ELECTRON BEAM AS A NEXT GENERATION VACCINE PLATFORM: MICROBIOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF AN ELECTRON BEAM BASED VACCINE AGAINST *Salmonella* Typhimurium

A Dissertation

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ABSTRACT

Vaccines against infectious diseases are a corner stone of public health globally. Vaccine technology based on attenuation, conventionally "killed" or cellular sub-units suffer from significant drawbacks. Live attenuated vaccines impart strong cellular and humoral immune response compared to inactivated or subunit vaccines, but there is an increased risk of infection from the potential virulence reversion in such formulations. The current study explores the use of eBeam irradiation as a novel tool for vaccine generation. The value of eBeam based vaccines is that, they combine the safety of "killed" vaccines, yet retain the immunogenicity of "attenuated" vaccines. The principle of eBeam vaccine is eBeam irradiation will only irreversibly damage the target microorganism's nucleic acid without modifying the antigenic properties of surface macromolecules. In this study microbiological characterization of eBeam based *S*. Typhimurium (EBST) vaccine was carried out. The immunological correlates of protection induced by the EBST in dendritic cell (DC) (*in vitro*) and mice (*in vivo*) model were also assessed.

Results showed that eBeam inactivation preserved the potent proinflammatory and immunogenic properties of *S*. Typhimurium. The EBST remained metabolically active yet unable to multiply under favorable *in vitro* and *in vivo* conditions. The EBST potently stimulated innate pro inflammatory response (TNFα) and maturation (MHC-II, CD40, CD80 and CD86) of DC. Immunostimulatory potential of EBST was on par with live *Salmonella*, and most importantly on par with a commercial *Salmonella* vaccine.

Results from the mice challenge studies demonstrated the involvement of $CD4^{+}$T cells as key player in cell mediated immune response of EBST immunized mice, triggering the production of Th1 cytokine IFNγ. This indicates the stimulation and development of robust *Salmonella* specific T cell response similar to that caused by the live attenuated vaccine ($AroA^{-}$ ST) formulation. The colonization of virulent *Salmonella* was also reduced in EBST immunized mice similar to AroA immuninized mice. The EBST retained stable immunogenic properties for several months at room temperature, 4°C, -20°C and also after lyophilization. These findings highlight the potential of eBeam technology as an effective and affordable next generation vaccine platform to address global public health issues.

DEDICATION

To my dearest Unnana Ammumma, my Guardian angel!

To my beloved husband Praveen!

To my ever loving Amma & Achan!

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NOMENCLATURE

ST	*Salmonella* Typhimurium
EBST	Electron beam irradiated *Salmonella* Typhimurium
HKST	Heat killed *Salmonella* Typhimurium
CFU	Colony Forming Units
PBS	Phosphate Buffered Saline
DC	Dendritic cell
BMDC	Bone marrow derived dendritic cell
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
IFNγ	Interferon gamma
TNF/TNFα	Tumor necrosis factor

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CHAPTER I

INTRODUCTION

The National Center for Electron Beam Research (NCEBR) at Texas A&M University has been at the forefront of developing different applications for high energy electron beam (eBeam) irradiation technology. These include applications in the development of high value pharmaceuticals, food safety, food security, phytosanitary applications, polymer modifications and wastewater treatment technologies. The Center's guiding principles are to exploit this technology across different disciplines so as to "*clean, heal, feed, and shape this world and beyond*". Over the past few years, the Center in collaboration with the USDA-ARS in College Station has demonstrated that eBeam irradiation can be used to develop immuno-modulators that can have commercial value as vaccines (Jesudhasan et al., 2010; Kogut et al., 2012).

The basis of eBeam vaccines is that eBeam (i.e., ionizing) radiation will only destroy the target microorganism's nucleic acid, without affecting any of the surface macromolecules. Ionizing radiation inactivates microorganisms by directly causing breaks in DNA strands, or indirectly by the generation of radiolytic byproducts that interact with DNA causing DNA breakages. The eBeam irradiation has been used for decades for microbial inactivation for sterilization and pasteurization (Dziedzic-Goclawska et al., 2005; Poon et al., 2004; Rivera et al., 2011 and Cabeza et al., 2010). The eBeam irradiation at appropriate doses can be used to inactivate large volumes of microbial cultures or sterilize materials (Pillai et al., 2007; Sommers and Fan, 2006 and

Brahmakshatriya et al., 2009). There are a number of salient advantages of eBeam based vaccines including its suitability for vaccine formulations that are either in a liquid, lyophilized, or semi-solid state (Pillai et al., 2007). Also, importantly, the technology to accurately deliver and measure varying eBeam energies and doses provides the added possibility of being able to customize this technology for scaling up vaccine production processes.

In order to understand the immune response mechanisms triggered by eBeam irradiated vaccines, *Salmonella* was chosen as the model vaccine. *Salmonella enterica* serovars consists of group of gram negative facultative intracellular bacteria that causes a variety of infections ranging from acute gastroenteritis to systemic enteric fever, and is regarded as a leading cause of food borne infections in humans as well as livestock (CDC, 2009; Mittrucker et al., 2000). Among the serovars of *Salmonella enterica*, *S.* Typhimurium (ST) gives rise to systemic infection and gastroenteritis in different animal models (Eisenstein, 1999), hence accepted as an experimental model for more virulent *Salmonella* serovars. Immune response of *S.* Typhimurium is widely studied in mice model (Mittrucker et al., 2000), hence would serve as an ideal candidate to understand immune mechanisms induced by eBeam irradiated vaccine preparations. Preliminary studies conducted in laying hens, support the ability of irradiated vaccine to reduce bacterial colonization during challenge studies and provided evidence of the stimulation of the immune response (Jesudhasan et al., 2010).

Several attenuated strains of *Salmonella* are currently being considered as live vaccine candidates as they are capable of inducing humoral as well as cellular immune responses (Mittrucker et al., 2000). Studies have revealed that live vaccines are more effective than killed or non-replicating vaccines especially against pathogens that require cell mediated defenses like *Salmonella* (Thatte et al., 1993; Harrison et al., 1997). But, safety concerns limit the utilization of live/attenuated preparations as vaccine (Datta et al., 2006), especially considering the increasing number of chronically immunocompromised individuals, with a high risk of contracting vaccine induced diseases after immunization (McFarland, 1999, Wolfe and Bhat, 2005). In this context, eBeam irradiated vaccine serves as an ideal alternative to live attenuated vaccine provided, they are capable of inducing the cellular and humoral immune response similar to or better than that by the live vaccine preparations.

The long term goal of this research is to use high energy electron beam (eBeam) technology to develop effective, low-cost irradiated vaccines, which can impart long term immunity against pathogens. The central hypothesis is that high energy electron beam (eBeam) irradiation can inactivate *Salmonella*, but would preserve the immunomodulatory properties enabling it to serve as an efficacious vaccine. In this study, we conducted detailed microbiological characterization of eBeam based *S*.Typhimurium (EBST) vaccine and assessed the immunological correlates of protection induced by the EBST using *in vitro* and *in vivo* assays. Dendritic cell lines (DC) and mice studies were used for in vitro and in vivo assays respectively. These studies were

performed to gain a deeper understanding and develop new knowledge about the mechanisms by which eBeam vaccines confer immunity.

The specific objectives of the research were:

(1) To develop eBeam irradiated ST that has effective immune-modulation potential.

(2) To perform microbiological characterization of the eBeam inactivated vaccine to determine the stability and longevity of vaccine.

(3) Evaluate the immunogenicity of eBeam irradiated vaccine and understand the underlying immune response mechanism triggered by the vaccine.

CHAPTER II

LITERATURE REVIEW

Vaccines- background

Vaccines are considered as a revolutionary advancement in the history of human and animal health care. Ever since the use of first cowpox virus vaccine by Edward Jenner in 1796, vaccination has played a major role in the conquest of infectious diseases and in improving public health. One of the greatest benefits of vaccination to the modern society is the increase in life expectancy. Increased life expectancy is due to the greater control of infectious diseases, decreased early mortality and decreased exposure to acute and chronic inflammatory processes (Rappuoli et al., 2011; Wilson and Marcuse, 2001). Key contributions of vaccination include small pox eradication, almost complete elimination of poliomyelitis, reduction in incidence of diphtheria, pertussis, tetanus, measles, mumps, rubella, hepatitis A, hepatitis B, *Haemophilus influenzae* type b, pneumococcus etc. (Rappuoli et al., 2011).

There are several criteria for an effective vaccine (Adapted from Janeway et al., 2001)

- Vaccines must be safe - When administered, the vaccine itself must not cause illness or death.

- Vaccines should be protective - Vaccines must protect the host against subsequent re-exposure to pathogen.
- Vaccine should provide sustained protection - The protection imparted by vaccine should be long lasting.
- Induction of neutralizing antibodies - Vaccines that develop neutralizing antibodies prevent infection of cell, which is particularly useful in case of vaccines like polio vaccine where the virus infects irreplaceable cells such as neurons.
- Induction of protective T cells - Development of cell mediated immune responses is effective especially for control of intracellular pathogens.
- Vaccines should have practical value - This includes cost-effective production, and biological stablity. Vaccines should also be easy to administer with negligible to low side effects.

Effectors of vaccine response

In order to control or eliminate diseases, vaccines must mediate long term protective immunity. Long term immunity is conferred by antigen specific immune effectors and by the induction of immune memory cells that are capable of rapid reactivation upon re-exposure to pathogen (Siegrist, 2008). Major vaccine immune effectors include antibodies, cytotoxic $CD8^+$ T lymphocytes (CTL) and $CD4^+$ T helper lymphocytes (Th). Antibodies produced by B lymphocytes in response to antigen stimulation, are capable of neutralizing or binding to specific pathogens or toxins.

Cytotoxic T cells recognize and lyse the infected cells directly or secrete specific antiviral cytokines. Helper T cells provide adequate growth factors and signals for generation and maintenance of both B cells and CTL. The $CD4^+$ helper T cells are divided into Th1 and Th2 subtypes. Upon activation, Th1 cells differentiate into cells mainly secreting Th1 cytokines such as IFNγ, TNFα, IL-2 that exert antimicrobial functions by supporting cytotoxic T cells and macrophages. On the other hand, activated Th2 cell differentiate into cells secreting IL-4, IL-6, IL-10 providing support essentially to B lymphocytes. Immune memory T cells are also considered important determinants of vaccine efficiency. Memory T cells are divided in to effector memory cells (T_{em}) and central memory cells (T_{cm}) based on their phenotype and function. Effector memory T cells traffic through non lymphoid organs monitoring specific microbial peptides. They act immediately upon pathogen recognition and have high cytotoxic potential. Central memory T cells, on the other hand, traffic through lymph nodes, bone marrow and recognize antigens transported by activated dendritic cells. Their action is delayed but exhibits high proliferative potential and limited cytotoxic capacity (Siegrist, 2008; Sallusto et al., 2004).

Classification of vaccines

For a long time, , empirical approaches such as use of live attenuated or killed organisms and partially purified components of organisms were used for developing vaccines (Plotkin, 2003). Broadly, vaccines can be classified into live attenuated and inactivated vaccines (CDC, 2012).

Live attenuated vaccines are derived by modifying wild type or natural disease causing pathogen. The pathogen is "attenuated" or "weakened" by different methods which reduces its ability to cause disease, but retains the ability to replicate and produce immune response (CDC, 2012). There are different approaches used for generating live attenuated vaccines. One such approach pioneered by Edward Jenner is to use a natural pathogen in another host to serve as a vaccine in a different host system. As a result, there occurs an abortive infection in the recipient host which triggers immune response. This approach was used in case of cowpox vaccine, parainfluenza vaccines etc. (Plotkin, 2003). Attenuation of pathogens can also be achieved by extensively passaging them in an *in vitro* tissue culture model or an animal host till there reaches a balance between the loss of virulence and retention of immunogenicity. The BCG, oral polio vaccine, flu, measles and yellow fever vaccines were developed using this approach (CDC, 2012; Plotkin and Plotkin, 2013). Use of a naturally occurring attenuated strain of pathogen is also possible, such as the use of Type 2 polio virus (Arnon, 2011). The most common method of attenuation is to select mutants that are generated by cold adaptation (rubella, live flu), temperature sensitivity (respiratory syncytial virus), auxotrophy (Ty21a typhoid vaccine) (CDC, 2012; Plotkin, 2003; Rappouli et al., 2011).

Live attenuated vaccines replicate like the original pathogenic strains and therefore cause immune responses similar to a natural infection. Thus they confer effective long term immunity to host. They are required in only lower doses and works effectively with different routes of vaccine administration (Siegrist, 2008). But, live attenuated vaccines can cause severe reactions in case of immunocompromised

individuals making it unsafe for administration. The reversion of attenuated organisms to pathogenic form has been reported in case of poliomyelitis referred as vaccine associated paralytic poliomyelitis (VAPP) (Wilson and Marcuse, 2001). The immune response of live attenuated vaccines can be affected by interfering circulatory antibodies present in the host system, which prevents replication of attenuated vaccine. As a result, such live vaccines can mount only a poor immune response and thus causes vaccine failure. Live attenuated measles vaccines are known to be very sensitive to circulating antibodies (CDC, 2012). Being live organisms makes the attenuated vaccines very fragile and prone to damage by heat or light. Hence, proper storage and handling is required for live attenuated vaccines (Plotkin and Plotkin, 2013).

As the name suggests, inactivated vaccines are obtained by inactivating the whole disease causing organisms using physical methods such as heat or chemical methods such as formalin inactivation (Plotkin, 2003). For developing fractional inactivated vaccines, the organisms are further treated and different immunogenic components are purified. Such fractional inactivated vaccines include subunit as well as toxoid vaccines. Polysaccharide vaccines are also inactivated vaccines that contain polysaccharide molecules present in surface capsules of bacteria. Conjugate vaccines have also been produced by combining protein molecules with polysaccharide to develop vaccines with increased immunogenicity. Recombinant vaccines are developed using genetic engineering technology where gene segments from pathogenic organisms are inserted to nonpathogenic host. Table 2-1 provides the list of different vaccine classifications and examples of vaccines developed under each category.

Table 2-1. List of different vaccine classifications and examples of vaccines developed under each category

Vaccine development strategy	Examples
Live attenuated vaccine	
Viral	measles, mumps, rubella, vaccinia, varicella, zoster, yellow fever, rotavirus, intranasal influenza, oral polio
Bacterial	BCG, oral typhoid
Inactivated vaccines	
Whole cell vaccines	
Viral	polio, hepatitisA, rabies, influenza
Bacterial	pertussis, typhoid, cholera, plague
Fractional vaccines	
Subunit	hepatitis B, influenza, acellular pertussis, human papillomavirus, anthrax
Toxoid	diphtheria, tetanus
Polysaccharide vaccine	pneumococcus, meningococcus, *Salmonella* Typhi (Vi)
Conjugate polysaccharide	*Heaemophilus influenzae* typeb, pneumococcus, meningococcus
Recombinant vaccine	hepatitis B, human papillomavirus

Since inactivated vaccines are incapable of replication, they are considered safe even for immunocompromised individuals. Inactivated vaccines are also less affected by the circulating antibodies present in the host system. But, multiple doses of inactivated vaccines need to be administered to obtain immune response. In contrast to live vaccines, inactivated vaccines confer poor cell mediated immune response, hence are considered less immunogenic compared to live attenuated vaccines (Siegrist, 2008; Plotkin, 2003). Additional components such as adjuvants can be added to enhance the immunogenicity of inactivated vaccine.

