

YEAST VACCINE VECTOR INCLUDING IMMUNOSTIMULATORY AND ANTIGENIC POLYPEPTIDES AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

5 The present application claims the benefit of priority to United States Provisional Patent Application No. 62/331,044, filed on May 3, 2016, the contents of which are incorporated herein by reference in its entirety.

SEQUENCE LISTING

10 This application is being filed electronically via EFS-Web and includes an electronically submitted Sequence Listing in .txt format. The .txt file contains a sequence listing entitled "2017-05-01_5658-00379_ST25.txt" created on May 2, 2017 and is 118,371 bytes in size. The Sequence Listing contained in this .txt file is part of the specification and is hereby incorporated by reference herein in its entirety.

INTRODUCTION

15 Vaccines are used to initiate an adaptive immune response against antigens, in particular antigens from pathogens in order to ameliorate or prevent disease. Inactivated or attenuated microorganism vaccines are often effective at stimulating a robust immune response that is fully protective, but in some cases these vaccines are not protective or only partially protective and other strategies must be used to develop protective vaccines. Microorganism based vaccines
20 cannot post-translationally modify proteins by glycosylation to properly express large antigenic proteins, such as viral proteins; therefore, development of a yeast vaccine vector that can glycosylate and result in properly folded large antigenic proteins that is safe and effective at stimulating a lasting protective immune response is needed.

SUMMARY

25 Yeast vaccine vectors are provided herein. The vaccine vectors are suitable for oral administration and produce rapid and long-lasting immunity to the antigens and protection from subsequent infection with the targeted microorganism. In particular the immune response generated is an IgA response suitable for protection from mucosal infections.

30 In one aspect, a yeast vaccine composition is provided. The composition includes a yeast comprising an immunostimulatory polynucleotide encoding an immunostimulatory polypeptide selected from an HMGB1 polypeptide or a CD40 ligand. The yeast is engineered to express the

HMGB1 polypeptide or the CD40 ligand on the surface of the yeast. The compositions may also include an antigenic polypeptide, suitably expressed on the surface of the yeast as well. The compositions may be combined with pharmaceutically acceptable carriers and/or adjuvants to generate pharmaceutical compositions. The compositions may include more than one antigenic polypeptide and the more than one antigenic polypeptides may be derived from the same or different organism or species.

In another aspect, methods of enhancing an immune response in a subject by administering the vaccine compositions and pharmaceutical compositions provided herein to the subject in an amount effective to enhance the immune response of the subject to the vaccine composition and the infectious agent related to the antigenic polypeptides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph comparing the fluorescence intensity of RAW 264 macrophages after co-culture with fluorescent labeled with *Salmonella* Enteritidis *aroA/htrA*, *Salmonella* Enteritidis *aroA/htrA*-AgA-CD154, and *Salmonella* Enteritidis *aroA/htrA*-AgA-AgB-HMGB1.

Figure 2 is a schematic depiction of the pPICZ plasmid map engineered to include *Gallus gallus* specific high mobility group box 1 (HMGB1) protein expression on the *Pichia pastoris* using the glycosylphosphatidylinositol anchored *Saccharomyces cerevisiae* alpha agglutinin cell surface expression method. pPICZ is methanol inducible using the AOX1 promoter.

Figure 3 is a set of photographs showing HMGB1 cell surface expression on *Pichia pastoris* (X33). *Pichia pastoris*-HMGB1 construct #4 (Fig. 3D: DIC only, Fig. 3E: fluorescence only, Fig 3F: DIC/fluorescence overlay) and *Pichia pastoris* (X33; Fig. 3A: DIC only, Fig. 3B: fluorescence only, Fig. 3C: DIC/fluorescence overlay) backbone were stained using rabbit polyclonal HMGB1 156-177 diluted 1:5 in phosphate buffered saline (PBS) with the F(ab)² portion of goat anti-rabbit IgG conjugated with Alexa 488 at 1:1000 in 1% goat serum in PBS. HMGB1 protein expression was optimally expressed on three of the nine *Pichia pastoris*-HMGB1 constructs that were transformed.

Figure 4 is a graph showing the *Pichia pastoris* specific serum antibody sample/negative control serum ratio. We observed a typical dose response curve of *Pichia pastoris* specific antibodies determined using an ELISA. *Pichia pastoris* administered SQ at 10⁷/broiler was too little to mount an immune response, and 10⁹/broiler was too much resulting in seroconversion to even the X33 construct backbone. *Pichia pastoris* administered SQ at 10⁸/broiler was the best

dose that resulted in all HMGB1+ *Pichia pastoris* constructs significantly elevating *Pichia pastoris* specific serum antibodies ($P < 0.001$).

Figure 5 is a graph showing the *Pichia pastoris* specific serum antibody normalized to non-vaccinated control broilers (Group 1) serum ratio. The dose response that we expected repeated. We observed a typical dose response curve. *Pichia pastoris* administered SQ at 10^7 /broiler was too little to mount an immune response, and 10^9 /broiler was too much resulting in seroconversion to even the X33 construct backbone. *Pichia pastoris* administered SQ at 10^8 /broiler was the best dose that resulted in all HMGB1+*Pichia pastoris* constructs significantly elevating *Pichia pastoris* specific serum antibodies ($P = 0.049$).

Figure 6 is a graph showing the percentage of animals having a lesion score of 4 on day 6 post-challenge and the percentage indicated within each bar shows the percent mortality at day 6 post-challenge.

Figure 7 is a graph showing the distribution of all lesion scores was also decreased in the vaccinated animals. The vaccinated animals demonstrated lower lesion scores.

DETAILED DESCRIPTION

A vaccine composition capable of eliciting an immune response against the vaccine composition or against an antigenic polypeptide expressed by the vaccine composition is provided herein. In particular the vaccine composition includes a yeast engineered to express an immunostimulatory polypeptide on its surface. The yeast may also be engineered to express additional antigenic polypeptides on the surface of the yeast. In particular embodiments, a *Pichia pastoris* vaccine vector is provided. The vaccine vector includes an immunostimulatory polynucleotide sequence encoding an immunostimulatory polypeptide which is displayed or expressed on the surface of the yeast. The immunostimulatory polypeptide may be a high mobility group box 1 (HMGB1) immunostimulatory polypeptide or a CD40 ligand such as CD154 polypeptide or a fragment thereof or other CD40 agonist such as a CD40 agonistic antibody. The immunostimulatory polypeptide may be expressed on the surface of the yeast, e.g., *Pichia pastoris*, using any means available to those of skill in the art. In the examples the immunostimulatory polypeptide is attached to the surface of the yeast via a glycosylphosphatidylinositol (GPI)-anchored mechanism encoded by the 3' end of *Saccharomyces cerevisiae* α -agglutinin. Those skilled in the art will readily appreciate that other

expression systems may be used to obtain surface expression of the immunostimulatory and/or antigenic polypeptides included in the yeast to generate the vaccine compositions.

