targeting to MR [17]. Yeast-derived recombinant ovalbumin (OVA) carrying branched N- and O-linked mannoses were shown to be far more immunogenic than non-mannosylated OVA [18]. Enhancement of antigen uptake was achieved by extensive O-mannosylation of proteins, and deglycosylation strongly inhibited T cell responses [19].
Furthermore, MR-mediated endocytosis of OVA has been shown to be essential for cross-presentation to CD8+ T cells [20].

In contrast to protein-protein interactions, individual protein-carbohydrate interactions are characterized by a low affinity binding with Kd values many times in the mM range [21-23]. Instead the overall binding strength in such interactions is to a large extent accomplished by multivalency, i.e. several carbohydrate determinants on for example a cell binding several copies of a carbohydrate-specific receptor or domain on another cell. Multivalency can increase the affinity of a particular protein-carbohydrate interaction several thousand-fold resulting in nano- and picomolar Kd values [24, 25]. This is exemplified by the asialoglycoprotein receptor, which recognizes serum glycoproteins with increasing affinity as the number of exposed terminal galactoses increases with age [26]. In addition to multivalency, the molecular fit between the carbohydrate and its receptor can add to the affinity. In this respect the fact that both the inner core saccharide chain as well as the protein backbone may influence the presentation of the carbohydrate determinant is of particular importance [27-29].

P-selectin glycoprotein ligand-1/mouse IgG2b (PSGL-1/mIgG2b) is a mucin-like immunoglobulin fusion glycoprotein with 106 potential sites for O-linked glycosylation and six potential sites for N-linked glycosylation in its dimeric form [30]. With the aim of interfering with or promoting protein-carbohydrate interactions of biomedical importance, we have used this fusion protein as a scaffold for multivalent presentation of various tailored carbohydrate determinants of diagnostic or therapeutic significance [30]. By displaying multiple oligomannose chains in various combinations for MR, DC-SIGN and mannan binding lectin (MBL), we have shown that recombinant PSGL-1/mIgG2b produced in the yeast Pichia pastoris can target these receptors with high affinity by engaging multiple
carbohydrate recognition domains of MR and MBL or multiple/oligomerized DC-SIGN receptors [31].

We hypothesize that a mucin-type fusion protein carrying multiple O-linked oligomannose structures has the potential of working as a universal antigen presenting cell (APC)-targeting molecule for a broad repertoire of protein antigens in different vaccine compositions. As such, it may amplify both humoral and cellular immune responses and may be used together with different antigens for which already established manufacturing bioprocesses can be maintained. Here, we present data on the OVA-specific immune responses in mice immunized with OVA, OVA-mannosylated PSGL-1/mIgG2b conjugates or mixtures, with or without an additional adjuvant in the form of Imject®Alum or AbiSCO®-100.
Materials and methods

Mice

Inbred C57Bl/6J (H-2b) were bred and housed at the Unit for Embryology and Genetics at Karolinska Institutet, Karolinska University Hospital Huddinge, Sweden. The animals were caged at five to ten mice per cage and fed a commercial diet with free access to food and water. All animals were six to eight weeks of age at the start of the experiment. All animal experiments were approved by the regional committee (Stockholms södra djuretiska nämnd) on animal ethics, S-184-06 and S-132-09.

Proteins, peptides and adjuvants

For ELISpot and proliferation assays different proteins and peptides at varying concentrations were used: the OVA-SIINFEKL peptide (OVA 257-264, SP-O257-5; Innovagen, Lund, Sweden) was used at a concentration of 1 μg/mL – 0.0001 μg/mL, the FILKSINE control peptide at 1 μg/mL, the Th-OVA peptide (OVA 323-339, SP-O323A-5; Innovagen) at 10 μg/mL – 0.01 μg/mL, the OVA protein (SIGMA A7641-250 mg, grade VII, lot: 066K7020) was used at a concentration of 625 μg/mL – 5 μg/mL, BSA (SIGMA A8806) at 25 μg/mL and Concanavalin A (SIGMA L7647) at 5 and 1 μg/mL.

The LC-SPDP linker was from Pierce (cat. no. 21650, lot: IG112740; Thermo Fischer Scientific, Waltham, MA, USA). Imject Alum was from Thermo Fischer Scientific (77161, lot: 1H118004E) and AbiSCO®-100 was from Isconova AB (Lot: 132107; Uppsala, Sweden).
Mannosylated PSGL-1/mIgG2b (PPM) was produced in *P. pastoris*, purified, quantified and characterized by Western blotting and mass spectrometry as described [31].

PSGL-1/mIgG2b with mono and disialylated core 1 structure (CP) was produced in a stable CHO (Chinese hamster ovary) cell line given the name, C-P55.2. The cells were cultured in serum-free ProCHO4 medium (Lonza) in repeated batch mode in a 20L Wave bioreactor (Wave System 20/50 EH, GE Health Care, Uppsala, Sweden). The bioreactor was inoculated at 0.8 x 10^6 viable cells/mL in a volume of 5.2 L. At regular intervals, fresh cultivation medium with 2mM glutamine was added as a bolus until the final volume in the bioreactor reached 10.3 L. The culture was harvested when the final cell density was 4.6 x 10^6 total cells/mL and the viability dropped to 88%. The glucose, glutamine and pH levels were monitored daily and adjusted to optimal levels. Total cultivation time was 11 days.

Cell culture supernatants were clarified using a 540 cm² Millistak+ POD C0HC filter (Millipore, Billerica, MA, USA) connected to the Quattroflow pump on the Cogent M TFF system (Millipore). The clarified supernatant was subsequently concentrated 22x using a 0.11 m² Pellicon 3 ultrafilter (Millipore) on the Cogent M TFF system, and then further diafiltered against six volumes of PBS. Finally, 1 mL/L of protease inhibitor cocktail (Sigma P8215, St.Louis, MO, USA) and 0.02% NaN₃ (Sigma 71289) were added to the product solution, which was stored at 4°C until purification.

All chromatographic procedures were carried out on an ÄKTAExplorer 100 (GE Healthcare, Uppsala, Sweden) controlled by the Unicorn software (v. 5.11). The clarified supernatants were sterile filtered with a 0.22µm polyethersulfone (PES) filter (TPP, Trasadingen,
Switzerland) before loading onto a MabSelect SuRe column (GE Healthcare) pre-equilibrated with PBS. The column was washed with ten column volumes (CV) of PBS, and elution of recombinant fusion protein was achieved using 5 CV of 0.1 M sodium citrate, pH 3.0. After elution, selected fractions were pooled, neutralized with 250 μL per mL of 1 M Tris-HCl, pH 9.0 and then dialyzed extensively (12-14 kD cut-off) against MilliQ water at 4°C. After dialysis, the samples were frozen, lyophilized and stored at -80°C before further purification.

Lyophilized samples were dissolved at a concentration of approximately 5 mg/mL in gel filtration buffer (0.1 M sodium phosphate pH 7.2, 0.5 M sodium chloride). Gel filtration of PSGL-1/mIgG2b was carried out on a pre-equilibrated HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare). Typically, 5 mL of sample was applied to the gel filtration column and eluted with a flow rate of 1 mL/minute. Eluted fractions were kept at 4°C until pooling was done on the basis of Western blot analysis. Pooled fractions were dialyzed as above, frozen, lyophilized and stored at -80°C.

