Comparative Study of Using Different Yeast Genera as Vehicles for Protein Delivery to Antigen-Presenting Cells

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Abbreviations

amp^R	ampicillin resistance gene (β-lactamase)
aox	alcohol oxidase
APC	antigen-presenting cell
APS	ammonium persulfate
ars	autonomous replication sequence
ATP	adenosine triphosphate
BMG	buffered minimal glycerol
BMM	buffered minimal methanol
bp	base pairs
BSA	bovine serum albumin
CD(#)	cluster of differentiation (number)
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CO_2	carbon dioxide
Cy7	cyanine dye 7
Da	Dalton
DC	dendritic cell
DOC	deoxycholate
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	deoxynucleoside triphosphate
d/o	dropout
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
eGFP	enhanced green fluorescent protein
EMM	Edinburgh minimal medium
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour(s)
HBSS	Hank's buffered salt solution
HCMV	human cytomegalovirus
his	histidine
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranosid

kan^R	kanamycin resistance gene (aminoglycoside 3'-phosphotransferase)
kb	kilobase
kDa	kilodalton
KRE	killer resistant
LB	Luria Bertani
leu	leucine
LPS	lipopolysaccharide
M	molar
Mg^{2+}	magnesium
Mb	mega base pairs
MCS	multiple cloning site
MHC	major histocompatibility complex
mRNA	messenger RNA
nmt	no message in thiamine
OD	optical density
ORF	open reading frame
ori	origin of replication
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBSE	phosphate-buffered saline, EDTA
PBSES	phosphate-buffered saline, EDTA, sorbitol
PCR	polymerase chain reaction
PE	R-phycoerythrin
PEG	polyethyleneglycol
PerCP	peridinin chlorophyl protein
PGK	phosphoglycerate kinase
PMA	phorbol myristate acetate
PMSF	phenylmethanesulfonyl fluoride
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
RLU	relative luminescence units
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
s	second(s)
s.c.	subcutaneously
SC	synthetic complete
SD	standard deviation
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis

SOC	Super Optimal Broth with Glucose
SOE	splicing by overlapping extention
Taq	*Termus aquaticus*
TBE	tris, boric acid, EDTA
TBS	tris-buffered saline
TCA	2,2,2-trichloroacetic acid
TCR	T cell receptor
TE	tris, EDTA
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	tumor necrosis factor
Tris	tris-(hydrohymethyl)-aminomethane
ura	uracil
U	unit(s)
UV	ultraviolet
VLP	virus-like particles
v/v	volume per volume
w/o	without
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YCB	yeast carbon base
YEp	yeast episomal plasmid
YNB	yeast nitrogen base
YPD	yeast extract pepton dextrose
YPGal	yeast extract peptone galactose

ABSTRACT

The use of yeasts as vehicle for protein antigens has been demonstrated to be a highly effective vaccination approach. In part, this can be attributed to the intrinsic adjuvant properties of yeast cell wall components. Moreover, the correct processing of recombinantly expressed proteins and the safety status of many yeast genera has encouraged the onset of preclinical and clinical trials using yeast vectors. However, the vast majority of such studies focused the attention on yeasts of the genus *Saccharomyces* as candidate T cell vaccine. In this work, different yeast genera were evaluated as potential antigen carrier in view of the development of novel yeast-based vaccines. For this purpose, yeasts were initially assessed for their ability to induce maturation and activation of human dendritic cells. Next, the internalization profile of selected yeasts by mammalian phagocytes was analyzed, as well as the involvement of pattern recognition receptors in the uptake process. Subsequently, yeasts engineered to express foreign proteins were assessed for their antigen delivery capacity. *In vitro* antigen presentation and *ex vivo* whole blood assays showed that recombinant yeast genera differently activate antigen-specific T cells. Furthermore, antigen localization played a decisive role in T cell activation. The data presented here strongly support the potential of recombinant yeast in the development of novel vaccine strategies in order to induce antigen-specific T cell responses.

Der Einsatz von Hefen als Vehikel für Proteinantigene stellt eine vielversprechende Vakzinierungsstrategie dar, was u.a. auf adjuvante Eigenschaften der Hefe-Zellwandkomponenten zurückzuführen ist. Weiterhin haben der Nachweis der korrekten Prozessierung rekombinanter Proteine und der unbedenkliche Status vieler Hefegattungen ihren Einsatz in präklinischen und klinischen Studien gefördert. Bislang hat sich die Mehrzahl dieser Studien auf Hefen der Gattung *Saccharomyces* als Vakzinkandidaten für zellvermittelte Immunantworten konzentriert. Im Rahmen dieser Arbeit wurden verschiedene Hefegattungen als potentielle Antigenvehikel zur Etablierung neuartiger Hefe-basierter Vakzinen untersucht. Zunächst wurden Ausreifung und Aktivierung von Dendritischen Zellen durch diverse Hefegattungen analysiert. Danach wurden sowohl die Aufnahme bestimmter Hefegattungen durch Säuger-Phagozyten als auch die Beteiligung spezifischer Rezeptoren in diesem Prozess untersucht. Anschließend wurde die Fähigkeit rekombinanter Hefen zum Antigen „Delivery“ evaluiert. Durch *in vitro* Antigenpräsentation und *ex vivo* Vollblut-Assays konnte gezeigt werden, dass verschiedene Hefegattungen Antigen-spezifische T-Zellen unterschiedlich aktivieren. Des Weiteren spielt die Antigenlokalisierung eine wichtige Rolle bei der T-Zellaktivierung. Die vorliegenden Ergebnisse unterstreichen das Potenzial rekombinanter Hefen bei der Entwicklung neuartiger Impfstrategien zur Induktion Antigen-spezifischer T-Zell-Immunantworten.

CONTENTS

I. INTRODUCTION

1. The immune system – a short overview

The immune system consists of a set of molecules, cells, tissues, and organs that work together to provide protection against foreign organisms. The initial phases of the host defense against infection depend on the mechanisms of innate immunity. It comprises epithelial barriers, phagocytic cells (dendritic cells (DCs), macrophages), soluble factors, such as complement proteins, granulocytes (basophils, eosinophils and neutrophils), mast cells, natural killer cells, and cytokines that coordinate the functions of the cells of the innate immunity. The innate immunity is always present in all individuals, reacts rapidly and equally to repeated infections (without developing an immunologic memory with repeated exposure to a given pathogen), and is able to distinguish between a group of related pathogens. Adaptive immunity is a response to antigen mediated by $CD4^+$ and $CD8^+$ T lymphocytes and B lymphocytes. Adaptive immune responses develop more slowly and are characterized by specificity for distinct molecules and development of immunological memory, which is the capability of responding more vigorously to repeated exposure to the same antigen (Janeway *et al*., 2001; Abbas *et al*., 2007).

Adaptive immunity can be divided into humoral and cell-mediated immune responses, which have different mediators and functions. In the humoral immunity, antibodies produced by B lymphocytes recognize microbial antigens and contribute to immunity in three main mechanisms: (i) neutralization, *i.e*. preventing pathogens from entering cells; (ii) opsonization, in which antibodies coat the pathogen surface promoting particle phagocytosis via recognition of a portion of the antibody (Fc) by a Fc receptor in the phagocytic cell; and (iii) complement activation, which results in binding of complement proteins to the microbe, promoting either enhanced opsonization, recruitment of phagocytes to the site of infection, or microbe lysis via pore formation. Cell-mediated immunity involves the action of T lymphocytes, which induce destruction of pathogens residing in phagocytic cells or killing of infected cells. T lymphocytes comprise functionally different subpopulations such as helper T cells, cytotoxic T lymphocytes (CTLs) and regulatory T cells. Helper T cells, upon antigenic stimulation, produce cytokines that promote the proliferation and differentiation of T cells and activate, among other cell types, macrophages and B lymphocytes. CTLs kill

cells displaying foreign antigens, such as cells infected with virus or intracellular pathogens. Regulatory T cells are mainly involved in inhibiting immune responses (Janeway *et al*., 2001; Abbas *et al*., 2007).

Protective immunity against a specific pathogen can be elicited upon exposure to the microbe, in a process called active immunization, or by transferring serum or lymphocytes from an immunized host without any contact of the recipient with the antigen, a process named passive immunization (Abbas *et al*., 2007).

1.1. Antigen recognition and presentation to T lymphocytes

Antigen-presenting cells

Antigen-presenting cells (APCs) are specialized elements which take up and display antigens to T cells. DCs, macrophages and B cells are called professional APCs, since they express MHC molecules and co-stimulators. However, DCs are considered the most effective APCs as they are the only cell type which can stimulate naïve T cells and, consequently, initiate T cell responses (Abbas *et al*., 2007).

DCs comprise a heterogeneous cell population derived from bone marrow precursors and are widely distributed, in an immature state, into lymphoid and nonlymphoid tissues. In peripheral tissues, immature DCs sense and capture microbes and other antigens. After engagement of antigens with receptors, as well as detection of pro-inflammatory cytokines, DCs migrate to lymph nodes and undergo maturation. Maturation is characterized by reduced ability in internalizing antigens but increased antigen presentation capacity. Furthermore, MHC class II molecules are redistributed from intracellular compartments to the cell surface, and the expression of costimulatory molecules (such as CD80, CD86), MHC class I, and T cell adhesion molecules (*e.g*. CD58) is upregulated. DCs also adjust their profile of chemokine receptors that enable homing to lymphoid organs. Besides, the cells show “dendritic” projections, which enable a large contact surface to the surroundings and may enhance the odds for T cell interaction. In lymphoid organs, mature DCs stimulate naïve T cells priming immune responses. DCs are able to elicit distinct types of T cell responses, depending on their lineage, maturation status and activation signals (Huang *et al*., 2001; Liu *et al*., 2001; Mellman and Steinman, 2001; Guermonprez *et al*., 2002; Reis e Sousa, 2006).

Immature DCs and macrophages can internalize antigens by distinct mechanisms. Antigen capture via receptor-mediated endocytosis enables efficient

antigen delivery to the processing compartment. Antigens that do not associate with cell surface receptors can be internalized by fluid phase pinocytosis and presented by APCs, although with lower efficiency. Fluid phase uptake can happen through different means: (i) micropinocytosis, in which clathrin-coated pits invaginate to form small vesicles (< 0.1 μm). Soluble proteins that enter the vesicles associate with receptors in the coated pit, being further trafficked to lysosomes and degraded; and (ii) macropinocytosis, in which small particles enter larger vesicles (0.5-3 μm) formed at sites of membrane ruffling mediated by the actin cytoskeleton. Micropinocytosis is a constitutive process which occurs in nearly all cells, whereas macropinocytosis is restricted to distinct cell types (APCs), for example macrophages and immature DCs (Sallusto *et al*., 1995, Steinman and Swanson, 1995, Lam *et al*., 2007).

In phagocytosis, particles larger than 1.0 μm are internalized in a receptor triggered, actin-based mechanism which involves membrane extension or addition in order to create an intracellular acidified compartment (Morrissette *et al*., 1999). After internalization, phagosomes undergo maturation by a sequence of fission and fusion events with components of the endocytic pathway, resulting in mature phagolysosomes (Aderem and Underhill, 1999).

A number of surface receptors has been reported to recognize pathogen-associated molecular patterns (PAMPs) present on microorganisms. These so-called pattern-recognition receptors (PRRs) recognize, among others, bacterial liposaccharides, unmethylated CpG motifs of bacterial DNA, viral double-stranded RNA, β-glucans, chitin, mannans, and fungal nucleic acids. PAMPs are highly conserved structures, since they are indispensable for the survival of the microorganisms. Recognition by PRRs results in microbial ingestion and killing through respiratory burst, among other mechanisms. PRRs activate signaling pathways, stimulating several cellular responses, such as cytokine and chemokine production, and therefore connect innate and adaptive immunity (Figdor *et al*., 2002; Tsoni and Brown, 2008; Brown, 2011; Romani, 2011). A broad variety of PRRs help in the identification of potential pathogens, for example Toll-like receptors (TLRs), C-type lectin receptors (CLRs), integrins, or scavenger receptors (Brown, 2011). The observation that glycosylated protein antigens are more efficiently internalized than nonglycosylated ones indicates that CLRs are abundantly expressed in DCs (Yokota *et al*., 2001; Figdor *et al*., 2002). Macropinocytosis and mannose receptor-mediated endocytosis lead to antigen delivery to MHC class II-containing compartments, but particles engulfed via

mannose receptors are considerably more effective at increasing T cell proliferation (Tan *et al*., 1997; Lam *et al*., 2005).

Antigen processing and presentation

Antigen peptides recognized by T cells are displayed by specialized glycoproteins that are encoded by genes of the major histocompatibility complex (MHC), which fall into two classes, I and II. MHC classes I and II molecules exhibit different structures, functions and distribution patterns among cells. Each MHC molecule can associate with a broad variety of peptide antigens, enabling the formation of diverse peptide-MHC complexes. Therefore, the immune system can specifically react to a large diversity of challenges (Madden, 1995; Klein and Sato, 2000; Janeway *et al*., 2001; Abbas *et al*., 2007).

Basically, MHC molecules consist of a peptide-binding groove, an IgG-like region, a transmembrane domain, and a cytoplasmic region (Abbas *et al*., 2007). MHC class I molecules consist of two polypeptide chains, a heavy chain (α chain) which spans the membrane, and the non-covalently attached β_2-microglobulin (β_2m; Bjorkman *et al*., 1987). These molecules are expressed by almost all nucleated cells, but the expression level varies according to the tissue. In contrast, MHC class II molecules are generally found on a subgroup of immune cells, such as DCs, macrophages, B lymphocytes, thymic epithelial cells (Klein and Sato, 2000). MHC class II molecules are composed of two transmembrane polypeptide chains, α and β, which are noncovalenty associated (Janeway *et al*., 2001).

In order to enable proper T cell recognition, MHC molecules must be capable of retaining the bound peptide for a sufficient time period. Even though such interaction normally requires high specificity, peptide-MHC binding is both stable and promiscuous (Madden, 1995).

Generally, MHC class I molecules present peptides derived from degraded proteins synthesized by the cell or entering the cytosol, such as tumor antigens or proteins encoded by intracellular pathogens. Since MHC class I is expressed by the majority of cells, these can be inspected by circulating $CD8^+$ cytotoxic T cells for potential infection or improper protein expression. Cellular proteins are degraded in the cytosol, essentially by the proteasome, and the resulting peptides are translocated by TAP (transporter associated with antigen processing) into the lumen of the endoplasmic reticulum (ER), where an aminopeptidase trims the peptides down to a length of 8-10

amino acids for association with class I molecules. After this association, the MHC class I molecule completes its folding and the peptide-MHC I complex leaves the ER, being transported via Golgi apparatus to the cell surface, where it can interact with $CD8^+$ T cells (Germain and Margulies, 1993; Ackerman and Cresswell, 2004; Cresswell *et al*., 2005).

Exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins are degraded within acidified vesicular compartments, giving rise to peptides that can associate with MHC class II molecules. MHC II molecules are expressed mainly by B cells, DCs, and monocytes/macrophages. MHC II molecules assemble as αβ heterodimers in the ER and associate with an invariant chain (Ii), preventing the binding of ER peptides. An Ii peptide called CLIP (class II-associated invariant-chain peptide) occupies the peptide-binding groove. Further, in MHC class II-containing compartments, Ii undergoes degradation and CLIP is removed afterwards so that a high-affinity peptide can bind (reviewed by Rocha and Neefjes, 2008). Peptide-MHC-II complexes travel to the cell surface and interact with specific $CD4^+$ T cells. Peptides generated from degradation of internalized exogenous antigens normally do not bind to MHC class I molecules, preventing that CTL lyse normal cells which may have engulfed antigens from tumor or virus-infected cells (Harding and Geuze, 1992; Germain and Margulies, 1993; Rodriguez *et al*., 1999; Ackerman and Cresswell, 2004).

In macrophages and DCs, exogenous antigens can escape from vacuolar compartments into the cytosol and enter the endogenous pathway for processing and presentation by MHC-I molecules (Fig. 1). This process of providing engulfed proteins access to proteasomes and their resulting oligopeptides entry into the MHC class I processing machinery is known as “cross-presentation” and constitutes a requirement for induction and expansion of $CD8^+$ T cell responses (Harding and Song, 1994; Rodriguez *et al*., 1999; Ackerman *et al*., 2005; Wadle *et al*., 2006).

Once antigens are found in phagosomes, different mechanisms can lead to MHC class I presentation. In the phagosome-to-cytosol pathway, the antigen escapes from the phagosome into the cytosol in an unknown process and is degraded by proteasomes. Some of the generated peptides are then transported by TAP and loaded onto MHC class I molecules in the ER or “ER-phagosome” vesicles. Newly synthesized MHC class I molecules transport peptides to the cell surface for further presentation. Several evidences suggest that the phagosome-to-cytosol pathway is the main one operating *in vivo*. In the vacuolar pathway, antigenic peptides are generated from internalized

antigens hydrolysed by proteases in the phagosome and then associate with recycling MHC class I molecules. A third mechanism has been proposed, the "endosome to ER pathway", which would explain the cross-presentation of soluble proteins. It has been reported that some exogenous soluble proteins can be internalized by DCs and transported into the ER. Since proteins in the ER can gain access to the cytosol for degradation by the ER-associated degradation pathway (ERAD), this mechanism would elucidate the phenomenon of cross-presentation of soluble proteins (reviewed by Rock 2003; Rock and Shen, 2005).

The process of exogenous antigen capture by APCs, processing into the MHC class I presentation pathway, and subsequently priming of CTL is termed 'cross-priming' (Heath and Carbone, 2001). Cross-priming is considered to be inefficient, but it has been postulated that dendritic cells cross-prime $CD8^+$ T cells with more efficiency than other APCs. Furthermore, particulate antigens elicit, in general, more efficient cross-priming than their soluble counterparts. Cross-presentation is desirable in vaccine approaches based on proteins that aim at the generation of $CD8^+$ T cell responses. Therefore, numerous attempts to enhance the effectiveness of this process have been made in the field of vaccine development (Maecker *et al*., 2001).

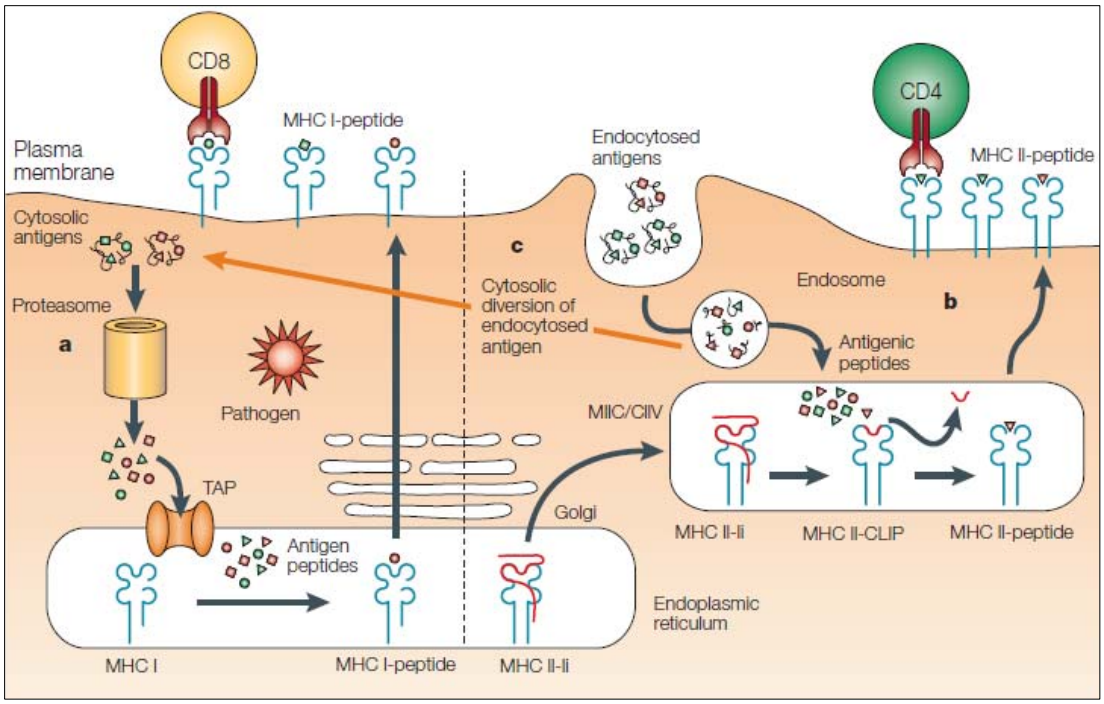


Fig. 1. Antigen-processing pathways. Simplified mechanisms for presentation of peptides associated with MHC I or MHC II molecules are shown. From Heath and Carbone, 2001.

T lymphocytes

Naïve T cells are inexperienced cells that have not encountered an antigen. In lymphoid tissues, T cells interact with APCs in a process mediated by adhesion molecules such as CD54 and CD58 on the APC and LFA-1 and CD2 on the T cell. This enables T cells to sample MHC molecules for the presence of a certain peptide. After binding of the T cell receptor (TCR) and the co-receptor (CD4 or CD8) to a peptide-MHC complex and co-stimulation, the T cell becomes activated and can proliferate and differentiate. Most of the T cells that undergo proliferation differentiate into effector T cells, others can differentiate into memory cells. Among the effector T cell population, the majority of the cells undergo apoptosis after antigens have been cleared, while others become long-lived memory T cells (Abbas *et al*., 2007; Janeway *et al*., 2001).

Naïve CD4 T cells can differentiate into distinct cytokine-producing subsets with different immuneregulatory functions. Effector $CD4^+$ T helper (T_H) cells have been divided into some lineages, including T_H1, T_H2, and T_H17 cells. Development of a specific T_H subset is determined by various stimuli, such as the characteristic of the peptide-MHC ligand, the costimulatory molecules involved and cytokines. In addition to ligation of the TCR and costimulatory receptors (as described before), IFN-γ and IL-12 are the main inducers of T_H1-cell differentiation and IL-4 triggers T_H2-cell differentiation. T_H17 differentiation is stimulated by TGF-β, IL-6, IL-1, and possibly other pro-inflammatory cytokines (Janeway *et al*., 2001; Dong, 2006; Abbas *et al*., 2007). Other T_H subsets have been described in the past few years, such as T_H9, T_H22, and T_{FH} (follicular helper T cells), but this theme is beyond the scope of the present work.

Infections by intracellular bacteria and some parasites as well as by viruses induce reactions of the innate immune system that are involved in the production of IL-12, IL-18 and type I interferons. IL-12 is the main cytokine involved in T_H1-cell differentiation. T_H1 cells produce characteristic cytokines, especially IL-2 and IFN-γ, driving the development of strong cell-mediated immunity, most notably in combating intracellular pathogens. IFN-γ activates macrophages, elicits intracellular killing of microorganisms by phagocytes and stimulates B cells to produce opsonising and complement-fixing IgG antibodies. Additionally, IFN-γ induces further T_H1 differentiation and inhibits T_H2-cell proliferation (Janeway *et al.,* 2001; Farrar *et al*., 2002; Dong, 2006; Abbas *et al*., 2007).

T_H2 differentiation is driven by chronic T cell stimulation, which can be triggered by allergens or helminths, for example. T_H2 cells produce a set of cytokines, such as IL-4, IL-5, and IL-13, mediating allergic responses and humoral immunity. IL-4 stimulates B cells to produce neutralizing antibodies; IL-4 and IL-13 induce the production of IgE antibodies against helminths and also activate macrophages to express mannose receptors (alternative macrophage activation); IL-5 activates eosinophils in the neighbourhood of helminths. Mast cells are activated by IgE-opsonized helminths, leading to degranulation. IL-4 and IL-10 inhibit T_H1-cell differentiation (Farrar *et al*., 2002; Dong, 2006; Abbas *et al*., 2007).

T_H17 cells produce IL-17, a regulator of inflammatory responses, and may be involved in the induction of neutrophil-rich inflammation. T_H17-cell differentiation is induced by TGF-β in combination with IL-1 and IL-6, and inhibited by IL-4 and IFN-γ. IL-23 is associated with maintenance and survival of the T_H17 cell subset (Dong, 2006; Abbas *et al*., 2007).

Naïve $CD8^+$ T cells differentiate into CTLs, wich play a crucial role in combating infections caused by viruses and intracellular pathogens. CTLs kill target cells which display the same peptide-MHC class I complex that has driven the proliferation and differentiation of the naïve $CD8^+$ T cells. CTL-mediated killing of target cells can occur by two main mechanisms and requires direct contact between the effector and target cells. In one mechanism, cytotoxic proteins such as perforin (a membrane-disrupting molecule) and granzymes (serine-proteases) are released by exocytosis, inducing apoptosis of the target cell via activation of cell-death pathways. Another mechanism involves interaction of surface molecules on the CTLs and the target cells. The target-cell death receptor Fas binds to Fas ligand (FasL) on CTL, leading to caspase-dependent apoptosis of target cells. CTLs can also secrete cytokines such as IFN-γ, TNF-α, and TNF-β. IFN-γ, among other functions, restrains viral replication, promotes increased MHC class I expression in infected cells, recruits macrophages to the site of infection and can act synergistically with TNF-α or TNF-β, for instance, in macrophage activation (Janeway *et al*., 2001; Trapani and Smyth, 2002; Abbas *et al*., 2007).

Memory T cells

After clearance of an infection by means of T-cell mediated immunity, part of the population of T cells specific for a given antigen remains for years or for the whole life

and ensures protection upon reinfection. These memory T cells respond quickly and in an amplified manner if they reencounter the same antigen. However, memory T cells might also be maintained in the presence of antigen, for example in some infections such as those caused by cytomegalovirus. In this case, the immune responses result in control of pathogen growth but not complete eradication (Rocha and Tanchot, 2004; Abbas *et al*., 2007; Gerlach *et al*., 2011).

Memory T cells can be generated at distinct stages of T cell differentiation. Both maintenance and survival of memory $CD4^+$ and $CD8^+$ T cells depends on certain cytokines, such as IL-7 which promotes low-level proliferation of memory T cells. IL-15 is also necessary for maintenance of memory $CD8^+$ T cells (Abbas *et al*., 2007).

Depending on their effector functions and homing characteristics, $CD4^+$ and $CD8^+$ memory T cells can be divided into central memory and effector memory T cells. Central memory T cells, which express CCR7 and CD62L, home to lymph nodes and proliferate quickly, expanding the effector lymphocyte population after reencountering an antigen. Effector memory T cells home to peripheral tissues and secrete effector cytokines such as IFN-γ upon antigen re-exposure, combating invading microbes at the site of infection. Development of vaccine approaches aiming at the induction of both sets of memory cells is highly desirable, particularly against persistent or chronic pathogens (Esser, 2003; Abbas *et al*., 2007).

T cell activation

Activation of naïve T cells by APCs requires distinct independent signals which further dictate the T cell fate. In the lymph node, the first signal for T cell activation implies interaction of the TCR with a specific MHC-peptide complex on the APC. It is antigen-specific and makes the T cell enter the cell cycle; the TCR is then internalized and degraded. The second signal is provided by the interaction of costimulatory molecules on the surface of professional APCs with their ligands on T cells. A number of molecules on APCs have been described to provide the costimulatory signals for T-cell activation. For example, CD80/CD86 (B7.1/B7.2), the main costimulatory molecules, bind CD28 on T cells; CD54 (intercellular adhesion molecule-1, ICAM-1) on APCs binds to LFA-1; CD58 (leukocyte function-associated antigen-3, LFA-3) associates with CD2 on T cells. Interaction between CD40 on APCs and CD40L on T cells is also important during co-stimulation as it further activates the APC to express CD80/86. Signal 2 promotes the synthesis of IL-2 by the T cell which drives clonal expansion of

antigen-specific T cells. TCR binding in the absence of co-stimulation leads to either apoptosis or anergy. A third step involved in T cell activation is provided by signals delivered by the APCs to T cells, such as cytokines (either pro- or anti-inflammatory), driving T cell differentiation into effector cells. According to the activation status of the APC, a particular cytokine profile dictates which type of T cell response is to be primed. This concept for T cell activation has played an important role in the elucidation of the mechanisms involved in discrimination between self and nonself antigens (Hodge *et al*., 2000; Alegre *et al*., 2001; Coyle and Gutierrez-Ramos, 2001; Janeway *et al*., 2001; Reis e Sousa, 2006; Mays and Wilson, 2011).

In situations that usually generate weak innate immune reactions, such as latent viral infections, tumors, and organ transplants, T_H cells may be required for inducing complete activation of naïve $CD8^+$ T lymphocytes and differentiation into CTLs. Importantly, this process is only effective if both T_H and $CD8^+$ T cells recognize antigen on the same APC. Activation of $CD8^+$ T cells by T_H cells can occur by distinct mechanisms. In one pathway, T_H cells secrete specific cytokines upon antigen recognition that further promote $CD8^+$ T cell differentiation, such as IL-2. Another mechanism implicates activation of APCs by antigen-stimulated $CD4^+$ T_H cells via CD40-CD40L. Activated APCs subsequently express cytokines and costimulatory molecules, resulting in stimulation of CTL development. CD40 signaling seems to be unique in the ability to induce the generation of memory CTLs (Clarke, 2000; Abbas *et al*., 2007).

2. Recombinant yeasts in biotechnological approaches

In the past decades, the choice of a single yeast species as host for the expression of recombinant proteins relied heavily on the baker's yeast *S. cerevisiae*. This dependence is nowadays circumvented by a set of nonconventional yeasts which have become available as expression systems for a wide range of recombinant proteins (Gellissen and Hollenberg, 1997; Müller *et al.*, 1998). The spectrum of yeasts as organisms for the expression of heterologous gene products includes, among others, the fission yeast *Schizosaccharomyces pombe* (Giga-Hama *et al.*, 1994; Tohda *et al.*, 1994), the methylotrophic yeast *Pichia pastoris* (Tschopp *et al.*, 1987; Sreekrishna *et al*., 1989), and the budding yeast *Kluyveromyces lactis* (Das and Hollenberg, 1982).

The use of recombinant yeasts as vaccine strategy has become a very attractive means for the induction of robust immune responses. Yeast is a versatile eukaryotic microorganism as it can be easily engineered, grows rapidly to high cell densities in inexpensive culture media and properly performs post-translational processing of the expressed proteins (Valenzuela *et al*., 1982; Smith *et al*., 1985). This latter ability is especially advantageous in immunotherapy because the expressed antigens can be recognized by the host as native antigens, thus contributing to the generation of more efficient immune responses (Bucarey *et al*., 2009).

Saccharomyces cerevisiae

S. cerevisiae has been used for thousands of years for fermenting food and beverages and is considered a GRAS (**g**enerally **r**ecognized **a**s **s**afe) organism (Gellisen and Hollenberg, 1997; Sicard and Legras, 2011). This yeast has been extensively studied in the past decades, and the vast knowledge about its genetics, biochemistry, physiology, and fermentation properties makes this yeast species the best characterized until today (Gellisen and Hollenberg, 1997; Porro *et al*., 2005). Its genome has been completely sequenced, revealing almost 6,000 ORFs distributed in 16 chromosomes and 12 Mb (Goffeau *et al*., 1996). For all these reasons, *S. cerevisiae* has become the organism of choice for the expression of the majority of recombinant proteins.

However, *S. cerevisiae* has been reported to have some disadvantage as host for recombinant protein production. Low yields of heterologous proteins, plasmid instability, retention of the products within the periplasmic space, and hyperglycosylation have been repeatedly observed (Müller *et al*., 1998; Porro *et al*., 2005). Hyperglycosylation in *S. cerevisiae* can hinder protein folding. Glycoproteins with 50–150 mannose residues have been described in *S. cerevisiae*. Furthermore, this budding yeast adds outer α-1,3-linked mannose residues to its N-glycans, which is considered allergenic in humans and thus prevents the pharmaceutical use of some proteins expressed in *S. cerevisiae* (Varki *et al*., 2009).

Kluyveromyces lactis

K. lactis is one of the most important non-*Saccharomyces* yeasts in biotechnology. This yeast has been used as a source of lactase. Enzymes produced in *K. lactis* have GRAS status, so that they can be used in several food and feed applications (Dominguez *et al*., 1998; van Ooyen *et al*., 2006). *K. lactis* can grow on media containing lactose as sole

energy and carbon source and, under this condition, the enzymes involved in the lactose utilization pathway are strongly induced. A β-galactosidase encoded by the *lac4* gene metabolizes lactose to glucose and galactose. With the establishment of a transformation technique for *K. lactis* (Das and Hollenberg, 1982), the strong inducible *LAC4* promoter turned out to be a widely used element in recombinant protein expression. *K. lactis* can grow to very high cell densities and a large number of proteins from diverse sources have been successfully expressed in this organism (Gellissen and Hollenberg, 1997; Colussi and Taron, 2005; van Ooyen *et al*., 2006). The *K. lactis* genome is 10.6 Mb in size, organized into 6 chromosomes and more than 5,300 coding sequences (Dujon *et al*., 2004).

Schizosaccharomyces pombe

The fission yeast *Sz. pombe* was discovered in 1893 by P. Lindner. The name "pombe" comes from the Swahili word for beer, since it was first isolated in millet beer from eastern Africa (Wixon, 2002). This rod-shaped yeast grows by increasing length but constant diameter and divides by medial fission, generating equal-sized daughter cells. Although *Sz. pombe* is evolutionarily distant from common ascomycetes such as *S. cerevisiae*, it is nowadays also widely used for genetic manipulations (Wixon, 2002; Forsburg and Rhind, 2006; Varki *et al*., 2009). *Sz. pombe* has a fully-sequenced 13.8 Mb genome, with 3 chromosomes and approximately 4,800 ORFs (Wood *et al*., 2002). A number of plasmids containing *Sz. pombe*-specific elements has been developed for recombinant protein production (Giga-Hama *et al*., 1994; Tohda *et al*., 1994). The cell wall of *Sz. pombe* differs from the one of *S. cerevisiae* in terms of carbohydrate composition, for example due to the presence of galactomannan and alkali-soluble α-1,3-glucan (Kopecká *et al*., 1995; Varki *et al*., 2009).

Pichia pastoris

P. pastoris is a nonpathogenic microorganism capable of utilizing methanol as sole carbon source. It was discovered in 1969 in a screen for yeasts able to use methanol. *P. pastoris* can grow to extremely high cell densities in minimal media (Varki *et al*., 2009). In peroxisomes, methanol is converted to formaldehyde by the alcohol oxidase (AOX) in a reaction in which oxygen is simultaneously reduced to hydrogen peroxide. The formaldehyde leaves the peroxisome and is oxidized in a series of reactions to carbon dioxide and water (Ellis *et al*., 1985). The major enzymes involved in methanol

metabolism can comprise up to 30% of the total intracellular protein content in cells cultivated in methanol-containing media. The genes coding for these enzymes are regulated by strong inducible promoters, which can be used for expression of heterologous genes, such as the methanol-inducible *AOX* promoter. The *Pichia* genome contains the two alcohol oxidase genes, *aox1* and *aox2*, which are highly homologous (Koutz *et al*., 1989).

After the establishment of transformation methods, high-yield systems were developed for recombinant protein production. Moreover, *Pichia* secretes relatively low levels of endogenous proteins, facilitating purification of secreted recombinant proteins. The fact that N-glycosylated proteins produced in *P. pastoris* contain only 5–15 mannose residues and that this yeast does not add outer α-1,3-linked mannose residues to its N-glycans, makes *P. pastoris* one of the most efficient yeasts for biotechnological applications (Varki *et al*., 2009). The *P. pastoris* expression system has been extensively used for production of several recombinant proteins, impressing not only due to the expression levels that can be attained, but also by virtue of the bioactivity of the heterologous proteins (Gellissen and Hollenberg, 1997; Macauley-Patrick *et al*., 2005). The genomes of some *P. pastoris* strains have been sequenced (De Schutter *et al*., 2009; Mattanovich *et al*., 2009; Küberl *et al*., 2011), for instance, the 9.43 Mb-genome of the GS115 strain, with 5,313 protein-coding genes distributed in 4 chromosomes (De Schutter *et al*., 2009).

2.1. Yeasts and the immune system

Immune recognition of fungi mostly depends on the interaction of receptors at the surface of the phagocytic cells with structural components of the fungal cell wall. In general, yeast cell walls consist of an outer layer of mannosylated proteins and an inner layer of β-glucans and chitin. However, β-glucans may be permanently exposed at the surface in specific regions, which is sufficient for Dectin-1 sensing. The cell wall composition in fungi is species- and morphology-dependent. In addition, the cell wall represents a highly dynamic structure which is considerably remodeled during the particular yeast life cycle (Gantner *et al*., 2005; Brown, 2011).

Recognition of fungal particles relies on diverse PRRs including lectin receptors such as mannose receptor (MR) and Dectin-1, Toll-like receptors (TLRs), scavenger receptors, as well as integrins, like the complement receptor type 3 (CR3). The

distribution of these receptors for sensing of potential pathogens depends on the cell type (Brown, 2011).

Many of the lectin receptors, such as MR and Dectin-1, belong to the calcium-dependent (C-type) lectin family which is classified into two types, I and II, depending on their structure. Type I surface lectins contain multiple carbohydrate recognition domains (CRDs) on their N-termini, whereas type II surface lectins consist of a single CRD on their C-terminal regions, as shown in Fig. 2 (Keler *et al*., 2004). CRDs contain calcium-binding pockets that are fundamental for binding of the carbohydrate ligand. Although some indications suggest preferential binding of C-type lectin receptors (CLRs) to carbohydrates derived from pathogens, there is no further evidence for discrimination between self and non-self antigens (Figdor *et al*., 2002).

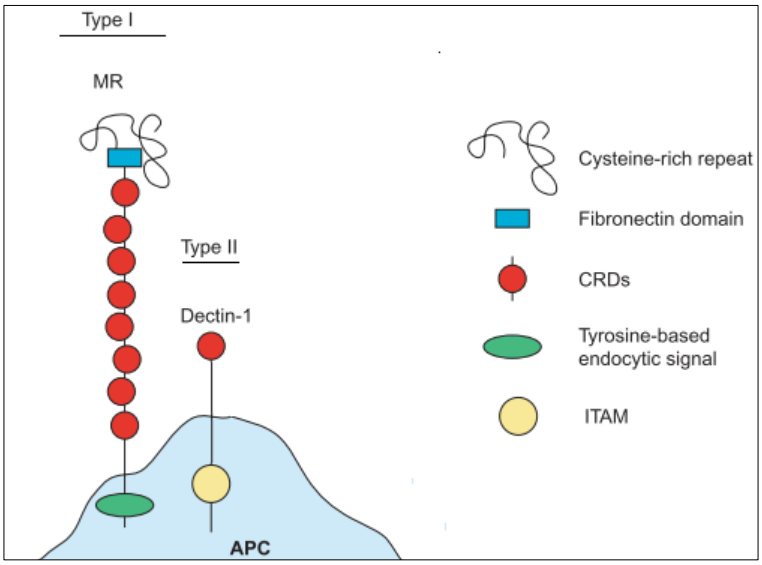


Fig. 2. Schematic representation of the basic structures of the C-type lectin receptors (CLRs) mannose receptor (MR) and Dectin-1 expressed by APCs. MR is a type I CLR with eight carbohydrate recognition domains (CRDs), whereas Dectin-1 bears a single CRD, being therefore classified as type II CLR. Adapted from Keler *et al*., 2004.

Dectin-1 is a small membrane-integrated type II surface CLR. Besides the extracellular CRD, this receptor also contains an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence in its cytoplasmic domain (Ariizumi *et al*., 2000; Yokota *et al*., 2001). Dectin-1 is predominantly expressed in DCs, macrophages and neutrophils, and recognizes diverse fungi- and plant-derived β-1,3-linked and β-1,6-linked glucans (Ariizumi *et al*., 2000; Yokota *et al*., 2001; Brown and Gordon, 2001; Willment *et al*., 2001; Taylor *et al*., 2002). Dectin-1 has been demonstrated to recognize intact yeasts,

including *Candida albicans*, *S. cerevisiae*, and *Pneumocystis carinii* (Brown and Gordon, 2001; Steele *et al*., 2003; Gantner *et al*., 2005).

Dectin-1 induces phagocytosis, killing via production of reactive oxygen species, and the expression of chemokines and cytokines including IL-1β, IL-2, IL-10, IL-12, and TNF-α (Brown *et al*., 2003; Gantner *et al*., 2003; Steele *et al*., 2003; Underhill *et al*., 2005; Brown, 2006). These events result from activation of intracellular signaling pathways, the Syk (spleen tyrosine kinase)-CARD9 (caspase recruitment domain-containing protein 9) pathway, and the Raf-1 kinase pathway. Both pathways act synergistically and are activated through the ITAM-like motif of Dectin-1. Also, Dectin-1 collaborates with TLRs to modulate cytokine production (Brown, 2011; Romani, 2011). Dectin-1 recognizes various types of β-1,3-linked and β-1,6-linked glucans as well as intact yeast cells (Brown and Gordon, 2001).

The mannose receptor (MR, also CD206) is a type I CLR expressed, among others, on macrophages, DCs, subsets of endothelial cells and myeloid cells. MR contains eight CRDs, a fibronectin type II repeat domain, a cysteine-rich domain and a short cytoplasmic tail (Keler *et al*., 2004; Hollmig *et al*., 2009; Brown 2011). This receptor has been shown to specifically bind to mannose, fucose and N-acetylglucosamine in many fungal species and to mediate subsequent particle internalization (Avraméas *et al*., 1996; Ezekowitz *et al*., 1991; Giaimis *et al*., 1993; Mansour *et al*., 2002). Even though classical signaling motifs are missing in the cytosolic region of MR, this receptor has been shown to mediate a number of cellular events, such as NF-κB signaling and cytokine production (*e.g*. GM-CSF, IL-1β, IL-6 IL-10, IL-12). The majority of the MR molecules is located within the intracellular endocytic pathway (Hollmig *et al*., 2009; Brown, 2011). MR has been demonstrated to recognize several organisms, including *C. albicans* (Ezekowitz *et al*., 1990), *C. neoformans* (Syme *et al*., 2002), *P. carinii* (Ezekowitz *et al*., 1991), and *M. furfur* (Buentke *et al*., 2000). Furthermore, MR connects innate and adaptive immunity as it has been demonstrated that selective targeting of an antigen fused to a specific anti-MR antibody and uptake by the MR on DCs induced MHC-restricted antigen-specific $CD4^+$ and $CD8^+$ T cell responses (Ramakrishna *et al*., 2004).

TLRs are also capable of recognizing fungal components, such as zymosan (a derivative of yeast cell walls), O-linked mannans, phospholipomannan and fungal DNA (Nakamura *et al*., 2008; Romani, 2011). The TLRs comprise a well-characterized family of PRRs which contain an extracellular domain for ligand binding and a

conserved cytoplasmic domain that triggers specific signaling pathways, driving specific patterns of gene expression and the synthesis of cytokines and chemokines that activate innate and adaptive immune responses (Brown, 2006; Diebold, 2009; Hollmig *et al*., 2009). TLR2, TLR4 and TLR9 are the major TLRs implicated in recognition of fungal elements (Romani, 2011). The influence of TLRs on phagocytosis of fungal particles remains unclear but seems to depend on the host cell type, the fungal species and morphotypes, the route of infection and receptor cooperativity. TLRs have been shown to facilitate the presentation of fungal-derived antigens by DCs and modulate T cell responses (Brown, 2006; Romani, 2011).

The complement receptor 3 (CR3, Mac-1) has also been demonstrated to interact with zymosan and yeasts such as *S. cerevisiae* (Ross *et al*., 1985) and *C. albicans* (Forsyth *et al*., 1998). CR3 is a heterodimeric integrin of CD11b (α_M) and CD18 (β_2) subunits and recognizes β-glucan, N-acetyl-D-glucosamine, mannose-containing polysaccharides and glucose via a cation-independent lectin region situated C-terminal to the I-domain of CD11b (Thornton *et al*., 1996). CR3 mediates phagocytosis both in a complement-dependent and -independent manner and is expressed by several cell types, including DCs, neutrophils, monocytes and macrophages (Ross *et al*., 1985; Tsoni and Brown, 2008; van Bruggen *et al*., 2009). It has been shown that phagocytosis of β-glucan-bearing particles by human neutrophils is completely CR3 dependent (van Bruggen *et al*., 2009).

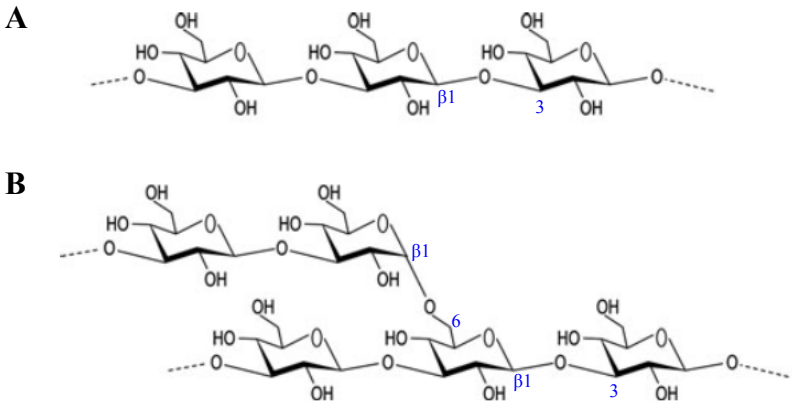
Several other PRRs have been shown to associate with fungal PAMPs, in a process which is dependent on the cell type involved and on fungal characteristics such as species and morphology. The final host immune response to a certain fungus will depend on distinct factors, like the relative degree of stimulation of each receptor, the level of receptor cooperativity and cellular localization (Romani, 2011).

It is well established that yeast cell wall components are able to activate the immune system. For instance, β-1,3-D-glucan and mannan have been demonstrated to act as natural adjuvants (Suzuki *et al.*, 1989, Toda *et al.*, 1997). Fungal β-glucan has been shown to possess immunomodulating (acting on both innate and adaptive immunity) and antitumor activities, which depend on polymer structure, length and degree of branching. By virtue of their anti-cancer effects, β-glucans have been tested in pre-clinical and clinical trials (Suzuki *et al*., 1989; Tsoni and Brown, 2008; Chan *et al*., 2009; Li *et al*., 2010). Fungal β-glucans comprise a group of heterogeneous glucose polymers consisting of a linear 1,3-β-glycosidic chain core with β-1,6-linked branches

of varying length and structure (Fig. 3 A and B). The branching pattern seems to be species-specific. Evidences indicate that higher structural complexity is associated with stronger immunmodulatory and antitumor effects (Akramiene *et al*., 2007; Chan *et al*., 2009).

Mannans are polymers of α-linked mannose residues (Fig. 3 C-F). Studies on *S. cerevisiae* mannan have revealed a highly branched polysaccharide with α-1,2- and α-1,3-linked side chains attached to an α-1,6-linked backbone (Stewart *et al*., 1968; Jones and Ballou, 1969). Mannan has also been analyzed for its effect on the immune system. Toda *et al*. (1997) demonstrated that mannan-coated liposomes showed potent adjuvant activity in eliciting cell-mediated immunity and activated T_H1-mediated immune responses *in vivo*, when administered in combination with a DNA vaccine (Toda *et al*., 1997). Other groups have also shown that fungal mannosylated antigens enhanced antigen presentation and stimulated antigen-specific $CD4^+$ and $CD8^+$ T cell responses (Lam *et al*., 2005; Lam *et al*., 2007; Luong *et al*., 2007).

Chitin, a polymer of β-1,4-linked N-acetylglucosamine (Fig. 3 G), forms chains generaly larger than 1,000 residues that are primarily found at the bud neck or septa in fungi (Varki *et al*., 2009). Contradictory data exist on the receptor(s) involved in chitin recognition. Chitin particles have been demonstrated to be phagocytosed by murine macrophages most probably via MR (Shibata *et al*., 1997). Recently, it has been shown that Dectin-1 is required for chitin recognition, however without directly binding this polysaccharide (Mora-Montes *et al*., 2011). In the past years, chitin has been shown to be a potent adjuvant stimulating adaptive T_H1, T_H2, and T_H17 immune responses in mice (Da Silva *et al*., 2010).



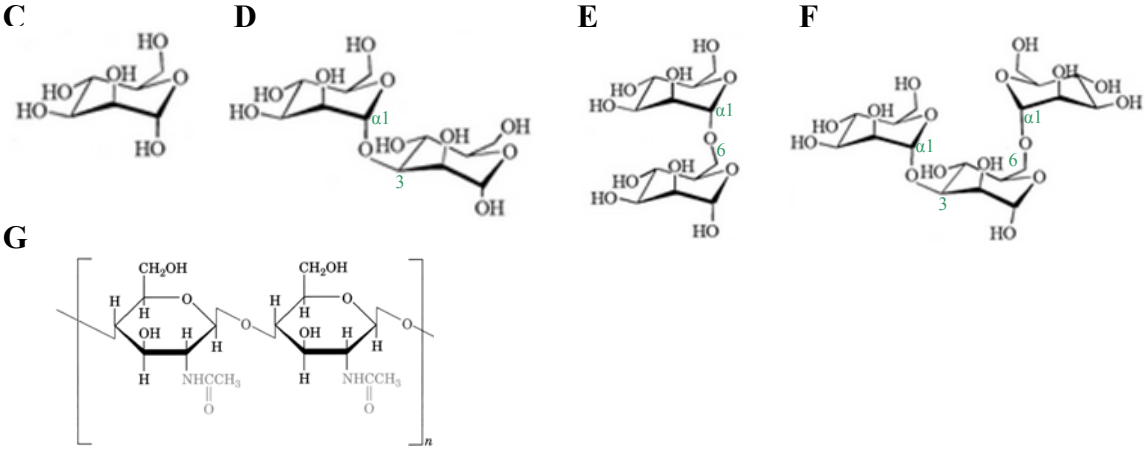


Fig. 3. Schematic representation of some β-glucan, mannose (Man) and chitin structures. **(A)** Linear β-1,3-glucan; **(B)** β-1,3-glucan with β-1,6 branching; **(C)** Mannose; **(D)** Man-α-1,3-Man; **(E)** Man-α-1,6-Man; **(F)** Man-α-1,3-Man-α-1,6-Man; **(G)** chitin. Adapted from Rani *et al*., 1999, Tsoni and Brown, 2008; Voet *et al*., 2010.

3. General concepts of vaccines

Some of the first attempts to protect induviduals from pathogen-associated diseases date from ancient China where healthy people were given smallpox scabs or clothes from infected patients. This practice reached Europe in the 18th century. In 1796, Edward Jenner, performing human experimentation, showed that individuals immunized with the cowpox virus were protected from disease caused by the smallpox virus (Hilleman, 2000; Esser *et al*., 2003). This well-succeeded vaccination (Latin *vaccinus* = from or of cows) was worldwide accepted and smallpox was declared eradicated in the early 1980s.

Hitherto, vaccination remains the most effective means to avoid infectious diseases. A number of prophylactic vaccines against bacteria and viruses have contributed to a significant decrease in morbidity and mortality worldwide.

The following vaccination approaches are nowadays routinely used: live attenuated, inactivated, subunit, conjugate, and toxoid vaccines. More recent techniques, such as those employing DNA vaccines, autologous cells and recombinant vectors are still being evaluated.

Attenuated vaccines consist of a weakened version of the microbe through repeated passaging in cells which are not its natural host. Since the microbe has to adapt to the new environment, it will not be capable of replicating at sufficient levels in

humans to cause disease. Classical examples include vaccines against polio, mumps, measles, rubella and varicella (Cáceres and Sutter, 2001; Hanna *et al*., 2009).

Inactivated vaccines are obtained by treating a certain pathogen with chemicals, radiation or heat. The pathogen cannot replicate but remains intact. Vaccines against hepatitis A and polio are examples of inactivated vaccines (Just and Berger, 1992; Simizu *et al*., 2006).

Toxoid vaccines consist of chemically inactivated toxins which are produced by some bacteria. In this case, the disease is not caused by the microorganism itself but by the toxin it secretes. Examples are the vaccines against tetanus and diphtheria (Smith, 1969).

Subunit vaccines contain specific antigens from a certain pathogen instead of the whole microbe. This can be achieved by isolating a protein (or a portion of it) from the microorganism or by expressing the antigen recombinantly, such as in the case of the vaccines against hepatitis B virus or human papilomavirus (Poland and Jacobson, 2004; Barr and Tamms, 2007).

Conjugate vaccines are a type of subunit vaccine, for instance when a polysaccharide from a bacterial coating is covalently linked to a more immunogenic proteinaceous antigen in order to elicit stronger immune responses, especially in immature immune systems. A classical example is the vaccine against *Haemophilus influenzae* type B (Ojo *et al*., 2010).

DNA vaccines resort to plasmids containing pathogen- or tumor-associated antigens (and additional immunostimulatory factors) under the regulation of an appropriate eukaryotic promoter. Upon inoculation of the plasmid, the DNA is taken up by some cells and the specific proteins are synthesized in the cytosol for subsequent presentation to lymphocytes (Abbas *et al*., 2007, Eschenburg *et al*., 2010).

Immunization with autologous cells is used, for example, for combating cancer. The classical example employs DC-based vaccines, which can be generated by genetic modification or by loading DCs with tumor-associated antigens or whole dead tumor cells (Janikashvili *et al*., 2010).

“Live” vectors are a recent approach for delivering antigens. In general, attenuated viruses or bacteria are used as antigen carrier inducing humoral and cellular immune responses. Viral vectors, such as vaccinia virus and bacteria (*e.g*. *Listeria monocytogenes* or *Yersinia enterocolitica*) can be engineered to deliver recombinant molecules (Wiedig *et al*., 2005; Loeffler *et al*., 2006; Embry *et al*., 2011). In the 21th

century, antigen delivery strategies using whole recombinant yeasts have been demonstrated a promising approach (reviewed by Ardiani *et al*., 2010).

3.1. Vaccines for cellular immune responses

The major purpose of vaccines is to provide long-term immunological protection. Traditionally, most vaccine strategies in the 20th century have driven the attention to the generation of high titers of antibodies, with modest understanding of the role of the cellular immune responses (Kaech *et al*., 2002; Esser *et al*., 2003). Since the vast majority of vaccines routinely used today elicit humoral immunity, many efforts to promote cell-mediated immunity through vaccination have been made in the past years.

However, combating tumors and pathogens that cause chronic or persistent infections implicates eliciting the mechanisms of cellular immunity. For this reason, development of vaccines that stimulate long-lived cellular immune responses is critical to fighting against a number of diseases, including those caused by cytomegalovirus, hepatitis C virus, human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (Esser *et al*., 2003).

Long-term protection is based on the quantity and the quality of the memory T cells that are produced. Ideally, vaccines should elicit the formation of a very large effector T-cell population, which represents a challenge (Kaech *et al*., 2002). Elimination of pathogen-infected or malignant cells by T-cell mediated immune responses mainly relies on cell-mediated cytotoxicity via $CD8^+$ CTLs. However, vaccines must also be capable of inducing $CD4^+$ T cell responses because they secrete several immunomodulatory cytokines to subsequently trigger the generation and proliferation of the robust $CD8^+$ CTL responses required (Ardiani *et al*., 2010).

Distinct approaches have been employed in order to increase the activation of CTLs specifically against affected cells. DCs have been considered an interesting target due to their unique ability to cross-present antigens. Therefore, strategies aiming at the delivery of antigens to DCs and inducing their maturation and activation are critical in stimulating cell-mediated responses against diseased cells. Among the different approaches for activating cell-mediated immunity are DNA vaccines, viral vectors, autologous and allogeneic cells, and delivery of molecules by virulence-attenuated bacterial strains and yeast (reviewed by Franzusoff *et al*., 2005). DNA vaccines have low boosting capacity; viral vectors are neutralized by antibodies after repetitive

applications; besides being very expensive and complex, immunotherapy with tumor cells is patient-specific, and cell-mediated immunity is poorly activated due to the lack of danger signals; bacterial vectors can cause unwanted side effects associated with the host-bacteria interactions (Franzusoff *et al*., 2005; Pálffy *et al*., 2006). The benefit of using yeast as vaccine carrier has been demonstrated in recent pre-clinical and clinical trials, making this approach very attractive.