The HMGB1 protein was first identified as a DNA-binding protein critical for DNA structure and stability. It is a ubiquitously expressed nuclear protein that binds DNA with no sequence specificity. The protein is highly conserved and found in organisms ranging from plants to mammals. The chicken, zebrafish, human, mouse, rat, crab-eating macaca, cow, horse, canine, pig, rabbit, red drum, catfish, humphead snapper, goldfish, king cobra, brine shrimp and other HMGB1 amino acid sequences are provided. See SEQ ID NOs: 2-30 and 94-105. The sequence throughout mammals is highly conserved with 95% amino acid identity and the amino acid changes are conservative. Thus an HMGB1 protein from one species may likely substitute for that from another species functionally. The full-length HMGB1 protein or a portion thereof may be used as the HMGB1 polypeptide in the vaccine vectors described herein. HMGB1 has two DNA binding regions termed A1 and A2 and B1 and B2. See Andersson and Tracey, *Annu. Rev. Immunol.* 2011, 29:139-162.

HMGB1 is a mediator of inflammation and serves as a signal of nuclear damage, such as from necrotic cells. HMGB1 can also be actively secreted by cells of the monocyte/macrophage lineage in a process requiring acetylation of the protein, translocation across the nucleus, and secretion. Extracellular HMGB1 acts as a potent mediator of inflammation by signaling via the Receptor for Advanced Glycated End-products (RAGE) and via members of the Toll-like Receptor family (TLR), in particular TLR4. The RAGE binding activity has been identified and requires the polypeptide of the HMGB1 RAGE binding domain. TLR4 binding requires the cysteine at position 106 of the chicken HMGB1 sequence (SEQ ID NO: 2), which is found in the B box region of HMGB1.

Suitably, the vaccine vector contains a polynucleotide encoding a polypeptide including amino acids 150-183 and 89-109 of the chicken HMGB1 polypeptide or a homolog thereof. See SEQ ID NO: 2. In the Examples, a 190 amino acid polypeptide of HMGB1 was used. Suitably, the polynucleotide encodes a HMGB1 polypeptide from the same species as the subject in which the vaccine composition will be used. Heterologous combinations of HMGB1 polypeptides and subjects (i.e. a human HMGB1 polypeptide for use in a chicken vaccine) may be useful in the methods of the invention because HMGB1 is highly conserved through a wide number of species as discussed above. The HMGB1 polypeptide may be used to enhance the immune response in

the subject to any foreign antigen or antigenic polypeptide present in or on the yeast vaccine compositions. One of skill in the art will appreciate that the HMGB1 polypeptide could be used to enhance the immune response to more than one antigenic polypeptide present in a yeast vaccine composition. The polypeptide from HMGB1 stimulates an immune response at least in part by activating dendritic cells and macrophages and thus stimulating production of cytokines such as IL-1, IL-6, IFN- γ and TNF- α . In the Examples, a polypeptide of HMGB1 was expressed on the surface of the vaccine compositions.

The inflammatory activities of HMGB1 do not require the full-length protein and functional fragments have been identified. The B box has been shown to be sufficient to mediate the pro-inflammatory effects of HMGB1 and thus HMGB1 box b1 and HMGB1 box b2 are HMGB1 polypeptides or functional fragments thereof within the context of the present invention. See e.g. SEQ ID NO: 35 and 36. In addition, the RAGE binding site and the pro-inflammatory cytokine activity have been mapped. See SEQ ID NO: 37 and 38, respectively. Thus, these polypeptides are functional fragments of HMGB1 polypeptides in the context of the present invention. See SEQ ID NOs: 31-38.

Those of skill in the art are capable of identifying HMGB1 polypeptides and fragments thereof capable of stimulating pro-inflammatory cytokine activity, using methods such as those in International Publication No. WO2002 092004, which is incorporated herein by reference in its entirety. Suitably, the HMGB1 polypeptide includes the RAGE binding domain at amino acids 150-183 of the chicken HMGB1 sequence (HMGB1 RAGE binding domain or a homolog thereof) and the pro-inflammatory cytokine activity domain between amino acids 89-109 of the chicken HMGB1 sequence (SEQ ID NO: 2; HMGB1 proinflammatory cytokine activity or a homolog thereof). In particular, HMGB1 polypeptides and functional fragments or homologs thereof include polypeptides identical to, or at least 99% identical, at least 98% identical, at least 95% identical, at least 90% identical, at least 85% identical, or at least 80% identical to the HMGB1 polypeptides of the chicken HMGB1 sequence or HMGB1 box a1, HMGB1 box a2, HMGB1 box b1, HMGB1 box b2, HMGB1 RAGE binding domain, or HMGB1 proinflammatory cytokine activity, respectively.

The immunostimulatory polypeptide may also be a CD40 ligand or CD40 agonist. A CD154 polypeptide that is capable of binding CD40 in the subject and stimulating the subject to respond to the vaccine composition and its associated foreign antigenic polypeptide may be used

as the immunostimulatory polypeptide. The CD154 polypeptide may be full-length CD154 or may be fewer than 50 amino acids long, more suitably fewer than 40, fewer than 30 or fewer than 20 amino acids in length. The polypeptide may be between 10 and 15 amino acids, between 10 and 20 amino acids or between 10 and 25 amino acids in length. The CD154 sequence and CD40 binding region are not highly conserved among various species. The CD154 sequences of chicken and human are provided in SEQ ID NO: 106 and SEQ ID NO: 107, respectively.

The CD40 binding regions of CD154 have been determined for a number of species, including human, chicken, duck, mouse and cattle and are shown in SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, and SEQ ID NO: 112, respectively. Also included are polypeptides identical to, or at least 99% identical, at least 98% identical, at least 95% identical, at least 90% identical, at least 85% identical, or at least 80% identical to the CD154 sequences provided in SEQ ID NOs: 106-112. Although there is variability in the sequences in the CD40 binding region between species, cross-species binding of CD154 to CD40 has been reported. For example, the human CD154 polypeptide was able to enhance the immune response in chickens. Therefore, one may practice the invention using species specific CD154 polypeptides or a heterologous CD154 polypeptide.

In another alternative, the CD40 ligand may be a CD40 agonistic antibody or portion thereof. Such CD40 agonistic antibodies are disclosed at least in International Application No. WO2015/187969. CD40 antibodies and agonistic CD40 antibodies are also commercially available for several species, in particular mouse and human. An antibody is agonistic for CD40 if it is capable of inducing signaling within the target cell expressing CD40. The signalling via CD40 results in increased expression of CD40 and TNF receptors on the surface of the antigen-presenting cells and induces production of reactive oxygen species and nitric oxide, and B cell activation leading to isotype switching.