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**Conjugation of PSGL-1/mIgG2b to ovalbumin**

A 5 mg/mL (113 μM) solution of OVA was prepared in conjugation buffer (0.1 M sodium phosphate pH 7.2, 0.15 M sodium chloride, 1 mM EDTA). A 20 mM solution of the LC-SPDP linker was prepared in MilliQ water immediately prior to activation of OVA. For activation of OVA, 300 μL of the linker solution was added to 2.1 mL of OVA solution at a 25x molar excess of linker. The mixture was allowed to react under rotation for 30 minutes at room temperature, yielding activated OVA (OVA*). The solution was applied to a PD-10 desalting column (17-0851-01, lot 10009977; GE Healthcare, Uppsala, Sweden) and OVA* eluted in 3.5 mL conjugation buffer thereby removing free linker. The eluted OVA* had a
concentration of 3 mg/mL (68 µM) as determined with the BCA method (Pierce 23225, lot HJ106350) using BSA as standard.

A 10 mg/mL (67 µM) solution of the PSGL-1/mIgG2b fusion protein was prepared in conjugation buffer. To obtain the fusion protein in a form suitable for conjugation with OVA*, the fusion protein solution was reduced with 100x molar excess of DTT (0.5 M solution). The reduction was carried out in a heat block at 37°C for 15 minutes. The DTT was removed from the fusion protein by passing the reaction solution twice through a PD MiniTrap G25 column (28-9180-07, lot 356228; GE Healthcare).

To assess the amount of linker present on OVA*, a pyridine-2-thione assay was performed. Fifty µL OVA* was mixed with 950 µL PBS in a plastic cuvette and the absorbance at 343 nm was measured. Two µL 0.5 M DTT was added to the cuvette to cleave the chromophore from the LC-SPDP linker. After 15 minutes at room temperature the absorbance at 343 nm was measured again. The molecular substitution ratio (MSR) was calculated as follows:

$$\text{MSR} = \frac{A_{343, \text{after}} - A_{343, \text{before}}}{\epsilon} \times \frac{\text{MW}_{\text{OVA}}}{[\text{OVA}^*]}$$

The reactions were repeated once. The activation of OVA for conjugation with PPM (study A), PPM (study B) and CP afforded an MSR of 6.0, 6.2 and 5.6, respectively. This indicates that there were on average 6.0, 6.2 and 5.6 linkers, respectively, on each OVA molecule for the PPM (study A and B) and CP conjugations.

To estimate the number of accessible thiol groups on the reduced fusion protein, Ellman's assay was performed. Two µL reduced fusion protein, 998 µL Ellman's buffer (0.1 M sodium
phosphate pH 8.0, 1 mM EDTA) and 50 µL 10 mM Ellman's reagent (5,5'-Dithio-bis-[2-
nitrobenzoic acid], Pierce 22582) was mixed in a cuvette. A blank was prepared in the same
way, replacing the fusion protein with buffer. After 15 minutes at room temperature the
absorbance at 412 nm was measured. Duplicate samples were analyzed. The concentration of
accessible thiols was calculated as follows:

\[
[-\text{SH}] = \frac{A_{412}(\text{sample}) - A_{412}(\text{blank})}{\varepsilon} \times \frac{V_{\text{total}}}{V_{\text{sample}}}
\]

Dividing [-SH] with the concentration of reduced, monomeric fusion protein, a calculated
number of 10.4 (PPM study A), 7.8 (PPM study B) and 5.9 (CP) thiol groups per monomer of
fusion protein was obtained (cp. 8 cysteines in each monomer).

For conjugation, 2.816 mL OVA* and 1.0 mL reduced PPM (study A), 3.15 mL OVA* and
0.8 mL reduced PPM (study B), and 2.412 mL OVA* and 1 ml reduced CP, respectively,
were mixed and split into two parallel reactions. The reaction was carried out at room
temperature over night under rotation. See Table 1 for the molar quantities of the respective
species for the different conjugations.

After the conjugation reaction, the sample was centrifuged at 4,250 x g and applied to a 26/60
HiPrep Sephacryl S300 gel filtration column (17-1196-01, GE Healthcare; lot 10019010) pre-
equilibrated with 0.1 M sodium phosphate pH 7.2 with 0.5 M sodium chloride. Eluted
fractions were kept at 4°C until pooling was done on the basis of Western blot analysis.
Pooled fractions were then dialyzed as above, frozen, lyophilized and stored at -80°C.
Quantification of OVA and fusion proteins

Quantification of conjugated OVA used for immunizations in study A was done by anti-OVA Western blot analysis using OVA of known concentration as standard. The OVA standard was determined with the BCA method (Pierce 23225, lot HJ106350) using BSA as standard. The concentration of OVA in the stock solution was 2.0 mg/mL. A dilution series of reduced (dithiothreitol-treated) samples was heat-inactivated for ten minutes at 70°C prior to separation on a 4-12% Novex Bis-Tris gel (MES buffer, 200 V for 45 minutes). Two identical SDS-PAGE gels were run, blotted and analyzed as described below. Blotting was performed in an Invitrogen iBlot device for ten minutes using an iBlot Transfer Stack (SKU# IB1001EU; Invitrogen, San Diego, CA, USA) with a nitrocellulose membrane (SKU# IB3010-01; Invitrogen). After washing of the membrane in PBS-Tween (PBS-T; two times five minutes), it was incubated in blocking solution (3% BSA in PBS-T) for one hour at room temperature (RT). After additional washes (three times five minutes), the membrane was incubated over night at +4°C with an anti-OVA antibody (Sigma A6075) diluted 1:20,000 in 3% BSA/PBS-T. The membrane was after washing (three times five minutes in PBS-T) incubated for one hour at RT with a horseredish peroxidase (HRP)-conjugated secondary goat anti-mIgG Fab antibody (Sigma A2304) diluted 1:25,000 in 3% BSA/PBS-T. The membranes were washed three times before they were incubated with chemo-luminescent HRP substrate for five minutes (Immobilon Western WBKLS0050; Millipore, Billerica, MA, USA). The membranes were then exposed to Amersham Hyperfilm ECL for 30 seconds to five minutes for visualization of anti-OVA staining. The films were scanned using a Fluor-S MAX MultiImager (BioRad, Hercules, CA, USA) and the concentration of OVA released from the conjugate was quantified using the Quantity One v. 4.6.5 software (BioRad). The anti-OVA staining of the known OVA samples and the dilutions of the reduced conjugate were integrated and a standard curve was created. By fitting the integrated volumes of the
anti-OVA staining of the reduced conjugate samples to the standard curve, the concentration of OVA in the conjugate estimated from the two gels was 2.4 and 2.5 mg/mL, respectively. With a volume of 2.5 mL this amounts to 6.0 mg of OVA conjugated to the PSGL-1/mIgG2b fusion protein. The final amount of conjugated PSGL-1/mIgG2b fusion protein was assumed to be equal to the amount applied in the conjugation reaction.

