Developmental and Comparative Immunology 37 (2012) 107–114

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

# Yeast-surface expressed BVDV E2 protein induces a Th1/Th2 response in naïve T cells

Robert Patterson [a], Jessica Nerren [b,1], Michael Kogut [b], Pinar Court [c], Bernardo Villarreal-Ramos [c], Hans-Martin Seyfert [d], Paul Dalby [e], Dirk Werling [a,*]

[a] Royal Veterinary College, Department of Pathology and Infectious Diseases, Hawkshead Lane, Hatfield AL9 7TA, UK
[b] USDA-ARS-SPARC-FFSRU, 2881 F&B Road, College Station, TX 77843, USA
[c] TB Research Group, Veterinary Laboratory Agency – Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK
[d] Leibniz Institute for Farm Animal Biology, Wilhelm-Stahl-Allee 2, D-18196 Dummerstorf, Germany
[e] Department of Biochemical Engineering, University College London, Gower Street, London WC1E 6BT, UK

ARTICLE INFO

*Article history:*
Received 9 September 2011
Revised 18 October 2011
Accepted 22 October 2011
Available online 31 October 2011

*Keywords:*
*Saccharomyces cerevisiae*
Dectin-1
TLR2
BVD
E2
Cattle

ABSTRACT

Yeast species such as *Saccharomyces cerevisiae* are known to be potent activators of the immune system. *S. cerevisiae* activates the innate immune system by engaging pattern recognition receptors such as toll like receptor 2 (TLR2) and dectin-1. In the current project, we express the immunogenic envelope protein E2 of bovine viral diarrhoea virus (BVDV) on the surface of *S. cerevisiae*. After successful expression, components of the innate and adaptive immune response induced by the recombinant *S. cerevisiae in vitro* were analysed to determine if expression in yeast enhances the immunogenicity of the viral protein. Recombinant *S. cerevisiae* stimulated production of the chemokine CXCL-8 in primary bovine macrophages, but did no stimulate production of reactive oxygen species (ROS) in the same cells. Additionally, bovine macrophages primed with *S. cerevisiae* expressing viral envelope proteins had a greater capacity for stimulating proliferation of CD4+ T-cells from BVDV-free animals compared to macrophages primed with envelope protein alone or *S. cerevisiae* without envelope protein expression. Heat inactivation of recombinant *S. cerevisiae* increased ROS production and capacity to stimulate CD4+ T-cells in macrophages but did not alter CXCL-8 release compared to the live counter-part. Additionally, heat-inactivation of recombinant *S. cerevisiae* induced less INFγ and IL-4 but equal amounts of IL-10 compared to live yeast T-cell cultures. Our studies demonstrate a use for *S. cerevisiae* as a vehicle for transporting BVDV vaccine antigen to antigen-presenting cell in order to elicit cell-mediated immunity even in naïve animals.



## 1. Introduction

Recognition of invading antigen by the immune system is key for tailoring an appropriate immune response in order to quell infection. This recognition is facilitated through pattern recognition receptors (PRRs), which recognise conserved structures on invading microbes termed pathogen associated molecular patterns (PAMPs). Ligation of these PAMPs to their corresponding PRRs induces a very specific immune response. Of the many PRRs now known, the toll like receptors (TLRs) are the best characterised and most extensively studied.

TLRs 1–10 have been identified in humans and in cattle, and bovine TLRs have been shown to share high sequence homology with human TLRs (ranging between 77% and 86% sequence similarity at the nucleotide level) (Werling et al., 2006). Of these TLRs, TLR2 seems to be the one involved in the recognising antigens from Gram-positive bacteria and fungi, and ligation of TLR2 induces cellular signalling via NF-kB.

Another group of PRRs are the c-type lectin receptors (CTLRs), of which dectin-1 is the best characterised. This receptor has been shown to bind to β-glucan, a component of yeast cell wall. Engagement of dectin-1 facilitates internalisation of yeast particles by phagocytosis (Dennehy and Brown, 2007; Herre et al., 2004). Dectin-1 and TLR2 have previously been shown to co-operate in mounting an immune response to yeast antigen. Receptors co-localise on the surface of RAW-D1 macrophages (MØ) at the point of zymosan contact, and also in nascent zymosan-induced phagosomes (Brown et al., 2003). In addition to these observations, previous work in our lab has demonstrated that stimulating HEK293 cells transfected with bovine dectin-1 and TLR2 receptors with zymosan induces co-localisation of receptors at points of zymosan contact (Willcocks and Werling, unpublished results).

* Corresponding author. Tel.: +44 1707 666 358; fax: +44 1707 652 090.
*E-mail address:* dwerling@rvc.ac.uk (D. Werling).
[1] Current address: Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843, USA.

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doi:10.1016/j.dci.2011.10.009

Although dectin-1 and TLR2 both recognise and bind yeast antigen and co-operate in initiating the ensuing immune response, they appear to play very different roles. Experiments by others also suggest that TLR2 is mainly responsible for the inflammatory response to yeast antigen (Gantner et al., 2003; Gersuk et al., 2006; Underhill, 2007). Cells expressing a mutated murine TLR2 receptor fail to show NF-kB activity or produce TNF-α in response to zymosan compared to cells expressing the wild-type receptor. However, cells expressing the mutated receptor still engulfed zymosan particles and produced reactive oxygen species (ROS) with equal efficiency to those expressing the wildtype receptor indicating that TLR2 is not obligatory for phagocytosis or production of ROS (Gantner et al., 2003; Underhill et al., 1999a,b).