3.2. Yeast as vehicle for generating antigen-specific immune responses

The use of recombinant yeast as vaccine strategy has become a very promising means for the induction of robust immune responses. The ability of yeasts to properly perform post-translational processing on the expressed proteins is especially advantageous in immunotherapy because the expressed antigens can be recognized by the host as native antigens, thus contributing to the generation of high-quality immune responses (Bucarey *et al*., 2009). Furthermore, antigen processing by DCs after administration of whole recombinant yeast carrying recombinant proteins provides a wide spectrum of epitopes derived from the delivered proteins, which allows the presentation of epitopes relevant for the MHC repertoire from a certain individual (Bui *et al*., 2010).

It has been postulated that protein-based vaccines have the major disadvantage of insufficient immunogenicity and, as a consequence, the majority of them requires co-administration with adjuvants to elicit protective immune responses (Petrovsky and Aguilar, 2004). Thus, some efforts have been made in an attempt to develop more effective vaccine strategies, in particular for inducing potent T cell-mediated immune responses needed against viral and tumor antigens. Among the different approaches, the use of recombinant yeast cells as antigen delivery system turned out to be an auspicious strategy.

The first experiments performed in order to examine the potential use of whole recombinant *S. cerevisiae* as a vaccine to elicit antibody responses came with Schreuder *et al.* (1996a). In a cell-surface display system, an antigen bearing two major hydrophilic regions of the hepatitis B surface antigen (HBsAg) was fused to the C-terminal region of α-agglutinin from *S. cerevisiae* and exposed on the yeast cell surface. Heat-killed yeasts were used to immunize mice intraperitoneally and serum immune responses were analyzed. Very low specific immune responses against the recombinant

protein were observed, in contrast to strong immune responses elicited against the yeast carrier itself.

The observations of Schreuder *et al*. (1996a) demonstrating the stimulation of the immune system by yeasts alone, combined with other reports showing the immunnogenic characteristics of fungal β-glucan and mannan, have encouraged the onset of several approaches using whole yeasts expressing recombinant antigens as carrier systems for generation of specific immune responses (Suzuki *et al*., 1989; Williams *et al*., 1992; Toda *et al*., 1997). Additionally, uptake of fungal particles by phagocytic cells has been demonstrated (Newman *et al*., 1990; Suzuki *et al*., 1998; Newman and Holly, 2001; Breinig *et al*., 2003), so that many studies have directed their attention to the use of yeasts carrying proteinaceous antigens in immunotherapy.

In the first study analyzing cell-mediated immunity after vaccination of mice with live yeasts, Stubbs *et al*. (2001) showed that *S. cerevisiae* cells expressing Ova were capable of stimulating protective $CD8^+$ T cells *in vivo*. Mice vaccinated with recombinant yeast and challenged with Ova-expressing lymphoma cells were protected from tumor formation. They also demonstrated that whole heat-killed yeast led to DC maturation and IL-12 secretion and to increased presentation of Ova to antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes. When they tested a clinically relevant antigen, such as the envelop protein $_{SF2}$-gp160 from HIV-1, they observed that cells expressing this protein were killed by cytotoxic T lymphocytes derived from mice vaccinated with yeast/$_{SF2}$-gp160, but not with PBS or yeast carrying an empty vector. Proliferative responses of T lymphocytes specific for HIV-1-gp120 (gp160 devoid of gp41) were also reported for mice immunized with yeast/$_{SF2}$-gp160. It was the first demonstration that whole recombinant yeast could induce potent cell-mediated responses (Stubbs *et al*., 2001).

After that study, many other groups have resorted to this strategy, with different antigens and mouse models. For example, cellular immune responses against the hepatitis C virus (HCV) were examined in mice using heat-inactivated whole yeasts expressing NS3/Core fusion protein. In this study, Haller and colleagues (2007) demonstrated antigen-specific and dose dependent *in vitro* cytotoxicity. They have also shown that repeated weekly immunization did not induce neutralization or tolerance, and that immunized mice were protected from challenge with tumor cells expressing HCV NS3. Further, mice vaccinated after NS3-positive tumor cell implantation showed no or reduced tumor growth compared to an untreated group (Haller *et al*., 2007).

Many studies using tumor-bearing mice have analyzed the therapeutic effect of recombinant *S. cerevisiae* expressing tumor-associated antigens (TAAs), such as mammalian mutant Ras proteins, the carcinoembryonic antigen (CEA), and a melanocyte/melanoma antigen (MART-1), with promising results. Besides tumor regression, increased survival time, cytotoxicity and cytokine production, no toxicity or autoimmunity related to the yeast-based vaccine was observed (Lu *et al*., 2004; Wansley *et al*., 2008; Tanaka *et al*., 2011). Clinical trials of immunotherapy with yeast carrying Ras or CEA have been conducted with patients bearing Ras mutation^{+} or CEA^{+} tumors (ClinicalTrials.gov Identifiers: NCT00300950, NCT00924092; Ardiani *et al*., 2010).

The use of recombinant yeast in prophylactic vaccine approaches has also been investigated, for example with yeasts carrying MART-1 or BCR-ABL^{T315I}, a mutated protein in drug-resistant leukemia cells. Protection against tumor challenge, *in vitro* cytotoxic activity and production of cytokines, as well as prolonged survival were observed (Riemann *et al*., 2007; Bui *et al*., 2010).

A number of studies using yeasts displaying antigens on the cell surface to immunize animals have also been conducted, most of them aiming at the development of humoral immune responses (Zhu *et al*., 2006; Upadhyaya and Manjunath, 2009; Kim *et al*., 2010).

Although *S. cerevisiae* has been used in the vast majority of studies, other well-accepted yeast species have rarely been tested. A vaccine using live *P. pastoris* as protein delivery system was tested by Saiki and colleagues (2005). The pcd-17, an antigen associated with the paraneoplastic cerebellar degeneration, an autoimmune disease, was chosen for recombinant expression in *Pichia*. Lymph node cells of mice immunized with yeast/pcd-17 showed proliferative responses and IFN-γ production after peptide restimulation, in contrast to cells derived from mice receiving the wild type yeast only. The presence of autoantibodies in serum of vaccinated mice has also been confirmed. However, these responses have only been observed in one out of four mouse strains tested and no cytotoxicity assay was performed. In another study, heat-treated *H. polymorpha* expressing cytosolic HBsAg was used to vaccinate mice. Yeasts led to an increase in the number of T cells and DCs in the mouse spleen and elicited DC maturation. Furthermore, yeasts/HBsAg induced the generation of anti-HBsAg antibodies in a greater extent than HBsAg alone or administered with an alum adjuvant. Cytotoxicity and cytokine secretion was also observed. This report confirmed the idea

that recombinant yeasts stimulate both cellular and humoral immunity and promote mixed Th1/Th2-type immune responses (Bian *et al*., 2010).

Recently, an approach for delivering nucleic acids by recombinant yeast to antigen-presenting cells has been developed. In that work, *S. cerevisiae* and *Sz. pombe* were analyzed as carrier for antigen-encoding DNA and mRNA. Recombinant protein expression (*e.g.* eGFP and Ova_{cyt}) in mammalian APCs was confirmed after yeast internalization, and *ex vivo* activation of pp65-specific memory T cells was demonstrated using *S. cerevisiae* as DNA/RNA delivery system (Walch, 2009; Walch *et al*., 2011).

As previously described, activation of naïve T cells by APCs involves a set of distinct signals. Yeasts lead to augmented expression of MHC class I and II (needed for the first signal), as well as of the costimulatory and adhesion molecules CD40, CD80/CD86, CD54, CD58 (necessary for the second signal), and stimulate secretion of cytokines by DCs (providing the third signal).

In sum, the use of inherently nonpathogenic yeast as antigen delivery system turned out to be an especially versatile technique. The main points in this issue are: (i) phagocytic cells, especially DCs, are able to ingest a wide variety of antigens, including whole yeasts; (ii) after internalization of fungal particles, DCs mature and efficiently process the antigens in context of MHC class I and II molecules for presentation, express a set of costimulatory molecules and secrete cytokines, stimulating the differentiation of T lymphocytes into distinct classes of effector cells, controlling the quality of the T cell response; (iii) upon using whole yeasts, all three signals necessary for T cell activation are increased, due to the natural adjuvant characteristics of the yeast PAMPs. This important feature facilitates the generation of robust adaptive immunity. Therefore, since antigen-specific, adaptive responses play a critical role in combating infections and tumors (Mellman *et al*., 2001; Heath and Carbone, 2001), the use of whole yeasts is a promising vaccine strategy (Fig. 4). All efforts that have been made in the vaccination field to target and activate DCs and consequently providing the potential to generate both $CD4^+$ and $CD8^+$ T cells immune responses are worth.

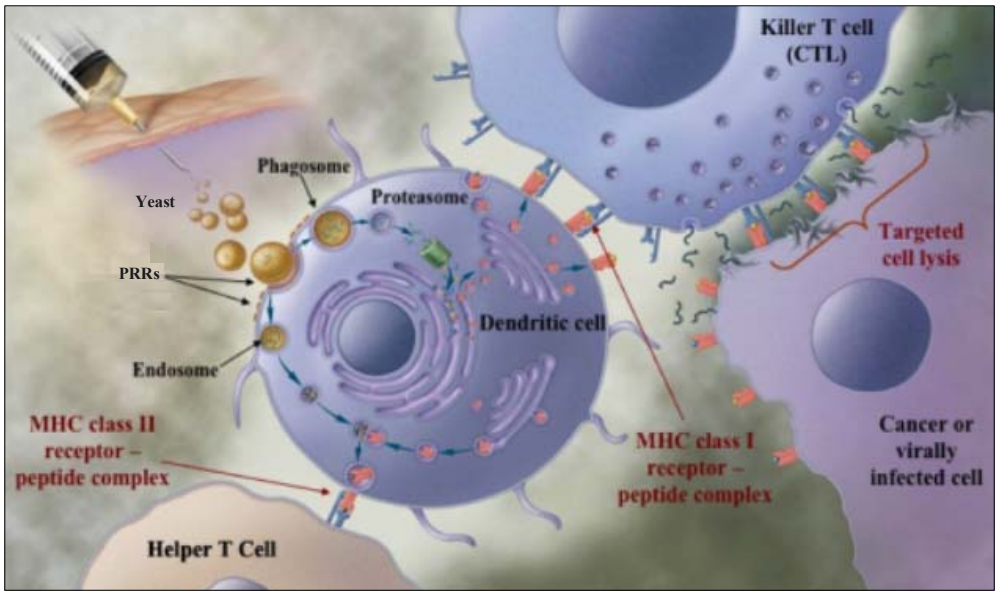


Fig. 4. Yeast as antigen carrier. Simplified mechanism of action. Adapted from Ardiani *et al*., 2010.

4. Experimental systems

As part of this work, recombinant yeasts were analyzed for their ability to deliver different antigen variants to antigen-presentig cells and influence the generation of specific T cell responses due to their adjuvant properties.

4.1. Ovalbumin as model antigen

Ova is a monomeric protein from chicken egg-white with a molecular weight of approximately 43 kDa. This protein contains an internal secretion signal possibly located between residues 21 and 47 (Nisbet *et al*., 1981; Huntington and Stein, 2001). Ova belongs to the serpin superfamily (**ser**ine **p**rotease **in**hibitors), but possesses no inibitory activity. Rather, its function has not been elucidated (Hunt and Dayhoff, 1980; Huntington and Stein, 2001). An immunodominant MHC class I epitop in the Ovalbumin sequence between amino acid residues 257 and 264 has been characterized (Rötzschke *et al*., 1991). This octamer SIINFEKL (Serine-Isoleucine-Isoleucine-Asparagine-Phenylalanine-Glutamate-Lysine-Leucine) epitope is restricted to mouse H-$2K^b$ MHC class I molecules, rendering Ova a model antigen for analysis of antigen presentation via the MHC class I pathway, as well as generation of $CD8^+$ T cell immune responses. For this reason, Ova is widely used in immunologic approaches.

In order to target Ova to different subcellular compartments, the following Ova derivatives were constructed and analyzed: i) full-length Ova which contains an internal signal peptide that directs the protein into the secretory pathway for secretion of the protein (Ova), ii) a derivative which lacks this signal, but still contains the SIINFEKL epitope, and that is expressed intracellularly within the yeast cytosol (Ova_{cyt}), and iii) a fusion of Ova_{cyt} with the yeast cell wall protein Sed1p which targets the protein to the cell surface (Ova_{cyt}/Sed1p). To direct this fusion into the yeast secretory pathway, a genus-specific ER-import signal was added at the N-terminus. In another strategy, Ova_{cyt} was fused to the Gag protein from the *S. cerevisiae* L-A virus and intracellularly expressed as Ovacyt-containing virus-like particles.

The efficiency with which different Ova derivatives in different yeast genera can trigger antigen-specific $CD8^+$ T cell responses was subsequently tested *in vitro* by means of IFN-γ detection.

4.2. Pp65, a matrix protein from human cytomegalovirus, as model antigen

The human cytomegalovirus (HCMV) is a betaherpesvirus with high prevalence in the population in the whole world. The virus consists of an icosahedral capsid containing a linear, double-stranded, 235-kb DNA genome encoding approximately 165 genes and surrounded by a matrix or tegument within a lipid envelope. Primary infection with HCMV is usually asymptomatic, but latent infection remains for the entire life, with eventual reactivation and shedding of virus particles from mucosal sites (Mocarski Jr and Courcelle, 2007; Khanna and Diamond, 2006).

The phosphoprotein 65 (pp65, also known as lower matrix protein or UL83) from HCMV is the major constituent of viral particles and has been demonstrated to be a relevant T cell antigen (reviewed by Kalejta, 2008). This structural protein associates with host and viral kinases, but it is not clear whether pp65 itself possesses kinase activity (Mocarski Jr and Courcelle, 2007; Kalejta, 2008). It has been shown that pp65 can be found in infected cells all times after viral infection, although pp65 is produced in the late phase of viral gene expression, *i.e.* 24 h post infection (Grefte *et al.*, 1992). The recognition and lysis of HCMV-infected cells by the majority of virus-specific CTL without the requirement of *de novo* viral protein synthesis indicates that structural proteins are introduced into the cytoplasm of infected cells in sufficient amount to enter the class I pathway after viral penetration (Riddell *et al.*, 1991; Grefte *et al.*, 1992).

McLaughlin-Taylor and co-workers (1994) have shown that pp65 is a target antigen for HCMV-specific MHC class I-restricted CTL. In one study, when fourteen HCMV antigens were compared in their abilities to stimulate helper T cells *in vitro*, pp65 was by far the most reacting protein (Beninga *et al*., 1995). Kern and colleagues (2002) determined the frequencies of $CD4^+$ and $CD8^+$ T cell responses to pp65 in healthy HCMV-positive individuals. They found that 63% and 83% of the donors tested showed a $CD4^+$ or a $CD8^+$ T cell response, respectively, to pp65-derived peptides. In addition, the frequencies of pp65-specific T cells among responders showed a great variation. In another study conducted by Wills *et al*. (1996) it was demonstrated that, among all CTL which were able to recognize HCMV-infected cells, 70-90% were specific for pp65. They hypothesized that since this high frequency of pp65-specific memory CTL was maintained for 18-22 months, this structural protein is probably continuously expressed. This fact might indicate that either episodic reactivation or continual replication of the virus at low levels enables restimulation of pp65-specific CTL precursors and preservation of the pool of memory CTL.

The repeated observation that a number of HCMV-seropositive donors does not respond to some established pp65 dominant epitopes, combined with the suggestions that these individuals might respond to unpredicted or subdominant peptides makes the use of a full-length protein more promising in terms of stimulating CTL expansion (Solache *et al*.,1999; Vaz-Santiago, 2001; Kern *et al*., 2002).

Cell-mediated immunity plays a crucial role in control of latent HCMV infection. However, in individuals with compromised or immature immune systems, such as transplant recipients and neonates, HCMV is constantly associated to morbidity and mortality in a number of disease patterns. Despite many efforts that have been made in the past decades, no vaccine against HCMV is available to date. Thus, vaccine development is nowadays a main biomedical research concern, especially in the following scenarios: congenital primary infection, primary infection in immunosuppressed individuals and virus reactivation in immunosuppressed adults (Mocarski Jr and Courcelle, 2007; Schleiss, 2005; Khanna and Diamond, 2006). Recently, a phase I clinical trial has been performed, in which a vaccine consisting of a two-component alphavirus replicon vector system expressing both the pp65 fused to a 72-kDa immediate-early protein (IE1) and the major HCMV surface glycoprotein (gB) was evaluated in HCMV-seronegative individuals. The vaccine elicited cytokine responses in $CD8^+$ and $CD4^+$ T cells against all three proteins and induced the

generation of neutralizing antibodies to HCMV, most likely against gB (Bernstein *et al*., 2010). However, a vaccine with promising therapeutic potential for HCMV-positive individuals is still missing.

4.3. Virus-like particles (VLP) as antigen carrier

The *S. cerevisiae* L-A virus is a double-stranded RNA virus which replicates in the cytoplasm of yeast cells without a known phenotype. Its viral icosahedral particles are composed of a 76-kDa major protein (Gag) and a 180-kDa minor Gag-Pol fusion protein. The 4.6-kb viral genome consists of two overlapping open reading frames (*gag* and *pol*). A -1 ribosomal frameshift is responsible for the formation of the Gag-Pol fusion protein. The L-A capsid has a diameter of approximately 39 nm, comprising 118 Gag molecules and 2 Gag-Pol molecules (Wickner, 1996). N-terminal Gag acetylation by the *MAK3* gene product is crucial for VLP assembly (Tercero and Wickner, 1992). Gag alone has been demonstrated to be sufficient for the assembly of VLP, therefore strategies aiming at the development of biotechnologically or pharmaceutically relevant chimeric Gag-VLP have been employed (Fujimura *et al*., 1992; Wickner, 1996; Powilleit *et al*., 2007).

VLP have been shown to function as adjuvants, eliciting the generation of innate and cell-mediated immune responses against both the carrier itself and the delivered antigen. VLP are considered a promising setting in the field of vaccine production, not only because of their particulate structure and size range (40-50 nm), which have been shown to facilitate their capture by DCs, but also because of the repetitive, high-density display of epitopes, which is generally efficient in stimulating robust immune responses. Furthermore, VLP are optimal delivery systems in prophylactic and therapeutic approaches by virtue of their intrinsic ability to break self-tolerance (Fifis *et al*., 2004; Grgacic and Anderson, 2006; Schumacher *et al*., 2007; Crisci *et al*., 2009).

In the L-A virus, Pol projects into the inner side of the capsid, participating in replication and transcription of the viral genome (Fujimura *et al*., 1992). In this work, the interior of the capsid was modified by substituting Pol for Ova_{cyt} or full-length pp65. Nonetheless, in contrast to *pol*, *ova*$_{cyt}$ and *pp65* were cloned in frame with *gag*, so that all molecules produced are Gag/Ova_{cyt} or Gag/pp65 fusions, respectively. For expression of Gag-variants, the virus-free *S. cerevisiae* strain S86c was chosen as host

(Powilleit, 2004). S86c derives from the S86 strain, which was heat-treated for elimination of the viral genome (Schmitt, 1995).

5. Aim of the study

The main objective of this work was to compare different yeast strains as carrier for recombinant protein antigens as well as to analyze the influence of the subcellular localization of the heterologous protein on activation of antigen-specific T lymphocytes for a possible application in vaccination approaches.

First, the effect of distinct yeast genera, species, strains, or cell wall mutants on maturation and activation of human dendritic cells was investigated by analyzing upregulation of diverse cell surface markers and cytokines. The major components of the yeast cell wall, mannan and β-glucan, which are known to possess adjuvant properties, were examined for establishment of a potential association with the results observed. Further, the biotechnologically relevant yeasts *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Pichia pastoris* were assessed for the interaction kinetics with human DCs or murine macrophages, and the involvement of major pattern recognition receptors MR and Dectin-1 in their internalization was determined. The influence of heat-treatment of yeast on phagocyte activation, as measured by production of reactive oxygen species in human whole blood, was also investigated.

Next, the different yeasts were used for the expression of model proteins such as Ova and the clinically significant antigen pp65 from human cytomegalovirus, and activation of antigen-specific T lymphocytes was examined *in vitro* and *ex vivo*. The efficiency of yeast-delivered pp65 or pp65 VLP to activate pp65-specific memory $CD4^+$ and $CD8^+$ T lymphocytes was measured in blood from cytomegalovirus-seropositive donors by means of an *ex vivo* whole blood assay. In the case of Ova, *in vitro* antigen presentation assays using murine bone marrow macrophages and antigen-specific $CD8^+$ T lymphocytes were used to assess the MHC I-restricted antigen presentation after protein delivery by the distinct yeast genera. Moreover, the influence of the subcellular localization of Ova on the degree of T cell activation was investigated, in which yeasts harbouring different antigen variants – intracellular, secreted or cell-wall anchored – were compared. Finally, a preliminary *in vivo* experiment using a selected yeast species

and a single antigenic variant was performed in order to obtain basic information with respect to the establishment of future immunization protocols in an animal model.

II. MATERIALS

1. Organisms

The organisms used within this work, as well as their respective source and characteristics, are listed below. Bacteria, yeasts and cell lines utilized belong to the culture collection of the Institute for Molecular and Cell Biology at Saarland University, unless otherwise stated.

1.1. *Escherichia coli* strains

Table 1. *E. coli* strains used in this work, as well as their respective genotype and source.

Strain	Genotype	Reference or Source
TOP10	F´*mcr*A Δ(*mrr-hsd*RMS *mcr*BC) Φ80*lac*ZΔM15 ΔlacX74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen (Germany)
DH5α	*F-, recA1, endA3, gyrA96, thi-1, hsdR17, supE44, relA1, deoR,* Δ*(argF lacZYA)U196,* φ*80dlacZΔM15*	Hanahan (1983)
Nova Blue Singles™ Competent Cells	*endA1 hsdR17* (r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[$proA^+B^+$ $lacI^qZ\Delta M15$::Tn*10*] (Tet^R)	Novagen (Germany)

1.2. Yeast strains

Table 2. Yeast strains used throughout this work, their respective genotype and source.

Strain	Genotype	Reference or Source
Saccharomyces cerevisiae S86c	*MATα ura3-2 leu2 his3 pra1 prb2 prc1 cps1 (heat cured)*	Schmitt (1995)
Saccharomyces cerevisiae W303-1a	*MATa leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*	Parlati *et al*. (1995)
Saccharomyces cerevisiae BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al*. (1998)
Saccharomyces cerevisiae BY4742 Δ*mnn11*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YJL183w::kanMX4*	EUROSCARF (Germany)
Saccharomyces cerevisiae BY4742 Δ*ost3*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YOR085w::kanMX4*	EUROSCARF (Germany)

Table 2 continuation

Strain	Genotype	Reference or Source
Kluyveromyces lactis GG799	No auxotrophies or genetic markers	Industrial isolate. New England Biolabs (Germany)
Pichia pastoris GS115	*his4*	Invitrogen (Germany)
Pichia pastoris KM71	*his4*, *arg4, aox1*::ARG4	Invitrogen (Germany)
Schizosaccharomyces pombe PW260	*h⁻leu1.32 ura4. dl18 ade 6.210*	Peter Wagner (Saarland University, Germany)
Hansenula polymorpha	*wild type*	Institut Pasteur (France)
Yarrowia lipolytica	*wild type*	Institut National de la Recherche Agronomique (France)
Candida glabrata	*wild type*	National Collection of Yeast Cultures (UK)

1.3. Cell lines

IC21 murine macrophages

This cell line is derived from C57BL/6 mouse peritoneal macrophages transformed with simian virus 40 (Walker and Gandour, 1980). These cells share many features with normal mouse macrophages, like phagocytic and cytolytic activities, and also express macrophage specific antigens. Cells were kindly provided by Dr. Gernot Geginat (Institute for Medical Microbiology and Hygiene, Faculty for Clinical Medicine Mannheim of the University Heidelberg, Germany).

1.4. Mice

OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb) express a transgenic T-cell receptor (TCR) that recognizes $OVA_{257-264}$ peptide (SIINFEKL) in context of MHC class I H-$2K^b$ (Hogquist *et al*., 1994). This TCR can be specifically identified with the anti-mouse Vα2 and Vβ5.5, 5.2 monoclonal antibodies. Experiments with OT-I mice were performed at the Medical Clinic for Rheumatology and Clinical Immunology (Charité, Berlin), in cooperation with Elisabeth Kenngott.

2. Culture Media and Supplements

The chemicals for the following media and supplement stock solutions were solubilized in distilled water and autoclave-sterilised, unless otherwise noted. Glucose as well as galactose solutions were autoclaved separetely. Liquid media were generally stored at room temperature. Solid agar media were stored at 4°C.

2.1. Bacterial culture media

SOC Medium

Yeast extract	0.5% w/v
Peptone	2% w/v
Glucose	20 mM
Potassium chloride	2.5 mM
Magnesium chloride	10 mM
Magnesium sulphate	10 mM
Sodium chloride	10 mM

The medium was stored at 4°C.

Luria Bertani (LB)

Peptone	1% w/v
Yeast extract	0.5% w/v
Sodium chloride	1% w/v
(Agar	1.5% w/v)

Ampicillin

Ampicillin	50 mg/ml

in 50 % ethanol

The solution was filter-sterilized and stored at -20°C.

Kanamycin

Kanamycin	25 mg/ml

The solution was filter-sterilized and stored at -20°C.

IPTG

IPTG	100 mM

The solution was filter-sterilized and stored at -20°C.

X-gal

X-gal	40 mg/ml
in dimethylformamide	

The solution was filter-sterilized and stored in the dark at -20°C.

2.2. Yeast culture media

YPD

Yeast extract	1% w/v
Peptone	2% w/v
Glucose	2% w/v
(Agar	1.5% w/v)

10× Yeast nitrogen base (YNB) standard stock solution

YNB without aminoacids and ammonium sulfate	17 g/l

The solution was filter-sterilized before use.

Synthetic Complete Medium (SC Medium)

10× YNB standard stock solution	10% v/v
Ammonium sulphate	0.5% w/v
Glucose	2% w/v
Bases and amino acids mix	0.087% w/v

Bases and amino acids mix

Adenine sulphate	0.2 g
Uracil	0.2 g
L-tryptophan	0.2 g
L-histidin HCl	0.2 g
L-arginin HCl	0.2 g
L-methionin HCl	0.2 g
L-tyrosin HCl	0.3 g

L-lysin	0.3 g
L-isoleucin HCl	0.3 g
L-phenylalanin	0.5 g
L-leucin HCl	1.0 g
L-valin	1.5 g
L-threonin	2.0 g
L-serin	4.0 g
L-aspartic acid	6.0 g
L-glutamic acid	6.0 g

Powders were ground into a homogeneous mixture and stored at 4°C. In the dropout (d/o) media described below uracil or histidin, respectively, were omitted in the mix.

2.2.1. *S. cerevisiae* media

Ura d/o Glucose

10× YNB standard stock solution	10% v/v
Ammonium sulphate	0.5% w/v
Bases and amino acids mix without uracil	0.087% w/v
Glucose	2% w/v
(Agar	1.5% w/v)

2.2.2. *P. pastoris* media

10× YNB stock for *P. pastoris*

YNB	34 g/l
Ammonium sulphate	100 g/l

The YNB amount in this solution is twice as concentrated as the standard YNB stock solution. The solution was filter-sterilized before use.

BMG (Buffered Minimal Glycerol)

1 M potassium phosphate buffer	10% v/v
10× YNB stock for *P. pastoris*	10% v/v
500× Biotin stock	0.2% v/v

Glycerol	1% v/v

BMM (Buffered Minimal Methanol)

Potassium phosphate, pH 6.0	100 mM
10× YNB stock for *P. pastoris*	10% v/v
500× Biotin stock	0.2% v/v
Methanol	0.5% v/v

1 M Potassium Phosphate Buffer, pH 6.0

Potassium dihydrogenphosphat	118.1 g/l
Di-potassium hydrogenphosphat	23 g/l

500× Biotin stock

Biotin	20% w/v

The solution was filter-sterilized and stored at 4°C

His d/o Glucose

10× YNB standard stock solution	10% v/v
Ammonium sulphate	0.5% w/v
Bases and amino acids mix without histidin	0.069% w/v
Glucose	2% w/v
(Agar	1.5% w/v)

2.2.3. *Sz. pombe* media

Yeast extract with supplements (YES)

Yeast extract	0.5% w/v
Glucose	3.0% w/v
Agar	2% w/v
10× supplements	10% v/v

Edinburgh minimal medium (EMM) with supplements

Potassium hydrogen phthalate	14.7 mM

Di-sodium hydrogenphosphate	15.5 mM
Ammonium chloride	93.5 mM
Glucose	2% w/v
(Low glucose	0.5% w/v)
50× Salt stock	2% v/v
1,000× Vitamin stock	0.1 % v/v
10,000× Mineral stock	0.01 % v/v
10× Supplements	10% v/v
(2,000× Thiamin stock	0.05% v/v)
(Agar	2% w/v)

50× Salt stock

Calcium chloride dihydrate	4.99 mM
Magnesium chloride hexahydrate	0.26 M
Potassium chloride	0.67 M
Sodium sulfate	14.1 mM

The solution was filter-sterilized and stored at 4°C

1,000× Vitamin stock

Biotin	40.8 µM
Inositol	55.5 mM
Nicotinic acid	81.2 mM
Pantothenic acid	4.2 mM

The solution was filter-sterilized and stored at 4°C

10,000× Mineral stock

Boric acid	80.9 mM
Citric acid monohydrate	47.6 mM
Copper(II)-sulfate pentahydrate	1.6 mM
Iron(III)-chloride hexahydrate	7.4 mM
Manganese sulfate monohydrate	23.7 mM
Molybdic acid	2.47 mM
Potassium iodide	6.02 mM
Zinc sulfate heptahydrate	13.9 mM

The solution was filter-sterilized and stored at 4°C

10× Supplements

Adenine	2.25 g/l
L-histidine	2.25 g/l
L-leucine	2.25 g/l
L-lysine	2.25 g/l
Uracil	2.25 g/l

The solution was filter-sterilized and stored at 4°C. For preparation of EMM leu d/o medium, leucine was omitted.

2,000× Thiamin stock

Thiamin	10 g/l

The solution was filter-sterilized and stored at -20°C

2.2.4. *K. lactis* media

YPGal

Yeast extract	1% w/v
Peptone	2% w/v
Galactose	3% w/v

YCB acetamid

Yeast carbon base	1.17% w/v
Acetamid	1% v/v
Sodium phosphate buffer, pH 7.0	3% v/v
Agar	1% w/v

The sodium phosphate buffer was prepared separetely. The medium was cooled to approximately 40°C before addition of acetamide.

1 M sodium phosphate buffer pH 7.0

Sodium dihydrogen phosphate	1 M
Di-sodium hydrogen phosphate	1 M

The buffer was prepared mixing 423 ml of the 1 M sodium dihydrogenphosphate solution with 577 ml of the 1 M di-sodium hydrogenphosphate solution.

100× Acetamid stock solution

Acetamid	500 mM

The solution was filter-sterilized and stored in the dark at 4 °C.

2.3. Mammalian cells culture media

RPMI 1640 was used as the basic medium for all mammalian cells. This culture medium contains 2 mM L-glutamine, sodium bicarbonate, and phenol red. Fetal calf serum included in all media was inactivated for 10 minutes at 42°C.

2.3.1. IC-21 murine macrophages

RPMI 1640	
Fetal calf serum	10% v/v

2.3.2. Human monocytes, dendritic cells

RPMI 1640	
Fetal calf serum (LPS-free)	10% v/v
Penicillin	100 U/ml
Streptomycin	100 μg/ml

2.3.3. Human lymphocytes

RPMI 1640	
Fetal calf serum	10% v/v
Penicillin	100 U/ml
Streptomycin	100 μg/ml

2.3.4. Freezing medium

RPMI 1640	20% v/v

DMSO	10% v/v
FCS	60% v/v

3. Buffers and Solutions

3.1. Common buffers

10× TE

Tris-HCl	10 mM
EDTA (disodium)	1 mM

pH 7.5

10× Phosphate-buffered saline (PBS)

Sodium chloride	1.5 M
Disodium hydrogen phosphate	0.1 M

pH 7.4

Hank's balanced salt solution (HBSS)

- Stock 1

Sodium chloride	8% w/v
Potassium chloride	0.4% w/v

- Stock 2

Disodium hydrogen phosphate	0,36% w/v
Potassium dihydrogen phosphate	0.6% w/v

- Stock 3

Calcium chloride	1.44% w/v

- Stock 4

Magnesium sulphate heptahydrate	2.46% w/v

- Stock 5

Sodium hydrogen carbonate	3.5% w/v

All stock solutions were filter-sterilized and stored at 4°C. Prior to use, solutions were mixed as follows:

Stock 1	10% v/v

Stock 2	1% v/v
Stock 3	1% v/v
Stock 4	1% v/v
Stock 5	1% v/v

250× Luminol solution

Lminol	100 mM

in DMSO

Aliquots were stored at -20°C.

Lysis buffer

Tris	20 mM
Magnesium chloride	10 mM
Ammonium sulphate	0.3 M
EDTA	1 mM
Glycerin	5% v/v
Dithiothreitol	1 mM
Protease inhibitor cocktail	1×

Isolation buffer

Tris-HCl, pH 7.8	10 mM
100× PMSF	1% v/v

100× PMSF

PMSF	100 mM

in isopropanol

The solution was stored at -20°C.

1,000× DTT

DTT	1 M

in 10 mM sodium acetate, pH 5.2.

The solution was filter-sterilized and stored at -20°C.

3.2. Buffers for agarose gel electrophoresis

GLB (Gel Loading Buffer)

Glycerol	50% w/v
SDS	1% w/v
EDTA	125 mM
Bromophenol blue	0.05% w/v
Xylenecyanol	0.05% w/v

10× TBE

Tris base	0.89 M
Boric acid	0.89 M
EDTA	20 mM

3.3. Buffers for isolation of plasmid DNA

GTE

Glucose	50 mM
Tris-HCl, pH 8.0	25 mM
EDTA	10 mM

5 M Potassium acetate

Acetic acid (98%)	29.5% v/v
pH 4.8	

NaOH/SDS

NaOH	200 mM
SDS	1% w/v

3.4. Solutions for transformation of *Sz. pombe*

10× TE

Tris	100 mM
EDTA	10 mM

pH 7.5

Lithium acetate / EDTA

Lithium acetate	100 mM
EDTA	1 mM

pH 4.9, filter-sterilized

PEG / lithium acetate / EDTA

Polyethylene glycol 3350	40% w/v
EDTA	1 mM
Lithium acetate	100 mM

3.5. Solutions for transformation of *P. pastoris*

10 × Bicin-NaOH pH 8.3

Bicin	100 mM

pH 8.3

BEDS Buffer

10 x Bicin-NaOH pH 8.3	10% v/v
Ethylene glycol	3% v/v
DMSO	3% v/v
Sorbitol	1 M

1 M Sorbitol

Sorbitol	1 M

The solution was filter-sterilized and stored at 4 °C.

3.6. Solutions for transformation of *S. cerevisiae*

10× lithium acetate

Lithium acetate	1 M

pH 7.5

The solution was filter-sterilized and stored at room temperature.

10× TE

Tris	100 mM
EDTA	10 mM
pH 7.5	

Lithium acetate / TE

10× TE	10% v/v
10× lithium acetate pH 7.5	10% v/v

PEG solution

10× Lithiumacetat	10% v/v
10× TE pH 7,5	10% v/v
PEG 4000 50% (w/v)	80% v/v

3.7. Solutions for transformation of *K. lactis*

Yeast Transformation Reagent (New England Biolabs)

3.8. Buffers for VLP preparation

Spheroplasting buffer

Tris-HCl pH7.5	10 mM
Sorbit	0.8 M
$CaCl_2$	10 mM
1,000× DTT	0.2% v/v
Zymolyase 20T	200 µg/ml

DTT and zymolyase were added separately.

PBSE(S)

NaCl	150 mM
Na_2HPO_4	10 mM
Na_2EDTA	10 mM
(Sorbitol	1M)
pH 7.4	

Sucrose gradient solutions

Sucrose	20%, 45%, or 70% w/v
in PBSE	

The 20-70% sucrose linear gradient was prepared with a gradient former (BioRad) using equal volumes of 20% sucrose and 70% sucrose solutions.

3.9. Buffers and solutions for SDS-PAGE

Tris-Glycin

Tris	25 mM
Glycin	250 mM
SDS	0.1% w/v

10× SDS gel loading buffer

Tris-HCl pH 6.8	1 M
SDS	10% w/v
Bromophenol blue	0.5% w/v
Glycerol	50% v/v
β-Mercaptoethanol	1 M

3.10. Buffers and solutions for western blotting

10× TBS

Tris	1 M
NaCl	1.5 M
pH 7.5	

Transfer buffer

Tris-HCl, pH8.4	25 mM
Glycine	190 mM
Methanol	20% v/v
SDS	0.1% w/v

Wash buffer

10× TBS	10% v/v
Tween 20	0.05% v/v

Blocking buffer

10× TBS	10% v/v
Tween 20	0.05% v/v
Low-fat milk	5% w/v

3.11. Buffers and solutions for cell culture

Erythrocyte lysing solution

Potassium bicarbonate	0.01 M
Ammonium chloride	0.155 M
EDTA	0.1 mM

The pH was adjusted to 7.5 and the solution was filter-sterilized.

FACS buffer

Fetal calf serum	5% v/v
BSA	0.5% w/v
Sodium azide	0.07% w/v
in PBS	

FACS Fix

Paraformaldehyde	1% w/v
Sodium chloride	8.5 g/l
pH 7.4	

MACS buffer

BSA	0.5% w/v
EDTA	2 mM
in PBS	

4. Primers

Table 3 shows the oligonucleotide primers used in this work for PCR amplification. Primers were synthesized by Sigma.

Table 3. Oligonucleotide primers used and their respective sequences. Restriction enzyme recognition sites are underlined; start and stop codons are written in bold; mutations are written in italics.

Primer name	Sequence (5' – 3')
5'*Hind*III_*Eco*RI_Ova	AAG CTT GAA TTC **ATG** GGC TCC ATC GGC GCA G
3'*Bgl*II_*Not*I_Ova	GCG GCC GC A GAT CT**T TA**A GGG GAA ACA CAT CTG CCA AAG AAG
5'*Eco*RI_*Xho*I Ova (Walch, 2009)	GAA TTC CTC GAG **ATG** GGC TCC ATC GGC GCA G
3'*Sal*I_*Bgl*II_Ova (Walch, 2009)	GTC GAC AGA TCT **TTA** AGG GGA AAC ACA TCT GCC AAA GAA G
5'*Avr*II_Sed1 (Müller, 2008)	CCT AGG TTT TCC AAC AGT ACA TCT GCT TCT TCC ACC
3'*Not*I_Sed1 (Müller, 2008)	GCG GCC GC**T TA**T AAG AAT AAC ATA GCA ACA CCA GCC AAA CC
3'*Bgl*II_Sed1	AGA TCT **TTA** TAA GAA TAA CAT AGC AAC ACC AGC CAA ACC
3'Ova_*Avr*II_ohneStop	CCT AGG AGG GGA AAC ACA TCT GCC AAA GAA GAG
5' Ovacyt_mut	TAT TCA AGG AGC T*T*A TCA ATT CCT GGG TA
3' Ovacyt_mut	TAC CCA GGA ATT GAT *A*AG CTC CTT GAA TA
5' *Xba*I*Sac*I_Ovacyt	TCT AGA GAG CTC **ATG** GGC TCC ATC GGC GCA GCA
5'pp65 (Walch, 2009)	GAA TTC CTC GAG GAG CTC **ATG** ATA TCC GTA CTG GGT CCC ATT TCG
3' pp65 (Walch, 2009)	TCT AGA GTC GAC **TCA** ACC TCG GTG CTT TTT GGG C
3'*Bam*HIpp65 (Schumacher, 2001)	GGA TCC **TCA** ACC TCG GTG CTT TTT GGG C
5'*Sac*I pp65	GAG CTC **ATG** ATA TCC GTA CTG GGT CCC
5'Prepropp65	AGC AGG GCT TAG AAG AAC GT**A TG**A TAT CCG TAC TGG GT
3'Prepropp65	ACC CAG TAC GGA TAT CAT ACG TTC TTC TAA GCC CTG CT
5'pp65mut*Bst*XI	TTC GTG TTT CCC AC*A* AAG GAC GTG
3'pp65 mut*Bst*XI	CAC GTC CTT *T*GT GGG AAA CAC GAA
5' K28 (Sendzik, 2006)	CTC GAG GAA TTC **ATG** GAG AGC GTT TCC TCA TTA TTT AAC ATT TTT TC

5. Antibodies

5.1. Primary antibodies

All primary antibodies used in this work are listed in Table 4.

Table 4. Primary antibodies utilized throughout this work, their main characteristics and sources.

Antigen	Clone	Host	Isotype	Conjugation	Manufacturer
Chicken Ovalbu-min (polyclonal)	-	rabbit	-	-	Sigma
HCMV pp65	2 and 6	mouse	IgG1 κ	-	Leica
β-1,3-glucan		mouse	IgG κ	-	Biosupplies Australia
Mouse CD8a	53-6.7	rat	IgG2a κ	FITC	Becton Dickinson
Mouse IFN-γ	XMG1.2	rat	IgG1 κ	PE-Cy7	Becton Dickinson
Mouse Vβ5.1/5.2	MR9-4	mouse	IgG1 κ	PE	Becton Dickinson
Mouse Dectin-1	2A11	rat	IgG2b	-	Serotec
Mouse/human Mannose receptor	15-2	mouse	IgG1	-	Abcam
Human Dectin-1	GE2	mouse	IgG1	-	Serotec
Human IFN-γ	4S.B3	mouse	IgG1 κ	FITC	Becton Dickinson
Human CD1a	NA1/34	mouse	IgG2a κ	FITC	Dako
Human CD3	UCHT1	mouse	IgG1	FITC	Beckman Coulter
Human CD4	SK3	mouse	IgG1 κ	PerCP	Becton Dickinson
Human CD8	SK1	mouse	IgG1	PerCP	Becton Dickinson
Human CD14	TÜK4	mouse	IgG2a κ	PE	Dako
Human CD19	J3-119	mouse	IgG1	PE	Beckman Coulter
Human CD28	L293	mouse	IgG1	-	Becton Dickinson
Human CD40	5C3	mouse	IgG1 κ	FITC	Becton Dickinson
Human CD49d	9F10	mouse	IgG1 κ	-	Becton Dickinson
Human CD54	HA58	mouse	IgG1 κ	PE	Becton Dickinson

Table 4 continuation

Antigen	Clone	Host	Isotype	Conjugation	Manufacturer
Human CD58	1C3	mouse	IgG2a κ	FITC	Becton Dickinson
Human CD69	TP1.55.3	mouse	IgG2b	PE	Beckman Coulter
Human CD80	MAB104	mouse	IgG1	PE	Beckman Coulter
Human CD83	HB15a	mouse	IgG2b	PE	Beckman Coulter
Human CD86	2331 (FUN-1)	mouse	IgG1 κ	FITC	Becton Dickinson
Human CCR7	3D12	rat	IgG2a κ	PE	Becton Dickinson
Human HLA A,B,C	W6/32	mouse	IgG2a κ	PE	Dako
Human HLA DP, DQ, DR	CR3/43	mouse	IgG1 κ	FITC	Dako

5.2. Secondary antibodies

The secondary antibodies used in this work are listed in Table 5.

Table 5. Secondary antibodies used in this work, their main characteristics and manufacturers.

Antigen	Host	Conjugation	Manufacturer
Mouse IgG	goat	FITC	Sigma
Mouse IgG	goat	PE	Sigma
Mouse IgG	goat	horseradish peroxidase type VI	Sigma
Rabbit IgG	goat	FITC	Sigma
Rabbit IgG	goat	horseradish peroxidase type VI	Sigma
Rat IgG	goat	FITC	Sigma

6. Proteins and peptides

Chicken ovalbumin, wheat germ agglutinin-FITC	Sigma-Aldrich (Schnelldorf, Germany)
Concanavalin A-FITC	Invitrogen (Darmstadt, Germany)
Pp65 from HCMV	Miltenyi Biotec (Bergisch Gladbach, Germany)

SIINFEKL peptide	Eurogentec (Cologne, Germany)

7. Enzymes

DNase-free RNase, restriction enzymes, T4 DNA Ligase	Fermentas (St. Leon-Rot, Germany)
Klenow Polymerase	Roche (Mannheim, Germany)
Laminarinase	Sigma-Aldrich (Schnelldorf, Germany)
Zymolyase 20T (*A. luteus*)	AMS Biotechnology (Abingdon, UK)

8. Molecular weight markers

PageRuler prestained protein ladder	Fermentas (St. Leon-Rot, Germany)
Quick-Load 1 kb DNA Ladder	New England Biolabs (Frankfurt, Germany)
Smart Ladder DNA ladder	Eurogentec (Seraing, Belgium)

9. Reagents for cell culture

Brefeldin A, ionomycin, LPS, penicillin/streptomycin, PMA, RPMI1640 with phenol red, saponin, *Staphylococcus* enterotoxin B	Sigma-Aldrich (Schnelldorf, Germany)
HCMV lysate	Virion\Serion (Würzburg, Germany)
FACS lysing solution	Becton Dickinson (Heidelberg, Germany)
Lymphocyte separation medium, Fetal calf serum	PAA (Cölbe, Germany)
Recombinant human GM-CSF (rhGM-CSF)	Berlex Laboratories (Richmond, USA)
Recombinant human IL-4 (rhIL-4)	Miltenyi Biotec (Bergisch Gladbach, Germany)

10. Kits

Expand High Fidelity PCR System	Roche Diagnostics (Mannheim, Germany)
E.Z.N.A. Gel Extraction Kit	OMEGA bio-tek (Norcross, USA)
K. lactis Protein Expression Kit	New England Biolabs (Frankfurt, Germany)

MACS CD14 microbeads, separation columns, and separation unit	Miltenyi Biotec (Bergisch Gladbach, Germany)
peqGOLD Plasmid Miniprep Kit II	PEQLAB Biotechnologie (Erlangen, Germany)
pSTBlue-1 AccepTor Vector Giga Kit	Novagen (Darmstadt, Germany)
SuperSignal West Dura Substrat	Thermo Scientific (Reinach, Switzerland)

11. Other chemicals and materials

Agar, peptone, yeast extract	Marcor (New Jersey, USA)
Agarose	Biozym (Oldendorf, Germany)
Ampicillin, bromophenolblue	Sigma-Aldrich (Schnelldorf, Germany)
Complete protease inhibitor cocktail, PVDF Membrane	Roche (Mannheim, Germany)
Dialysis filter	Millipore (Schwalbach, Germany)
Difco YNB w/o amino acids and ammonium sulphate	Becton Dickinson (Heidelberg, Germany)
Electroporation cuvette, extra thick blot paper	BioRad (Munich, Germany)
Luminol, salmon sperm DNA, β-mercaptoethanol	Serva (Heidelberg, Germany)
Vivaspin	Sartorius (Goettingen, Germany)
All other chemicals and materials	Merck (Darmstadt, Germany); Roth (Karlsruhe, Germany)

12. Plasmids

The source and main characteristics of the plasmids used as PCR template or for cloning, subcloning, or sequencing purposes are listed in Table 6. Constructed plasmids used for expression of recombinant proteins in this work are listed in Table 7.

Table 6: Plasmids used throughout this work and their main properties.

Plasmid (size)	Source	Properties
pSTBlue-1 (3.8 kbp)	Novagen	Linearized vector for TA cloning, amp^R, kan^R, T7 and SP6 promoters, *lacZα*-reporter gene for blue/white screening,
pKLAC1 (9.0 kbp)	New England Biolabs	*K. lactis* integrative expression vector, LAC4 promoter, *amdS* gene, α-mating factor, amp^R
pREP1 (8.9 kbp)	Maundrell, 1993	*Sz. pombe* episomal expression vector, *nmt1* promoter, *leu2* gene, autonomous replicating sequence, amp^R

Table 6 continuation

Plasmid (size)	Source	Properties
pREP-BD (9.0 kbp)	Diehl, 2008	pREP1 vector with improved multiple cloning site
pPIC3.5 (7.8 kbp)	Invitrogen	*P. pastoris* integrative expression vector, *aox1* promoter, *his4* gene, amp^R
pPIC9 (8.0 kbp)	Invitrogen	*P. pastoris* integrative expression vector, *aox1* promoter, *his4* gene, α-mating factor, amp^R
pPIC9/*sed1*	Müller, 2008	pPIC9 vector containing the *sed1* sequence
pPGK (6.1 kbp)	Kang *et al*., 1990	*S. cerevisiae* episomal expression vector, *pgk* promoter, *ura3* gene, 2µ origin, amp^R
pPGK-6His/Xa/GST (6.7 kbp)	Bernardy, 2006	pPGK vector containig a *6×His/Xa/GST/TGA* gene fusion
pPGK-M28-I (6.9 kbp)	Schmitt and Tipper, 1995	pPGK vector containing a *K28preprotoxin* coding sequence
pFB2 (6.3 kbp)	Breinig, 2002	pPGK-M28-I-derived vector containing *Kre1*/*HA*/*Cwp2* genes
pYES/OVA (7.0 kbp)	Walch, 2009	*S. cerevisiae* expression vector pYES (Invitrogen), *ura3* gene, GAL1 promoter, 2µ origin, amp^R; containing the *ova* gene
pYES/OVAcyt (6.7 kbp)	Walch, 2009	*S. cerevisiae* expression vector pYES (Invitrogen), *ura3* gene, GAL1 promoter, 2µ origin, amp^R; contains the Ova_{cyt} coding sequence
pPGK/pp65 (7.7 kbp)	Schumacher, 2001	pPGK vector containing the sequence coding for full-length pp65
pG[0]G (8.9 kbp)	Powilleit, 2004	pPGK vector containing *gag*/*gfp* gene fusion
pG (8.1 kbp)	Powilleit, 2004	pPGK vector containing *gag* gene
YEp 352 (5.2 kbp)	Hill *et al*., 1986	*S. cerevisiae* vector, *ura3* gene, 2µ origin, amp^R

Table 7: Plasmids constructed in this work for recombinant protein expression and their properties.

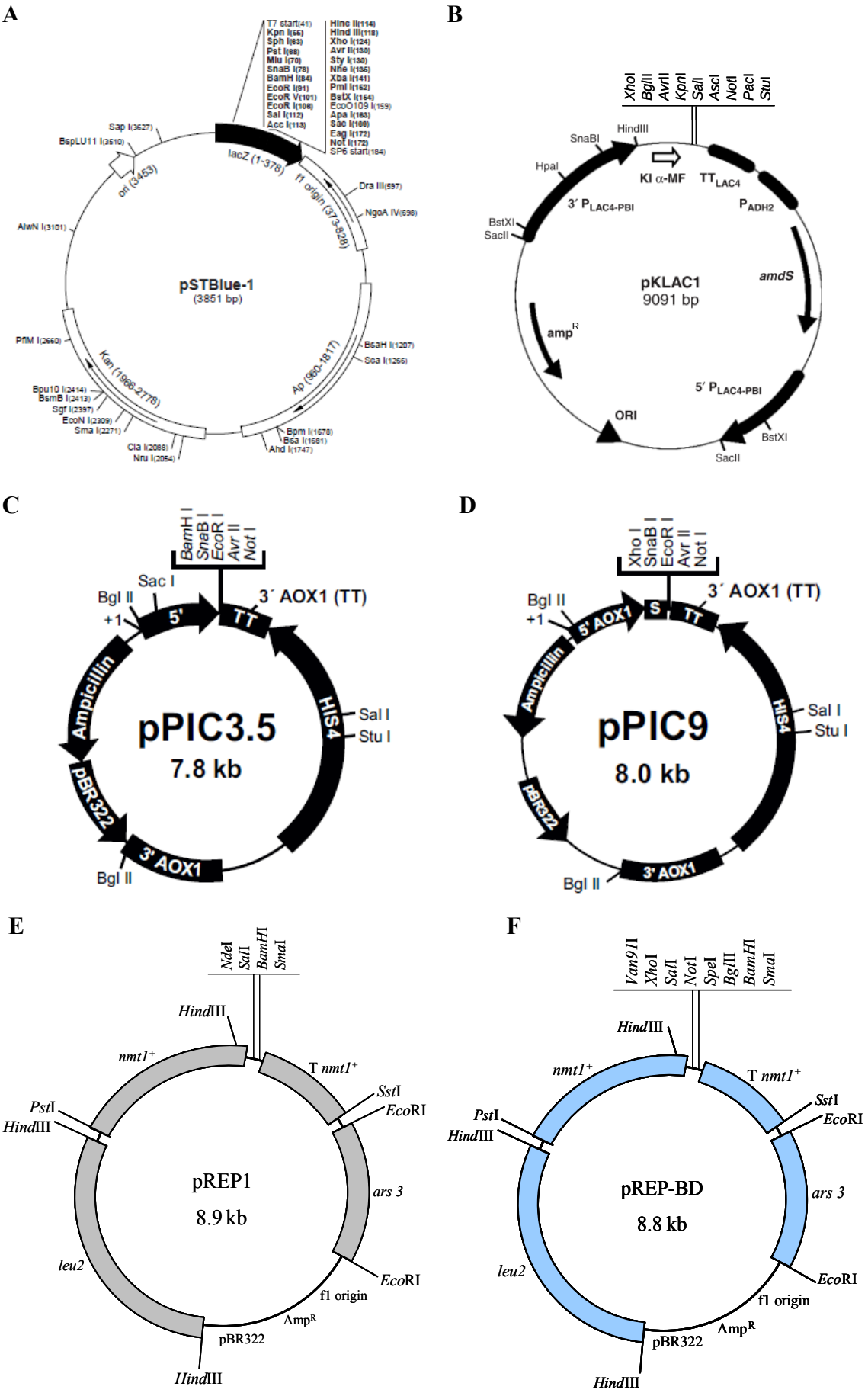
Plasmid (size)	Properties
pPGK/*ova* (7.2 kbp)	pPGK vector containing the sequence coding for full-length Ova
pPGK/ova_{cyt} (6.9 kbp)	pPGK vector containing the Ova_{cyt} coding sequence
pFB2/ova_{cyt}/*sed1* (7.9 kbp)	pFB2 vector containing the OVA_{cyt}/sed1 open reading frame downstream of the Kre1 leader sequence
pPGK/*kre1*/*pp65* (7.8 kbp)	pPGK vector containing the sequence coding for full-length pp65 downstream of the Kre1 leader sequence

Table 7 continuation

Plasmid (size)	Properties
pG[0]G/*ova*$_{cyt}$ (9.0 kbp)	pG[0]G vector containing the Ova$_{cyt}$ coding sequence downstream of the *gag* gene (replacing *gfp*)
pG[0]G/*pp65* (9.8 kbp)	pG[0]G vector containing the sequence coding for full-length pp65 downstream of the *gag* gene (replacing *gfp*)
pREP-BD/*ova* (9.9 kbp)	pREP-BD vector containing the sequence coding for full-length Ova
pREP-BD/*ova*$_{cyt}$ (9.6 kbp)	pREP-BD vector containing the sequence coding for Ova$_{cyt}$
pREP1/*pp65* (10.6 kbp)	pREP1 vector containing the sequence coding for full-length pp65
pREP1/*prepropp65* (10.7 kbp)	pREP1 vector containing the sequence coding for full-length pp65 downstream of a K28preprotoxin leader sequence
pPIC3.5/*ova* (8.9 kbp)	pPIC3.5 vector containing the sequence coding for full-length Ova
pPIC3.5/*ova*$_{cyt}$ (8.6 kbp)	pPIC3.5 vector containing the sequence coding for Ova$_{cyt}$
pPIC3.5/*pp65* (9.4 kbp)	pPIC3.5 vector containing the sequence coding for full-length pp65
pPIC9/*ova*$_{cyt}$/*sed1* (9.7 kbp)	pPIC9 vector containing the sequence coding for Ova$_{cyt}$ downstream of the α-mating factor sequence and upstream of the *sed1* coding sequence
pPIC9/*pp65* (9.6 kbp)	Contains the sequence coding for full- length pp65 downstream of the α-mating factor sequence
pKLAC1/*ova* (10 kbp)	pKLAC1 vector without the α-mating factor sequence and containing the sequence coding for full-lehgth OVA
pKLAC1/*ova*$_{cyt}$ (9.7 kbp)	pKLAC1 vector without the α-mating factor sequence and containing the Ova$_{cyt}$ coding sequence
pKLAC1/*ova*$_{cyt}$/ *sed1* (10.7 kbp)	pKLAC1 vector containing the *ova*$_{cyt}$/*sed1* fusion downstream of the α-mating factor sequence
pKLAC1/*pp65mut* (10.5 kbp)	pKLAC1 vector without the α-mating factor sequence and containing the sequence coding for pp65
pKLAC1/ *secpp65mut* (10.7 kbp)	pKLAC1 vector containing the sequence coding for pp65 downstream of the α-mating factor sequence

12.1. Plasmid maps

The maps of the plasmids used in this work for sequencing or transformation purposes are given below (Fig. 5).