Components in vaccine

Apart from the active component (antigen), vaccine formulations include various additional components that help in increasing, maintaining, and preserving immunogenicity of vaccine (Eldred et al., 2006). The components of vaccine include active component, adjuvant, diluent, stabilizer, preservatives and trace compounds.

Antigens are the active components of the vaccine. These could be either live attenuated, inactivated or subunits of the antigen. Adjuvants are compounds that are used to increase the immune response of vaccine. Adjuvants can be divided into delivery systems or immunomodulators (Coffman et al., 2010). Delivery system adjuvants help in prolonging the antigen deposition at the site of injection, recruiting more dendritic cells (DC). Immunomodulators provide additional activation and differentiation signals to DC and monocytes (Siegrist, 2008). Commonly used adjuvants include aluminium salts, Freund's adjuvant, oil –in- water emulsion, toll like receptor ligands. Even though

progress is being made in the use of adjuvants, none of the currently used adjuvants trigger the immune response to the same degree as that of live vaccines (Siegrist, 2008).

Diluents are usually sterile liquids such as water or saline that is provided with the vaccine for diluting the vaccine to appropriate concentration for administration. Stabilizers refer to the additives that help in maintaining effectiveness of vaccine by keeping the vaccine components stable. Commonly used stabilizers include lactose, sucrose, monosodium glutamate (MSG), glycine, bovine serum albumin, gelatin etc. (Eldred et al., 2006). Preservatives are added to vaccine formulations to prevent the fungal or bacterial contamination. A mercury containing compound, Thimerosal was used as a preservative. Use of these compounds are now highly restricted owing to the mercury content in the formulation. Preservatives are added mostly to multidose vials to prevent contamination. Trace compounds refers to the minute quantities of substances remaining from the production process of vaccine such as formalin traces from formalin inactivated vaccines, cell culture fluids, egg proteins, antibiotics etc. Sometimes antibiotics are also added to the formulation during the process of manufacturing to prevent bacterial contamination. Some of the commonly used antibiotics include neomycin, polymyxin B and gentamicin. It is always recommended to properly scrutinize for specific vaccine components before vaccine administration to avoid any potential allergic or adverse reactions.

Risks and public concerns associated with vaccination

Recent years witnessed an increase in public concerns over the safety and net benefits of vaccines. This might be because, the fear of vaccine preventable disease waned among public and there is increased awareness about potential adverse effects of vaccines (Wilson and Marcuse, 2001). Vaccines are not completely free from any side effects or adverse effects, but most of them are mild, rare, or could be due to coincidence. As part of the evaluation of vaccine safety, researchers conduct assessment of any possible causal relationship of vaccination to any specific adverse effect (NRC, 2012). One of the major vaccine safety issues arose when vaccine induced poliomyelitis was reported around 1955. It was attributed to the insufficient inactivation of poliovirus in certain lots of inactivated polio vaccine (IPV) which resulted in almost 260 cases of vaccine induced polio (Wilson and Marcuse, 2001). Another huge public outcry was reported when IPV was found contaminated with simian virus- 40 (SV-40), which is oncogenic in several species. The contamination occurred from the use of rhesus monkey kidney cells which were previously contaminated with SV-40. Sporadic cases of vaccine associated paralytic poliomyelitis (VAPP) from the oral administration of live attenuated polio vaccine (OPV) were also reported (Strebel et al., 1992). Another case of vaccine recall was reported in case of a new generation live recombinant viral vaccine, Rhesus-human reassortment rotavirus vaccine. Almost 13 cases of serious intestinal obstructive disorder (intestinal intussusception) was reported in vaccinated children which led to suspension of vaccine in 1999, one year after its licensing (Chang et al., 2001). Several physical conditions and diseases have been reported to be associated with

Table 2-2. List of new strategies for vaccine development (Adapted from Rappouli et al., 2011)

Vaccine development strategy	Examples
Cell culture	Rabies, Japanese encephalitis virus, varicella zoster, hepatitis A, rotavirus, Avian influenza, H1N1 influenza, small pox, yellow fever virus
Recombinant DNA, virus- like particles	Hepatitis B, acellular pertussis, lyme disease, HPV
Reverse vaccinology	meningococcus B, Staphylococcus aureus
Conjugation	H. influenzae type b (Hib), meningococcus, pneumococcus, GroupB Streptococcus, typhoid
Combination	Diphtheria-pertussis-teteanus-Hib, Hib- hepatitis B, DTaP-Hib, hepatitisB-hepatitis A, diphtheria-tetatnus-acellular pertussis-poliovirus-hepatitis B
New adjuvants	Influenza, HPV, H1N1 influenza, vaccines from superior alum formulation, TLR agonists

certain vaccines in recent years, which have no sound epidemiological evidence to support the association. Few of such hypothetical associations include autism due to MMR vaccine (Wakefield et al., 1998) and use of thimerosal in the vaccine (Ball et al., 2001), multiple sclerosis associated with hepatitis B vaccine (Ascherio et al., 2001), allergy due to adjuvant alum (Kristensen et al., 2000; Wilson and Marcuse, 2001). To address such risks and public concerns, it is highly important to develop effective and safer vaccine particularly for diseases of global importance. Stringent vaccine safety and efficacy trials should be conducted pre and post licensing of vaccines. There is an ever increasing demand for newer approaches in developing safer and effective vaccines to address the public concerns.

New approaches in vaccine development

Recent years have witnessed a greater interest for next generation vaccine development technologies. Some of the revolutionary technologies include use of recombinant DNA technology for development of hepatitis B vaccine, conjugation technology for *Haemophilus influenza*e type b vaccine, reverse vaccinology for meningococcus B vaccine, virus like particles (VPL) against human papilloma virus and the relatively new nucleic acids and synthetic vaccine technology (Plotkin, 2003; Rappuoli et al., 2011). Table 2-2 provides a brief list of some of the new and improved strategies used for vaccine development (Rappouli et al., 2011). This review focusses on the approach involving the use of ionizing radiation for vaccine development, with emphasis on electron beam (eBeam) technology.

Electron beam radiation (eBeam) – background and terminologies

There are majorly two sources of ionizing radiation- radioisotopes and linear accelerators. Both these sources produce short wave high energy radiation which have similar effects. Radioactive isotopes such as ^{137}Cs and ^{60}Co emit gamma radiation, whereas, electron beam is machine generated (linear accelerator), which does not involve any radioactive sources. The eBeam uses near-speed-of-light electrons generated from electricity as ionizing radiation source (Tahergorabi et al., 2012). In eBeam, electrons are accelerated under controlled electric and magnetic fields in a single direction to penetrate the target. Many public acceptance studies on ionizing radiation products showed that consumers preferred machine generated eBeam compared to radio isotope derived gamma radiation (Mitchell, 1994; Gehringer, 2003). The effect caused by eBeam depends upon certain key factors such as dose, dose distribution, stopping power, range and penetration of the eBeam.

When electrons travel through a target material, they tend to lose energy due to excitation and ionization of atoms present within the material. This average linear rate of energy loss of these electrons in a particular medium is referred to as "stopping power". Stopping power is generally expressed in terms of MeV per cm. It is also referred to as the linear energy transfer (LET) of the particle with units generally expressed as keV per μm (Turner, 1995). Stopping power and LET are closely related to the dose imparted to the material by the electrons traveling through that material. Dose refers to the energy deposition per electron colliding with an atom in the target surface thereby generating

secondary, tertiary electrons to dissipate off the energy. Absorbed dose is defined as the quantity of the ionizing radiation energy which is imparted per unit mass of the specified material being irradiated (Sommers and Fan, 2006). The SI unit of absorbed dose is the gray (Gy). Conveniently expressed as kilogray (kGy). One Gy represents one joule of energy deposited per kilogram of material. One kilogray refers to 1000 Grays.

Depth dose distribution profile depicts the energy absorbed by a unit mass at particular depth. This dose- depth distribution curve consists of energy absorbed from the incident electron or the absorbed dose as the ordinate and the depth of penetration as the abscissa. The dose distribution profile over different depths varies based upon the source of electron. Since the electron distribution is not constant, certain positions in the target material receives more energy compared to other positions. The uniformity of electron deposition is thus represented by a D_{max}/D_{min} ratio, where D_{max} and D_{min} correspond to depths receiving maximum and minimum dose respectively.

The electron distribution pattern is markedly different in case of single and double sided eBeam. In case of a double sided dose distribution curve energy deposition occurs from both the sides of the target material. Hence the range of the particle can be increased by using a dual e-beam which allows for the electrons to travel more distance inside the target material before it comes to rest (Turner, 1995). The dual beams overlap and would result in a synergistic effect at the point of convergence which maintains the dose distribution uniform throughout the thickness of the material.

Microbial inactivation by electron beam radiation

When microbial cells are exposed to ionizing radiations as in case of electron beam, it causes several direct as well as indirect effects that results in microbial inactivation. The primary effects of the eBeam is due to the collision of electrons in the microbial cell which results in breakdown of the vital cellular components such as nucleic acid whereas the secondary effects are due to the production of radiolytic species causing indirect damage to the cell.

The primary target of ionizing radiation is the genetic material of cell as they form the major component of the cell (Urbain et al., 1996). Damage to proteins, lipids and RNA contributes only to a lesser extent to microbial lethality (Sonntag, 1987). Upon entering the cell, eBeam induces nonspecific collision of electrons with atoms of the microbial cell that causes single and double stranded breaks in microbial DNA resulting in disintegration of DNA double helix (Tahergorabi et al., 2012; Smith and Pillai, 2004). Disintegration of DNA affects the cell division, resulting in cellular reproductive death of microbe (Tahergorabi et al., 2012). The damage is directly proportional to the genome size and has an inverse relationship with the radiation dose (Alsharafi and Mullbacher 2009). Microbial cell is capable of single strand as well as most of the double stranded (not directly opposed) DNA repair with the help of the DNA polymerase enzyme owing to its proof reading activity. Directly opposed double stranded DNA repair is hard to undertake as there does not exist a corresponding complementary base to provide the correct nucleotide information to repair the strand breakage. This kind of DNA strand

damage could possibly accumulate together to bring about lethal mutations in the nucleic acid of the microbial cell (Bartek and Lukas, 2003).

Apart from the DNA damage there also occurs production of a range of radiolytic species as a result of the series of oxidation reduction reactions occurring in the course of electron transfer from one atom to another. Water constitutes a major portion of the microbial cell and hence would suffer from the radiolytic breakdown by the incoming electrons producing several reactive oxygen species (ROS). The ROS thus produced are toxic to the cell and causes damage to the other cell components (Grecz et al., 1983). Major ROS produced includes hydroxyl radical, superoxide radical, hydrogen peroxide molecules etc. Cells do have innate systems such as catalase, peroxide reductase, superoxide dismutase which scavenges these ROS being formed (Arena, 1971). If there occurs sufficient indirect damage, cell death can occur by cellular leakage and complete cell lysis.

Another important effect is the production of mutagenic lesions in the DNA such as the GO lesion (Michaels and Miller, 1992). The 8 hydroxyguanine (GO) or the 7, 8 dihydro-8-oxoguanine (8-oxoG) refers to the damaged form of the Guanine residue in the nucleic acid. The GO lesions are very stable product of oxidative damage of DNA which can be due to ionic radiation, electron transport or lipid peroxidation. Cells have evolved different strategies such as GO system comprising of mutM, mutT and mutY genes to enhance the proof reading of the DNA polymerase III to encounter such lesions in the nucleic acid (Michaels and Miller, 1992).

Irradiated vaccines

Ability of ionizing radiation to induce microbial inactivation has been used in the past to develop irradiated vaccines. Most of the studies with irradiated vaccines used gamma irradiation for generating inactivated bacterial or protozoan vaccines. Some of the early studies were conducted using gamma radiation attenuated *Plasmodium berghei* sporozoites (Nussenzwig et al., 1967), *Rickettsia* (Eisenberg and Osterman, 1978; Jerrels, 1983), *Trypanosoma rhodensiense* (Wellde etal., 1973). It was shown that use of radiation attenuated pathogens that retain their ability to infect host cells was helpful in improving the immune response (Bain et al., 1999). Among the various vaccines developed, the most promising results were obtained from gamma attenuated *Plasmodium* sporozoites, causative organism for malaria in humans. It was shown that infecting murine model system with gamma irradiated mosquitoes harboring infectious sporozoites imparted protection (Nussenzwig et al., 1967). Rodent studies provided great impetus towards human studies involving radiation attenuated *P. falciparum* sporozoites (Clyde et al., 1990; Rieckmann, 1990). Immunization of human volunteers with bites of irradiated mosquitoes harboring *P. falciparum* and *P. vivax* sporozoites imparted protective immunity against challenge with infectious *P. falciparum* or *P. vivax* sporozoites (Hoffmann et al., 2001). This proved that gamma attenuated malaria vaccine provided sterile protective immunity. Recent clinical trials conducted by Seder et al. (2013), using intraveneous injection of gamma attenuated *P. falciparum* sporozoite imparted consistent, high level vaccine induced protection against human malaria, thus

becoming the first malarial vaccine to provide 100% protection against disease (Seder et al., 2013).

Studies using gamma irradiated *Listeria monocytogenes* have shown that, unlike heat killed or formalin treated bacteria, irradiated *L. monocytogenes* preserved antigenic and adjuvant properties (Datta et al., 2006). It was shown that irradiated *Listeria* induced protective T cell responses, previously thought to require live infection. Irradiated *Brucella abortus* and *Brucella neotomae* was also found to induce high levels of cellular immune response and imparted protection against virulent challenge in mice model system (Sannakkayala et al., 2005; Moustafa et al., 2011; Magnani et al., 2009). Encouraging results were obtained from studies using gamma irradiated influenza A virus vaccine (γ-A/PC) in mice, which showed that γ-A/PC was highly immunogenic and elicited cytotoxic T cell responses, most likely responsible for the long lasting cross protective immunity against highly lethal influenza A infections (Furuya et al., 2010; Alsharifi, 2009). Intranasal administration of single dose of γ-A/PC conferred significant protection in mice against homologous and heterosubtypic virus challenge (Furuya et al., 2010).

With the background obtained from the gamma irradiated vaccine, the current study focused towards exploring a relatively novel area of using environmentally safe electron beam irradiation for vaccine development. Proof of concept studies were conducted using a widely studied pathogenic bacterium *Salmonella enterica* serovar Typhimurium.

Salmonella

Salmonella are enteric bacteria that are of major clinical relevance in both developed and developing countries, where it causes food-borne illnesses (Scallan et al., 2011). *Salmonella* are found as commensal organism or as a pathogen in various warm and cold blooded animals (Dougan et al., 2011). The microbe is capable of surviving freely for extended time period in different environments such as seawater, sewage, river etc. *Salmonella* infects both humans and animals via fecal- oral route. *Salmonella* is commonly associated with acute gut-associated non systemic gastroenteritis or Salmonellosis (Dougan et al., 2011, Fabrega and Vila, 2013). Certain serovars of *Salmonella* can also cause systemic infection- typhoid fever in humans and animals. According to CDC, approximately 40,000 confirmed cases of Salmonellosis are reported in the United States annually (CDC). Illness is mostly predominant in elderly, young children and immunocompromised individuals that results in death of approximately 400 people per year (Scallan et al., 2011).