In study B, quantification of conjugated OVA, PPM and CP (in conjugates OVA-PPM and OVA-CP) used for immunizations was done by SDS-PAGE and SilverQuest analysis using OVA, PPM and CP of known concentration as standard. The OVA standard was determined with the BCA method (Pierce 23225) using BSA as standard. The concentration of OVA in the stock solution was 2.3 mg/mL. Concentrations of stock solutions of PPM and CP was determined by ELISA to 4 mg/mL and 0.33 mg/mL, respectively. A dilution series of reduced (dithiothreitol-treated) samples was heat inactivated for ten minutes at 70°C prior to separation on a 4-12% Novex Bis-Tris gel (MES buffer, 200 V for 45 minutes). Two SDS-PAGE gels were run, stained and analysed as described below. SilverQuest staining was performed according to the manufacturer’s instructions. Briefly, the gels were rinsed in ultrapure water and fixed in 40% ethanol and 10% acetic acid in Ultrapure water, for 20 minutes with gentle rotation. The gels were washed in 30% ethanol for ten minutes. The ethanol was decanted and sensitizing solution added to the gels and incubated for ten minutes. The sensitizing solution was decanted, the gels washed in 30% ethanol for ten minutes, and further washed in ultrapure water for ten minutes. The gels were incubated in staining solution for 25 minutes, after which it was decanted and the gels washed with ultrapure water for 30 seconds. The gels were incubated in developing solution for eight minutes both for the fusion protein and the OVA gels. Stop solution was then immediately added to the gels that were agitated for ten minutes. The stop/developing solution was decanted and the gels washed in ultrapure water for ten minutes. The gels were scanned using
the Fluor-S MAX MultImager (BioRad) and the concentration of OVA, PPM and CP released from the conjugate was quantified by fitting the integrated volumes of the OVA/PPM/CP staining of the reduced conjugate samples to the standard curve. For OVA the concentrations were 3.8 mg/mL and 3.4 mg/mL for the OVA-PPM and OVA-CP conjugates used in study B, respectively. PPM in OVA-PPM was estimated to 11.3 mg/mL and CP in OVA-CP was estimated to 7.9 mg/mL. The amounts and coupling yields for the conjugations as well as the molar ratios of OVA to fusion protein are shown in Table 1. To measure the endotoxin level in the vaccine preparations, Limulus amoebocyte lysate tests were performed with the gel-clot method (Apotekens Produktion och Laboratorier, Stockholm, Sweden).

Coupling yield(%) = \frac{\text{amount in conjugate}}{\text{amount in reaction}} \times 100

Molar ratio = \frac{\text{amount OVA in conjugate}}{\text{amount fusion protein in conjugate}}

Study design

Two separate studies were performed; A and B. In study A, 8 mice/group were immunized subcutaneously (s.c.) in the base of the tail using a dose of 50 μg OVA, either free or conjugated 1:1 with mannosylated PSGL-1/mIgG2b (PPM) produced in Pichia pastoris.
The antigen and antigen conjugate were either given alone or in combination with AbiSCO®-100 or Alum (Imject®). The mice were immunized three times with three-week intervals (Fig. 1). The day before the first immunization, mice under Isofluran (IsoFlo® vet, Orion Pharma) anesthesia were bled by retro-orbital bleeding. All immunizations were done under Hypnorm® (VetaPharma) anesthesia (20-30 μL intraperitonelly) by s.c. injections at the base of the tail vein. Mice were also bled two weeks after each immunization (w.2, w.5 and w.8). Boost immunizations were done at week three and five. Two weeks after the final immunization all mice were sacrificed and the spleen was recovered for cell isolation and further in vitro analyses of cellular immune responses (cell proliferation, cytokine ELISpot and CTL assays).

The following groups of mice were included in study A: 1) 50μg OVA in PBS; 2) 50μg/140μg OVA-PPM in PBS; 3) 50μg OVA + 12μg AbiSCO®-100 in PBS; 4) 50μg/140μg OVA-PPM + 12μg AbiSCO®-100 in PBS; 5) 50μg OVA in a 1:1 mixture of PBS:Imject® Alum; 6) 50μg/140μg OVA-PPM in a 1:1 mixture of PBS:Imject® Alum; and 7) 140μg PPM in PBS. All material used for the immunizations had an endotoxin level below 1 EU/dose.

In study B, 8-10 mice/group were immunized as above using a dose of 35 μg OVA either free or conjugated 1:1 with mannosylated PSGL-1/mIgG2b (PPM) produced in P. pastoris or with PSGL-1/mIgG2b carrying mono and disialylated core 1 structures (CP) produced in CHO cells. The antigen and antigen conjugate were either given alone or in combination with AbiSCO®-100. The same immunization scheme as described above was used (Fig. 1). The following groups of mice were included in study B: 1) 35μg/89μg OVA-PPM; 2) 35μg/89μg OVA-CP; 3) 35μg OVA + 12μg AbiSCO®-100; 4) 35μg OVA + 89μg PPM + 12μg AbiSCO®-100; 5) 89μg PPM + 12μg AbiSCO®-100; and 6) PBS. All groups had an
endotoxin level below 1 EU/dose except the groups immunized with OVA-PPM that had 3.8-7.6 EU/dose and OVA-CP had 4.8-9.6 EU/dose.

**Detection of total IgG and IgG subclasses IgG1, IgG2a, IgG2b, IgG3 specific for OVA**

Mouse sera analyzed with regard to antibody levels and isotypes were collected before the first immunization (w0) and then 2 weeks after each immunization (w.2, w.5 and w.8) by retro-orbital bleeding of isofluorane-anesthetized mice. To free the sera of cells, the samples were centrifuged twice at 6,000 x g. OVA-specific mouse immunoglobulins were quantified by ELISA. ELISA plates (Corning 3590) were coated using a 10 μg/mL OVA (SIGMA A7641) solution, which was incubated in the plates o/n at +4°C. After every incubation step the plates were washed 4 times with 400 μL of wash solution (9 g NaCl/L H₂O + 0.05% Tween). Plates were blocked with 1% BSA in PBS for 1 hour at 37°C. Serial dilutions of sera in 1% BSA/PBS were analyzed in duplicates or triplicates and incubated for 1 hour at 37°C. Anti-mouse IgG (Southern Biotech 1030-05), IgG1 (Southern Biotech 1070-05), IgG2a (Southern Biotech 1080-05), IgG2b (Southern Biotech 1090-05) or IgG3 (Southern Biotech 1100-05)-HRP conjugates diluted 1:2,000-8,000 and incubated for 1 hour at 37 °C was used for detection of the different IgG subclasses. The TMB (tetramethylbenzidine, SIGMA T3405) substrate (1 tablet) was dissolved in 10 mL phosphate citrate buffer, pH 5.0 containing 3 μL 30% H₂O₂ per 10 mL buffer and was used for detection of HRP conjugates. The reaction was stopped after 3-5 minutes with 2 M H₂SO₄. Optical density (OD) was measured in a TECAN Sunrise spectrophotometer (TECAN; Männedorf, Switzerland) at 450 nm within 2 hours after addition of H₂SO₄. An antibody titer was considered positive if the OD value was three times that of the animal serum collected prior to the first immunization.
A hyperimmune serum of known titer was used as positive control. Pooled serum from non-immunized wt C57Bl/6J mice was used as negative control.