A
pSTBlue-1
(3851 bp)
lacZ (1-378)
f1 origin (373-828)
ori (3453)
Kan (1966-2778)
Ap (960-1817)
B
pKLAC1
9091 bp
amdS
amp^R
ORI
TT_LAC4
P_ADH2
3′ P_LAC4-PBI
5′ P_LAC4-PBI
C
pPIC3.5
7.8 kb
Ampicillin
pBR322
3′ AOX1
HIS4
5′
TT
Sal I
Stu I
Bgl II
Sac I
D
pPIC9
8.0 kb
5′ AOX1
S
TT
3′ AOX1 (TT)
E
pREP1
8.9 kb
nmt1+
T nmt1+
ars 3
leu2
f1 origin
pBR322
F
pREP-BD
8.8 kb
nmt1+
T nmt1+
ars 3
leu2
f1 origin
pBR322

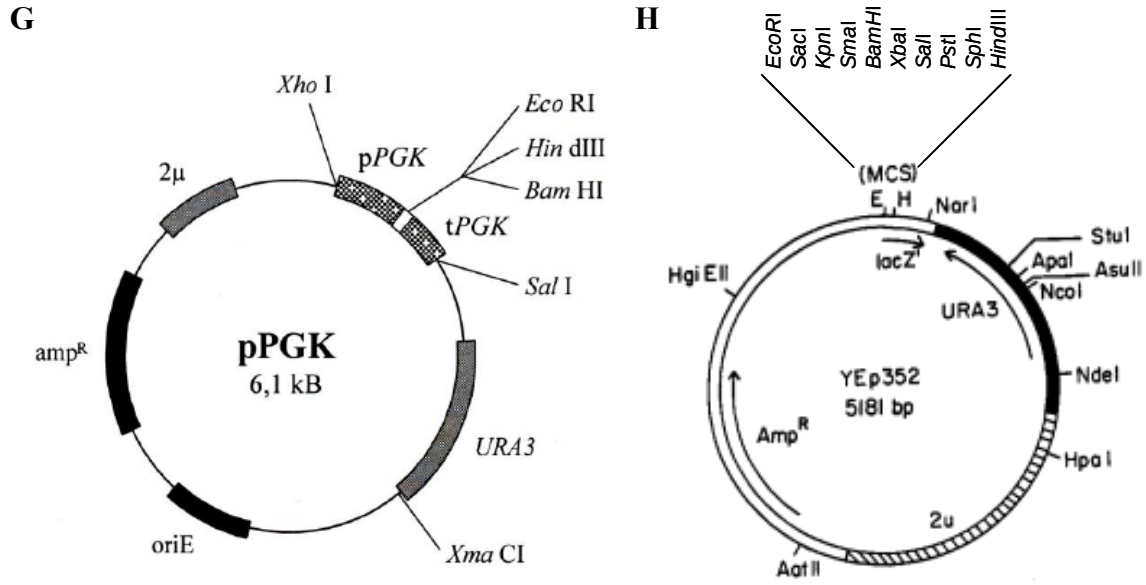


Fig. 5. Schematic representation of DNA plasmids used for sequencing **(A)** or for heterologous gene expression in *K. lactis* **(B)**, *P. pastoris* **(C and D)**, *Sz. pombe* **(E and F)**, and *S. cerevisiae* **(G)**. The plasmid YEp352 **(H)** was only used as control vector in *S. cerevisiae*.

III. METHODS

1. Molecular Biology Methods

1.1. DNA amplification by polymerase chain reaction

The polymerase chain reaction (PCR) allows the amplification of a specific DNA fragment flanked by regions of known sequence. This method relies on the selective replication of a certain DNA segment catalyzed *in vitro* by a heat-stable DNA polymerase in a series of cycles (Saiki *et al*., 1985). Two oligonucleotides which are complementary to the 3' ends of the region to be amplified are used as primers. These can also contain additional restriction sites, in order to permit further cloning of the DNA fragment into specific plasmid vectors. A typical reaction mixture contains the template DNA, a thermostable DNA polymerase, at least two oligonucleotide primers, and the four deoxynucleotides in a reaction buffer containing Mg^{2+}. First, the reaction mixture is heated to 94°C in order to denature the DNA, than cooled down below the melting temperature of the primers (40-60°C) for hybridization of these synthetic oligonucleotides to the complementary DNA strands (annealing). Next, when the optimum temperature for the DNA polymerase is reached (68-72°C), elongation occurs. In this step, DNA is synthesized by polymerization, starting from the oligonucleotide primers. The most widely used polymerase is derived from the thermophilic bacterium *Thermus aquaticus* and referred to as *Taq* polymerase. In order to minimize the occurrence of mutations, a high-fidelity DNA polymerase with proofreading activity was used.

A typical 50 µl-reaction was prepared as follows:

Template DNA (1:10 dilution)	1.0 µl
10 mM dNTP mix	0.5 µl
100 µM Forward primer	0.5 µl
100 µM Reverse primer	0.5 µl
Taq DNA Polimerase 5U/µl	0.7 µl
10× reaction buffer containing Mg^{2+}	5.0 µl
Distilled water	ad 50 µl

PCRs were performed in a Peqlab or an Eppendorf thermocycler. A classical reaction was run as follows:

Initial denaturation	94ºC	3 min	
Denaturation	94ºC	30 s	30 cycles
Annealing	45-65ºC	30 s	
Elongation	72ºC	1 min/kb	
Terminal elongation	72ºC	6 min	

SOE PCR

The "splicing by overlap extention" (SOE) method is used to construct a DNA fragment encoding a fusion protein. In this technique, the sequences are fused in frame without the use of restriction sites. A total of four oligonucleotide primers are used, so that the reverse primer of the first sequence has an overlapping region with the forward primer of the second coding sequence.

First, the genes to be fused are amplified in separate PCRs. Then, the PCR products are isolated and brought together in a reaction tube containing the forward primer of the first sequence and the reverse primer of the second sequence, among the other reagents necessary for a classical reaction, as described above. In the course of the reaction, the strands with the matching sequences overlap, acting as primers for each other and allowing extension by DNA polymerase. Therefore, the resulting molecule is a product of recombined genes, whose original sequences were 'spliced' together (Ho *et al*., 1989; Horton *et al*., 1989). Reactions were basically run as described above in this section.

1.2. Agarose gel electrophoresis

Agarose gel electrophoresis is a method used for determination of length and purity of DNA molecules that migrate in an electric field (Aaij and Borst, 1972). Double-stranded DNA molecules pass through the gel at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. Large DNA fragments migrate through the matrix pores with less efficiency in comparison to smaller molecules (Sambrook *et al.*, 1989). Migration patterns of DNA molecules also depend on molecular conformation and

agarose concentration. Since the phosphate backbone confers negative charge to nucleic acids, these molecules migrate toward the positive electrode under the selected buffer conditions (TBE, pH 8.3).

An appropriate amount of agarose (0.8-1% w/v) was diluted in 1× TBE buffer and heated in a microwave oven until it was completely dissolved. After cooling to 50-60°C, the solution was poured into a mould and allowed to gelatinize. After solidification, the gel was submerged in 1X TBE running buffer. The DNA samples were mixed with "Gel Loading Buffer" (5:1) before being loaded on the agarose gel. This buffer also contains glycerol, which enhances the density of the samples, rendering them to sink into the pockets. Electrophoresis was performed at approximately 8 V/cm. The electrophoretic separation was monitored by the position of dyes (bromophenol blue and xylene cyanol). Smart Ladder (range 200 bp – 10,000 bp, Fermentas) or Quick Load 100 bp DNA Ladder (range 100 bp – 1517 bp, New England Biolabs) were used as molecular weight markers.

After electrophoresis, the gel was incubated for 10 minutes in a water bath containing 0.3 ‰ (v/v) ethidium bromide. This dye intercalates between base pairs of nucleic acids, generating fluorescent complexes which can be visualised under UV light.

1.3. Elution of DNA fragments from agarose gels

Reisolation of DNA fragments was performed with the "E.Z.N.A. Gel Extraction Kit" (Omega Biotek). This procedure relies on the melting of agarose and subsequent binding of the DNA to a membrane in the presence of chaotropic ions. The desired DNA band was excised from the ethidium bromide stained agarose gel with a scalpel and placed into a 2 ml tube. The gel slice was melted in 400 µl of XP2 binding buffer at 65°C for 7 minutes. The mixture was transferred to the silica column and centrifuged at 13,000 rpm for 1 minute. Then, the column was washed with 300 µl of XP2 binding buffer, followed by 700 µl of SPW wash buffer. The column was further centrifuged for 2 min at 13,000 rpm to dry the matrix and 30 µl of sterile distilled water were added. After 2 minutes, the DNA was eluted by centrifugation at 13,000 rpm.

1.4. Restriction enzyme digestion of plasmid DNA

Restriction enzymes are endonucleases of bacterial origin that bind to double-stranded DNA at specific sites, breaking phosphodiester bonds inside or beyond the recognition sequence. Restriction endonucleases have been divided into three groups (type I, type II, type III), and type II enzymes are the most routinely used in molecular cloning, since they digest the DNA within the recognition sequence. Some restriction endonucleases generate staggered cuts, leaving short, 5' or 3' single-stranded overhanging ends at the tails of the fragments, which are known as cohesive or sticky ends. Other DNA endonucleases generate blunt-ended fragments. The majority of the recognition sequences are 4-8 base pairs long and palindromic, *i.e.*, rotationally symmetrical (Bigger *et al*., 1973; Sambrook *et al*., 1989).

Restriction digestion was performed using 5 U of the specific enzyme, 0.5–2 µg plasmid DNA, the correspondent buffer properly diluted (10-fold or 5-fold dilutions, depending on the enzyme), and sterile distilled water to 20 µl. If DNA preparations were derived from alkaline lysis, 5 U of RNase A was added in order to degrade any remaining RNA in the sample. Reactions were incubated at the optimum temperature for each enzyme (generally 37°C) for 1.5 h, then mixed with "Gel Loading Buffer" and analyzed by agarose gel electrophoresis.

1.5. Cloning of PCR products using AccepTor Vector Kit

PCR amplification products were ligated to pSTBlue-1 vector (Novagen) for sequencing purposes. This linearized vector contains single 3'-dU overhangs, providing optimal conditions for ligation with PCR fragments generated by some thermostable DNA polymerases, like *Taq* polymerase, which add single 3'-dA to the amplified products. The dU residues are substituted for dT residues as the plasmid is replicated within bacteria.

A typical ligation reaction of a PCR product in the pSTBlue-1 vector was performed as follows: 0.5 µl (25 ng) of pSTBlue-1 vector were mixed with 1 µl of the purified PCR product and 2.5 µl of "Clonables 2× Ligation Premix". Water was added to 5 µl and the reaction tube was incubated for 1 h at 16°C. After that, the ligation product was used to transform "NovaBlue Singles Competent Cells" (Novagen), as described in section 4.1.

Colonies were screened for the presence of insert in the pSTBlue-1 plasmid following the method for blue/white selection. The pSTBlue-1 plasmid contains a gene which codes for a *lacZ* α-peptide that complements the *lacZ* ω-fragment produced by the *E. coli* strain supplied with the kit. The resulting product, β-galactosidase, hydrolizes the galactoside substrate X-gal (5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside) yielding 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue pigment, which confers the colony a blue phenotype. Inserts successful cloned into pSTBlue-1 vector interrupt the sequence encoding the α-peptide, which generates a white colony on X-gal/IPTG plates.

1.6. DNA ligation

Ligation of two DNA molecules involves the formation of a phosphodiester bond between the 3'-hydroxyl group at the terminus of one strand and a 5'phosphate at the end of another strand, in a reaction that consumes ATP. In molecular cloning, bacteriophage T4 DNA Ligase is the most routinely used enzyme, as it can efficiently link blunt-ended DNA segments under normal reaction conditions (Sambrook *et al*., 1989; Nelson and Cox, 2005).

Sticky-end ligation

A previously digested and purified DNA fragment was mixed with the correspondent vector DNA carrying compatible cohesive ends. Normally, a threefold molar excess of the insert in comparison to the vector was used. For a typical 20-µl reaction, 1 U of T4 DNA Ligase and 2 µl of 10× ligation buffer containing ATP were added. If necessary, sterile distilled water was added up to 20 µl. Reactions were incubated overnight at 16°C or for 2 h at room temperature. After that, the ligation mixture was dialyzed against 10% glycerine on a nitrocellulose membrane (0.025 µm mean pore size, Millipore) at room temperature, for 1 h.

Blunt-end ligation

In order to fill recessed 3' termini or to digest protruding 3' ends created by digestion of DNA with restriction enzymes, the large fragment of DNA polymerase I (Klenow Fragment) was used. Klenow Fragment is a product of proteolytic cleavage of *E. coli*

DNA polymerase I which possesses polymerization and 3'→ 5' exonuclease activities, but no 5'→ 3' exonuclease activity (Klenow and Henningsen, 1970). Klenow was used to generate blunt ends by removing of 3´ overhangs or filling-in of 5´ overhangs. For removal of protruding 3´ ends, 2 U of Klenow enzyme were added to the digestion reaction after 1.5 h restriction, and the volume was brought up to 30 µl with sterile distilled water and restriction enzyme buffer. Samples were incubated for 1 h at 37°C and subsequently purified with "E.Z.N.A. Gel Extraction kit" for further digestion. For filling 5´ overhangs, 4 µl 10 mM dNTPs were also added to the reaction mixture.

Ligation was performed as follows: for a 20-µl reaction, specific volumes of insert and vector were mixed with 1 µl of 10 mM ATP, 1 µl of 40% PEG 8000, and 1 U of T4 DNA Ligase. PEG 8000 is a condensing agent, which enhances macromolecular crowding, increasing the efficiency of ligation reactions (Zimmerman and Pheiffer, 1983). Reactions were incubated overnight at 16°C and dialyzed on a filter membrane, as described previously for sticky-end ligation.

1.7. Determination of DNA concentration and purity

Spectrophotometric determination of DNA concentration relies on the fact that nucleic acids strongly absorb UV light at 260 nm. Since the aromatic amino acids phenylalanine, tyrosine and tryptophane absorb strongly at 280 nm, purity of DNA preparation can be assessed by the quotient A_{260}/A_{280}. An optimum A_{260}/A_{280} ratio ranges between 1.8-2.0 for a cuvette spectrophotometer. Values under 1.8 indicate protein contamination, whereas values above 2.0 suggest the presence of RNA in the sample.

One microliter of a DNA solution was mixed with 59 µl of sterile distilled water and transferred to a 1 cm-thick quarz cuvette. Water was used as reference. Concentrations were calculated in µg/ml.

Alternatively, the DNA concentration was roughly estimated by comparing the intensity of the DNA band to a molecular marker of known concentration. Two microliters of DNA solution were mixed with GLB and water to a final volume of 10 µl, applied on 1% agarose gel and allowed to migrate a certain distance, so that the bands of the marker would be well separated. After incubation of the gel in ethidium bromide bath for 30 minutes (for sufficient staining of the less concentrated bands), bands were visualized under UV light for direct comparison.

1.8. DNA sequencing

DNA sequence analysis by the dideoxy chain termination method (Sanger, 1977) makes use of labeled dideoxynucleotides as chain terminating agents. A new DNA strand is synthesized, starting from a specific primer, and synthesis is interrupted after incorporation of a dideoxynucleotide. The reactions are base-specific, so that the sizes of the fragments are used to determine the exact positions in which a specific base appears. At the very end of the procedure, four sets of labeled fragments are present, which are then separated on a gel matrix and identified by a detector (Nelson and Cox, 2005).

Aliquots of miniprep-purified plasmid DNA (75 ng/µl) were subjected to sequence analysis to confirm the predicted nucleotide sequences. For verification of inserts cloned into the pSTBlue-1 vector the oligonucleotide primers T7 and SP6 were used. Analyzes were performed on an automated DNA sequencer (GATC Biotech, Konstanz, Germany). Data analysis was carried out using the softwares Chromas Lite (Technelysium Pty Ltd) and GeneRunner (Hastings Software, Inc.).

1.9. Plasmid DNA extraction

1.9.1. Alkaline lysis

The alkaline lysis is the most routinely used method for isolation of plasmid DNA. Bacteria are first resuspended in the osmotic stabilizing solution GTE. EDTA destabilizes cell membranes by complexing with divalent cations. Then, cells are lysed with SDS and NaOH denatures chromosomal DNA (unlike close circular DNA, linear chromosomal DNA denatures upon exposure to alkaline pH). By adding potassium acetate, the pH is neutralized, causing chromosomal DNA to aggregate, and protein-SDS complexes and high-molecular weight RNA to precipitate. Thus, plasmid DNA can be separated from the major contaminating macromolecules by centrifugation and subsequently concentrated by ethanol precipitation (Birnboim and Doly, 1979).

A single bacterial colony was picked and inoculated in 5 ml of LB medium supplemented with the appropriate antibiotic. The culture was incubated overnight at 37°C and 220 rpm. Solutions needed for isolation are described in Section 3.3 of *Materials*. Cells from 1.5 ml culture were centrifuged at 13,000 rpm for 30 s. The bacterial pellet was resuspended in 100 µl of GTE solution by vigorous vortexing. After

3 minutes of incubation at room temperature, 200 µl of NaOH/SDS were added. The contents were mixed by inverting the tube rapidly and the tube was stored on ice for 5 min, after which 150 µl of potassium acetate were added. The tube was inverted for 10 seconds, stored for another 5 minutes on ice and then centrifuged at 13,000 rpm for 5 minutes at 4°C to pellet cell debris and chromosomal DNA. The supernatant was transferred to a fresh 1.5 ml tube. The double-stranded plasmid DNA was precipitated with 1 ml of ethanol at room temperature. After mixing, the mixture was incubated for 3 minutes at room temperature and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was removed and the DNA pellet was allowed to dry at 60°C for 10 minutes. The pellet was dissolved in 20 µl of distilled water at 60°C and stored at -20°C. For restriction endonuclease digestion purposes, residual low-molecular weight RNA was degraded by DNase-free RNase.

1.9.2. Plasmid DNA isolation with Miniprep-kit

By exploiting the selective binding of plasmid DNA to a silica membrane, this method allows the isolation of plasmid DNA without RNA. After a modified alkaline lysis of bacterial cells and removal of cellular debris by centrifugation, the supernatant is loaded onto a silica membrane. Chaotropic salts lead to denaturation of contaminants, which are washed away with wash buffers. Selectively bound circular DNA can then be eluted from the membrane.

A bacterial colony grown on selective LB agar medium was inoculated in 5 ml of liquid LB containing the appropriate antibiotic and incubated overnight at 37°C, under agitation of 220 rpm. Cells from 4 ml culture were centrifuged at 13,000 rpm for 1 minute. Supernatant was discarded and the bacterial pellet was treated with reagents of the "peqGOLD Plasmid Miniprep Kit II" (Peqlab) for plasmid DNA purification. First, the pellet was thoroughly resuspended in 500 µl Solution I containing RNase. Then, 500 µl of Solution II was added for lysis and the reagents were mixed by inversion. After a short incubation period at room temperature (~ 3 minutes), 700 µl of Solution III were added for neutralization. The tube was repeatedly inverted and centrifuged at 13,000 rpm for 10 minutes (room temperature). The clarified supernatant was transferred to the silica membrane and centrifuged for 1 minute at 10,000 rpm. This step was followed by two sequential washes, with 600 µl HB buffer and 850 µl wash buffer, respectively. The membrane was dried by further centrifugation at 13,000 rpm

for 2 minutes and the column was transferred to a 1.5 ml eppendorf tube. The plasmid DNA was eluted after incubation with 40 µl sterile water for 2 minutes at room temperature and a centrifugation step at 13,000 rpm for 2 minutes. The purified DNA was kept at -20°C for any downstream application.

1.10. Isolation of genomic DNA from yeast

For PCR amplification of sequences within yeast genomic DNA, yeast cells were mechanically broken and the DNA was isolated upon extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation. This protocol was adapted from Hoffman and Winston (1987).

One mililiter from a yeast culture grown overnight was centrifuged at 11,000 rpm for 3 minutes (room temperature), washed once with 1.5 ml distilled water and resuspended in 100 µl of lysis buffer. Then, approximately 0.03 g of glass beads were added, followed by addition of 100 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v). Yeasts were lysed by vortexing for 3 minutes. Subsequently, 100 µl of TE were added, briefly vortexed, and the mixture was centrifuged at 12,500 rpm for 5 minutes. The aqueous phase was carefully transferred to another microcentrifuge tube and incubated with 1 ml of 99% ethanol. The tube was kept at -20°C overnight and then centrifuged at 12,500 rpm for 3 minutes at 4°C. The DNA pellet was washed twice with 70% ethanol, air-dried, dissolved in 30 µl distilled water and kept at -20°C until use.

2. Culture conditions

2.1. *E. coli*

E. coli was cultured in LB medium at 37°C and 220 rpm. Plates containing LB agar medium were incubated overnight at 37°C. For culture of bacteria carrying recombinant plasmid DNA, LB medium was supplemented with 100 µg/ml ampicillin or 25 µg/ml kanamycin. For blue/white screening of colonies, 20 µl of X-gal stock solution (40 mg/ml) and 20 µl of IPTG stock solution (100 mM) were spread on agar plates containing kanamycin.

2.2. Yeasts

All yeasts were cultivated aerobically either in YPD or in complete SC medium at 30°C, under agitation of 220 rpm. In order to induce hyphal growth of *C. albicans*, a culture grown in YPD medium was shifted to YPD containing 10% FCS and incubated at 37°C and 220 rpm for 6 h. With respect to solid media, untransformed yeasts were grown in YPD, except for *Sz. pombe*, which was grown in YES medium.

When recombinant protein production was necessary, the species *S. cerevisiae*, *Sz. pombe*, *K. lactis* and *P. pastoris* were cultivated as follows:

S. cerevisiae transformants were selected on uracil-deficient (ura d/o) SC agar medium. Recombinant protein expression was achieved by culturing yeasts for 3 days in ura d/o-glucose medium at 30 °C and 220 rpm.

For transformation purposes, *Sz. pombe* was grown in EMM low glucose medium. Transformants were selected on EMM plates containing 15μM thiamine. To induce expression from the *nmt1* promoter, transformants were grown in EMM medium lacking leucine for 3 days at 30 °C and 220 rpm.

K. lactis transformants were selected onto YCB agar medium. For expression of heterologous proteins cells were cultured in inducing YPGal medium at 30 °C for 72 h.

After transformation of *P. pastoris*, cells were plated onto selective SC agar medium lacking histidine (his d/o). Colonies were cultivated in BMG medium for 3 days. Then, cultures were centrifuged at 7,000 rpm for 5 min, resuspended in BMM medium and cultivated for another 3 days. Methanol (final concentration = 0.5%) was added every 24 h to induce recombinant protein expression.

2.3. Mammalian cells

All mammalian cells were cultured in tissue culture flasks at 37°C under 5% CO_2 atmosphere, unless otherwise stated.

2.3.1. IC-21 mouse peritoneal macrophages

For routine passaging of IC-21, cells were grown until approximately 80% confluence, then the culture medium was aspirated and cells were detached from flasks with cold PBS for approximately 10 minutes. Subsequently, cells were splitted 1:3 with pre-warmed culture medium in 25 cm^2 tissue culture flasks and incubated for 3-4 days.

2.4. Freezing and thawing cells

2.4.1. Bacteria and yeasts

Bacteria and yeast stocks were prepared by mixing equal volumes of a grown culture and 99% sterile glycerol. Cryogenic vials were stored at -80°C. For recovering of a stored microorganism, the strain was scraped with a pipette tip and streaked onto a proper agar plate.

2.4.2. Freezing mammalian cells

Cells were counted, centrifuged at 300× *g* at 4°C, and resuspended with culture medium in one half the volume needed to give the desired cell concentration. Subsequently, the same volume of 2× freezing medium was added dropwise and the suspension was carefully mixed. Cells were aliquoted in sterile cryogenic vials, frozen at -80°C overnight, and then transferred to a liquid nitrogen tank.

2.4.3. Thawing mammalian cells

Cells in a storage vial were thawed in a water bath at 37°C for 2 minutes and then transferred to a 15 ml tube containing 5 ml pre-warmed culture medium. After centrifugation at 300× *g* for 10 minutes at 4°C, supernatant was discarded and the pellet was resuspended with 5 ml culture medium. After that, cells were plated at a density of 1×10^6 cells/ml in 75 cm^2.

3. Cell number determination

3.1. Optical density

The optical density of a bacterial or yeast culture in liquid medium was determined spectrophotometrically at the wavelength λ = 600 nm in a 1 cm-thick cuvette against sterile medium.

3.2. Determination of yeast cell number

The total number of yeast cells in a culture was determined by light microscopy with a Neubauer hemacytometer (0.1 mm depth). Yeast cells were properly diluted in PBS and counted in 80 squares. The cell number was then calculated using the formula: yeast cells/ml = number of yeasts counted in 5 large squares × number of large squares in the entire chamber × dilution × (chamber volume)$^{-1}$. The chamber volume was 10^{-4} ml and there were 5 large squares in the chamber. Therefore, yeasts/ml = number of yeasts in 5 large squares × 5 × dilution factor × 10^4.

3.3. Determination of mammalian cell number

The total number of mammalian cells was determined using a hemacytometer "Neubauer improved" (0.1 mm depth). Cells were diluted in PBS as needed and clumps were dispersed. Cells were counted in the four corner squares and the mean value was determined. The total number of cells per milliliter was calculated as follows: cells/ml = average count per square × dilution factor × 10^4 (Phelan, 2007).

4. Transformation methods

4.1. *E. coli* transformation

Preparation of electrocompetent bacteria

Electrocompetent cells become permeable to nucleic acids upon exposure to high-strength electric fields. Bacteria are grown to mid-log phase and washed thoroughly in a low conductivity medium such as glycerol to reduce the ionic strength of the cell suspension (Miller and Nickoloff, 1995).

Six mililiters of an *E.coli* DH5α or TOP10 overnight culture were inoculated in 300 ml LB medium and cultured at 37°C, 220 rpm, until OD_{600} = 0.6-0.8. The culture was placed on ice for 5 minutes, then centrifuged at 6,000 rpm for 10 minutes at 4°C and the pellet was washed twice with 100 ml of ice-cold 10% glycerol. Next, cells were resuspended in the remaining fluid and transferred to sterile 1.5 ml eppendorf tubes on ice. After centrifugation for 5 minutes at 8,000 rpm (4°C), the supernatant was

discarded, cell pellets were resuspended with the remaining fluid and stored as 40-µl aliquots at -80°C.

Transformation of competent bacteria by electroporation

In the electroporation process, short electric impulses of high frequency produce transient pores in the outer membranes of exponentially grown cells, which are then able to take up high molecular mass molecules, such as DNA (Calvin and Hanawalt, 1988; Dower *et al*., 1988). This method allows highly efficient transformations, up to 10^9-10^{10} cfu/µg plasmid DNA (Dower *et al*., 1988).

A 40-µl aliquot of competent *E. coli* DH5α or TOP10 was thawed on ice and incubated with plasmid DNA (50-200 ng) for 5 min. If cells were incubated with the product of a ligation reaction, this latter had to be previously dialyzed against a 10% glycerol solution for 1 h in order to reduce the salt content. Too high salt or DNA concentrations in the electroporation cuvette result in arcing, which damages the cells. The mixture was then transferred to a prechilled electroporation cuvette (2 mm gap) and the cells were pulsed at 2,5kV/cm, 200Ω, 25 µF in an electroporator ("Gene Pulser II", BioRad). Immediately after the pulse, cells were incubated with 500 µl SOC medium at 37°C under agitation of 220 rpm for 1 h. After this period, the transformed cells were plated in suitable dilutions on selective LB agar plates and incubated overnight at 37°C.

Transformation of Nova Blue Singles™ competent bacteria

Nova Blue Singles™ competent bacteria were transformed with pSTBlue-1 vectors containing PCR-derived inserts. These chemically competent bacteria belong to the AccepTor™ kit.

Competent cells were thawed on ice for 5 minutes. Then, 1 µl of the ligation reaction was added directly to the cells and the tube was incubated on ice for 5 minutes. Subsequently, tubes were heated for 30 s at 42°C in a water bath and immediately placed on ice. After 2 minutes, cells were given 250 µl of SOC medium at room temperature and shaken at 220 rpm for 45 minutes before being plated on LB agar medium supplemented with kanamycin, X-gal, and IPTG, as described in section 2.1 of *Materials*. Plates were incubated overnight at 37°C.

4.2. Transformation of *S. cerevisiae*

The most common procedure for introducing plasmid DNA into *S. cerevisiae* cells is the lithium-acetate method. This technique was first described by Ito *et al*. (1983), who observed that alkali metal ions, especially Li^+, are able to induce the uptake of DNA by yeast cells. Addition of PEG and a heat-shock by 42°C have been shown to improve transformation (Ito *et al*., 1983). Schiestl and Gietz (1989) demonstrated that adding heat-denatured salmon sperm DNA to LiAc competent cells led to increased transformation efficiency (approximately 10^5 transformants per µg DNA), since it functions as carrier DNA. Interactions between negatively charged PEG, monovalent cations, and the yeast cell surfaces alter membrane charges, which may lead to conformational changes. The effectiveness of monovalent cations might be attributed to a mild chaotropic effect (Kawai *et al*., 2010).

A volume of 2 ml of an overnight *S. cerevisiae* culture grown in YPD was centrifuged at 7,000 rpm for 5 min. Solutions for *S. cerevisiae* transformation are described in Section 3.6. The cell pellet was washed with 500 µl of LiAc/TE solution and resuspended in 200 µl of this solution. To this yeast suspension 20 µl of salmon sperm DNA, 2 µl of plasmid DNA (from an alkaline lysis), 600 µl of PEG solution and 50 µl of 10× LiAc were added. The cells were incubated at 30°C under agitation at 220 rpm for 2 h and then heat-shocked at 42°C for 15 min. After that, yeasts were spun at 13,000 rpm, washed twice with 500 µl of TE solution and resuspended in 500 µl of TE. Cells were plated as 250 µl aliquots on ura d/o plates and incubated at 30°C for 3 days.

4.3. *K. lactis* transformation (New England Biolabs)

Transformation of chemical competent *K. lactis* cells was performed using the kit "*K. lactis* Competent Cells" and "NEB Yeast Transformation Reagent" (New England Biolabs). A linearised pKLAC1-derived expression cassette is introduced into chemically competent cells and integrates at the *LAC4* locus in the *K. lactis* genome. Selection of transformants is based on growth onto acetamide-containing medium. The *amdS* gene in the pKLAC1 vector codes for acetamidase, which breaks down acetamide to ammonia, which in turn can be used by *K. lactis* as a nitrogen source.

A tube of *K. lactis* GG799 Competent Cells was thawed on ice. One hundred and fifty five microliter of NEB Yeast Transformation Reagent was added to the cells. The solution was homogenized by inverting the tube. Linearized pKLAC1-DNA

containing the gene of interest (1 μg) was added to the cell mixture. After an incubation time of 30 minutes at 30°C, the cell mixture was heat-shocked by incubation at 37°C for 1 hour in a water bath. Cells were pelleted by centrifugation at 7,000 rpm for 2 minutes and washed with 1 ml sterile deionized water. Cells were pelleted by centrifugation at 7,000 rpm for 2 minutes, resuspended in 1 ml YPD medium, transferred to a sterile culture tube and incubated with shaking (220 rpm) at 30°C for 30 minutes. The cell mixture was transferred to a sterile 1.5 ml microcentrifuge tube and pelleted by microcentrifugation at 7,000 rpm for 2 minutes. The cell pellet was resuspended in 1 ml sterile deionized water. One, 10 and 50 μl of the cell suspension were removed to separate fresh sterile tubes each containing 50 μl of sterile deionized water. The entire cell mixture from each tube was spread onto separate YCB Agar Medium plates containing 5 mM acetamide. Plates were incubated at 30°C for 3 days.

4.4. *Sz. pombe* transformation by the rapid lithium acetate method

Of the several existing methods for transformation of *Sz. pombe* cells, the one that yields the highest transformation frequency utilizes alkali cations. Kanter-Smoler *et al.* (1994) have described for *Sz. pombe* an adaptation of the LiAc method developed for *S. cerevisiae*.

Sz. pombe PW260 cells were grown in 5 ml EMM low glucose medium to a density of 0.5 - 1.0 x 10^7 cells/ml (2 - 3 days). Growth in low glucose medium has been shown to increase transformation efficiency for this transformation method (Kanter-Smoler *et al*., 1994). The cells were then harvested at 7,000 rpm for 5 min at room temperature and washed twice with 1 ml of sterile water. Solutions for *Sz. pombe* transformation are described in section 3.4. The cells were washed with 200 μl of lithium acetate/EDTA and resuspended in 50 μl of lithium acetate/EDTA solution. After that, 30 μl of TE buffer containing 1 μg of plasmid DNA and 300 μl of lithium acetate/EDTA/PEG solution were added. Cells were incubated under agitation at 220 rpm at 30°C, for 30 min and then heat-shocked at 42°C for 15 min. The cells were centrifuged at 7,000 rpm, resuspended in 1 ml of TE buffer, plated on EMM leu d/o thiamine solid medium as 200 μl-aliquots and incubated for 5 days at 30°C.

4.5. *P. pastoris* transformation

Preparation of competent cells

P. pastoris (GS115 or KM71) was inoculated in 5 ml of YPD. The culture was incubated at 30°C, under agitation at 220 rpm, for 16 hours. After this period, culture was re-inoculated in 50 ml of YPD. This culture was cultivated at 30°C, 220 rpm, until an O.D. of 0,8-1,0 was reached. After that, culture was centrifugated at 500 *g*, 5 minutes at room temperature. Supernatant was discarded and cells were resuspended in 9 ml of cold BEDS and 1 ml 1M DTT. The suspension was incubated at 30°C, under agitation at 100 rpm, and centrifuged again as previously. Cells were ressuspended in 1 ml of BEDS and frozen at -80°C as 55-µl aliquots.

Transformation

A 55 µl-aliquot of competent cells was mixed with 5 µl of linearized plasmid DNA and incubated on ice for 5 minutes. The mixture was transferred to a previously cooled electroporation cuvette (2 mm) and subjected to a pulse of 200 Ω, 25 µF, 1.5 kV/cm. Imediatelly after the pulse, cells were resuspended in 1 ml of cold 1M sorbitol and 0.5 ml of YPD, transferred to a sterile 15 ml tube and incubated at 220 rpm, 30°C, for 3 hours. After that, 750 µl of culture were plated onto his d/o agar medium. Plates were incubated for 3 days at 30°C.

5. Protein Methods

5.1. Protein extraction from yeast cells or from cell-free culture medium

Mechanical shearing of yeasts by vortexing with glass beads releases their cytoplasmatic contents by virtue of the abrasive action of the vortexed beads.

Protein extracts were prepared from 3×10^8 yeast cells collected by centrifugation (7,000 rpm for 5 minutes), washed three times with PBS and resuspended in 200 µl of disruption buffer containing a cocktail of protease inhibitors (Complete, Roche). One volume of glass beads (0.5 mm diameter) was added and cells were disrupted using a Beadbeater (Retsch) for 10 min at maximum speed at 4 °C. After cell breakage, the soluble protein fraction was recovered by centrifugation at 13,000 rpm for 10 min at 4°C, transferred to new microcentrifuge tubes, and precipitated as described in section 5.3.

For analysis of secreted proteins, a volume of culture medium corresponding to 3×10^8 cells was centrifuged at 7,000 rpm for 5 minutes to remove the cells. Supernatants were transferred to new microcentrifuge tubes and concentrated by employing one of the methods described in section 5.3.

5.2. Protein isolation from yeast cell walls

Glucanase extraction of proteins from yeast cell walls was performed as described by Schreuder *et al*. (1993), with minor modifications. Yeast cells grown for three days under inducing conditions were counted, and 3×10^8 cells were centrifuged and washed three times in ice-cold isolation buffer. Then, 3 ml of isolation buffer and 10 g of glass beads were added per gram of cells (fresh weight) and yeasts were lysed in a Beadbeater at maximum speed for 10 minutes. Cell extracts were transferred to a new tube, and glass beads were extensively washed. Raw cell extracts and washes were pooled and centrifuged for 5 minutes at 1,000 × *g*. Pellets were washed three times with 1 ml of 100 mM-sodium acetate, pH 5.0, containing 1 mM PMSF, and resuspended in the same buffer (20 µl of buffer for 10 mg of cell walls). To this volume was added 0.5 mU of β-1,3-glucanase (laminarinase), and the tubes were incubated for 2 h at 37°C. Then, another 0.5 mU of laminarinase was added and the mixtures were further incubated for 2 h at 37°C. Afterwards, samples were centrifuged for 5 minutes at 12,000 rpm and the supernatant was precipitated as described in section 5.3.

5.3. Protein precipitation

A number of precipitation techniques has been routinely used for recovery of proteins. In order to concentrate proteins present in a large volume of a certain solution, precipitation can be induced by addition of organic solvents or by changes in the pH, for example.

Ethanol precipitation

Dehydration caused by ethanol makes proteins attract each other in such an extent that they become insoluble in ethanol-water mixtures (van Oss, 1989). One volume of culture supernatant was mixed with 3 volumes of 99% ethanol. Samples were kept

overnight at -80°C and centrifuged at 13,000 rpm for 30 min at 4°C. Pellets were allowed to dry and subsequently resuspended in SDS-loading buffer.

Deoxycholate (DOC) and 2,2,2-trichloroacetic acid (TCA) precipitation

TCA is one of the most efficient agents used for protein precipitation, although its mechanism of action has not been completely elucidated yet. Rajalingam *et al*. (2009) suggested that the tricholoroacetate ions lead to protein unfolding by disrupting electrostatic interactions. Partial unfolding of proteins gives rise to exposure of nonpolar surfaces, resulting in intermolecular coalescence of protein molecules and consequently precipitation. Addition of the detergent DOC improves recovery (Bensadoun and Weinstein, 1976). To one volume of protein solution, 0.01 volume of 2% sodium deoxycholate was added. Samples were vortexed and incubated for 30 minutes at 4°C. Then, 0.1 volume of 100% trichloroacetic acid was added, and samples were incubated overnight at 4°C after brief vortexing. Next, tubes were centrifuged at 15,000 rpm for 15 minutes at 4°C and the pellets were washed twice with 1 ml of cold acetone (kept at -20°C). Samples were centrifuged for 5 minutes at 15,000 rpm between washes, allowed to dry at room temperature, and resuspended in a minimal volume of SDS-loading buffer.

5.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is a method that allows protein separation by applying an electric field. This procedure is carried out under conditions that permit dissociation of the proteins into their individual subunits and minimize aggregation. Samples are denaturated at high temperatures with the anionic detergent sodium dodecyl sulfate (SDS), which interacts with the hydrophobic amino acid residues of the proteins, disrupting their folded structure and allowing them to adopt an extended conformation. Since the amount of SDS bound is proportional to the molecular weight of the polypeptide and independs on its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels at rates that reflect their sizes. The denatured polypeptides become negatively charged and migrate towards the positive electrode (anode). A thiol reducing agent such as β-mercaptoethanol is often used to reduce disulfide bonds within or between molecules (Sambrook *et al*. 1989).

Following the classical system developed by Laemmli (1970), tris-glycine polyacrylamide gels (8-12%) were assembled in a "Mini-PROTEAN 3" system (BioRad) and run as a stacking gel bottomed by a running gel, in a discontinuous buffer system, according to Sambrook *et al*. (1989). The resolving gel (10% polyacrylamide) were prepared as follows (final volume = 10 ml):

Distilled water	4.0 ml
30% polyacrilamide mix	3.3 ml
1.5 M tris pH 8.8	2.5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.004 ml

The mixture was pippeted between two assembled glass plates (1.0 or 1.5 mm spacer), followed by addition of 1 ml isopropanol. After polymerization, the isopropanol was removed, plates were carefully dried, the stacking gel was poured and the comb was inserted.

The stacking gel (5% polyacrylamide) was prepared in the following manner (final volume = 5 ml):

Distilled water	3.4 ml
30% polyacrilamide mix	0.83 ml
1.0 M tris pH 6.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

After complete polymerization, the plates were loaded into the cassette, which in turn was placed inside an electrophoresis chamber. Protein samples were mixed with SDS buffer containing β-mercaptoethanol, incubated at 100°C for 10 minutes and loaded on the gel. The "Page Ruler Prestained Protein Ladder" (Fermentas) was applied as a molecular mass standard. After applying the samples, the gel was run in tris-glycine buffer at a constant voltage of 150 V.

5.5. Western blotting

Western blotting is a method for immunologic detection of a specific protein immobilized on a membrane. Proteins separated by electrophoresis are transferred to a membrane, such as PVDF, which is then incubated with a specific antibody that recognizes the protein of interest. Subsequently, the primary antibody is bound by a secondary antibody (anti-immunoglobulin), which is normally coupled to an enzyme. Addition of an appropriate substrate generates a detectable product (Towbin *et al.*, 1979).

After SDS-PAGE, the gel was incubated with transfer buffer for removal of salts and detergents. The PVDF membrane was previously immersed in methanol and then equilibrated with transfer buffer. One piece of transfer buffer-soaked Whatmann filter paper was placed on the lower electrode of a semi-dry transfer apparatus, followed by the PVDF membrane, the gel and another buffer-soaked filter paper. Transfer was carried out at 0.85 mA/cm^2, in a "Trans Blot SD Electroforectic Transfer Cell" (BioRad) for 1.5 h.

The membrane was blocked overnight at 4°C in blocking buffer in order to prevent nonspecific binding of the antibodies and then incubated for 1 h at room temperature with a properly diluted primary antibody. The membrane was washed with TBST for 15 min and then incubated for 1 h at room temperature with specific peroxidase-conjugated anti-IgG diluted in blocking buffer, under agitation. This step was followed by 15-min washes in TBST and TBS. The membrane was further incubated with reagents of an enhanced chemiluminescence detection kit consisting of luminol and peroxide solutions. The horseradish peroxidase coupled to the secondary antibody catalyzes the oxidation of luminol by peroxide. This reaction generates a product (3-aminophthalate) in an excited state, which emits light at 425 nm upon decay to a lower energy state. Luminescence was detected and recorded by a ChemiDoc XRS System (BioRad).

Protein quantitation was performed by comparison of the relative intensity of the bands from 3×10^8 cells or the corresponding amount of culture medium to a standard curve of commercially available protein of known concentration by densitometry using QuantityOne software (version 4.6.2, Bio-Rad).

5.6. VLP preparation and transmission electron microscopy (TEM)

The protocols for VLP isolation and preparation for TEM analysis were performed as described by Powilleit *et al*., 2004. Yeasts are treated for spheroplast formation, lysed, and the cell extract is submitted to two ultracentrifugation steps and dialysis before the electron microscopy.

S. cerevisiae S86c transformed with pG[0]G/*ova*$_{cyt}$*mut*, pG[0]G/*pp65*, and pG were inoculated in 8 ml ura d/o glucose for 72 h, transferred to 400 ml ura d/o medium and grown to a density of 4-6×10^7cells/ml at 30°C, 220 rpm. Next, cells were centrifuged at 6,000 rpm for 5 minutes, washed with 50 ml of prechilled water and subsequently with the same volume of spheroplasting buffer, and resuspended in 50 ml spheroplasting buffer containing 2 mM DTT and 200 µg/ml zymolyase 20T. After 1.5 h of incubation at 120 rpm and 30°C, spheroplasts were centrifuged for 20 min at 2,200 rpm (4°C) and washed with 50 ml of cold PBSES. Then, cells were resuspended in 10 ml PBSE and 10 g of micro glass beads (0.25–0.50 mm diameter) were added. Cells were lysed by vortexing six times for 1 min, with 1 min intervals on ice. Total yeast extracts were supplemented with 5 ml PBSE and pelleted at 10,000 rpm for 1 h at 4°C. The supernatant (15 ml) was layered onto a 15-ml cushion of 45% sucrose and ultracentrifuged at 19,000 rpm overnight at 4°C using a Beckman SW28 rotor. In this step, only high molecular weight structures can pass the cushion and form a pellet. The cushion pellet was gently resuspended in 1 ml PBSE, layered onto a linear density gradient of 20–70% sucrose, and further ultracentrifuged at 20,000 rpm overnight at 4°C. After that, the gradient was fractionated into 2-ml fractions and the gradient pellet was resuspended in 2 ml PBSE. Aliquots of each fraction were analyzed by SDS-PAGE and western blotting. For reisolation of VLP, a maximum of 6 gradient fractions was pooled and dialyzed against 5 l PBSE overnight (4°C) in membranes with an exclusion limit of 14 kDa. The dialysed solution was concentrated to 200–500 µl using a 10 kDa cut-off Vivaspin and examined for the presence of VLP by TEM analysis.

For TEM analysis, samples were adsorbed to poly-L-lysine-coated cupper grids for 5 min. The grids were rinsed twice with distilled water, negatively stained with 2% uranyl acetate for 5 min, and allowed to to air-dry. Samples were imaged with diverse magnifications with a TECNAI 12 (FEI) transmission electron microscope at the Department of Anatomy and Cell Biology of Medical Faculty at the Saarland University (Homburg, Germany).

6. Cell- and immunobiology methods

6.1. Immunolabelling of yeasts

Immunostaining of yeasts was performed as follows: 100 µl of an exponentially growing yeast culture were pelleted, washed three times with phosphate-buffered saline (PBS, 10 mM potassium phosphate buffer, 150 mM sodium chloride, pH 7.4), and resuspended in 100 µl PBS. The cell suspension was incubated with the primary antibody at a dilution rate of 1:100 for 1 h at room temperature in an orbital shaker. After the cells had been washed three times with PBS, the second antibody (FITC- or PE-conjugated) was diluted to 1:100 and allowed to react with the cells at room temperature for 1 h in the dark. The cells were then washed three times with PBS and examined using either a fluorescence microscope (Keyence), or a confocal laser scanning microscope equipped with a 560-615 nm filter (Zeiss LSM 510 meta). Microscopic observations were carried out using 100× objectives. When necessary, cells were analyzed by flow cytometry. Data for 50,000 events were collected.

For detection of mannose or chitin in yeast cell walls, staining was performed as described above, except for the incubation steps with antibodies, which were substituted for a one-step incubation with 200 µg/ml of Concanavalin A-FITC or wheat germ agglutinin-FITC, respectively, in the dark (Buck and Andrews, 1999).

6.2. Staining cell surface antigens in mammalian cells

Cells were centrifuged at 1,400 rpm for 6 minutes, washed with PBS containing 10% FCS and resuspended in 100 µl at a concentration of at least 10^6 cells/ml. Then, an appropriate volume of antibody (previously determined by titration) was added to the cells, and samples were vortexed and incubated on ice for 45 minutes in the dark. After this period, cells were washed with 3 ml PBS containing 10% FCS, fixed with 150 µl of FACS-Fix and kept at 4°C in the dark until flow cytometric analysis.

6.3. Intracellular cytokine staining in mammalian cells

Detection of intracellular molecules presupposes permeabilization of the cell membrane in order to allow access of the antibodies to the antigens within the cells. Cells are

initially stimulated in the presence of a secretion inhibitor. Brefeldin A from *Penicillium brefeldianum* destroys the structure and function of the Golgi apparatus, so that secretion of proteins no longer occurs. Therefore, generally secreted proteins remain in the cell interior and can be detected. Cells are then fixed to prevent leakage of the proteins, permeabilized, and incubated with a specific conjugated antibody, which penetrates the cell, subsequently binding to its target. Cells were made permeable upon incubation with saponin, a surfactant from Quillaya saponaria bark that increases the penetration of macromolecules through cell membranes (Jacob *et al*., 1991).

Cells were incubated in FACS-buffer containing 0.5% saponin for 10 minutes at room temperature. Then, cells were centrifuged at 1,400 rpm for 6 minutes, supernatant was discarded and the antibody was added to the cells in the remaining buffer. After brief vortexing, cells were incubated on ice for 45 minutes in the dark, then washed with 2 ml of FACS-buffer, fixed with 150 µl of FACS-Fix and kept at 4°C in the dark until flow cytometric analysis.

When both surface and intracellular staining in the same cell population was necessary, stainings were carried out successively, *i.e*., cells were first stained for surface molecules (as described above) and thereafter permeabilized with saponin for intracellular staining.

6.4. Flow cytometry

Flow cytometry is a powerful method for detecting individual cells from a certain cell population or subset, based on their morphologic characteristics (such as size and granularity) and the presence of specific surface or intracellular molecules, which are previous stained with fluorescent substances. A mixture of cells is forced with a sheath fluid through a nozzle, which generates a stream of single cells. These cells pass through a laser beam, scattering the light, and the fluorochrome molecules are excited, emitting fluorescence. Photomultipliers sense fluorescence emissions and scattered light. The fluorescence intensity gives information on the presence of a certain molecule in the cell interior or on the cell surface. Scattered light is collected as forward scatter (FSC) and side scatter (SSC). FSC measures the light scattered in the same axis of the laser beam path and is proportional to the particle size. SSC is detected at approximately 90 degrees to the laser path and is directly proportional to cell granularity (Janeway *et al*., 2001).

It is possible to set a gate on a FSC vs. SSC plot so that the resulting information refers only to the cell population inside it. For two or three colour analysis, spectral overlap compensation was performed.

Cells were stained as described in section 2.29 and examined on a FACScan or a FACScalibur (Becton Dickinson). Data were analyzed using CELLQuest software (Becton Dickinson).

6.5. Isolation of peripheral blood mononuclear cells (PBMC)

Dendritic cells can be obtained from isolated monocytes cultured with appropriate cytokines. Mononuclear cells are isolated from peripheral whole blood by density gradient centrifugation (Ficoll-Paque, 1.077 g/ml), which is a widely used method. Lymphocytes and monocytes, according to their respective densities, accumulate in the interphase between supernatant (which contains plasma and thrombocytes) and Ficoll (where erythrocytes and granulocytes sediment by virtue of their higher densities). Sallusto and Lanzavecchia (1994) demonstrated the generation of DCs from adult peripheral blood in the presence of IL-4 and GM-CSF. This protocol has been widely used, with some minor modifications.

Fresh human anticoagulated blood (100 ml) was obtained from the blood bank of the Winterberg Klinik in Saarbrücken. Human blood was carefully transferred to five sterile 50 ml tubes containing 15 ml PBS (Ca^{2+}- and Mg^{2+}-free) until a final volume of 35 ml each, and carefully mixed. The diluted cell suspension was gently transferred to tubes containing 15 ml of the lymphocyte separation medium Ficoll-Paque. Tubes were centrifugated at 1,600 rpm for 25 minutes at room temperature without brake, in order to preserve the layering. Ten millilitres of plasma from each tube were discarded. The white blood cell ring fraction (buffy coat with PBMC) was carefully aspirated and transferred to new 50 ml tubes. The volume was adjusted to 50 ml per tube using PBS. Samples were centrifuged for 6 minutes at 1,600 rpm (brake switched on) and the supernatant was carefully discarded. Each pellet was resuspended in 5 ml of sterile water to lyse remaining erythrocytes, immediately adding enough PBS to the PBMC to make up 50 ml. Cells were centrifuged and this step was repeated until no red blood cells could be seen. Cells were then joined in one tube by pipetting PBS from tube to tube. Tubes were rinsed with PBS and volume was adjusted to 50 ml. After 10 minutes of centrifugation at 900 rpm in order to eliminate trombocytes, the pellets were

resuspended in PBS and cells were counted under the light microscope, as described in section 3.3.

6.6. Monocytes isolation and differentiation into dendritic cells

Monocyte isolation by adherence to plastic surfaces

The isolated PBMC were suspended in RPMI 1640 containing 10% heat-inactivated FCS (LPS-free), 100 U/ml penicillin and 0.1 mg/ml streptomycin at a density of 7.5×10^6 cells/ml, plated onto cell-culture flasks (20 ml culture for 125 cm^2 area), and incubated for 1.5 h at 37°C. Then, the peripheral blood lymphocytes (PBL), which do not adhere to plastic surfaces, were harvested, centrifuged at 1,400 rpm for 6 minutes, resuspended in PBS, counted, and properly frozen as described in section 2.4.2. Adherent cells (monocytes) were gently rinsed with PBS to remove remaining nonadherent cells. Monocytes were cultured at 7.5×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated FCS (LPS-free), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 U/ml recombinant human IL-4, and 800 U/ml recombinant human GM-CSF. Immature monocyte-derived dendritic cells (MDDCs) were obtained after cells had been incubated at 37 °C with 5% CO_2 for 7 days, and re-fed on day 3. Cells harvested at day 7 were analyzed by flow cytometry for typical immature DC surface phenotype ($CD1a^+$, $CD14^{low}$, $CD80^+$, $CD86^+$, HLA-I^+, HLA-II^+, $CD83^{low}$) and contamination with $CD3^+$ T cells or $CD19^+$ B cells.

Monocyte isolation by positive selection using magnetic microbeads

Alternatively, monocytes were isolated from PBMC using magnetic microbeads conjugated with anti-CD14 antibody. Taking advantage of the fact that CD14 is strongly expressed on the surface of monocytes, these cells can be labeled with anti-CD14 micro beads. In the presence of a magnetic field, the cell suspension is applied onto a column, and the magnetically labeled cells are retained, whereas the other cells pass through. When the column is removed from the magnetic field, the $CD14^+$ cells can be eluted. Binding of antibody to CD14 does not trigger any signal transduction.

PBMC isolated as described in section 7.1 were counted and resuspended in MACS buffer (80 µl per 10^7 cells). For 10^7 cells, 20 µl of anti-CD14 micro beads were added, and the cell suspension was incubated for 15 minutes at 4°C. Then, 2 ml MACS buffer were added per 10^7 cells. After centrifugation for 10 minutes at 1,200 rpm, the

supernatant was discarded and cells were resuspended in MACS buffer (50 µl per 10^7 cells). A column was placed in the magnetic field of a separator and rinsed with 3 ml of MACS buffer. Then, the cell suspension was applied onto the column and the cells that passed through were collected. The column was washed three times with 3 ml of MACS buffer and subsequently placed on a sterile tube. Afterwards, 5 ml of buffer was pipetted onto the column and the magnetically labeled cells were forced through the column with a plunger. Cells were then incubated in tissue culture flasks containing the appropriate medium to induce differentiation into DCs as described above in this section.