Salmonella belongs to the family Enterobacteriaceae and are gram negative, motile, facultative anaerobic, non-lactose fermenting rod shaped bacteria (Mastroeni et al., 2001; Dougan etal., 2011). The genus *Salmonella* comprises of two species which are pathogenic to humans, *Salmonella enterica* and *S*. bongori. Among the different species, *Salmonella enterica* is of prime importance as it harbors multiple serotypes that are pathogenic to humans and animals such as Typhimurium, Enteritidis, Typhi and Paratyphi. Although *Salmonella* can infect wide range of hosts, certain serovars are

restricted to specific host species and causes varied clinical symptoms. *Salmonella* Typhi causes typhoid symptoms in humans and other primates and it does not cause infection in mice or other rodents. Similarly, *S*. Paratyphi A is also restricted to humans, *S*. Gallinarum and *S*. Pullorum causes fowl typhoid. *Salmonella* Typhimurium causes invasive typhoid like symptoms in susceptible mice, which helps to utilize murine *S*. Typhimurium as a surrogate model for typhoid and general Salmonellosis (Dougan eal., 2011; Hormachae, 1979).

Salmonella pathogenesis

Most of the current understanding on *Salmonella* infection and immune response mechanisms are obtained from murine typhoid model (Dougan et al., 2011). Several studies have also been carried out in other model systems such as poultry, cattle and even human clinical observations (Villareal ramos et al., 2000; Pullinger etal., 2007; Hornick et al., 1970). *Salmonella* is acquired in host system via ingestion of contaminated food or water or from a carrier. Following ingestion, a proportion of bacteria resists the low pH prevalent in stomach environment and reaches the small intestine. The adaptive acid tolerance mechanism of *Salmonella* helps in survival of the bacteria in the low pH conditions of the stomach (Muller et al., 2009). *Salmonella* establishes a luminal colonization of small and large intestine and eventually causes bacterial shedding for significant time period. *Salmonella* effects the invasion of host system by penetrating the mucosal epithelia. *Salmonella* employs a number of virulence genes or pathogenicity genes defined as *Salmonella* Pathogenicity Island- I (SP-1) to

gain access to epithelial cells (Dougan et al., 2011). *Salmonella* mediates mucosal penetration through different routes. The bacteria can enter through specialized epithelial cells called microfold cells (M cells) with the contribution of SP-1 genes (Jensen et al., 1998; Jepson and Clark, 2001). An alternate penetration strategy involves direct phagocytosis of *Salmonella* by $CD18^{+}$ cell such as dendritic cells and macrophages, which reaches through the epithelial barrier and pulls the bacteria to sub epithelial layers (Vazquez-Torres et al., 1999). Another mechanism utilizes the ability of *Salmonella* to disrupt the tight junction of epithelial layers to gain entry to mucosal epithelia (Rescigno et al., 2001)). Thus, this bacterim employs a a combination of different routes to effect mucosal penetration.

During systemic infection, *Salmonella* gains entry to nonphagocytic cell such as epithelial cell, or phagocytic cells like macrophage and dendritic cells. *Salmonella* subverts the normal maturation of phagosomes and transforms it to *Salmonella* containing vacuole (SCV), where the bacteria survives and eventually undergoes replication (Petrovska et al., 2004; Wick, 2002). Expression of a functional natural resistance associated macrophage protein 1 (Nramp-1) in macrophage is essential to restrain the *Salmonella* invasion *in vivo* (Blanden et al., 1996; Monack et al., 2004; Mittrucker et al., 2000). In order to survive in the endosomal compartment *Salmonella* tightly regulate virulence determinants encoded by SPI-2 genes (Ohl and Miller, 2002). Following infection, bacteria spread from infected cells to new infection foci such as lymphatic tissues, liver spleen, bone marrow where they undergo further intracellular replication (Mastroeni et al., 2001; Dougan et al., 2011). During fatal infection with

higher bacterial loads, there occurs secondary bacteremia, endotoxic shock and rapid death (Makela and Hormaeche, 1997). Non-fatal infection is characterized by splenomegaly, general macrophage mediated immune suppression which results in plateauing of bacterial titers. Finally, there adaptive immune response occurs imparting long lasting immunity against reinfection (Mittrucker et al., 2000).

In vivo immune response to *Salmonella* Typhimurium

The immune response mechanisms elicited by the host reflects the different stages of *S*. Typhimurium infection. Primary immune response to *S*. Typhimurium occurs in different phases which include early innate response and adaptive immune response (Makela and Hormaeche, 1997; Eisenstein, 1999).

Innate immune response mechanisms are triggered during initial stages of *Salmonella* infection. When *Salmonella* gains entry to the epithelium, host cell invokes first set of innate immune responses targeted to ensure bacterial killing and control of bacterial replication. Phagocytic cells like macrophages and neutrophils are the major players in this phase which mediates bactericidal activities (Mittrucker et al., 2000). Proinflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor (TNFα) are considered as prerequisites for activating macrophages (Nauciel and Espinasse-Maes, 1992; Gulig et al., 1997). Activated phagocytic cells control the growth of virulent *Salmonella* using reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Shiloh et al., 1999; Mastroeni et al., 2001). The cytokines IFNγ and TNFα also help in rendering the *Salmonella* containing vacuole accessible to lytic

effector molecules from lysosomes (Mittrucker et al., 2000). During the infection, cell wall components like lipopolysaccharide (LPS), triggers Toll like receptor (TLR) mediated inflammatory response by the production of proinflammatory cytokines such as TNFα, Interleukin (IL)-12, IL-18, IFNγ by phagocytes such as DC, macrophage, and natural killer (NK) cells (McCormick et al., 1998). TNFα is involved in granuloma formation, whereas IFNγ is crucial for macrophage activation and also contributes to the formation of focal granulomas in tissues (Mastroeni et al., 2000). Both IL-12 and IL-18 help to regulate IFNγ production by NK cell and also aid in down regulation of anti-inflammatory cytokines such as IL-10 (Mastroeni et al., 1999; Mastroeni et al., 2000). Production of inflammatory cytokines stimulates a positive feedback loop by activating phagocytes to further produce inflammatory cytokines. Although the innate response mechanisms effectively restrict the initial bacterial growth, it alone is not sufficient to mediate complete bacterial elimination from the host. Moreover, *Salmonella* adapts to the innate immune mechanisms by expressing various virulence factors that provide resistance to bactericidal host mechanisms (Cirillo et al., 1998; Mittrucker et al., 2000). Hence, for effective bacterial elimination and for protection against subsequent pathogen encounter, it is necessary to generate *Salmonella* specific adaptive immune response mechanisms.

Dendritic cells (DC) serve as crucial link between innate and adaptive immune response to *Salmonella*. DCs are the most efficient antigen presenting cells (APC) capable of internalizing and processing the bacteria, and presenting the pathogen derived peptide antigens to naïve T cells to initiate antigen specific adaptive immune response

(Sundquist et al., 2004; Yrlid et al., 2000). Upon pathogen encounter, DC undergoes maturation process indicated by increased surface expression of co-stimulatory molecules and production of proinflammatory cytokines IL-12 and TNFα. Mature DC migrate from peripheral tissues to secondary lymphoid organs such as lymph nodes, spleen, where they present *Salmonella* antigens to prime naïve T cells (Sundquist et al., 2004). Antigen specific activation of T cells differentiates naïve $CD4^+$ T cells to Th1 cells. The major contributor of adaptive response is $CD4^+\alpha\beta TCR^+$ T cells with Th1 phenotype (Dougan et al., 2011). Th1 cells produce inflammatory cytokine IFNγ, aiding in recruitment of effector macrophages for antigen specific pathogen clearance (Mastroeni et al., 2001; Mastroeni and Menager, 2003). Th1 cells also mediate regulation of *Salmonella* specific B cell activation and maturation, resulting in production of isotype switched antibodies against bacterial polysaccharide and bacterial protein antigens (Sinha et al., 1997; Dougan et al., 2011). But, role of $CD8^+$ T cells and B cells are dispensable in case of primary response. Effector B cells and T cells transforms to memory cells after pathogen clearance, that provide immunological memory against *Salmonella* (Hess et al., 1996; Dougan et al., 2011). Apart from Th1 cells other T cells such as Th17 cells are also involved in immune response. Th17 help in recruiting neutophils, chemokines and defensins during early phase of immune response to trigger neutrophil influx to infection site (Liu et al., 2009).

Secondary immune response is characterized by relatively shorter innate phase followed by antigen specific adaptive phase resulting in faster bacterial clearance. Infection triggers activation of memory cells to transform to effector cells resulting in

faster induction of antigen specific adaptive immune response. Concerted action of both $CD4^+$ and $CD8^+$ T cell along with anti- *Salmonella* antibodies is required in secondary immune response (Mastroeni and Menager, 2003). $CD4^+$ T cell mediates protection through IFNγ mediated macrophage activation. $CD8^+$ T cell differentiates into cytotoxic T cells mediating lysis of infected cell and presence of antigen specific antibodies help in bacterial clearance, early in the infection phase (Dougan et al., 2011).

Vaccines against *Salmonella* infections

Vaccination is an effective tool for prevention and control of *Salmonella* infections (Mastroeni et al., 2001). Broadly, *Salmonella* vaccines can be classified as whole cell killed vaccines, subunit vaccines and live attenuated vaccines (Mastroeni and Menager, 2003).

Whole cell killed vaccine – Killed whole cell *Salmonella* vaccines are developed by inactivating the bacterial cultures using heat, β-propiolactone, glutaraldehyde or formaldehyde (Singh, 2009). Whole cell killed vaccines have been found to be immunogenic and induce poor cell mediated immunity (Collins, 1974; Harrison et al., 1997). Killed vaccines were found to induce good humoral immune response, but insufficient Th1-type response (Thatte et al., 1993; Harrison et al., 1997). In a mice model, killed whole cell vaccines conferred protection against virulent challenge (Collins, 1974; Eisenstein et al., 1984). Killed whole cell vaccine imparted partial protection in chicken against intestinal colonization, fecal shedding, systemic pathogen spread and egg contamination (Gast et al., 1992; Mastroeni et al., 2000). In calves, killed

Salmonella vaccines were found to be less protective compared to live attenuated vaccines owing to impaired cell mediated immunity (Bairey et al., 1978; Robertsson et al., 1983). Killed *S*. Typhi elicited good antibody response and conferred moderate degree of protection in humans (Levine et al., 1989).

Subunit vaccines/ Bacterial fractions – Subunit vaccines are relatively safe and immunogenic. Subunit vaccines like Vi polysaccharide of *S*. Typhi are currently licensed for human use (Acharya et al., 1987; Klugman et al., 1996). The immunogenicity and protective ability of Vi subunit vaccine can be increased by binding to certain proteins like tetanus toxoid, cholera toxin or diphtheria toxin (Kossaczka et al., 1999; Singh et al., 1999). Immunization with bacterial fractions have shown moderate protection which is mostly achieved effectively in innately resistant animals (Mastroeni et al., 2001). Immunization of innately resistant mice with porins imparted protection against virulent challenge (Svenson et al., 1979), whereas innately susceptible mice were protected against challenge with moderately virulent organisms (Kuusi et al., 1979). Administration of *S*. Enteritidis outer membrane protein (OMP) with adjuvant elicits high antibody titer and prevents *Salmonella* shedding in chicken (Meenakshi et al., 1999). Repeated immunization with naïve or alkali treated lipopolysaccharide (LPS) imparted protection only for innately resistant mice (Ding et al., 1990). Acid hydrolyzed LPS, *Salmonella* O-polysaccharides (O-PS) are less immunogenic in nature, but the immunogenicity of O-PS was found to increase when conjugated with protein carriers like tetanus toxoid, diphtheria toxin, porin etc. (Watson et al., 1992).

Inactivated *Salmonella* vaccines like killed whole cell vaccines and subunit vaccines confer protection against reinfection by inducing strong antibody response. They induce IL-4 dominated Th2 type immune response with low levels of delayed type hypersensitivity (DTH) and high levels of *Salmonella* specific antibodies of IgG1 isotype (Thatte et al., 1993; Galdiero et al., 1998; Mastroeni and Menager, 2003).

Live attenuated *Salmonella* vaccines – Live attenuated vaccines are immunogenically superior to inactivated vaccine due to their ability to induce cell-mediated immune response in addition to antibody response. Attenuated live *Salmonella* are generated either by inducing undefined mutations or defined mutations. Availability of the complete genome sequence of *S*. Typhimurium and *S*. Typhi has helped in generating very specific defined mutants which serve as potential vaccine candidates. Various *Salmonella* genes that have been targeted for attenuated vaccine development. A detailed list of various live attenuated *Salmonella* vaccine candidates is provided in table 2-3. Immunization with live attenuated *Salmonella* induces long lasting protective immunity and involves recall of immunological memory (Killar and Eisenstein, 1985; Hormaeche et al., 1991; Mastroeni etal., 1992). Cellular responses are of Th1 type, indicated by the DTH and production of proinflammatory cytokine IFNγ upon restimulation by immune T cells (Harrison et al., 1997; Sztein et al., 1994; Mastroeni and Menager, 2003). For long lasting protective immunity, live attenuated vaccine employs both humoral and T cell mediated immunity which is responsible for the superior potential of vaccines compared to inactivated *Salmonella* vaccines (Mastroeni et al., 2001; Mittrucker and Kaufmann, 2000).