**Detection of OVA-specific CTL responses/activity**

Spleens from OVA immunized C57Bl/6J mice were collected two weeks after the final immunization and single cell suspensions were prepared in RPMI-1640 medium containing 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO, 15140). Red blood cells were removed using Red blood cell lysing buffer (SIGMA, R7757). Immune spleen cells (25 x 10⁶) were stimulated *in vitro* by co-cultivation in 25 mL-flasks containing 12 mL complete RPMI-1640 medium with 10% FBS (SIGMA, F7524), 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO, 15140), 10 mM HEPES, 2 mM L-glutamine (GIBCO, 25030), 1 mM nonessential amino acids (GIBCO, 1114035), 1 mM Sodium puruvate (GIBCO, 11360039) and 50 µM 2-mercaptoethanol for 5 days with an equal number of irradiated (2,000 rad) syngeneic splenocytes and the MHC class I-specific SIINFEKL peptide at a concentration of 0.5 mM. Effector cells were harvested at day 5 and a 4 hour ⁵¹Cr release assay was performed in V-bottomed 96-well plates. RMA-S target cells (1 x 10⁶) were incubated with the SIINFEKL, or as negative control the FILKSINE, peptide at a concentration of 50 µM for 90 minutes at 37 °C in a 5% CO₂ atmosphere. The cells were carefully mixed every 15 minutes. Peptide-loaded target cells were incubated for 1 hour at 37 °C with 30 µL ⁵¹Cr (5 mCi/mL) and washed 3 times in PBS before use. Target cells were added to the plates in a total volume of 200 µL at effector:target (E:T) ratios of 80:1, 40:1 and 8:1. The cytotoxic activity was determined after a 4 hour incubation at 37 °C in a 5% CO₂ atmosphere. Twenty-five µL supernatant were harvested and transferred to 200 µL OptiPhase super mix (PerkinElmer, Waltham, MA, USA; 1200-439) in a 96-well Isoplate (Wallac/PerkinElmer, 1450-514), and
the radioactivity was measured in a γ-counter TRILUX 1450 MicroBeta counter (Wallac, PerkinElmer, Waltham, MA, USA). Results were determined using the formula: percent specific lysis = (experimental release – spontaneous release) / (maximum release – spontaneous release). Experimental release is the mean count/minute released by target cells in the presence of effector cells. Maximum release was calculated from lysed incubated target cells. Spontaneous release was calculated from spontaneous release from incubated target cells. All samples were run in triplicates.

**Detection of OVA-specific CTLs and Th-cells producing IFN-γ, IL-2, IL-4 and IL-5**

Spleen cells from four to five individuals in each group were pooled and immediately tested for the presence of OVA-specific T cells. Spleen cells from the other four to five individuals in each group were used to repeat the experiment with consistent results. The ability of OVA-specific Th and CTLs to produce IFN-γ, IL-2, IL-4 and IL-5 after exposure to different peptides [SIINFEKL (OVA 257-264; Innovagen SP-O257), FILKSINE (Innovagen), and ISQAVHAAHAEINEAGR (OVA 323-339; Innovagen SP-O323A)], proteins (OVA, Sigma A7641 and grade VII, BSA Sigma A8806), Concanavalin-A (SIGMA L7647) and media was assessed. The production of the different cytokines was determined by a commercially available ELISpot assay. In brief, ELLIP plates (cat. no MAIPSWU; Millipore, Billerica, MA, USA) with PVDF membranes were treated with 70% ethanol for 1 minute, washed in dH₂O and coated o/n at +4 °C with 10 μg/mL of monoclonal antibodies specific for IFN-γ (AN18), IL-2 (1A12), IL-4 (11B11) or IL-5 (TRFK5) (Mabtech AB, Nacka strand, Sweden) in PBS. After washing 5 times in PBS, the plates were blocked for 2 hours with complete RPMI-1640 medium. All stimulations (36 hours at 37 °C, 5% CO₂) were carried out using 250,000 immune cells/well. Various concentrations of the different antigens were added in
triplicates to a total volume of 200 µL. After stimulation, the wells were washed and incubated for 2 hours at 37 °C with the following biotinylated antibodies, respectively: anti-IFN-γ (R4-6A2-biotin), anti-IL-2 (5H4-biotin), anti-IL-4 (BVD6-24G2-biotin) and anti-IL-5 (TRFK-biotin) (Mabtech AB) at 2 µg/mL in 0.5% FBS/PBS (F6178; SIGMA, St Louis, MO, USA). After washing, Strep-ALP (Mabtech 3310-10) diluted 1:1,000 in 0.5% FBS/PBS was added and incubated for 1 hour in RT. Sterile-filtered substrate (BCIP/NBT) was used to develop spots; IFN-γ and IL-2 for 10 minutes, IL-4 for 12 minutes and IL-5 for 14 minutes. The substrate reaction was stopped by rinsing extensively with dH2O, after which the plates were left to dry. The number of spots was counted using the AID ELISpot reader and software ver. 3.2.3 (AID, Strassberg, Germany). The number of spots (cytokine producing cells) was determined at each concentration of peptide or protein and the results given as the number of IFN-γ, IL-2, IL-4 or IL-5-producing cells per 10⁶ cells. A mean number of cytokine-producing cells of <50 per 10⁶ cells was considered as negative.

**CTL and Th cell-proliferation in response to OVA and OVA peptides**

Proliferative responses to OVA and OVA peptides were determined by stimulation of splenocytes from groups of mice immunized with the different vaccine compositions. A total of 600,000 cells/well in complete RPMI-1640 medium were seeded in 96-well flat bottom plates with lid (Corning 3595). Stimulation was carried out for 48 hours at 37°C in a 5% CO2 atmosphere using the same antigens and concentrations as in the ELISpot assay. At 48 hours, 0.1 Ci/mL ³H-thymidine (TRA120-5MC; GE Heathcare, Chalfont St. Giles, United Kingdom) was added and 16-20 hours later the cells were harvested onto filtermat A filters (Wallac 145-421) and the radioactivity counted in a TRILUX 1450 MicroBeta counter (Wallac). Proliferation was determined by dividing the radioactivity as counts per minute...
(cpm) of cells incubated with an Ag with the cpm of the cells incubated with medium alone (sample to negative (S/N) ratio). Groups were compared by the mean S/N ratios at each time point after subtraction of proliferative responses seen in the negative control group receiving PPM alone. All samples were run in triplicates.

**Statistical analyses**

The ANOVA with Tukey post-hoc test was used for statistical analyses using the JMP version 8.0.1 for PC software (SAS Institute Inc., Cary, North Carolina, USA). P-values <0.05 were considered statistically significant.
Results

Conjugation of OVA to mannosylated PSGL-1/mIgG₂b boosts anti-OVA IgG responses

Total IgG was compared between all experimental groups at week 0, 2, 5 and 8 using mouse serum from individual mice. In study A (Fig. 2A) only the groups including the AbiSCO®-100 adjuvant had detectable antibody titers at week two. The group that received OVA-PPM + AbiSCO®-100 had significantly higher IgG titers as compared to all other groups. At week five after two immunizations, all groups except the group immunized with OVA alone had detectable IgG titers. Five out of eight mice immunized with OVA-PPM had IgG titers over 1:100, while none of the mice immunized with OVA alone had detectable IgG titers. The OVA-PPM + AbiSCO®-100 group had significantly higher titers compared to the OVA + AbiSCO®-100 group. Imject®Alum showed a significantly weaker adjuvant effect on the induction of anti-OVA IgGs than AbiSCO®-100. No difference in the anti-OVA IgG response could be detected between the two groups immunized with OVA + Imject®Alum or OVA-PPM + Imject®Alum at week five indicating that our mannosylated fusion protein does not potentiate the adjuvant effect of Imject®Alum. At week eight, after three immunizations, titers over 1:1,000 was achieved in the group immunized with OVA-PPM. At this time point, the IgG pattern between the groups was similar to that seen at week five but with even higher IgG titers peaking 1:10⁶ in the group immunized with OVA-PPM + AbiSCO®-100.

