Killed but metabolically active vaccines

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Beginning in the 20th century and continuing into the new millennia, vaccines against numerous diseases have had an unquestioned principal role of both enhancing the quality of life and increasing life expectancy (Rappuoli R, Mandl CW, Black S, De Gregorio E: **Vaccines for the twenty-first century society**. *Nat Rev Immunol* 2011, **11**:865–872). Despite this success and the development of sophisticated new vaccine technologies, there remain multiple infectious diseases including tuberculosis, malaria and AIDS that await an effective prophylactic vaccine. In addition, there have been recent clinical successes among individuals with cancer using vaccine treatment strategies — so-called therapeutic vaccines — that stimulate tumor specific immunity and increase survival (Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, *et al*.: **Sipuleucel-T immunotherapy for castration-resistant prostate cancer**. *New Engl J Med* 2010, **363**:411–422). Here we summarize a new class of vaccines termed Killed But Metabolically Active (KBMA). KBMA vaccines are whole pathogenic or attenuated organisms killed through photochemical inactivation and cannot cause disease, yet retain sufficient metabolic activity to initiate a potent immune response. KBMA vaccines have two broad applications. First, recombinant KBMA vaccines encoding selected antigens relevant to infectious disease or cancer can be used to elicit a desired immune response. In the second application, KBMA vaccines can be derived from attenuated forms of a targeted pathogen, allowing for the presentation of the entire antigenic repertoire to the immune system, of particular importance when the correlates of protection are unknown.

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Introduction

Killed But Metabolically Active — KBMA — refers to a new class of vaccines based on whole microbes that have been inactivated by defined genotoxic methods that render the organism incapable of productive growth — and of causing disease — but retain metabolic activity sufficient to induce immunity [1**]. In principal, KBMA vaccines address vaccinologists' dilemma, defined generally by the observation that vaccines based on live-attenuated pathogens while immunogenic can have unacceptable toxicity, and subunit vaccines, while safe can have weak potency [35]. This review discusses two broad applications of KBMA vaccines: (1) recombinant KBMA vaccines based on attenuated strains of the intracellular bacterium *Listeria monocytogenes* (Lm), engineered to express designated antigens (Ags) derived from to targeted infectious and malignant diseases; and, (2) KBMA vaccines derived from attenuated forms of virulent pathogens. In both applications the vaccine is inactivated by a defined and limited disruption of the vaccine chromosome using photochemical treatment with a psoralen cross-linking agent, impacting an absolute block to DNA replication and possible vaccine outgrowth. However, the lesions are limited and random within the genome, such that in the context of a full dose containing multiple logs of the inactivated vaccine (e.g., 1×10^8 units), the expression of any one gene in the population is largely unaffected. As a result, KBMA vaccines are capable of expressing the genetic repertoire necessary to proceed through the life-cycle of the parent organism, including infecting target host cells (e.g., dendritic cells) and expressing Ags *de novo* for processing and presentation to the immune system. In contrast to killed vaccines that are inactivated by heat or formalin treatment, KBMA vaccines are sensed as 'live,' by the vaccinated host which in turn generates a functional immune response that protects against subsequent challenges with the fully pathogenic organism. The development of KBMA vaccines may, in concept, be most desirable for pathogens in which the correlates of protective immunity are unknown or poorly understood, as knowledge of neutralizing target Ags of the pathogen is not, *a priori*, a prerequisite for an effective vaccine. While the term 'KBMA' was coined to describe vaccines inactivated by a photochemical process combining treatment with a synthetic psoralen and long-wave UV light, vaccines can be inactivated by alternative methods that also preserve metabolic activity — and immunogenicity — such as γ-irradiation, and will be discussed.