Bovine viral diarrhoea virus (BVDV) is a single stranded positive RNA pestivirus from the family Flaviviridae (Schweizer et al., 2006). Infection of cattle with BVDV presents a wide spectrum of disease, ranging from mild acute infection to fatal mucosal disease, and causes significant loss to the live-stock industry. The USDA licence over 180 commercially available vaccines for BVDV with varying levels of success and duration of protection (Kelling, 2004). In general, the use of modified live vaccine induces high titres of virus neutralising antibodies and gives the animal longer protection from clinical disease than inactivated vaccines. However, there is a risk that modified-live vaccines can revert to a virulent form and cause disease and indeed calves vaccinated with modified live vaccines often develop transient viraemia and shed vaccine virus (Fulton et al., 2003; Kelling, 2004). Neither modified-live nor inactivated vaccines allow for discrimination by clinical test as to which animals have been vaccinated and which animals have contracted BVDV by natural infection. This failure to discriminate could be rectified by development of an effective subunit vaccine for BVDV and the bovine immune response to individual BVDV antigens has been the subject of much research recently (Thomas et al., 2009). The 53 kDa E2 protein of BVDV is part of the viral envelope and is widely accepted as being the most immunogenic protein of this virus with high titres of neutralising antibodies against E2 being found in the host after infection (Baxi et al., 2000; Pande et al., 2005; Thomas et al., 2009). For any vaccine to be successful, the host immune system must recognise it as antigenic.

In the current study we chose to assess the value of using recombinant *Saccharomyces* (S.) *cerevisiae* yeast expressing the BVDV envelope protein 2 (E2) on its surface as a vaccine candidate for BVDV, by targeting dectin-1/TLR2 expressed on the surface of bovine macrophages. Due to the immunogenicity of the E2 protein and ability of yeast species to activate both inflammatory and phagocytic receptors on antigen presenting cells (APCs) our recombinant yeast represents an ideal expression system to determine if the efficiency of protein sub-unit vaccine may be improved by expression in yeast, and prevents the negative effects seen when expressed in baculovirus (Pande et al., 2005).

## 2. Methods and materials

### 2.1. Animals

Whole blood was collected from healthy Friesian–Holstein bullocks into 10% acid citrate dextrose buffer as anti-coagulant in accordance with Home Office regulations. All animals were tested for the absence of BVDV antibodies and viral RNA by the Veterinary Laboratories Agency (Weybridge, UK) and tested negative for both. Thus, these animals were naïve with regards to recognising BVDV derived peptides.

### 2.2. Cloning of BVDV E2 sequence

The partial coding sequence of the BVDV Ky1203ncp E2 gene in pSecTag (Invitrogen) was kindly provided by Dr. C. Thomas (Royal Veterinary College) already. To obtain the full coding sequence, this sequence was amplified by PCR adding start and stop codon on the 5′ and 3′ end respectively using PCR and forward (5′-ATGCACGACTGCAAACC-3′) and reverse (5′-TGGCCCTAATGAAGCGGTCA-3′) primers. The following PCR conditions were used: 95 °C for 2 min followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 2 min. After successful cloning of the full E2 sequence was confirmed by sequencing, the pYD1 vector was linearised by a double restriction digest using BamHI and NotI (New England Bio-labs) and the E2 gene was ligated into the linearised pYD1 vector using T4 DNA ligase (Promega). Presence of the E2 insert in the correct orientation and in frame with the pYD1 vector was confirmed by PCR and sequencing (Gene service, Cambridge, UK).

### 2.3. Transformation of competent EBY100 S. cerevisiae with pYD1-E2

Competent EBY100 cells pre-prepared using the *S. cerevisiae* EasyComp kit (Invitrogen) according to the manufacturer's instructions. Stored competent cells were thawed and 50 μl was pipetted into a sterile Eppendorf tube for each transformation reaction. 1 μg of pYD1 or pYD1-E2 was added to the cells. An Eppendorf tube containing competent cells but no plasmid DNA was set up as a negative control. 500 μl of solution 3 of the *S. cerevisiae* EasyComp kit (Invitrogen) was added to each transformation tube and tubes were vortexed for 5 s each. Tubes were incubated at 30 °C for 60 min and during this incubation were vortexed every 15 min for 5 s per tube. After incubation, 100 μl of transformation reaction from each tube was spread on a separate minimal dextrose plates pre-spread with 100 μl of 10 mg $ml^{-1}$ L-leucine (Sigma–Aldrich). Plates were incubated at 30 °C for 2–4 days and checked for growth of transformed colonies.

### 2.4. Surface protein induction and staining in EBY100 S. cerevisiae

Wild-type EBY100 and EBY100 transformed with the pYD1 plasmid (Invitrogen) containing the E2 gene insert of BVDV or the empty pYD1 plasmid were grown overnight in 10 ml of YNB-CAA media containing 2% glucose at 30 °C and 250 rpm. The absorbance of the overnight cultures was read at OD600 and the cells were pelleted by centrifugation at 4000*g* for 10 min at room temperature. The cell pellet was re-suspended to an OD600 of 0.5 in YNB-CAA medium containing 2% galactose and a volume of cells equivalent to 2 U OD600 was removed and stored on ice as a zero time point. Cell cultures were incubated for 48 h at 20 °C and 250 rpm with a volume of cells equivalent to 2 U OD600 being removed every 12 h and stored on ice until all time-points were collected. Samples were pelleted and washed with PBS (Sigma) at 4000*g* at 4 °C for 10 min. Cells were suspended in 250 μl of PBS with 1 mg $ml^{-1}$ BSA (PAA) and 1 μg of X-press antibody (Invitrogen) or WB162 antibody (provided by VLA, Weybridge) against the BVDV E2 protein. Cells were incubated on ice for 30 min with occasional mixing. Antibody was removed with a centrifugation step and cells were again washed in PBS. After washing, cell pellet was re-suspended in 250 μl PBS with 1 mg $ml^{-1}$ BSA and 1 μg donkey anti-mouse-Alexafluor 488 antibody (Invitrogen). Samples were incubated on ice and away from light for 30 min with occasional mixing. Antibody was removed with a centrifugation step and cells were washed twice in PBS. After washing, cells were re-suspended in 100 μl of PBS and 10 μl of this was spotted on a microscope slide. A cover slip was applied and cells were viewed by fluorescent microscopy using 530 nm band-pass filter and 20× magnification lens.