Suitable chicken CD40 agonistic antibodies include the antibody provided herein as SEQ ID NO: 113 (heavy chain) and SEQ ID NO: 114 (light chain) referred to as 2C5 or SEQ ID NO: 115 (single chain variable fragment (scFv)) referred to as DAG-1). These antibodies may be made in a "chickenized" form such that the Fc portion and the non-CDR regions may be replaced with homologous host-compatible antibody backbone sequences to minimize the immune response to the antibody backbone itself. In addition, the antibodies may be made either recombinantly or via enzyme digestion (*i.e.* papain or pepsin) into smaller portions of the

antibodies and include only the F(ab) portion of the antibody, such as an F(ab)₂ fragment. The CDR regions for both chicken CD40 antibodies have been identified. For the antibody designated as 2C5 and provided in SEQ ID NO: 113 and SEQ ID NO: 114, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 116, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 117, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 118 and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 119, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 120, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 121. For the antibody designated as DAG-1 and provided in SEQ ID NO: 115, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 122, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 123, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 124 and wherein the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 125, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 126, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 127. Also included are polypeptides identical to, or at least 99% identical, at least 98% identical, at least 95% identical, at least 90% identical, at least 85% identical, or at least 80% identical to at least one of SEQ ID NOs: 113-127.

The vaccine compositions provided herein comprise a yeast. The yeast may be selected from any of the following yeast genus: *Saccharomyces*, *Candida*, *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Yarrowia*. The yeast may be of a species selected from the group consisting of *Saccharomyces cerevisiae*, *Candida albicans*, *Hansenula polymorpha*, *Pichia pastoris* and *Schizosaccharomyces pombe*. In the Examples *Pichia pastoris* was used. The yeast include an immunostimulatory polynucleotide encoding an immunostimulatory polypeptide and may further comprise an antigenic polynucleotide encoding an antigenic polypeptide. Suitably the immunostimulatory polypeptide and the antigenic polypeptide are not natively expressed by the yeast. The yeast are engineered to express the immunostimulatory polypeptide and the antigenic polypeptide and display or express these polypeptides on the surface of the yeast.

At least a portion of the antigenic polypeptide and at least a portion of the immunostimulatory polypeptide are present on the surface of the *Pichia pastoris* or other yeast-

based vaccine composition. Present on the surface of the vaccine composition includes polypeptides that are comprised within a transmembrane protein, interacting with, covalently or chemically cross-linked to a transmembrane protein, a membrane lipid or membrane GPI-anchored carbohydrate or protein. A polypeptide can be comprised within a transmembrane protein by having the amino acids comprising the polypeptide linked via a peptide bond to the N-terminus, C-terminus or anywhere within the transmembrane protein (i.e. inserted between two amino acids of the transmembrane protein or in place of one or more amino acids of the transmembrane protein (i.e. deletion-insertion)). Those skilled in the art will appreciate that a non-native immunostimulatory polynucleotide or an antigenic polynucleotide may be inserted in frame within an extracellular loop of a transmembrane or cell wall protein to obtain surface expression of the immunostimulatory or antigenic polypeptide. In the Examples, the α -agglutinin C-terminal anchoring method that uses a covalently linked GPI-anchoring system of yeast is used, but other similar anchoring methods are available to those of skill in the art.

Alternatively, the polypeptides may be covalently or chemically linked to proteins, lipids or carbohydrates in the membrane through methods available to persons of skill in the art. For example, di-sulfide bonds or biotin – avidin cross-linking could be used to present the antigenic and immunostimulatory polypeptides on the surface of a yeast in the vaccine compositions. Suitably, the antigenic polypeptide and the immunostimulatory polypeptide are part of a fusion protein. The two polypeptides may be directly linked via a peptide bond or may be separated by a linker peptide encoded by a polynucleotide or a section of a third protein into which they are inserted. In some embodiments a fusion protein comprising more than one copy of each of the immunostimulatory polypeptide and/or the antigenic polypeptide is included in the yeast. In some embodiments, the multiple copies of the immunostimulatory polypeptide include more than one copy of the same immunostimulatory polypeptide or different polypeptides. The same is true for the antigenic polypeptides in that multiple copies of the same or analogous antigenic polypeptides may be included in the yeast, e.g., multiple copies of the avian influenza M2e antigen possibly having single or only a few amino acid sequence differences. Alternatively the yeast may be engineered to express multiple different and distinct antigenic polypeptides to allow for a single administration of a vaccine composition to elicit or enhance the immune response to different antigens from different species. For example, the vaccine composition may be prepared to enhance the immune response to subsequent infection with *Campylobacter* and

Eimeria by including antigenic polypeptides of SEQ ID NO: 55 and SEQ ID NO: 61 may be included in the same vaccine composition.

Polynucleotides encoding the antigenic polypeptide or immunostimulatory polypeptides may be inserted into the yeast of the vaccine composition and expressed to generate the antigenic polypeptide and the immunostimulatory polypeptide. The polynucleotides may be inserted into the chromosome of the vaccine composition or encoded on plasmids or other extrachromosomal DNA such as on a YAC (yeast artificial chromosome). Suitably, polynucleotides encoding the antigenic polypeptide and/or the immunostimulatory polypeptide may be expressed independently or are inserted into a yeast vaccine polynucleotide that is expressed. The yeast vaccine polynucleotide may encode a polypeptide expressed on the surface of the yeast vaccine such as a transmembrane protein. The polynucleotide encoding the antigenic polypeptide and/or the immunostimulatory polypeptide may be inserted into the yeast vaccine polynucleotide sequence to allow expression of the antigenic polypeptide and/or the immunostimulatory polypeptide on the surface of the yeast.

Alternatively, the polynucleotide encoding the antigenic polypeptide and/or the immunostimulatory polypeptide may be inserted into a secreted polypeptide which is displayed or presented on the surface of the yeast vaccine through association with a protein, lipid or carbohydrate on the surface of the yeast vaccine. Those of skill in the art will appreciate that the polynucleotide encoding the antigenic polypeptide and/or the immunostimulatory polypeptide could be inserted in a wide variety of yeast polynucleotides to provide expression and presentation of the antigenic polypeptide and/or the immunostimulatory polypeptide to the immune cells of a subject treated with the yeast.

As noted in the discussion above, the vaccines described herein may also include an antigenic polynucleotide encoding an antigenic polypeptide. An antigenic polypeptide is a polypeptide that is capable of being specifically recognized by the adaptive immune system. The antigenic polypeptide may be natively expressed by the yeast chosen as the vector to vaccinate against the yeast acting as the vaccine vector. Alternatively, a yeast vaccine vector may carry a heterologous polynucleotide encoding a heterologous polypeptide not natively associated with the vaccine vector as the antigenic polypeptide. An antigenic polypeptide includes any polypeptide that is immunogenic. The antigenic polypeptides include, but are not limited to, antigens that are pathogen-related, allergen-related, tumor-related or disease-related. Pathogens

include viral, parasitic, fungal and bacterial pathogens as well as protein pathogens such as the prions. The antigenic polypeptides may be full-length proteins or portions thereof.