KBMA approach

Photochemical inactivation of KBMA vaccines utilizes a technology that was developed initially for killing undetected microbes contaminating plasma and platelet blood products, known as the INTERCEPT Blood System (Cerus Corporation, Concord, CA), and has been adopted

Figure 1

(a)

8-Methoxy Psoralen
(8-MOP)

(b)

S-59 Psoralen

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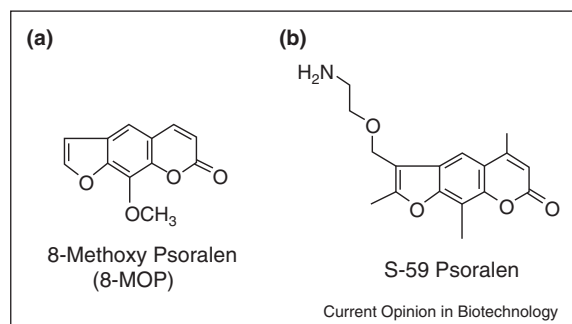
Structure of psoralens used for photochemical inactivation. Psoralens are natural compounds isolated from plants that induce interstrand crosslinks in combination with long-wave UV (UVA) light, blocking DNA replication and transcription by preventing strand separation [2]. Psoralen derivatives, including **(a)**, 8-methoxypsoralen, used to treat psoriasis [29]; and **(b)**, S-59 psoralen, used for pathogen activation and to produce KBMA vaccines [1••,3••].

by blood processing centers in several European countries [2,3••]. The process combines sequential treatment of a highly active synthetic psoralen known as amotosalen hydrochloride (S-59) and illumination with long-wave UV (UVA) light to form covalent monoadducts and crosslinks with pyrimidine bases of DNA and RNA (Figure 1). The extent of nucleic acid modification is dependent on psoralen dose, and micromolar amounts of S-59 are used to ensure broad inactivation of pathogens of diverse origin that may contaminate blood, and have variable sensitivity to photochemical inactivation. In contrast, the goal of KBMA vaccines is to use the lowest concentration of S-59 psoralen that results in complete inactivation, as the number of DNA crosslinks is inversely correlated to gene expression (Figure 2). The primary mechanism in bacteria for repair of psoralen crosslinks is nucleotide excision repair (NER), a process initiated by the ABC exinuclease, a DNA nuclease encoded by the ultraviolet light response (*uvr*) genes [4]. The exinuclease senses DNA crosslinks and cleaves at flanking sites, leading to removal of the damaged DNA region and repair. Photo-

Figure 2

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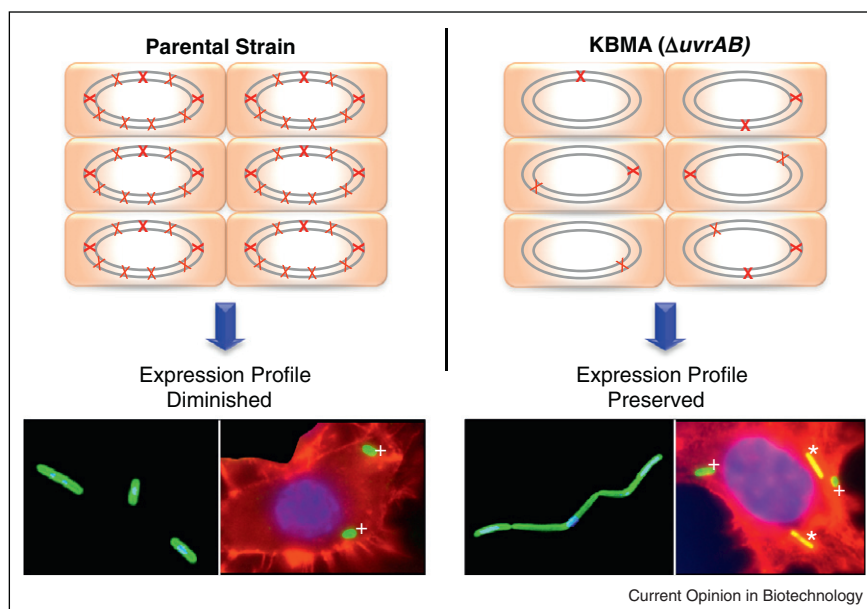
KBMA vaccines derived from nucleotide excision repair (NER) mutants retain metabolic activity. Top panels: Schematic representation of photochemical inactivation of parental and NER mutant (Δ*uvrAB*) vaccine strains, showing reduced level of interstrand crosslinks required to inactivate DNA repair mutant strains. Bottom panels: Immunofluorescence microscopy of photochemically inactivated Lm parental and KBMA Lm vaccine strains. Each lower panel shows photomicrographs of photochemically inactivated Lm incubated in broth culture (left picture) or 6 hours post-infection of mouse DC2.4 dendritic cells (right picture), respectively. Bacteria were stained with a FITC-labeled (green) Lm-specific antibody, and DNA with DAPI. To distinguish phagolysosome-confined Listeria from metabolically active Lm that escaped to the cytosol, bacteria residing in infected DC2.4 cells were stained with rhodamine-labeled (red) phalloidin which binds to actin produced by Lm in the cytoplasm, which subsequently decorates the bacterial cell surface. Bacteria confined in the vacuole appear green and are marked with a '+' in the figure, and Listeria in the cytoplasm appear yellow and are marked with a '*' in the figure. The photomicrographs demonstrate that photochemically inactivated parental strains lack metabolic activity sufficient to grow in broth culture or to access the cytosol of infected cells (left lower photomicrographs). In contrast, photochemically inactivated KBMA vaccines grow in broth culture (but cannot divide because crosslinked genomes cannot segregate), and access the cytosol of infected cells (right lower photomicrographs).