In order to examine the importance of the oligomannose residues on the fusion protein for its adjuvant effect on the IgG antibody response, we included in study B a control fusion protein (CP) lacking oligomannose residues. At week two the group immunized with OVA-PPM + AbiSCO®-100 had significantly higher antibody titers than the other groups including the group immunized with a fusion protein (CP) conjugate lacking oligomannose residues (Fig. 2B). However OVA-PPM + AbiSCO®-100 was not significantly different from the
OVA + PPM + AbiSCO®-100 group. At week five the group that had received OVA conjugated to PPM + AbiSCO®-100 had significantly higher IgG titers compared to all other groups. The adjuvant effect of PPM was also seen when it was mixed with OVA and AbiSCO®-100 (Fig. 2B). At week eight the OVA-PPM + AbiSCO®-100 group had significantly higher titers compared to all groups except the OVA + AbiSCO®-100 group.

The OVA – PSGL-1/mIgG2b conjugate induces a strong and broad anti-OVA IgG isotype response

The IgG isotype distribution within each group was evaluated at week eight using sera from individual mice (Fig. 2C and D). In study A, the IgG subclasses in the control group (PPM alone) was not analyzed since there were no anti-OVA IgG detected in this group. IgG1 followed by IgG2b was shown to be the most predominant IgG subclasses in all tested groups. IgG2a was found only in the group immunized with OVA-PPM + AbiSCO®-100 with titres of 1:1,000. Interestingly the other AbiSCO®-100 group without the fusion protein had no detectable IgG2a at all. Anti-OVA IgG2b was detected with titers over 1:10,000 in the OVA-PPM + AbiSCO®-100 group that represents about ten times higher titers than in the OVA + AbiSCO®-100 group. The same relationship was seen in the Imject®Alum groups where mice immunized with the fusion protein conjugate had titers of about 1:1,000, while the OVA + Imject®Alum group had anti-OVA IgG2b titers below 1:100. These results suggest that the mannosylated fusion protein supports an anti-OVA IgG2b response. Anti-OVA IgG3 titers were low or undetectable in all examined groups, peaking at a 1:100 titer in the OVA-PPM + AbiSCO®-100 group. The group immunized with OVA-PPM + AbiSCO®-100 had significantly higher IgG1-, IgG2a- and IgG2b titres than all other groups.
In study B (Fig. 2D), the IgG subclasses in the control groups were not analyzed as no anti-OVA IgG were detected in these groups. Supportive of study A, IgG1 followed by IgG2b was shown to be the most predominant IgG subclasses in all tested groups. The OVA-PPM + AbISCO®-100 group had significantly higher IgG1 titers compared to all groups except the OVA + AbISCO®-100 group. IgG2a was again only found in the OVA-PPM + AbISCO®-100 group with titers of 1:500 and this result was significantly higher than in the other groups. IgG2b was detected with titers of 1:8,500 in the OVA-PPM + AbISCO®-100 group, which represents about five times higher titers than in the OVA + PPM + AbISCO®-100 group or the OVA + AbISCO®-100 group and eight times higher titers than the OVA-CP + AbISCO®-100 group. Anti-OVA IgG3 titers were low or undetectable in all examined groups (data not shown). These results suggest that the mannose structures in the fusion protein play a decisive role for an early onset of antibody production, for inducing high antibody titers and also for inducing an anti-OVA IgG2a response. When comparing conjugated OVA with just mixing, conjugation of OVA to PPM appears to give both stronger and broader antibody responses than when OVA is just mixed in with PPM.

**Induction of OVA-specific cytotoxic T-lymphocytes**

To study if the different immunizations were able to induce cellular immune responses with detectable OVA-specificity, lytic activity mediated by cytotoxic T lymphocytes were tested in $^{51}$Cr release assays using *in vitro* primed splenocytes. In this assay specific lysis of $^{51}$Cr-fed TAP-deficient cells (RMAs) loaded with an MHC class I-binding OVA peptide (SIINFEKLF) and induced by peptide-stimulated splenocytes represent the effectiveness of an induced CTL response. The CTL activity was studied in individual mice within all groups (Fig. 3A and B). In study A, a specific lysis of target cells over 10%, was only detectable in groups of mice.
injected with compositions including the AbISCO®-100 adjuvant (Fig. 3A). Interestingly, there was significantly higher specific lysis in the OVA – PPM + AbISCO®-100 group compared to the group that received OVA + AbISCO®-100. At an 80:1 effector:target (E:T) ratio, 25.1±10.5 lysis was detected in mice immunized with OVA + AbISCO®-100 as compared to 46.0±8.7; p<0.05 lysis in the group receiving OVA linked to the mannosylated fusion protein. At an 8:1 E:T ratio, the difference was even more clear with a specific lysis of 5.9±3.9 compared to 29.3±10.6; p<0.05.

In Study B, there was significantly higher specific lysis at E:T ratios of 80:1 and 40:1 in the groups immunized with OVA-PPM + AbISCO®-100 (mean 80.2±12.5; p<0.05 lysis and 73.3±14.8;p<0.05 lysis, respectively) and OVA mixed with PPM + AbISCO®-100 (mean 72.2±16.6;p<0.05 lysis and 62.9±17.0;p<0.05 lysis, respectively) compared to the other groups (Fig. 3B). Although significantly lower than the OVA-PPM + AbISCO®-100 and OVA mixed with PPM + AbISCO®-100 groups, the OVA-CP + AbISCO®-100 group had a significantly higher CTL response (mean 42.8±12.6;p<0.05 lysis) at the 80:1 E:T ratio than the other groups. At the 8:1 E:T ratio, the group that received OVA-PPM + AbISCO®-100 had a mean specific lysis of 45±22.1;p<0.05, which was significantly higher compared to all groups except the OVA + PPM + AbISCO®-100 group (mean 26.4±10.6). These results support the antibody results and again show that the mannose structures in the fusion protein play a decisive role for inducing a broad immune response including a strong CTL response.

*Induction of OVA-specific T-cells producing IFN-γ, IL-2, IL-4 and IL-5*
To further characterize and quantify the type of immune response elicited, we studied the \textit{in vitro} secretion of IFN-\(\gamma\), IL-2, IL-4 and IL-5. Spleen cells from a pool of splenocytes isolated from four to five immunized mice were stimulated for 36 hours with various antigens and the number of cells producing a particular cytokine was assessed by the ELISpot assay.