### 2.5. Heat inactivation of EBY100 S. cerevisiae

EBY100-pYD1 or EBY100-pYD1-E2 cells were suspended in 500 μl sterile PBS (Sigma) and incubated at 56 °C for 60 min. Heat-killed yeast was termed ΔEBY100-pYD1 and ΔEBY100-pYD1-E2. The viability of heat-killed cells was determined by plating on YPD plates.

### 2.6. Culture of bovine macrophages (MØ)

MØ were prepared as described (Werling et al., 2004). Briefly, blood was collected from animal in a vacuum bottle containing 10% final volume of ACD anticoagulant buffer, the buffy coat harvested, cells washed with citrate-buffer, and red blood cells lysed. Subsequently, PBMC were generated by density-gradient centrifugation over Histopaque ($d$ = 1.083 g $dl^{-1}$; Sigma) for 45 min, 1200$g$ at room temperature. The interphase was carefully removed, washed, cells counted in trypan blue and adjusted to a concentration of $5 \times 10^6$ cells per ml, placed into Teflon bags and incubated at 37 °C and 5% $CO_2$ for 7 days.

### 2.7. ELISA for CXCL8

ELISA for CXCL-8 was performed according manufacturer's recommendation (R&D Systems). Optical density of each well was determined using a Spectra Max M2 plate reader (Molecular Devices) set to 450 nm with wavelength correction of 540 nm. Results were analysed in Excel (Microsoft).

### 2.8. Measurement of ROS production

$1 \times 10^7$ MØ in a volume of 1 ml of HBSS (Gibco) were placed in sterile 1.5 ml Eppendorf tubes. Cells were stimulated with 1 mg $ml^{-1}$ zymosan, 390 μg $ml^{-1}$ isolated E2 protein, or the equivalent amount of E2 expressed by yeast, resulting in 5ppc EBY100-pYD1, 5ppc EBY100-pYD1-E2, 5ppc ΔEBY100-pYD1, 5ppc ΔEBY100-pYD1-E2, or HBSS as a negative control. ROS production was analysed as recently described (Conejeros et al., 2011), and plates were read at 485 nm excitation and 530 nm emission in a Spectra Max M2 plate reader (Molecular Devices) and results recorded over a 135 min period with readings being taken every 15 min. Results were analysed in Excel (Microsoft).

### 2.9. T-cell presentation assay

Bovine MØ were seeded at a density of $1 \times 10^4$ per well in 200 μl RPMI (Gibco) supplemented with 10% FCS and 200 U $ml^{-1}$ penicillin and streptomycin in 96-well round bottom plates (Cellstar). Plates were incubated at 37 °C and 5% $CO_2$ for 2 h after which 100 μl of media was removed from each well. Cells were primed with either 5ppc EBY100-pYD1, 5ppc ΔEBY100-pYD1, 5ppc EBY100-pYD1-E2, 5ppc ΔEBY100-pYD1-E2 or 390 μg $ml^{-1}$ E2 in a volume of 100 μl media per well. Plates were incubated at 37 °C and 5% $CO_2$ for 2 h after which 100 μl was removed from each well. CD4+ autologues T cells were prepared by MidiMacs separation using mAb specific for bovine CD4 (CC8), as described (Werling et al., 2002). The purity of the cells was evaluated by flow cytometry and shown to be >98%. Subsequently, $1 \times 10^5$ autologous CD4+ T-cells were added to each well. Plates were incubated at 37 °C and 5% $CO_2$ for up to 5 days, pulsed with 0.04 MBq of tritium labelled thymidine (3-H-TdR) (Perkin–Elmer) in 25 μl media and plates were incubated for another 16 h before being stored at −20 °C. Subsequently, cells were harvested (Tomtec 96, Mach II) onto glass-fibre mats (Perkin–Elmer) and analysed by liquid-scitillation counting using a a1450MicroBeta counter (Perkin–Elmer).

### 2.10. MSD cytokine measurement

To evaluate the presence of IFNγ, IL-4 and IL-10 in culture supernatants, the Meso-Scale Discovery (MSD) technology (Gaithersburg, MD, USA) was used as previously described (Coad et al., 2010). Custom coated 7-plex 96-well plates were supplied by MSD. In brief, MSD plates were blocked with MSD's proprietary assay buffer prior to addition of sample or standards. Standards were serially diluted in MSD dilution buffer with top concentration of standard shown in parenthesis: IFNγ (Endogen, 100 ng $ml^{-1}$); IL-4 (bovine IL-4 calibrator (MSD), 2 ng $ml^{-1}$) and IL-10 (IAH, 30 U $ml^{-1}$). 25 μl of cell culture supernatant or standards were added to duplicate wells. Incubations were performed at room temperature. Following a 2 h incubation, plates were washed and then incubated for a further 2 h with a combined cocktail of secondary antibodies conjugated to SulfoTag TM(MSD) (IFNγ, IL-4 and IL-10 all from MSD) in MSD dilution buffer. After a final wash, plates were coated with MSD Buffer-T and luminescence signal measured on an MSD-6000 reader. Results are shown as calibrated units extrapolated from standard curves.

### 2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 5) by means of 2-way analysis of variance (ANOVA) and post hoc analyses for multiple significance test comparisons were provided by Bonferroni correction. Graphical data are presented as the mean ± SEM. Significance values were set at $p < 0.05$ for 95% confidence intervals.

## 3. Results

### 3.1. Display of the mature E2 protein on the surface of EBY100 S. cerevisiae

EBY100 cells transformed with the pYD1-E2 vector and untransformed EBY100 cells were grown overnight, stained and visualised using fluorescent microscopy. Fig. 1 shows expression of the X-press epitope on the surface of EBY100 cells transformed with pYD1-E2 (Fig. 1, right panels) can be detected in as little as 12 h after induction with galactose. This expression of the X-press epitope increased for up to 48 h and then decreased over a period of time not being detectable at 96 h (data not shown). No X-press epitope was detected on the surface of wild-type EBY100 cells (Fig. 1, left panels) for any time point analysed. All further experiments inducing protein expression on the surface of EBY100 cells were completed using 48 h as the time point at which surface expression was tested for.