6.7. Maturation of dendritic cells by different stimuli and analysis of cytokine secretion

For maturation studies, day 7-immature dendritic cells were harvested, resuspended at 1×10^6 cells/mL, plated in 24-well plates and incubated either with yeasts at a ratio of 1:1, or with other maturation factors (250 ng/ml LPS and 32 µl/ml of a lysate of HCMV-infected fibroblasts), or left untreated, for 48 h, at 37°C in 5% CO_2. Cells were then analyzed by dual-color flow cytometry for classical surface maturation markers using the following monoclonal antibodies: anti-HLA DP/DQ/DR FITC, anti-HLA A/B/C PE, anti-CD80 PE, anti-CD83 PE, anti-CD86 FITC, anti-CD58 FITC, anti-CD54 PE, anti-CD40 FITC, anti-CCR7 PE. Cells were stained as described in section 6.2 and fixed with 1% paraformaldehyde before flow cytometric analysis (section 6.4). DCs were gated following characteristic forward/side scatter patterns. Data were collected from 10,000 cells and expressed as median of fluorescence intensity of all cells, or percentage of positive cells.

For quantitation of diverse cytokines secreted by stimulated DCs, supernatants from DCs incubated with the various stimuli were collected and stored at -80°C until analysis with a multiplex cytokine kit (PROGEN Biotechnik, Heidelberg, Germany).

6.8. Incubation of immature dendritic cells with yeast vectors

Human MDDCs were generated as described in section 6.6, collected on day 7 by centrifugation at 1,400 rpm for 6 minutes, counted, and aliquoted at 10^6 cells/ml. For phagocytosis of yeasts carrying pp65, 2×10^6 DCs from HCMV-infected individuals

were incubated with yeasts expressing recombinant pp65 or harbouring empty vectors (MOI 5) in sterile 50 ml tubes. Samples were briefly centrifuged, incubated for 2 days at 37°C and subsequently added to autologous T lymphocytes (*Methods*, section 6.9).

6.9. Stimulation of antigen-specific memory T lymphocytes by autologous dendritic cells

For stimulation of antigen-specific memory T lymphocytes by autologous dendritic cells from HCMV-seropositive donors, a protocol based on Scheller *et al*. (2008) was used. Frozen lymphocytes obtained after monocyte isolation by adherence to plastic (section 6.6) were thawed, washed twice with medium, and counted. Subsequently, 2×10^6 cells were incubated in 15-ml tubes with 1 µg/ml of the costimulatory antibodies anti-CD28 and anti-CD49d and with DCs (1:1) which had been previously incubated with yeasts expressing pp65 or control yeasts. Samples were incubated for 2 h at 37°C, then 10 µg/ml Brefeldin A were added and tubes were further incubated at 37°C for 4 h. Subsequently, EDTA was added (final concentration = 0.4 mM), followed by incubation at room temperature for 15 minutes. Thereafter, samples were incubated for 5 minutes with 0.008% EDTA in PBS. After centrifugation at 1,400 rpm for 6 minutes, supernatants were discarded and 300 µl of 4% paraformaldehyde were added. After incubation for 5 minutes at room temperature, 2 ml of FACS-buffer were added and samples were centrifuged for 6 minutes at 1,400 rpm. Supernatants were discarded and cells were stained for surface and intracellular antigens as described in sections 6.2 and 6.3 of *Methods*.

6.10. Measurement of ROS production in whole blood

The ability of phagocytes to produce reactive oxygen species (ROS) is a critical aspect in host immune responses against potential pathogens. ROS play an important role as antimicrobial agents by killing microorganisms directly and as signaling molecules involved in various physiological pathways in neutrophils and macrophages (Fialkow *et al*., 2007; Kelly *et al*., 2010). Chemiluminescence is a low-cost method used to examine the respiratory burst of phagocytes. Luminol reacts with diverse ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals, generating aminophthalate ion in

an excited state, which emits light when returning to the ground state (Marquette and Blum, 2006).

Production of ROS by phagocytic cells in whole blood after incubation with the yeasts *S. cerevisiae*, *Sz. pombe*, *K. lactis* and *P. pastoris* was examined. Yeasts were grown in SC medium, washed three times with HBBS, counted and aliquoted at 10^8 yeasts cells/ml in HBBS. Then, yeasts were incubated at 65°C for 1 h or left at room temperature before the chemiluminescence assay.

Fresh heparinised whole blood was 1:10 diluted in HBSS containing 0.1% gelatine. Diluted blood samples were divided into three groups: stimulated, resting and blank. In stimulated samples 90.5 µl diluted blood were incubated with 7.5 µl (2.5×10^5 yeasts) of a yeast suspension (10^8 yeasts cells/ml in HBSS) and 2 µl of luminol solution (final concentration = 400 µM/well); in resting samples the same volume of blood was incubated with 7.5 µl HBSS and 400 µM luminol; in blank samples blood was incubated with 2.5×10^5 yeasts and 2 µl DMSO. All samples were plated in triplicates (100 µl/well) in a white Lumitrac Greiner 96-well plate. Luminescence was recorded at 37°C over a 150-minute interval with 1s integration time, using a plate reader (PARADIGM, BeckmanCoulter). Data were analyzed with the software Multimode Analysis version 3.3.0.9 (BeckmanCoulter).

6.11. *In vitro* yeast phagocytosis assays

6.11.1. Determination of percent phagocytic cells and phagocytic index

Human dendritic cells or murine IC21 macrophages were seeded in 24-well plates at a density of 1×10^5 cells/well. In the case of macrophages, cells were allowed to adhere overnight. Phagocytes were then incubated with yeasts at various MOI for 4 h or at MOI 5 for various periods of time, in 1 ml culture medium per well, at 37 °C in 5% CO_2. Plates were briefly centrifuged to ensure that yeasts and cells were brought together. After incubation, samples were vigorously resuspended to detach loosely bound yeasts from cells. The cells were then washed with PBS and fixed in 1% paraformaldehyde at 4 °C. At least 100 cells were examined per well for attachment and uptake of yeasts by direct visual enumeration using a light microscope equipped with a 40× objective lens (Olympus) to determine the percentage of phagocytes containing at least one yeast cell (% phagocytic cells) or the number of yeasts in 100 phagocytes

(phagocytic index). Experiments were performed in triplicate and results are shown as mean ± standard deviation (SD).

6.11.2. Effect of blocking antibodies on yeast phagocytosis

Immature DCs or murine IC21 macrophages (1×10^5cells/ml in the respective culture media) were pre-incubated with 25 μg/ml of the mouse monoclonal antibodies anti-human Dectin-1, or anti-human mannose receptor, as well as with a correspondent isotype control antibody, in 24-well plates for 1 h at 37°C. All assays were performed in media supplemented with heat-inactivated serum to exclude contributions of complement. Yeasts were added at a 5:1 ratio and incubated for another 4 h. Mean ± SD of triplicate experiments is shown. Student's *t* test was used to establish the statistical significance of differences between mean values using GraphPad Prism 4. One-sided p values less than 0.05 were considered statistically significant.

6.12. Whole blood assay

The presence of antigen-specific memory T cells in human blood can be quantified by means of a whole blood assay. The antigens are incubated with aliquots of whole blood where they are taken up by phagocytic cells, processed and presented to CD4 and CD8 T lymphocytes in context of MHC class I and II, respectively. Activated T cells produce certain molecules, such as CD69 on the cell surface and secreted IFN-γ. Simultaneous staining of CD4 or CD8, CD69, and IFN-γ allows identification and determination of the frequency of antigen-specific activated T lymphocytes. Incubation of cells with Brefeldin A avoids secretion of IFN-γ, which remains intracellular and can be stained for flow cytometric analyses after cell permeabilisation with saponin. The incubation time of the assay (6 h) is not enough for activation of naïve T cells, so that only effector and memory T cells are specifically stimulated (Breinig *et al*., 2006).

Whole blood assays were performed as described by Breinig *et al*. (2006). Aliquots of 450 μl heparinized human blood were incubated in 15-ml polypropylene tubes and mixed with 1 μg/ml of the costimulatory antibodies anti-CD28 and anti-CD49d. As positive control, 2.5 μg/ml *Staphylococcus* enterotoxin B were used and cells incubated with costimulatory antibodies only served as negative control. A lysate of HCMV-infected fibroblasts (3.8 μl) was used to confirm whether donors were positive for this virus.

The polypropylene tubes were incubated at 37°C and 5% CO_2. After 2 h, 10 µg/ml of Brefeldin A were added and samples were further incubated for 4 h. After this period, the blood was incubated for 15 min in the presence of 2 mM EDTA and then for 10 min with "FACS lysing solution" to lyse erythrocytes and fix leukocytes. Cells were washed with FACS buffer (PBS, 5% FCS, 0.5% BSA, 0.07% NaN_3) and let overnight at 4°C. Cells were then permeabilized with FACS buffer containing 0.1% saponin for 10 min and immunostained using saturating conditions of the antibodies anti-CD4 (4 µl), anti-CD8 (4 µl), anti-IFNγ (5 µl, 1:10 diluted) and anti-CD69 (2 µl).

Cells were washed with FACS buffer, fixed in 1% paraformaldehyde and analyzed (at least 25,000 of CD4- and CD8-positive lymphocytes each) on a FACScan flow cytometer (Beckton Dickinson). Antigen-specific activated T lymphocytes were identified and quantified as CD69 and IFN-γ double-positive cells.

6.13. *In vitro* antigen presentation assay

These experiments were performed in collaboration with Dr. Gernot Geginat at the Institute for Medical Microbiology and Hygiene (Faculty for Clinical Medicine Mannheim of the University Heidelberg).

Bone marrow macrophages from C57BL/6 mice were plated at a density of 1×10^5 cells per well (100 µl DMEM high glucose supplemented with L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) in 96-well microtiter plates and loaded with the different yeast genera (MOIs 1.75, 3.5, and 7), as well as with synthetic $OVA_{257–264}$ peptide (SIINFEKL), or left untreated. Phagocytes and yeasts were brought together after centrifugation at 300 rpm for 3 min. After overnight incubation at 37°C, infected cells were washed twice and resuspended in culture medium supplemented with nystatin (200 U/ml) to kill any remaining uninternalized yeast cells. T cells specific for the H-$2K^b$-restricted epitope SIINFEKL were added 16 h after infection (3×10^4/well). Culture supernatants were harvested 18 h after addition of T cells and IFN γ protein content was assessed using a specific enzyme-linked immunosorbent assay (ELISA) kit with a detection limit of 0.05 pg (Bazan *et al*., 2011). The ELISA was carried out as directed by the manufacturer. The amount of IFN-γ in individual wells was determined based on a supplied recombinant mouse IFN-γ standard.

6.14. Immunization of OT-I mice

These experiments were carried out in cooperation with Elisabeth Kenngott at the Medical Clinic for Rheumatology and Clinical Immunology (Charité, Berlin).

OT-I transgenic mice were immunized subcutaneously (s.c.) at the tail base with 5×10^7 yeasts (either expressing Ova_{cyt} or carrying empty vector), in 100 µl PBS, or with PBS alone. Boost injections were given 7 and 14 days after priming. On day 21, inguinal lymph nodes and spleens were collected for *ex vivo* restimulation and further analysis of IFN- γ production.

6.15. Analysis of cellular immune responses in lymph nodes and spleen cells from immunized OT-I transgenic mice

These experiments were performed in collaboration with Elisabeth Kenngott at the Medical Clinic for Rheumatology and Clinical Immunology (Charité, Berlin).

Immunized OT-I mice were killed by cervical dislocation. Inguinal lymph nodes and spleens were harvested one week after the last immunization. Single cell suspensions were obtained by grinding the lymph nodes or spleens with a syringe plunger against cell strainers in RPMI 1640 containing 10% FCS. Spleen cells preparations were incubated with erythrocyte lysing solution for 3 minutes at 4°C. Cells were centrifuged at 1,200 rpm at room temperature for 8 minutes, washed with PBS and counted. Subsequently, 2×10^6 lymphoid cells in 1 ml culture medium were restimulated in sterile 15-ml tubes with 10 µg/ml of SIINFEKL peptide or PMA/ionomycin, at 37°C under 5% CO_2 atmosphere. After 2 h of incubation, 10 µg/ml Brefeldin A were added and SIINFEKL-stimulated cells were incubated for further 4 h, and PMA/ionomycin were incubated for further 2h. Then, cells were harvested, washed with PBS/BSA, fixed with 2 % paraformaldehyde in PBS, washed twice with PBS/BSA and stained for the surface molecules CD8 and Vβ5 TCR as described in section 6.2. Samples were left overnight at 4°C in the dark. Afterwards, cells were centrifuged and resuspended in 100 µl PBS containing 0.5% saponin, 10 µg/ml rat IgG and anti-IFN-γ PECy7 (100-fold diluted) or PECy7-conjugated isotype control. After incubation at room temperature for 30 minutes, cells were washed with 1 ml of saponin buffer, then with 1 ml PBS, and resuspended in 200 µl PBS prior to flow cytometric analyses on a FACSCanto (BD Biosciences).

For determination of the frequencies of activated (IFN-γ-producing) cells, $CD8^+$ $V\beta5^+$ lymphocytes were gated. Samples were measured using a FACSCanto flow cytometer equipped with a FACSDiva software (BD Biosciences) and analyzed using Flowjo (Tree Star, Inc).

IV. RESULTS

1. Effects of different yeast genera on maturation and activation of human immature DCs

Interaction of non-pathogenic as well as disease-associated yeasts with mammalian APCs such as DCs has been repeatedly demonstrated (Buentke *et al*., 2000; Newman and Holly, 2001; Heintel *et al*., 2003). Here, the expression profile of diverse cell surface markers and cytokines by DCs after incubation with distinct yeast genera, species, strains, or yeast mutants was examined and compared in an attempt to identify attractive antigen delivery vehicles for potential vaccination approaches.

1.1. Maturation of human DCs by diverse yeast genera

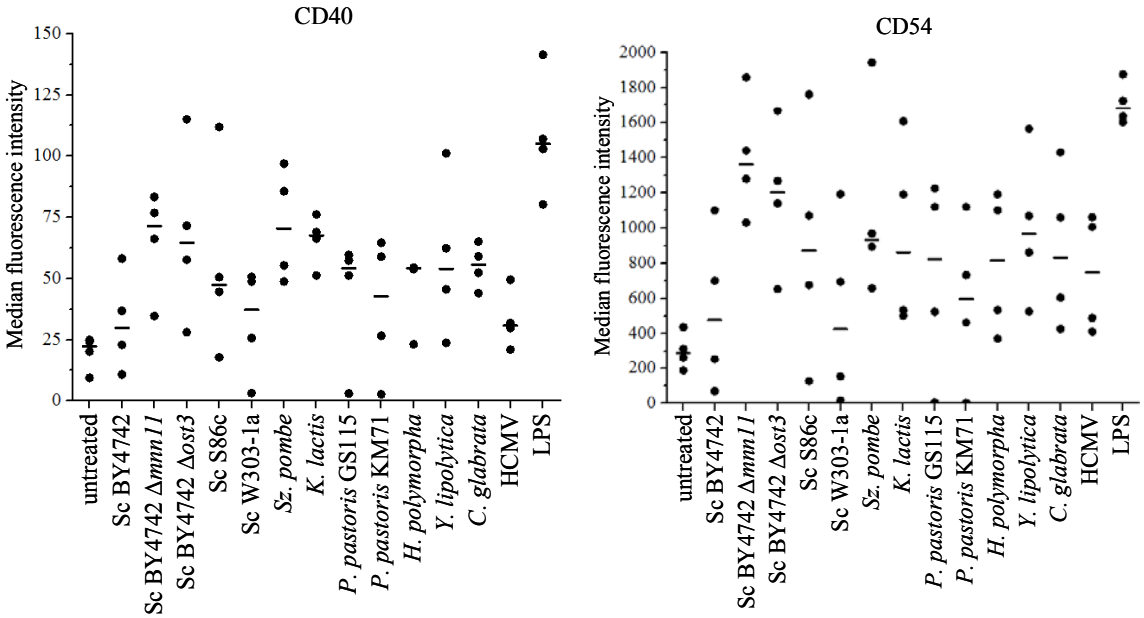
Maturation of human and murine DCs stimulated with whole yeasts or yeast components has been described. Among the species analyzed are the baker's yeast *S. cerevisiae,* and the potentially pathogenic yeasts *C. albicans, M. furfur and C. neoformans* (Buentke *et al*., 2001; Romani *et al*., 2004; Stubbs *et al*., 2001; Pietrella *et al*., 2005). However, no study comparing several yeast strains with respect to their ability to stimulate DCs has so far been performed.

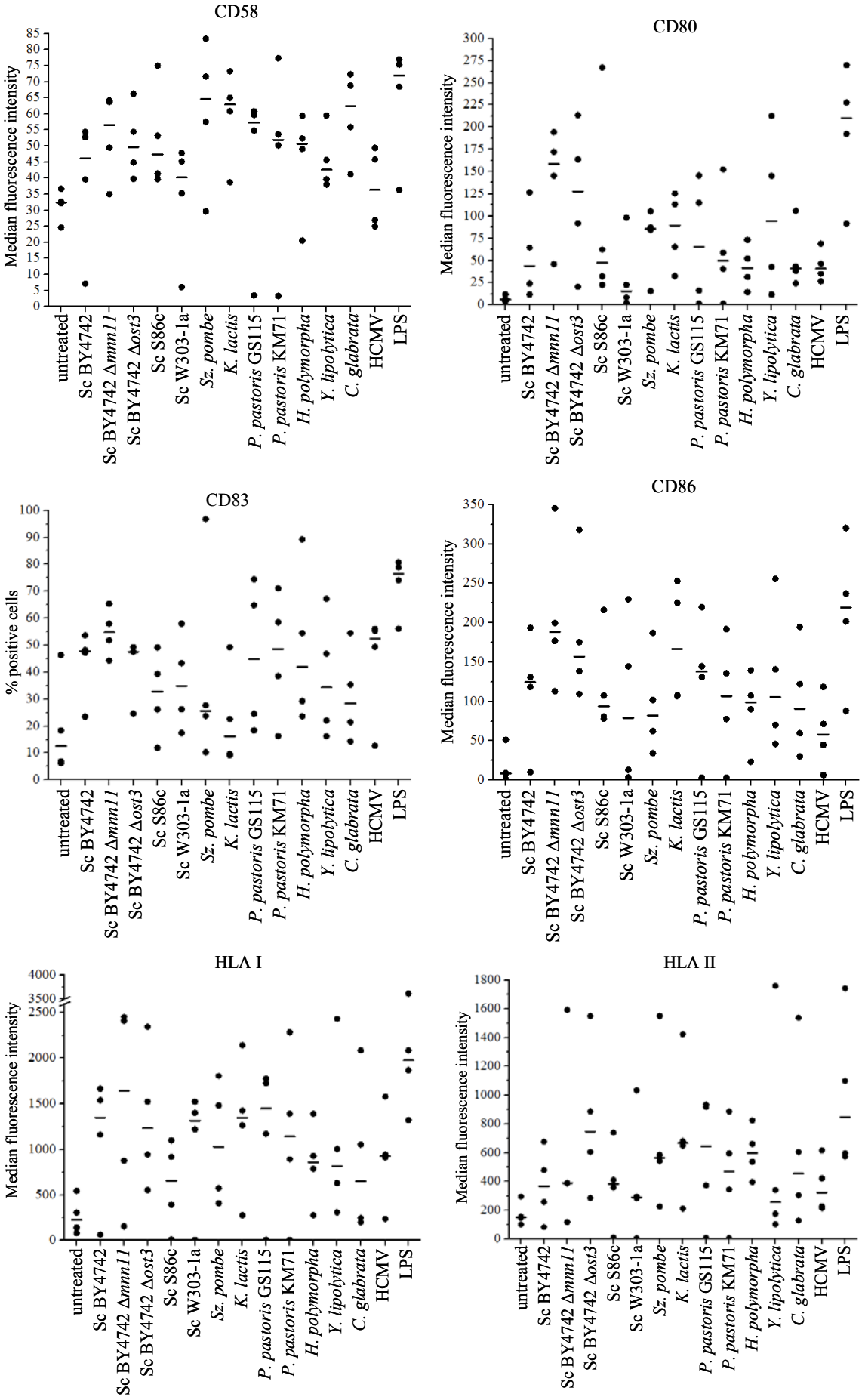
Other inflammatory stimuli, such as bacteria and viruses, have also been demonstrated to induce DC maturation (Rescigno *et al*., 1998; Rudolf *et al*., 2001). In this way, DC responses to fungal, bacterial and viral stimuli were examined in the present work. Immature DCs derived from PBMC of four individuals were cultured for 48 h with various yeast genera, *E. coli* LPS, a lysate from HCMV-infected fibroblasts, or left untreated. Then, phenotypic analysis of DCs was performed by flow cytometry using specific antibodies. The influence of each stimulus on DC maturation was determined by analyzing changes in the expression of 9 surface markers (CD40, CD54, CD58, CD80, CD83, CD86, CCR7, HLA I, and HLA II molecules).

The expression of all these molecules is upregulated in mature DCs. The initial association of DCs with naïve T cells is mediated by cell-adhesion molecules, such as CD54 and CD58 on DC surface and LFA-1 and CD2, respectively, on the T cell. Costimulatory molecules, including CD80 and CD86, provide the second (costimulatory) signal needed for the clonal expansion of antigen-specific T cells.

Interaction between CD40 on DCs and CD40L on T cells transmits activating signals to the T cell and stimulates the APC to express CD80 and CD86, promoting further T cell proliferation. Mature DCs also produce high levels of MHC molecules for enhanced peptide presentation. Moreover, activated DCs express the chemokine receptor CCR7 for sensing of lymph node-derived chemokines and migration into lymphoid tissues (Janeway *et al*., 2001; Abbas *et al*., 2007).

As seen in Fig. 6, DCs showed increased expression of the surface markers CD80, CD86, HLA I, HLA II, CD40, CD54, CD58 and CCR7 (as median of fluorescence intensity levels) after 48 h of incubation with the appropriate stimulus compared to untreated DCs. Similarly, the percentage of CD83 positive cells also increased after treatment. Notably, yeast genera differently up-regulated the surface expression of maturation markers in human DCs. The lysate from HCMV-infected fibroblasts also led to an increase in MFI levels for all surface markers analyzed; LPS, the classical inducer of DC maturation *in vitro*, elicited the highest increases in median fluorescence of the same molecules.




CD58
Median fluorescence intensity
CD80
Median fluorescence intensity
CD83
% positive cells
CD86
Median fluorescence intensity
HLA I
Median fluorescence intensity
HLA II
Median fluorescence intensity
untreated
Sc BY4742
Sc BY4742 Δmnn11
Sc BY4742 Δost3
Sc S86c
Sc W303-1a
Sz. pombe
K. lactis
P. pastoris GS115
P. pastoris KM71
H. polymorpha
Y. lipolytica
C. glabrata
HCMV
LPS

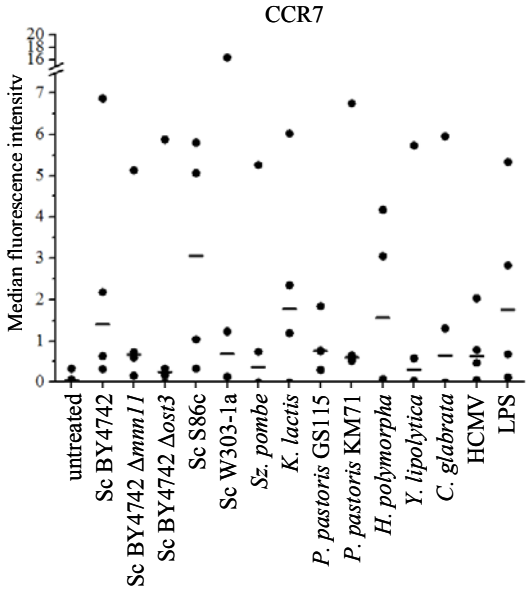


Fig. 6: Upregulation of various DC surface markers induced by fungal, bacterial and viral components. Human immature DCs were incubated for 48 h with diverse yeasts (MOI 1), *E. coli* LPS, a HCMV lysate, or left untreated, and subsequently stained with specific antibodies for flow cytometric analyses. Results are depicted as percent positive cells (for CD83) or median values of fluorescence intensities (for all other markers). Bars represent median values from four donors. Sc = *S. cerevisiae*.

In order to allow comparisons based on the yeast genus tested, additional values for each genus were calculated for each donor from the median values relative to each strain (in the case of species in which two or more strains were analyzed, such as *P. pastoris* and *S. cerevisiae*). For practical reasons, these values are not shown in the graphics.

DC maturation caused by different *S. cerevisiae* strains

All *S. cerevisiae* strains analyzed were able to induce, though in some cases to different degree, upregulation of DC surface molecules. Among the three *S. cerevisiae* strains BY4742, S86c and W303-1a, BY4742 and S86c alternated as inducers of higher expression levels of DC surface markers (or higher levels of CD83-positive cells). For example, levels of CD86, HLA I and percentage of $CD83^+$ cells were generally higher after DC incubation with BY4742, whereas levels of CD40, CD54 and CCR7 were higher in the case of S86c. Levels of CD58, CD80 and HLA II were similar for BY4742 and S86c, and higher than for W303-1a. Thus, in terms of DC maturation, the *S. cerevisiae* strain W303-1a exhibits some degree of drawback compared to strains S86c and BY4742.

DC maturation caused by different *S. cerevisiae* mutants

In order to verify the role of the yeast cell wall composition in DC maturation, two cell-wall mutants of *S. cerevisiae* BY4742 were also tested, the Δ*mnn11* mutant, which lacks an α-1,6-mannosyltransferase and cannot elongate mannan chains, and the Δ*ost3* mutant, in which glycoproteins show an underglycosylated pattern due to the absence of an oligosaccharyltransferase (Karaoglu *et al*., 1995; Jungmann and Munro, 1998). In general, the mutants were more efficient than the parental strain in terms of upregulation of DC maturation markers, except in the case of CCR7. Higher surface marker levels (or percentage of positive cells) were observed after incubation of DCs with Δ*mnn11*, except for HLA II, which was detected in higher levels after stimulation with Δ*ost3* (Fig. 6). This higher activation induced by cell-wall mutants compared to the wild-type strain indicates that the degree of maturation depends on antigen nature. Also, shorter mannan chains on yeast cell surface seem to affect maturation in a more explicit manner than the presence of underglycosylated proteins.

DC maturation caused by different *P. pastoris* strains

Increases in the expression of DC surface markers induced by the two *P. pastoris* strains examined (GS115 and KM71) showed similar patterns, indicating that both strains share similar characteristics in terms of promoting DC maturation. The median of fluorescence intensity for most of the markers analyzed were slightly higher in the case of GS115 (CD40, CD54, CD58, CD80, CD86, HLA I, HLA II and CCR7), whereas the percentage of $CD83^+$ cells was a little higher when KM71 was added to DCs. However, these differences are not distinct enough to place one strain in a more favoured position than the other with respect to DC maturation.

DC maturation caused by different yeast genera

When only the yeast genera are compared, it can be assumed that there is no preponderance of one genus among the set of genera analyzed. For instance, the highest levels of CD86, HLA I and HLA II were induced by *K. lactis*. *Y. lipolytica* caused the strongest upregulation of CD54 and CD80, *Sz. pombe* of CD40 and CD58, and *S. cerevisiae* of CCR7, whereas the percentage of $CD83^+$ cells was more elevated after incubation with *P. pastoris*.

Comparing the viral and bacterial stimuli with the yeast genera, LPS led to stronger upregulation of all markers examined. In relation to the classical DC maturation marker, CD83, the viral stimulus led to a higher number of positive cells than fungal stimuli. When the adhesion molecules CD54 and CD58 are analyzed, HCMV was similar to many yeast genera in promoting upregulation of CD54, whereas all fungal stimuli were more potent in inducing upregulation of CD58 than the viral stimulus. This latter finding was also true for the costimulatory molecules CD40 and CD86. For the HLA classes I and II, as well as for CD80 and CCR7, higher variations were observed among different stimuli, and no explicit pattern could be defined.

In sum, all yeast genera were able to effectively induce DC maturation, as observed by the upregulation of the adhesion molecules CD54 and CD58, the costimulatory molecules CD40, CD80 and CD86, the chemokine receptor CCR7, the classical maturation marker CD83, and the HLA molecules. Based on the analyses of these surface markers, differences among yeast strains could be observed, and they were greater among the *S. cerevisiae* strains tested than among the *P. pastoris* strains. Yeasts bearing mutations that affect their glycan structures also differ in their abilities to mature DCs, and the maturation was positively modulated when the two mutants were compared to the wild-type counterpart. Fungal stimuli were indeed less potent than the bacterial stimulus LPS in eliciting upregulation of expression of these surface markers, but, in many situations, stronger than the viral stimulus.

These results suggest that some differences in the immunological responses might be observed after *in vivo* application of distinct yeast genera as antigen carriers.

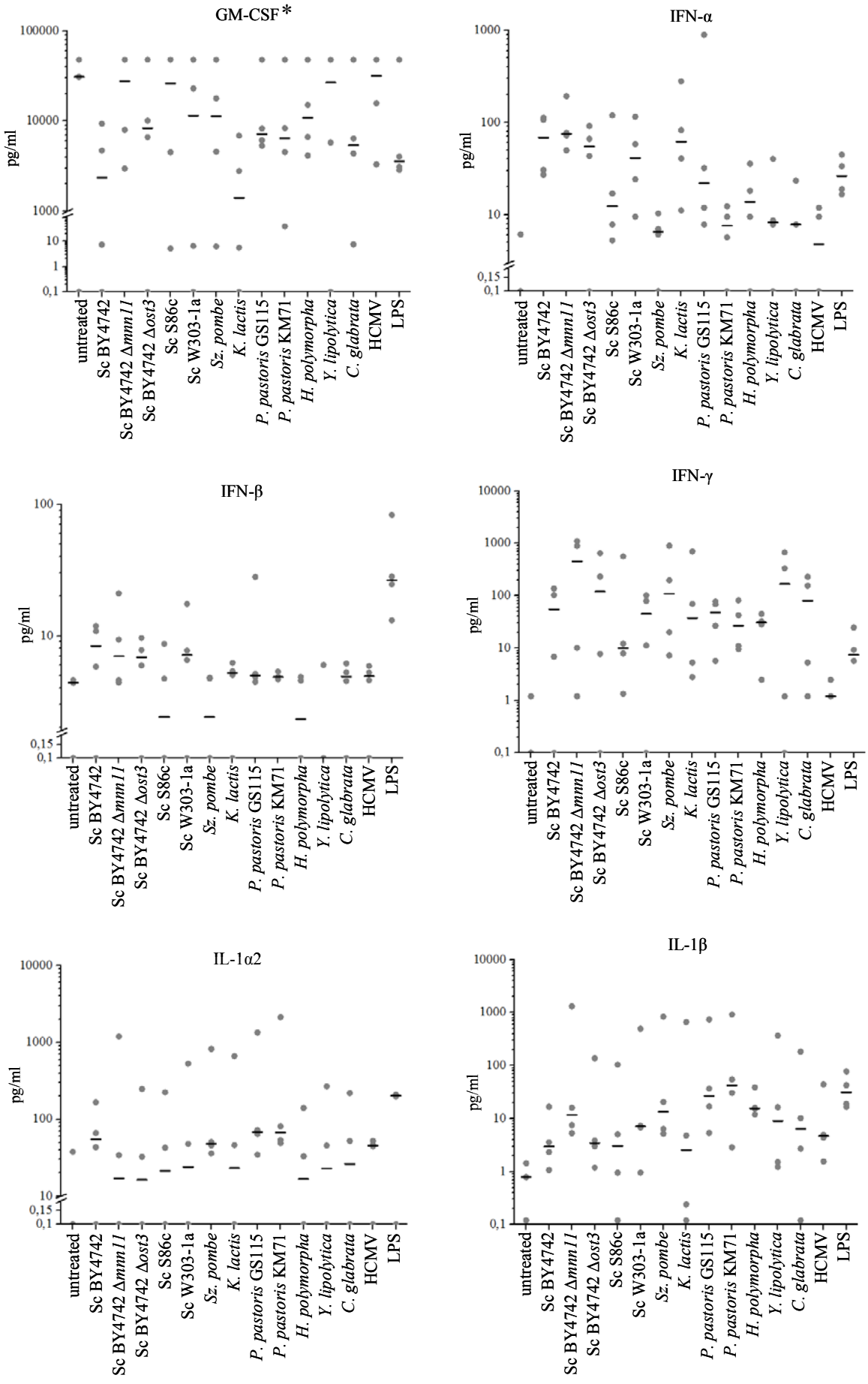
1.2. Cytokine detection in DC culture supernatants

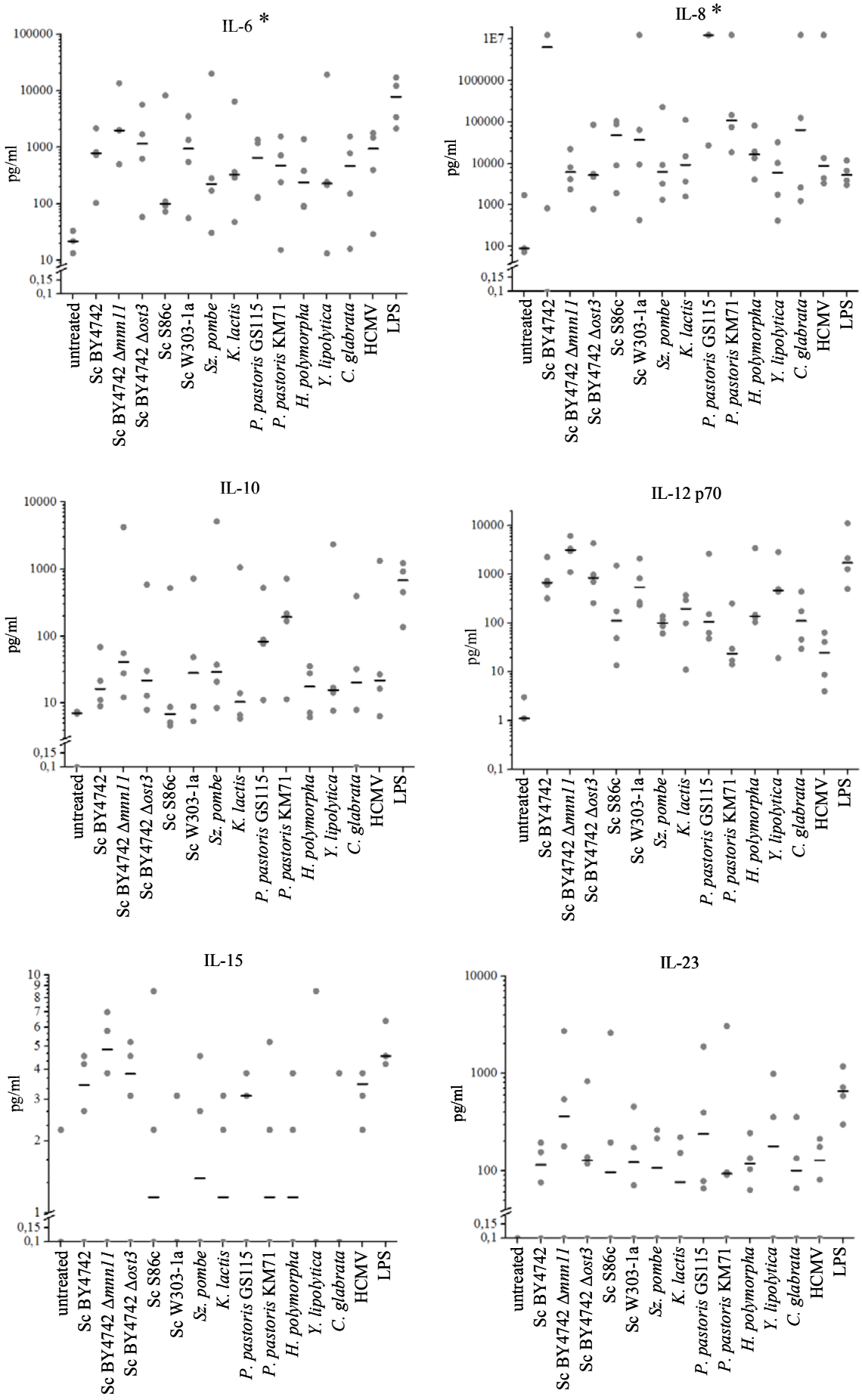
Besides the elevated expression of maturation markers, DCs were assessed for secretion of a number of cytokines that are known to be associated with their particular activation status. Again, the nature of antigens (fungal, bacterial and viral) on cytokine secretion by DCs was examined. For this purpose, immature DCs were incubated with distinct yeast genera, LPS, HCMV lysate, or left untreated. After 48 h incubation, culture supernatants were collected and assessed for the presence of the cytokines GM-CSF, IFNα, IFNβ, IFNγ, TNFα, IL-1α2, IL-1β, IL-6, IL-8, IL-10, IL-12 p70, IL-15, IL-23 and IL-27. The main functions of these cytokines are summarized and outlined below:

- GM-CSF is involved in the production and functional activation of hematopoietic cells, particularly granulocytes and monocyte/macrophages (Metcalf, 2008; Hercus *et al*., 2009). GM-CSF is also crucial for the proinflammatory activity of T_H17 cells (McGeachy, 2011).
- IFN-α and IFN-β belong to a family of cytokines (type I IFNs) with pleiotropic activities on nearly all somatic cells. Secretion of IFN-α/β is mainly induced upon viral infection, but can also occur in response to other biological stresses. One remarkable function of IFN-α/β is its interference with virus replication and spread (Huber and Farrar, 2011). IFN-α promotes a number of biological effects, such as induction of apoptosis and inhibition of cell growth, as well as induction of differentiation and activity of host immune cells (Rizza *et al*., 2010). IFN-β and IFN-γ inhibit upregulation of CCR7 expression in mature DCs, reducing their migratory capacity. However, unlike IFN-γ, IFN-β significantly inhibits production of IL-12 (Szabo *et al*., 2000; Yen *et al*., 2010). Human T cells, in contrast to mice T cells, induce T_H1 development in response to type I IFNs (Moser and Murphy, 2000). IFN-γ is a type II IFN, whose functions play an essential role in immunity towards intracellular microorganisms. IFN-γ is critical for T_H1 cells responses, inhibiting differentiation of T_H2 cells. It also stimulates APCs to upregulate expression of MHC class I and class II as well as costimulatory molecules. IFN-γ activates NK cells and stimulates macrophages to kill internalized microbes (reviewed by Abbas *et al*., 2007; Miller *et al*., 2009).
- IL-1 is a proinflammatory cytokine comprising IL-1α and IL-1β, which are products from different genes and exert distinct immunological activities, but are mutually inductive (Horai *et al*., 1998; Nakae *et al*., 2001a). Both IL-1α and IL-1β induce inflammation and activate lymphocytes. IL-1β strongly activates humoral immune responses, whereas IL-1α is involved in the development of antigen-specific memory T cells (Nakae *et al*., 2001a; Nakae *et al*., 2001b).
- IL-6 stimulates production of neutrophils, the growth of antibody-producer B cells and induces the proliferation and differentiation of T_H17 cells (Abbas *et al*., 2007).
- IL-8, a constituent of the chemokine family, exerts various functions on leukocytes, especially neutrophils, playing an important role in the establishment of acute inflammation. Among other functions, it is involved in leukocyte chemotaxis and induction of neutrophils to release lysosomal enzymes (Harada *et al*., 1994; Vlahopoulos *et al*., 1999).

- IL-10 possesses anti-inflammatory properties and targets different leukocytes. This cytokine functions to repress excessive inflammatory responses and to limit subsequent tissue damage. In phagocytes, IL-10 reduces antigen presentation and expression of inflammatory mediators, but enhances antigen uptake. Moreover, IL-10 is critical in the biology of B and T cells and contributes to induced tolerance (Sabat *et al*., 2010; Ouyang *et al*., 2011).
- IL-12 plays a crucial role in priming T_H1 cell responses and stimulating IFN-γ production and cytotoxic activity by T lymphocytes and NK cells. Furthermore, it functions as a third signal in activation of $CD8^+$ T cells and is involved in the reactivation and survival of memory $CD4^+$ T cells (DelVecchio *et al*., 2007). IL-12 and type I IFN-α/β are important in responses against viruses due to their antimicrobial and immunoregulatory activities (Dalod *et al*., 2002).
- IL-15 has been associated with the stimulation and survival of $CD8^+$ memory T cells and NK cells (Zhang *et al*., 1998; Abbas *et al*., 2007).
- IL-23 might expand the pool of IL-17-producing cells from activated and memory T-cell populations, but cannot trigger differentiation into T_H17 cells from naive T-cell precursors (Bettelli *et al*., 2006). Leibundgut-Landmann and colleagues (2007) showed that Dectin-1 signaling in DCs through Syk kinase strongly triggers T_H cell differentiation into T_H17 cells, and also observed that Dectin-1 agonists induce T_H17 and T_H1 responses *in vivo*.
- IL-27 inhibits development of T_H17 and T_H2 cells, inducing $CD4^+$ T cell differentiation into T_H1 cells that produce IFN-γ (Baten *et al*., 2006; Yoshimoto *et al*., 2007).
- Secretion of the pluripotent cytokine TNF-α activates a cytokine cascade needed for proper cellular responses in the affected tissue. In monocytes, TNF-α leads to enhanced cytotoxic capacity and increased secretion of other inflammatory mediators, such as IL-1 and IL-8 (Vlahopoulos *et al*., 1999).

As illustrated in Fig. 7, DCs incubated with yeasts up-regulated the production of several cytokines, in some cases even more pronounced than after LPS treatment. Results from four different blood donors showed great variability for a specific cytokine. This observation has also been described by others (Buentke *et al*., 2001; Barron *et al*., 2006; Remondo *et al*., 2009).


GM-CSF*
IFN-α
IFN-β
IFN-γ
IL-1α2
IL-1β
pg/ml
untreated
Sc BY4742
Sc BY4742 Δmnn11
Sc BY4742 Δost3
Sc S86c
Sc W303-1a
Sz. pombe
K. lactis
P. pastoris GS115
P. pastoris KM71
H. polymorpha
Y. lipolytica
C. glabrata
HCMV
LPS


IL-6 *
IL-8 *
IL-10
IL-12 p70
IL-15
IL-23
pg/ml
untreated
Sc BY4742
Sc BY4742 Δmnn11
Sc BY4742 Δost3
Sc S86c
Sc W303-1a
Sz. pombe
K. lactis
P. pastoris GS115
P. pastoris KM71
H. polymorpha
Y. lipolytica
C. glabrata
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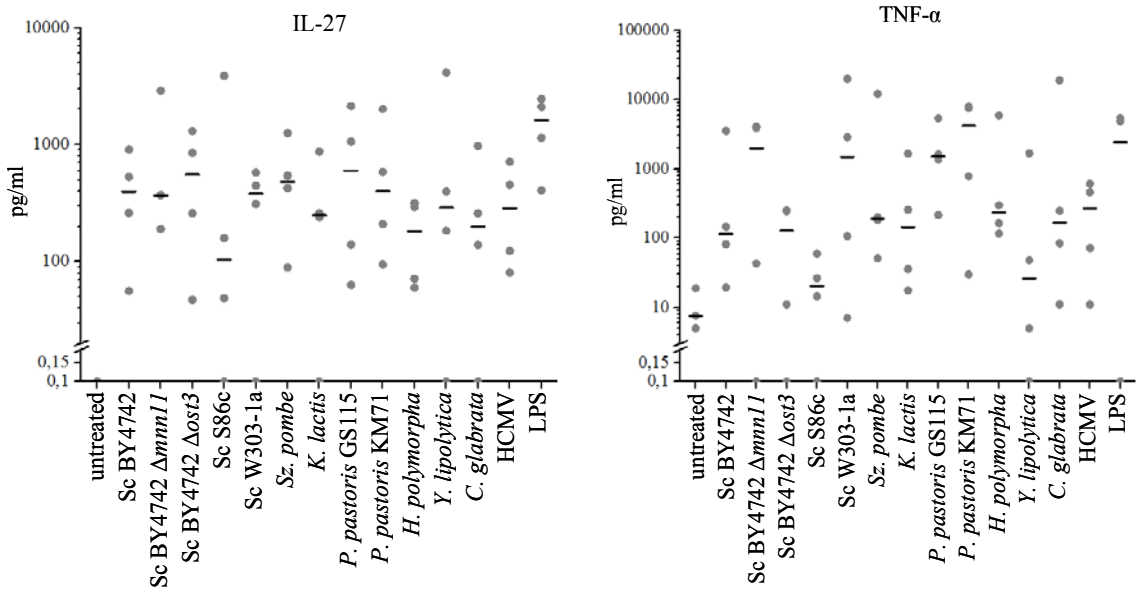


Fig. 7: Cytokine profile secreted by DCs after incubation with different stimuli. Human immature DCs were incubated with various yeasts (MOI 1), LPS, a lysate from HCMV-infected fibroblasts, or left untreated. After 48 h, supernatants were collected and cytokine concentrations were determined using a multiplex cytokine/chemokine kit (PROGEN Biotechnik, Heidelberg, Germany). Graphics show cytokine concentration in pg/ml. Bars indicate median values from four donors. Sc = *S. cerevisiae*. Graphics identified with an asterisk (*) show values above the detection limit (47,836 pg/ml for GM-CSF; 20,000 pg/ml for IL-6; 12,647,444.85 pg/ml for IL-8).

Stimuli were compared using the median values only. For comparison among single yeast genera, median values were calculated as described above for the surface markers. The high level of GM-CSF detected in supernatants of untreated DCs might have resulted from the presence of this cytokine in the culture medium to induce monocyte differentiation into immature DCs (Sallusto and Lanzavecchia, 1994).

Cytokine secretion by DCs in response to different *S. cerevisiae* strains

The *S. cerevisiae* strains S86c, BY4742 and W303-1a differently stimulated the secretion of cytokines by DCs, as observed for the maturation markers. Interestingly, the strain BY4742 led to secretion of higher levels of IFN-α, IFN-β, IFN-γ, IL-12 p70 (T_H1-driving cytokines), as well as of IL-1α2, IL-8, IL-15 and IL-27. Strain W303-1a elicited production of higher amounts of IL-1β, IL-6, IL-23 (T_H17-driving cytokines), IL-10 and TNF-α. S86c, in turn, caused the highest secretion levels of GM-CSF among the three *S. cerevisiae* strains. These results show a different pattern than the findings related to DC maturation, in which W303-1a showed some disadvantage compared to

the other two strains. However, a significantly higher number of donors needs to be tested in order to confirm these observations.

Cytokine secretion by DCs in response to *S. cerevisiae* BY4742 and its mutants

Among the tested *S. cerevisiae* mutants, the wild-type strain BY4742 provoked superior secretion levels of IFN-β, IL-1α2, and IL-8 by DCs. Its isogenic Δ*ost3* mutant induced higher expression levels of IL-27 only, whereas the Δ*mnn11* knock-out was more effective in inducing DC expression of GM-CSF, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-12 p70, IL-15 and IL-23. This bias for the Δ*mnn11* mutant confirms the results observed for DC maturation. Again, these results demonstrate a positive influence of shorter mannose structures within yeast cell wall components on immune recognition and activation in comparison to normal yeasts or yeasts carrying glycosylation defects.

Cytokine secretion by DCs in response to the *P. pastoris* strains

As observed for the DC surface maturation markers, no outstanding difference in cytokine production was seen after incubation of DCs with either *P. pastoris* GS115 or KM71, except for IL-8, whose levels were more than hundred fold higher in supernatants from DCs incubated with GS115. Levels of GM-CSF, IFN-β, IL-1α2, IL-1β and IL-6 were very similar in supernatants from DCs incubated with either strain GS115 or KM71. Higher production of IL-10 and TNF-α was detected after incubation of DCs with KM71, whereas slightly higher levels of IFN-α, IFN-γ, IL-8, IL-12 p70, IL-15, IL-23 and IL-27 were measured after incubation with GS115. The much higher levels of IL-8 observed after incubation of DCs with strain GS115 could be advantageous, since IL-8 acts as chemoattractant and inflammatory mediator, and it has been demonstrated that its synthesis precedes upregulation of the activation marker CD40 (Hellman and Eriksson, 2007).

Cytokine secretion by DCs in response to different yeast genera

Comparing the single yeast genera only, a slight bias could be observed towards *P. pastoris*, which induced the highest expression levels of IL-1α2, IL-1β, IL-8, IL-10, IL-15, IL-27 and TNF-α by DCs, while *Y. lipolytica* led to secretion of the highest levels of GM-CSF, IFN-γ and IL-23, *S. cerevisiae* of IFN-β, IL-6 and IL-12, and *K. lactis* of

IFN-α. However, it has to be underscored that levels of IFN-β, IL-1α2, IL-23 and IL-27 in culture supernatants were very similar for all yeast genera analyzed. Yeast genera upregulated the production of almost all cytokines analyzed. Secretion of IL-15 and IFN-β by DCs was, in some cases such as after incubation with *Y. lipolytica*, not upregulated in comparison to the median value of the untreated control. However, no data in the literature describing secretion of both cytokines in response to yeast could be found.

Comparing all yeast genera with the viral and the bacterial stimuli, it could be observed that LPS was responsible for the highest levels of IFN-β, IL-1α2, IL-6, IL-10, IL-12, IL-15, IL-23 and IL-27. The lysate of HCMV-infected fibroblasts provoked more GM-CSF secretion than any other stimulus. Besides GM-CSF, the only cytokine whose level was higher in response to HCMV than to LPS was IL-8. Secretion of IL-8 was markedly high for almost all yeasts tested, especially *P. pastoris*, which was by far the most effective stimulus, with DCs exhibiting IL-8 levels above 10^6 pg/ml (Fig. 7). Levels of IFN-α, IFN-γ, IL-1β and TNF-α, as well as the already mentioned IL-8, were higher in response to at least one yeast stimulus than for the viral or bacteria stimulus. IFN-γ levels in response to LPS and especially to HCMV were several times lower than to any yeast genus tested. Interestingly, lowest secretion of IFN-α was observed when DCs were incubated with the viral stimulus, and IFN-β levels did not significantly increase after DC incubation with HCMV in comparison to the untreated samples.

From these results it could be inferred that a wide spectrum of cytokines can be produced by DCs after incubation with different yeast stimuli, and that yeasts differ in their ability to stimulate cytokine production by DCs. Moreover, for a given set of cytokines, fungal stimuli can promote stronger DC activation than bacterial or viral antigens.

2. Fungal cell wall staining

The observation that distinct yeast genera lead to maturation of human DCs and production of a variety of cytokines, and that the composition of the fungal cell wall plays an important role in these events (as demonstrated previously), provided the rationale for investigating the variations in yeast cell wall constitution more closely. For this purpose, three of the major yeast cell wall components, mannan, β-glucan and

chitin, were examined in each one of the yeast species and strains used for DC maturation and activation.

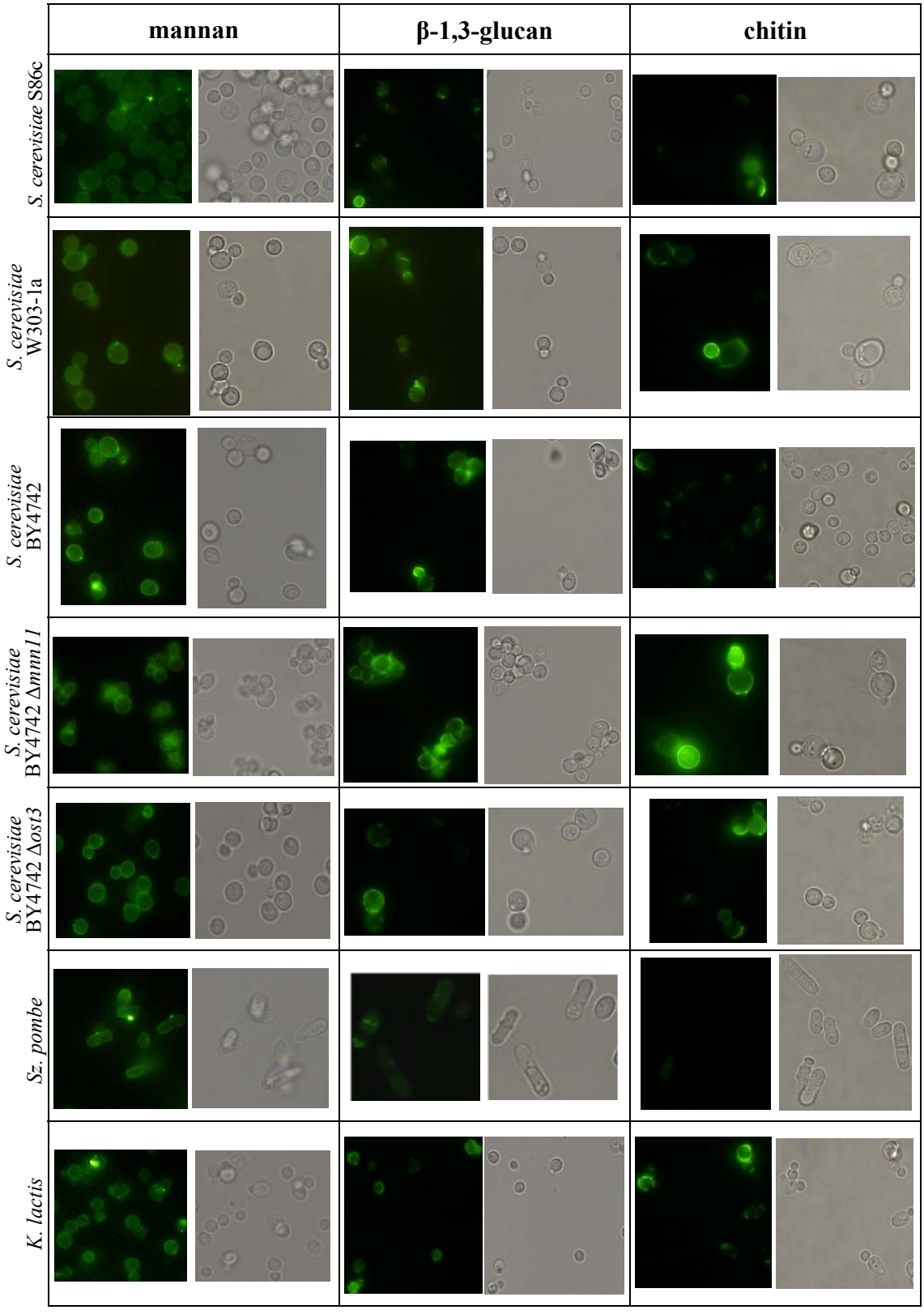
Concanavalin A (Con A), a lectin of the jack bean (*Canavalia ensiformis*), has binding affinity for terminal α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl, or α-D-arabinofuranosyl residues. Con A does not interact with yeast glucan or chitin, therefore FITC-labeled Con A can be used to specifically stain α-mannan in yeast cell walls (Goldstein and So, 1965; So and Goldstein, 1968; Tkacz *et al*., 1971).

The mouse monoclonal anti-β-1,3-glucan antibody recognizes linear β-1,3-oligosaccharides present in β-glucans. When an anti-mouse IgG-FITC antibody is subsequently used after incubation of yeasts with the primary antibody, linear cell wall β-1,3-glucans can be detected by fluorescence microscopy or flow cytometry.

Wheat germ agglutinin (WGA) is a lectin from *Triticum vulgaris* which specifically interacts with β-1,4-N-acetylglucosamine oligomers, such as those found in fungal chitin. WGA-FITC was used in this study to stain chitin on yeast cell walls, as described by others (Buck and Andrews, 1999; Mora-Montes *et al*., 2011).