chemical inactivation of organisms with intact NER requires a concentration sufficient to overwhelm DNA repair mechanisms. Deletion of any one of the three *uvr* genes renders bacteria exquisitely sensitive to photochemical inactivation. While 2.5 mM of S-59 psoralen was required to completely inactivate parental Lm strains with intact NER, 200 nM was sufficient to inactivate 1×10^8 colony forming units (cfu), of Lm NER mutants deleted of *uvrAB* [1••]. Remarkably, direct quantitation of DNA crosslinks in photochemically inactivated Lm *uvrAB* mutants revealed that the psoralen adduct frequency was comparable to the theoretical crosslink frequency calculated by Poisson distribution where a single crosslink in a DNA repair mutant bacteria is lethal, by providing an absolute block to DNA replication. The *uvr* genes are highly conserved, allowing for the KBMA approach to be broadly applied among bacterial-based vaccines. For example, KBMA vaccines developed from *Bacillus anthracis* [5••] and *Salmonella typhimurium* [6•] vaccines by deleting *uvrAB*, like KBMA Lm, had increased sensitivity to photochemical inactivation, which correlated with enhanced metabolic activity and vaccine potency, as compared to the isogenic parental strains with intact NER.

Recombinant KBMA *L. monocytogenes* (Lm) vaccines

L. monocytogenes (Lm) is a facultative intracellular bacterium characterized by its ability to induce a profound innate immune response that leads to robust and highly functional CD4 and CD8 T cell immunity specific for vaccine-encoded Ags [7]. We have completed three separate Phase 1 clinical trials in settings of cancer and chronic infection and we are currently conducting a Phase 2 clinical trial, all under US IND, with each trial utilizing a genetically defined live-attenuated Lm vaccine platform strain [8•,9]. While this live-attenuated strain was shown to be safe and well-tolerated in these clinical settings, Lm is a food-borne bacterium with increased pathogenicity in immune-compromised populations [10]. Therefore, recombinant KBMA Lm platforms may have application for treatment or prevention of diseases having a risk-benefit profile not appropriate for live-attenuated vaccines. To prime CD8 T cells, Lm must first escape from the vacuole of infected dendritic cells (DCs) in a process mediated by expression of a pore-forming cytolysin known as listeriolysin O (LLO) [11,12], and desired antigens are engineered to be expressed and secreted from bacteria in the cytoplasm, where they are subsequently processed and presented on MHC class I molecules (Figure 3). Heat-killed Lm is not metabolically active and as a result cannot prime CD8 T cells that confer protective immunity [13,14]. Photochemically inactivated Lm NER mutants thus represent an acid-test for the KBMA approach, as gene expression must occur post-immunization for recombinant Lm vaccines to be effective. As shown in Figure 2, in contrast to parental

strains with intact NER (and therefore treated with higher doses of S-59), photochemically inactivated KBMA Lm *uvrAB* mutants are able to access the cytosol of infected DCs. As a result, recombinant KBMA Lm vaccines were shown to elicit protection against lethal virus or bacterial challenge, and to provide a survival benefit when used to treat tumor-bearing syngeneic mice, all models requiring functional cytotoxic T lymphocytes for efficacy. While effective, KBMA Lm vaccines were less potent than their analogous live-attenuated counterparts. To overcome this deficiency, we subsequently engineered KBMA Lm vaccines to constitutively synthesize bacterial proteins critical for intracellular growth and Ags, which are normally induced after infection by a transcription regulator known as Prf [15]. So-called PrfA* mutants express these determinants constitutively, and the PrfA* modification conferred a significant improvement to the potency of KBMA Lm vaccines [16•]. The PrfA* KBMA Lm vaccine platform may form the basis for future clinical evaluation.