As seen in Fig. 2, EBY100 cells transformed with the pYD1-E2 vector (EBY100-pYD1-E2) (Fig. 2, left hand panels) and grown in media containing galactose for 48 h stained positive for both the X-press epitope (Fig. 2, lower panels) and the E2 protein (Fig. 2, upper panels) as tested using the WB162 antibody. EBY100 cells transformed with the pYD1 vector (EBY100-pYD1) (Fig. 2, middle panels) tested positive for the X-press epitope but negative for the E2 protein. Wild-type EBY100 cells tested negative for both the X-press epitope and the E2 protein. The data suggest that working E2 protein is displayed on the surface of EBY100 cells transformed with the pYD1-E2 vector.

### 3.2. CXCL8 and ROS production by bovine MØ is dependent on the form of S. cerevisiae expressing BVDV E2 protein

CXCL8 is produced predominantly by MØ and acts as a potent chemo-attractant for neutrophils and other granulocytes inducing

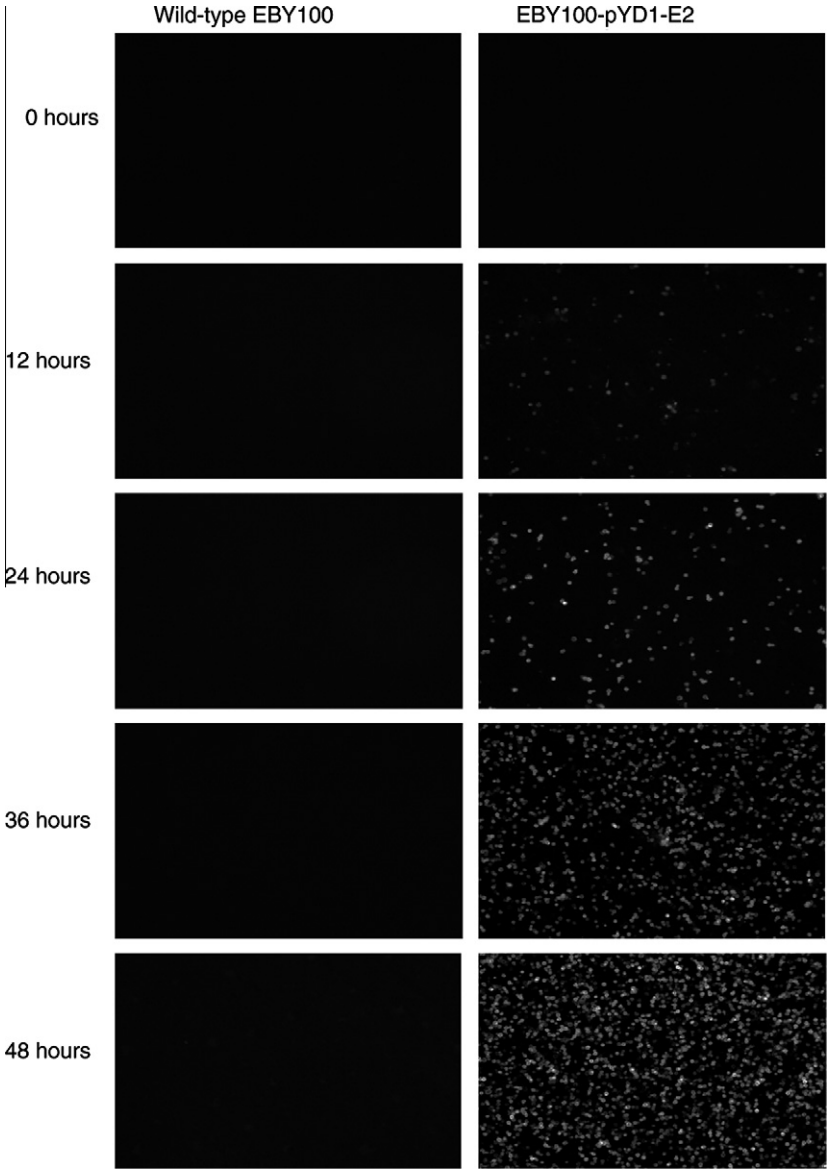


**Fig. 1.** Fluorescent microscope pictures showing staining of X-press epitope on the surface of Wild-type EBY100 and EBY100 transformed with pYD1-E2. Wild-type EBY100 *S. cerevisiae* and EBY100 *S. cerevisiae* transformed with pYD1-E2 were grown as described, and stained using an anti-X-press antibody followed by a secondary anti-mouse-Alexafluor488 antibody. Cells were viewed by fluorescent microscopy using a 20× objective.

their migration to areas of infection in body tissue. Once at the site of infection neutrophils, MØ and other phagocytes engulf antigen and destroy it by pathways including the ROS pathway. We therefore assessed whether recombinant *S. cerevisiae* induces CXCL-8 and ROS production in bovine MØ. Bovine MØ stimulated with both, life or heat-inactivated EBY100-pYD1 and EBY100-pYD1-E2 produced significant amounts of CXCL-8 compared with those stimulated E2 protein alone (Fig. 3A). In contrast, neither EBY100-pYD1 nor EBY100-pYD1-E2 induced large amounts of ROS production by MØ. In contrast, ΔEBY100-pYD1 and ΔEBY100-pYD1-E2 induced significantly amounts of ROS.