It is well established that immune system recognition of many proteins is based on a relatively small number of amino acids, often referred to as the epitope. Epitopes may be only 8-10 amino acids. The term antigenic polypeptide may include an epitope to which an antibody or T cell immune response is generated in the subject. The term epitope and antigen or antigenic polypeptide may be used interchangeably. Thus, the antigenic polypeptides described herein may be full-length proteins, 8 amino acid long epitopes or any portion between these extremes. In fact the antigenic polypeptide may include more than one epitope from a single pathogen or protein. Antigenic polypeptides may include but will not be limited to large segments of bacteria or small polypeptides of bacteria such as those associated with Mastitis infection, *Salmonella*, *Clostridium*, *Campylobacter*, *Escherichia*, *Shigella*, *Helicobacter*, *Vibrio*, *Plesiomonas*, *Edwardia*, *Klebsiella*, *Staphylococcus*, *Streptococcus*, *Aeromonas*; viral proteins including but not limited to influenza, Foot and Mouth virus, porcine epidemic diarrhea virus (PEDv), and Porcine reproductive and respiratory syndrome virus (PRRSV); parasitic infections including but not limited to *Eimeria* spp, *Toxoplasma*, malaria, or other parasites; and tumor antigens. For example, the antigens or epitopes identified in US Patent No. 8,604,198, International Publication Nos. WO2009/059018, WO2009/059298, WO2011/091255, WO2011/156619, WO2014070709, WO 2014/127185 or WO 2014/152508 may be used. Antigenic polypeptides may include any one or more of those provided in SEQ ID NOs: 39-93 and include polypeptides identical to, or at least 99% identical, at least 98% identical, at least 95% identical, at least 90% identical, at least 85% identical, or at least 80% identical to those provided in SEQ ID NOs: 39-93. Those skilled in the art recognize that some of the peptides included in SEQ ID NO: 39-93 are longer than is likely required to act as an antigenic epitope, thus fragments of these antigenic polypeptides are also included herein. Those skilled in the art will also recognize that the antigenic polypeptides may also include additional amino acids or may be linked to each other or to the immunostimulatory polypeptide via linker amino acids to form a sort of fusion protein. The linker amino acids may be any amino acids but serine and glycine are most commonly used. The linker may be as short as one or two amino acids, but may be 4, 5, 6, 8, 10, 12, 14, 15 or more amino acids long.

Multiple copies of the same epitope or antigenic polypeptide or multiple epitopes from different proteins may be included in the vaccine vector. It is envisioned that several epitopes or antigens from the same or different pathogens or diseases may be administered in combination in the yeast vaccine vector to generate an enhanced immune response against multiple antigens.

- 5 The yeast vaccine vector may encode antigens from multiple pathogenic microorganisms, viruses or tumor associated antigens. Administration of vaccine vectors capable of expressing multiple antigens has the advantage of inducing immunity against two or more diseases at the same time.

The polynucleotide encoding an immunostimulatory polypeptide capable of enhancing the immune response to an antigenic polypeptide may also encode the antigenic polypeptide.

- 10 The polynucleotide encoding an immunostimulatory polypeptide may be linked to the polynucleotide encoding the antigenic polypeptide, such that in the vaccine vector the immunostimulatory polypeptide and the antigenic polypeptide are encoded by the same polynucleotide. At least a portion of the antigenic polypeptide and the immunostimulatory polypeptide are present on the surface of the yeast vaccine vector. The vaccine composition may
15 include an antigenic polynucleotide encoding the antigenic polypeptide and an immunostimulatory polynucleotide encoding the immunostimulatory polypeptide. The immunostimulatory polypeptide and the antigenic polypeptide may be linked, such as in a fusion protein. The immunostimulatory polypeptide and the antigenic polypeptide may both be inserted within an external loop of a transmembrane protein or may be attached to the surface through a
20 GPI-anchoring mechanism.

- Heterologous polynucleotides include, but are not limited to, polynucleotides encoding antigens selected from pathogenic microorganisms or viruses other than the yeast vaccine vector. Such heterologous or antigenic polynucleotides may be derived from pathogenic viruses such as influenza (e.g., M2e, hemagglutinin, or neuraminidase), herpesviruses (e.g., the genes encoding
25 the structural proteins of herpesviruses), retroviruses (e.g., the gp160 envelope protein), adenoviruses, paramyxoviruses, coronaviruses and the like. Heterologous polynucleotides can also be obtained from pathogenic bacteria, e.g., genes encoding bacterial proteins such as toxins, and outer membrane proteins. Further, heterologous polynucleotides from parasites, such as Eimeria are attractive candidates for use in a yeast vectored vaccine composition.

- 30 Additional immunostimulatory polypeptides involved in triggering the immune system may also be included in the vaccine compositions described herein. The polynucleotides may

encode immune system molecules known for their stimulatory effects, such as an interleukin, Tumor Necrosis Factor or an interferon, or another polypeptide involved in immune-regulation such as a CD40 ligand or CD40 agonist. Thus the yeast vaccine vectors may contain more than one immunostimulatory polypeptide or more than one antigenic polypeptide. This includes more
5 than one copy of the same polypeptide to increase the expression level of the polypeptide. Alternatively multiple different immunostimulatory polypeptides or nucleotides encoding the same or multiple antigenic polypeptides or nucleotides encoding the same may be included in a single recombinant yeast. The multiple antigenic polypeptides may be multiple copies of a similar antigen such as two different epitopes of the M2e antigen (SEQ ID NO: 41 and 42). The
10 antigenic polypeptides may be antigens directed to completely different antigens but related to the same infectious agent such as M2e and HA5. See SEQ ID NOs: 41-44. The antigenic polypeptides may also be antigens directed to different species in order to vaccinate against more than one pathogen with a single unitary vaccine. Such as SEQ ID NO: 41 to Influenza M2e in combination with SEQ ID NO: 54 directed to PAL from *E. coli* or SEQ ID NO: 61 directed to
15 MPP from *Eimeria*. In the Examples, a vaccine composition comprising a yeast expressing MPP-TRAP-HMGB-1 (SEQ ID NO: 61 linked to SEQ ID NO: 65 linked to SEQ ID NO: 2) was generated and shown to reduce both morbidity and mortality associated with challenge with *Eimeria maxima*.

Compositions comprising the vaccine compositions and a pharmaceutically acceptable
20 carrier are also provided. A pharmaceutically acceptable carrier is any carrier suitable for *in vivo* administration. Suitably, the pharmaceutically acceptable carrier is acceptable for oral, nasal or mucosal delivery. The pharmaceutically acceptable carrier may include water, buffered solutions, glucose solutions or bacterial culture fluids. Additional components of the compositions may suitably include excipients such as stabilizers, preservatives, diluents,
25 emulsifiers and lubricants. Examples of pharmaceutically acceptable carriers or diluents include stabilizers such as carbohydrates (e.g., sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein-containing agents such as bovine serum or skimmed milk and buffers (e.g., phosphate buffer). Especially when such stabilizers are added to the compositions, the composition is suitable for freeze-drying or spray-drying.

30 The vaccine compositions may not be capable of replication in the subject. The yeast may be incapable of growth outside of a laboratory environment, such as an attenuated form of

the yeast. Suitably the yeast is inactivated or killed prior to addition to the vaccine composition. The vaccine compositions may also include an adjuvant. Adjuvants are known in the art and in the Examples a mannosylated chitosan adjuvant was used. See WO 2014/070709.