In study A, IFN-\(\gamma\) producing cells were mainly seen in groups of mice immunized with compositions containing AbISCO\textsuperscript{®}-100 (Fig. 4A). In the OVA + AbISCO\textsuperscript{®}-100 group, IFN-\(\gamma\) producing cells were detected after stimulation with the MHC class I restricted SIINFEKL peptide (1 \(\mu\)g/mL) with up to 500 spot-forming colonies (SFC)/\(10^6\) cells. The corresponding value in the group of mice receiving the OVA-PPM + AbISCO\textsuperscript{®}-100 was over 2,500 SFC/\(10^6\)cells even when using 100 times lower concentration of the peptide (0.01 \(\mu\)g/mL). In the OVA-PPM + AbISCO\textsuperscript{®}-100 group, not only IFN-\(\gamma\) producing CTLs but also Th-cells responded and secreted IFN-\(\gamma\) when stimulated with an OVA-Th peptide or the intact OVA protein. Although it seems that AbISCO\textsuperscript{®}-100 is needed for the mice to mount a cellular immune response including induction of IFN-\(\gamma\) producing cells, the response is clearly higher when OVA is combined with the mannosylated PSGL-1/mIgG\(_{2b}\) than when it is used alone. Negative controls (FILKSINE, BSA and medium alone) did not induce any production of IFN-\(\gamma\) and the positive control (Con-A) showed similar responses between groups.

In study B, the IFN-\(\gamma\) response was shown to be highest in the group of mice immunized with OVA-PPM + AbISCO\textsuperscript{®}-100, which was the only group that responded to the OVA-Th peptide (Fig. 4B). The response to the SIINFEKL peptide was similar in both groups that received OVA and PPM (conjugated or mixed) + AbISCO\textsuperscript{®}-100. The group that received OVA conjugated to CP + AbISCO\textsuperscript{®}-100 had about 500 spots less at 1\(\mu\)g/mL SIINFEKL than these two groups, while the OVA + AbISCO\textsuperscript{®}-100 group had about 1,000 spots less. The OVA-CP + AbISCO\textsuperscript{®}-100 group showed a similar response to the group that received OVA.
+ AbISCO®-100 alone, except in case of the SIINFEKL peptide. Negative controls (FILKSINE, BSA and medium alone) did not induce any production of IFN-γ and the positive control (Con-A) showed similar responses between groups.

In study A, the differences observed with regard to the number of IFN-γ secreting cells were also seen when the number of IL-2 producing cells was assessed (Fig. 5A). Both stimulation with the MHC class II-binding OVA peptide and the MHC class I-binding peptide at a concentration of 1 μg/mL induced over 1,000 SFC when splenocytes from mice immunized with the mannosylated PSGL-1/mIgG2b conjugate were used. In the OVA + AbISCO®-100 group, few (under 100 SFC) or no IL-2 producing SFC were detected. All controls behaved as expected.

In study B, the differences observed between the groups with regard to the number of IFN-γ secreting cells was not as clear when the number of IL-2 producing cells was assessed (Fig. 5B). The group that received the OVA conjugated to PPM + AbISCO®-100 again showed the highest response overall and was the only group that responded to the T-helper peptide. However, the other AbISCO®-100 and OVA containing groups showed similar responses when stimulated with OVA protein, MHC-I- (SIINFEKL) or MHC-II- (Th-peptide) binding peptide. All controls behaved as expected.

In relation to the Con-A control, the IL-4 ELISpot assay revealed fewer SFC as compared to both the IFN-γ and IL-2 ELISpot (Supplementary Fig. 1A and 1B). There was also a slightly higher background with up to 50 SFC in the medium controls. However, there was a slight increase in the number of splenocytes secreting IL-4 in the OVA-PPM +
AbISCO®-100 group following stimulation with recombinant OVA and the OVA Th peptide (1-10 μg/mL) suggesting a weak Th-2 activation.

IL-5 producing cells were detected in low numbers and only when using OVA concentrations of 25-625 μg/mL (Supplementary Fig. 2A and 2B).

Proliferation

In an attempt to compare the proliferative responses between the groups, a ³H-thymidine proliferation assay was performed. In this assay, the ratio between the cpm value of splenocytes stimulated with the different antigens (in triplicate) and the cpm value of splenocytes incubated in medium alone was first calculated. These values were then compared to the proliferation seen in the control group immunized with PPM alone. Each graph describes the proliferative responses from a pool of splenocytes isolated from four mice within each group. The experiment was repeated twice with consistent results. The analysis revealed that OVA-specific proliferation was detectable mainly in the groups of mice immunized with antigen compositions containing AbISCO®-100 (Fig. 6). Consistent with the detection of anti-OVA antibodies, lytic CTLs and cytokine-producing (IFN-γ, IL-2) T-cells, the proliferative responses were higher when the OVA-PPM conjugate was used (Fig. 6). Alum did not have any effect on the CD4+ T-cell proliferation.
Numerous strategies have been suggested aiming at developing vaccine compositions targeting antigen presenting cells (APC) in order to improve antigen immunogenicity and elicit a Th1 response with the development of cytotoxic T lymphocytes (CTLs). The ability to generate CTL responses, and the killing of tumor cells and cells harboring intracellular pathogens, is maybe the most important feature of a therapeutic vaccine. One APC-directed strategy involves targeting mannose-binding receptors on macrophages and dendritic cells in order to improve vaccinogen uptake and MHC presentation (reviewed in [4, 32]). The engineered mucin-type immunoglobulin fusion protein, which when expressed in *Pichia pastoris* carried O-glycans comprised of linear oligomannose structures with up to nine residues and bound the mannose-specific receptors MMR, DC-SIGN and mannose-binding lectin (MBL) with high affinity [31], was evaluated here with regard to its effect on humoral and cellular anti-OVA immune responses *in vivo.*

In combination with AbiSCO®-100, the OVA – mannosylated PSGL-1/mIgG2b conjugate elicited a significantly faster and stronger antibody response compared to when OVA alone was used as antigen. Mannosylated PSGL-1/mIgG2b improved the anti-OVA IgG response also without AbiSCO®-100, but the response was weaker. The anti-OVA response was broader with regard to the IgG subclasses being induced and only the OVA – mannosylated PSGL-1/mIgG2b conjugate with AbiSCO®-100 induced an IgG2a response. IgG1 was the predominant IgG subclass detected indicating a strong Th2 type of immune response. IgG2a and IgG2b antibody titers were, however, only detectable after inclusion of AbiSCO®-100 and was stronger in the OVA – mannosylated PSGL-1/mIgG2b + AbiSCO®-100 groups. These IgG subclasses would indicate a Th1 immune profile. The Th1 response is further evidenced by the generation of a strong OVA-specific CTL response and increased numbers
of IFN-\(\gamma\) and IL-2 producing splenocytes in groups immunized with the OVA – fusion protein conjugate in the presence of AbiSCO\(^\circ\)-100. Increased CD4\(^+\) T-cell proliferation was also found, further confirming the ability of the OVA – mannosylated PSGL-1/mIgG\(_{2b}\) + AbiSCO\(^\circ\)-100 combination to strongly activate the immune system.