The cell walls of different yeast genera were analyzed both by flow cytometry and fluorescence microscopy with respect to the presence and distribution of mannan, β-1,3-glucan and chitin. Among some yeast species, different strains (as for *S. cerevisiae* and *P. pastoris*) were analyzed, and among one *S. cerevisiae* strain (BY4742), two cell wall mutants were evaluated. As depicted in Fig. 8, mannan, β-glucan and chitin staining patterns varied among yeast genera, species, strains and cell-wall mutants, to greater or lesser extents. Unstained yeasts showed no fluorescence (data not shown). All yeasts could be stained for mannan and β-glucan. Both *S. cerevisiae* mutants, Δ*mnn11*, which bears a mutation that affects mannan biosysnthesis, and Δ*ost3*, which contains underglycosylated glycoproteins, could also be specifically stained by Con A on their cell surfaces. These results were not unexpected, since the mutations do not exclude mannans from the cell walls. With respect to chitin, fluorescence microscopy of yeast cells stained with WGA-FITC revealed a higher discrepancy among the yeasts analyzed. Some yeast species, such as *P. pastoris* and *Y. lipolytica*, exhibited green fluorescence outlining the cells, whereas others showed fluorescence only in discrete patches (*e.g. K. lactis*) or almost no fluorescence (*Sz. pombe*). In the case of β-glucan staining, the budding yeasts showed the existence of restricted regions on the cell wall where this structure is exposed. In fission yeast, linear β-1,3-glucan is restricted to the

septum (Humbel *et al*., 2001). In contrast, mannan structures are more homogeneously distributed over the cell walls (Fig. 8).



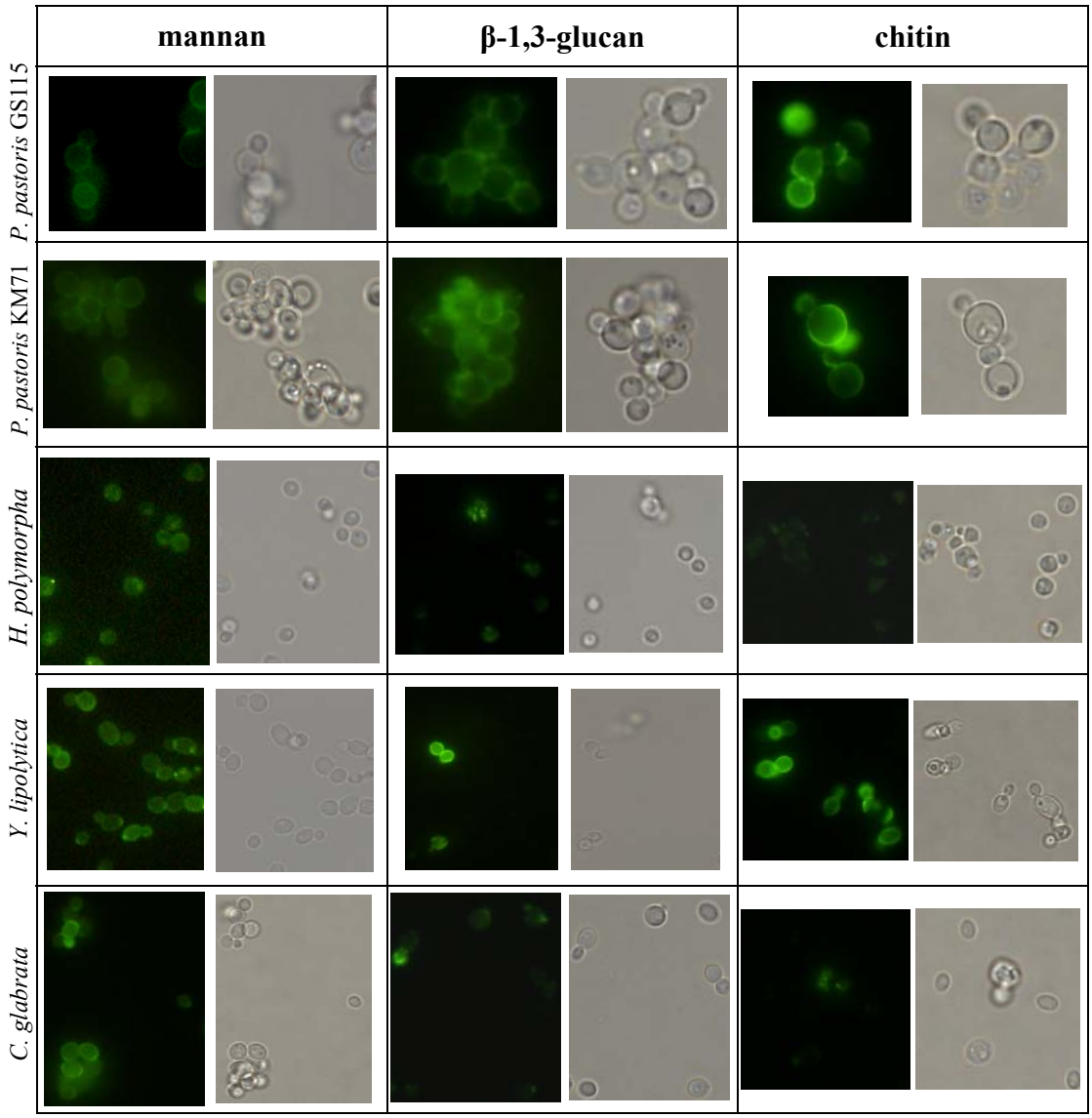


Fig. 8: Fluorescence microscopy after mannan, β-1,3-glucan and chitin staining of diverse yeast genera. Yeasts exponentially grown in SC medium were stained for mannan with Con A-FITC, β-glucan with mouse anti-β-1,3-glucan and anti-mouse IgG-FITC, and chitin with WGA-FITC, and analyzed with a fluorescence microscope (GFP channel). Each column shows fluorescence (left) and the matching bright field (right) micrographs. 50× zoom.

Additional flow cytometric analyses confirmed that the majority of all yeast genera, species, strains or mutants stained positive for mannan (*i.e.* O- and N-linked mannose residues) and β-1,3-glucan but, in some cases, not for chitin (Fig. 9). Different staining patterns were observed, also between strains. Among the three *S. cerevisiae* strains analyzed (S86c, BY4742 and W303-1a) mannan and β-glucan staining resulted in a similar number of positive cells, while chitin distribution varied dramatically (about 37%, 72% and 98% positive cells of BY4742, S86c and W303-1a, respectively). The BY4742 mutants Δ*mnn11* and Δ*ost3* showed a similar percentage of positive cells after

Con A binding (~84% and ~86%, respectively), which was not only lower than in the parental strain BY4742 (~93%), but also than in any other strain tested. The two *P. pastoris* strains examined showed no relevant difference in relation to β-glucan and chitin, whereas the percentage of KM71 cells positive for mannan was about 10% lower in comparison to GS115. *Sz. pombe* showed the lowest percentage of cells positive for β-glucan (28,6%) and chitin (~5%). The first observation is in agreement with the fact that growth by elongation, unlike budding, does not unmask β-glucan. The presence of chitin in *Sz. pombe* is controversial. Little amounts of this polymer in *Sz. pombe* have been found by some authors, whereas other investigators failed to detect this structure (Horisberger *et al*., 1978; Sietsma and Wessels, 1990).

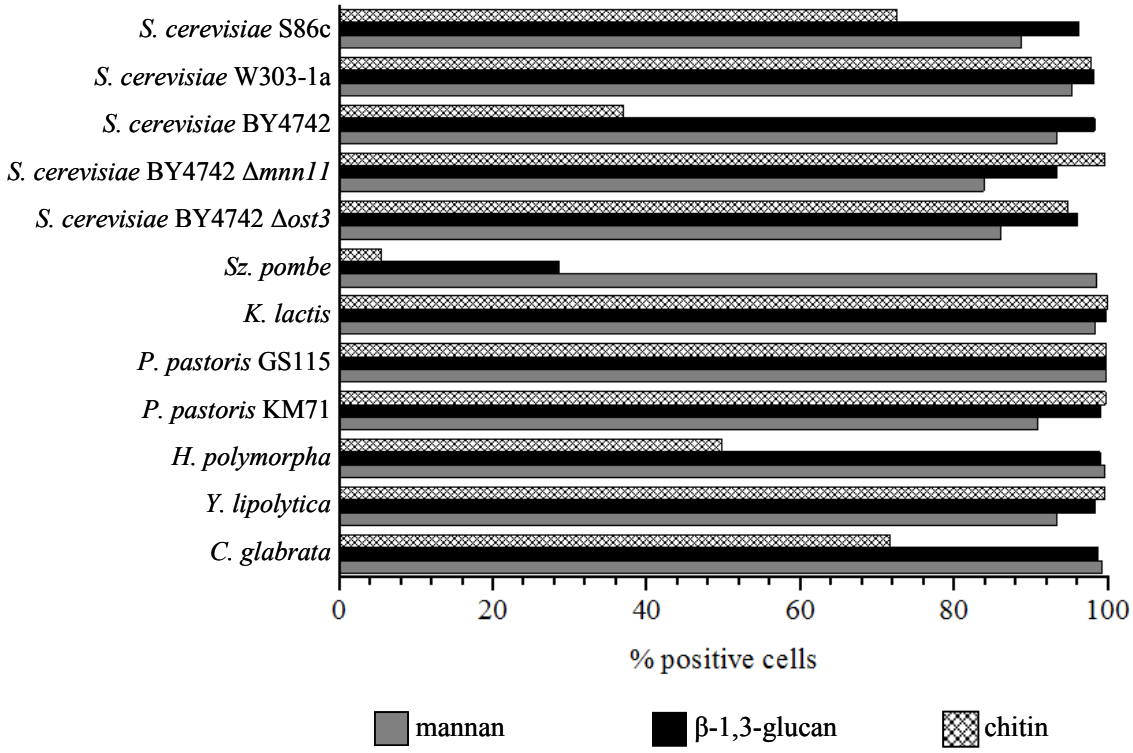


Fig. 9: Flow cytometric analysis of mannan, β-1,3-glucan and chitin from various yeast genera. Yeasts exponentially grown in SC medium were stained with Con A-FITC for mannan, WGA-FITC for chitin, or with anti-β-glucan and FITC-conjugated secondary antibody for β-1,3-glucan, and analyzed by flow cytometry. Bars indicate the percentage of positive cells. Values relative to unstained yeasts (negative controls) were subtracted.

Thus, each yeast strain has its peculiarities in terms of cell wall composition. Evidently, other yeast cell wall components, like β-1,6-glucan and β-1,6-branched β-1,3-glucan, also play an important role in yeast recognition by the immune system. However,

detection of such structures was not possible in this study due to the lack of commercial antibodies or specific lectins.

In this way, the particular set of PAMPs of a specific yeast strain should be taken into account in terms of immune recognition and interaction with host cells.

3. Uptake of different yeast genera by mammalian phagocytic cells

A necessary feature of a possible antigen carrier is its interaction with cell surface receptors of antigen-presenting cells in order to trigger its subsequent internalization. In this study, the ability of murine macrophages and human DCs to phagocytose four representative yeast genera (*S. cerevisiae*, *Sz. pombe*, *P. pastoris* and *K. lactis*) that are among the most widely used expression systems in biotechnology were characterized.

3.1. Uptake of whole yeast cells by IC-21 murine macrophages

The pattern of *S. cerevisiae*, *K. lactis*, *P. pastoris*, and *Sz. pombe* uptake by IC-21 murine macrophages was microscopically monitored using 24-well plates and a light microscope. First, yeasts were incubated with macrophages at MOI 5 for varying periods of time, following the same protocol described in the *Methods* section. Fig 10 A shows that *S. cerevisiae*, *K. lactis*, and *P. pastoris* were phagocytosed by IC-21 in a similar fashion, in which these yeasts were quickly internalized by macrophages, whereas recognition and engulfment of *Sz. pombe* occurred more slowly (for example, after 4 h 65% ± 4%, 65% ± 3%, 65% ± 6%, and 31% ± 5% of the macrophages had ingested at least one single yeast cell of *S. cerevisiae*, *K. lactis*, *P. pastoris*, and *Sz. pombe*). However, after 12 h of incubation, a plateau level was reached, and all yeast genera showed almost the same pattern of internalization (81% ± 4% for *S. cerevisiae*, 82% ± 3% for *K. lactis*, 82% ± 3% for *P. pastoris*, and 79% ± 3% for *Sz. pombe*). Some macrophages ingested a high number of yeast cells, especially in the case of *P. pastoris*. The presence of several yeast cells inside a single macrophage was not due to yeast growth within the phagosome, as the total yeast cell number remained constant in the course of the experiment (data not shown). Upon microscopic visualization, only yeast cells which were localized inside the boundaries of the macrophage cell membrane were counted, ensuring that yeasts that had not been internalized were excluded (Fig. 10 B).

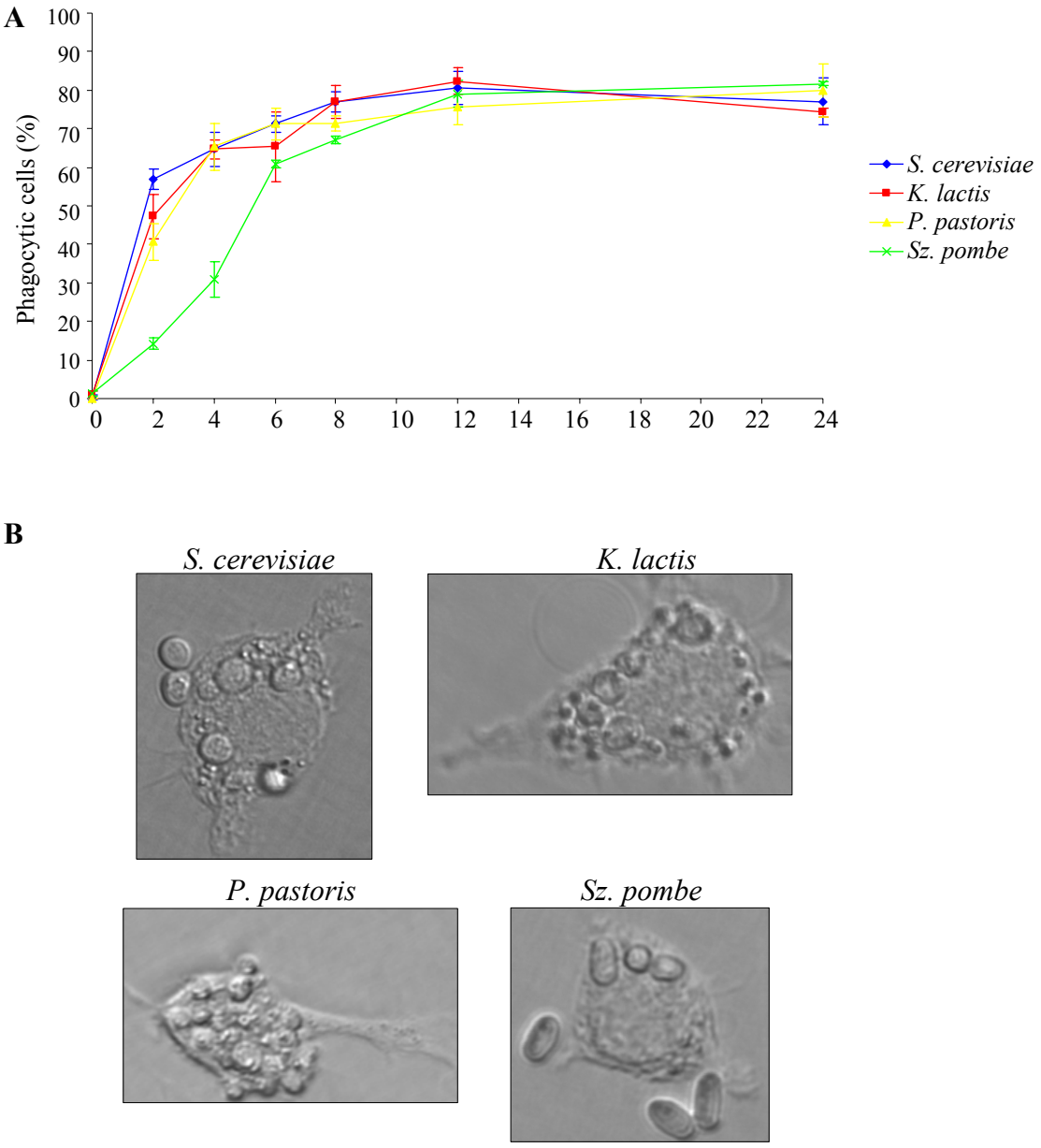


Fig. 10: (A) Uptake kinetics of yeasts by mouse macrophages. The yeasts *S. cerevisiae*, *K. lactis*, *P. pastoris* and *Sz. pombe* were incubated at a 5:1 ratio with mouse macrophages for different periods. After each time point indicated, medium was aspirated and macrophages were extensively washed to remove unbound yeasts. Then, cells were fixed and examined under light microscopy for the percentage of phagocytes binding at least one single yeast cell. **(B)** Confocal laser scanning microscopy after 4 h co-cultivation of IC-21 macrophages and yeasts at MOI 5. Micrographs show that *S. cerevisiae*, *K. lactis*, and *P. pastoris* are rapidly internalized, whereas many *Sz. pombe* cells are found in the neighbourhood of the macrophages.

Next, the effect of the MOI on the uptake of different yeast genera by IC-21 macrophages was examined. Initially, ingestion of yeast cells increased proportionally with the MOI (Fig. 11 A), and reached a maximum at MOI 7 for *K. lactis* (65% ± 1% of macrophages ingesting) or 9 for *S. cerevisiae* (69% ± 5%), *P. pastoris* (73% ± 3%) and

Sz. pombe (30% ± 4%). When the phagocytic index was determined, *i.e.*, the number of internalized yeast cells in 100 macrophages, it could be seen that the average number of yeast cells per phagocyte enhanced continuously as MOIs increased. This observation was valid for all yeast species tested and, again, internalization of *Sz. pombe* showed to be less effective in comparison to the other species. In all MOIs tested, uptake of *S. cerevisiae*, *K. lactis* and *P. pastoris* by IC-21 macrophages was equivalent, and the difference to *Sz. pombe* became higher each time when MOI was increased. At MOI 11 and after 4 h of co-incubation, there was approximately one single fission yeast cell per macrophage, and 3.5 yeasts/macrophage in the case of the other yeasts (Fig. 11 B).

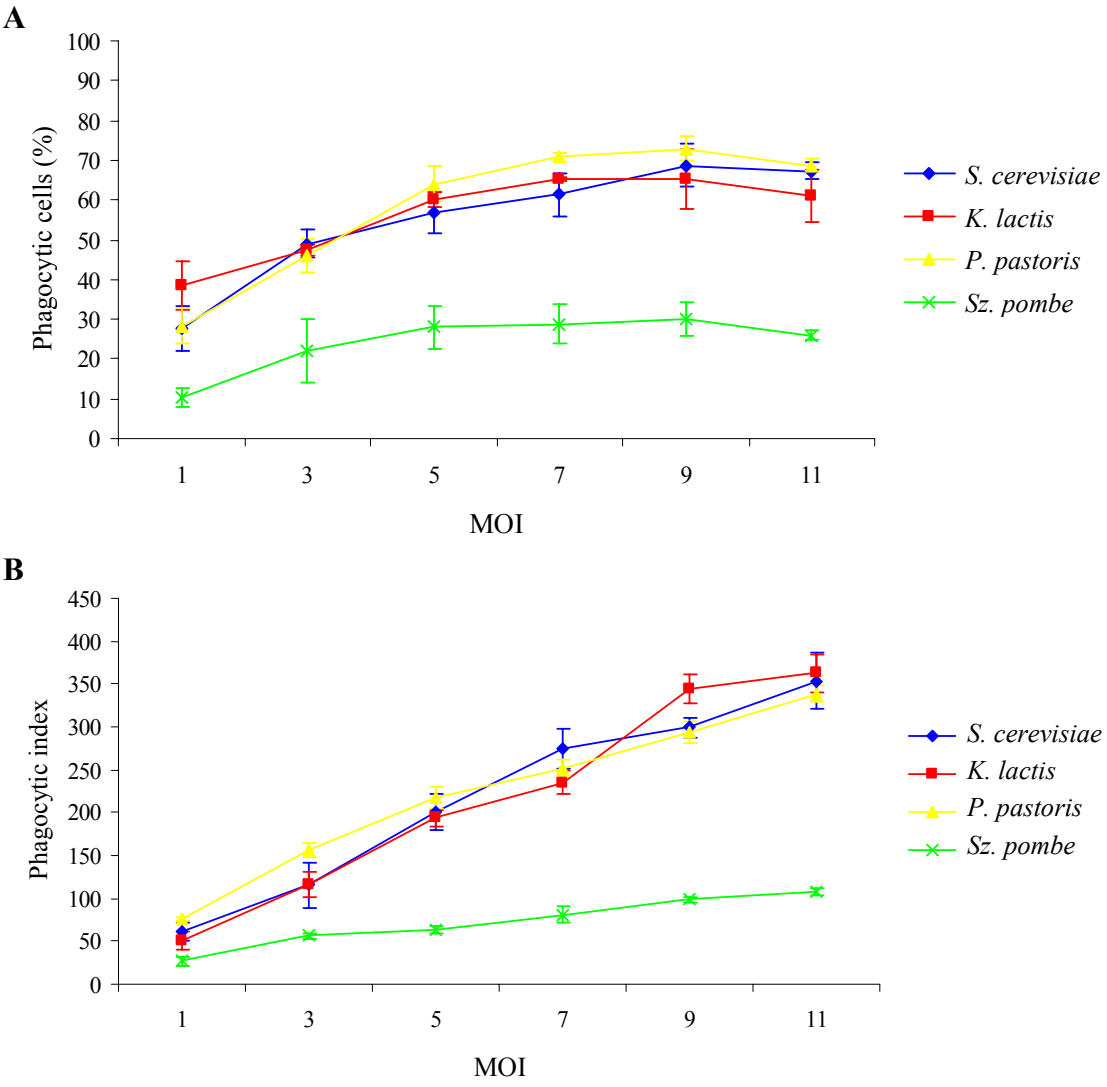


Fig. 11: (A) Effect of yeast/macrophage ratio on phagocytosis. IC-21 murine macrophages were incubated with the yeasts *S. cerevisiae*, *Sz. pombe*, *K. lactis* and *P. pastoris* at varying MOIs for 4 h. After this period, cells were thoroughly washed to remove unbound or attached yeasts, fixed, and analyzed under a light microscope for assessment of percent ingestion. **(B)** Alternatively, the total number of yeast cells in 100

macrophages was assessed for each MOI. Results are presented as mean values ± standard deviation from triplicates.

3.1.1. Role of Dectin-1 on yeast uptake by IC-21 murine macrophages

Since some macrophages do not bear the mannose receptor (MR) on the cell surface, such as resident peritoneal mouse macrophages (Taylor *et al*., 2002) and the J774 murine macrophage cell line (Martinez-Pomares *et al*., 2003), the presence of this receptor in IC-21 cells was investigated.

Murine macrophages were incubated with rat anti-mouse MR or mouse anti-mouse Dectin-1 and subsequently with anti-rat IgG-FITC and anti-mouse IgG-FITC, respectively, before being analyzed by flow cytometry. As shown in Fig. 12 A, IC-21 macrophages do not express MR on their surface. In contrast, Dectin-1 is highly expressed in this cell line (Fig. 12 B).

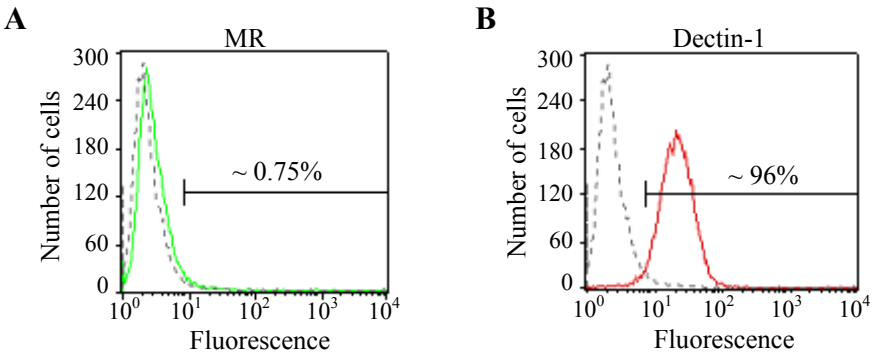


Fig. 12. IC-21 murine macrophages were assessed for surface expression of MR **(A)** and Dectin-1 **(B)**. Cells were first stained with monoclonal anti-MR, anti-Dectin-1, or an isotype control antibody, and then with a FITC-conjugated anti-IgG secondary antibody. Dashed lines represent cells stained with the isotype control antibody. Green solid lines represent cells stained for the mannose receptor and red solid lines represent cells stained for the Dectin-1 receptor. A total of 25,000 cells was analyzed.

Next, the effect of Dectin-1 on yeast uptake by IC-21 was examined. Cells were incubated with 25 µg/ml anti-Dectin-1 for 1 h at 37°C, prior to the addition of *S. cerevisiae*, *K. lactis*, *P. pastoris* and *Sz. pombe*, at MOI 5. This yeast:macrophage ratio was chosen because it provided sufficient yeast-phagocyte interactions, and MOI values higher than 5 are very difficult to analyze microscopically. The appropriate antibody concentration was previously determined (not shown). After 4 h of incubation, cells were vigorously washed, fixed, and analyzed by light microscopy. As seen in Fig. 13, blocking of Dectin-1 caused a decrease in uptake of all yeast species tested. The

percentage of macrophages ingesting at least a single yeast cell decreased from 65% ± 5% to 49% ± 4% for *S. cerevisiae*, 63% ± 5% to 46% ± 5% for *K. lactis*, 60% ± 2% to 42% ± 2% for *P. pastoris*, and from 31% ± 2% to 19% ± 3% for *Sz. pombe*. This diminished internalization was statistically significant in all cases. No significant difference was observed between untreated cells and cells incubated with an isotype control antibody.

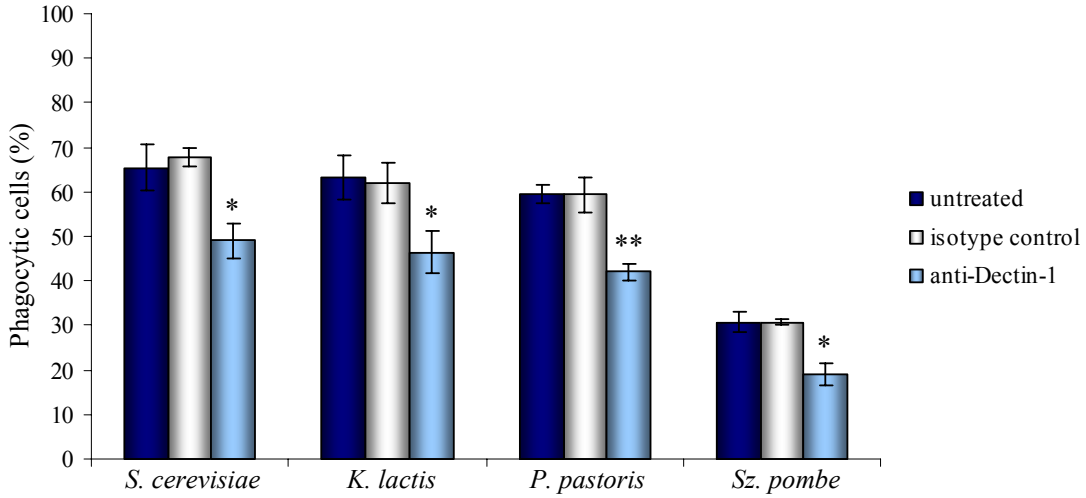


Fig. 13: Dectin-1 in murine IC-21 macrophages recognizes the yeasts *S. cerevisiae*, *K. lactis*, *P. pa storis* and *Sz. pombe*. IC-21 cells were pre-treated with anti-Dectin-1 for 1 h, then incubated with yeasts (MOI 5) for another 4 h. After extensive washing, cells were fixed and analyzed by light microscopy. Results show mean values ± standard deviation from triplicates. Asterisks indicate significant differences compared to untreated macrophages (* $p < 0.05$, ** $p < 0.01$).

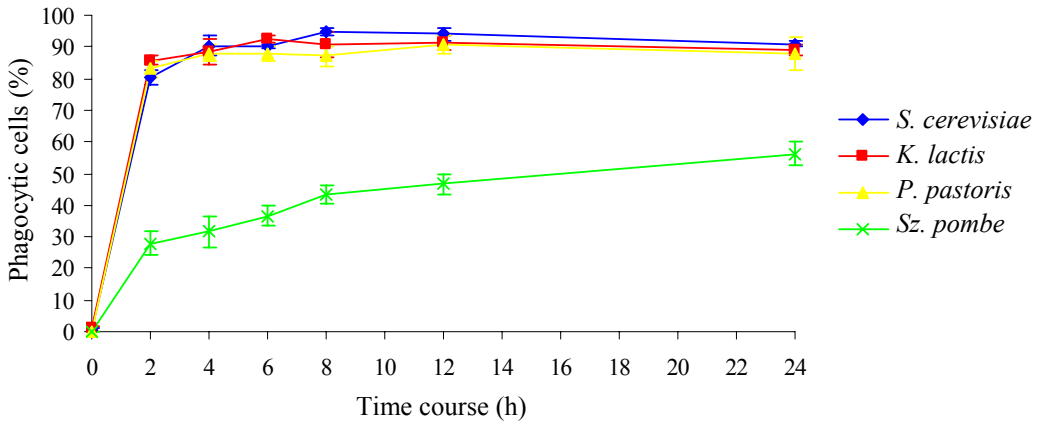
In sum, recognition of the four tested yeast genera by IC-21 murine macrophages involves Dectin-1, but not MR. Many other PRRs are associated with yeast recognition by phagocytes, nevertheless analyzing the participation of each of them is a task outside the scope of this work.

3.2. Uptake of whole yeast cells by human dendritic cells

Previous studies have demonstrated that several yeast genera are effectively recognized and phagocytosed by immature human DCs, among them *S. cerevisiae* (Stubbs *et al*., 2001; Barron *et al*., 2006), *Histoplasma capsulatum* (Gildea *et al*., 2001), *Malassezia furfur* (Buentke *et al*., 2000) and *Candida albicans* (Newman and Holly, 2001; Cambi *et al*., 2003).

In order to analyze and compare the internalization patterns of the four biotechnologically relevant yeasts *S. cerevisiae*, *Sz. pombe*, *K. lactis* and *P. pastoris*, immature monocyte-derived DCs were incubated with unopsonized live yeast cells at MOI 5 for varying time periods. As shown in Fig. 14 A, approximately 80 % of DCs had ingested one or more yeast cell after 2 h in the case of *S. cerevisiae* (80 ± 2 %) *K. lactis* (86 ± 1 %), and *P. pastoris* (83 ± 3 %), and the number of phagocytozing DCs reached a plateau level after 6 h (90 ± 1 %; 93 ± 1 %; 88 ± 2 %, respectively). In contrast, ingestion of *Sz. pombe* by DCs occurred less effective and in a more time-dependent manner. After 2 h, the number of cells ingesting at least a single yeast cell was 28 ± 4 %, and increased gradually at least until 24 h after the co-incubation (56 ± 4 %). The same was true when DCs were examined for their ability to engulf live yeasts at various MOIs ranging from 1 to 11 after 4 h at 37 °C. As expected, the number of cells engulfing at least one yeast was enhanced by increasing the MOI, reaching a plateau level at MOI 7 for *S. cerevisiae* (92 ± 1 %), *K. lactis* (93 ± 3 %) and *P. pastoris* (86 ± 2 %), and was maximum for *Sz. pombe* at MOI 9 (54 ± 1 %; Fig. 14 B). Analysis of phagocytic indices revealed a similar pattern as obtained with murine macrophages, with *S. cerevisiae*, *K. lactis* and *P. pastoris* being much more efficiently internalized than *Sz. pombe* (Fig. 14 C). However, human DCs appear to have a greater capacity of phagocytosis than murine IC-21 macrophages. When MOI 1 was examined, practically all yeasts had been taken up after 4 h of incubation with DCs. However, and as observed for IC-21, increasing MOI values implied greater differences of internalization between *Sz. pombe* and the other species.

A



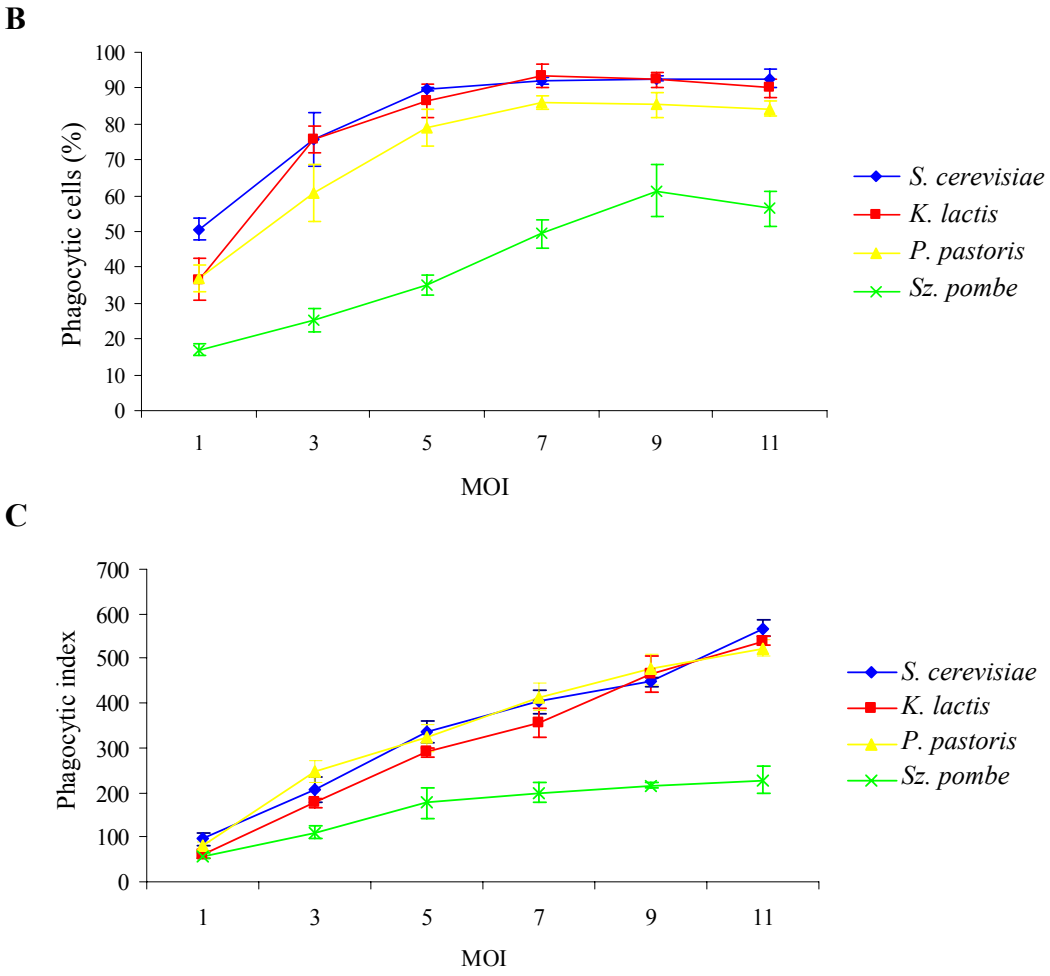


Fig. 14: Uptake of different yeast genera by human dendritic cells. Immature DCs were incubated with *S. cerevisiae*, *K. lactis*, *P. pastoris* and *Sz. pombe* cells at MOI 5 **(A)** for varying periods of time or **(B)** for 4 h with varying MOIs. The data are presented as the percentage of phagocytic cells (*i.e.* percentage of DCs containing at least one ingested yeast cell). **(C)** The phagocytic indices (number of internalized yeast cells per 100 DCs) were determined for distinct MOIs after 4 h incubation. Uptake was verified by light microscopy. The data represent the mean ± standard deviation of triplicates. One representative experiment is shown [Bazan *et al.*, 2011].

3.2.1. Involvement of Dectin-1 and mannose receptor in yeast uptake

Next, the effect of two major receptors recognizing fungal structures (Dectin-1 and MR) on phagocytosis of the different yeast genera by human DCs was determined and compared. As shown in Fig. 15, internalization of all yeast genera was significantly inhibited when DCs were pre-incubated with anti-human Dectin-1 or anti-human MR monoclonal antibodies. No significant differences were observed relative to untreated cells or cells incubated with an isotype control antibody. In detail, inhibition of Dectin-1 led to a greater reduction in yeast uptake than blockage of MR for *S. cerevisiae*, *Sz. pombe*, and *P. pastoris*. Interestingly, the opposite effect was observed for *K. lactis*,

although the cell wall composition of this yeast species does not significantly differ from that of *S. cerevisiae* or *P. pastoris*. Noteworthy, and in contrast to the other yeast genera tested, blockage of Dectin-1 dramatically impaired phagocytosis of fission yeast cells. After incubation of DCs with anti-MR, the percentage of DCs internalizing at least a single yeast cell was reduced from 84% ± 4% to 64% ± 2% for *S. cerevisiae*, 76% ± 2% to 43% ± 6% for *K. lactis*, 75% ± 2% to 52% ± 5% for *P. pastoris*, and from 32% ± 1% to 25% ± 3% for *Sz. pombe*. With respect to inhibition of Dectin-1, yeast uptake dropped to 50% ± 9% for *S. cerevisiae*, 57% ± 2% for *K. lactis*, 42% ± 6% for *P. pastoris* and 4% ± 2% for *Sz. pombe*.

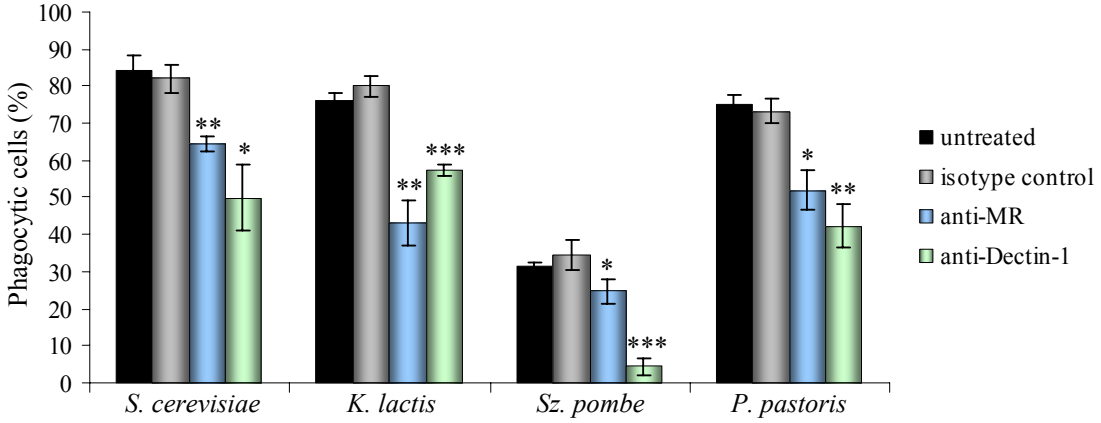


Fig. 15. Dectin-1 and mannose receptor (MR) are involved in phagocytosis of S. *cerevisiae*, *K. lactis*, *Sz. pombe* and *P. pastoris* by human dendritic cells. DCs were preincubated with anti-MR- or anti-Dectin-1-antibodies for 1 h, yeast cells were added at MOI 5 and the number of phagocytic DCs was determined after 4 h at 37 °C. Ingestion was verified by light microscopy. Results are expressed as average of triplicates with error bars indicating standard deviation. Asterisks indicate significant differences compared to untreated macrophages (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) [Bazan *et al*., 2011].

Taken together, these results show that both MR and Dectin-1 are involved in recognition of the four yeast genera analyzed, and that *Sz. pombe* cells, in contrast to *S. cerevisiae*, *P. pastoris* and *K. lactis*, are taken up less effectively by human DCs.

4. Recombinant expression of model antigens in different yeast genera

After demonstrating the abilities of several yeast genera to mature human DCs and induce cytokine secretion, and having shown that some selected yeast genera are endocytosed by mammalian phagocytic cells, the activation of antigen-specific T cells

after protein delivery by yeast to APCs was determined. For this purpose, the model antigens Ova and pp65 were used for recombinant expression in the yeasts *S. cerevisiae* S86c, *Sz. pombe* PW260, *K. lactis* GG799 and *P. pastoris* GS115.

4.1. Heterologous expression of Ova-derivatives in yeast

4.1.1. Construction of yeast expression vectors for heterologous expression of Ova variants

The *ova* and *ova$_{cyt}$* coding sequences were PCR-amplified from vectors pYES/Ova and pYES/Ova$_{cyt}$ using the following oligonucleotide primer pairs: 5'*Hind*III_*Eco*RI_Ova and 3'*Bgl*II_*Not*I_Ova, or 5'*Eco*RI_*Xho*I Ova and 3'*Sal*I_*Bgl*II_Ova. Ova$_{cyt}$ lacks the internal secretion signal sequence within the *ova* open reading frame (Jochen Stritzker, University of Würzburg, Germany). For cell surface display strategies, the *ova$_{cyt}$*-coding sequence without stop codon was inserted upstream of the anchor gene *sed1* from *S. cerevisiae* and downstream of a genus-specific secretion signal sequence. The *ova$_{cyt}$* gene without stop codon was amplified with the oligonucleotides 5'*Hind*III_*Eco*RI_Ova and 3'Ova_*Avr*II_ohneStop. The anchor DNA fragment encoding the C-terminal 319 amino acids of Sed1p was PCR-amplified from *S. cerevisiae* genomic DNA using oligonucleotide primers 5'*Avr*II_Sed1 and 3'*Not*I_Sed1 (Müller, 2008). The amplified segments were inserted into the pSTBlue-1 vector for routine sequencing, as decribed previously. The Ova derivatives used in this work are depicted in Fig. 16.

The glucanase-extractable cell wall protein Sed1p contains a glycosylphosphatidylinositol (GPI) attachment signal. Two signal sequences are present in the primary translation product of GPI-anchored proteins: an N-terminal sequence targeting the product to the ER membrane, and a C-terminal sequence directing the attachment of a GPI anchor. Both sequences are removed during processing, and the C-terminal GPI sequence is replaced with GPI by a GPI transamidase in the ER. The GPI anchor is then partially cleaved within its glycan moiety, losing its inositol-containing lipid moiety. The glycosyl remnant of the GPI structure, together with the attached protein, becomes covalently linked to cell-wall β-1,6-glucans (Orlean and Menon, 2007; Varki *et al*., 2009).

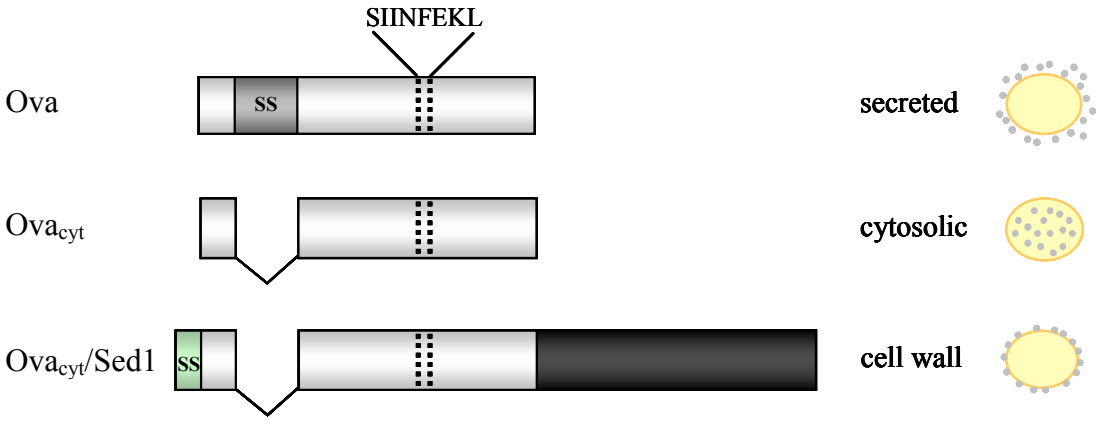


Fig. 16. Schematic representation and subcellular localization of the different Ova derivatives used in this study. Ova is secreted due to the presence of an internal secretion signal (SS), which is removed for intracellular expression (Ova$_{cyt}$). Alternatively, Ova$_{cyt}$ was fused to the C-terminal part of the cell wall protein Sed1p leading to a anchoring of the fusion within the cell wall; in this derivative, the particular genus-specific SS was used. The position of the epitope SIINFEKL is indicated.

Cloning into yeast expression vectors was performed as follows:

S. cerevisiae

The *ova* and *ova$_{cyt}$* coding sequences were digested from the cloning vector pSTBlue-1 with *Xho*I and *Bgl*II for inserting into *Xho*I/*Bgl*II-digested *S. cerevisiae* pFB2 expression vector. The resultant plasmids were named pPGK/*ova* and pPGK/*ova$_{cyt}$*, respectively. For cell-surface display of Ova$_{cyt}$, the fusion *ova$_{cyt}$/sed1* was excised from vector pPIC9/*ova$_{cyt}$/sed1* (described below) by digestion with *Not*I, end-filling with Klenow polymerase, and then restricting with *Eco*RI. This fragment was cloned into pFB2 digested with *Bgl*II, blunted with Klenow and finally digested with *Eco*RI. This pPGK-derived expression vector contains a *KRE1* signal peptide sequence upstream of the cloning region. The resultant plasmid was named pFB/*ova$_{cyt}$/sed1*.

Sz. pombe

The multiple cloning site of the thiamine-repressible expression vector pREP1 was modified and the plasmid is referred to as pREP-BD (Diehl, 2008). This *Sz. pombe* expression vector was *Xho*I and *Bgl*II digested for ligation with both full-length *ovalbumin* and *ova$_{cyt}$* which were isolated from *Xho*I/*Bgl*II-digested pSTBlue-1 vector. The resulting plasmids were named pREP-BD/*ova* and pREP-BD/*ova$_{cyt}$*, respectively.

K. lactis

For cloning into the *K. lactis* expression vector, full-length *ova* was digested with *Hind*III and *Not*I and ligated to pKLAC1 previously digested with the same restriction endonucleases. The gene encoding ova_{cyt} was restricted with *Hind*III/*Bgl*II and inserted into the *Hind*III and *Bgl*II sites of pKLAC1. The fusion ova_{cyt}/*sed* was digested from pPIC9/ova_{cyt}/*sed1* with *Eco*RI, filled with Klenow polymerase and subsequently digested with *Not*I for cloning into pKLAC1 restricted with *Sal*I, blunt-ended and digested with *Not*I, giving rise to pKLAC1/ova_{cyt}/*sed*. The constructed vectors pKLAC1/*ova*, pKLAC1/ova_{cyt} and pKLAC1/ova_{cyt}/*sed* were linearized with *Sac*II prior to integrative transformation of chemically competent *K. lactis* GG799. The expression cassette can therefore integrate into the *K. lactis* genome at the *LAC4* locus by means of homologous recombination.

P. pastoris

The genes encoding *ova* and ova_{cyt} were excised from pSTBlue-1 by digestion with *EcoR*I and *Not*I, and ligated to the *EcoR*I/*Not*I sites in pPIC3.5 vector, giving rise to pPIC3.5/*ova* and pPIC3.5/ova_{cyt}. To link ova_{cyt} to *sed1*, the sequence encoding ova_{cyt} without stop codon was isolated as an *Eco*RI-*Avr*II fragment from pSTBlue-1 and inserted upstream of the *sed1* coding sequence in vector pPIC9/*sed1* previously digested with *Eco*RI and *Avr*II, generating pPIC9/ova_{cyt}/*sed1*. The vectors were linearized with *Sal*I before transformation of *P. pastoris* cells in order to direct genomic integration at the *his4* locus.

4.1.2. Analysis of recombinant expression of Ova_{cyt} by different yeast genera

In order to verify cytosolic expression of recombinant Ova_{cyt}, lysates from 3×10^8 yeast cells cultured for until 72 h in the respective media were precipitated with DOC/TCA and applied to SDS-polyacrylamide gels. Western blots were performed using rabbit anti-chicken egg albumin and goat anti-rabbit IgG-peroxidase.

After transformation of *S. cerevisiae* S86c with pPGK/ova_{cyt}, some transformant colonies were inoculated in ura d/o medium and then analyzed for cytosolic expression of the recombinant protein. Foreign protein expression in *S. cerevisiae* was driven from the constitutive promoter *PGK*. As seen in Fig. 17 A (lane 1), a single protein band in the expected size of ~28 kDa could be detected by the anti-Ova antibody in cell lysates

of *S. cerevisiae* transformed with pPGK/*ova*$_{cyt}$. This band was absent in the cell extract of *S. cerevisiae* carrying the empty vector YEp352 (Fig. 17 A, lane 2).

Sz. pombe strain PW260 was transformed with the expression vector pREP-BD/*ova*$_{cyt}$, as well as with the plasmid pREP-BD containing no insert. To induce expression from the *nmt1* promoter, transformants were grown in EMM medium lacking leucine. Fig 17 B shows a 28 kDa protein band specific to the cell extract of yeast cells transformed with pREP-BD/*ova*$_{cyt}$ (lane 2). It was observed that some lower molecular weight proteins present in the cell lysates of *Sz. pombe* containing pREP-BD/*ova*$_{cyt}$ reacted positively with the antibody, probably being produced or created during recombinant protein expression, and presumably resulting from degradation of Ova$_{cyt}$, since these bands are not present in lysates of PW260 cells carrying the empty vector (Fig. 17 B, lane 3).

K. lactis GG799 transformant colonies selected on YCB agar plates were cultivated in YPGal medium for *LAC4*-driven expression of Ova$_{cyt}$. As a control, *K. lactis* was also transformed with empty pKLAC1. As shown in Fig. 17 C, a 28-kDa protein band in total yeast extracts reacted positively with the anti-Ova antibody (lane 2). This band was absent in cell lysates from *K. lactis* harbouring the empty vector pKLAC1 (Fig. 17 C, lane 1). Some proteins above the Ova$_{cyt}$ band reacted unspecifically with the anti-Ova antibody, as they also appeared in the negative control (*K. lactis* carrying the empty vector pKLAC1).

Colonies of *P. pastoris* GS115 transformed with the expression vector pPIC3.5/*ova*$_{cyt}$ and selected onto his d/o agar medium were grown in BMG medium and shifted to inducing BMM medium for proper protein expression. Methanol was added every 24 h (final concentration = 0.5% v/v) to maintain induction. As shown in Fig. 17 D, cell lysates of induced *P. pastoris* cells showed a specific 28-kDa protein band corresponding to Ova$_{cyt}$ (lane 2), which was absent in total extracts from methanol-induced control cells (lane 3). Additional bands present in the lysates from cells harboring pPIC3.5/*ova*$_{cyt}$ and pPIC9 reacted unspecifically with the anti-Ova antibody. Another lower molecular weight protein band could be seen in the lysate from Ova$_{cyt}$-expressing cells (~15 kDa, lane 2), which might indicate degradation of the recombinant protein.

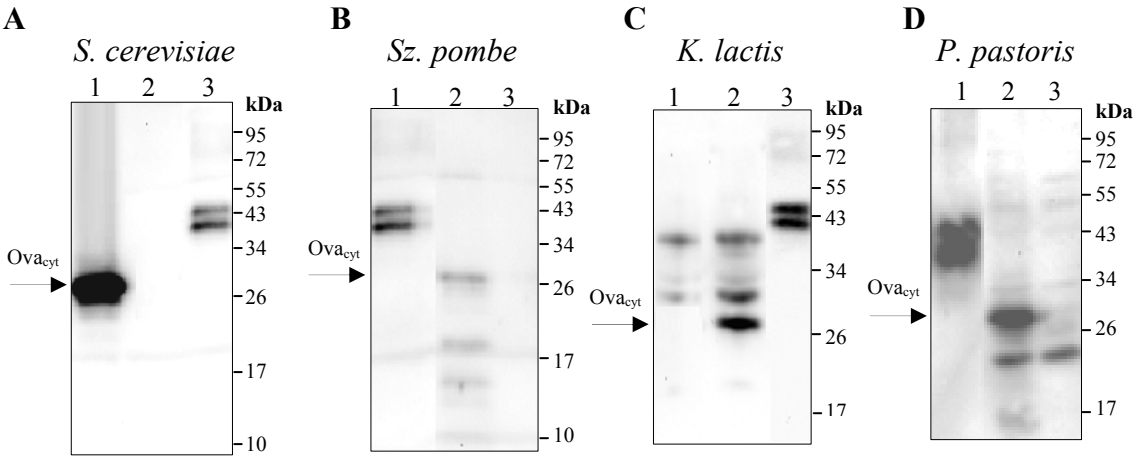


Fig. 17: Expression of Ova$_{cyt}$ by different yeast genera. Immunoblots of cell extracts from 3×10^8 yeast cells. Analyses were performed using monoclonal anti-Ova antibody. Commercially available Ova was used as positive control. The Ova$_{cyt}$ 28-kDa band is indicated by an arrow. **(A)** *S. cerevisiae* S86c. Lane 1, pPGK/*ova$_{cyt}$*; lane 2, empty vector Yep352; lane 3, positive control. **(B)** *Sz. pombe* PW260. Lane 1, positive control; lane 2, pREP-BD/*ova$_{cyt}$*; lane 3, empty vector pREP-BD. **(C)** *K. lactis* GG799. Lane 1, *empty vector* pKLAC1; lane 2, pKLAC1/*ova$_{cyt}$*; lane 3, positive control. **(D)** *P. pastoris* GS115. Lane 1, positive control; lane 2, pPIC3.5/*ova$_{cyt}$*; lane 3, empty vector pPIC9. The positions of the bands of the molecular weight marker ("Pre-stained protein ladder") as well as their sizes in kDa are indicated.

The amount of recombinant Ova$_{cyt}$ produced by each yeast strain was quantified by western blotting. For this purpose, protein bands from yeast lysates (3×10^8 cells) were compared to a standard curve of known concentration of Ova. Fig. 18 shows that the particular protein levels in cell lysates varied significantly among the yeast genera, i.e., the Ova$_{cyt}$ amount in 3×10^8 cells varied between 52.7 ng in *S. cerevisiae*, 37.1 ng in *P. pastoris*, 11.6 ng in *K. lactis* and 11.4 ng in *Sz. pombe* after 72 h of cultivation (mean values from experiments performed in triplicate). No secretion of Ova$_{cyt}$ was detected (data not shown).

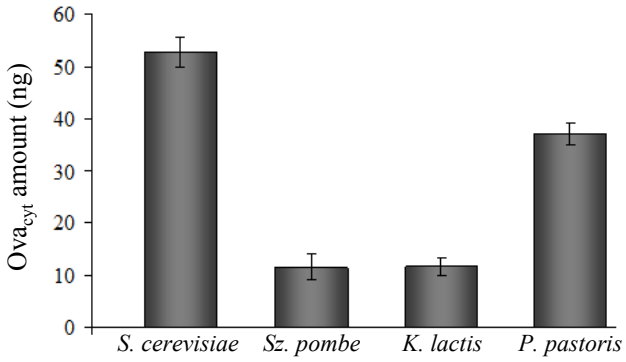


Fig. 18: Amount of recombinant Ova_{cyt} after recombinant expression by the different yeast genera. The values correspond to the total cell extract of 3×10^8 yeast cells after 72 h cultivation. Expression levels were quantified against a standard curve of commercially available Ova. Bars represent mean values with standard deviations from triplicate determinations.

4.1.3. Analysis of Ova secretion by different yeast genera

For detection of secreted Ova into the culture supernatants, a medium volume corresponding to 3×10^8 yeast cells was precipitated with DOC/TCA and the resulting products were applied to SDS-polyacrylamide gels. Western blots were performed using a specific anti-Ova antibody, as described before for Ova_{cyt}. Cultivation conditions were the same as above for all yeast strains.