5 The compositions described herein may be used to enhance an immune response such as an antibody response to the antigenic polypeptide or to the vaccine vector itself. The compositions and vaccine vectors described herein may reduce the severity of subsequent disease by decreasing the length of disease, decreasing the morbidity or mortality associated with the disease or reducing the likelihood of contracting the disease. The morbidity or mortality associated with the disease after administration of the vaccine vectors described herein may be
10 reduced by 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or even 100% as compared to similar subjects not provided the vaccine vector.

Methods of enhancing immune responses in a subject by administering the vaccine composition are also provided. The vaccine composition may contain a full length immunostimulatory polypeptide or portion thereof capable of stimulating the immune response
15 to the vaccine composition and its associated antigenic polypeptide. The vaccine composition comprising an immunostimulatory polypeptide is administered to a subject in an amount effective to enhance the immune response of the subject to the vaccine and in particular to the antigenic polypeptide. Enhancing an immune response includes, but is not limited to, inducing a therapeutic or prophylactic effect that is mediated by the immune system of the subject. The
20 effect may be measured by testing a response to the antigenic polypeptide or to an infectious or cancerous agent that expresses the antigenic polypeptide. Specifically, enhancing an immune response may include, but is not limited to, enhanced production of antibodies, enhanced class switching of antibody heavy chains, maturation of antigen presenting cells, stimulation of helper T cells, stimulation of cytolytic T cells or induction of T and/or B cell memory.

25 The compositions may be administered by a variety of means including, but not limited to, subcutaneously, orally, intranasally, and mucosally. For example, the vaccine compositions or vaccine vectors may be delivered by aerosol, by spraying, by addition to food or water, by oral gavage, or via eye drops. In some embodiments, the compositions are administered by injection such as intradermally, parenterally, subcutaneously, intraperitoneally, intravenously,
30 intracranially, or intramuscularly. For chickens or other poultry, the compositions may be administered *in ovo*. Combinations of administration means may also be used. In the Examples

a sub-cutaneous vaccination was followed by a boost of the vaccine composition given orally. Other combinations may also be used, such as intranasal or delivery via aerosols or spraying followed by oral gavage or inclusion in the feed or drinking water.

Subjects include, but are not limited to, a vertebrate, suitably a mammal, suitably a human, cows, cats, dogs, pigs, aquaculture, suitable catfish, snapper, goldfish, or birds, suitably poultry such as chickens or turkeys. Other animal models of infection may also be used. Enhancing an immune response includes, but is not limited to, inducing a therapeutic or prophylactic effect that is mediated by the immune system of the subject. Specifically, enhancing an immune response may include enhanced production of antibodies, such as demonstrated in Figures 4 and 5. In some embodiments, an IgA response is produced.

The useful dosage to be administered will vary depending on the age, weight and species of the subject, the mode and route of administration and the type of pathogen or disease against which an immune response is sought. The composition may be administered in any dose of yeast vaccine vector sufficient to evoke an immune response. It is envisioned that doses ranging from 10^5 to 10^{10} yeast vector copies are sufficient. Specifically, the dosage of 10^8 *Pichia pastoris*-HMGB1 vaccine vector copies determined by counting the number of yeast in a cubic mm using a hemacytometer under 400X magnification was optimal for inducing a vaccine vector specific immune response ultimately signifying stimulation of an immune response to antigenic cargo adjacent to HMGB1 on the yeast cell surface.

The composition may be administered only once or may be administered two or more times to increase the immune response. If the composition is administered more than one time, the composition may be administered via different routes of administration each time the vaccine is administered as discussed above. For example, the composition may be administered two or more times separated by one week, two weeks, or by three weeks, one month, two months, three months, six months or more. The yeast may be viable prior to administration, but in most embodiments the yeast will be killed or inactivated prior to administration. In some embodiments, the yeast may be able to replicate in the subject, while in other embodiments the yeast may not be capable of replicating in the subject. As shown in the Examples, the yeast vaccine vector may be inactivated prior to administration using formalin, glutaraldehyde, ethanol, acidification, heat or antibiotics. One skilled in the art would appreciate other means of inactivating yeast vaccine vectors could be used as well.

The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings, should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms "including," "comprising," or "having," and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as "including," "comprising," or "having" certain elements are also contemplated as "consisting essentially of" and "consisting of" those certain elements.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word "about" to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human

error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless
5 otherwise stated, reference to any document herein does not constitute an admission that any of these documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference, unless explicitly
10 indicated otherwise. The present disclosure shall control in the event there are any disparities between any definitions and/or description found in the cited references.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

15 We have developed a *Pichia pastoris* vaccine vector that expresses full length high mobility group box 1 (HMGB1) to significantly increase the immune response to antigenic cargo. Previously, we inserted a truncated CD154, or CD40 ligand, polypeptide and/or full length HMGB1 into a double attenuated *Salmonella* Enteritidis (SE) and compared phagocytic uptake of the SE by Raw 264 murine macrophage cells (Figure 1). Relative fluorescence
20 intensity within Raw 264 was measured using fluorescence activated cell sorting (FACS) analysis. The fluorescence intensity was measured because the fluorescence signal on the *S. aureus* pHrodo particles increases at a more acidic pH. A phagolysosome is created within the macrophage once the bacteria are taken into the macrophage by phagocytosis. The macrophage breaks down bacteria by acidifying the pH inside of a phagolysosome. Therefore, the
25 macrophages that were actively breaking down bacteria would have greater fluorescence intensity. The data in Figure 1 demonstrate that murine macrophages preferentially phagocytosed the SE vaccine vector containing HMGB1 as compared to the attenuated SE vaccine vector alone (P=0.011) and CD154 did not significantly alter phagocytic uptake by murine macrophages (P=0.057).

30 We have engineered *Pichia pastoris* for cell surface expression of HMGB1 using a plasmid integrated system to chromosomally insert the HMGB1 protein into *Pichia pastoris*

increasing the immune response to the vaccine cargo. *Pichia pastoris* yields 10- to 100-fold higher protein expression than *Saccharomyces cerevisiae*. HMGB1 sends a danger signal to the immune system triggering the RAGE response. The phagocytosis assay described above shows that HMGB1 is a potent immune stimulatory molecule that can increase uptake of the carrier system, for example, *Salmonella* Enteritidis, into murine macrophages. HMGB1 expression on the cell surface of the yeast should enhance uptake of the yeast-vectored vaccine into macrophages by phagocytosis as observed in the *Salmonella* Enteritidis construct. We obtained an EasySelect *Pichia* Expression Kit from Invitrogen® that includes the pPICZ expression vector for expression in *Pichia pastoris*. A cell surface expression kit for *Pichia pastoris* is not currently available, but several researchers have used GPI-anchored proteins to initiate cell surface expression in a yeast system (Wasilenko et al., 2009). The GPI-anchored protein that was used is the C-terminus portion of the α -agglutinin from *Saccharomyces cerevisiae*. HMGB1 connected to the C-terminus portion of *Saccharomyces cerevisiae* α -agglutinin by a serine spacer region was spliced into the pPICZ intracellular expression plasmid (Figure 2 and SEQ ID NO: 1). pPICZ is a methanol inducible plasmid for fast and high levels of protein expression. Cell surface expression of HMGB1 was confirmed in Figure 3, which shows immunofluorescence of the presence of HMGB-1 only on the transformed yeast cells (Fig. 3E and Fig. 3F).