Table 2-3. List of live attenuated *Salmonella* vaccine candidates developed and tested in humans and animals

Type of mutation/ attenuation	***Salmonella* strains/ serovars**	**Host**	**Comments**	**Reference**
Early live attenuated vaccines				
Chemical or ultra violet mutagenesis (Temperature sensitive mutants- TS)	*S*. Enteritidsis, *S*. Typhimurium, *S*. Typhi	Mice, humans	Induced anti-*Salmonella* antibodies, imparted protection against virulent *Salmonella* challenge in mice. TS S. Typhi was poorly immunogenic in humans	Gheradi etal., 1993; Nauciel, 1990; Bellanti et al., 1993
Streptomycin dependent mutants	*S*. Typhi	Humans	Safe and immunogenic (increased antibodies) in humans. Lyophilization drastically reduced vaccine efficiency	Levine etal., 1976
Undefined *Salmonella* mutant (*S*. Gallinarum 9R)	*S*. Gallinarum	Chicken	Afforded protection against virulent *S*. Gallinarum and *S*. Enteritidis. Induced only poor protection against *S*. Typhimurium	Smith, 1956; Silva et al., 1981
Adaptation in nonspecific host or some specific growth media	*S*.Dublin Strain 51	Calves	Induced cell mediated and humoral response and provided protection in calves compared to killed bacterins	Meyer et al., 1992

Table 2-3. Continued, List of live attenuated *Salmonella* vaccine candidates developed and tested in humans and animals

Galactose epimerase-less mutants				
galE mutant	*S.* Typhimurium	Mice, calves	Significantly reduced fecal shedding with homologous challenge. No significant humoral immune response	Hone et al., 1987; Wray et al., 1977
Nitrosoguanidine (NTG) induced undefined galE mutant	*S.* Typhi (Ty21a)	Humans	Currently used as live vaccine in humans. Elicits local and systemic humoral immunity and cellular immune response. Confers significant although incomplete protection from typhoid. Being an undefined mutant, it's difficult to determine genetic basis of attenuation	Gilman et al., 1977; Cryz et al., 1993; Kantele et al., 1986; Levine et al., 1989
Auxotrophic mutants				
aroA mutant	*S.* Typhimurium, *S.* Enteritidis. *S.* Dublin, *S.* Gallinarum	Mice, calves, chicken, calves, Swine	Induces good cell mediated and humoral immunity. Imparts long lasting protection against re-challenge. Can cause slow progressive lethal infection in severely immunocompromised animals.	Hoiseth and Stocker, 1981; Killar and Eisenstein, 1985; Cooper et al., 1994; Griffin and Barrow, 1993; Lumbsden et al., 1991
aroA aroC double mutant	*S.* Typhimurium, *S.* Typhi (Ty2)	Mice, humans	Introduction of mutations of more than one genes reduces chance of virulent reversion. Induces protection against virulent challenge.	Dougan et al., 1988; Chatfield et al., 1992

Table 2-3. Continued, List of live attenuated *Salmonella* vaccine candidates developed and tested in humans and animals

aroA aroD double mutant	*S.* Typhimurium	Calves	Provided efficient and safer protection to calves	Jones et al., 1991
aroC aroD double mutant	*S.* Typhi (Ty2)	Humans	Provided humoral and cell mediated immunity. But, induced undesirable reactions with mild transient bacteremia in a proportion of human subjects	Tacket et al., 1992; Sztein et al., 1994
Purine gene mutants				
purA mutant	*S.* Typhimurium	Mice	Induced cell mediated and antibody response, but was ineffective as oral or parenteral vaccines	McFarland and Stocker, 1987
aroA purA double mutants	*S.* Dublin, *S.* Typhi	Mice, humans	Reduced ability to colonize, poorly immunogenic	Sigwart et al., 1989; Levine et al., 1987; Stocker et al., 1988
purE mutant	*S.* Typhimurium	Mice	Moderately attenuated, can induce formation of liver abscesses	Everest et al., 1997

Table 2-3. Continued, List of live attenuated *Salmonella* vaccine candidates developed and tested in humans and animals

htr mutants				
htrA mutants	*S.* Typhimurium,	Mice, calves	Imparted protection against virulent challenge in mice. But, oral administration resulted in fatal disease in calves	Strahan et al., 1992; Villareal-Ramos et al., 2000
htrA aroaA double mutants	*S.* Typhimurium	Mice	Confers good protection against challenge in mice	Chatfield et al., 1992
aroC aroD htrA triple mutants	*S.* Typhi	Humans	Induced *Salmonella* specific antibodies as well as cell mediated responses. Relatively safer in human volunteers	Tacket et al., 1997
cya crp mutants				
cya crp double mutants	*S.* Typhimurium, *S.* Choleraesuis, *S.* Typhi	Mice, chicken, swine, humans	Imparted humoral and cell mediated response in chicken. Impaired ability to invade/ survive in mice spleen. Developed cell mediated response, but impaired humoral response in swine. A small proportion of human volunteers developed fever and bacteremia after vaccination	Curtiss and Kelly, 1987; Hassan and Curtiss, 1990; Stabel et al., 1993; Tacket et al., 1992

Table 2-3. Continued, List of live attenuated *Salmonella* vaccine candidates developed and tested in humans and animals

phoP/phoQ mutants				
phoP/phoQ mutants	*S*. Typhiurium, *S*. Typhi	Mice, humans	Induces cell mediated immunity in mice, but inefficient in growing in mice spleen. Found to be relatively safe and immunogenic in human volunteers	Galan and Curtiss, 1989; Miller, 1991;
aroA phoP/phoQ mutants	*S*. Typhi	Humans	Found to over attenuated and poorly immunogenic	Hohmann et al., 1996
Other mutants				
dam mutants	*S*. Typhimurium	Mice	Totally avirulent and induce effective protection	Heithoff et al., 1999
surA mutants	*S*. Typhimurium	Mice	Induce protective immunity in mice	Sydenham et al., 2000
nuoG mutants	*S*. Gallinarum	Chicken	Reduces colonization in caeca, liver and spleen. Reduces mortality	Zhang-Barber et al., 1998

Electron beam irradiated *Salmonella* vaccines

The National Center for Electron Beam Research (NCEBR) at Texas A&M University has been at the forefront of developing different applications for high energy electron beam (eBeam) irradiation technology. Over the past few years, the Center in collaboration with the USDA-ARS in College Station has demonstrated that eBeam irradiation can be used to develop immuno-modulators that can have commercial value as vaccines in poultry (Kogut *et al*., 2012 and Jesudhasan *et al*., 2010). The basic technology is currently under US patent protection (US patent 8,173,139).

Studies have been conducted with eBeam irradiated *S*. Typhimurium (ST) and *S*. Enteritidis (SE) to check the effectiveness of the immunomodulators in chicken (Kogut et al., 2012; Jesudhasan et al., 2010). It was showed that intramuscular administration of eBeam irradiated SE to laying hens, imparted protection against virulent SE challenge (Jesudhasan et al., 2010). Significant reduction in colonization of SE in liver, ceca, spleen and ovary was observed which was attributable to the increased *Salmonella* specific IgG levels in the birds. Studies by Kogut et al. (2011) were focused towards reducing intestinal colonization and shedding of ST in neonatal chickens. The study demonstrated that *in ovo* administration of eBeam irradiated ST provided protective intestinal colonization inhibition effect against homologous *Salmonella* challenge. They observed that the increased resistance to *Salmonella* infections by vaccinated birds was concomitant with the augmented functional innate immune response by chicken heterophils. Findings from the bird studies indicate that eBeam vaccine technology is an

enabling platform technology to create high potency formulations that can serve as veterinary and human vaccines. Therefore, the next step was to study microbiological and immunological characterization of the eBeam irradiated vaccine to gain deeper understanding about the immune response mechanism by which protection is provided by eBeam vaccines.

CHAPTER III

MATERIALS AND METHODS

Bacterial strains

The *Salmonella enterica* serovar Typhimurium strain ST14028 was used for eBeam irradiation (EBST). The *S*. Typhimurium strain 14028, *aroA*$^{-}$ (del-STM0978:: KanR; aroA null) was used as the live attenuated bacteria control (AroA) for mice immunization studies. The stationary phase cultures were prepared by growing bacterial cells overnight at 37°C in Tryptic Soy Broth (BD Diagnostic Systems, Sparks, MD). The overnight bacterial cultures were washed twice and re-suspended in phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO). The optical density of the bacterial cultures was adjusted to ~1.0 (~1x 10^9 CFU/ml) and used for eBeam irradiation studies and heat inactivation studies. Bacterial enumeration was carried out by plating the serially diluted samples on Tryptic soy agar plates. For heat killed antigen (HKST) preparation, bacterial suspension containing ~1x 10^9 CFU/ml was heat inactivated at 70°C for 1 h.

Animals

Six to eight weeks old female C57BL/6J mice were obtained from Jacksons Laboratory (Bar Harbor, Maine). All the mice were housed in specific pathogen free

conditions and were cared according to the Texas A&M University Institutional Animal Care and Use Committee guidelines.

Electron beam irradiation and dose optimization

For eBeam irradiation trials, stationary phase *S*. Typhimurium (~ 1x 10^9 CFU/ml) cells that were resuspended in PBS were used. Aliquots (10 ml) of the bacterial suspension was packaged (in triplicate) in Whirl-Pak® bags (Nasco, New York, NY) and heat sealed. Each heat-sealed Whirl-Pak® bag in turn was triple bagged to meet the University regulations regarding handling potentially bio hazardous samples at the eBeam irradiation facility. The eBeam irradiations were performed at the National Center for Electron Beam Research on Texas A&M University campus using a 10 MeV linear accelerator. In order to study the inactivation kinetics, samples were subjected to different target eBeam doses ranging from 0.2 – 8 kGy. Irradiation dose measurements were performed using alanine dosimetry that was validated to international standards. The dosimeters (Harwell Dosimeters, Oxfordshire, UK) were measured using the Bruker E-scan spectrometer (Bruker, Billerica, MA). The measured doses were used for data plotting and calculation of inactivation kinetics (Praveen et al., 2013).

Confirming inability of bacterial growth

The inability of lethally irradiated bacteria to replicate was verified under *in vitro* and *in vivo* conditions. The eBeam irradiated *S*. Typhimurium cells were incubated in TSB at 37°C and room temperature (25°C) for up to 10 days and scored for visual evidence of cell multiplication. The multiplication of eBeam irradiated *Salmonella* cells

in culture media was verified by plating aliquots (200 µl) on TSA plates. Counts of EBST were taken using Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) at defined times during culture to check multiplication of non culturable *S*. Typhimurium. Bacterial cells were loaded to Petroff-Hausser counting chamber and visualized under dark field using a phase contrast microscope, Nikon Optishot 2. Based on inactivation kinetics as well as the inability of bacterial regrowth, eBeam dose of 7 kGy was used for generating eBeam irradiated *S*. Typhimurium vaccine (EBST).

The possible multiplication of EBST under *in vivo* condition was studied using C57BL/6J mice, an innately susceptible mice model for *S*. Typhimurium (Pie et al., 1997). Three groups of mice (5 mice/ group) were orally gavaged with 1 x 10^8 CFU of EBST (EBST mice), 1 x 10^8 CFU live *Salmonella* Typhimurium (live ST mice) and 200 µl of PBS (sham mice). The mice were observed for presence of any disease symptoms and mortality. The fecal samples (~0.1g/ mouse) were collected from individual mouse on day 3, 7, 10 and 14, post oral gavage. Homogenized fecal samples were plated on Xylose Lysine deoxycholate (XLD) plates, selective for *Salmonella* sp.. After 14 days of oral gavage, mice were euthanized. The liver, spleen, mesenteric lymph nodes and cecum from the EBST fed mice were homogenized and plated on XLD plates.

Cell membrane integrity assessment

The integrity of the cell membranes of the EBST was visually verified using the LIVE/DEAD® assay. This assay was performed using the BacLight™ Bacterial viability kit for microscopy (Molecular Probes, Eugene, OR). The green fluorescent SYTO®9 and

the red fluorescent propidium iodide nucleic acid stains were used to stain the EBST, HKST and live ST cell preparations as per manufacturer's protocol. Briefly, components A (SYTO®9) and B (propidium iodide) were mixed in ratio 1:1 and 3µl of dye mixture was added to 1 ml of bacterial suspension. The bacteria-dye suspension was mixed thoroughly and incubated at room temperature for 15 min. After incubation, 5 µl of bacterial suspension was placed on a clean glass slide, covered with a cover slip and observed under Nikon Eclipse 80i fluorescence microscope.

Electron microscopic examination of cell surfaces

To understand the ultrastructural changes that occur after eBeam irradiation, the EBST, live and HKST cells were examined using Scanning Electron microscopy (SEM) and Transmission electron microscopy (TEM). Live and heat-killed *S*. Typhimurium samples were examined for comparing the structural differences. All samples were preserved by mixing equal volume of sample with a fixative containing 4% glutaraldehyde prepared in 100mM phosphate and 100mM sucrose, pH 7.4. The fixed samples were rinsed twice in a wash buffer (50 mM phosphate, 50 mM sucrose, pH 7.4) by centrifugation at 1600 x g for 10 min and resuspended in a small wash buffer volume. A 50 µl aliquot of the sample was placed on 0.1% poly-L-lysine coated glass coverslips and incubated at room temperature for 60 min. The samples were fixed to the coverslips by immersion in 3% glutaraldehyde fixative for 60 min, followed by post-fixation in 1% OsO_4 prepared in 100 mM phosphate and 100 mM sucrose, pH 7.4 for 90 min at 4°C. The coverslips were dehydrated and critical point dried in CO_2. The dried samples were

then sputter-coated with gold and examined in a Hitachi H7110. The remaining portions of the samples were pelleted in 2.5% agar, post-fixed in 1% OsO_4 as above, and post-stained overnight in 0.5% aqueous uranyl acetate, dehydrated and embedded in Mollenhauer's mixture of epoxy resin (Mollenhauer et al., 1964). Thin sections of embedded samples were examined in a Hitachi H7000.

Determination of bacterial motility

Motility assessment was carried out by means of a comparative scoring between EBST and live *S*. Typhimurium. Wet mounts of bacterial samples were visualized using Phase contrast microscope (Nikon Optishot 2). Negative staining was carried out to specifically examine presence of bacterial flagella on EBST. A drop of EBST bacterial suspension was placed on a carbon coated 300 mesh copper grid and allowed to stand for 30 seconds. Further, the grid was decanted and replaced with a drop of negative stain 1% phosphotungstic acid (Sigma Aldrich, St. Louis, MO) for 30 seconds, and excess stain was removed and the grid was allowed to dry. The stained grids were examined using a Hitachi H-700 Transmission electron microscope.

Immunoreactivity profile of EBST to *Salmonella* antibodies

Salmonella specific immunoreactivity profile of EBST was compared with non-irradiated and heat killed *S*. Typhimurium using immunoblotting. After eBeam irradiation, bacterial samples were stored at room temperature, 4°C, -20°C and lyophilized condition. After one month of storage at indicated conditions, total soluble protein was extracted using B-PER Bacterial protein extraction reagent (Thermo

Scientific, Rockford, IL) according to manufacturer's protocol. Briefly, bacterial cells were pelleted by centrifugation at 5000 x g for 10 minutes. To the bacterial pellet, B-PER reagent (Thermo Scientific, Rockford, IL) was added (4 ml reagent/ g of cell pellet), supplemented with lysozyme (2 µl/ ml) and DNase I (2 µl/ ml). After mixing, the bacterial suspension was incubated for 10 min at room temperature. The lysate was obtained by centrifuging the bacterial suspension at 12,000 x g for 5 min. Protein extraction was also carried out from overnight culture of *S*. Typhimurium and heat killed *S*. Typhimurium for comparison. The extracted proteins were quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to manufacturer's instruction. The extracted proteins were separated under reducing conditions on an SDS PAGE gradient (4-20%) gel (Precise Protein Gel, Thermo Scientific) and subsequently transferred to PVDF membrane for western blot analysis (Laemmli, 1970). The membrane was blocked overnight at 4°C using 5% BSA in TBST (Sigma Aldrich, St Louis, MO). The membrane was washed twice with TBST and incubated with primary antibody for 2 h at room temperature. Immune serum from mice, previously immunized with *Salmonella* Typhimurium (a kind gift from Dr. R.C. Alaniz, Texas A&M Health Science Center, College Station, TX) was used as primary antibody (1/1000 dilution). Following incubation, membrane was washed 4 times in TBST and incubated at room temperature for 1 h, with 1/ 20,000 dilution of alkaline phosphatase conjugated sheep anti mouse IgG $F(ab`)_2$ fragment (Sigma Aldrich, St Louis, MO). Further, the membrane was washed 4 times with TBST and developed using chromogenic reagent, NCP/ BCIP

(Thermo Scientific, Rockford, IL). Excess of chromogenic reagent was washed off using deionized water and the membrane was dried and digitally recorded.

Determination of bacterial metabolic activity

The metabolic activity of EBST was assessed using Alamar Blue® reagent (Life Technologies, Carlsbad, CA) which quantitatively measures the reducing environment present within the cell (Magnani et al., 2009; Moustafa et al., 2011). Alamar Blue® system incorporates a non-toxic, cell permeable, non-fluorescent, blue colored redox indicator, resazurin. In metabolically active cells maintaining a reduced cell environment, resazurin gets reduced to highly fluorescent red colored compound called resorufin. The conversion of resazurin to resorufin by the metabolically active cells can be quantitatively measured by measuring the increase in fluorescence. After eBeam irradiation, bacterial samples were stored at 4°C and the persistence of metabolic activity in the eBeam irradiated *S*. Typhimurium was monitored for 10 days. Non-irradiated *S*. Typhimurium and heat killed *S*. Typhimurium were used as controls for metabolic activity monitoring. Ninety microliters of EBST, HKST and live ST samples were mixed with 10µl of Alamar blue reagent and incubated at 37°C for 1 h. After incubation fluorescence was monitored at 530-560 nm excitation wavelength and 590 nm emission wavelength using Wallac 1420 VICTOR 2™ plate reader (PerkinElmer, Waltham, MA).