As seen in Fig. 19 A, Ova could be secreted in the culture medium by *S. cerevisiae* S86c transformed with pPGK/*Ova* (lane 2); no bands were detectable in culture supernatants of negative control cells (lane 1). Fig 19 B shows a ~45 kDa protein band in the culture supernatant of *Sz. pombe* transformed with pREP-BD/*ova* (lane 1), which is absent in the culture medium of *Sz. pombe* carrying the empty vector pREP-BD (lane 2). From Fig. 19 C (lane 2), it was observed that a protein band of the predicted size, ~45 kDa, was detected in the supernatant from *K. lactis* transformed with pKLAC1/*ova*. This band was not observed in culture supernatants of *K. lactis* carrying the empty vector pKLAC1 (lane 1). Fig. 19 D shows that Ova could also be detected in the culture supernatant of *P. pastoris* transformed with pPIC3.5/*ova* (lane 1). Culture medium of induced cells carrying the empty vector pPIC9 showed no reaction with the anti-Ova antibody (lane 2).

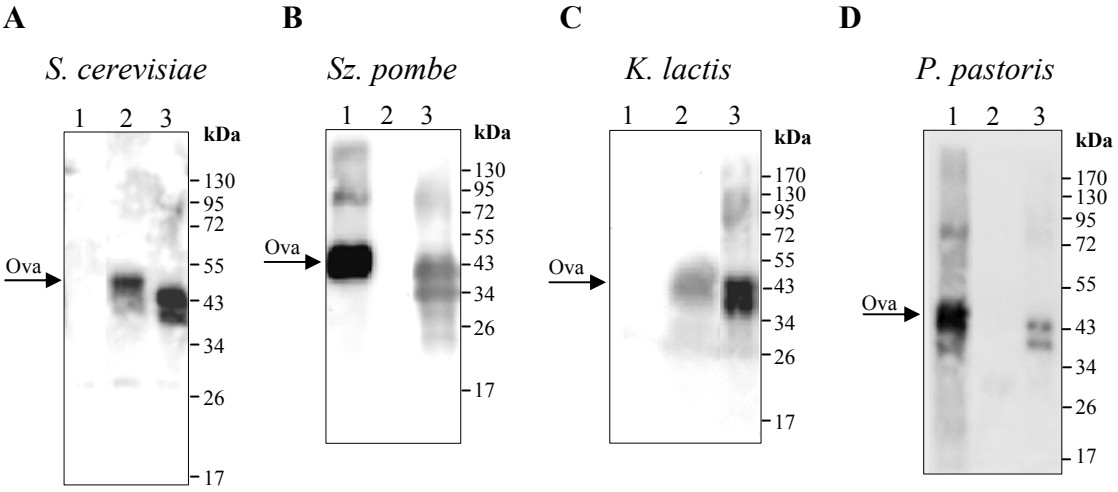


Fig. 9: Secretion of full-length Ova by different yeast genera. Culture supernatants from 3×10^8 cells. The ~45 kDa Ova band is indicated by an arrow. The positions of the bands

of the molecular weight marker ("Pre-stained protein ladder") and their sizes in kDa are given. **(A)** S86c Lane 1, empty vector Yep352; lane 2, pPGK/*ova*; lane 3, positive control. **(B)** Lane 1, PW260/pREP-BD/*ova*; lane 2, PW260/pREP-BD; lane 3, positive control. **(C)** *K. lactis* GG799 Lane 1, /pKLAC1/*ova*; lane 2, *K. lactis*/pKLAC1; lane 3, positive control. **(D)** *P. pastoris* GS115. Lane 1, pPIC3.5/*ova*; lane 2, empty vector pPIC9, lane 3, positive control.

As shown in Fig. 19, yeast-expressed Ova has a slightly higher molecular weight than the commercially available Ova (used as positive control), although both are derived from chicken (*Gallus gallus,* GenBank accession number NP_990483). Chicken Ova contains two putative N-glycosylation sites, and both mono- and di-glycosylated forms were reported to be secreted by yeast (Ito and Matsudomi, 2005). Since only one protein band was seen in the four yeast genera analyzed, it might represent the di-glycosylated form, as Ova from egg white is mono-glycosylated only (Nisbet *et al*., 1981; Ito *et al*., 2007). The two bands visualized in the positive control may be the result of other post-translational modifications which have been reported for this protein (acetylation and phosphorylation; Nisbet *et al*., 1981).

The amount of recombinant Ova secreted by each yeast strain was also quantified. Culture media from 3×10^8 yeast cells were analyzed by western blot, as described before. As observed for Ova_{cyt}, the level of secreted Ova showed great variation among the yeast genera (Fig. 20). The amount of Ova present in the culture supernatant from 3×10^8 cells after 72 h cultivation was 857 ng for *Sz. pombe*, 497 ng for *S. cerevisiae*, 438 ng for *P. pastoris*, and 39 ng for *K. lactis* (mean values from three different experiments).

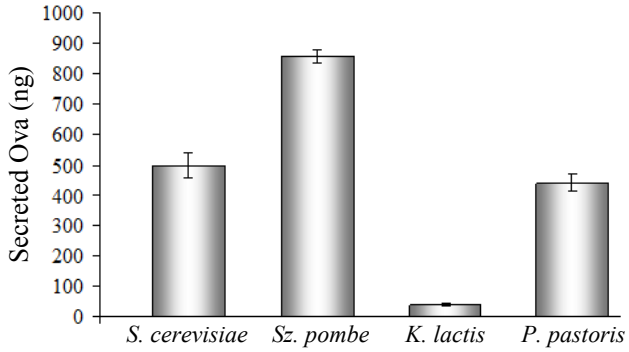


Fig. 20: Amount of recombinant Ova secreted into the culture supernatant of 3×10^8 yeast cells after 72 h cultivation. Recombinant Ova secretion by the different yeast genera was quantified by western blotting based on a standard curve of commercially available Ova. Shown are the means and standard deviation from triplicate determinations.

4.1.3.1. Ova secretion in a phagolysosome-like milieu

Next, it was investigated whether yeasts would be able to deliver secreted Ova to macrophages. Lorenz and Fink (2001) demonstrated that the glyoxylate cycle, a metabolic pathway that allows cells to use molecules containing two carbon atoms as carbon source, was induced in live yeast isolated from macrophage phagolysosomes. Although the exact composition of the phagolysosome is not completely elucidated, this finding indicates the existence of simple carbon sources in this milieu. In order to examine whether yeasts carrying plasmid vectors containing full-length Ova would be able to secrete this recombinant protein inside the macrophage phagolysosomes, yeasts were cultivated for 72 h in the respective culture medium under inducing conditions, washed, then transferred to an acetate solution, and incubated for another 8 h. After that, supernatants corresponding to 3×10^8 cells were collected, precipitated with DOC/TCA, and analyzed by western blotting. As shown in Fig. 21 A, Ova could be detected in the supernatant of all yeast species after 8 h incubation, although some degree of degradation was observed. Again, the amount of recombinant Ova protein in the supernatants varied considerably among the different yeasts (Fig. 21 B).

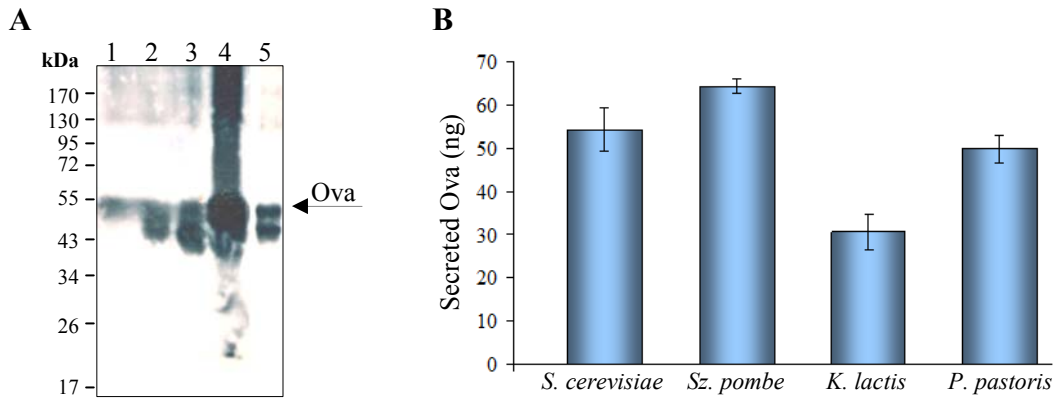


Fig. 21. Secretion of Ova in acetate-containing medium. **(A)** Western blot analysis reveals Ova secretion by all yeast genera tested after 8 h incubation in a medium containing a two-carbon compound as C-source. Yeasts were grown for 72 h in the respective medium, then washed and shifted to acetate medium. A volume corresponding to 3×10^8 cells was centrifuged and precipitated prior to analysis. Lane 1, *K. lactis* pKLAC1/*ova*; lane 2, *P. pastoris* pPIC3.5/*ova*; lane 3, *S. cerevisiae* pPGK/*ova*; lane 4, *Sz. pombe* pREP-BD/*ova*; lane 5, positive control (commercial Ova). **(B)** Quantification of Ova secreted by the diverse yeast genera after 8 h in acetate-containing medium. Ova was quantified by western blotting, in which the band intensities were compared to those from commercial Ova of known concentration. Shown are mean values and standard deviation from three different determinations [Bazan *et al*., 2011].

In *S. cerevisiae* and, to a lesser extent, also in *P. pastoris*, the amount of secreted Ova in acetate solution corresponded roughly to the intracellular level, whereas *Sz. pombe* and *K. lactis* secreted significantly more Ova protein. Of note, the expression profile of secreted Ova after 8 h in acetate-containing medium was very similar among the yeast genera to that after growth in the conventional culture media after 72 h, indicating that expression under artificial 'phagosomal' conditions resembles the expression after optimal cultivation.

4.1.4. Recombinant expression of Ova_{cyt} on yeast cell walls as Sed1p-fusions

Yeasts transformed with vectors harboring the *ova*$_{cyt}$/*sed1* fusion were cultivated in the respective medium for 3 days, re-inoculated (5% v/v) and allowed to grow for approximately 2 h. Detection of cell-surface displayed Ova_{cyt} was carried out by indirect immunofluorescence using rabbit anti-chicken egg albumin and goat anti-rabbit IgG-PE. Fluorescent labelling was measured by fluorescence microscopy and flow cytometry. Figure 22 (A and B) shows the fluorescence, bright field and merge micrographs of immunolabeled Ova_{cyt}/Sed1p-expressing *S. cerevisiae* and *P. pastoris* cells, as well as the correspondent control yeasts (yeasts expressing an irrelevant Sed1p-fusion protein). The red fluorescence of the immunostained Ova_{cyt}/Sed1p fusion protein was observed on the cell surface of *S. cerevisiae* carrying pPGK/*ova*$_{cyt}$/*sed1* and *P. pastoris* harboring pPIC9/*ova*$_{cyt}$/*sed1*. No fluorescence was observed outlining yeast cells harboring irrelevant Sed1p-fusion protein (Figure 10 A and B, lower panels). This result indicates that the fusion protein Ova_{cyt}/Sed1p is anchored and successfully displayed on the cell surface of *S. cerevisiae* and *P. pastoris*. Again secretion of OVA_{cyt} was not detected (data not shown). No fluorescence could be detected in *K. lactis* cells transformed with pKLAC1/*ova*$_{cyt}$/*sed1* (not shown).

Variation in fluorescence intensity among the cell population of yeasts expressing the Ova_{cyt}/Sed1p fusion protein was observed for both genera, indicating differences in the amount of anchored protein. To investigate this assumption, expression levels of Ova_{cyt}/Sed1p on the cell wall of *S. cerevisiae* and *P. pastoris* were compared using flow cytometry (Fig 22 C and D) and showed a strong variation among both genera, with approximately 38% in *S. cerevisiae* and 66% in *P. pastoris*. Comparison of mean fluorescence values of *Pichia* to *Saccharomyces* cells indicated a ~1.3-fold higher amount of OVA_{cyt} at the cell surface of *Pichia*, which is in accordance

with a quantification performed by western analyses after glucanase extraction of cell walls (Fig. 23).

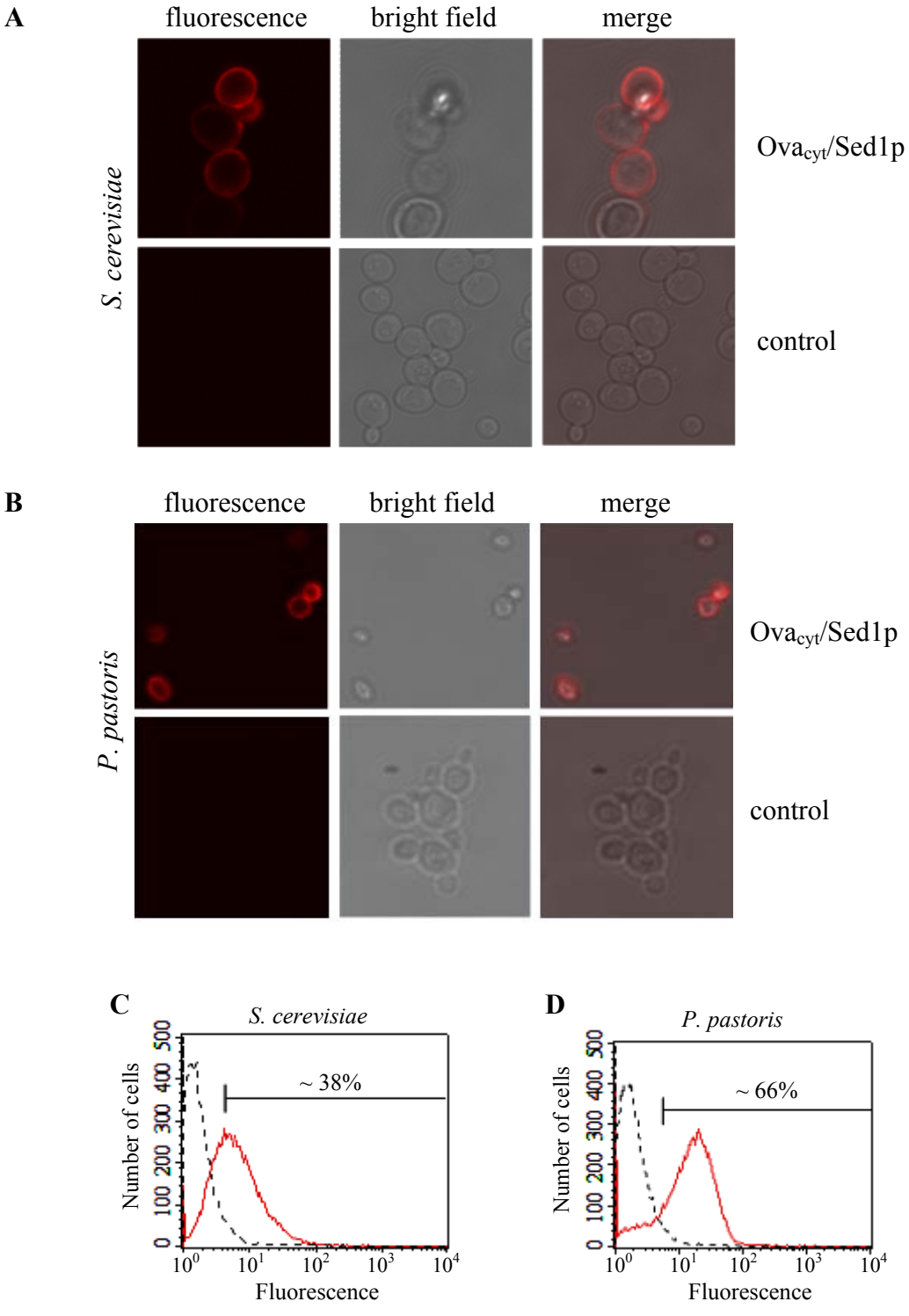


Fig. 22: Visualization of displayed proteins on the yeast cell surface. Cells were immunostained with rabbit anti-Ova IgG and PE-labeled goat anti-rabbit IgG. **(A and B)** Confocal laser scanning microscopy. Fluorescence micrographs (left panels), light micrographs (central panels) and merge (right panels) of *S. cerevisiae* **(A)** and *P. pastoris* **(B)**. Upper panels, cells expressing Ova$_{cyt}$ on the cell wall; lower panels, cells harboring negative control plasmids. Magnification: 63×20. **(C and D)** Histograms

showing flow cytometric analysis of *S. cerevisiae* **(C)** and *P. pastoris* **(D)**. Dashed lines represent yeasts expressing an irrespective Sed1p fusion protein. Red solid lines show transformants displaying Ova_{cyt}/Sed1p. Vertical axis represents total cell number and horizontal axis indicates PE fluorescence intensity. The percentage of positive cells is shown. A total of 50,000 cells was analyzed.

In order to confirm that Ova_{cyt} was anchored on the cell surfaces of *S. cerevisiae* and *P. pastoris*, 3×10^8 yeast cells expressing the fusion protein Ova_{cyt}/Sed1p, as well as yeasts expressing an unrelated Sed1p-fusion protein, were lysed with glass beads, and cell walls were extensively washed prior to treatment with β-1,3-glucanase (laminarinase). Then, supernatants were concentrated by precipitation with DOC/TCA and the presence of Ova_{cyt}/Sed1p was analyzed by western blotting using the monoclonal anti-Ova antibody. As seen in Fig. 23 A, a very faint band of approximately 64 kDa could be detected in cell wall extracts of *S. cerevisiae* carrying pPGK/*ova_{cyt}*/*sed1* (lane 2), but not in the negative control (*S. cerevisiae* expressing another Sed1p-fusion protein, lane 3). This protein band corresponds to the Ova_{cyt}/Sed1p protein, which has a theoretical molecular weight of ~64 kDa. Additional low molecular weight bands could be seen in the blot, some of them reacted unspecifically with the anti-Ova antibody, since they are also present in the negative control extracts. The other bands that appear only in the cell wall extracts of the Ova_{cyt}/Sed1p-producing yeast are probably degradation products of Ova_{cyt}/Sed1p. In Fig. 23 B, a slight band was seen in the cell wall extract of *P. pastoris* carrying pPIC9/*ova_{cyt}*/*sed1* (lane 2), corresponding to the 64-kDa Ova_{cyt}/Sed1p. This band was not detected in the negative control (*P. pastoris* expressing an irrelevant Sed1p-fusion protein, lane 3).

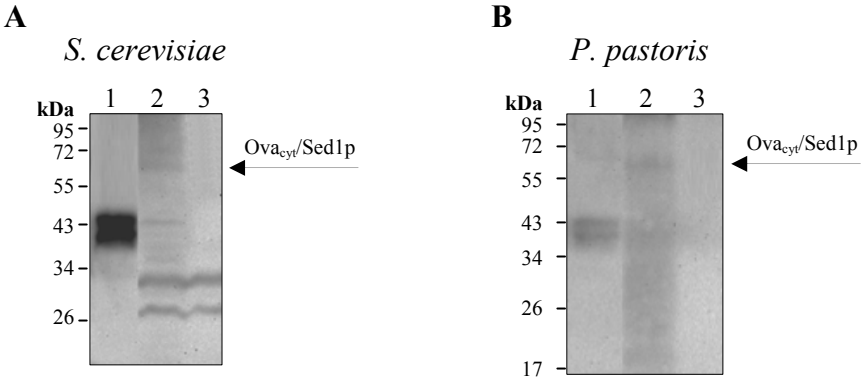


Fig. 23: Laminarinase extraction of Ova_{cyt}/Sed1p from yeast cell walls. The Ova_{cyt}/Sed1p bands are indicated with arrows. As positive control, commercially available Ova was used. **(A)** Lane 1, positive control; lane 2, laminarinase extracts of cell walls of *S. cerevisiae*/Ova_{cyt}/Sed1p; lane 3, laminarinase extracts of cell walls of *S. cerevisiae* expressing an irrelevant Sed1p-fusion protein. **(B)** Lane 1, positive control;

lane 2, laminarinase extracts of *P. pastoris* expressing Ova_{cyt}/Sed1p; lane 3, laminarinase cell wall extracts of *P. pastoris* expressing an irrelevant Sed1p-fusion protein.

4.2. The HCMV pp65 tegument protein as model antigen

4.2.1. Construction of yeast expression vectors for recombinant expression of pp65

The sequence encoding pp65 was amplified by PCR with the oligonucleotide primers 5'pp65 and 3'pp65 using the vector pPGK/*pp65* as template. The resulting PCR product was cloned into pSTBlue-1 for sequencing analysis (pSTBlue-1/*pp65*) and subsequently used to clone the gene pp65 into expression vectors for *S. cerevisiae, Sz. pombe, K. lactis* and *P. pastoris* for intracellular, secreted, and cell-wall anchored expression. However, the latter approach turned out to be infeasible, since fusion of *pp65* to a secretion signal sequence did not lead to protein secretion, as described later in this section. Fig. 24 shows the pp65 variants used for recombinant expression in yeast.

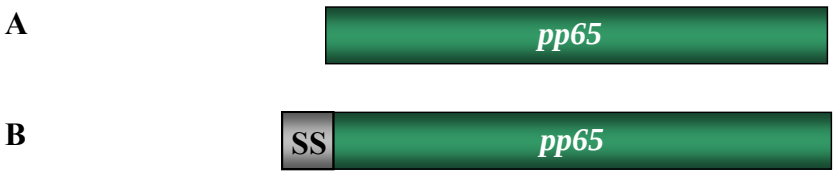


Fig. 24. Schematic representation of *pp65* variants used throughout this work. **(A)** Full-length *pp65* for intracellular expression. **(B)** The *pp65* gene also fused to a secretion signal (SS) sequence in order to direct the protein for secretion.

The gene encoding full-length pp65 was cloned into the yeast expression vectors as follows:

S. cerevisiae

The construct pPGK/*pp65* for foreign expression in *S. cerevisiae* was already available (Schumacher, 2001). In order to drive secretion of pp65 in *S. cerevisiae*, the pp65 open reading frame was inserted downstream of the sequence encoding the *KRE1* signal peptide. For this purpose, the gene *pp65* was digested from the pSTBlue-1/pp65 vector with *Sal*I, end-filled with Klenow polymerase, and subsequently digested with *Eco*RI. The expression vector pFB2 (Breinig and Schmitt, 2002) was restricted with *Bgl*II, blunt-ended with Klenow polymerase, and further digested with *Eco*RI. After ligation, the resulting vector, named pPGK/*kre1*/*pp65*, was used to transform *S. cerevisiae* S86c.

Sz. pombe

For cloning into the *Sz. pombe* expression vector pREP1, *pp65* was digested from pSTBlue-1/*pp65* with *Xho*I and *Bam*HI for inserting into the *Sal*I/*Bam*HI-digested pREP1, generating pREP1/*pp65*.

The signal peptide of the K28 preprotoxin was used to direct pp65 into the secretion pathway of *Sz. pombe*. This secretion signal has been shown to efficiently drive the secretion of recombinant proteins in this yeast species (Heintel *et al*., 2001; Eiden-Plach *et al*., 2004). Therefore, the K28 signal peptide/*pp65* fusion was amplified by PCR using the oligonucleotide primers 5'K28, 3'Prepropp65, 3'pp65 and 5'Prepropp65, and the plasmids pPGK-M28-I and pPIC9/*pp65* as templates. The final PCR product, named *prepropp65*, was cloned into pSTBlue-1 for sequence analysis. Subsequently, the *prepropp65* coding sequence was restricted from pSTBlue-1/*prepropp65* with *Xho*I and *Bam*HI and ligated to the *Sal*I/*Bam*HI-linearized pREP1 vector, giving rise to pREP1/*prepropp65*.

K. lactis

The *K. lactis* expression vector pKLAC1 requires linearization prior to transformation to allow integration into the yeast genome. Linearization can be performed with either *Sac*II or *Bst*XI restriction enzymes. Since both restriction sites are present in the pp65 sequence, a site-directed mutagenesis was performed in order to eliminate one recognition sequence, without altering the amino acid composition of the resulting protein. Given that the *Sac*II regognition sequence appears twice in the pp65 gene, the *Bst*XI restriction site was chosen for the single nucleotide exchange. Using the primers 5'pp65mut*Bst*XI, 3'pp65*Xba*I*Sal*I, 5'pp65_*Eco*RI*Xho*I*Sac*I and 3'pp65mut*Bst*XI, and the plasmid pSTBlue-1/pp65 as template, the pp65 gene was amplified by SOE-PCR as described in section 1.1 of *Methods*. After amplification, the final DNA fragment (*pp65mut*) was cloned into pSTBlue-1 for sequence analysis, which revealed the desired mutation (substitution of a cytosine for an adenine at position 540, so that codon ACC became ACA, but still coding for threonin).

Intracellular protein expression in *K. lactis* implies digesting the pKLAC1 expression vector with *Hind*III to remove the α-mating factor signal sequence. Therefore, for cloning into the *K. lactis* expression vector, *pp65mut* was digested from pSTBlue-1/*pp65mut* with *Eco*RI, blunt-ended with Klenow polymerase and then digested with *Sal*I. The vector pKLAC1 was digested with *Hind*III, end-filled with

Klenow and digested with *Sal*I. For integrative transformation of chemically competent *K. lactis*, the construct pKLAC1/*pp65mut* was linearized with *Bst*XI.

Full-length *pp65* was also cloned downstream of the α-mating factor signal peptide for protein secretion. For this purpose, *pp65* was digested from pSTBlue-1 with *Xba*I, blunt-ended with Klenow polymerase, and then restricted with *Xho*I. The vector pKLAC1 was digested with *Not*I, blunt-ended with Klenow polymerase, and digested with *Sal*I. The ligation product, pKLAC1/*secpp65mut*, was used to transform competent *K. lactis* cells.

P. pastoris

Cloning of *pp65* into *P. pastoris* expression vectors pPIC3.5 for intracellular expression and pPIC9 for protein secretion was performed by digesting the gene encoding *pp65* from pSTBlue-1/*pp65* with *Eco*RI and *Xba*I and ligating to the *Eco*RI/*Avr*II sites in pPIC3.5 and pPIC9 vectors. The resulting plasmids were named pPIC3.5/*pp65* and pPIC9/*pp65*. Both expression vectors were linearized with *Sal*I before transformation of competent *P. pastoris* cells.

4.2.2. Intracellular pp65 expression by different yeast genera

For detection of pp65 in the cytoplasm of the tested yeast genera, lysates from 3×10^8 yeast cells cultured for 72 h in the respective medium were precipitated with DOC/TCA, as described before. Western blots of cell extracts were performed using mouse monoclonal anti-pp65 antibody and goat anti-mouse IgG-peroxidase.

Colonies of *S. cerevisiae* S86c transformed with pPGK/*pp65* and selected based on uracil prototrophy were cultured in ura d/o medium for recombinant protein expression. Lysates from yeast cells carrying both pPGK/*pp65* and the empty vector YEp352 were analyzed. As seen in Fig. 25 A, pp65 was detected as a ~65-kDa band in the total extract of *S. cerevisiae* carrying the expression vector pPGK/*pp65* (lane 3). No bands were seen in the sample corresponding to *S. cerevisiae* harboring YEp352 vector (lane 2).

Sz. pombe PW260 cells harbouring the pREP1/*pp65* vector were grown in EMM leu d/o medium to induce pp65 expression from the *nmt1* promoter. Cells of *Sz. pombe*/pREP1/*pp65* and *Sz. pombe*/pREP-BD were subjected to expression analysis. As seen in Fig. 25 B, the corresponding western blot result showed a single 65-kDa protein

band in the lysate of *Sz. pombe*/pREP1/*pp65* (lane 1). The band corresponds to pp65, as it reacted specifically with the anti-pp65 monoclonal antibody. This protein was absent in *Sz. pombe* lysates carrying the empty vector pREP-BD and cultivated under the same conditions (lane 2).

After pKLAC1/pp65mut had been integrated into the *K. lactis* genome, cells were grown in YPGal medium for 72 h at 30°C, under agitation at 220 rpm. Total extracts from 3×10^8 cells were precipitated with DOC/TCA and analyzed by western blotting using monoclonal anti-pp65. No specific signal could be detected in *K. lactis* lysates, while the 65-kDa band of the positive control sample reacted specifically with the anti-pp65 antibody (data not shown). Many clones were analyzed for pp65 expression, however without success. The genomic DNA of some *K. lactis* clones was isolated and subjected to PCR analysis using the oligonucleotide primers 5'pp65 and 3'pp65. The presence of the pp65 gene integrated in the *K. lactis* genome was confirmed (data not shown), ruling out the possibility that the heterologous protein could not be detected because the recombinant DNA had not been integrated into the genome. This fact may indicate that the expression level of pp65 in *K. lactis* was below the detection limit, or that this protein was rapidly degraded by proteases, or even that pp65 is not expressed at all by this yeast strain.

P. pastoris colonies grown on selective agar medium lacking histidine and cultivated in BMG medium were shifted to BMM medium in order to induce *AOX1*-driven protein expression. As shown in Fig. 25 C, an immunoreactive band of pp65 (~65 kDa) was observed in the cell lysate of *P. pastoris* carrying the expression vector pPIC3.5/*pp65* (lane 1). This band was not observed in total extracts from yeast cells carrying the empty vector pPIC9 (lane 2).

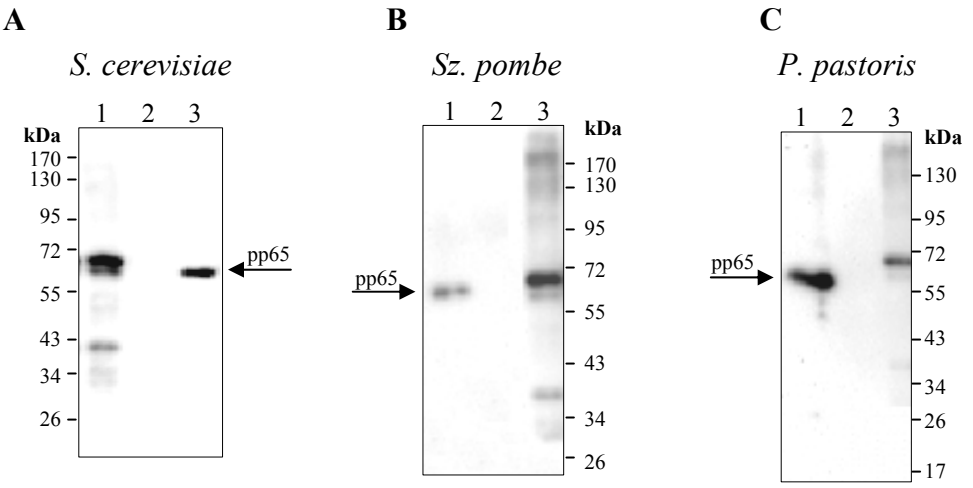


Fig. 25. Intracellular expression of pp65 by distinct yeast genera. Shown are western blots of yeast lysates from 3×10^8 yeast cells probed with monoclonal anti-pp65. As positive control, commercial pp65 was used. The yeast-derived 65 kDa protein bands identified as pp65 are indicated by arrows. The positions of the bands of the molecular weight marker ("Prestained protein ladder") are indicated. **(A)** *S. cerevisiae*. Lane 1, positive control; lane 2, lysate from cells harbouring the empty vector YEp352; lane 3, cell extracts from cells transformed with pPGK/*pp65*. **(B)** *Sz. pombe*. Lysates from cells carrying pREP1/*pp65* (lane 1) or empty vector pREP-BD (lane 2); lane 3, positive control. **(C)** *P. pastoris*. Extracts from cells transformed with pPIC3.5/pp65 (lane 1) or empty vector pPIC9 (lane 2); lane 3, positive control.

4.2.3. Pp65 secretion by different yeast genera

The presence of pp65 in culture medium collected after 24, 48 or 72 h was investigated as follows: a volume of culture medium equivalent to 3×10^8 yeast cells was collected and concentrated with DOC/TCA prior to SDS-PAGE and western analysis using monoclonal anti-pp65, as mentioned in the previous section.

S. cerevisiae transformants carrying pPGK/*kre1*/*pp65* were cultivated as described. Supernatants from a culture of *S. cerevisiae* carrying the empty vector YEp352 served as negative control; the commercially available pp65 served as positive control. No detectable pp65 could be seen in lanes corresponding to yeast transformants harboring pPGK/k*re1*/*pp65*, or in negative control cells. As expected, the positive control revealed a protein signal at 65 kDa, representing pp65 (data not shown). To determine whether pp65 was present in the cytoplasm of yeasts transformed with pPGK/*kre1*/*pp65*, transformants were lysed with glass beads and total cell extracts were analyzed by SDS-PAGE and probed with monoclonal anti-pp65. Recombinant pp65 could be detected in the cytosolic extracts analyzed, indicating that secretion of this protein was for some reason impaired.

The presence of pp65 in culture supernatants of *Sz. pombe* transformed with pREP1/*prepropp65* was verified. Supernatants from a culture of *Sz. pombe* harbouring pREP-BD were used as negative control and commercial pp65 as positive control. Western blot revealed no pp65-specific signal (12 lanes, each representing one clone), whereas the positive control band promptly reacted with the antibody (data not shown). *Sz. pombe* cells transformed with PREP1/*prepropp65* were then lysed with glass beads and analyzed for the presence of pp65 in the cytoplasm. This protein could be detected in the cell lysates (not shown), indicating that its absence in culture medium was not due to lack of expression.

K. lactis transformed with pKLAC1/*secpp65* was inoculated in YPGal medium and supernatants were assessed for the presence of pp65. After western analysis, no recombinant protein could be detected in the supernatant. Cell pellets (3×10^8 cells) were subjected to lysis to check if pp65 was retained in the cytoplasm but, again, no signal was verified after wester blotting using anti-pp65 (data not shown), as already reported for *K. lactis* carrying pKLAC1/*pp65*.

P. pastoris carrying pPIC9/*pp65* induced with methanol were analyzed for pp65 secretion. As for the other yeast strains tested, no pp65 could be detected in the culture supernatant, whereas the 65-kDa band appeared in the positive control lane (not shown). Some clones were lysed with glass beads and assessed for the presence of pp65 in the cytoplasm. Western analysis revealed a pp65-specific signal in all transformants examined, confirming that this protein was expressed in *P. pastoris* transformed with the pPIC9/*pp65* vector, but not secreted (data not shown).

In this way it must be concluded that cell-wall anchoring of full-length pp65 would most probably be unsuccessful, since none of the tested yeasts was able to secrete pp65.

4.3. Cloning and expression of Gag-fusions in *S. cerevisiae*

Delivery of antigens by virus-like particles has been considered a versatile approach. Since VLP are inherently highly immunogenic, strategies employing chimeric particles are considered very promising in immunological applications.

Fusion of L-A Gag with a truncated version of pp65 (Δ*pp65*) has been demonstrated by Powilleit and colleagues (2007). In that study, Δ*pp65* was fused to the 3' end of *gag* in the [0]-frame position, and the resulting Gag/Δpp65 fusion self-assembled *in vivo* into VLP. In addition, purified chimeric Gag/Δpp65 VLP have been shown to stimulate pp65-specific memory T cells in a whole blood assay (Powilleit *et al*., 2007).

In this work, the sequences encoding Ova_{cyt} or full-length pp65 were cloned downstream of *gag* in the [0]-frame to prevent *gag*/ova_{cyt} or *gag*/*pp65* frameshift occurrence in yeast (Fig. 26). The resulting recombinant proteins were partially purified and assessed for correct assembly into VLP. Since Ova represents an easy applicable system for a number of *in vitro* and *in vivo* settings, it would be interesting to examine the delivery of Ova_{cyt} by VLP in comparison to Ova_{cyt} alone. Furthermore, yeast

assembled Gag/pp65 VLP were analyzed in terms of activation of pp65-specific memory T lymphocytes in a whole blood assay and compared to pp65 alone.

Fig. 26: Schematic representation of *gag* fusions performed in this work. The sequences coding for *ova*$_{cyt}$ **(A)** and *pp65* **(B)** were cloned in frame and downstream of the L-A *gag* sequence for recombinant expression in the virus-free yeast strain *S. cerevisiae* S86c.

4.3.1. Cloning and expression of Gag/Ova$_{cyt}$ in *S. cerevisiae*

To insert the *ova*$_{cyt}$ gene downstream of *gag* without causing frameshift formation *in vivo*, *Sac*I restriction was used for cloning. However, given that *Sac*I cleaves the *ova*$_{cyt}$ sequence at position 56, and that there was no available conventional restriction enzyme which would leave compatible cohesive ends, site-directed mutagenesis was performed in order to destroy this site in the *ova*$_{cyt}$ gene without altering the amino acid composition of the resulting protein. Using pPIC3.5/*ova*$_{cyt}$ as template and the oligonucleotide primers 5'Ova$_{cyt}$mut 3'*Bgl*II_*Not*I_Ova, 3'Ova$_{cyt}$mut, and 5' *Xba*I*Sac*I_Ova$_{cyt}$, the *ova*$_{cyt}$*mut* sequence was amplified by SOE-PCR, as described before. The resulting PCR product was cloned into pSTBlue-1 and subjected to sequence analysis to confirm the correct sequence. A silent single base mutation could be obtained, in which a cytosine at position 57 was replaced by a thymine. Therefore, the codon CTC was changed into CTT, but the correspondent amino acid, leucine, was still the same.

The *ova*$_{cyt}$*mut* coding sequence was recovered from pSTBlue-1 by *Sac*I and *Bgl*II restriction and subsequently inserted into the *Sac*I/*Bam*HI-digested *S. cerevisiae* pPG[0]G expression vector, yielding pPG[0]G/*ova*$_{cyt}$*mut*.

After transformation of *S. cerevisiae* S86c with pPG[0]G/*ova*$_{cyt}$*mut*, some transformants were inoculated in ura d/o medium and cultured for 72 h for *PGK*-driven recombinant protein expression. For detection of Gag/Ova$_{cyt}$ in yeast cell lysates, crude extracts from 3×10^8 cells were precipitated with DOC/TCA and applied to SDS-polyacrylamide gels. Western blots were performed using rabbit anti-Ova antibody and

goat anti-rabbit IgG-peroxidase. Yeasts transformed with the pG vector (*i.e.*, expressing Gag only) were used as negative control; commercial Ova served as positive control.

As observed in Fig. 27, western analysis of total cell extracts from yeasts transformed with pG[0]G/*ova$_{cyt}$mut* revealed a ~103 kDa protein fusion (lane 1), which was absent in lysates from pG-carrying yeasts (lane 2). In lane 1, additional low-molecular weight immunoreactive bands were detectable which probably represent degradation products of full-length Gag/Ova$_{cyt}$.

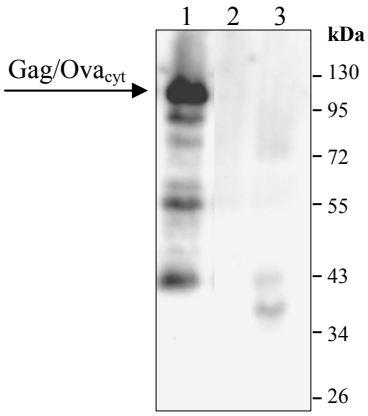


Fig. 27. Expression of Gag/Ova$_{cyt}$ in *S. cerevisiae* S86c. Total extracts from 3×10^8 cells were separated by SDS-PAGE and probed with anti-Ova. Lane 1, lysates from *S. cerevisiae* carrying pG[0]G/*ova$_{cyt}$mu*t; lane 2, lysates from *S. cerevisiae* carrying pG; lane 3, positive control (Ova). The fusion protein Gag/Ova$_{cyt}$ is indicated by an arrow. The positions of the bands of the molecular weight marker ("Prestained protein ladder") are indicated.

Next, the Gag/Ova$_{cyt}$ fusion protein expressed in *S. cerevisiae* was evaluated for its ability to assemble into VLP. For this purpose, cell lysates were applied onto a 45% sucrose cushion and after ultracentrifugation the resulting pellet was dissolved and applied onto a sucrose gradient (20-70%). A total of 18 fractions were recovered from the gradient after overnight ultracentrifugation. Aliquots from each fraction were separated by SDS-PAGE and analyzed by western blotting using an anti-Ova antibody (Fig. 28). Protein bands of approximately 103 kDa could be detected in all aliquots analyzed, showing a similar distribution pattern as previously described for Gag/GFP particles (Powilleit, 2004). Additional low molecular weight bands were observed in some fractions and in the gradient pellet, which most probably represent degradation products.

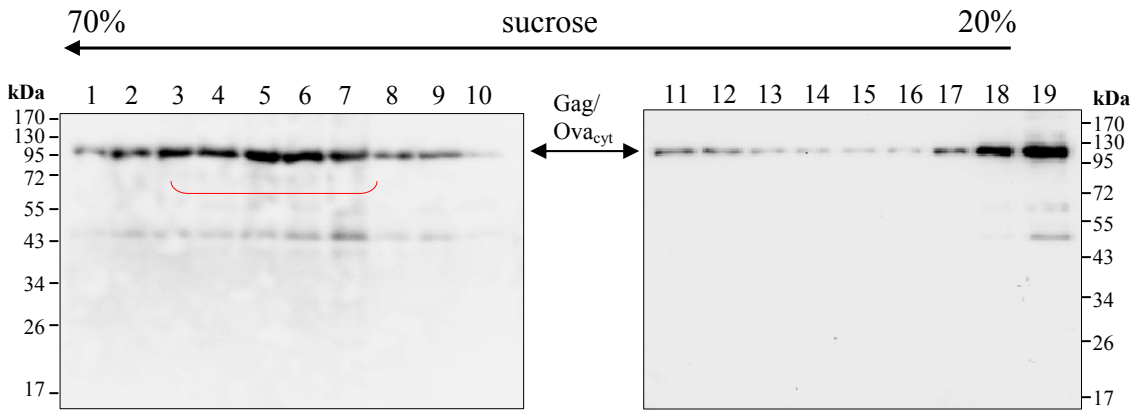


Fig. 28. Western blot analysis for the detection of Gag/Ova$_{cyt}$ in sucrose gradient fractions 1-18 and in the gradient pellet. Lanes 1-18, fractions 1-18; lane 19, gradient pellet. Pooled fractions are indicated with a red line. Sucrose concentrations are shown.

Some of the Gag/Ova$_{cyt}$ samples that reacted positively with anti-Ova were pooled (fractions 3-7), dialyzed against PBSE overnight, concentrated to 200 µl with Vivaspin™ and analyzed by transmission electron microscopy (TEM). TEM analysis identified correctly assembled VLP in samples purified from *S. cerevisiae* pG[0]G/*ova$_{cyt}$mut*. Gag/Ova$_{cyt}$ particles appeared symmetric and homogeneous in size, with a diameter of approximately 40 nm (Fig. 29). These particles were not seen in cell extracts from *S. cerevisiae* carrying the empty vector YEp352 subjected to the same procedure (data not shown).

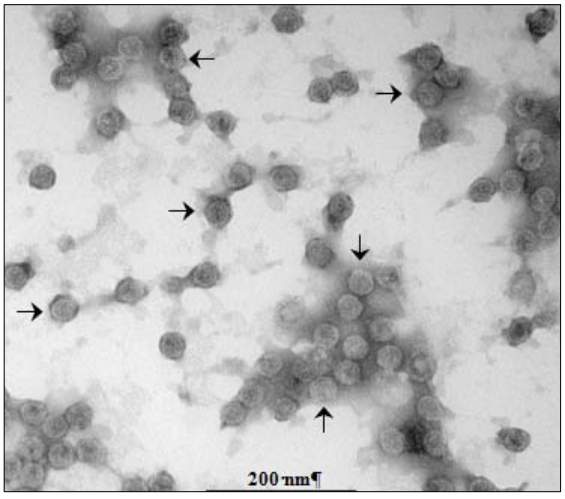


Fig. 29. Transmission electron microscopy of Gag/Ova$_{cyt}$ chimeric particles. Electron micrograph of sucrose gradient-purified Gag/Ova$_{cyt}$ derived from *S. cerevisiae*. Some particles are indicated by arrows. Sample was negatively stained with 2% uranyl acetate. Magnification of 98,000×. Bar = 200 nm.

As seen in Fig. 29, recombinant Gag/Ova$_{cyt}$ VLP exhibit a conformation which closely resembles those from Gag/GFP particles recombinantly expressed in the same yeast strain (Powilleit, 2004; data not shown). In general, particles appear relatively dark, with a clear margin.

4.3.2. Cloning and expression of Gag/pp65 in *S. cerevisiae*

The gene encoding pp65 was PCR-amplified using the primers 5' *Sac*Ipp65 and 3'*Bam*HIpp65 and plasmid pREP1/*pp65* as template. After cloning into pSTBlue-1 and further sequencing, the mutation-free *pp65* coding sequence was recovered from pSTBlue-1 by treatment with *Bam*HI and *Sac*I and subsequently inserted into *Bam*HI/*Sac*I-digested pPG[0]G, yielding the yeast expression vector pPG[0]G/*pp65*.

Selected *S. cerevisiae* S86c transformants carrying pPG[0]G/*pp65* were inoculated in ura d/o medium and cultured for 72 h recombinant protein expression. For detection of Gag/pp65 in yeast cell lysates, crude extracts from 3×10^8 cells were precipitated with DOC/TCA, separated by SDS-PAGE and probed with monoclonal anti-HCMV pp65.

Fig. 30 shows that the Gag/pp65 fusion protein was detected as a immunoreactive 141-kDa band in the cell lysates of *S. cerevisiae* carrying pG[0]G/*pp65* (lane 2). This band was not detected in cell extracts from clones lacking pp65 (*S. cerevisiae* transformed with pG, lane 3). Commercially available pp65 was used as positive control (lane 1).

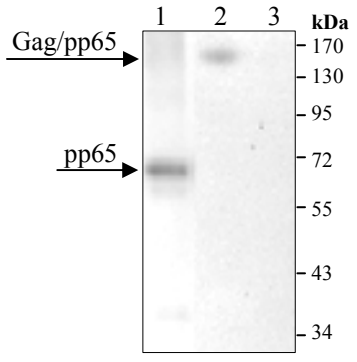


Fig. 30. Expression of Gag/pp65 in *S. cerevisiae* S86c. Total cell extracts were separated by SDS-PAGE and probed with monoclonal mouse anti-HCMVpp65. Lane 1, positive control (commercial pp65); lane 2, lysate from cells carrying pG[0]G/*pp65*; lane 3, lysate from cells harbouring pG. Positions of Gag/pp65 and pp65 are indicated. The positions of the bands of the molecular weight marker ("Prestained protein ladder") are given.

VLP preparations were performed as described for Gag/Ova$_{cyt}$ and aliquots of the gradient fractions were applied to SDS-polyacrylamide gels and analyzed by western blotting using monoclonal anti-pp65 (Fig. 31).

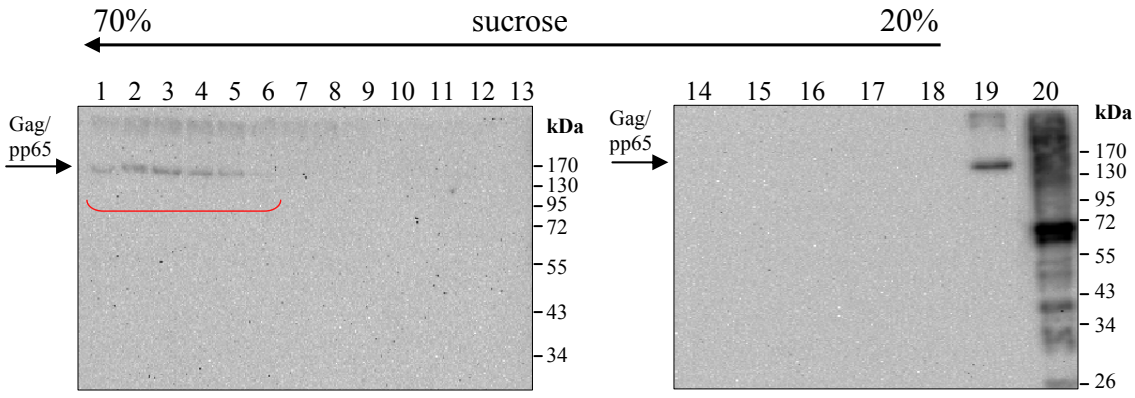


Fig. 31. Western analysis of Gag/pp65 in gradient fractions 1-18 and gradient pellet. Lanes 1-18, fractions 1-18; lane 19, gradient pellet; lane 20, positive control (commercial pp65). Pooled fractions are indicated with a red line.

Recombinant Gag/pp65 shows a different sedimentation profile as Gag/Ova$_{cyt}$ VLP. As seen in Fig. 31, the fusion protein (~141 kDa) could only be detected in fractions containing higher sucrose concentrations, and in the gradient pellet. This fact possibly reflects the higher molecular weight of Gag/pp65 in comparison to Gag/Ova$_{cyt}$.

In order to investigate whether Gag/pp65 assembled into VLP, fractions 1-6, which were positive for the presence of Gag/pp65, were pooled, dialyzed overnight against PBSE, concentrated to 200 µl and analyzed by transmission electron microscopy (Fig. 32).

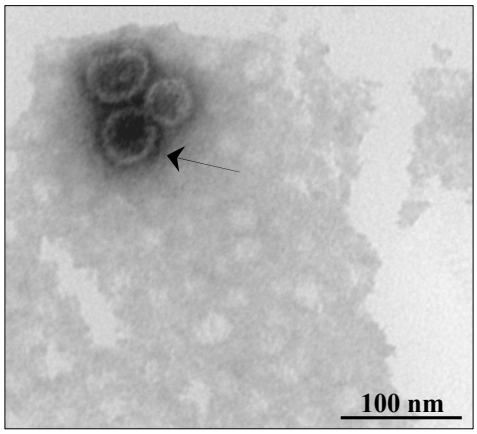


Fig. 32. TEM analysis of Gag/pp65 chimeric VLP. Electron micrograph of sucrose gradient-purified Gag/pp65 derived from *S. cerevisiae.* Particles are indicated by an arrow. Sample was negatively stained with 2% uranyl acetate. Magnification of 150,000. Bar = 100 nm.

As shown in Fig. 32, TEM analysis revealed the presence of VLP, which appear heterogeneous in size (35-55 nm in diameter) and symmetry. The dark core surrounded by a clear margin correlates well with the previous findings for Gag/Ova_{cyt} VLP. White globules that also appear in the sample may indicate the presence of other proteins, or even unassembled Gag/pp65 fusions. This latter possibility would explain the occurrence of only a limited number of assembled VLP, suggesting that Gag/pp65 assembly efficiency is somehow limited.

5. Activation of antigen-specific T cells after delivery of recombinant yeasts

Having characterized the heterologous expression of model proteins in yeasts, the selected recombinant yeast genera were tested *in vitro* (Ova) or *ex vivo* (pp65) for priming of antigen-specific T lymphocytes.

5.1. Influence of antigen localization on activation of Ova-specific $CD8^+$ T cells

The yeast genera expressing the Ova derivatives were tested in an *in vitro* antigen presentation assay using murine BMM as antigen-presenting cells and the activation of Ova (SIINFEKL)-specific $CD8^+$ T lymphocytes as read-out system. In this well-defined system, the recombinant yeast cells act as protein delivery vectors that feed antigens into the cytosolic MHC class I-restricted antigen presentation pathway. These experiments were performed in collaboration with Dr. Gernot Geginat at the Faculty for Clinical Medicine Mannheim of the University Heidelberg (Mannheim, Germany).

BMM were incubated with four different yeast:macrophage ratios (MOI 14, 7, 3, 1.5) or left untreated. Uptake kinetics of the diverse yeast genera by murine macrophages were previously analyzed (section 4.1).

As shown in Fig. 33, infection of mouse BMM with Ova_{cyt}-expressing yeast led to the activation of T cells recognizing the Ova epitope SIINFEKL and the consequent production of IFN-γ. Optimal activation of Ova-specific $CD8^+$ T cells was obtained at MOI 7 in the case of *S. cerevisiae* and *P. pastoris*. In contrast, *K. lactis* provoked only a slight activation, whereas *Sz. pombe* hardly activated any T cells, not even at the highest MOI 14. Interestingly, regarding the subcellular localization of the Ova antigen, the pattern of activation differed considerably between *S. cerevisiae* and *P. pastoris*. Although *S. cerevisiae* contained only a roughly 1.4 times higher amount of

intracellular Ova_{cyt} than *P. pastoris*, the former activated a several times higher number of T lymphocytes. In contrast, and in agreement with the lower amount of cytosolic Ova_{cyt} as well as (in the case of fission yeast) the diminished uptake by BMM, *K. lactis* and *Sz. pombe* activated only a small population of $CD8^+$ T cells. Notably, only a minor activation by *S. cerevisiae* and *P. pastoris* secreting Ova was observed, although the amount of secreted Ova was comparable to or even higher than the intracellular Ova_{cyt}. Here, *K. lactis* and *Sz. pombe* failed to induce any detectable amount of IFN-γ. In the case of cell-surface displayed Ova_{cyt}, activation of antigen-specific lymphocytes was significantly higher using *P. pastoris* as carrier system in comparison to *S. cerevisiae*. Noteworthy, at all MOIs applied, *P. pastoris* expressing Ova_{cyt} on the cell surface induced an even higher $CD8^+$ T cell response in comparison to cytosolic Ova_{cyt}, despite the considerably lower level of protein. In contrast, in *S. cerevisiae*, activation by intracellular Ova_{cyt} exceeded the cell wall-bound form at all MOIs. As expected, yeasts harbouring empty vectors caused no activation of Ova-specific lymphocytes as well as untreated macrophages.

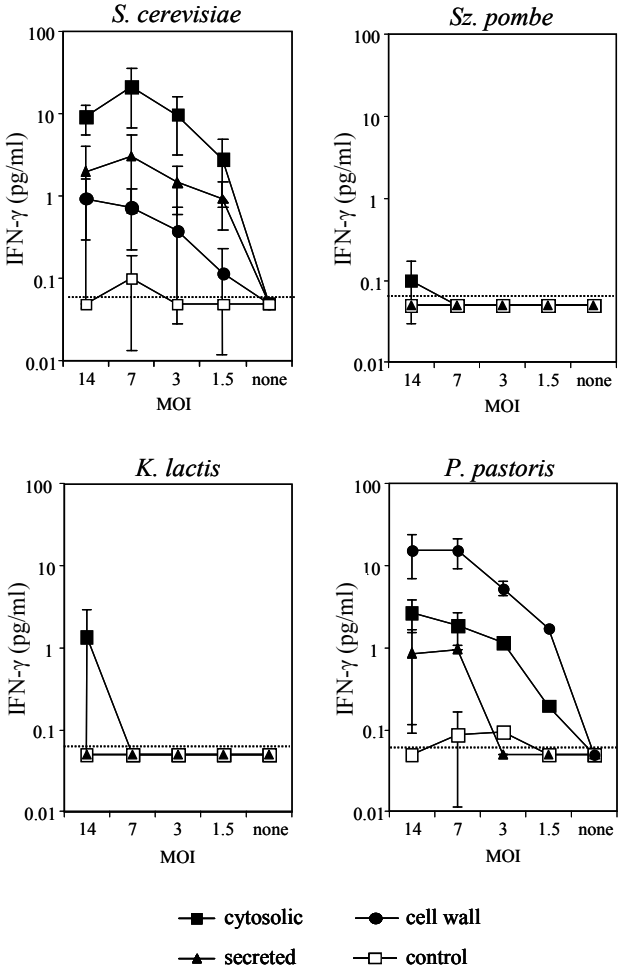


Fig. 33: Activation of Ova-specific $CD8^+$ T lymphocytes after yeast-mediated delivery of Ova-derivatives by bone marrow-derived macrophages *in vitro*. Adherent BMM from C57BL/6 mice were loaded with yeast genera expressing the different Ova derivatives. After 16 h, $CD8^+$ T cells specific for the MHC class I-restricted SIINFEKL epitope were added and incubated for another 18 h. Antigen presentation was measured by T cell activation which was assessed by measuring the amount of IFN-γ in culture supernatants by ELISA. Results are expressed as mean and SD values of three independent experiments [Bazan *et al*., 2011].

This assay could no longer be repeated within this work in order to test antigen delivery when Ova_{cyt} VLP were recombinantly expressed in *S. cerevisiae*.