After HMGB1 protein expression confirmation on the cell surface of *Pichia pastoris*, we chose three HMGB1 positive *Pichia pastoris* clones to test in broiler chickens (n=15 chicks/group). The *Pichia pastoris*-HMGB1 positive clones #3, 4, and 6 were inactivated using 0.3% glutaraldehyde in sterile water and mixed 1:2 in mannosylated chitosan adjuvant. See WO 2014/070709 which is incorporated herein by reference in its entirety. We administered three doses, 10^7 - 10^9 , of each *Pichia pastoris*-HMGB1 positive construct by subcutaneous injection, 0.25mL of vaccine/chick (Table 1). Broiler chicks were vaccinated on day of hatch and on day 14. Serum was collected for IgG antibody titer measurement on day 21. A direct ELISA measuring IgG specific for *Pichia pastoris* was optimized in our laboratory. Sample/negative control serum ratios were reported to account for plate to plate variability within the ELISA assay. Antibody titers specific for *Pichia pastoris* were determined from each broiler chick. *Pichia pastoris*-HMGB1 vaccinated chicks' IgG were compared to non-modified *Pichia pastoris* (X33) vaccinated chicks' IgG. The results are shown in Figure 4.

Table 1: *Pichia pastoris* vaccination dose strategy in broiler chicks

	Group	Dose
1	Control-No <i>Pichia pastoris</i>	-
2	<i>Pichia pastoris</i> w/o HMGB1 (X33)	10^7
3	<i>Pichia pastoris</i> -HMGB1 3	10^7
4	<i>Pichia pastoris</i> -HMGB1 4	10^7
5	<i>Pichia pastoris</i> -HMGB1 6	10^7
6	<i>Pichia pastoris</i> -HMGB1 (X33)	10^8
7	<i>Pichia pastoris</i> -HMGB1 3	10^8
8	<i>Pichia pastoris</i> -HMGB1 4	10^8
9	<i>Pichia pastoris</i> -HMGB1 6	10^8
10	<i>Pichia pastoris</i> -HMGB1 (X33)	10^9
11	<i>Pichia pastoris</i> -HMGB1 3	10^9
12	<i>Pichia pastoris</i> -HMGB1 4	10^9
13	<i>Pichia pastoris</i> -HMGB1 6	10^9

We then vaccinated three-week-old broiler chickens with the *Pichia pastoris*-HMGB1 constructs # 3, 4, or 6 to determine whether a similar IgG antibody response would be observed.

5 We vaccinated three-week-old broiler chickens with 0.25mL of each *Pichia pastoris* vaccine (Table 1: n=10 chickens/group). The *Pichia pastoris*-HMGB1 positive constructs #3, 4, or 6 were inactivated using 0.3% glutaraldehyde in sterile water and mixed 1:2 in mannosylated chitosan adjuvant. Broiler chickens were vaccinated on day 21 and on day 35. Serum was collected for IgG antibody titer measurement on day 21. A direct ELISA measuring IgG specific
10 for *Pichia pastoris* was optimized in our laboratory. Absorbance at 450nm normalized for non-vaccinated chickens (group 1) were reported to account for plate to plate variability within the ELISA assay. Antibody titers specific for *Pichia pastoris* were determined from each broiler chick. *Pichia pastoris*-HMGB1 vaccinated chicks' IgG were compared to non-modified *Pichia pastoris* (X33) vaccinated chicks' IgG. The results are depicted in Figure 5.

15 HMGB1 significantly elevated IgG antibody titers specific for *Pichia pastoris* in broilers injected SQ with inactivated HMGB1+*Pichia pastoris* as compared to those injected with non-modified *Pichia pastoris* (Figure 4 and Figure 5). HMGB1 increased the immune response to the

Pichia pastoris vaccine vector suggesting that any antigenic cargo expressed by the same *Pichia pastoris* would elicit a higher immune response than if the antigenic cargo were expressed in *Pichia pastoris* without HMGB1.

Materials and Methods

5 *Pichia pastoris* X-33 (wild type) was obtained from Invitrogen (Carlsbad, CA, USA) as part of the Easy Select™ *Pichia* Expression Kit. The HMGB1 coding sequence- optimized for expression in *Pichia pastoris*-was synthesized by Genscript (Piscataway, NJ, USA), and supplied to our lab in a pUC57 cloning vector. TOP10 electrocompetent *E. coli* (Invitrogen) was used for all necessary plasmid propagation during vaccine construction. Following transformations with
10 plasmid DNA, *E. coli* was propagated at 37°C using either LB medium supplemented with 100 µg/mL Ampicillin, or low salt LB medium containing 50 µg/mL Zeocin. Routine propagation of *Pichia pastoris* was done at 30°C using YPD medium supplemented with 50 µg Zeocin when appropriate. Minimal medium for yeast containing histidine (MMH) and minimal medium for yeast containing dextrose (MDH) were used for subsequent screening of the recombinant vaccine
15 strains. Minimal medium containing glycerol (MGY) and minimal medium containing methanol (MM) were used to induce expression of HMGB1 from *Pichia pastoris* in cultures.

Construction of vaccine vector.

To make the *Pichia pastoris*-HMGB1 expression vector, pPICZ was digested with *KpnI* and *PmeI* to prepare the vector backbone for cloning. pUC57/HMGB1-alpha agglutinin was
20 digested with *KpnI* and *EcoRV* and the 1.6 kb HMGB1-alpha agglutinin insert was subsequently gel purified. Following ligation and transformation into TOP10 *E. coli*, colony PCR was performed to identify colonies carrying the proper pPICZ/HMGB1-alpha agglutinin ligated plasmid. Primers for this PCR- AOXSeqF (5' GACTGGTTCCAATTGACAAGC 3'; SEQ ID NO: 128); AOXSeqR (5' GCAAATGGCATTCTGACATCC 3'; SEQ ID NO: 129) were
25 provided in the Easy Select™ kit. Amplicons were produced using KOD DNA polymerase (Millipore; Darmstadt, Germany). Cycling parameters for this reaction are as follows: 98 °C, 10 minutes; followed by 25 cycles of 98°C, 15 seconds; 55°C, 5 seconds. The ligated plasmid pPICZ/HMGB1-alpha agglutinin was further verified with sequencing at the University of Arkansas DNA core laboratory facility (Fayetteville, AR). pPICZ/HMGB1-alpha agglutinin was
30 subsequently linearized via *PmeI* digestion and purified to prepare for electroporation in to *Pichia pastoris* X-33.