Biochemical assays were performed to confirm the presence of metabolic activity in irradiated S. Typhimurium. The ability of bacteria to ferment specific carbohydrate in the media was tested by incubating EBST and HKST in Phenol Red Broth supplemented

with Sucrose at 37°C for 2 days. Overnight cultures of live *S*. Typhimurium and *E*. coli were used as controls. The color change in the media, turbidity and gas production as a result of bacterial metabolism were monitored. Presence of catalase enzyme in the irradiated cells was detected using catalase test. Catalase enzyme converts hydrogen peroxide to water and oxygen resulting in bubble formation. On a clean glass slides, EBST and live ST cells were smeared and a drop of hydrogen peroxide was added. Bubble formation from the bacterial preparations was monitored to detect catalase activity.

Dendritric cell culture and Bone marrow dendritic cell harvest

The murine dendritic cell line DC2.4 was cultured in Dulbecco's Modified Eagles Medium (DMEM) with high glucose and L-Glutamine supplemented with 5% fetal calf serum, penicillin and streptomycin (100U/ml), Non-essential amino acids and 10mM HEPES at 37°C, 5% CO_2. All the media components for dendritic cell (DC) culture and harvest were obtained from Life Technologies, Carlsbad, CA.

The mouse bone marrow derived dendritic cells (BMDC) were cultured from the bone marrow obtained from the femurs of naïve C57BL/6J mice. Under aseptic conditions, the bone marrow from femurs was flushed into RPMI Complete media (RPMI-C). The RPMI-C medium consisted of RPMI 1640 Medium containing GlutaMAX™ Supplement and HEPES, supplemented with 10% heat inactivated FCS, 50 µM 2-mercaptoethanol, 100 U/ ml penicillin and 100 U/ml streptomycin (Pen/Strep) and 50µg/ml Gentamicin. The cells were further spun and re-suspended in ACK lysis

buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA-Na_2) (Sigma Aldrich, St Louis, MO) for RBC lysis. After the lysis of RBC, bone marrow cells were centrifuged, resuspended in RPMI-C media supplemented with 40 ng/ml Recombinant Mouse Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (R&D Systems, Inc., Minneapolis, MN) and strained using a 70 μm cell strainer to obtain single cell suspension. On day 0, ~4x 10^6 cells/well were plated on 6 well cell culture plates (BD, Franklin Lakes, NJ) with RPMI-C+ GM-CSF (40ng/ml) media and cultured at 37°C, 5% CO_2. On day 2, 4 and 6 granulocytes and non-adherent cells were removed from the wells by aspirating portions of spent media. The wells were replenished with fresh RPMI-C+ GM-CSF (40 ng/ml) media and cultured at 37°C, 5% CO2. On day 7, semi-adherent BMDC were harvested from the wells, centrifuged, re-suspended in RPMI-C + GM-CSF (20 ng/ml), and used for BMDC stimulation assays.

***In vitro* dendritic cell (DC) stimulation assay**

DCs (DC2.4 cells or BMDC) were seeded in 96 well U bottom tissue culture plate (BD, Franklin Lakes, NJ) at 2 x 10^5 cells per well. DCs were co-incubated with indicated titrations of stimulants such as eBeam ST, heat killed ST, and viable live ST. A commercial ST vaccine (Salmune®, Ceva biomune, Lenexa) formulation was also included as a control for BMDC stimulation. Plates with DCs pulsed with the stimulants were incubated at 37°C, 5% CO_2 for 24 h. For intracellular cytokine staining (ICS), the DCs were co-incubated with all the above mentioned stimulants along with 1/1000 dilution of Protein transport inhibitor containing Brefeldin A (BD GolgiPlug™, BD

Biosciences, San Jose, CA) at 37°C, 5% CO_2 for 4 h. After indicated incubation time, DCs were surface stained or subjected to ICS to detect the expression levels of surface markers such as CD11c in case of BMDC, MHC-II, CD40, CD80, CD86 and proinflammatory cytokine TNFα.

Vaccine potency assessment

The potential of EBST to induce maturation of dendritic cells was used as a parameter to assess the vaccine potency upon storage at different temperature conditions over longer period of time. After eBeam irradiation, EBST samples were stored at room temperature, 4°C, -20°C and lyophilized condition for up to 6 months. Two independent lots of irradiated samples were stored. The EBST lots were tested for vaccine potency on a monthly basis using *in vitro* DC stimulation assays with DC2.4 cell line.

Mice immunization and challenge

Mice were immunized with $2x10^9$ EBST and the live attenuated, AroA suspended in PBS at 8 weeks of age. The immunization was performed by oral gavage with feeding needles (22 x $1^{1/2}$ with 1.25 mm ball; Popper & Sons, NY). Mice were diet restricted 5 h before oral infection. A SHAM control group and naïve mice control group were also maintained, which were fed with PBS. For the EBST immunized mice, 2 booster doses were given with $2x10^9$ EBST at 2 weeks intervals. The mice were observed for the appearance of symptoms of infection or scored for mortality. After 14 weeks of primary immunization, mice from EBST, AroA and SHAM group were challenged via oral route with ~ $1x10^8$ virulent nalidixic acid resistant *Salmonella*

Typhimurium strain ST14028. At 3 days and 7 days post challenge, the mice were sacrificed and liver, spleen, mesenteric lymph nodes (MLN), cecum and serum were harvested. Organs from individual mice were homogenized using motorized homogenizer (Omni International, Kennesaw, GA) in sterile PBS with 1% TritonX-100 (Sigma Aldrich, St. Louis, MO). The homogenates were serially diluted and plated in TSA supplemented with 30 mg/L of nalidixic acid (Sigma Aldrich, St. Louis, MO).

In vitro restimulation

The spleens harvested from the mice were divided into equal halves and one half was used for harvesting splenocytes. Splenocytes were harvested from individual mouse from the EBST, AroA, and the SHAM treatment groups. The spleen was squeezed between frosted ends of sterilized glass slides and the cells were collected in RPMI-C media supplemented with 10% FCS. The splenocytes were centrifuged and subjected to RBC lysis using ACK lysis buffer. After RBC lysis, splenocytes were centrifuged at 1300 rpm and strained using 40 μm cell strainer to obtain a single cell suspension of splenocytes. The splenocytes were counted after trypan blue staining using hemocytometer. Approximately, $2x10^6$ splenocytes were seeded per well on 96 well U bottom tissue culture plate and coincubated with indicated titers of stimulants such as EBST, HKST and anti-murine CD3e antibody (clone 145-2C11) at 37°C, 5% CO_2 for 24 h.

Surface staining and intracellular cytokine staining

The upregulation of cell surface markers on DCs (DC2.4 cells or BMDC) and splenocytes in response to the stimulants were detected using surface staining. The ICS was used to detect cytokine production in response to *in vitro* stimulation. After indicated incubation time of 24 h (surface staining) and 4 h (ICS), the DCs were washed twice with PBS supplemented with 0.5% BSA (Sigma Aldrich, St. Louis, MO) (PBSA). Surface staining was done with monoclonal anti-mouse antibodies such as Phyco erythrin (PE) labeled murine CD11c (clone N418), Allo phycocyanin (APC) labeled CD40 (clone 3/23), APC labeled CD80 (clone 16-10A1), Alexa Fluor® 700 labeled CD86 (clone GL1), eFluor® 450 labeled MHCII (clone AF6-120.1). Surface stained cells were fixed using 2% paraformaldehyde and permeabilized with Perm/wash buffer (BD Biosciences, San Jose, CA). Monoclonal anti-mouse antibodies against proinflammatory cytokine TNF, Alexa Fluor® 700 labeled TNF (clone MP6-XT22) were used for staining the permeabilized DCs. Stained DCs were further washed and re-suspended in PBSA and stored at 4°C in dark until analysis by flow cytometry.

In case of *in vitro* restimulation of splenocytes, Brefeldin A was added at 1/1000 dilution during the final 6 h of incubation. After 24 h of incubation, splenocytes were washed twice with PBSA and surface stained in the presence of Fc block (clone 2.4G2) with monoclonal anti mouse antibodies Pacific Blue™ labeled CD4 (clone RM4-5) and FITC labeled CD8a (clone 53-6.7). Following staining, splenocytes were fixed and permeabilized with Cytofix/Perm buffer (BD Biosciences, San Jose, CA). Monoclonal

anti mouse antibodies Alexa Fluor® 647 labeled IFNγ (clone XMG1.2) and PE labeled TNF (clone MP6-XT22) were used to stain the permeabilized splenocytes for detecting the cytokines. Stained cells were finally washed and resuspended in PBSA and stored at 4°C in dark. For all the *in vitro* stimulation assays, unstimulated cells and unstained cells were maintained as controls. All the flow cytometry reagents and antibodies were purchased from BD Pharmingen, San Jose, CA.

Flow cytometric analysis

Multi parameter flow cytometry was performed using BD FACSAria™II (BD, Franklin Lakes, NJ) and data acquired with BD FACSDiva™ software (BD, Franklin Lakes, NJ). Flow cytometric data analysis was carried out with FlowJo software (Tree Star, San Carlos, CA).

Serum IgG measurement

Blood collected from individual mice from EBST, AroA and SHAM group were allowed to clot at room temperature. After centrifugation, serum was collected without disturbing the pelleted clots and stored at -20°C until use. Indirect ELISA was performed for detection of antigen specific antibodies in mice sera. Nunc Maxi Sorp® flat bottom 96 well plates (eBioscience, Inc., San Diego, CA) were coated with EBST ($2x10^7$/well) using coating buffer (Sigma Aldrich, St. Louis, MO) and incubated overnight at 4°C. Plates were washed using PBS with 0.5% Tween 20 (PBST) (Sigma Aldrich, St. Louis, MO). After washing, the plates were blocked using 5%Bovine Serum Albumin (BSA) in PBST and incubated at room temperature for 1 h. Appropriate dilutions (1/500) of mice

serum samples were added to the wells (150 µl/well) in triplicates and incubated at 37°C for 1 h. Serum from previously immunized *Salmonella* immune mouse was used as a positive control for ELISA. Following incubation, plates were washed 4 times with PBST and 150 µl/well secondary HRP conjugated anti mouse IgG (Jackson ImmunoResearch, West Grove, PA), was added to wells at appropriate dilutions (1/5000) in blocking buffer. The plates were incubated with secondary antibody at room temperature for 1 h and washed 4 times with PBST. Acetate buffer was added to the wells for 2-5 minutes and removed. Plates were further developed by adding 100 µl/well TMB substrate (BD, Franklin Lakes, NJ) and incubating for 30 min at room temperature. Reaction was stopped using 2M H_2SO_4 and the color development was measured by measuring the absorbance at 450nm (OD_{450}) on Wallac 1420 VICTOR 2™ plate reader (PerkinElmer, Waltham, MA). Sample to positive control ratio (S/P) was calculated as per the following equation. Normalizing the ELISA absorbance readouts as S/P ratio reduces the plate variability when comparing the absorbance readings between different ELISA plates.

$$\frac{S}{P} ratio = \frac{(Sample\ OD450 - Negative\ control\ OD450)}{(Positive\ control\ OD450 - Negative\ control\ OD450)}$$

Statistical analysis

Unless otherwise stated all the data graphs and statistical analysis were generated using GraphPad Prism version 5.0 (GraphPad Prism software, La Jolla, CA). For calculating the inactivation kinetics of *S*. Typhimurium subjected to different eBeam doses, a survivor curve was plotted. The plate counts were transformed into $\log_{10}$ values.

These log_{10} values of the survivors were plotted against the measured eBeam doses and a linear regression analysis was performed. The D_{10} value was calculated by calculating the negative reciprocal of regression slope. For the mice challenge study, Kaplan meir curve was plotted to do the survival analysis. Kruskal Wallis one way ANOVA was used for statistical comparison of bacterial colonization between different treatment mice groups. In case of *in vitro* re-stimulation assays, for determining antigen specific T cell responses, two tailed student's t-test was used to compare different treatment mice groups for each antigen at indicated time points. For all the statistical analysis significance was set at $p \leq 0.05$.

CHAPTER IV

RESULTS

Electron beam inactivation of *Salmonella* Typhimurium

The first step in characterizing the eBeam irradiated vaccine was to determine the optimum eBeam dose that will ensure complete inactivation of *S.* Typhimurium (ST). Inactivation kinetics of eBeam irradiation was studied by exposing high titers of ST (~1x 10^9 CFU/ml) to incremental eBeam doses. Reduction in viable colony forming units (CFU) of ST was observed with an increase in eBeam dose exposure (Figure 4-1). The dose required for achieving 90% reduction (D_{10} value) of ST in PBS was calculated as 0.19 kGy. In order to study complete inactivation, bacterial cultures (~1x 10^9 CFU/ml) were subjected to higher eBeam doses of 6, 7 and 8 kGy. No surviving viable ST was detected after irradiation with any of the high eBeam doses, which ensured complete inactivation of ST.

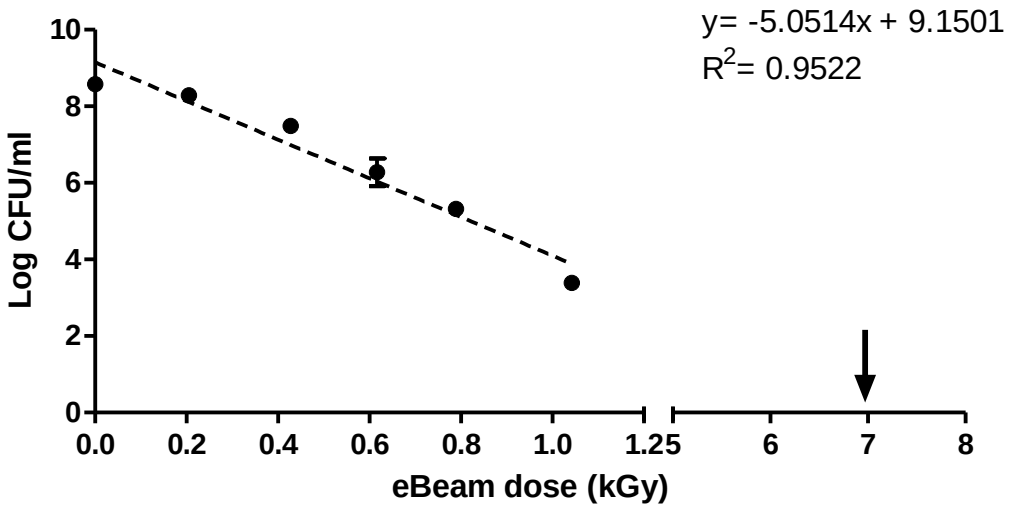


Figure 4-1. Electron beam irradiation induces *S*. Typhimurium inactivation.

Inactivation kinetics of *S*. Typhimurium exposed to different doses of eBeam irradiation. For each indicated doses of eBeam exposure, viable bacterial counts were obtained by plating tenfold serial dilution of irradiated bacterial samples on TSA plates.D_{10} value was calculated using the negative reciprocal of regression slope of the inactivation curve. The arrow represents the lethal eBeam dose used to generate eBeam vaccine.