In conclusion, delivery of a protein antigen by different yeast genera was able to activate antigen-specific $CD8^+$ T lymphocytes. Moreover, lymphocyte activation was influenced by antigen localization in yeast.

5.2. Activation of pp65-specific memory T lymphocytes from HCMV-positive donors in an autologous system

To simulate a condition more closely resembling an *in vivo* situation, the yeast-based delivery system was also tested using human DCs, the most effective inducers of T cell-mediated immunity. By means of an *ex vivo* T cell stimulation assay, human DCs loaded with distinct yeast genera expressing pp65 were assessed for efficient activation of antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes. The incubation time of DCs with yeasts (48 h) is sufficient to induce DC maturation and activation, as previously shown in section 1 of *Results*.

Immature DCs derived from monocytes isolated from HCMV-positive donors were incubated for 48 h with yeasts expressing pp65 or yeasts carrying empty vectors (MOI 5); as positive and negative controls a lysate from HCMV-infected fibroblasts or untreated cells were used, respectively. Then, autologous lymphoctes were added at a ratio of 1:1 (lymphocyte:DC), as well as anti-CD49d and anti-CD28. Cells were co-cultured for 6 h, during the last 4 h in the presence of Brefeldin A to inhibit protein secretion. Cells were stained for the surface molecules CD4 or CD8 and CD69 and intracellular IFN-γ. Activation of pp65-specific $CD4^+$ and $CD8^+$ memory T lymphocytes was analyzed by flow cytometry.

Fig. 34 shows the frequencies of $CD8^+$ T cells expressing CD69 and IFN-γ in response to pp65. Responses to yeasts delivering pp65 were higher than those obtained

against yeasts harbouring empty vectors, and even higher than after using a HCMV lysate. Some degree of unspecific responses to the yeast vectors were also observed, and were higher for *S. cerevisiae* (0.07%), followed by *P. pastoris* (0.06%), and *Sz. pombe* (0.02%). The percentage of activated pp65-specific memory $CD8^+$ T cells was 0.20% when *S. cerevisiae* was used as pp65 delivery system, 0.18% when *Sz. pombe* was used as vector and 0.14% when *P. pastoris* delivered pp65. Remarkably, these responses were significantly stronger compared to the response against a HCMV lysate (0.09%). The value corresponding to unspecific activation caused by untreated DCs was 0.01%.

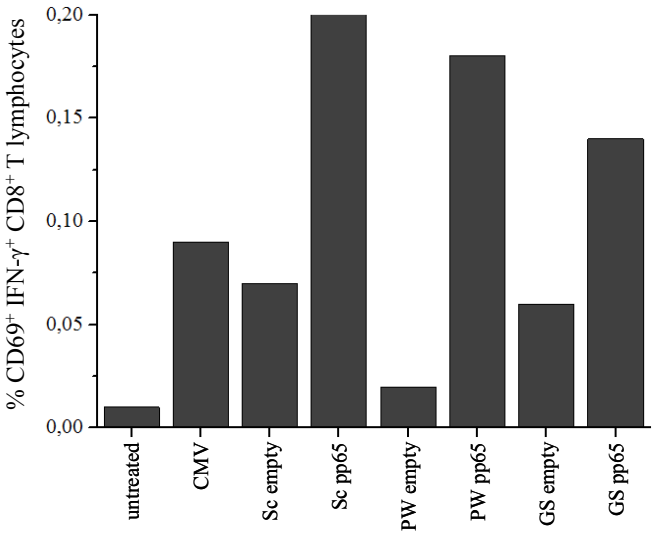


Fig. 34: Activation of antigen-specific memory $CD8^+$ T lymphocytes in an autologous system, as measured by CD69 and IFN-γ expression. DCs incubated with yeasts producing recombinant pp65 or harbouring empty vectors for 48 h were incubated with autologous T lymphocytes for antigen presentation. The HCMV lysate was used as positive control (CMV). Sc = *S. cerevisiae*; PW = *Sz. pombe*; GS = *P. pastoris*. Results of a single experiment are shown.

With respect to activated pp65-specific $CD4^+$ T cells, the observed difference between yeasts carrying pp65 and empty vectors was not significant (data not shown). Although the percentage of activated $CD8^+$ T cells in response to yeast-delivered pp65 were above the threshold of significant T cell responses (0.05%, Breinig *et al*., 2006), levels of double positive cells were still relatively low. Many other donors were tested, but since the lymphocytic activation observed was not significantly better, activation of T cells was subsequently analyzed in whole blood assays.

5.3. Activation of pp65-specific T lymphocytes in whole blood assay

It is well established that yeasts act as adjuvant and serve as efficient protein delivery system, stimulating potent immune responses (Stubbs *et al*., 2001; Ardiani *et al*., 2010). Activation of pp65-specific memory T lymphocytes using a cell lysate of *Sz. pombe* expressing recombinant pp65 in an *ex vivo* whole blood assay has been reported (Breinig *et al*., 2003). The same method was used here to investigate the ability of different yeast genera to stimulate antigen-specific memory T cells.

S. cerevisiae S86c, *Sz. pombe* PW260, and *P. pastoris* GS115 were used in this *ex vivo* stimulation assay, and their efficiencies as delivery vectors were compared. For this purpose, yeasts were cultivated under the respective inducing conditions for expression of recombinant pp65 (also Gag/pp65 in the case of *S. cerevisiae*). As negative controls, yeasts carrying empty vectors (or a Gag-encoding vector) were used. For all yeast species analyzed, three different variants were tested: an ethanol-precipitated yeast cell lysate; heat-treated yeasts (65°C, 1 h); and whole yeasts without receiving any treatment. Under each condition, the equivalent of 7.5×10^5 yeast cells was incubated with 450 µl blood. This optimal condition (1.7×10^6 yeast cells/ml) had been previously established by Breinig *et al*. (2003).

Expression of recombinant proteins was achieved for 72 h. For protein precipitation purposes, yeasts were resuspended in PBS and lysed with glass beads and crude extracts were precipitated with ethanol, as described in the *Methods* section. The resulting pellets were then resuspended in PBS. Whole yeasts were incubated at 65°C for 1 h, or left untreated, before being used in the whole blood assay. The frequencies of activated pp65-specific T lymphocytes were determined by means of three-colour flow cytometric analysis. As negative control, assays using whole blood from HCMV seronegative donors were also performed.

As depicted in Fig. 35, flow cytometric analyses of T cells from a HCMV positive donor revealed positive IFN-γ and CD69 responses against the HCMV lysate, in contrast to the HCMV negative donor. This latter exhibited strong T cell activation in response to the superantigen SEB, eliminating the possibility that the absence of response to HCMV antigens could result from potential experimental flaws. In both donors, incubation of the blood with only anti-CD28 and anti-CD49d did not cause any relevant effect on IFN-γ and CD69 production by T cells.

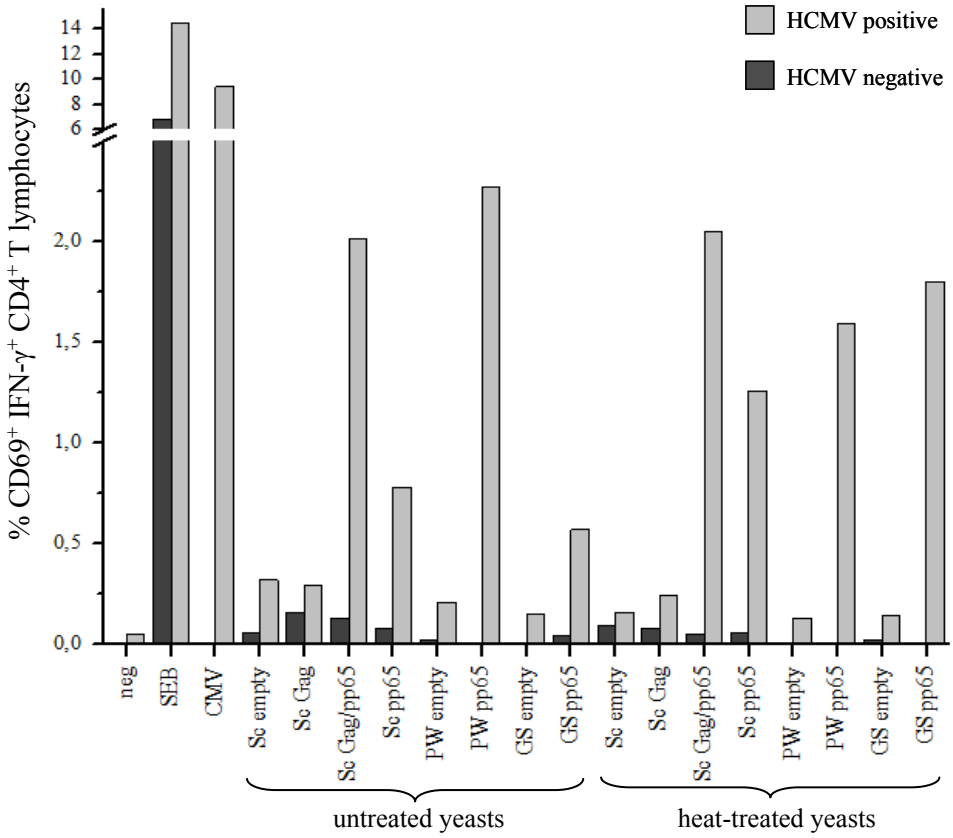
Lysates from all yeast genera failed to induce pp65-specific responses in HCMV-positive donors (data not shown), contradicting previous findings (Breinig *et*

al., 2003). IFN-γ/CD69 expression in the samples incubated with yeasts harboring empty vectors or unmodified Gag protein alone was considerably low. In contrast, live or heat-treated *S. cerevisiae*, *Sz. pombe* and *P. pastoris* engineered to express pp65 (or Gag/pp65) showed the ability to activate memory $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 35).

Comparing untreated yeasts expressing pp65 derivatives, *Sz. pombe* expressing pp65 provoked the highest $CD4^+$ T cell stimulation (2.27%), followed by *S. cerevisiae* expressing the fusion protein Gag/pp65 (2.01%) and pp65 alone (0.78%). Whole *P. pastoris* cells were less potent in stimulating antigen-specific $CD4^+$ T lymphocytes (0.57%). Among the heat-treated yeasts carrying pp65, *S. cerevisiae* expressing Gag/pp65 caused the highest activation of $CD4^+$ T lymphocytes (2.05%), followed by *P. pastoris* (1.8%), *Sz. pombe*/pp65 (1.59%) and *S. cerevisiae*/pp65 (1.26%). Thus, in terms of $CD4^+$ T cell activation, heat treatment was beneficial in the case of *S. cerevisiae* and *P. pastoris*, but not in *Sz. pombe*. Unspecific T cell activation, as measured upon incubation with yeasts harbouring control plasmids, was not higher than 0.32% (Fig. 35 A).

In relation to $CD8^+$ T cell responses (Fig. 35 B), *S. cerevisiae* delivering Gag/pp65 induced greater frequencies of pp65-specific lymphocytes (0.7%), followed by *Sz. pombe*/pp65 (0.45%) and *S. cerevisiae*/pp65 (0.31%). Production of cytokines induced by *P. pastoris* carrying pp65 was very similar to the values obtained with this same yeast harbouring the empty vector (0.18% and 0.17%, respectively). However, when *P. pastoris* was heat-treated, this difference increased dramatically (0.39% *vs*. 0.06%). This synergistic effect was not observed for the other yeasts in the case of $CD8^+$ T cell activation, in which the frequency of positive cells was markedly reduced (Fig. 35 B).

(A) CD4$^+$ T Lymphocytes



(B) CD8$^+$ T Lymphocytes

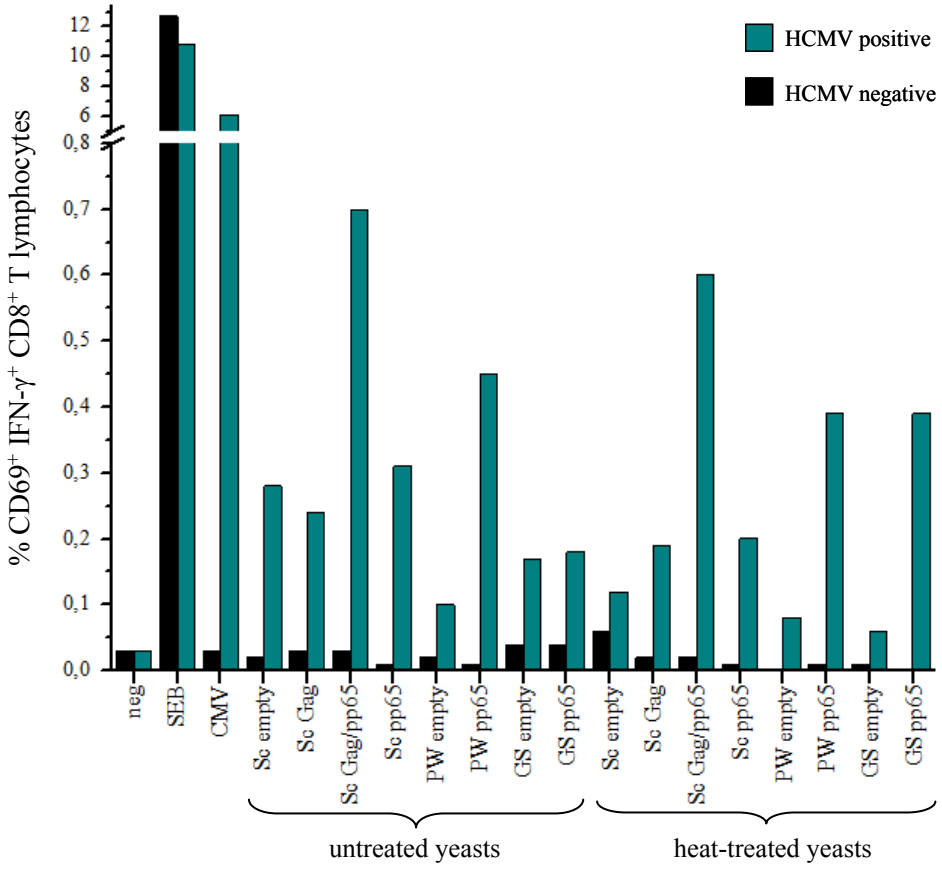


Fig. 35. Recombinant pp65 within whole yeasts is able to activate specific memory T cells in HCMV seropositive, but not seronegative, donors. Frequencies of antigen-specific $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell activation after stimulation by yeasts expressing pp65 are shown. Antigens were added to whole blood of HCMV seropositive and HCMV negative donors. Whole blood was stimulated for 6 h. Activated T lymphocytes were identified by expression of CD69 and IFN-γ using flow cytometry. Neg = negative control (sample incubated with costimulatory antibodies only); SEB = *Staphylococcus* enterotoxin B (positive control); CMV = sample incubated with a lysate of HCMV-infected fibroblasts; Sc = *S. cerevisiae*; PW = *Sz. pombe*; GS = *P. pastoris*. Data from a single representative experiment are shown.

Higher frequencies of antigen-specific memory $CD4^+$ T cells in comparison to $CD8^+$ T cells were obtained in all assays performed throughout this work. Nevertheless, in a whole blood assay using total extracts of *Sz. pombe* producing pp65 higher frequencies of pp65-specific $CD8^+$ T cells related to $CD4^+$ T cells have been described (Breinig *et al*., 2003). Unspecific responses to the yeast vehicles observed in all donors tested have also been described, and are directly linked to yeast cell wall components, as pointed out by Heintel *et al*. (2003).

Using *S. cerevisiae* as antigen carrier, the Gag/pp65 fusion has proven to be more effective in stimulating pp65-specific lymphocytes than pp65 alone. Since both proteins were synthesized in equivalent amounts (Fig. 36), these results support previous findings from Powilleit *et al.* (2007).

For comparison, expression levels of recombinant pp65 by the different yeast strains were quantified. After 72 h of expression, extracts from 3×10^8 cells were analyzed by western blotting. As shown in Fig. 36, *P. pastoris* expresses the highest levels of pp65 (mean value = 222.3 ng in 3×10^8 cells), followed by *Sz. pombe* (19.3 ng). Recombinant protein production was lower in *S. cerevisiae*, in which pp65 and Gag/pp65 were expressed at similar levels (10.8 ng and 10.6 ng in 3×10^8 cells, respectively).

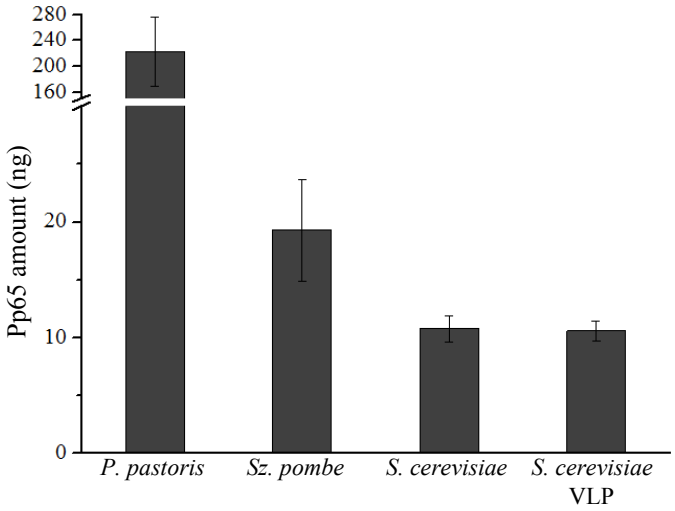


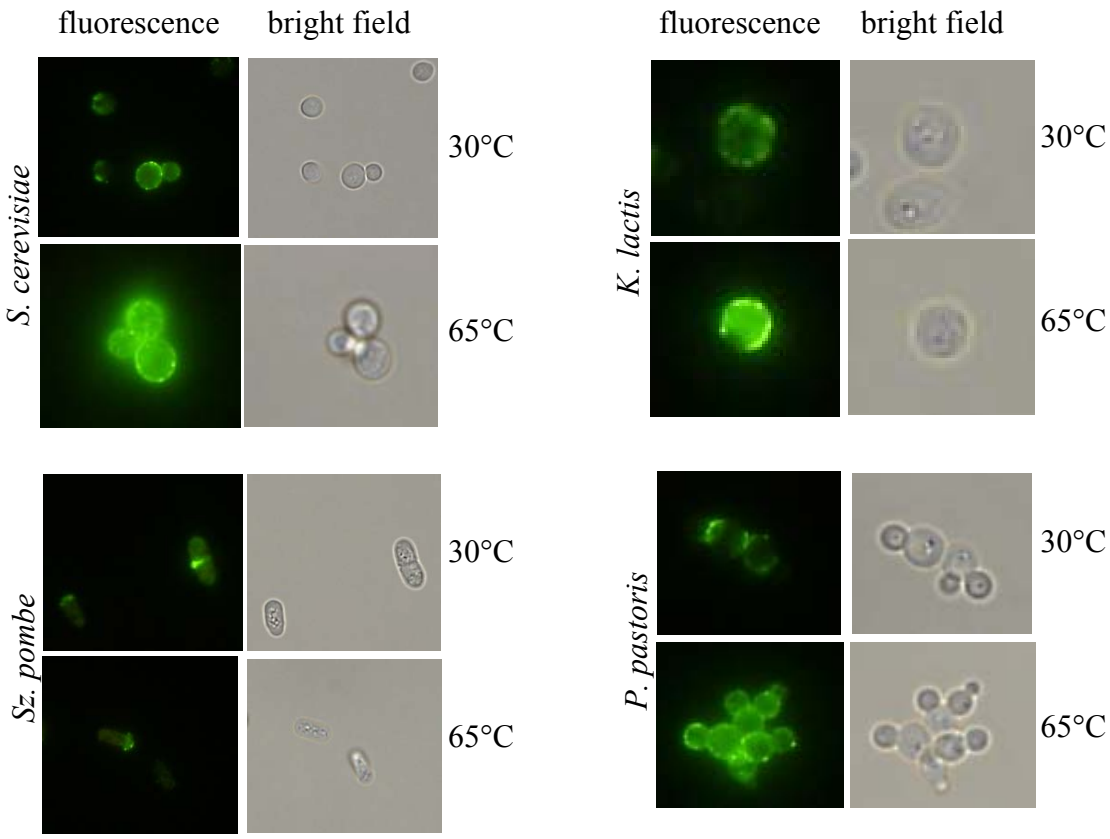
Fig. 36. Expression levels of recombinant pp65 produced in different yeast genera. Quantification was performed based on a standard curve of purified recombinant pp65 of known concentration. Shown are mean values and standard deviations from triplicate determinations.

From these data it can be concluded that activation of antigen-specific memory T lymphocytes does not correlate with the amount of recombinant protein expressed by each yeast, indicating that the yeast vehicle itself can influence cell-mediated responses. These findings are in agreement with the results observed for delivery of cytosolic Ova.

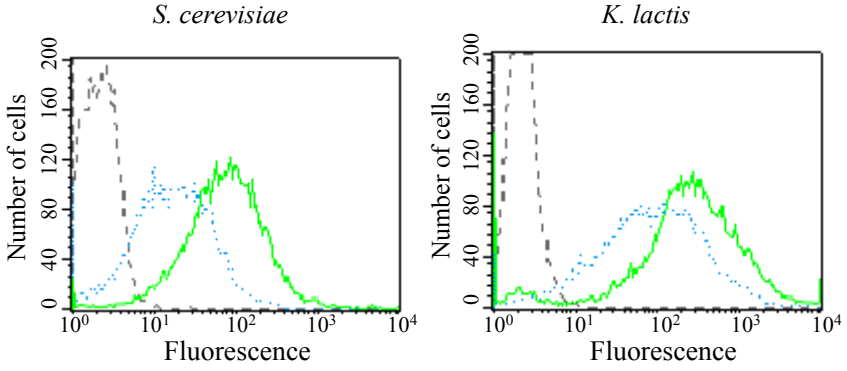
6. Effect of heat treatment on β-glucan exposure on yeast cell walls

The findings from the whole blood assays, in which heat treatment of yeasts led to a different T cell activation profile, encouraged a deeper investigation of the basis of the observed effects. Gantner and colleagues (2005) have demonstrated that heat-killed *C. albicans* expose more β-glucan at the cell surface. Normally, β-glucan is hidden by the outer mannose layer of yeast cell walls, and becomes only apparent in surface deformities such as bud and birth scars. Therefore, Dectin-1 binding to living yeast cells is restricted to these localized patches. Upon heating, β-glucan is uniformly exposed on the cell wall, in a manner that it is recognized by Dectin-1. Since *C. albicans* hyphae do not expose β-glucan by virtue of their filamentous growth, host Dectin-1 is not activated, which plays an important role in *C. albicans* pathogenicity. Therefore, Dectin-1 activation is a necessary step in the development of immune responses such as cytokine and chemokine production, as well as killing mechanisms against a variety of microorganisms (Gantner *et al*., 2005).

Exponentially grown yeasts were incubated for 1 h at 65°C, or left untreated at room temperature. Afterwards, cells were incubated with a monoclonal anti-β-1,3-glucan antibody and subsequently with a FITC-conjugated secondary antibody. Yeasts were analyzed by fluorescence microscopy and flow cytometry. Cells incubated with an isotype control antibody and FITC-coupled secondary antibody served as negative control (Fig. 37).



B



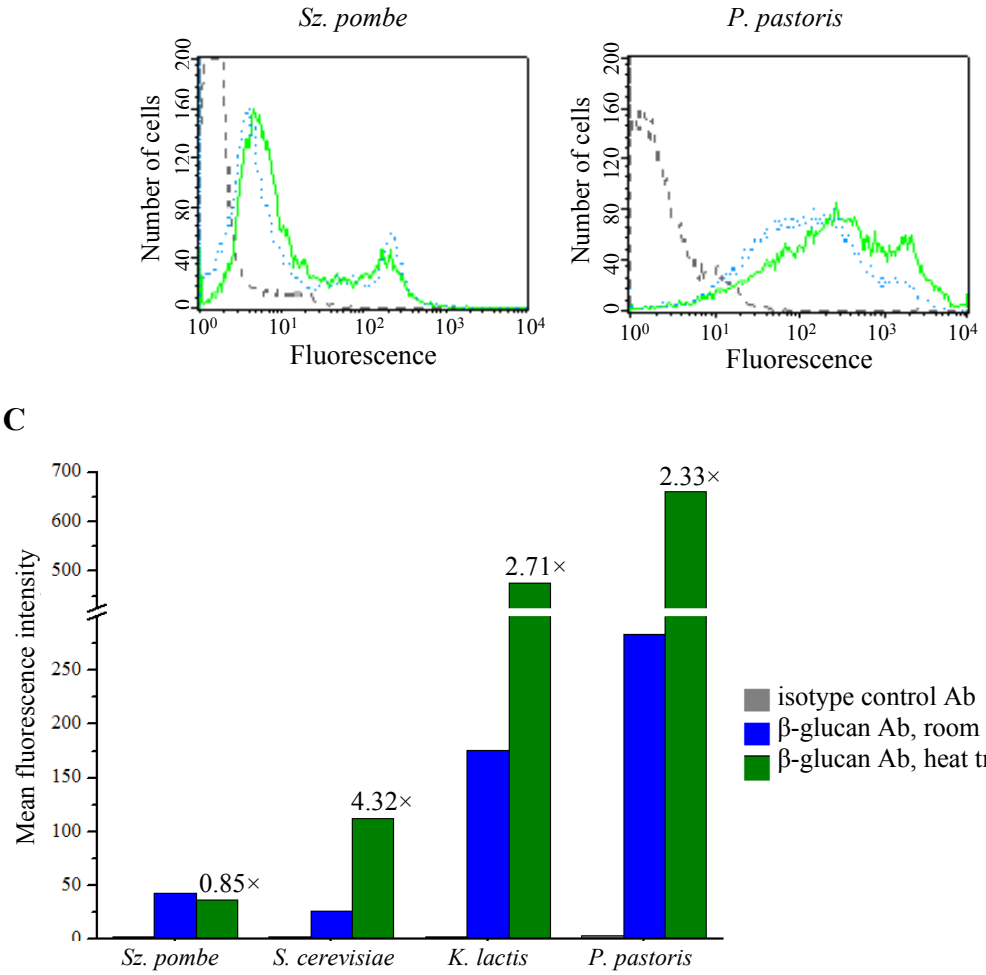


Fig. 37. Effect of heat-treatment on the exposure of β-glucan on the yeast cell surface. The yeasts *S. cerevisiae*, *K. lactis*, *P. pastoris* and *Sz. pombe* were incubated at 65°C for 1 h, or left untreated, before being incubated with anti-β-glucan or an irrelevant antibody (isotype control). Then, cells were incubated with a FITC-coupled secondary antibody and analyzed by fluorescence microscopy **(A)** and flow cytometry **(B and C)**. **(A)** Fluorescence micrographs (left panels) and light micrographs (right panels) of yeasts stained for β-glucan. **(B)** Histograms showing the fluorescence pattern of β-glucan staining among the yeast genera. Dashed lines represent cells incubated with an isotype control antibody; blue lines represent cells incubated at room temperature and stained for β-glucan; green solid lines indicate heat-treated cells stained for β-glucan. **(C)** Mean fluorescence intensity from untreated (blue bars) or heat-treated (green bars) yeast cells incubated with anti-β-glucan antibody, or untreated cells incubated with the isotype control antibody (grey bars). The fold increase in fluorescence observed after heat treatment in comparison to no treatment is shown. Ab = antibody.

As shown in Fig. 37, heat-treatment significantly increased β-glucan exposure on the cell surface of *S. cerevisiae*, *K. lactis* and *P. pastoris*. In contrast, *Sz. pombe* cells showed a slight reduction in mean fluorescence after incubation at 65°C.

In Fig. 37 A, fluorescence microscopy analyses show that β-glucan staining was observed in localized patches on the cell walls of untreated *S. cerevisiae*, *K. lactis* and

P. pastoris, such as birth and bud scars. Heat-treatment triggered an increase in fluorescence of cell walls of the three budding yeasts analyzed, whose entire cell walls became homogeneously stained. These results support earlier findings of Gantner *et al*. (2005), who demonstrated such a staining pattern for *C. albicans* and *S. cerevisiae*. Nonetheless, these observations are not valid for fission yeast. In the case of untreated *Sz. pombe* cells, a signal could be seen in the septum, where linear β-1,3-glucan is present in this species (Humbel *et al*., 2001). However, when this yeast was subjected to heating, fluorescence was not augmented and was detected only in punctuate regions. The microscopic examinations corroborate with the flow cytometric analyses (Fig. 37 B). The lines depicting the heat-treated yeasts (green solid lines) show a shift to the right in comparison to the lines representing untreated yeasts (blue lines) in the histograms of *S. cerevisiae*, *K. lactis* and *P. pastoris*, but not *Sz. pombe*. As expected, no fluorescence was observed in cells stained with the isotype control antibody. Heat-treated cells were also stained with the same irrelevant antibody, and also showed no detectable fluorescence (data not shown). Fig. 37 C shows that the greatest increase in mean fluorescence of heat-treated cells in comparison to untreated cells was observed for *S. cerevisiae* (112.62 *vs*. 26.06), followed by *K. lactis* (476.59 *vs*. 175.96) and *P. pastoris* (660.86 *vs*. 283.33). The opposite effect was observed for *Sz. pombe* (36.67 *vs*. 43.28).

In sum, heat-treatment strongly enhances β-glucan exposure on the cell wall of budding yeasts, but not of fission yeast.

6.1. Production of reactive oxygen species in whole blood after incubation with yeasts

The effect of heat-treatment on β-glucan exposure and its consequences were further analyzed. Gantner *et al*. (2005) showed that β-glucan sensing is a prerequisite for production of reactive oxygen species (ROS) by phagocytic cells in response to fungal stimuli. In this regard, yeasts were subjected to heating (65°C, 1 h) or left untreated and the production of ROS in whole blood was determined by means of a luminol chemiluminescence assay.

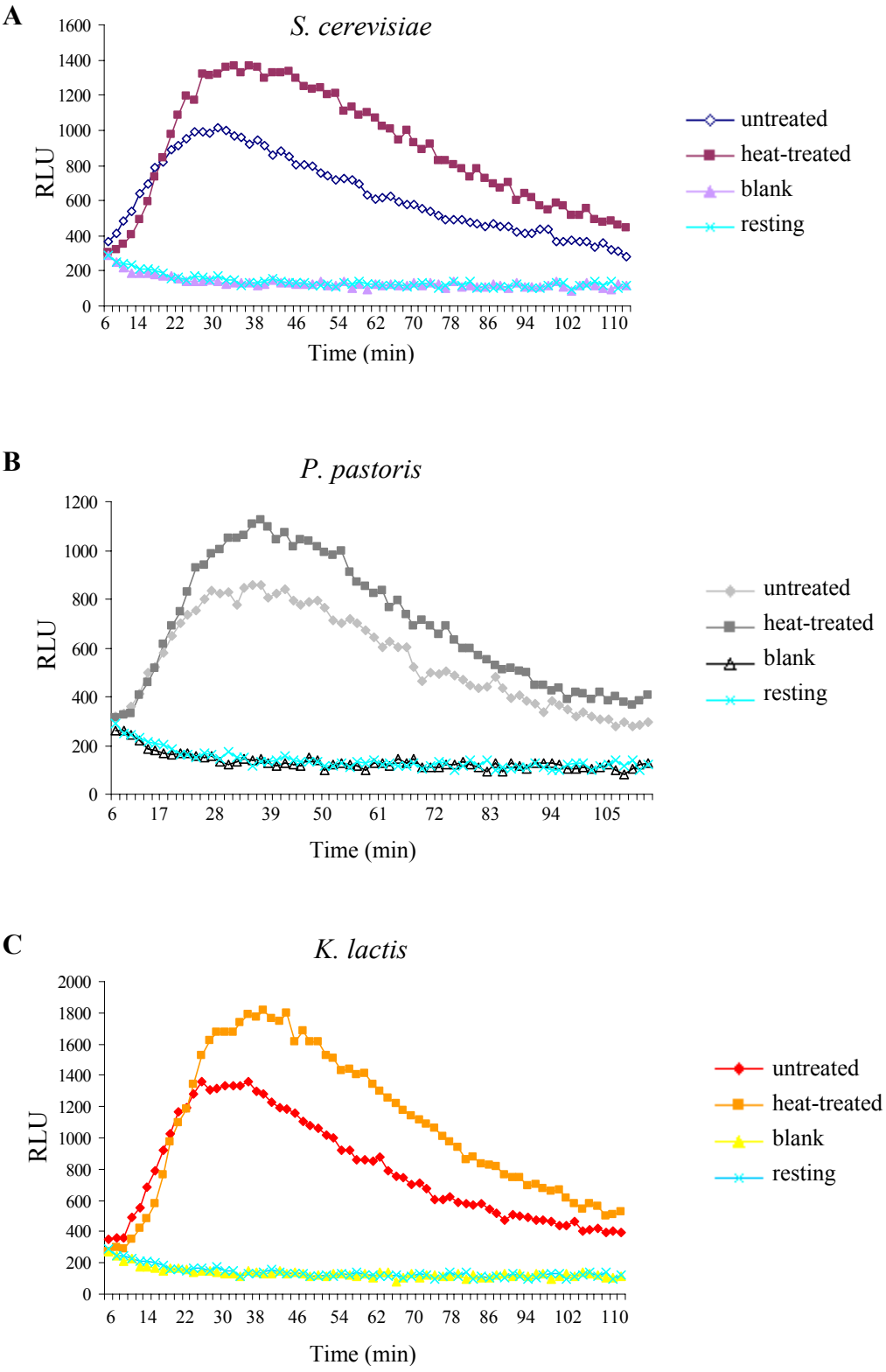
Generation of ROS by phagocytes is a significant aspect in the response against fungi. ROS production by macrophages in response to the yeast form of *C. albicans* and *S. cerevisiae* zymosan (a preparation containing β-glucans, mannans, mannoproteins

and chitin) has been demonstrated (Gantner *et al*., 2005; Wellington *et al*., 2009). However, generation of ROS in whole blood after incubation with yeasts has not been described. The choice for this system was based on the fact that incubation of yeasts with IC-21 macrophages did not result in any detectable luminescence (data not shown). Furthermore, whole blood chemiluminescence exhibits some advantage in comparison to chemiluminescensce assays performed on isolated PBMCs, as preactivation of leukocytes as a consequence of the purification process is avoided. Additionally, whole blood chemiluminescensce assays permit evaluation of cellular responses in a physiologic environment that more closely resembles the *in vivo* situation in comparison to isolated cells (Kopprasch *et al*., 1996).

In this experiment, untreated and heat-treated *S. cerevisiae*, *Sz. pombe*, *K. lactis*, and *P. pastoris* were compared with respect to their ability to stimulate ROS production by blood leukocytes. Yeasts were exponentially grown in SC medium. Subsequently, 2.5×10^5 yeast cells were added in each well to 10-fold diluted heparinised fresh venous blood previously mixed with luminol. In the blank samples, luminol was substituted for DMSO, and in the resting samples, yeasts were replaced by the equivalent volume of buffer. A series of blood dilutions was tested in this assay (undiluted, 10-, 50-, 100-, and 500-fold), and the 10-fold dilution has proven to be the most appropriate experimental set-up (data not shown). The final volume was 100 µl/well. Chemiluminescence was continuously monitored for 150 minutes at 37°C, with 1 s integration time.

As seen in Fig. 38 (A-C), chemiluminescensce was detectable approximately 6 minutes after incubation with *S. cerevisiae*, *K. lactis*, and *P. pastoris*, reaching a maximum after ~30 minutes and declining thereafter. Chemiluminescence was higher when these three yeast species had been previously incubated at 65°C. Interestingly, in the case of fission yeast, untreated yeasts caused a higher ROS production by blood phagocytic cells then heat-treated cells (Fig. 38 D). Also, chemiluminescence signals occurred after approximately 20 minutes of incubation, and reached a maximum after 45 minutes for the untreated yeasts, whereas incubation with heat-treated yeasts showed a different pattern, reaching a plateau after 64 minutes. Slower kinetics of *Sz. pombe* uptake by phagocytic cells was demonstrated in other experiments with human and murine phagocytic cells in this work, and might be the cause of the delay in ROS production observed. In contrast to the other yeasts examined, ROS production by blood phagocytic cells after incubation with heat-treated *Sz. pombe* was considerably lower than with untreated yeasts.

When either luminol or yeast was absent (blank or resting samples), no significant chemiluminescence was detected (Fig. 38). With respect to the blank samples, both untreated and heat-treated yeasts incubated with blood and DMSO resulted in the same chemiluminescence pattern (data not shown).



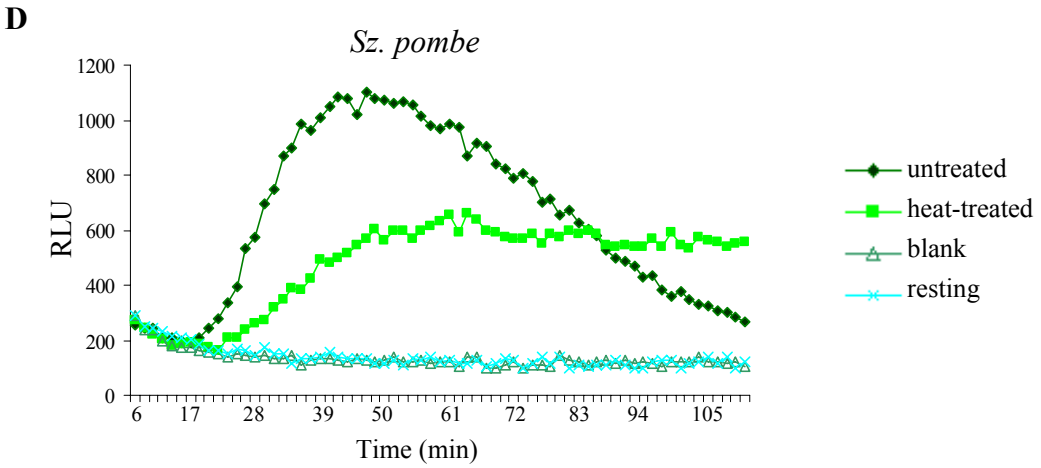


Fig. 38. ROS production detected by chemiluminescence kinetics of whole blood depends on the extent of β-glucan exposure on yeast cell surface. Whole blood was incubated with heat-treated, untreated, or no yeast, in the presence of luminol. Blood samples incubated with yeasts and DMSO served as blank. Blood samples incubated with luminol without yeasts were referred to as resting. Chemiluminescence was recorded at 37°C over a 150-minute interval. RLU = relative luminescence units. Results represent mean values of triplicate determinations.

All yeast genera analyzed are able to stimulate phagocytic cells in the blood to produce reactive oxygen species, a host's defence mechanism of the innate immunity. The results observed in these chemiluminescence studies are in agreement with the findings reported for flow cytometry and fluorescence microscopy, *i.e.*, budding yeasts expose more β-glucan on their cell surface after being subjected to heating, whereas fission yeast seems somehow to react in a different way.

Altogether, these findings might help planning future experiments involving yeasts as delivery systems. The choice of a certain yeast species for specific purposes should take into account the manner this microorganism behaves after being subjected to a particular treatment.

7. Stimulation of $CD8^+$ transgenic cells from OT-I mice after vaccination

It could be demonstrated that yeast is an effective vehicle for delivery of cytosolic recombinant antigens (*Results*, section 5). To investigate whether yeast delivery of Ova_{cyt} would stimulate antigen-specific $CD8^+$ T cells in an *in vivo* system, the *S. cerevisiae* W303-1a strain was used as protein carrier in a preliminar experiment with OT-I transgenic mice. This *S. cerevisiae* strain has been employed in various studies using whole yeasts in vaccination protocols (Riemann *et al.*, 2007; Lu *et al.*, 2004;

Wansley *et al*., 2008). Furthermore, this strain was also able to induce DC maturation and activation, as previously demonstrated (*Results*, section 1).

Experiments with OT-I mice were performed in collaboration with Elisabeth Kenngott at the Medical Clinic for Rheumatology and Clinical Immunology (Charité, Berlin). OT-I mice express high levels of a transgenic T-cell receptor in $CD8^+$ T lymphocytes which recognizes $OVA_{257-264}$ (SIINFEKL peptide) in MHC class I H-$2K^b$ molecules (Hogquist *et al*., 1994).

OT-I mice were given recombinant *S. cerevisiae* expressing Ova_{cyt}, *S. cerevisiae* carrying an empty vector, or PBS, three times, s.c., with 1-week interval between each immunization. One week after the last immunization, spleens and inguinal lymph nodes were harvested, and single cell suspensions (2×10^6 cells/ml) were restimulated with 10 µg/ml PMA/ionomycin for 4 h, or SIINFEKL peptide for 6 h (spleen cells only). PMA and ionomycin are frequently used in immunological approaches to stimulate cytokine production in a non-specific manner (Picker *et al*., 1995). Production of IFN-γ by activated lymphocytes was assessed by flow cytometry (Fig. 39).

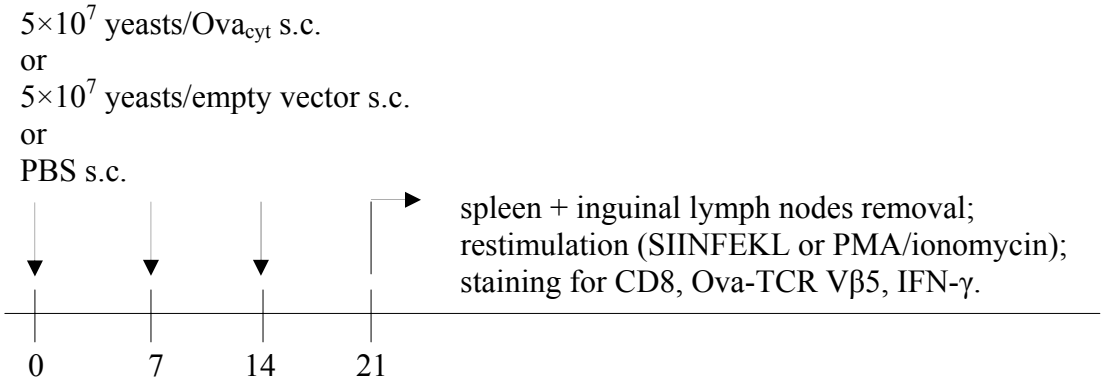


Fig. 39. Schematic representation of vaccination regimes and subsequent analysis of specific $CD8^+$ T cell immune response in spleen and lymph nodes. Yeasts expressing recombinant Ova_{cyt} or carrying an empty vector (5×10^7 cells in 100 µl PBS), or 100 µl PBS were injected three times s.c. in OT-I mice, with one-week intervals between doses. One week after the last immunization, spleens and inguinal lymph nodes were removed, and single-cell suspensions were restimulated with SINFEKL or PMA/ionomycin before being stained for CD8, Vβ5 and IFN-γ.

Fig. 40 A shows the production of IFN-γ by Ova-specific $CD8^+$ T cells after spleen cells had been incubated with 10 µg/ml SIINFEKL peptide. Mice receiving *S. cerevisiae*/Ova_{cyt} showed an increased frequency of IFN-γ^+-cells in comparison to the other groups (mean value = 3.34%). $CD8^+V\beta5^+$ lymphocytes isolated from spleen of mice receiving *S. cerevisiae* harbouring the empty vector also showed a certain degree

of background activation (mean value of IFN-γ^+ cells = 2.4%). Specific cells from mice receiving PBS showed a minimal IFN-γ production (mean value = 1.48%). When spleen cells were stimulated with PMA/ionomycyn, the frequency of IFN-γ-producing $CD8^+$ cells increased substantially, but this increase was observed for all groups (Fig. 40 B). Mean values of the frequencies of $CD8^+$ IFN-γ^+ cells were slightly higher in the group receiving *S. cerevisiae*/Ova_{cyt} than *S. cerevisiae*/empty vector (11.29% *vs*. 10.32%, respectively), whereas specific cells from mice receiving PBS only showed a frequency of 7.19% cells expressing IFN-γ.

Due to the low number of cells recovered after extraction of inguinal lymph nodes, cell suspensions were only stimulated with PMA/ionomycin. However, no significant increase in the frequency of specific cells producing IFN-γ could be seen (data not shown).

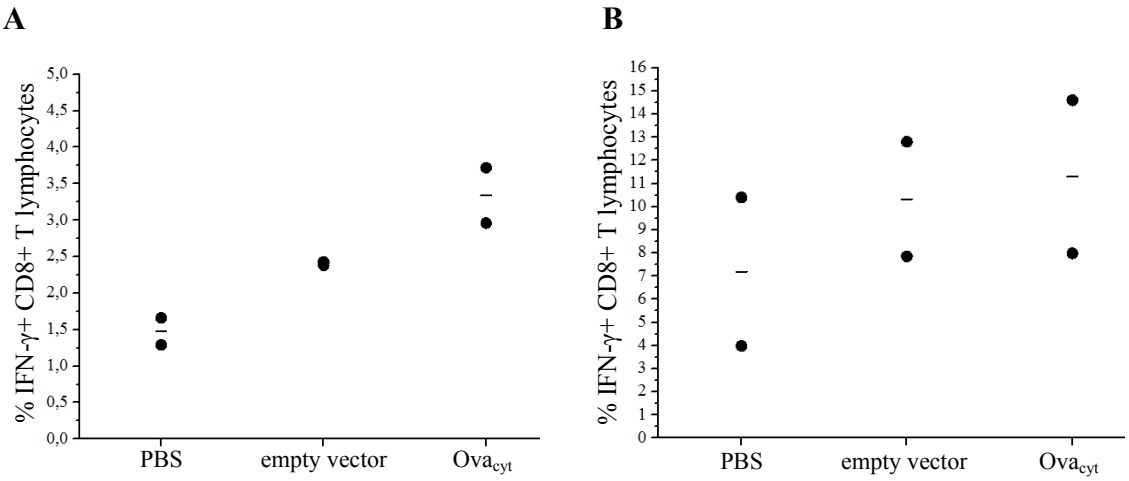


Fig. 40. OT-I transgenic mice were immunized with *S.cerevisiae*/Ova_{cyt}, *S. cerevisiae*/empty vector, or PBS. Then, spleens were harvested 7 days postvaccination and the percentage of transgenic $CD8^+$ T cells producing IFN-γ after *ex vivo* restimulation with 10 µg/ml of SIINFEKL peptide **(A)** or PMA/ionomycin **(B)** was determined by flow cytometry.

Although the differences between the Ova_{cyt}-receiving group and the control groups were not statistically significant, there was a tendency for enhanced IFN-γ production by spleen lymphocytes from Ova_{cyt}-vaccinated mice, especially when the cells were restimulated with the Ova peptide. This indicates that better results might be obtained by optimizing the immunization protocol.

V. DISCUSSION

Vaccination approaches aiming at inducing robust cell-mediated immune responses have been the focus of intense research. Strategies employing yeast as antigen carrier have been established in the past decade. A number of *in vitro* studies, as well as preclinical and clinical trials attest the intrinsic adjuvant properties of yeasts (Franzusoff *et al*., 2005; Ardiani *et al*., 2010). However so far, the overwhelming majority of these studies are restricted to the conventional yeast *S. cerevisiae*.

Hitherto, there is no study comparing the effectiveness of different yeast genera as vaccine vehicles. Although such comparison is sometimes difficult due to the difference in expression levels of the recombinant protein among the yeasts examined, testing the applicability of yeasts species alternative to *S. cerevisiae* in the development of novel antigen delivery systems could be of relevance.

The use of yeasts as antigen delivery system provides the following benefits: (i) yeasts can be engineered to express one or more recombinant proteins, and correctly perform post-translational modifications; (ii) cost-effectivenes of the system, also in large-scale productions; (iii) yeast cell wall components *per se* activate dendritic cells and amplify signals required for T cell activation; (iv) antigen presentation occurs via both MHC class I and MHC class II pathways, eliciting antigen-specific T cell responses; (v) absence of yeast-induced neutralizing immune responses, enabling repeated administration; and (vi) heat-killed and live yeasts trigger similar protective immunity, reducing the risks associated with the injection of live yeasts in immunocompromised individuals (Ardiani *et al*., 2010).

A set of "non-conventional", biotechnologically relevant yeast genera has been utilized in the past decades as alternative expression host to *S. cerevisiae*. Yeast species such as *K. lactis*, *P. pastoris*, and *Sz. pombe* have proven to be efficient cell factories and have offered some advantages over baker's yeast, for instance with respect to protein glycosylation. Hyperglycosylation and addition of allergenic α-1,3-linked mannose residues to N-glycans have been repeatedly reported in *S. cerevisiae* (Porro *et al*., 2005; Varki *et al*., 2009). This drawback, together with other limitations, such as plasmid instability, can be overcome in the majority of cases with the use of the previously mentioned alternative yeasts.

Yeast has been shown to be taken up by phagocytic cells through recognition of PAMPs, such as mannan and β-glucan, by pattern recognition receptors, such as Dectin-1 and the mannose receptor (Netea *et al*, 2006; Brown, 2011; Romani, 2011). After receptor-mediated phagocytosis, yeasts are killed and processed for antigen presentation (Gildea *et al*., 2001; Newman and Holly, 2001; Syme *et al*., 2002; Heintel *et al*., 2003). Besides, yeasts cause dendritic cells to undergo maturation (Buentke *et al*., 2001; Stubbs *et al*., 2001). For all these reasons, yeasts are appropriate vectors for delivering vaccine antigens.

The aim of this work was to compare four yeast strains – *S. cerevisiae*, *Sz. pombe*, *K. lactis*, and *P. pastoris* – as antigen delivery vector for activating T lymphocytes, as well as the influence of the subcellular localization of the recombinant protein on this activation. For this purpose, the interaction of different yeast strains with phagocytes was analyzed, as well as yeast-induced DC maturation. Subsequently, the genes coding for the model antigen Ovalbumin or the clinically relevant HCMV protein pp65 were cloned in expression plasmids specific for each yeast species and recombinantly expressed. In the case of full-length pp65, only intracellular expression was achieved (except for *K. lactis*), whereas Ova could be secreted, intracellularly expressed (Ova_{cyt}), or cell-wall anchored (Ova_{cyt}/Sed1p). This latter variant was only observed in *S. cerevisiae* and *P. pastoris*. Pp65-harboring yeasts, as well as *S. cerevisiae* assembling chimeric pp65 VLP, were analyzed in an *ex vivo* whole blood assay for their ability to stimulate pp65-specific $CD4^+$ and $CD8^+$ T lymphocytes from HCMV-seropositive donors. Yeasts carrying the plasmid-encoded Ova variants were analyzed *in vitro* by means of antigen-presentation assays using Ova-specific T lymphocytes. Finally, a preliminary *in vivo* experiment was performed in order to confirm the feasibility of the system.

1. Yeasts induce DC maturation and cytokine secretion

In order to efficiently stimulate T cells, DCs have to be in a mature state. Mature DCs in lymphoid tissues are the most powerful stimulators of naïve T cells. Maturation implies both functional and phenotypic changes. After antigen uptake and exposure to inflammatory stimuli, immature DCs in peripheral tissues are induced to migrate into the regional lymph nodes where, as mature cells, they express surface molecules that further activate T cells (Janeway *et al*., 2001; Lechmann *et al*., 2002).

DC maturation and activation can be elicited by diverse stimuli, such as microbial components, cytokines and costimulatory molecules. Maturation is accompanied by increased expression of adhesion, costimulatory and MHC molecules, altered expression of chemokine receptors (such as CCR7), production of some specific cytokines, such as IL-lβ and IL-12, and decreased capability of antigen uptake. In the lymph nodes, mature DCs present antigens to naïve T cells and activate these lymphocytes, thus connecting innate and adaptive immunity (Verhasselt *et al*., 1997; Akira *et al*., 2001; Abbas *et al*., 2007).

DCs are able to ingest a wide variety of antigens and efficiently process them in context of MHC class I and II molecules for presentation to T cells. Additionally, DCs express a set of costimulatory molecules that stimulate naïve T cells, and dictate the differentiation of naive lymphocytes into distinct classes of effectors, controlling the quality of the T cell response. These antigen-specific, adaptive responses play a critical role in combating infections and tumors (Mellman *et al*., 2001; Heath and Carbone, 2001). DCs can also efficiently process and present internalized, exogenous antigens via MHC class I, in a process known as "cross-presentation". Due to this remarkable ability, many efforts have been made in the vaccination field to target and activate DCs, thus providing the potential to generate both $CD4^+$ and $CD8^+$ immune responses.

In this work, it could be shown that incubation of monocyte-derived human DCs with different yeast genera resulted in increased expression of the DC surface markers CD83, CD80, CD86, CD54, CD58, CD40, MHC classes I and II, and led to secretion of GM-CSF, IFN-α, IFN-β, IFN-γ, IL-1α2, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-15, IL-23, IL-27, and TNF-α. These observations indicate that all yeasts examined are able to induce DC maturation and activation. Importantly, these data demonstrate that the yeast vehicle *per se* is able to augment all three signals required for T cell activation, since increased expression of MHC molecules (involved in signal 1), costimulatory molecules (which provide signal 2) and cytokines (which deliver signal 3) were observed.

With respect to DC maturation, upregulation of the expression of CD83, MHC class I, MHC class II, CD80, CD86, CD40, CD54, and CD58 was observed after incubation of DCs with all yeast genera and the HCMV lysate, as well LPS, which was used as positive control since its effects on DC maturation have been repeatedly demonstrated (Bernstein *et al*., 2008; Huang *et al*., 2009). However, expression of CCR7 after contact with yeasts or HCMV lysate, as well as with the classical stimulator

LPS, did not significantly increase, in contrast to all other surface markers analyzed. A possible explanation for this finding might be the presence of IFN-β and IFN-γ (which were released into the culture medium by DCs), as they have been shown to inhibit the expression of this homing receptor (Yen *et al*., 2010).

LPS, a component of Gram-negative bacterial outer membranes, is detected by TLR4 (Pålsson-McDermott and O'Neill, 2004). Maturation and cytokine production by DCs in response to LPS has been described. For instance, high-level secretion of IL-6, IL-8, IL-12 and TNF-α, and upregulation of MHC classes I and II, CD40, CD80, CD86, CD54 and CD58 have been shown (Sallusto *et al*., 1995; Verhasselt *et al*., 1997). In the present work, LPS induced higher expression levels of all surface markers and eight out of fourteen cytokines analyzed compared to yeast genera and the viral stimulus. Nevertheless, it has to be considered that yeasts and LPS, as well as viruses, stimulate and mature DCs through different mechanisms, *i.e.* different pathways are activated by distinct stimuli, leading to modulation of a particular set of genes (Huang *et al*., 2001).

With respect to immune response against viruses, DCs generally become activated after antigen uptake and synthesize a number of cytokines, such as IFN-α, IL-12 and IL-15 (Lambotin *et al*., 2010). However, viral infections can either stimulate or repress the maturation and functional activation of DCs (Moutaftsi *et al*., 2002). Responses to HCMV seem to depend on the DC subset. For example, in one study, HCMV did not lead to maturation of monocyte-derived DCs, as the expression of MHC class I, CD40 and CD80 diminished after HCMV infection (Moutaftsi *et al*., 2002). In contrast, another DC type (blood $CD11c^+$ DC) showed an elevated expression of MHC molecules and CD83, as well as IL-6, IL-10 and type I IFN secretion following HCMV exposure (Kvale *et al*., 2006). In the present study, upregulation of maturation markers and cytokine secretion could be detected following incubation of monocyte-derived DCs with the viral stimulus. However, since the stimulus used here consisted of a lysate of HCMV-infected fibroblasts, viral protein aggregates, instead of intact viral particles, might have contributed to the maturation and activation profile observed.