The fact that mannosylated PSGL-1/mIgG\(_{2b}\) binds to MMR, DC-SIGN and MBL in vitro [31], and that the OVA – fusion protein conjugate triggered a faster and stronger anti-OVA IgG response than OVA alone (Fig. 2), suggests that the mannosylated mucin-type fusion protein also without AbiSCO\(^\circ\)-100 has an immune-stimulating effect. It is clear from the data though that addition of AbiSCO\(^\circ\)-100 is required in order to generate an anti-OVA CTL response (Fig. 3). But also in the presence of AbiSCO\(^\circ\)-100, the OVA – fusion protein conjugate triggered a stronger anti-OVA CTL response than OVA alone (Fig. 3). The AbiSCO\(^\circ\)-100 adjuvant is an immunostimulatory complex matrix (ISCOMATRIX\(^\text{TM}\)) consisting of a selection of purified fractions of Quillaja saponins formulated with a mixture of cholesterol and phosphatidyl choline. This adjuvant is known to induce a broad immune response with strong antigen-specific cellular and humoral immune responses, and have been tested with numerous antigens both in humans and veterinary vaccine designs [33-35]. The adjuvant mechanisms of ISCOMATRIX\(^\text{TM}\) include the capability of antigen presentation by both MHC class I and MHC class II pathways [36] and the production of pro-inflammatory cytokines such as IL-12, IL-1, IL-6, IL-8 and IFN-\(\gamma\) with subsequent recruitment of lymphocytes [37, 38]. A recent study demonstrates that when OVA was incorporated in ISCOMATRIX forming ISCOMs this lead to a potent immune activation and antigen delivery to CD8\(^{a+}\) DCs \textit{in vivo} with effective cross-priming of CD8\(^{+}\) T cells and subsequent efficient induction of CTL responses [39]. In our study the synergistic effect between the mannosylated fusion protein and AbiSCO\(^\circ\)-100 was also reflected in the higher frequency of IFN-\(\gamma\)- and IL-2-producing splenocytes recovered from mice immunized with the conjugate.
plus AbiSCO®-100 compared to OVA alone plus AbiSCO®-100 (Fig. 4 and 5, respectively). The results obtained suggest that the mannosylated fusion protein improve the immunogenicity of the conjugated OVA, but that addition of AbiSCO-100® had a synergistic effect. Our hypothesis is that AbiSCO-100® induces a local inflammatory reaction that stimulates recruitment and activation of antigen-presenting cells at the site of injection, and that conjugating OVA to the mannosylated mucin-type fusion protein improves antigen uptake by APC. Other studies with mannosylated liposomes have shown that there is a need for the addition of either Quil A, Alum or TLR-ligands as adjuvant in order to induce a potent activation of immune cells and upregulation of costimulatory receptors such as CD80 and CD86 [40, 41]. However, mannosylated dendrimers have been shown to induce maturation of bone marrow DCs and to upregulate CD80, CD86 and CD40 [42]. They further demonstrated in a B16-OVA tumor model that tumors in mice pre-immunized with mannosylated OVA dendrimers did not grow, or displayed a more delayed onset and had slower kinetics of growth, than those of OVA-immunized mice [42]. The same group also published a report suggesting that there is, in fact, a concomitant need for TLR signaling for optimal function of DC subsets in antigen localization, processing and presentation [43].

Mannose receptor-mediated uptake of antigen has been shown to improve T-cell presentation a 100-fold compared to fluid phase uptake [44]. Similarly, antigen uptake by the endocytic receptor DC-SIGN has been shown to direct antigen to the late endosomal/lysosomal compartments and improve CD4+ T-cell presentation [45]. Although mannose-specific endocytic receptors may facilitate the transport of OVA to the compartments where antigen processing and MHC loading can occur, other processes may be involved which governs MHC loading. For example, it has been shown that the efficiency of antigen presentation on MHC class II molecules is dependent on the co-occurrence of Toll-like receptor (TLR) ligands and antigen in the same phagosome [46]. Furthermore, it has
been argued that TLR signaling might influence phagosome maturation in such a way as to remodel the late endosomal/lysosomal compartments for efficient antigen processing and MHC II loading [47]. The question remains whether the O-glycan oligomannoses of the fusion protein are able to directly engage TLR:s. There are reports on TLR4 recognizing mannans from *Saccharomyces cerevisiae* and *Candida albicans* [48], and that short linear O-linked mannans of *C. albicans* are recognized by TLR4 and induce proinflammatory cytokine production, such as TNF-α [49]. Though a recent study showed that only some *C. albicans* strains were recognized by TLR4 [50].

A role for mannose-binding receptor targeting and enhanced antigen uptake is also suggested by the fact that O-glycan oligomannoses are required on PSGL-1/mIgG2b for an optimal immune-stimulating effect. When OVA was conjugated to a fusion protein expressed in CHO cells and carrying mono and disialylated core 1 structures, weaker humoral and cellular anti-OVA responses were detected. When comparing conjugated OVA with just mixing, conjugation of OVA to mannosylated PSGL-1/mIgG2b appear to give more rapid, stronger and broader antibody responses than when OVA is just mixed with mannosylated PSGL-1/mIgG2b.

Antigen-specific CTL activities are important for control of virus infected cells and tumors [51-53]. Recombinant antigens frequently do not elicit CTL responses, possibly due to low incidence of MHC I presentation for exogenously internalized antigens [54]. However, under certain conditions and with some antigens cross-presentation may be more pronounced, which could serve to improve CD8+ T cell activation [55]. When conjugated to OVA and if given together with AbiSCO®-100, the mannosylated fusion protein appears to be able to skew the anti-OVA response towards a Th1 response and the generation of OVA-specific CTL:s. In addition, IgG2a antibody titers were only detectable in the group that received the
OVA – mannosylated PSGL-1/mIgG2b conjugate together with AbiSCO®-100. This suggests that OVA peptides may be more efficiently cross-presented when the OVA – mannosylated fusion protein conjugate is processed in APC. Alternatively, the conjugate stimulates cytokine (IL-12) secretion from APC that potentiates differentiation of activated Th cells to Th1 cells. Oxidized mannan coupled to a synthetic version of MUC1 has been found to activate macrophages leading to IL-12 secretion [13]. Other in vitro studies have found that OVA, when carrying multiple O-glycosylation sites and expressed in P. pastoris, is more potent in inducing CD8+ T-cell proliferation than when P. pastoris-expressed OVA carries mixed N- and O-glycans or N-glycans alone [16]. The majority of PSGL-1/mIgG2b glycans are O-glycans. Hence, extensive O-mannosylation may be particularly important for eliciting Th1 type of responses. Because the O-glycans of the mannosylated OVA used in the previous study were not characterized, it is difficult to try to identify an O-glycan determinant responsible for this effect. Collectively, the characterizations of O-glycans derived from P. pastoris-produced glycoproteins performed so far have demonstrated diversity and to suggest that P. pastoris-derived O-glycans have similar structures on different proteins may be misleading. P. pastoris O-glycans include Hex2, 3 structures, with or without phosphorylation, α1,2 and/or α1,3 glycosidic linkages, as well as terminal or subterminal mannoses linked by β1,2 glycosidic linkages [31, 56, 57]. We have shown with surface plasmon resonance techniques that PSGL-1/mIgG2b binds with similar high binding affinities to recombinant MBL, MR and DC-SIGN [31]. These results indicate that all of these receptors might be targeted in vivo. However, the specific signaling from one receptor and its contribution to subsequent events leading to the final immunological outcome of ligand binding is hard to assess. In one study MR−/− mice were used to demonstrate that the mannose receptor could direct soluble OVA for cross-presentation by dendritic cells suggesting that MR may have contributed to the enhanced CTL-activities observed in this study [20]. This is also supported
by other studies, which have suggested that targeting the MR by MUC1 coupled to oxidized mannan was important for obtaining high frequency anti-MUC1 CTL responses [58]. On the other hand, cross-talk with TLR:s by for example MBL and/or DC-SIGN may also gear the adaptive immune response towards a Th1 reaction making it difficult to assign one particular receptor to the final immunological outcome.