### *3.3. Bovine MØ primed with EBY100-pYD-E2 induce stronger CD4+ T-cell proliferation compared to EBY100-pYD1*

Antigen presenting cells such as MØ phagocytose and degrade antigen which is processed and presented at the cell surface in the context of MHC class II. Antigen specific CD4+ T cells recognise this presented antigen through their T-cell receptor and proliferate and release inflammatory cytokines. MØ primed with both EBY100-pYD1 and EBY100-pYD1-E2 induce significantly higher CD4+ T-cell proliferation than those primed with the E2 protein alone (Fig. 4A). Additionally, MØ primed with EBY100-pYD1-E2 induced significantly more CD4+ T-cell proliferation than those primed with EBY100-pYD1 indicating that a population of CD4+ cells specific for E2 antigenic peptides presented in the context of MHC class II had been expanded. Controls tested were media alone, CD4+ cells alone, MØ alone, CD4+ cells co-cultured with unstimulated MØ and MØ primed with either EBY100-pYD1, EBY100-pYD1-E2 or E2 alone. None of these controls displayed β-counts above 1500 cpm for any day tested (data not shown).

### *3.4. Bovine MØ primed with ΔEBY100-pYD1-E2 or EBY100-pYD1 induces stronger CD4+ T cell proliferation than those primed with live yeast*

Although live *S. cerevisiae* expressing hepatitis C virus proteins has been used in phase II clinical trials in humans (Habersetzer

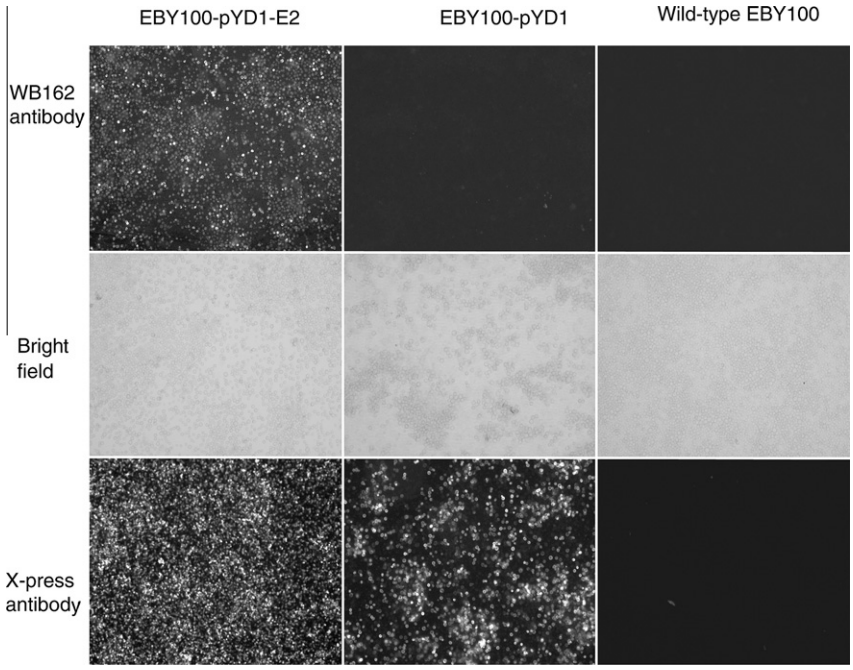


**Fig. 2.** Fluorescent microscope pictures showing staining of E2 protein of BVDV and X-press epitope on the surface of transformed EBY100. Wild-type EBY100 *S. cerevisiae* and EBY100 *S. cerevisiae* transformed with pYD1 or pYD1-E2 were grown overnight in YNB-CAA media containing 2% glucose. The following day, cells were pelleted and resuspended to an OD600 of 0.5–1.0 in YNB-CAA containing 2% galactose. Cultures were grown for 48 h and the E2 protein and X-press epitope were stained on the surface of the cells using WB162 and X-press antibody respectively followed by the use of a secondary anti-mouse-Alexafluor488 antibody. Cell were viewed by fluorescent microscopy using a 20× objective.

et al., 2009), inactivation of yeast would be an attractive and safe option for use in vaccines if it were to be as immunogenic as its live counter-part. For this reason we tested the capacity of MØ primed with ΔEBY100-pYD1-E2 to stimulate proliferation of CD4+ T-cells. Priming MØ with the ΔEBY100-pYD1-E2 increases their capacity to stimulate proliferation of CD4+ T-cells compared to live EBY100-pYD1-E2 at 5 days of incubation (Fig. 4B). No significant difference between the proliferative capacities of MØ primed with live or ΔEBY100-pYD1-E2 was observed for days 2–4 of incubation. Controls tested were media alone, MØ alone and CD4+ alone. None of these controls displayed β-counts above 1500 cpm for any day tested (data not shown).

### *3.5. Live EBY100-pYD1-E2 stimulates increased production of INFγ and IL-4*

Next, we wanted to determine the cytokine production by CD4+ T cells in response to MØ primed with live and ΔEBY100-pYD1-E2. Cell-free supernatants were collected from the same plates as shown in Fig. 4B, and assayed for INFγ, IL-4 and IL-10. Both, EBY100-pYD1-E2 and ΔEBY100-pYD1-E2 induced a higher production of all three cytokines tested compared to E2 protein alone. Surprisingly, live EBY100-pYD1-E2 induced more INFγ and IL-4 compared to ΔEBY100-pYD1-E2. This was statistically significant at days 2, 4 and 5 for INFγ and at days 2 and 4 for IL-4 (Fig. 5A and C). Very little difference is observed between the amount of IL-10 released in response to live or ΔEBY100-pYD1-E2 (Fig. 5B). Controls tested were media alone, MØ alone and CD4+ alone. The controls did not exceed 1 pg $ml^{-1}$ for INFγ, 160 pg $ml^{-1}$ for IL-10 and 0 pg $ml^{-1}$ for IL-4 for any of the days tested (data not shown).

## 4. Discussion

Several previous studies have highlighted the importance of dectin-1 as key innate microbial receptor. Dectin-1 collaborates with TLR2, as well as acting independently, to initiate a wide range of microbial responses, especially against fungal pathogens. Yeast such as *S. cerevisiae* are well documented as being potent activators of the immune system and therefore do not require the addition of an additional adjuvant (Romani et al., 2002). In the current study, we successfully expressed the E2 protein of BVDV on the surface of the *S. cerevisiae* EBY100 strain, and started to assess the potential of this recombinant yeast as a vaccine antigen for BVDV by measuring the bovine immune response *in vitro*. We used bovine MØ stimulated with the *S. cerevisiae* displaying the E2 protein at the surface and also *S. cerevisiae* with no expression of the E2 surface protein and measured their capacity to produce ROS, CXCL8 and also their capacity to induce proliferation of CD4+ T-cells.