Yeast-dependent upregulation of surface molecules indicative of DC maturation has been described for both non-pathogenic and opportunistic yeast species, such as *S. cerevisiae*, *C. albicans* and *M. furfur*. Cytokine secretion by DCs in response to fungi has been shown to be a more complex task, varying from one fungal stimulus to another (d'Ostiani *et al*., 2000; Buentke *et al*., 2001; Remondo *et al*., 2009). In the present study, upregulation of DC surface markers and cytokine secretion were, as expected,

variable among yeast genera. This observation might result from the particular composition of the cell wall of each yeast. This variability in DC responses might explain why some yeast species have been shown to induce T_H1-biased responses, such as *S. cerevisiae*, whereas others, such as *M. furfur*, have been reported to favour the production of T_H2-type cytokines (Buentke *et al*., 2001; Remondo *et al*, 2009). Also, murine DCs incubated with *C. albicans* yeasts have been shown to secrete IL-12 and prime T_H1 cells, whereas *C. albicans* hyphae inhibited T_H1 priming and led to a T_H2 response through induction of IL-4 production (d'Ostiani *et al*., 2000). One important finding of the present work was that all yeast genera analyzed led to release of IFN-γ and IL-12 by DCs, cytokines that drive T_H1 differentiation. Such outcome is desirable when considering the use of yeasts as vehicles to induce CTL responses. Nonetheless, it is not possible to affirm whether this scenario would also occur *in vivo*, since the microenvironment or cytokine milieu in the initial DC location in peripheral tissues may affect the antigen presentation in secondary lymphoid organs and T cell differentiation into the distinct T_H phenotypes (Liu *et al*., 1998).

The production of many pro-inflammatory cytokines has been linked to the NFκB signaling pathway. Recognition of β-glucans by Dectin-1 results in signaling through Syk kinase and Raf-1 pathways, inducing activation of NFκB and subsequently production of cytokines including IL-10, IL-1β, IL-6, IL-23, and GM-CSF (Rogers *et al*., 2005; Brown, 2011). TLRs detect several mannose-containing structures and also other components of the yeast cell wall yet to be determined. Nonetheless, the role of each TLR in immunity against fungi still remains to be determined, due to the large amount of contradictory literature for almost every TLR and fungus (Brown, 2011). Stimulation of TLRs also triggers the activation of signaling pathways and leads, for example, to the production of TNF-α, IL-8 and IL-12 (as a result from NFκB activation via MyD88-Mal) and type I IFNs (Netea *et al*., 2008; Romani, 2011). Dectin-1 has been shown to augment specific TLR-mediated signaling. As a consequence, production of TNF-α is enhanced due to a synergistic effect between Dectin-1 and TLR2 (Gantner *et al*., 2003). In contrast, these collaborative responses mediated by Dectin-1 and TLR2 lead to down-regulation of IL-12 expression in comparison to the levels obtained only by TLR ligation (Dennehy *et al*., 2009).

The signaling pathway activated by MR has not been completely elucidated since no classical signaling motifs are present in the cytoplasmic tail of this receptor (Willment and Brown, 2007). However, MR has also been demonstrated to elicit NFκB

activation and consequent cytokine production, including IL-12, IL-1β, IL-6, IL-10, GM-CSF, and TNF (Pietrella *et al*., 2005; Brown, 2011).

The recognition pathway of chitin has not been intensively studied, as well as its function in fungal infection (Netea *et al*., 2008). No study showing the specific role of chitin in DC maturation caused by entire yeasts or hyphae could be found. Nevertheless, it has been suggested that the signalling pathways involved after chitin recognition includes NF-κB (via TLR2 and Dectin-1) for TNF production as well as Syk and MR for IL-10 production (via Dectin-1-dependent and TLR2-dependent and -independent pathways; Da Silva *et al*., 2009).

The set of cytokines expressed by human DCs is based on the condition of activation and the cell subset (de Saint-Vis *et al*., 1998). Distinct DC subsets influence the initiation of adaptive antifungal responses. T_H17 and T_H2 cell responses towards fungi are initiated by inflammatory DCs, while tolerogenic DCs stimulate T_H1 and regulatory T cell responses. Prevailing T_H1 cell responses are associated with protective antifungal immunity and efficient vaccines against fungal infections. T_H1 cell activation can result from DC responses to a combination of signals provided by fungi via CLRs and TLRs (Romani, 2011).

Some studies analyzing the immune response against isolated yeast components have been conducted. Huang *et al*. (2009) showed that both particulate and soluble *S. cerevisiae*-derived β-glucans led to the production of high levels of TNF-α by DCs, while IL-12p70 levels were undetectable. Sheng *et al*. (2006) could demonstrate that mannan from *S. cerevisiae* induced maturation of murine DCs and cytokine production (including IL-6, IL-1β, GM-CSF and TNF-α). Production of IL-12, TNF-α and IFN-γ by murine spleen cells in response to chitin has been demonstrated (Shibata *et al*., 1997). Another investigation reported IL-10 and TNF-α secretion by murine macrophages after treatment with chitin particles (Da Silva *et al*., 2009). Moreover, this latter study showed that the effects observed depend on chitin size, since distinct combinations of receptors (such as TLR2, Dectin-1, and MR) and, consequently, different signaling pathways, were activated by different-sized chitin particles. However, it is complicated to draw conclusions about the role of each cell wall component in DC stimulation since, for a given cytokine, additive, antagonistic, and irrelevant effects can be observed when the combination of all yeast PAMPs is compared to the contribution of each individual PAMP (Huang *et al*., 2009). Moreover, it is tempting to speculate that the extent of DC activation caused by each stimulus is

strongly linked to the DC receptors involved in recognition of the referred antigen. In this way, since the composition of the yeast cell wall greatly varies among the yeast genera, the participation of a given receptor may also differ from one case to another.

The findings reported in this work indicate that all the yeast genera analyzed induce DC activation responses that *in vivo* would most probably lead to migration to secondary lymphoid organs and interaction with T lymphocytes for antigen presentation. Moreover, in an *in vivo* situation, a large spectrum of fungal PAMPs would be displayed in variable concentrations, leading to stimulation of several host PRRs. In this way, combinations of diverse ligands would result in complex patterns of inflammatory responses. The type of receptors stimulated in response to fungi and the extent of this stimulation strongly influence the nature of the immune responses (Levitz, 2010). In sum, the results of this work support the usage of yeasts as promising delivery system and suggest that different yeasts might lead to distinct responses. Furthermore, although each PRR alone stimulates the production of a particular pattern of cytokines (among other responses), the mechanisms by which all this is integrated remain to be determined (Brown, 2011).

2. Staining of yeast cell wall components

Stimulation of T lymphocytes in response to fungi is credited to fungal cell wall components (Heintel *et al*., 2003). Moreover, several studies have demonstrated the immunomodulatory effects of mannan and β-glucan (Suzuki *et al*., 1989; Toda *et al*., 1997). Particulate yeast β-glucan has been successfully used to stimulate robust CTL responses for combating tumors in animal models (Li *et al*., 2010; Qi *et al*., 2011). Li *et al*. (2010) demonstrated that yeast-derived, particulate whole glucan particles led to production of IL-12 and IFN-γ in the tumor environment and elicited antitumor T_H1 responses. Clinical trials combining β-glucan with antitumor therapy have also been conducted (ClinicalTrials.gov ID: NCT005455459). Alternatively, purified glucan particles from *S. cerevisiae* were used as vaccine delivery system, eliciting T cell responses, production of IFN-γ and IL-17 and secretion of antigen-specific antibodies (Huang *et al*., 2010).

Strategies exploiting yeast mannosylation of antigens for augmented immunogenicity have been aimed (Lam *et al*., 2005; Luong *et al*., 2007). Antigen mannosylation by yeast promoted increased antigen-specific $CD4^+$ and $CD8^+$ T cell-

mediated immune responses (Lam *et al*., 2005). Alternatively, mannan coating of a liposome preparation containing a DNA vaccine led to the activation of T_H1 cells (Toda *et al*., 1997).

Little has been investigated about the adjuvant effects of chitin. This component of the yeast cell wall has not been a focus of intense research, maybe due to the fact that it is a minor constituent of the fungal cell wall and has been, in some cases, not clearly identified in the cell wall of some yeast species (Arellano *et al*., 2000). However, chitin has been demonstrated to enhance antigen-specific T cell proliferation and to possess adjuvant properties in T_H1, T_H2 and T_H17 immune responses, as well as in IgE production (Da Silva *et al*., 2010).

Recently, *S. cerevisiae* has been employed as a complex antigen vaccine to induce cross-protection against systemic *aspergillosis* and *coccidioidomycosis* (Capilla *et al*., 2009; Stevens *et al*., 2011; Liu *et al*., 2011). Subcutaneous administration of heat-killed yeast protected mice against lethal systemic coccidioidomycosis challenge (Capilla *et al*., 2009). This protection was conferred by adaptive immune responses, which were mainly mediated by $CD8^+$ T cells and also involved production of IFN-γ, IL-6 and IL-17 (Liu *et al*., 2011).

Secretion and glycosylation of proteins, as well as the cell wall composition, differ among yeast genera. The Golgi apparatus of *S. cerevisiae* diverges significantly from those of *Sz. pombe* and *P. pastoris*, which resemble more closely the ones of higher eukaryotes. For instance, many *S. cerevisiae* glycans bear terminal α-1,3-mannose, whereas some mannose structures contain terminal α-galactose in *Sz. pombe*, α-1,2-linked mannose in *P. pastoris* and *N*-acetylglucosamine in *K. lactis* (Glick, 1996; Varki *et al*., 2009). Treatment of various fungal particles with Con A-FITC followed by flow cytometry or microscopic analyses does not allow distinction of these diverse structures.

Luong *et al*. (2007) showed that immunogenicity of an antigen recombinantly expressed by *P. pastoris* was enhanced by O-linked mannosylation and decreased by N-linked mannosylation, but the mechanisms by which this occurs are still unclear. An interesting aspect is that N-linked glycans in *Pichia* have a high-mannose configuration, whereas O-linked glycans are short (generally 2-3 mannose residues; Trimble *et al*., 2004; Luong *et al*., 2007). This fact might explain the observation that the *S. cerevisiae* BY4742 cell-wall mutants caused, in general, higher upregulation of DC surface markers and cytokine secretion in comparison to their wild-type counterpart. Since

Mnn11p is thought to function in outer chain elongation by adding α-1,6-mannose, the Δ*mnn11* mutant produces proteins with shortened, truncated mannan backbones (Dean, 1999). Yeasts lacking functional *OST3* gene have been described to underglycosylate soluble and membrane bound glycoproteins (Karaoglu *et al*., 1995). In this way, not only the type, but also the length of glycans seems to influence the host's immune responses.

Different β-glucan compositions also lead to distinct biological effects, as seen for zymosan, which contains 12-14% β-glucan, and purified whole glucan particles, composed of > 85% β-glucan (Li *et al*., 2010). Yeasts exposing more β-glucan should lead to increased Dectin-1-driven signaling. Mannan and α-1,3-glucan prevent or decrease β-glucan recognition via Dectin-1 (Rappleye *et al*., 2007; Netea *et al*., 2008). Therefore, it would be expected that yeasts bearing mutations in genes that result in reduction of mannan would promote different responses than yeasts containing, for instance, a dense mannan layer and α-1,3-glucan, such as *Sz. pombe*. With respect to chitin, it has been shown that this polymer associates with bud necks at the end of yeast cell division and become component of bud scars (Rodrigues *et al*., 2008). This fact might explain why chitin was poorly detected in fission yeast. The much higher percentage of cells positive for chitin in *S. cerevisiae* cell wall mutants compared to the parental strain might result from a compensatory mechanism to maintain cell wall stability, as hypothesized by Uccelletti *et al*. (2006). They observed increased chitin amounts in *K. lactis* mutants with reduced mannoprotein contents compared to wild-type.

It has to be pointed out that the yeast cell wall is a highly dynamic structure and, therefore, antigenically inconstant. Factors such as strain, age of cultured cells and type of growth medium have been described to exert influence on yeast antigenicity, *i.e.* on the display of epitopes on the cell suface (reviewed by Nelson *et al*., 1991). Thus, fluorescence detection of mannan and glucan as performed in this work may diverge from one experiment to another, also when attempts to keep the same experimental conditions are made. The different patterns of cell wall staining observed here are in accordance with literature data, which show that massive differences can be observed when the cell walls of different fungal species and also strains within a species are compared (Levitz *et al*., 2010).

To date, distinct molecules have been identified as receptors for mannose or β-glucans on APCs, but information about how each receptor discriminates its ligands is

still missing (Qi *et al*., 2011). Additionally, and as stated before, glycan structures vary from one yeast strain to another and the simultaneous presence of several yeast PAMPs as in whole yeasts may lead to different responses than those observed against each particular PAMP separately (Huang *et al*., 2009). In this work, staining of yeast cell wall components was performed in an attempt to determine if there was a causal relation between the yeast cell wall and DC maturation or activation. The results suggest that no direct correlation could be established between the mannan, β-1,3-glucan and chitin contents on the surface of yeast cells and the degree of stimulation of human DCs. This fact was not surprising by virtue of the dynamic nature of yeast cell walls and the consequent fluctuation in glycan contents.

3. Interaction patterns between yeasts and mammalian phagocytic cells

Immune recognition of fungal particles takes part mostly at the cell wall level. While the yeast cell wall consists of an interconnected structure of polysaccharides, glycoproteins and some glycolipids, mainly carbohydrate structures are involved in the interaction between yeast and mammalian host cells.

The findings reported in this study show that the interactions between human DCs and the biotechnologically relevant yeasts *S. cerevisiae, Sz. pombe, K. lactis and P. pastoris* are mediated by both mannan and glucan. Specific blocking of MR and Dectin-1 in DCs was able to impair, to a greater or a lesser extent, host cell binding of all yeast species tested. In general, yeast cell wall consists of an outer layer of mannosylated proteins and an inner layer of β-glucans and chitin. However, β-glucans may be permanently exposed at the surface in specific regions like bud and birth scars, which is sufficient for Dectin-1 sensing (Gantner *et al*., 2005). Specific blockage of Dectin-1 led to a greater reduction in yeast binding compared to MR blockage in three out of four species tested (*S. cerevisiae*, *Sz. pombe*, and *P. pastoris*). Interestingly, and in agreement with its low phylogenetic relationship to other yeast genera and its notable difference in cell wall architecture (Russell and Nurse, 1986), uptake of *Sz. pombe* by DCs was almost completely abrogated after anti-Dectin-1 treatment. The fission yeast β-D-glucan repertoire is basically composed of β-1,6-branched β-1,3- and β-1,6-glucans, while linear β-1,3-glucan is absent in the cell wall and exclusively found in the primary septum (Humbel *et al*., 2001). However, since Dectin-1 recognizes both β-1,3- and β-1,6-glucans (Brown and Gordon, 2001), the lack of linear β-1,3-glucan structures

in *Sz. pombe* does obviously not confer any advantage for this microorganism in terms of being less recognizable by Dectin-1. With respect to MR, blocking of this receptor does not mean that yeast mannan is no longer recognized, since alternative mannose receptors are present on the surface of DCs, such as DC-SIGN (Cambi *et al*., 2003).

As observed for human DCs, the kinetic pattern of *Sz. pombe* internalization was different from the other three yeast species analyzed. *S. cerevisiae*, *K. lactis* and *P. pastoris* are round-shaped yeasts, in contrast to the rod-like shape of fission yeast. It has been demonstrated that phagocytosis depends on both particle size and shape, and particles requiring only gradual actin ring expansion are more efficiently phagocytosed (Champion and Mitragotri, 2006). Since *Sz. pombe* was taken up more slowly, possibly the morphology of fission yeast does not favour internalization in such a rapid manner as for the other three yeast species analyzed.

Since IC-21 mouse macrophages do not express the mannose receptor, only the effect of Dectin-1 on yeast uptake was analyzed in this work. Lack of MR in some murine macrophage cell lines, such as J774 and RAW264.7, as well as resident peritoneal mouse macrophages has been described (Taylor *et al*., 2002; Martinez-Pomares *et al*., 2003; Lin *et al*., 2010). Incubation of IC-21 macrophages with anti-Dectin-1 diminished yeast uptake, indicating that recognition of yeasts by IC-21 cells involves Dectin-1. This interaction was specific, since addition of an isotype control antibody to the cells led to similar results than those for untreated cells. The differences among yeast genera observed after Dectin-1 blocking might reflect the amount of exposed β-glucan on cell walls of each genus.

Uptake of *S. cerevisiae* and *Sz. pombe* in whole blood has been analyzed (Breinig *et al*., 2003), with no significant differences among both yeasts. However, yeasts are avidly internalized in whole blood by a great sort of phagocytic cells, including neutrophils and monocytes, so that the different systems cannot be directly compared.

An important characteristic of the mannose receptor is that it recycles to the cell surface after having released its ligand. Thus, ligands can be delivered in much larger amounts than the number of receptors, which provides an unlimited antigen accumulation capacity (Stahl *et al*., 1980; Sallusto *et al*., 1995). Antigens internalized by MR might, after receptor dissociation, be trafficked to lysosomes rich in MHC class II molecules (Tan *et al*., 1997; Keler *et al*., 2004). Moreover, it has been demonstrated that mannosylated peptides are more efficiently presented to antigen-specific T cells

than non-mannosylated proteins (Engering *et al*., 1997; Tan *et al*., 1997). In this way, MR targeting has become an attractive approach to increase vaccine efficacy (Keler *et al*., 2004).

Dectin-1-mediated uptake has also been demonstrated to enhance antigen presentation via MHC class II and increase antigen-specific T cell activation, as well as cross-presentation (Xie *et al*., 2010; Weck *et al*., 2008). Other C-type lectin receptors on DCs, such as DC-SIGN, have also been targeted in order to improve antigen presentation (Adams *et al*., 2008) and consequently augment vaccine efficiency.

Therefore, and according to the findings of this work, yeasts naturally target CLRs on phagocytes, including macrophages and DCs, so that vaccination approaches using whole yeasts are of significant potential.

4. Expression and delivery of different Ova variants by yeasts

One line of investigation in this work was to study the efficiency of different yeast genera as protein delivery vector, and to analyze the influence of subcellular antigen (Ova) localization on its processing and presentation in context of MHC class I to specific $CD8^+$ T lymphocytes. An interesting question was to evaluate the antigen presentation efficiency of SIINFEKL epitope when Ova was located in different yeast compartments. In order to get basic information which system would be more advantageous in terms of vaccination approaches, recombinant *S. cerevisiae*, *P. pastoris*, *Sz. pombe*, and *K. lactis* expressing either full-length Ova, which is destined for secretion, and a truncated version of this protein, Ova_{cyt}, which lacks the internal secretion signal and remains intracellular, were used. Given that yeast cell-surface display is an attractive tool in vaccine approaches, as surface-anchored epitopes are stably maintained due to the rigid, thick cell wall architecture (Kim *et al*., 2010), a cell-wall anchoring system, in which Ova_{cyt} is exposed on the yeast cell surface of *S. cerevisiae* and *P. pastoris* as Sed1p fusions was also tested. The presence of Ova_{cyt} in the cell wall of *K. lactis* and *Sz. pombe* could not be detected (data not shown). No data was found in the literature showing the functionality of Sed1p from *S. cerevisiae* in *K. lactis*, and this type of anchoring system is likely to be inefficient in *Sz. pombe* due to its particular cell wall composition. *K. lactis* was transformed with another Sed1p-containing fusion protein, but the presence of the recombinant protein on cell surface was again undetectable (data not shown), indicating that the foreign protein was not the

cause of the anchoring failure. Cell-surface display of a recombinant protein (α-galactosidase) as α-agglutinin fusion has been described for *K. lactis* (Schreuder *et al*., 1996b), but to date no other GPI-anchoring system has been reported for this species.

Expression levels of cytosolic, secreted, and cell wall-anchored Ova varied drastically among the yeast genera tested in the present study. However, it is difficult to directly compare productivity between different yeast genera, since each expression system has its particularities and variations in plasmid copy number and stability, as well as codon bias index, influence recombinant protein levels (Buckholz and Gleeson, 1991).

In relation to the surface-display of Ova in *S. cerevisiae* and *P. pastoris*, it was observed that, although cell-surface expression could be detected by immunofluorescence and flow cytometric analyses, it was heterogeneously distributed over the cell population. This observation has been repeatedly reported for *S. cerevisiae* (Schreuder *et al.*, 1993; Schreuder *et al*., 1996a; Breinig and Schmitt, 2002; Feldhaus *et al*., 2003, Kim *et al*., 2010). In one study, it was hypothesized that this staining pattern could be attributed to variations in plasmid copy number and expression levels among individual cells, or even to differences in cell physiology (Kim *et al.*, 2010). Another reason for the observed uneven distribution of cell surface expressed proteins could be the polarized growth and varying morphology in yeast, so that mother cells do not express the fusion on their cell walls (Feldhaus *et al*., 2003).

Fusion of Ova_{cyt} to Gag led to the formation of abundant VLP which were homogeneous in size and shape, as confirmed by electron microscopy. Unfortunately, *S. cerevisiae* expressing Gag/Ova_{cyt} VLP could not be analyzed in the T-cell activation assay so far. However, this very promising approach can be useful in future applications. Crisci *et al.* (2009) reported that VLP from rabbit hemorrhagic disease virus delivering the Ova SIINFEKL epitope led to the induction of specific cytotoxic and memory T cells responses. Moreover, mice immunized with these chimeric VLP could resolve infection caused by a recombinant vaccinia virus expressing Ova (Crisci *et al.* 2009). These findings reaffirm the potential of VLP in vaccination approaches.

Activation of Ova-specific $CD8^+$ T lymphocytes, determined by the amount of released IFN-γ in supernatants, was also very variable among the yeast genera used. The data from this work suggest that the most efficient antigen loading strategy by *S. cerevisiae* and *P. pastoris* is cytosolic and cell surface bound antigen delivery, respectively. In contrast, secreted Ova caused only a weak activation of lymphocytes in

S. cerevisiae and hardly any in *P. pastoris*. This latter finding was unanticipated, because *in vitro* activation of antigen-specific T cells after delivery of proteins secreted by gram-positive bacteria has been demonstrated (Ikonomidis *et al.*, 1994; Pan *et al.*, 1995; Soussi *et al.*, 2000; Loeffler *et al.*, 2006). Furthermore, protein secretion most likely still occurs in macrophage phagolysosomes (as shown by western blotting after cultivating yeasts for 8 h in acetate-containing medium), indicating that the lack of lymphocyte activation observed in this case was not related to the absence of Ova in macrophages. A significant amount of protein, however, might be subsequently degraded in phagolysosomes and thus become unavailable for subsequent cytoplasmatic antigen processing steps. In contrast, antigen bound to the cell wall or antigen residing in the cytoplasm of the yeast cell might be more efficiently protected from degradation and thus be more efficiently cross-presented.

Cell-surface displayed Ova caused lymphocyte activation only when *P. pastoris* cells were used in the assay, and it proved to be better than cytosolic Ova at the highest MOI tested in this species. This observation confirms data from Howland and Wittrup (2008), who compared the cross-presentation of an antigen derived from the human cytomegalovirus phosphoprotein pp65 displayed on the cell-surface (as Aga2-fusion) or intracellularly expressed in *S. cerevisiae*. They concluded that antigen exposed on the yeast external surface, more accessible to proteases, is more efficiently cross-presented than antigen trapped inside the yeast cell wall, after observing that cross-presentation of the epitope expressed in the yeast cytoplasm by DCs was half of that from surface-anchored antigen, although the expression level of the recombinant peptide was much higher in the cytosol than on the cell surface. Thus, this seems also true for *Pichia pastoris*. However, the very weak T cell activation after cell-surface expression in *S. cerevisiae*, in particular compared to the intracellular derivative, is somehow surprising and is not consistent with the hypothesis of Howland and Wittrup. In this respect, both the slightly higher antigen amount in the cell wall of *Pichia* and the higher number of Ova-expressing cells within the population in comparison to *S. cerevisiae* could play a role. Alternatively, this observation may be explained by differences in the type of cell wall protein used for cell wall targeting. Whereas the Ova_{cyt}/Sed1p fusion used in our study is covalently linked to and embedded in the cell wall, Howland and Wittrup anchored their antigen via disulfide bonds to the yeast cell surface allowing the antigen to be released under reducing conditions.

The differences in T cell activation seen in this study can be attributed to many distinct factors. For example, it might be related to the levels of recombinant protein expressed by each yeast clone. However, it is not clear whether higher levels of antigen in a specific compartment necessarily lead to an increase in antigen-specific lymphocytic activation. For example, *S. cerevisiae* expressed 1.4 fold more Ova_{cyt} than *P. pastoris*, but the level of IFN-γ in the culture supernatant after lymphocytic activation was about 12.5 fold greater at MOI 7 in the case of the budding yeast, which suggests that other factors, like the particular composition of the yeast cell wall, might also be involved. MHC I-presentation of a certain CTL epitope in infected cells is affected, among other factors, by the degradation rate of the antigen in the cytoplasm and the efficiency with which the peptide is processed from the antigen by cellular proteases (Sijts *et al*., 1996). Ovalbumin contains a single disulphide bridge between Cys73 and Cys 120 (Nisbet *et al*., 1981), whose lack results in a less stable and more fluctuating protein conformation in comparison to intact Ova (Ishimaru *et al*., 2011). Given that in the Ova_{cyt} coding sequence the fragment encoding amino acids 20-145 is missing, degradation of Ova_{cyt} might be somehow easier, contributing to better antigen processing and presentation, which is in agreement with the results observed so far. This diminished stability of Ova_{cyt} could also explain the lower levels quantified for this protein in comparison to secreted Ova after the same expression period. Lower Ova_{cyt} levels in comparison to secreted Ova have been reported as being the result of its greater proteolysis and consequently shorter half-life (Shen and Rock, 2004; Rowe *et al*., 2006).

A multitude of studies showing the use of whole yeast as vaccine vehicles expressing recombinant proteins intracellularly (Stubbs *et al*., 2001; Lu *et al*., 2004; Barron *et al*., 2006; Haller *et al.*, 2007; Riemann *et al*., 2007; Wansley *et al.*, 2008; Remondo *et al*., 2009; Tanaka *et al*., 2011) or on the cell surface (Schreuder *et al*., 1996a; Wadle *et al.*, 2006; Zhu *et al*., 2006; Kim *et al*., 2010) have been reported. Nevertheless, almost all of them employ the conventional yeast *S. cerevisiae* as carrier system. In this study, all species tested proved to be appropriate for delivering intracellularly expressed Ova, although the efficiency of antigen presentation varied. Delivery of cell surface-anchored Ova was more effective in *P. pastoris* than in *S. cerevisiae*, and none of these yeasts showed to be efficient at delivering secreted Ova.

Bacterial delivery has also been used in immunologic approaches. The microorganisms used for such strategies, including *Listeria, Salmonella* and *Yersinia,*

are generally attenuated or contain a suicide gene that promotes bacterial destruction (Husseiny and Hensel, 2005; Wiedig *et al*., 2005; Loeffler *et al*., 2006). However, application of bacteria into a mammal usually implies unpredictable side effects that can only be seriously examined in large clinical trials (Pálffy *et al*., 2006). The use of non-pathogenic yeasts is therefore safer, and the benefits of using eukaryotic microbes, which can express a recombinant polypeptide in a manner that closely resembles the native protein, should also be taken into consideration.

Until now there is no study comparing the efficiencies of different yeast genera routinely used in biotechnology as protein delivery vectors on the activation of antigen-specific lymphocytes. Data from this work show that, besides *S. cerevisiae*, three additional yeast genera with biotechnological importance are able to deliver a protein antigen to mammalian antigen-presenting cells and to subsequently activate specific T cells, providing usefull basic knowledge for the design of superior yeast-based vaccination vectors. However, since peptide sequence *per se* has been described to exert influence on the efficiency of antigen presentation (Sijts *et al*., 1996), the most convenient approach for a specific therapeutic purpose has to be evaluated individually.

5. Expression of pp65 by different yeast strains and activation of pp65-specific memory T lymphocytes in HCMV-seropositive donors

Some advantages in choosing HCMV as model virus are that host specificity can be simply verified, validating the experiment (Suni *et al*., 1998) and that pp65-specific T cells are highly prevalent among HCMV-seropositive induviduals (Kern *et al*., 2002). The decision to use the entire pp65 protein instead of single $CD4^+$ and $CD8^+$ epitopes was based on the fact that some HCMV-seropositive individuals do not respond to some dominant epitopes, and response to subdominant or unpredicted epitopes might also occur (Solache *et al*., 1999, Kondo *et al*., 2004). Furthermore, immunodominant epitopes from viral proteins frequently undergo mutations *in vivo*, reducing their efficacy as therapeutic target (Bertoletti *et al*., 1994; Solache *et al*., 1999). Therefore, use of an entire polypeptide may provide a wide spectrum of epitopes deriving from the referred protein, increasing the odds that epitopes relevant for the MHC repertoire from a certain individual will be presented (Bui *et al*., 2010). The HCMV pp65 tegument protein is a good vaccine candidate, since it resembles an immunodominant antigen for T cells, and 70-90% of all CTL which are able to recognize HCMV-infected cells are

specific for pp65 (McLaughlin-Taylor *et al*., 1994; Beninga *et al*., 1995; Wills *et al*., 1996; Kern *et al*., 2002).

Thus, full-length pp65 was used for recombinant expression in different yeast genera, which were subsequently tested for their ability to deliver pp65 in an *ex vivo* assay. Recombinant pp65 could be detected in cell lysates of *Sz. pombe*, *P. pastoris* and *S. cerevisiae*, but not in *K. lactis*. A number of *K. lactis* transformants grown in selective medium and positive for the presence of integrated pp65 was tested under varying inducing conditions, however with no success. Some protease-deficient strains of *K. lactis* were also analyzed (YCT 389, YCT390, YCT569, YCT598, New England Biolabs), but the presence of pp65 in cell lysates could not be detected, indicating that the protein was either expressed under the detection level, or rapidly degraded or not expressed at all (data not shown).

The fact that pp65 could not be secreted into the culture medium by *Sz. pombe*, *P. pastoris* and *S. cerevisiae* might suggest that this protein is somehow not properly processed in the secretory pathway. Cytoplasmic retention of recombinant proteins that were engineered to be secreted has been reported (Emr *et al*., 1984; Chaudhuri and Stephan, 1995). As a consequence to this problem, cell wall-anchoring of whole pp65 also turned out inviable. Recombinant full-length pp65 expression in yeast has only been demonstrated in *Sz. pombe* (Breinig *et al*., 2003). In contrast, successful expression of pp65 epitopes in the cytoplasm or even on the cell surface *S. cerevisiae* as Aga2-fusion has recently been reported (Powilleit *et al*., 2007; Howland and Wittrup, 2008).

Whole recombinant yeasts expressing the HCMV pp65 matrix protein in the cytosol were assessed for protein delivery efficiency in a whole blood assay. It has been shown that full-length pp65 expressed in fission yeast or a fragment containing the major pp65 epitopes alone or in combination with Gag expressed in *S. cerevisiae* could elicit specific cellular immune responses, with higher frequencies of $CD8^+$ than $CD4^+$ T cells (Breinig *et al*., 2003; Powilleit *et al*., 2007).

It has also been reported that yeasts are rapidly internalized by blood phagocytes and efficiently processed for presentation to $CD4^+$ and $CD8^+$ T lymphocytes (Breinig *et al*., 2003; Heintel *et al*., 2003). In relation to pp65, antigen-specific memory $CD4^+$ T cells were stimulated by untreated and heat-treated pp65-expressing cells of *S. cerevisiae*, *Sz. pombe*, and *P. pastoris*. Significant IFN-γ expression by pp65-specific $CD8^+$ T lymphocytes was detected after incubation with untreated and heat-treated *Sz.*

pombe and heat-treated *P. pastoris*, whereas in *S. cerevisiae* neither untreated nor heat-treated cells caused any significant activation.

Heat treatment was particular advantageous for *P. pastoris*, in terms of both $CD4^+$ and $CD8^+$ T cell responses. In the case of *S. cerevisiae*, pp65-specific $CD4^+$ responses were more pronounced after heat treatment, whereas $CD8^+$ responses were lower for heat-treated yeasts, but the relation pp65/empty plasmid was higher in comparison to untreated cells of *S. cerevisiae*. For *Sz. pombe*, lower frequencies of activated lymphocytes were observed when cells expressing pp65were heat-treated, however the ratio pp65/empty vector was higher after heat treatment. This means that lower responses to the yeast vehicle were detected after heat treatment. Since T cell responses to yeasts is uniquely based on the recognition of epitopes derived from cell wall proteins (Heintel *et al*., 2003), mannosyl rather than glucosyl residues should play the most important role. Upon heat treatment, the β-glucan layer turns out to be densely and homogeneously exposed on yeast cell surface, possibly masking mannoproteins from being promptly processed and presented by APCs after yeast uptake. T cell activation caused by *C. albicans* supports this hypothesis. Heintel *et al*. (2003) showed that T cell responses to the unicellular form of *C. albicans* were poor, in contrast to the robust responses directed against the hyphal form. Since β-glucan is not functionally accessible on *Candida* filaments (Gantner *et al*., 2005), it is tempting to speculate that mannoproteins dictate the activation of memory T cells. For this reason, yeasts alone subjected to incubation at 65°C caused a lesser extent of lymphocyte activation than untreated yeasts.

When *S. cerevisiae*-delivered Gag/pp65 was analyzed, significant activation of both $CD4^+$ and $CD8^+$ T lymphocytes was observed, and IFN-γ levels were much higher than those caused by pp65 expression in the same yeast strain, although the amount of delivered protein was practically the same. This observation is in agreement with other reports, in that particulate antigens are highly efficient in inducing robust immune responses (Powilleit *et al*., 2007; Crisci *et al*., 2009). This fact can be attributed to the repetitive occurrence of epitopes and the particulate structure of VLP, which favour uptake by phagocytic cells (Fifis *et al*., 2004; Grgacic and Anderson, 2006). It has been shown that internalization of yeast-derived VLP by DCs occurs via macropinocytosis and receptor-mediated endocytosis involving mannose-recognizing receptors, though not the mannose receptor (Tsunetsugu-Yokota *et al*., 2003).

Although electron microscopy of pp65 N-terminally fused to Gag revealed the presence of only a few particles, which were heterogeneous in size and shape (maybe due to the large size of pp65), the fusion protein nevertheless was more efficient in stimulating pp65-specific memory T lymphocytes than pp65 alone. Heat treatment was slightly beneficial for $CD4^+$ responses, and exactly as observed for *S. cerevisiae* carrying unfused pp65, $CD8^+$ responses were a little lower for the heat-treated yeasts, but the relation Gagpp65/Gag was higher in the heat-treated yeasts. VLP have repeatedly proven their potential as vector for vaccination and demonstrated to be powerful CTL inducers compared to adjuvants and/or live recombinant vectors (Allsopp *et al*., 1996; Crisci *et al*., 2009). For this reason, strategies combining whole yeasts and VLP as antigen carrier should be an prospective attempt.

Responses to the HCMV lysate were notedly higher than any condition using yeast. However, this lysate contains all HCMV proteins and, consequently, a large variety of $CD4^+$ and $CD8^+$ T cell epitopes available for antigen presentation.

Frequencies of specifically activated memory T lymphocytes were higher for $CD4^+$ cells than for $CD8^+$ cells in all samples analyzed, which is consistent with previous observations (Suni *et al*., 1998; Sester *et al*., 2001). In the case of HCMV, the frequency of $CD8^+$ T cells fluctuates significantly, whereas the frequency of the $CD4^+$ T cell population is more stable. In addition, $CD4^+$ T cells are considered more important than $CD8^+$ T cells in terms of disease prediction (Sester *et al*., 2001).

The lower frequencies of $CD8^+$ T lymphocytes observed in this work may also result from the presence of Brefeldin A, which has been described to inhibit MHC class I-restricted presentation of some protein antigens (Yewdell *et al*., 1989; Brossart and Bevan, 1997). Although contradictory effects have been observed, the pathway through which antigens are presented in the context of MHC class I seems to play a critical role in Brefeldin A sensitivity. Antigens processed by the phagosome-to-cytosol pathway are loaded onto newly synthesized class I MHC molecules prior to their egress from the trans-Golgi complex. Brefeldin A hinders vesicular exit from the ER and Golgi, blocking the presentation of peptide-MHC I complexes (Yewdell *et al*., 1989; Kovacsovics-Bankowski and Rock, 1995; Brossart and Bevan, 1997). The vacuolar pathway seems to be Brefeldin A-insensitive (Harding and Song, 1994; Pfeifer *et al*., 1993), since peptides associate with recycling class I molecules in phagocytic vacuoles or after "regurgitation" and recapture of processed antigens (Harding and Song, 1994; Norbury *et al*., 1995). Tabi *et al*. (2001) showed that DCs cocultured with HCMV-

infected fibroblasts led to $CD8^+$ T cell responses to pp65, but $CD8^+$ T cell stimulation was inhibited if DCs were pretreated with Brefeldin A, indicating a phagosome-to-cytosol pathway. However, since the characteristics of the antigen might influence the pathway involved (Norbury *et al*., 1995), it is not possible to state whether Brefeldin A affected to any extent the results observed in the present work.

Low-level responses towards the yeast vehicle alone were sometimes observed, with varying extents among the donors and the yeast genera, but in all cases frequencies of activated lymphocytes were below 0.4%. In a study performed by Heintel *et al*. (2003), frequencies of yeast-specific memory T cells were determined using whole blood from five individuals. Activation of T lymphocytes was verified for most yeast genera, with frequencies up to 0.5% for $CD4^+$ T cells and up to 6.4% for $CD8^+$ T cells, depending on both the donor and the yeast species (Heintel *et al*., 2003). Despite some detectable response to the yeast carrier, frequencies of activated $CD4^+$ and $CD8^+$ cells were always higher for pp65-containing yeasts in the HCMV-seropositive donors, indicating host specificity.

Surprisingly, and in contrast to reports of Breinig *et al.* (2003), ethanol-precipitated total yeast extracts from cells expressing pp65, including *Sz. pombe*, were not able to sufficiently activate pp65-specific memory T lymphocytes. However, since pp65-specific memory T cell activation shows great variability among individuals (Kern *et al*., 2002; Breinig *et al.*, 2003; Powilleit *et al*., 2007), and giving that the donors analyzed in that study were different from those examined in this work, a direct comparison is not possible.

In this work, delivery of pp65 by yeasts was also analyzed by means of an autologous system, *i.e*., a method in which DCs from HCMV-seropositive individuals are isolated, incubated for 48 h with yeasts and then given autologous lymphocytes. However, despite many attempts, only poor $CD4^+$ and $CD8^+$ T cell activation could be observed, and this method proved to be less effective than the whole blood assay. Suni *et al*. (1998) compared frequencies of antigen-specific activated T cells in whole blood samples and isolated PBMC in autologous plasma. Since whole blood preparations resulted in higher responses, they hypothesized that, within the 6 h incubation time, whole blood cultures form an upper band of PBMC over a layer of red cells, probably promoting a more physiological millieu to respond to stimuli. This sedimentation pattern might also provide improved cell-cell interactions within the PBMC population, not influenced by the lower layer of red blood cells (Suni *et al*., 1998).

Significant activation of pp65-specific memory T lymphocytes after incubation with pp65-expressing yeasts, determined by means of both whole blood assay and autologous system, could not be detected for some HCMV-seropositive individuals. One possible explanation for this fact is that some HCMV-seropositive persons lack pp65-specific memory lymphocytes, as it has been reported by others (McLaughlin-Taylor *et al*., 1994; Kern *et al*., 2002). According to Kern *et al*. (2002), this lack of pp65-specific T lymphocytes in peripheral blood alone does not mean absence of protection from HCMV disease. In that study, they analyzed 40 healthy HCMV-exposed donors and 12.5% of them had no T cell response to peptides derived from pp65. These individuals were not sick, and HCMV reactivation presumably occured as often as in other healthy individuals (Kern *et al*., 2002).

In sum, all three yeast species (*S. cerevisiae, Sz. pombe, and P. pastoris*) carrying pp65 were able to deliver the antigen, and the activation of pp65-specific memory lymphocytes observed indicates that recombinant pp65 probably mimics the native antigen in terms of recognition by the host.

6. Effect of heat treatment of yeast on β-glucan exposure and ROS production in whole blood

"ROS" include several oxygen species, such as singlet oxygen, hydroxyl radical, superoxide and hydrogen peroxide. In most cell types, ROS influence signaling pathways in different manners (Fialkow *et al*., 2007). Production of ROS (respiratory burst) by phagocytes, a process mediated through the NADPH oxidase, is considered a major mechanism of defense against fungi. A number of PRRs can elicit the respiratory burst, such as Dectin-1, TLRs and Fcγ receptors (Brown, 2011).

According to previous studies, heat-treated *C. albicans* and *S. cerevisiae* expose more β-glucan at the cell surface than untreated cells (Fradin *et al*., 1996; Gantner *et al*., 2005). Gantner *et al*. (2005) observed that most β-glucan in the yeast cell wall is inaccessible to Dectin-1 and that β-glucan is exposed only in certain subdomains such as birth and bud scars. Upon heat treatment, matricial cell wall components are released (Fradin *et al*., 1996), bringing β-glucan uniformly to the outer layer and consequently increasing the chance of phagocyte sensing through β-glucan receptors.

The effect of heat treatment on β-glucan exposure in *S. cerevisiae*, *K. lactis*, *Sz. pombe*, and *P. pastoris* was analyzed by flow cytometry. All three budding yeasts

examined showed more than a two-fold increase in mean fluorescence after incubation at 65°C, indicating that heat treatment indeed enhances the amount of cell-wall exposed β-glucan. In contrast, this could not be observed in fission yeast, which showed even a slight reduction in mean fluorescence intensity. This observation was surprising, because although linear β-1,3-glucan is not found on the cell wall, it can be found in the primary septum of *Sz. pombe* (Humbel *et al*., 2001), so that at least a discrete increase in fluorescence would have been expected. However, the architecture of the cell wall in fission yeast might, for some reason, not allow significant β-glucan exposure upon heat treatment.

Untreated and heat-treated yeasts were then incubated with whole blood for determination of ROS production. Since recognition of β-glucan by Dectin-1 leads to the generation of ROS (Gantner *et al*., 2003), it was reasonable to suppose that yeasts subjected to heat would be more effective in eliciting ROS production due to augmented ligand availability. In fact, this was true for *S. cerevisiae*, *K. lactis* and *P. pastoris*. Noteworthy, the amount of ROS was lower when blood phagocytes were incubated with heat-treated *Sz. pombe* cells in comparison to untreated cells. Again, this result was unexpected, since the cell surface of this yeast contains β-1,6-branched β-1,3-glucan, which is also a target for Dectin-1 (Humbel *et al*., 2001; Adams *et al*., 2008; this work), so that at least a similar kinetic pattern of ROS production would have been expected for untreated and heat-treated fission yeast cells. These observations indicate that heat treatment may alter the distribution of cell wall components in *Sz. pombe* in a different fashion than in budding yeasts.

A series of pre-clinical and clinical studies has resorted to the use of heat-inactivated yeasts as protein delivery vectors, thus reducing the possible risks involved in using live cells, particularly in immunocompromised individuals (Lu *et al*., 2004; Franzusoff *et al*., 2005; Ardiani *et al*., 2010). Experiments employing either untreated or heat-treated yeasts have proven to be effective in terms of protective immunity (Stubbs *et al*., 2001; Franzusoff *et al*., 2005). Results from the whole blood assays performed in this work showed that heat treatment was beneficial in some cases, such as when *P. pastoris* was used as vector, but not always, as when *Sz. pombe* cells delivered the antigen. This fact indicates that the choice for such treatment should take into consideration the cell wall features of the yeast species being used in the specific approach. Nonetheless, a higher number of assays would have to be performed in order to confirm this hypothesis.

7. Immunization of OT-I transgenic mice with whole recombinant yeasts

Initiation of immune responses after vaccination depends on several factors, such as antigen dose, form of administration, antigen access to processing pathways, co-stimulation and cytokine milieu at the moment of antigen capture and presentation (Rush *et al*., 2002).

OT-I mice constitute an important model system for *in vivo* approaches. Analyses with mice bearing TCR-transgenic T cells eliminate the problem of low frequency of antigen-specific T cells and allow the isolation and quantitation of T cells as well as the determination of *in vitro* and *in vivo* antigen-specific responses (Miyagawa *et al*., 2010). In an attempt to examine $CD8^+$ T cell responses after administration of yeast expressing Ova_{cyt} to mice, an immunization protocol was established based on literature data, especially on Riemann *et al*. (2007). However, and in contrast to other works, yeasts were not heat-treated or frozen, but lyophilized to facilitate shipment. Since the majority of the studies published so far describing immunization approaches using yeast employ *S. cerevisiae* W303, this strain was also used for this experiment. Protocoll optimization and utilization of other yeast genera, as well as of a higher number of mice could no longer be performed in this work.

Splenic $CD8^+$ T lymphoctes from mice vaccinated with *S. cerevisiae* carrying Ova_{cyt} restimulated with $OVA_{257-264}$ peptide were able to produce IFN-γ. Yeasts alone also stimulated OVA-specific $CD8^+$ T lymphocytes, although to a lesser extent, still the difference between both conditions was not significant. Spleen $CD8^+$ T lymphoctes restimulated with PMA/ionomycin showed the highest levels of IFN-γ, but the difference among the groups was even more discrete. Since PMA and ionomycin are potent inducers of cytokine production (Picker *et al*., 1995), lymphocytes were strongly and non-specifically activated, so that eventual subtle differences among experimental groups could have been masked. Inguinal lymph node cells were stimulated only with PMA/ionomycin due to the low number of recovered cells, and activation of $CD8^+$ T lymphocytes from mice immunized with yeasts carrying Ova_{cyt} was even lower than in the PBS control group. This latter result was unexpected, since most of the subcutaneously administered yeasts are taken up by monocyte-derived inflammatory DCs (or, to a lesser extent, by skin-derived DCs) and then transported to draining lymph nodes (Ersland *et al*., 2010). However, Wang *et al*. (2003) demonstrated that non-self peptides, including SIINFEKL, led to the generation of higher responses in the spleen

than in draining lymph nodes after s.c. immunization of mice with antigens mixed with adjuvant or pulsed on DCs.

Particulate antigens are efficiently cross-presented *in vivo* and are likely to stimulate robust CTL responses. Particles larger than 1 μm are taken up by phagocytosis. The higher efficiency with which particulate antigens are cross-presented in comparison to soluble antigen arises from the fact that (i) the amount of antigen internalized by phagocytosis is much greater than when soluble proteins enter APCs by fluid-phase pinocytosis, and (ii) antigens internalized by phagocytosis are thought to access the cross-presentation pathway more efficiently (reviewed by Rock and Shen, 2005). Since yeast carrying Ova_{cyt} is also a form of particulate antigen, cross-presentation would be expected to occur, also when the antigen amount was not very high.

Despite the existence of several studies showing yeast as antigen carrier in immunization experiments, there is no data about IFN-γ production by antigen-specific $CD8^+$ T lymphocytes, which might reflect the complexity of this type of analysis. However, the results obtained after incubation of spleen cells with $Ova_{(257-264)}$ peptide indicate that yeasts were internalized and Ova_{cyt}-derived SIINFEKL could be processed for cross-presentation to $CD8^+$ T lymphocytes. The low lymphocyte activation observed after restimulation of splenocytes with $Ova_{(257-264)}$ peptide was most probably not due to the presence of Brefeldin A, since it has been shown that DC treatment with this substance had no influence on presentation of exogenously added SIINFEKL (Brossart and Bevan, 1997). Western blot analysis of the yeasts used in this immunization protocol revealed low expression of Ova_{cyt} (approximately 17 ng in 5×10^7 cells, data not shown), which might have difficulted the detection of more significant responses. Nevertheless, proliferation of $CD8^+$ T lymphocytes in response to Ova has been demonstrated with as little as 0.2 ng antigen when delivered in a cell-associated form (Li *et al*., 2001). Maybe determination of the frequency of IFN-γ-producing cells is not a very sensitive method to detect $CD8^+$ T cell responses when the antigen amount is low, so that analysis of T cell proliferation would, for example, represent an alternative method for such immunization experiments.

Nevertheless, these experiments clearly show the potential of whole, live yeast as antigen carrier and suggest that protocol optimization would most probably result in more convincing results. Moreover, the use of other yeast genera as examined

throughout this work could lead to the establishment of novel whole yeast-based vaccines.

8. Perspectives

Approaches using yeasts as vaccine vehicle exhibit a vast number of advantages over other carrier systems, and this field of research has made significant progress in the past decade. However, many issues remain to be explored or more profoundly investigated. In this work, the potential of different yeast genera as antigen carrier could be successfully demonstrated, as well as their ability to activate antigen-specific T cells. However, additional experiments, in particular involving *in vivo* models, are required in order to enable the establishment of more effective yeast-based antigen delivery systems.

With respect to the cytokines secreted by stimulated DCs, the T cell-biasing profile could probably be more clearly identified by analyzing a higher number of donors. Furthermore, the presence of IL-4 in the culture medium to induce differentiation of monocytes into DCs made it difficult to determine the amount of IL-4 secreted by DCs. Consequently, it was not possible to ascertain if DC activation by some of the stimuli tested would result in the priming of T_H2 cells. Repeated cell washings and resuspension in fresh medium depleted of cytokines would likely allow a more reliable IL-4 determination.

Recombinant protein expression in yeast could be optimized by altering distinct parameters. For example, gene optimization taking into account the codon usage bias of each yeast species and use of expression vectors that enable multi-copy integration into the yeast genome would most probably lead to higher protein yields. Additionally, different anchors for cell-surface expression could be tested in an attempt to identify the most effective anchoring system for a particular yeast species.

The shortcomings in the delivery of secreted Ova might have been a result of the extremely high stability of this protein. To test this hypothesis, ova_{cyt} could be cloned downstream of a proper signal sequence for recombinant secretion in yeast. If antigen presentation was detected after delivery of secreted Ova_{cyt}, a rather unstable Ova derivative, it would be in agreement with the assumption that highly stable proteins are lesser available for processing and presentation by virtue of their diminished degradation. In an immediate future, this finding could be helpful in terms of choosing

the most adequate protein variant to be delivered. However, the limited availability of T cell lines specific for a particular epitope restrains the applicability of such *in vitro* antigen presentation assays.

In relation to *in vivo* approaches involving recombinant yeasts, immunization schedules for the different yeast genera delivering all Ova derivatives (including Ova VLP) should be established. In this context, a higher number of yeast cells per injection and a higher number of mice per group, as previously suggested, should be employed. Furthermore, assessment of T cell proliferation would probably be a more appropriate method to verify T cell responses. Resorting to this technique, in which CFSE-labeled transgenic OT-I T cells were injected in mice which were then primed 1 day later with Ova, Li *et al*. (2001) could detect proliferation of OT-I cells in response to cell-associated Ova even when the antigen amount was as low as 0.2 ng/mouse. Moreover, a number of immunization strategies should be evaluated, such as the use of different yeast genera, distinct subcellular antigen localizations and antigen-loaded VLP. Also, the results from the whole blood assays performed in this work support the use of heat-treated yeasts in immunization approaches. One drawback is that, in the case of antigens designed to combating viral infections in humans, suitable animal models are frequently scarce, hampering the onset of several vaccination studies.

An ideal therapeutic vaccine should also stimulate the generation of $CD4^+$ T helper responses, since T_H cells can further promote the generation and proliferation of potent CTL responses via release of immunomodulatory cytokines (Ardiani *et al*., 2010). Since different yeast species can trigger the secretion of a different set of modulatory cytokines, analyses of both $CD4^+$ and $CD8^+$ T cell responses should be considered in yeast-based immunization approaches.

VI. SUMMARY

Yeasts represent promising vaccine vehicles and have been demonstrated to elicit robust innate and adaptive immune responses. Nonetheless, the vast majority of studies employing yeast as delivery system has so far been restricted to *S. cerevisiae*. Nowadays however, an additional set of yeast genera of biotechnological relevance is available which could be tested as potential antigen carrier. Therefore, in the present study, different yeast genera were assessed for APC activation and antigen delivery aiming at the stimulation of antigen-specific T cells.

Distinct yeast genera, species, strains and mutants were able to induce maturation of human DCs, as analyzed by the upregulation of various cell-surface markers, including costimulatory, adhesion and MHC molecules, as well as a major chemokine receptor. In general, the diverse fungal stimuli differently triggered DC maturation, and to different extents than bacterial and viral stimuli. Yeasts also promoted cytokine secretion by human DCs, and cytokine levels differed among genera in most situations. As observed for the cell-surface molecules, the nature of the stimulus (bacterial, fungal, viral) led to DC activation in different ways. Importantly, all yeasts tested promoted release of T_H1-type cytokines such as IL-12 and IFN-γ, demonstrating that different yeast genera bear the potential to function as efficient carrier for activation of naïve T cells in antigen delivery approaches.

Four biotechnologically relevant yeast genera (*S. cerevisiae*, *P. pastoris*, *Sz. pombe* and *K. lactis*) were assessed for interaction with mammalian phagocytic cells, such as human DCs and murine macrophages. Yeast phagocytosis was MR- and Dectin-1-dependent in human DCs, and Dectin-1-dependent in IC-21 mouse macrophages. Furthermore, kinetic patterns of yeast internalization differed among budding and fission yeasts.

In order to address the suitability of yeast as carrier for protein antigens, delivery of heterologously expressed Ova was investigated in a comparative study. Expression of recombinant Ova derivatives exhibited, in general, a great variation among yeast genera. *S. cerevisiae*, *P. pastoris*, *Sz. pombe* and *K. lactis* expressing full-length Ova (targeted to secretion) or Ova_{cyt} (intracellular), as well as *S. cerevisiae* and *P. pastoris* expressing Ova_{cyt}/Sed1p (cell-wall anchored) were assessed for antigen delivery by

means of an *in vitro* presentation assay. It could be shown that all yeasts tested varied in their ability to deliver protein antigens and, furthermore, that subcellular protein localization influenced the activation of antigen-specific $CD8^+$ T cells. Cytosolic and cell-surface displayed Ova_{cyt} were delivered by all yeast genera, albeit to different degrees, whereas secreted Ova led to slight lymphocyte activation in *S. cerevisiae* and *P. pastoris*.

Yeasts were also genetically modified in order to express the clinically relevant tegument antigen pp65 from HCMV. Again, foreign protein expression was highly variable among yeast genera, and failed in *K. lactis*. Pp65 VLP were also recombinantly expressed in *S. cerevisiae*. Specific activation of pp65-specific memory $CD4^+$ and $CD8^+$ T cells from HCMV-seropositive individuals could be observed when heat-treated and untreated whole yeasts served as antigen vehicle in an *ex vivo* whole blood assay. T cell stimulation was specific, since no activation was detected in blood from HCMV-seronegative donors and much lower responses against the yeast vectors were observed compared to yeasts carrying pp65. The extent of pp65-specific T-cell activation varied among yeast genera, and pp65 VLP were more effective than unmodified pp65 in *S. cerevisiae*. Further, heat-treatment of yeasts differently affected stimulation of T lymphocytes but showed, in general, a beneficial effect. The $CD4^+$ T cell population exhibited a higher percentage of $CD69^+$ IFN-γ^+ cells in comparison to the population of $CD8^+$ T cells in all cases analyzed. Also, detection of T cell activation by means of whole blood assay proved to be more advantageous than the method of T cell stimulation in an autologous system.

Heat treatment affected the response of blood phagocytes towards *S. cerevisiae*, *P. pastoris*, *Sz. pombe* and *K. lactis*, as measured by the production of ROS. In the case of the budding yeasts, ROS production in response to heat-treated yeasts was higher than for untreated yeasts, but the opposite effect was observed in fission yeast.

Finally, Ova-specific $CD8^+$ T lymphocytes from OT-I transgenic mice immunized with *S. cerevisiae* carrying Ova_{cyt} showed higher production of IFN-γ upon restimulation with an SIINFEKL peptide epitope than lymphocytes from mice receiving *S. cerevisiae* harbouring an empty vector or PBS only. This experimental procedure requires, however, further optimization.

VII. REFERENCES

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