For electroporation, 5mL of *Pichia pastoris* X-33 was grown overnight at 30°C in YPD broth. Five hundred milliliters of fresh YPD broth was inoculated the following day with the 5 mL culture and grown to an OD of 1.5. Cells were then washed twice with ice- cold, sterile water and once with ice-cold, sterile sorbitol (1M). Cells were ultimately resuspended in 1mL of ice-
5 cold sorbitol. Eighty microliters of the competent *Pichia pastoris* was mixed with 10 µg of linearized pPICZ/HMGB1-alpha agglutinin and pulsed once at 2.0KV to electroporate the yeast cells. Transformants that underwent successful chromosomal integration of the linear vaccine construct containing the Zeocin resistance gene were selected on YPD plates containing 100 µg/mL Zeocin. Again, colony PCR was used to analyze the transformants. The aforementioned
10 primers and cycling parameters were utilized.

Screening of Pichia pastoris pPICZ/HMGB1 AOX1 gene.

Resulting vaccine strains were tested to verify the presence and stability of the *AOX1* gene needed to drive expression of HMGB1-alpha agglutinin. Nine Zeocin resistant strains in addition to GS115 Mut⁻ (a negative control strain with a nonfunctional *AOX1* gene provided in
15 the Easy SelectTM *Pichia* Expression Kit) were tested by plating on MDH and MMH agar. *AOX1* deficient strains show much slower growth on MMH medium than on MDH. Growth times on these two mediums are used to identify *AOX1* deficient strains. Each of the nine strains plus the negative control GS115 Mut⁻ were replica plated on MMH and MDH mediums and incubated for 3 days. Cultures were checked every 24 hours and levels of growth were recorded.

20 *HMGB1-alpha agglutinin protein induction.*

To induce expression of HMGB1 in culture, a 50 mL *Pichia pastoris* pPICZ/HMGB1-alpha agglutinin culture was grown overnight at 30 °C in MGY broth from a single colony. Twenty-five milliliters of this overnight culture was transferred into 250 mL or pre-warmed MM broth and covered with sterilized cheese-cloth for proper aeration. This culture was then grown
25 with vigorous shaking (250 rpm) for 96 hours; with 100% methanol being added to a final concentration of 0.5% every 24 hours to maintain induction. Induction of the *HMGB1-alpha agglutinin* gene product was maximized after 96 hours.

After induction, each of the nine *Pichia pastoris*-HMGB1 positive constructs were tested for HMGB1 protein expression on the *Pichia pastoris* cell surface using an immunofluorescence
30 assay. *Pichia pastoris*-HMGB1 construct #1, 3, 4, 5, 6, 7, 8, 9, and 10 and X33 backbone were stained using rabbit polyclonal HMGB1 156-177 diluted 1:5 in phosphate buffered saline (PBS)

with the F(ab)² portion of goat anti-rabbit IgG conjugated with Alexa 488 at 1:1000 in 1% goat serum in PBS. HMGB1 protein expression was optimally expressed on three (#3, 4, and 6) of the nine *Pichia pastoris*-HMGB1 constructs that were transformed (Figure 3).

Evaluation of S. Enteritidis recovery after vaccination with a Yeast vectored vaccine candidate.

5 We next tested the efficacy of Yeast vectored PAL-HMGB1 (SEQ ID NO: 54 linked to SEQ ID NO: 2) and antibody guided PAL vaccine candidates to reduce recovery of *S. Enteritidis* (SE), a serogroup D *Salmonella*, after challenge. Sixty SPF leghorn chicks were hatched at the poultry farm. On the day of hatch, chicks were divided into two groups, control or vaccinated. Vaccinated chicks were vaccinated with 4.25×10^9 cfu/bird Yeast-PAL-HMGB1 subcutaneously and by oral gavage, both groups were then placed in batteries with wire floors, and feed and water were provided *ad libitum*. Prior to vaccination, ten 1-day-old chicks were euthanized by CO₂ inhalation for sampling to confirm the chicks were *Salmonella* negative. On day 7, vaccinated chickens were boosted with 5×10^9 cfu/bird subcutaneously and by oral gavage. On day 14, all chickens were challenged by oral gavage with 1.2×10^8 cfu/bird SE. On days 21 and 28, 10 cloacal swabs per group were obtained to assess shedding of challenge strains. On days 32, liver and spleen (LS), and ceca (CT) were collected from 20 chickens (control) or 10 chickens (vaccinated) and cultured by direct plating (CFU/g) and enrichment for SE incidence.

Summary of experimental procedure:

Group No. (n=30)	Group	Vaccination Dose DOH	Boost Dose D7	Challenge Dose D14	Cloacal Swabs
1	Control	NA	NA	SE@10 ⁸ /bird	D21, 28
2	Yeast-PAL-HMGB1-SC/oral	10 ⁹ /ml	10 ⁹ /ml	SE@10 ⁸ /bird	D21, 28

20 As shown in Table 2, significantly less *Salmonella* was recovered from chickens vaccinated with the Yeast-PAL-HMGB-1 vaccine by 18 days post-challenge. As shown in Table 3, significantly less *Salmonella* was also recovered from cloacal swabs and was completely cleared as early as 2 weeks post-challenge.

Table 2. Recovery of SE 18 days post-challenge.

	LS (%+)	CT (% + (# +))
Control	0	65 (13/20)
Yeast-PAL-HMGB1	0	30 (3/10)*

*Different from Control p<0.1

Table 3. Recovery of SE from cloacal swabs 1 and 2 weeks post-challenge.

	1 wk (% + (# +))	2 wk (% + (# +))
Control	70 (14/20)	25 (5/20)
Yeast-PAL-HMGB1	40 (4/10)	0 (0/10)*

*Different from Control $p < 0.1$

Evaluation of Coccidia infection after vaccination with a Yeast vectored vaccine candidate.

We next tested the efficacy of Yeast (*Pichia pastoris*) vectored MPP-TRAP-HMGB1 (SEQ ID NO: 61 linked to SEQ ID NO: 65 linked to SEQ ID NO: 2) to reduce morbidity and mortality after infection with *Eimeria maxima*, M6 strain, after challenge. The expression of MPP-TRAP-HMGB1 was confirmed to be surface expressed by immunofluorescence. Eighty chicks were obtained on the day of hatch from a commercial hatchery. On the day of hatch, chicks were randomly divided into four groups, negative control, positive control, vaccinated with the Pichia-MPP-TRAP-HMGB-1 in MCA vaccine by oral gavage at day of hatch and day 14 or vaccinated with the Pichia-MPP-TRAP-HMGB-1 in MCA vaccine in the drinking water at day 4 and day 14. Vaccinated chicks were vaccinated with 5×10^7 cfu/bird Yeast-MPP-TRAP-HMGB1 by oral gavage or in the drinking water. The mannosylated chitosan adjuvant (MCA) stock solution (1.5% chitosan w:v) was diluted 1:2 with the suspension of Pichia (0.5% final concentration). For oral gavage, the MCA (0.5%) plus Pichia construct (1×10^7 cells) was delivered in 0.25 mL for both the prime and the boost by oral gavage. For administration in the drinking water, the final concentration of MCA in the drinking water was 0.004% and the final concentration of the Pichia was 2.3×10^6 cells/mL of drinking water and this was used for both prime and boost administration. All chicks were individually tagged and all chicks (N=20 per group) were commingled except during drinking water vaccination and feed and water were provided *ad libitum*.