Hohmann and colleagues derived an alternative recombinant KBMA vaccine candidate based on *S. typhimurium* CK362 (St; KBMA St CK362), a recombinant strain evaluated previously in human volunteers that encodes HIV Gag, and is attenuated by deletions of the *phoP/phoQ* genes encoding virulence factors and *aroA*, a gene encoding an enzyme required for synthesis of aromatic amino acids [6•]. KBMA St CK362 synthesized bacterial proteins following photochemical inactivation with S-59 psoralen and UVA illumination, and induced bacterial-specific antibodies in vaccinated mice at equivalent levels measured to mice vaccinated with live St CK362 [6•]. However, induction of HIV Gag-specific antibodies was not observed in mice vaccinated with either KBMA St CK362 or live St CK362.

Pathogen-derived KBMA vaccines

A compelling feature of the KBMA technology is that photochemically inactivated vaccine candidates maintain the complete antigenic repertoire of the selected pathogen. This diversity of antigens allows for the development of vaccines without knowing the correlates of protective immunity. The property of continued gene expression differs fundamentally from heat or formalin-inactivated vaccines in that retained metabolic activity of KBMA vaccines post-inactivation allows for expression and secretion of proteins in the vaccinated recipient that can be lost during the manufacturing process. KBMA vaccines represent an attractive alternative particularly for pathogens from which live-attenuated vaccine candidates would face significant regulatory barriers due to safety and reactogenicity concerns. One possibility may be *Clostridium difficile* (Ct), a Gram-positive spore-forming anaerobic bacterium that can cause acute diarrhea and colitis and is particularly a problem among the elderly who are hospitalized for other underlying illness or other

Figure 3
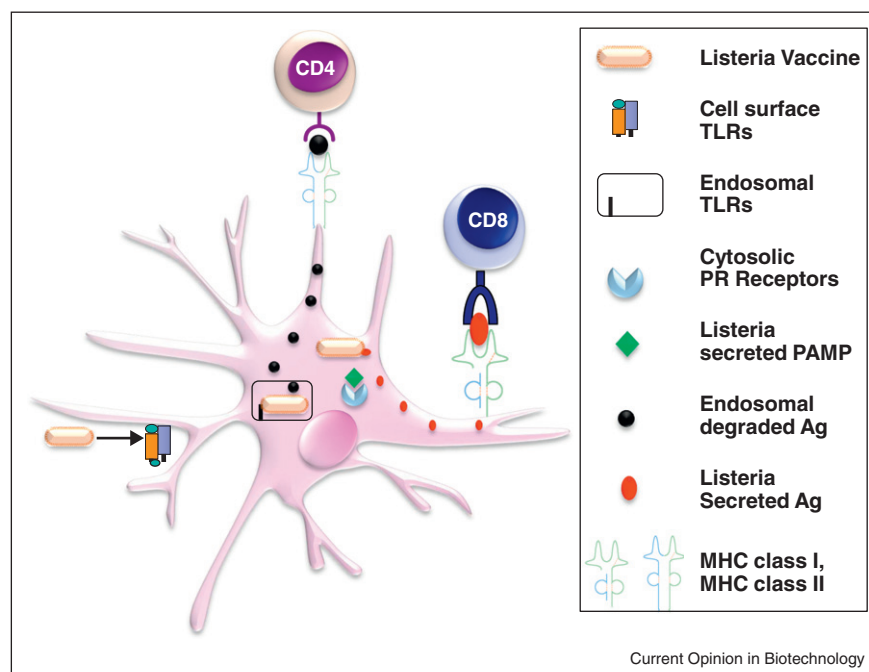
Schematic illustration demonstrating infection of dendritic cells with recombinant KBMA Lm vaccines. Lm vaccines induce a profound Th1-skewed innate immune response through interaction with both cell surface and endosomal TLRs [7]. Listeria in the cytoplasm produces a PAMP (pathogen-associated molecular pattern) known as cyclic-di-AMP which induces production of IFN-β through binding of the cytosolic PRR (Pattern Recognition Receptor) known as STING (Stimulator of Interferon Genes), and activating downstream signaling [30,31•,32]. Recombinant KBMA Lm vaccines are deleted of *uvrAB* genes, abolishing nucleotide excision repair (NER) capability, and are attenuated by deletion of two virulence genes, a*ctA* and *inlB* [33]. Antigen expression cassettes are integrated stably in the bacterial chromosome and are engineered to be induced and secreted from the bacterium in the infected dendritic cell [34]. The figure depicts Ags produced in the phagolysosome are degraded and processed by the MHC class II pathway; Ags secreted in the cytosol are subsequently processed by the MHC class I pathway.