EBST is unable to grow under *in vitro* and *in vivo* conditions

Regrowth studies were conducted to examine the ability of EBST to grow and multiply, by culturing the irradiated bacterial cells under different conditions to promote bacterial growth. Aliquots of ST irradiated at eBeam doses of 4, 5, 6 and 7 kGy were inoculated into TSB, a non-selective media and incubated at 37°C and room temperature. The bacterial regrowth was monitored on a daily basis by plating the incubated bacterial cultures on TSA plates. It was noticed that bacteria irradiated at eBeam doses of 4 and 5 kGy showed regrowth whereas, bacterial samples subjected to higher doses of eBeam irradiation such as 6 and 7 kGy did not regrow. Bacterial growth on TSA plates is an indication of the presence of culturable bacterial cells. Hence it was confirmed that no culturable ST was present, when exposed to an eBeam dose of 7 kGy. To test for the possible multiplication of non culturable EBST in TSB, bacterial counts were made using Petroff-Hausser counting chamber on day 0 and 2 (Table 4-1). Bacterial counts obtained from the Petroff-Hausser counting chamber were compared and it was observed that there was no increase in the number of eBeam irradiated bacteria upon incubation in TSB media for 2 days. Hence, it was concluded that at an irradiation dose of 7 kGy, ST is unable to multiply even under the most favorable *in vitro* conditions.

Table 4-1. Inability of eBeam irradiated *S*. Typhimurium to multiply *in vitro* in a regrowth media, determined using Petroff- Hausser counting chamber

Days in culture	**Bacterial counts**
Day 0	1.14×10^9 cells/ml
Day 2	7.1×10^8 cells/ml

The inability of lethally irradiated ST to multiply *in vivo* was tested using a *Salmonella* susceptible mice model, C57BL/6J (Pie et al., 1997). Five mice were orally inoculated with 1x 10^8 CFU of eBeam irradiated ST (EBST mice). Their fecal droppings were collected on regular intervals and plated on *Salmonella* selective media XLD to verify whether viable ST was shed by the mice. Two control groups of mice were also maintained which were gavaged with live S. Typhimurium (live ST mice) and PBS (sham mice) respectively. Results showed that *Salmonella* shedding was observed only in live ST mice (~ 4 log CFU/ g fecal pellet) (Table 4-2). In case of EBST mice and sham mice, no detectable *Salmonella* colonies were observed in any of the fecal samples collected over 14 days, indicative of the absence of viable bacterial regrowth. The *Salmonella* inoculum dose was also found to be safe for C57BL/6J mice as no mortality or disease symptoms were observed. After 14 days of inoculation, mice were euthanized and liver, spleen, mesenteric lymph nodes (MLN) and cecum were harvested, homogenized and plated on XLD plates. *Salmonella* colonies were recovered from liver, spleen, MLN and cecum of live ST mice. Both EBST mice and sham mice did not recover viable *Salmonella* from organs. Absence of detectable viable *Salmonella* colonies confirmed that lethally irradiated ST at an eBeam dose of 7 kGy does not regrow in a susceptible mice model. The eBeam irradiated ST (EBST) cannot be re-isolated from host and grown to pure culture and it is unable to cause any disease symptoms or mortality in susceptible host, thus rendering EBST to be non-pathogenic as per Koch's postulate (Koch, 1876). Thus, eBeam irradiated ST at 7kGy (SD ±0.38) (EBST) could serve as a non-replicating vaccine candidate.

Table 4-2. Inability of eBeam irradiated *S*. Typhimurium to multiply *in vivo* in a *Salmonella* susceptible mice model

(A) *Salmonella* recovery from fecal pellets (FP)

Days of FP collection	**Sham mice**	**Live ST mice (Log CFU/g FP)**	**EBST mice**
Day 3	BD*	4.79 ± 0.49	BD*
Day 7	BD*	4.79 ± 0.23	BD*
Day 10	BD*	4.61 ± 0.48	BD*
Day 14	BD*	4.46 ± 0.51	BD*

(B) *Salmonella* recovery from organs

Organ	**Sham mice**	**Live ST mice (Log CFU/organ)**	**EBST mice**
Liver	BD#	2.24 ± 1.12	BD#
Spleen	BD#	2.07 ± 1.03	BD#
Cecum	BD#	4.73 ± 2.36	BD#
MLN	BDϕ	3.31 ± 1.65	BDϕ

*, BD= Below detection limit of 1.69 log CFU
#, BD= Below detection limit of 1.17 log CFU
ϕ, BD= Below detection limit of 0.69 log CFU

EBST maintains an intact cell membrane

To investigate whether eBeam irradiation causes damage to the bacterial cell membrane, the membrane integrity of EBST, HKST and live ST was assessed using the BacLight™ assay. The BacLight™ assay utilizes the differential ability of two nucleic acid stains SYTO®9 and propidium iodide to penetrate healthy bacterial cells. SYTO®9 can penetrate bacterial cells with either intact or damaged membrane staining them green. In contrast, propidium iodide can enter only the cells with a damaged membrane, causing reduction in SYTO®9 stain fluorescence when both dyes are present, thus staining the membrane damaged cells red. Hence, bacteria with intact membrane would stain fluorescent green, whereas bacteria with damaged cell membrane would stain fluorescent red. EBST maintained an intact cell membrane similar to live ST indicated by green fluorescent bacterial cells (Figure 4-2). Heat killed ST (HKST) stained red indicating loss of membrane integrity due to the heat treatment. This observation was further confirmed by ultra-structural examination of EBST using electron microscopy. EBST showed no discernable differences when examined by scanning electron microscopy compared to live ST controls (Figure 4-3). Increased morphological unevenness was observed in case of HKST as cell membrane appeared to be shrunken or caved in comparison to live and EBST. For detailed analysis, cross sections of bacteria were examined by Transmission Electron Microscopy (TEM). The EBST and live ST showed no significant difference (visually) in the cellular organization. Cell contents of HKST seemed to be disorganized and the cell membrane appeared to be detached from the periplasmic contents (Figure 4-4). Membrane damage can be clearly observed in case

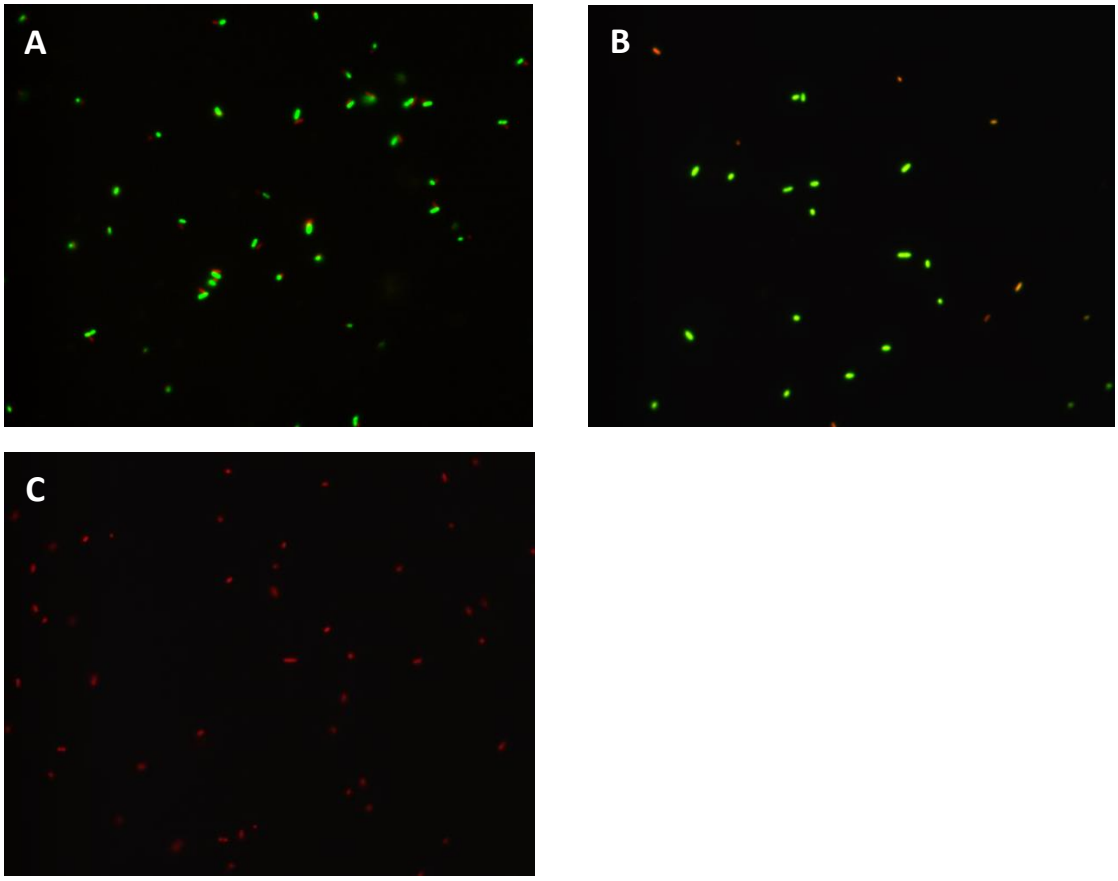


Figure 4-2. Electron beam irradiated ST (EBST) maintains cell membrane integrity.

Membrane integrity of *S*. Typhimurium remains unaffected by eBeam irradiation. Staining with Live/ Dead BacLight™ kit reveals the presence of intact cell membrane for (A) Live ST and (B) EBST indicated by the green labelled bacterial cells. Red colored staining of HKST (C) indicates disrupted cell membrane due to heat treatment.

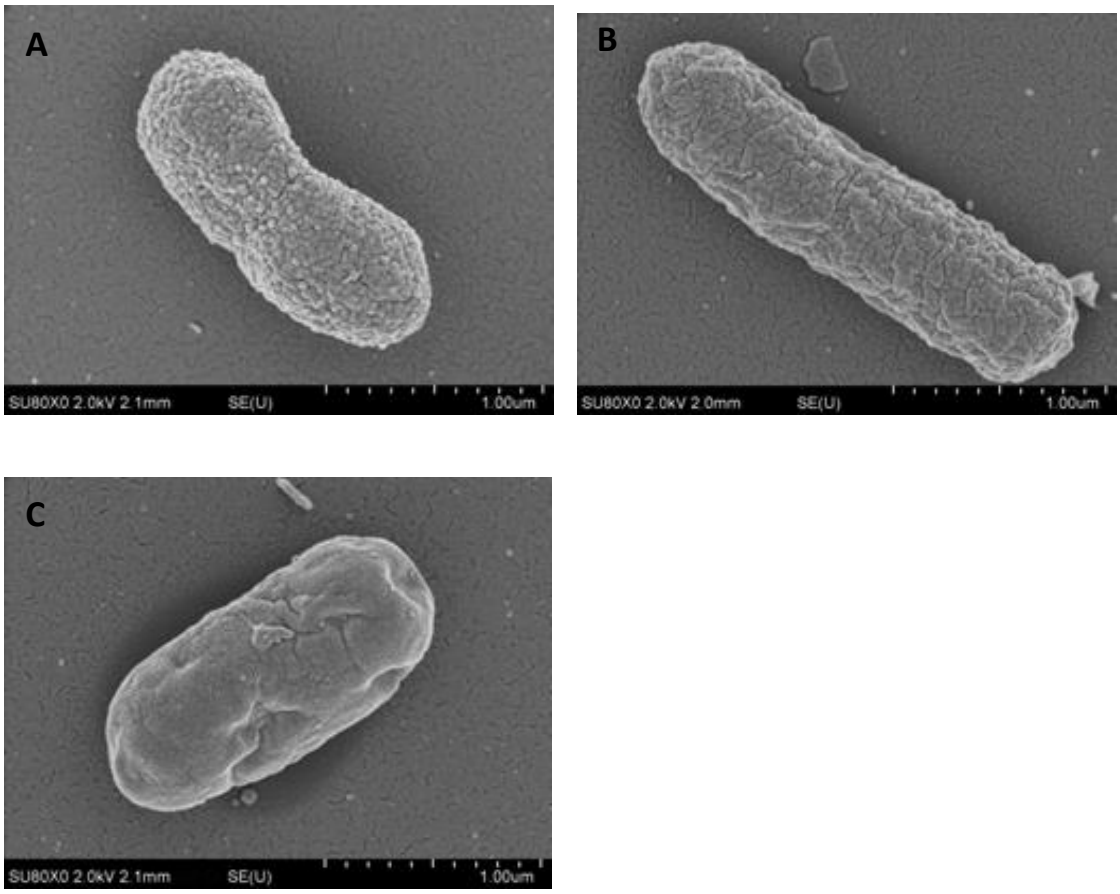
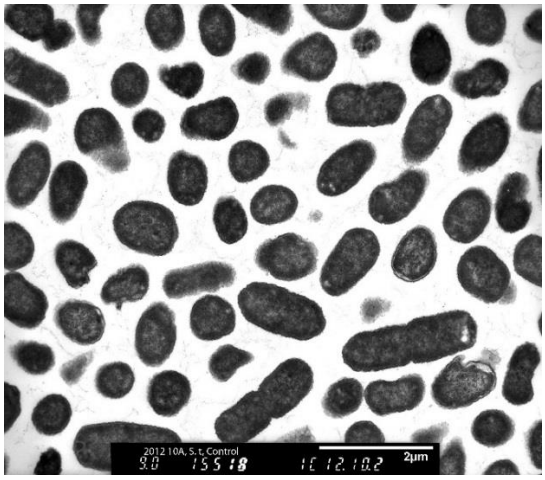
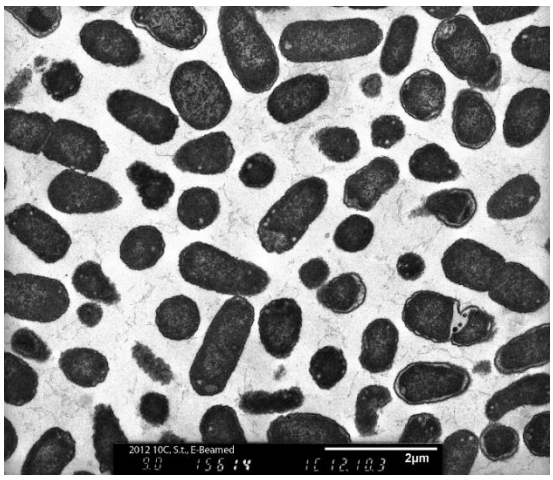


Figure 4-3. Membrane morphology of EBST is similar to live ST.

Scanning electron microscopic analysis shows presence of intact bacterial cell membrane for (A) Live ST and (B) EBST. HKST (C) appears to be morphologically uneven with shrunken cell membrane.

A **B**





C

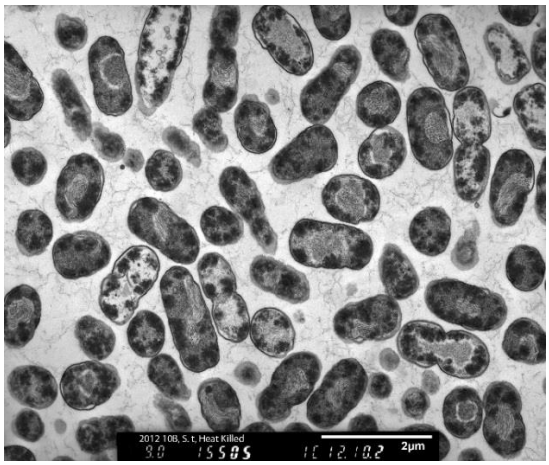


Figure 4-4. Electron beam irradiation does not alter bacterial cellular organization in ST.