Both yeast types induced significantly more CXCL8 than cells stimulated with equal amounts of isolated E2 fusion protein. These data indicate that in our system, the CXCL8 produced by MØ is due to stimulation of the cells with yeast components. This may be mainly as a result of TLR stimulation and subsequent NF-kB activation. However, the induction of CXCL8 may not be completely dependent on dectin-1, as shown recently in human neutrophils using depleted zymosan (van Bruggen et al., 2009). When neutrophils were stimulated with normal zymosan which contains both TLR2 and dectin-1 ligands, CXCL8 production were observed (van Bruggen et al., 2009). The small reduction in CXCL8 production observed in response to heat-killed yeast could be accounted for by a reduction in the quantity of mannan at the cell surface and therefore possibly less TLR activation (Gantner et al., 2005).

Interestingly, our data demonstrates that MØ produce very little ROS in response to stimulus with live version of EBY100-pYD1 and EBY100-pYD1-E2. However, when cells are stimulated with heat-killed versions of the same recombinant yeast, a significant rise in ROS production is observed. This increase in ROS production would indicate an increase in dectin-1 ligation as it is well documented that dectin-1 activation is responsible for ROS production in phagocytic cells (Gantner et al., 2003; Goodridge and Underhill,

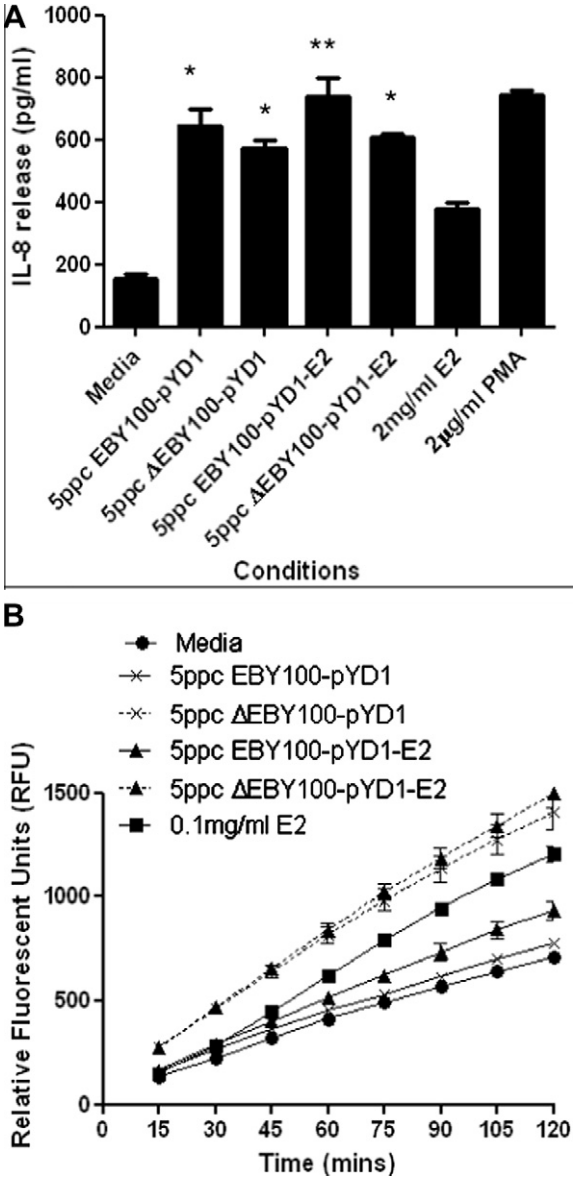


**Fig. 3.** Live EBY100-pYD1 and EBY100-pYD1-E2 induce CXCL8 whereas heat-inactivation induces ROS production in bovine MØ. (A) Bovine MØ were stimulated with media, 2 μg ml$^{-1}$ PMA, 5ppc EBY100-pYD1, 5ppc EBY100-pYD1-E2, 5ppc ΔEBY100-pYD1 5ppc ΔEBY100-pYD1-E2 or 2 mg ml$^{-1}$ E2 for 24 h and cell-free supernatant was harvested and assayed for CXCL8 content by ELISA. Asterisks denote a significant ($^{*}p < 0.05$, $^{**}p < 0.01$) difference from 2 mg ml$^{-1}$ E2 stimulated group. (B) Bovine MØ were stimulated with media, 5ppc EBY100-pYD1, 5ppc EBY100-pYD1-E2, 5ppc ΔEBY100-pYD1 or 5ppc ΔEBY100-pYD1-E2 and probed for ROS every 15 min for 2 h. Graphs are representative of 3 biological repeats.