In addition to the mentioned receptors, other lectins may also be involved. For example, Dectin-1 belongs to the C-type lectins like MBL, MR and DC-SIGN and has been shown to bind cell wall components and beta-glucans of fungal pathogens including C. albicans [59]. Dectin-1 can induce DC maturation, which subsequently may potentiate the differentiation of naïve CD4+ T cells to IL-17 secreting Th17cells important for anti-fungal responses [60, 61]. It is interesting to speculate that Dectin-1 may be involved in the shaping of the anti-OVA immune responses observed in the present study. However, it has been noted that Th1-associated cytokines repress Th17-differentiation in the mouse. Consequently, the anti-OVA Th1 type of responses elicited by the OVA-PPM conjugate in this study would contradict involvement of Dectin-1 [62, 63]. In addition, the O-glycans of P. pastoris derived PSGL-1/mIgG2b are not identified as ligands for Dectin-1 [31, 59]. Assaying for IL-23 and/or IL-17 amongst the splenocytes and lymph node cells would perhaps reveal involvement of Th17 cells and the Dectin-1 receptor.

In conclusion, we have shown that that the mannose structures in the fusion protein play a decisive role for inducing a broad immune response with a rapid and strong antibody response and a strong CTL response. When comparing conjugated OVA with just mixing, conjugation of OVA to mannosylated PSGL-1/mIgG2b appear to give a more rapid, stronger and broader antibody response than when OVA is mixed with mannosylated PSGL-1/mIgG2b. Hence this study demonstrates that conjugation of P. pastoris produced PSGL-1/mIgG2b to an antigen may indeed improve the antigen specific immune responses in vivo. Conjugation of
the mannosylated fusion protein to an antigen may be particularly interesting when a Th1 type of response is desired as in the cases of vaccination against virus infections and cancer.

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**Figure legends**

**Fig. 1.** Schematic picture of the experimental design and the different read-outs used in study A and B.

**Fig. 2.** Serum IgG antibody titers against OVA in study A (A) and B (B). Titres are given as mean +SD (n=8-9 in study A and 8-10 in study B) and are defined as the reciprocal endpoint dilution giving an optical density at 450 nm of 3 times (A) or ≥0.2 above (B) the background value. In study A (A), the asterisk (**) indicates a significantly higher IgG titer compared to all other groups and (*) indicates a significantly higher IgG titer compared to all groups except OVA-PPM + AbISCO-100 at that time point. In study B, the asterisk (*) indicates a significantly higher IgG titer compared to all other groups. P<0.05.

Serum IgG-isotype antibody titers against OVA in study A (C) and B (D). Titres are given as mean +SD (n=8-9 in study A and 8-10 in study B) and are defined as the reciprocal endpoint dilution giving an optical density at 450 nm of 3 times (C) or ≥0.2 above (D) the background value. The asterisk (**) indicates a significantly higher IgG titer compared to all other groups. P<0.05. An (*) indicates a significantly higher IgG titer compared to all groups except the group indicated N.S. (non significant) at that time point. P<0.05.

**Fig. 3.** Detection of OVA peptide-specific cytolytic activity of splenocytes isolated from the different groups of mice and restimulated *in vitro* with an MHC class I-binding OVA peptide (SIINFEKL) in study A (A) and B (B). The results are presented as mean percentage specific lysis (n=8-9 in study A and n=4-5 in study B) of peptide coated RMA-S cells. The asterisks (**, *) indicates a statistical difference compared to the other groups at the specific effector:target ratio. P<0.05. At effector:target ratio 8:1 in study B an (*) indicates a
significantly higher specific lysis compared to all groups except the group indicated N.S. (non significant). P<0.05.

**Fig. 4.** Quantification of OVA-specific, $\gamma$-IFN-producing spot forming cells (SFC) after a 36 hour *in vitro* stimulation of splenocytes (pooled from 4-5 spleens) with indicated antigens [study A (A) and B (B)]. Data has been given as SFC/10$^6$ splenocytes. More SFC than the cut-off of 50 SFC/10$^6$ cells indicates a positive response.

**Fig. 5.** Quantification of OVA-specific, IL-2-producing spot forming cells (SFC) after a 36 hour *in vitro* stimulation of splenocytes (pooled from 4-5 spleens) with indicated antigens [study A (A) and B (B)]. Data has been given as SFC/10$^6$ splenocytes. More SFC than the cut-off of 50 SFC/10$^6$ cells indicates a positive response.

**Fig. 6.** Proliferative OVA-specific responses after a 68 hour stimulation of splenocytes (pools) with indicated antigens. The values in each group, presented as the S/N (sample to negative) ratio, indicates the difference between antigen-induced and spontaneous proliferation.
Fig. 1
Fig. 2

A

Mean OVA IgG antibody titre

OVA
OVA-PPM
OVA + AbISCO-100
OVA-PPM + AbISCO-100
OVA + Alum
OVA-PPM + Alum
PPM

W 2
W 5
W 8

B

Mean OVA IgG antibody titre

OVA-PPM + AbISCO-100
OVA-CP + AbISCO-100
OVA + AbISCO-100
OVA + PPM + AbISCO-100
PPM + AbISCO-100
PBS

W 2
W 5
W 8

C

Mean OVA IgG antibody titre

IgG1
IgG2a
IgG2b
IgG3

D

Mean OVA IgG antibody titre

IgG1
IgG2a
IgG2b

Fig. 2
Fig. 3

A

B

% Specific Lysis

effector: target ratio

OVA
OVA-PPM
OVA + AbISCO-100
OVA-PPM + AbISCO-100
OVA + Alum
OVA-PPM + Alum
PPM

* N.S.

**

OVA-PPM + AbISCO-100
OVA + AbISCO-100
OVA + PPM + AbISCO-100
PPM + AbISCO-100
PBS

**

*
Number of IFNγ-producing cells per 10^6 cells

Fig. 4A
Fig. 5A
Fig. 5B
Fig. 6
In vitro antigen

Media
BSA 2.5 μg/mL
FILKINE 1 μg/mL
PPM 17 μg/mL
OVA-PPM 5 μg/mL
OVA 5 μg/mL
OVA 2.5 μg/mL
OVA 12.5 μg/mL
OVA 62.5 μg/mL
OVA-Th 0.01 μg/mL
OVA-Th 1 μg/mL
OVA-Th 10 μg/mL
SIINFEKL 0.01 μg/mL
SIINFEKL 0.1 μg/mL
SIINFEKL 1 μg/mL
Con-A 0.5 μg/mL

In vitro antigen

OVA
OVA-PPM
OVA + AbISCO-100
OVA + Alum
OVA-PPM + Alum
PPM

Number of IL-4-producing cells per 10⁶ cells

Supplementary Fig. 1A
Supplementary Fig. 1B

Number of IL-4-producing cells per 10⁶ cells
Supplementary Fig. 2A
Number of IL-5-producing cells per 10⁶ cells

Supplementary Fig. 2B