Transmission electron micrographs of bacterial cross sections of (A) live ST and (B) EBST shows no alteration in the cellular organization. Heat treatment induces cellular disorganization and detachment of cell membrane from the periplasmic contents in HKST (C).

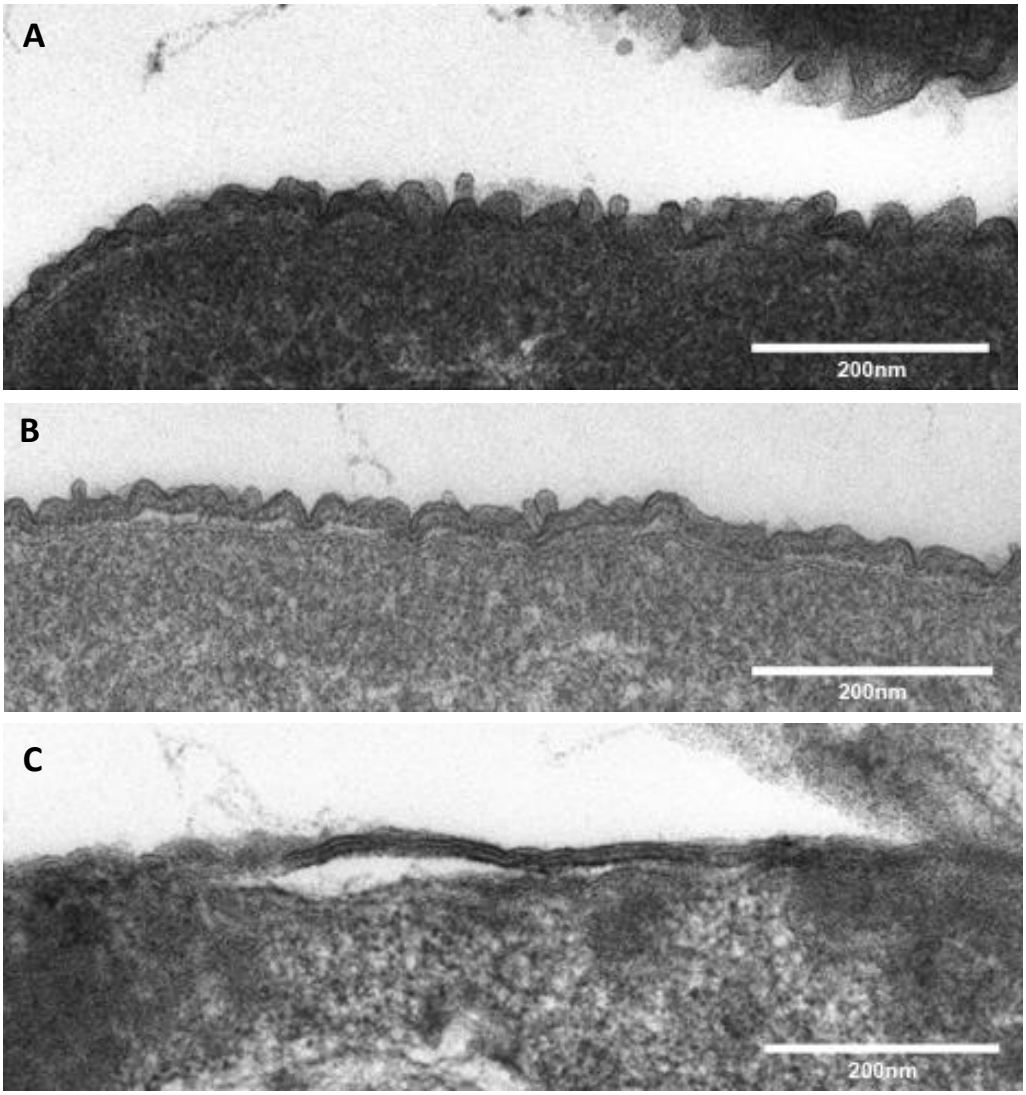


Figure 4-5. EBST retains cell surface molecules.

Transmission electron micrographs of (A) live ST (B) EBST and (C) HKST reveals the presence of surface molecules on bacterial cell membrane of both live and EBST. HKST shows a smoothened surface indicative of loss of surface molecules

of HKST with the loss of surface structures. The cell membrane appears to be "smoothed out" and detached in comparison to the live and EBST (Figure 4-5). Membrane integrity assay together with electron microscopic studies provide evidence for the presence of an intact bacterial cell membrane in EBST which is structurally similar to live ST.

EBST remains immotile but retains intact bacterial flagella

Motility agar has been used in the past to verify bacterial motility (Hine et al., 1988; Gomez et al., 1998). However, use of culture based motility agar was not applicable to EBST cells as they were non culturable in media. Hence, bacterial motility was assessed by observing wet mounts of bacterial cultures using phase contrast microscope. Care was taken to account for the motion due to Brownian movement. Brownian motion will allow bacterial cells to move along same direction in same speed, whereas truly motile bacteria would swim randomly or against the current across the slide. EBST was found to be non motile in comparison to live ST. In order to check whether the reduction in motility was due to loss of flagella by eBeam irradiation, the bacterial flagella was examined by TEM using negative staining. Results from negative staining revealed that bacterial flagella were still attached and intact even after eBeam irradiation (Figure 4-6).

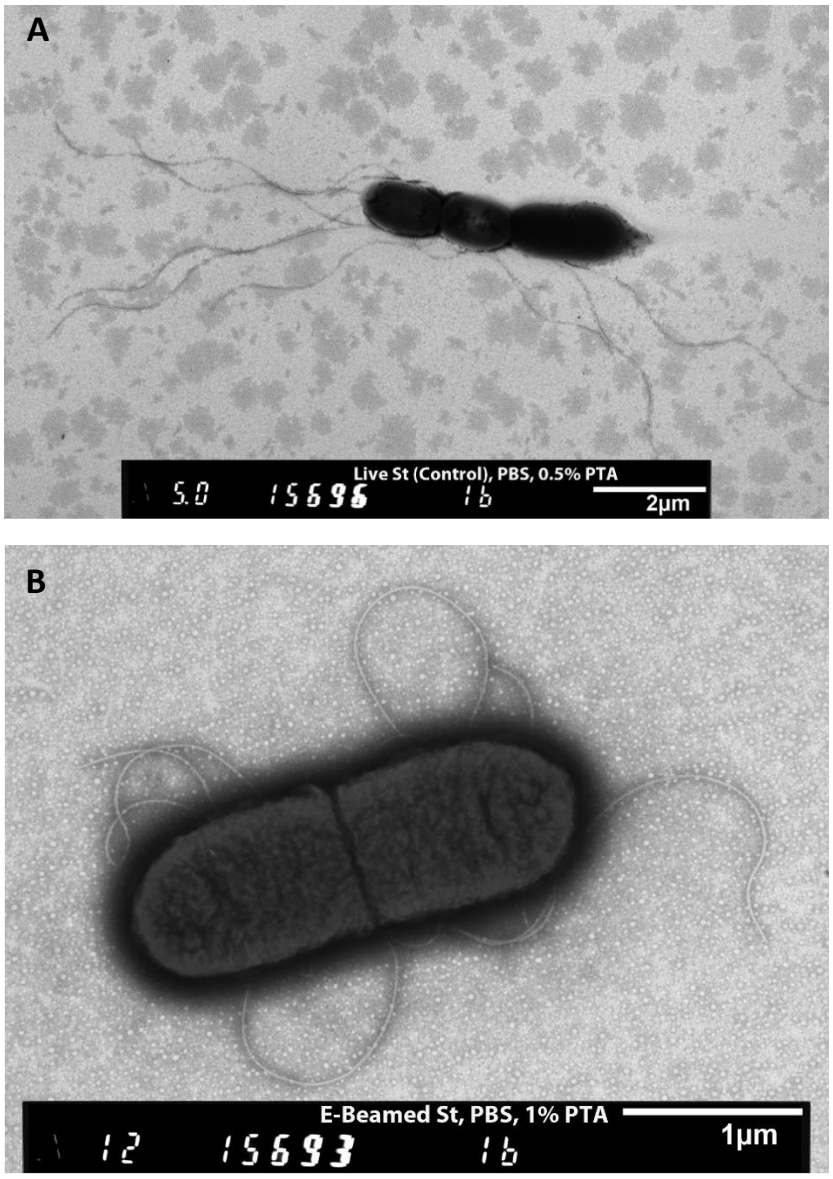


Figure 4-6. Electron beam irradiation preserves bacterial flagella in ST.

Transmission electron micrographs (negative staining) of (A) Live ST and (B) EBST show presence of intact bacterial flagella.

Immunoreactivity profile of EBST proteins is similar to live ST

To understand whether there is any loss of immunogenic *Salmonella* antigens in EBST preparation, immunoreactivity profile of EBST was developed and compared with live and heat killed ST. Using SDS PAGE, total soluble proteins from EBST, HKST and live ST were separated and immunoblotting was performed. Immune serum from mice, previously immunized with *Salmonella* was used as primary antibody to detect the *Salmonella* specific antigens in the respective bacterial preparation. The results showed that immunoreactivity profile of EBST was very similar to that of live ST which supports the absence of antigen loss in the EBST as a result of eBeam irradiation (Figure 4-7). However, marked difference in the banding pattern was observed in case of HKST immunoreactivity profile in comparison to live and EBST, indicative of the loss of antigenic proteins due to heat treatment. Proteins extracted from EBST stored at different temperature conditions such as room temperature, 4°C, -20°C and lyophilized EBST were also analyzed to detect the *Salmonella* specific protein profiles. Storage at different temperature regimes did not induce any alteration in the immunoreactivity profiles of EBST. These findings suggests that immunoreactive proteins in EBST remain stable during storage at different temperature conditions.

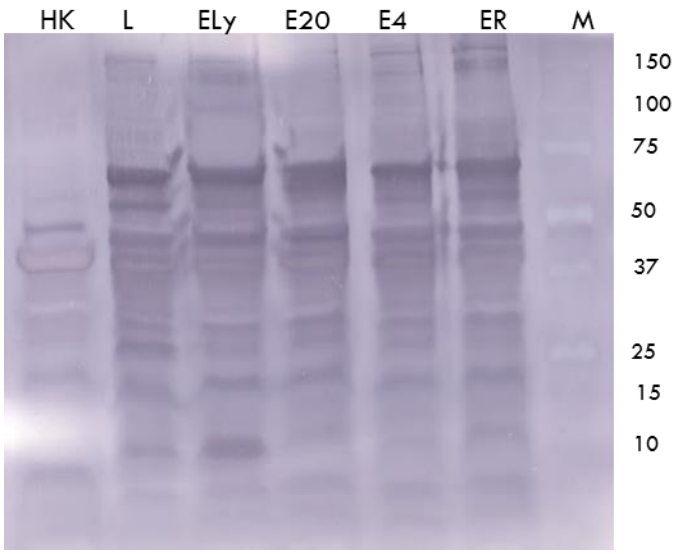


Figure 4-7. EBST retains immunogenic proteins that are detected by live ST immune mice serum.

After eBeam irradiation, ST was stored at multiple temperature conditions such as 4°C, -20°C, and room temperature and as lyophilized preparation. Total soluble protein was extracted from all different EBST preparations. Proteins from live ST and heat inactivated ST were also extracted as controls. Proteins were separated using SDS-PAGE and analyzed by western blotting. Immunodetction was carried out using 1/1000 dilution of *S*. Typhimurium infected mice serum as the primary antibody and 1/ 20,000 dilution of alkaline phosphatase conjugated sheep anti mouse IgG $F(ab`)_2$ fragment as secondary antibody. EBST stored at room temperature (ER), 4°C (E4), -20°C (E20), lyophilized (ELy), Live ST (L), heat killed ST (HK), Protein ladder (M).

EBST retains metabolic activity

Presence of enzymatic activity and cellular reducing conditions are considered as key indicators of metabolic activity of cells (Davey, 2011; Keer and Birch, 2003). In order to monitor the metabolic activity of *S*. Typhimurium post eBeam irradiation, the bacterial cells were subjected to Alamar blue® assay and biochemical assays (Rampersad, 2012). Fluorescence readings obtained from the Alamar Blue® assay indicate that EBST were metabolically active even after 10 days of storage at 4ºC (Figure 4-8). The EBST cells exhibited higher metabolic activity compared to live ST. As expected, the heat inactivated ST had no residual metabolic activity. To avoid the possibility of erroneous detection of reduced compounds generated during the process of eBeam irradiation, metabolic activity of EBST cells followed by heat inactivation, and HKST followed by eBeam irradiation were measured. The EBST cells subjected to heat treatment before or after eBeam irradiation did not exhibit changes in fluorescence which confirmed that the increased fluorescence detected by Alamar Blue® is due to the reduced environment present in the metabolically active irradiated cells.

The ability of bacteria to ferment specific carbohydrate in the media was tested by incubating EBST, HKST and live ST in Phenol Red Broth supplemented with sucrose. Media inoculated with EBST and live ST changed from red to yellow color whereas, no color change was observed in case of HKST (Figure 4-9A). The color change in media is due to acid production as a result of sugar fermentation by the bacteria. Despite the color change, EBST inoculated media lacked turbidity and gas

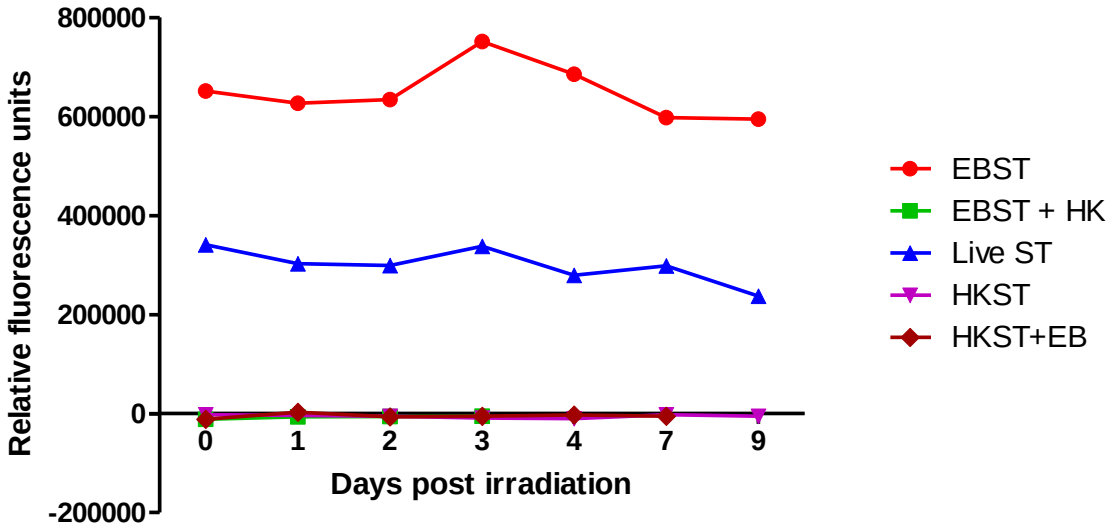
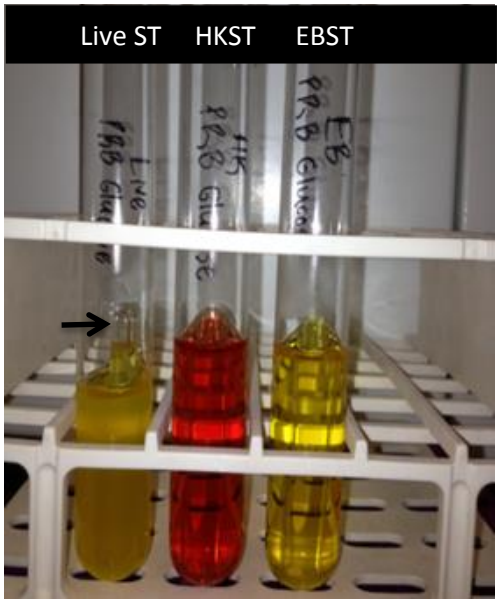


Figure 4-8. EBST remains metabolically active and retains the activity for extended time periods of storage at 4°C.