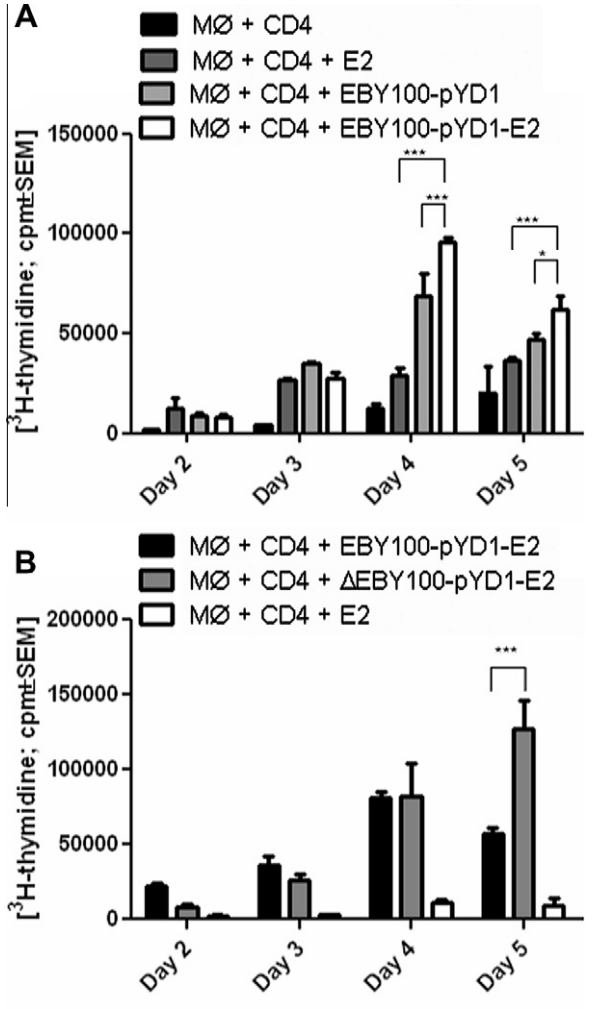


**Fig. 4.** Bovine MØ primed with EBY100-pYD1-E2 induces a stronger CD4+ T-cell proliferation compared to MØ primed with EBY100-pYD1, which is further increased by heat-inactivation. (A) Bovine MØ were seeded in 96-well plates and primed with 5ppc EBY100-pYD1, 5ppc EBY100-pYD1-E2 or 390 μg ml$^{-1}$ E2 before addition of CD4+ T-cells. Plates were incubated for 2–5 days before being pulsed with [3H]-thymidine. Cells were harvested and counts per minute were measured by liquid-scintillation counting. (B) To assess the effect of heat-inactivation, bovine MØ were incubated with either 5ppc EBY100-pYD1-E2 or 5ppc ΔEBY100-pYD1-E2 or 390 μg ml$^{-1}$ E2 and T cell proliferation analysed as described. Asterisks denote a significant difference between groups ($^{*}p < 0.05$, $^{***}p < 0.001$). Graphs are representative of 3 biological repeats.

2008). Nerren et al. have demonstrated that pre-treating chicken heterophils with the dectin-1 specific antagonist laminarin reduces their ability to produce ROS in response to stimulus with curdlan (a β-1,3-glucan) (Nerren and Kogut, 2009). Additionally, experiments from our lab have demonstrated a significant drop in ROS production by MØ in response to zymosan when pre-treated with laminarin (data not shown). The fact that heat-killed recombinant yeast is capable to stimulate ROS production can be explained by the fact that heat-inactivation exposes more of the dectin-1 ligand β-glucan at the yeast surface, as shown recently (Gantner et al., 2005). Furthermore, recent work by Wellington et al. has suggested that live *Candida albicans* not only inhibits ROS production by masking β-glucan at its surface but also actively suppresses ROS production. However, when using *S. cerevisiae*, no active suppression was observed. The authors concluded that this is due to the fact that *S. cerevisiae* is non-pathogenic (Wellington et al., 2009). In our system, this increased recognition of recombinant yeast by dectin-1 may lead to increased uptake and processing of both EBY100 and E2 antigen which would be advantageous from the perspective of using the recombinant yeast as a vaccine. The mature E2 protein expressed on the yeast surface did not appear to be affected by the heat-inactivation process as determined by fluorescent staining (data not shown).

The target of any vaccine is to give the recipient an immunological memory to the antigen being administered by activating the adaptive arm of the immune system. In the present study, we tested the ability of MØ primed with recombinant yeast expressing E2 protein to stimulate proliferation of purified autologous CD4+ T-cells derived from BVDV antibodies and virus-free animals, and have compared it to those primed with EBY100 yeast without surface E2 protein (Fig. 4A). MØ primed with either EBY100-pYD1 or