long-term care facilities. The hallmarks of Ct pathogenicity including colonic mucosal injury and inflammation mediated by secreted toxins A and B. Ct 'toxoid' vaccine candidates based on formalin-inactivated A and B toxins have been shown in early phase human trials to be safe and induce seroconversion [17]. Conceptually, a KBMA vaccine candidate based on an attenuated asporogenic Ct strain encoding toxins engineered to be non-functional will elicit toxin-specific antibodies as well as cellular and humoral immunity against additional bacterial proteins that may contribute to more durable protection.

There are several bacterial pathogens that represent possible biothreats for which safe and/or effective vaccines are not available, including species of *Bacillus*, *Francisella*, *Brucella*, and *Burkholderia*. KBMA vaccines based on an engineered de-toxified and asporogenic *B. anthracis* strain (Sterne), elicited a protective antigen (PA) specific antibody response that not only protected immunized rabbits against lethal challenge with *B. anthracis* (Ames) strain spores, but also induced antibodies against a broad panel of undefined bacterial proteins [5••]. These results provided evidence that the KBMA vaccine could

serve as an alternative strategy to engineered virulent *B. anthracis* strains designed to subvert existing recombinant PA-based vaccines. Another conceptually attractive example is a KBMA *Burkholderia* vaccine to prevent Glanders and Melliodosis (diseases endemic to Southeast Asia). To date, subunit vaccines have failed to illicit protective immunity and the most effective vaccine in animal models is heat killed *Burkholderia pseudomallei* which prolongs survival but does not provide sterilizing immunity. Given the increase in potency of KBMA vaccines over heat or formalin-inactivated vaccines, there are compelling reasons to investigate whether this approach can increase the potency of a whole-cell *B. pseudomallei* vaccine.

The KBMA technology can be applied to emerging pathogens or pathogens for which the correlates of immunity are unknown. A recent example includes KBMA-Lic, a vaccine candidate developed by Noah Craft and colleagues to prevent infection with *Leishmania infantum chagasi* (Lic), which can lead to visceral leishmaniasis, a deadly parasitic disease with significant mortality mostly affecting the developing world [18••]. 'Leishmanization,'

the practice of vaccinating high-risk individuals in endemic areas with live *Leishmania major* parasites, while used historically, has been largely abandoned due to the retained virulence of the immunizing parasite and the development of significant pathology in a high frequency of vaccinated individuals. In promising pre-clinical studies, KBMA-*Lic* promastigotes retained metabolic activity, induced Th1CD4 immunity and conferred protective immunity against challenge with virulent *Leishmania* in vaccinated mice. While KBMA-*Lic* vaccination was effective, the parasite was not engineered to abolish NER, suggesting the possibility that in contrast to bacteria, the comparatively larger genome of eukaryotic pathogens provides a therapeutic window of photochemical inactivation defined by the lowest amount of psoralen required for inactivation, combined with retained metabolic activity sufficient for vaccine potency. While the genes involved in protozoan NER are not as well characterized as in higher eukaryotes, it would be interesting to determine whether targeted deletion of putative genes associated with NER would increase the potency of the KBMA-Lic vaccine candidates, or as a general approach for generating KBMA-based vaccine candidates for other protozoan pathogens, including Trypanosome and Plasmodium species, for which there are no effective vaccines, and collectively account for a significant disease burden at a global scale [19–21].