All groups were challenged on day 20 and lesion scores were determined on day 6 post-inoculation. Some oocysts escape from the initial challenge (they pass unchanged) so a very modest challenge is expected in the unchallenged controls in these commingled chicks. On day 20 the positive control birds and both sets of vaccinated birds were challenged with 100,000 *Eimeria maxima* (strain M6) oocytes. At day 26 each bird was scored for lesions using the Johnson and Reid Lesion Score Index. Johnson, J. and W. M. Reid 1970. Experimental

Parasitology 28: 30-36. In this lesion score method the numerical scores indicate the following:
0: No gross lesions; 1: Small red petechiae may appear on the serosal side of the mid-intestine,
there is no ballooning or thickening of the intestine, though small amounts of orange mucus may
be present; 2: Serosal surface may be speckled with numerous red petechiae; intestine may be
filled with orange mucus; little or no ballooning of the intestine; thickening of the wall; 3:
Intestinal wall is ballooned and thickened, the mucosal surface is roughened; intestinal contents
filled with pinpoint blood clots and mucus; 4: the intestinal wall may be ballooned for most of its
length; contains numerous blood clots and digested red blood cells giving a characteristic color
and putrid odor; the wall is greatly thickened; dead birds are recorded with this score.

Figure 6 shows the percent of animals having a lesion score of 4 on day 6 post-challenge
and the percentage indicated in each bar shows the percent mortality at day 6 post-challenge.
Notably, none of the vaccinated birds died by day 6 as opposed to 15% of the positive control
animals. The lesion score was also reduced as shown by the calculated p value shown in figure 6
($p=0.037$ for drinking water vaccination, $p=0.067$ for oral gavage). When *Pichia* expressing
both MPP and TRAP antigens along with HMGB1 as the immunostimulatory polypeptide was
included in drinking water, the lesion scores were significantly reduced. The statistical analyses
were carried out as follows. The lesion data were analyzed using a PROC MIXED ANOVA
model in SAS, the assumption was made that the difference in severity between a score of 0 and
1 was similar to the difference in severity between a score of 1 and 2, and so on. Under this
assumption, score means may be analyzed for the PROC MIXED ANOVA analysis. Lesion
scores range from 0 to 4 as described by Johnson and Reid (1970). Tests of random and fixed
effects were performed. The differences of means were calculated to determine any significant
differences between lesion scores among treatment groups. The data was determined to have a
Poisson distribution and a Tukey Kramer test was used to determine whether there were any
statistically significant differences between treatment groups.

As shown in Figure 7, the distribution of all lesion scores was also decreased in the
vaccinated animals. The vaccinated animals demonstrated lower lesion scores. Thus vaccination
in either the drinking water or via oral gavage resulted in less mortality and less morbidity after
challenge with *Eimeria*.

CLAIMS

We claim:

1. A yeast vaccine composition comprising a yeast comprising an immunostimulatory polynucleotide encoding an HMGB1 polypeptide or a CD40 ligand, wherein the yeast
5 expresses the HMGB1 polypeptide or the CD40 ligand on the surface of the yeast.
2. The vaccine composition of claim 1, further comprising an antigenic polynucleotide encoding an antigenic polypeptide.
3. The vaccine composition of claim 2, wherein the yeast expresses the antigenic polypeptide on the surface of the yeast.
- 10 4. The vaccine composition of claim 2 or 3, wherein the antigenic polypeptide is selected from the group consisting of an Influenza polypeptide, a Campylobacter polypeptide, a Clostridium polypeptide, a Salmonella polypeptide, an Eimeria polypeptide and a tumor associated polypeptide.
5. The vaccine composition of claim 4, wherein the antigenic polypeptide is selected from
15 any of SEQ ID NOs: 39-93.
6. The vaccine composition of any one of claims 2-5, wherein the yeast comprises more than one antigenic polynucleotide.
7. The vaccine composition of claim 6, wherein the more than one antigenic polynucleotide is derived from more than one species.
- 20 8. The vaccine composition of any one of the preceding claims, wherein the yeast is a *Pichia*.
9. The vaccine composition of claim 8, wherein the *Pichia* is *Pichia pastoris*.
10. The vaccine composition of any one of the preceding claims, wherein the HMGB1 polypeptide is a full-length HMGB1 polypeptide.
- 25 11. The vaccine composition of claim 10, wherein the HMGB1 polypeptide is selected from the group consisting of HMGB1 sequences provided in SEQ ID NOs: 2-30 and 94-105 and sequences with 95% identity to those sequences.
12. The vaccine composition of any one of the preceding claims, wherein the CD40 ligand is selected from a CD154 polypeptide and a CD40 agonistic antibody or portion thereof.
- 30 13. The vaccine composition of any one of the preceding claims, wherein the CD40 ligand comprises at least one of SEQ ID NOs: 106-127.

14. The vaccine composition of any one claims 2-13, wherein the antigenic polypeptide and the HMGB1 polypeptide or CD40 ligand are part of a fusion protein.
15. The vaccine composition of any one of the preceding claims, wherein the HMGB1 polypeptide or the CD40 ligand is GPI-anchored.
- 5 16. The vaccine composition of claim 15, wherein the immunostimulatory polynucleotide is inserted into a polynucleotide encoding α -agglutinin from *Saccharomyces cerevisiae*.
17. The vaccine composition of any one of the preceding claims, wherein the immunostimulatory polynucleotide further comprises a linker oligonucleotide encoding at least two linker amino acids.
- 10 18. A pharmaceutical composition comprising the vaccine composition of any one of the preceding claims and a pharmaceutically acceptable carrier.
19. The pharmaceutical composition of claim 18, wherein the pharmaceutically acceptable carrier is acceptable for oral or nasal administration.
20. The pharmaceutical composition of claim 18 or 19, wherein the yeast is not capable of replication.
- 15 21. The pharmaceutical composition of any one of claims 18-20, wherein the yeast is inactivated or killed.
22. A method of enhancing an immune response in a subject comprising administering to the subject the vaccine composition of any one of claims 1-17 or the pharmaceutical composition of any one of claims 18-21 in an amount effective to enhance the immune response of the subject to the vaccine composition.
- 20 23. The method of claim 22, wherein the vaccine composition is administered orally or intranasally.
24. The method of any one of claims 22 or 23, wherein the subject is selected from the group consisting of human, cows, cats, dogs, pigs, fish, catfish, snapper, goldfish, birds, poultry, chickens, and turkeys.
- 25 25. The method of any one of claims 22-24, wherein the antibody response to the vaccine composition or to polypeptides in the vaccine composition is enhanced after administration of the vaccine composition.
- 30 26. The method of claim 25, wherein the immune response is an IgA antibody response.

27. The method of any one of claims 22-26, wherein the yeast in the vaccine composition is not capable of replication in the subject.
28. The method of any one of claims 22-27, wherein the yeast in the vaccine composition is inactivated or killed prior to administration to the subject.