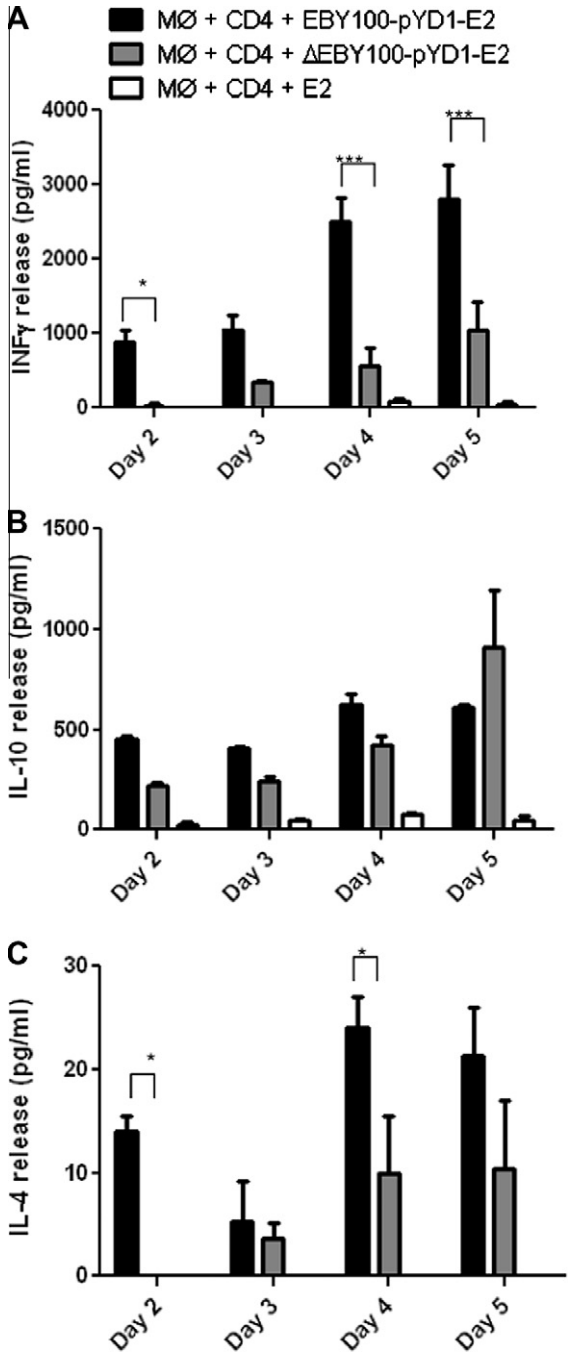


**Fig. 5.** Bovine MØ primed with live EBY100-pYD1-E2 induce a stronger INFγ and IL-4, but similar IL-10 production compared with those primed with ΔEBY100-pYD1-E2. Bovine MØ were seeded in 96-well plates and primed with 5ppc EBY100-pYD1-E2, 5ppc ΔEBY100-pYD1-E2 or 390 μg $ml^{-1}$ E2. CD4+ T-cells were added and plates were incubated for 2–5 days before cell-free supernatants were harvested and assayed for INFγ, IL-4 and IL-10 content as described. Asterisks denote a significant difference between groups. ($^{*}p < 0.05$, $^{***}p < 0.001$). Graphs are representative of 3 biological repeats.E2, 5ppc ΔEBY100-pYD1 or 5ppc ΔEBY100-pYD1-E2 and probed for ROS every 15 min for 2 h. Graphs are representative of 2 biological repeats.

EBY100-pYD1-E2 induced significant CD4+ proliferation compared with those primed with the E2 protein alone. We are fully aware of the fact that the stimulation of naïve CD4+ T cells should not occur when using MØ as antigen presenting cells. We can only assume at the moment that either the MØ prepared using the method described possess functions associated normally with dendritic cells, or that the use of yeast particles induces such a strong stimulatory signal for MØ that these are able to subsequently drive T cell proliferation. Our data indicate that either yeast antigen readily enters endocytic processing pathways and is expressed on the cell surface in the context of MHC II, or stimulates such a strong innate immune response with the secretion of pro-inflammatory cytokines that it drives T cell proliferation. Additionally, priming MØ with EBY100-pYD1-E2 induced a significantly stronger CD4+ proliferative response at day 4 and 5 than priming with EBY100-pYD1. Thus, in addition to the proliferation of potential yeast antigen specific CD4+ cell, an E2 specific CD4+ T cell population may expand. Our data are in line with recent data using murine dendritic cells primed with *S. cerevisiae* expressing recombinant ovalbumine (Stubbs et al., 2001). Here, dendritic cells primed with as little as 5 recombinant yeast particles induced CD4+ proliferation to the same degree as those primed with saturating amounts of ovalbumin peptide. The authors also found that stimulation of dendritic cells with yeast induced up-regulation of CD80, CD86, CD40, CD54 and MHC II as well as an increase in IL-12 production. These increases were comparable to stimulation with LPS. These data clearly demonstrate the adjuvant-like properties of *S. cerevisiae* yeast and offer some explanation of why conjugating protein antigen to whole yeast appears to increase the cellular immune response to this antigen. Given the differences seen between live and heat-inactivated yeast, and given the fact that a heat-inactivated delivery system still showing immunogenicity, we also assessed the ability of heat-killed EBY100-pYD1-E2 primed MØ to stimulate CD4+ proliferation, and compared the INFγ, IL-4 and IL-10 production induced in the responding T cells.

In correlation with the ROS data showing increased ROS production in response to heat-killed EBY100-pYD1-E2, a stronger CD4+ T-cell proliferative response was seen in MØ primed with heat-killed EBY100-pYD1-E2 after 5-days of incubation (Fig. 4B). Similar as for the ROS data, this may be a result of more β-glucan being exposed at the yeast surface (Gantner et al., 2005), resulting in increased dectin-1 ligation and therefore facilitate uptake of EBY100-pYD1-E2 by MØ. Indeed, work by Lu et al. and by Franzusoff et al. has demonstrated that immunisation with inactivated *S. cerevisiae* expressing tumour antigens elicits an equal anti-tumour response to immunisation with their live counter-parts (Ardiani et al., 2010; Franzusoff et al., 2005; Lu et al., 2004), but to our knowledge this is the first report that recombinant heat-inactivated *S. cerevisiae* may actually increase the adaptive immune. We were expecting to see an increased amount of INFγ produced by T-cell assays co-cultured with MØ exposed to ΔEBY100-pYD1-E2 compared to those exposed to live yeast, reflecting the increased CD4+ proliferation (Fig. 4B). Surprisingly this was not the case. In fact, higher INFγ and IL-4 concentrations were measured in supernatants of co-cultures where live EBY100-pYD1-E2 was used (Fig. 5A and C), whereas amounts of IL-10 were similar (Fig. 5B). Whereas we can currently not fully explain this observation, it is possible that heat-inactivated, but not live yeast induces an anti-inflammatory state in MØ, without affecting the ability of these cells to stimulate T cells. Such effect has been recently described for Murine macrophages (RAW 264.7) using either heat-inactivated or live *C.* albicans on the proteomics level (Martinez-Solano et al., 2009).

In conclusion, both live and heat-killed *S. cerevisiae* are an attractive vaccine vehicle for delivering the weakly immunogenic proteins to APC in order to stimulate cellular immunity directed against this protein. Such delivery system could not only improve current BVDV vaccine strategies, but potentially any vaccine which relies on such protein antigen. The ease with which *S. cerevisiae* can be genetically manipulated means that potentially any antigenic protein could be expressed at the yeast surface, potentially expressing even more than one immunogenic protein, either from

the same pathogen or derived from different pathogens. Furthermore, such delivery-systems could subsequently allow for the discrimination of vaccinated versus naturally infected animals.

## Acknowledgement

We thank Dr. C. Thomas (Royal Veterinary College) for providing the partial BVDV E2 protein in the pSeqTaq vector. The present work was funded through a BBSRC CASE studentship and a RCVS Charitable Trust Blue Sky Research Grant to D.W. with Pfizer Animal Health Ltd as industrial partner. This manuscript represents RVC publication P/PID/000120/.

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