Vaccines inactivated by irradiation

The notion of using alternative inactivation methods to formalin and heat in order to preserve antigenic structural integrity as well as to retain a basal level of gene expression is not new, and there are multiple examples of vaccine candidates based on inactivation by treatment with high doses of ionizing γ-irradiation. In contrast to the KBMA approach where a limited number of psoralen crosslinks are desired to maximize the genetic space available for expression of critical Ags, ionizing radiation induces extensive DNA damage and strand breakage, significantly diminishing the extent of continued gene expression post-inactivation. While not directly compared, mice vaccinated with γ-irradiated Lm provided only partial protection (1–2 logs) against challenge with wild-type Lm (wtLm), correlated with minimal secondary expansion of CD8 T cells following several vaccinations [22]. In contrast, Ag-specific CD8 T cells primed by KBMA Lm vaccines proliferated in response to secondary Ag challenge, correlated with >5 logs of protection against wtLm challenge, providing supportive evidence that KBMA may be a superior inactivation approach for preserving vaccine potency [16•]. A malaria vaccine candidate being developed by Sanaria based on γ-irradiated *Plasmodium falciparum* sporozoites (PfSPZ), when given by intradermal or subcutaneous immunization to human volunteers was safe, but had weak immunogenicity [23,24]. Based on results in animal models demonstrating

that intravenous vaccination of PfSPZ induced PfSPZ-specific CD8 IFN-γ T cells and protection, the company is sponsoring a clinical trial with γ-irradiated PfSPZ given by the intravenous route. Given the differences between photochemical and γ-irradiation inactivation methods, it would be interesting to compare the potency of KBMA PfSPZ NER mutant and γ-irradiated PfSPZ vaccine candidates.

Clinical advancement of KBMA vaccines

While there have been several proof-of-concept publications demonstrating that KBMA vaccines induce functional immune responses correlated with efficacy in animal models of infectious disease and cancer, this new technology has not been advanced to human trials. One possible clinical candidate was indicated from recent investigations conducted by Bhardwaj and colleagues, who demonstrated that human DCs infected *ex vivo* with KBMA Lm encoding selected melanoma-associated Ags (MAAs) stimulated the maturation of human DCs infected *ex vivo*, resulting in presentation of Ags on MHC class I molecules and recognition and priming of human CD8 T cells [25••]. The FDA approval of Provenge®, an autologous antigen presenting cell preparation combined with a recombinant protein consisting of prostatic acid phosphatase (PAP) fused with GM-CSF, for the treatment of metastatic prostate cancer has validated active cancer immunotherapy as a therapeutic area and reinvigorated the field [26,27]. Provenge® provides only a modest — albeit significant — survival benefit, leaving ample room for improvement. Human DCs infected *ex vivo* with KBMA vaccines encoding the PAP GM-CSF fusion protein or encoding selected MAAs may be interesting therapeutic candidates for evaluation in clinical settings of prostate cancer and melanoma, respectively.

For infectious disease applications, malaria may be an attractive indication to evaluate the safety, immunogenicity and potency of KBMA Lm vaccines. Malaria vaccinologists have long-tried to induce protective CD8 T cell immunity. The most advanced candidate vaccine is RTS,S, being developed by GSK, and is an adjuvanted recombinant *P. falciparum* protein [28]. The observed partial protection is thought to be mediated by CD4 T cells and antibodies. Boosting the RTS,S-induced immunity with KBMA Lm and adding robust, multi-functional CD8 T cell immunity might result in more effective protection. Initial clinical testing to demonstrate safety, immunogenicity and protection would be performed in healthy volunteers.

Conclusions

Both recombinant and pathogen-derived KBMA vaccine candidates have been developed by several groups, and have been shown to be safe, immunogenic and correlated with disease-specific prevention or reduction

in preclinical animal models of infectious disease and cancer. Results showing protection against challenge in animals given KBMA vaccines derived from photochemically inactivated protozoan parasites not engineered to abolish NER capability provides proof of concept evidence that this approach may be applicable to eukaryotic pathogens, including malaria. KBMA vaccines may have application as a rapid-response strategy for development of vaccines against biothreats or emerging pathogens. While the S-59 psoralen-based photochemical inactivation process has been used to inactivate possible pathogen contamination in thousands of platelet and plasma blood units that have been safely transfused into humans, the next phase of development will include the development of manufacturing process methods and safety-toxicology assessment to enable clinical evaluation of KBMA vaccines.

Conflicts of interest

Acknowledgement

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