Metabolic activity of *S.* Typhimurium post eBeam irradiation was measured using Alamar blue® assay. The reduced environment present in metabolically active cells were detected by redox indicator which fluoresces. The fluorescence readings obtained for EBST, live ST and heat killed ST (HKST) was measured on a daily basis up to 10 days and are reported as line graph. Metabolic activity of EBST cells followed by heat inactivation (EBST+HK) and HKST followed by eBeam irradiation (HKST+EB) were measured as process controls.

A



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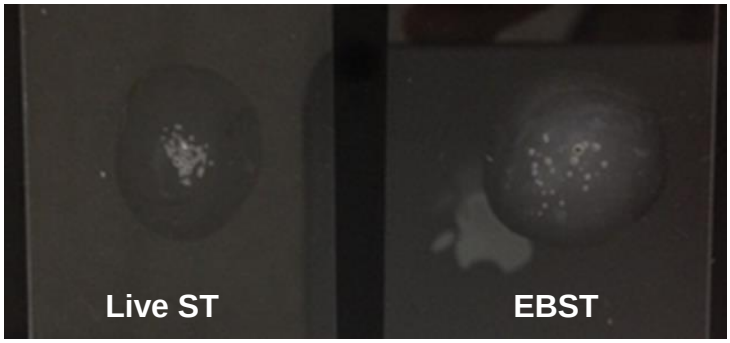


Figure 4-9. EBST remains metabolically active and tests positive for biochemical and enzymatic assays.

(A) Carbohydrate utilization ability of EBST was tested by inoculating EBST into phenol red broth containing glucose and incubating for 48 h at 37°C. Live and heat killed *S*. Typhimurium were used as controls. Media color change, turbidity and gas production is monitored for all the samples. Arrow indicates gas production. (B) To assess the catalase enzymatic activity in EBST, cells were smeared on clean glass slide and a drop of 3% hydrogen peroxide was added. Bubble formation was monitored for detecting catalase activity. Live *S*. Typhimurium was used as a positive control.

formation that was present in live ST inoculated media. Thus, it was clear that EBST was not replicating in the media, but the inoculated cells were metabolically active to induce the fermentation and color change. Presence of catalase enzyme in the irradiated cells was detected using catalase test. Catalase enzyme converts hydrogen peroxide to water and oxygen. The oxygen thus produced, forms bubbles which indicate the presence of intact catalase enzyme in the cells. Copious bubble formation was observed in both live and EBST cells (Figure 4-9B) which provided evidence for enzymatic activity in eBeam irradiated ST.

EBST induces maturation of dendritic cells

Dendritic cells (DC) are key antigen presenting cells (APC) which serve as functional junction between nonspecific innate immune response and antigen specific adaptive immune response mechanisms. *Salmonella* induces DC maturation, characterized by increased surface expression of MHC-II, co-stimulatory molecules and cytokine production (Pierre et al., 1997; Svensson et al., 2000). We tested the ability of EBST to trigger DC maturation *in vitro* using two murine DC models. The first model was a dendritic cell line DC2.4 (Shen et al., 1997) and second was a primary DC culture derived from bone marrow of C57BL/6J mice (BMDC) (Lutz et al., 1999). The DC2.4 cells were stimulated with EBST, HKST and live *S*. Typhimurium for 24 h and the surface expression of MHC-II, CD40, CD80 (B7-1) and CD86 (B7-2) was analyzed. Production of proinflammatory cytokine TNFα was measured followed by 4 h of co-incubation with stimulants. EBST induced efficient DC maturation indicated by the up-

regulation of MHC-II, CD40, CD80 and CD86 surface expression on DC2.4 (Figure 4-10). The level of DC maturation induced by EBST was similar to whole live ST and HKST. Increased production of TNFα by DC2.4 cells is indicative of the potent proinflammatory properties of EBST.

The potential of stimulants (EBST, HKST and live ST) to induce DC maturation and activation over a range of concentration was assessed by co-incubating stimulants and DC2.4 cells at a multiplicity of infection (MOI) of 100:1, 10:1, 1:1, 0.1:1, 0.01:1 (Bacteria: DC). It was observed that expression levels of surface molecules and TNFα followed similar trend for EBST, HKST and live ST at higher MOI. A drop in expression level of CD40, CD80 and TNFα was noticed at MOI of 1 and below whereas, levels of MHC-II and CD86 remained consistent even at lower MOI (Figure 4-11A-E). At lower MOI, EBST and HKST induce similar expression levels of CD80 and TNFα which was significantly higher than live ST (Figure 4-11C, E). These results indicate that EBST possess agonist activity over a wide range of concentration.

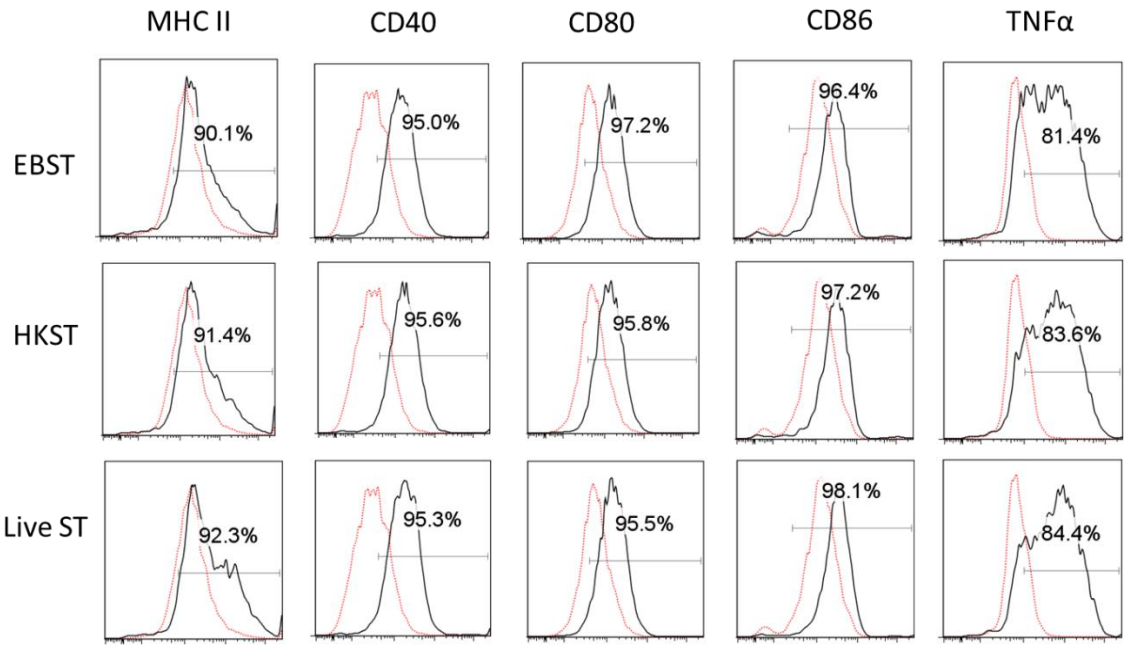


Figure 4-10. EBST induces efficient DC maturation and triggers proinflammatory cytokine production in DC2.4 cells.

DC2.4 cells were co-incubated with EBST, HKST and live *S*. Typhimurium (MOI 1:10 for 24 h or 4 h) as indicated to the left side of each row of histograms. Each column of histogram represents expression level of surface markers MHC-II, CD40, CD80, CD86 and proinflammatory cytokine TNFα. Percentages in the gated region indicate the proportion of DC expressing high levels of various surface markers and TNFα. Expression of levels of unstimulated DC (dotted red line) is compared to the antigen stimulated (thin black line) DC2.4 cells. Data are representative of three independent experiments.

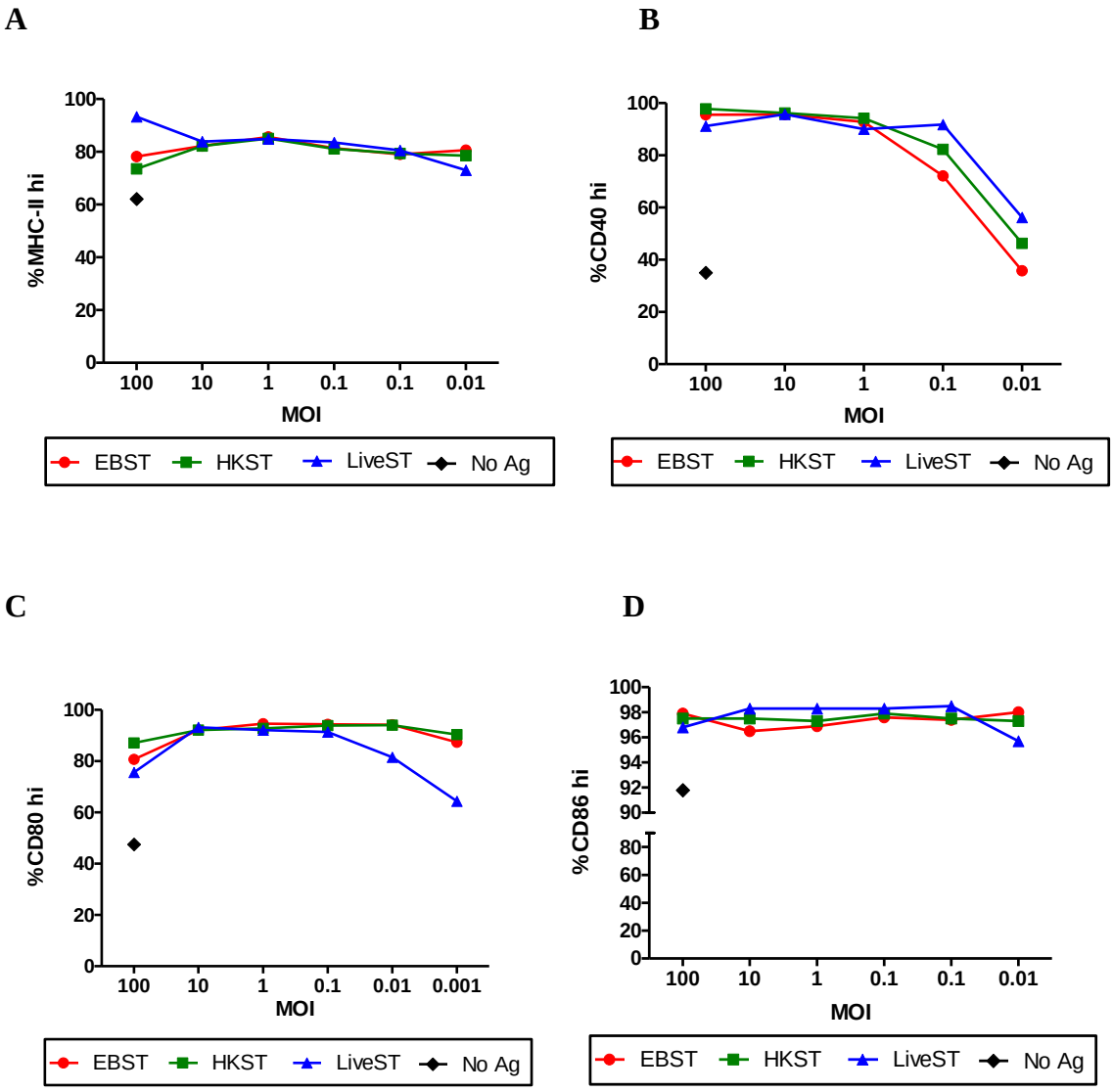


Figure 4-11. EBST induces maturation of DC2.4 cells and retains the agonist activity over wide range of concentration.

DC2.4 cells were coincubated with EBST, HKST and live *S.* Typhimurium at different multiplicity of infection (MOI) – 100, 10, 1, 0.1, 0.01, 0.001 for 24 h or 4 h. Line graphs represent the percentages of DC2.4 cells expressing high levels of (A) MHC-II, (B) CD40, (C) CD80, (D) CD86 and (E) TNFα. Unstimulated controls were included to assess the basal level of expression of surface markers and proinflammatory cytokine by DC2.4 cells in the absence of any external stimulation.

E

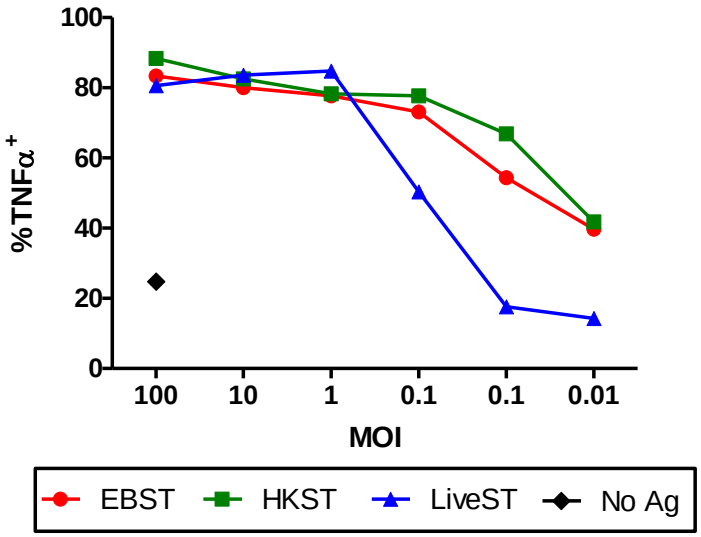


Figure 4-11 Continued.

Earlier reports have shown that DC2.4 cell line exhibits relatively higher level of constitutive expression of MHC-I, MHC-II, CD80, CD86 and ICAM-1 (Alshamsan et al., 2010). Hence, DC maturation and activation ability of EBST was validated *in vitro* using primary DC culture derived from bone marrow of C57BL/6J mice (BMDC). For BMDC activation studies, EBST, HKST, live ST and a commercial live attenuated ST vaccine formulation (Salmune®, Ceva biomune, Lenexa, KS) were used as stimulants. Results from BMDC activation studies support the findings from DC2.4 cell line studies. When compared to the unstimulated media control, co-incubation of BMDC with stimulants generated a clear shift towards DC population expressing higher levels of MHC-II, CD40, CD80, CD86 and TNFα (Figure 4-12). Expression levels of surface markers and proinflammatory cytokine followed similar trend for stimulation with EBST, HKST, live ST and commercial ST vaccine. These results demonstrate that EBST without any adjuvant addition, possess similar potential to that of the commercial formulation, in inducing the DC maturation. Thus, EBST preserves the immunogenic properties enabling them to induce DC maturation.

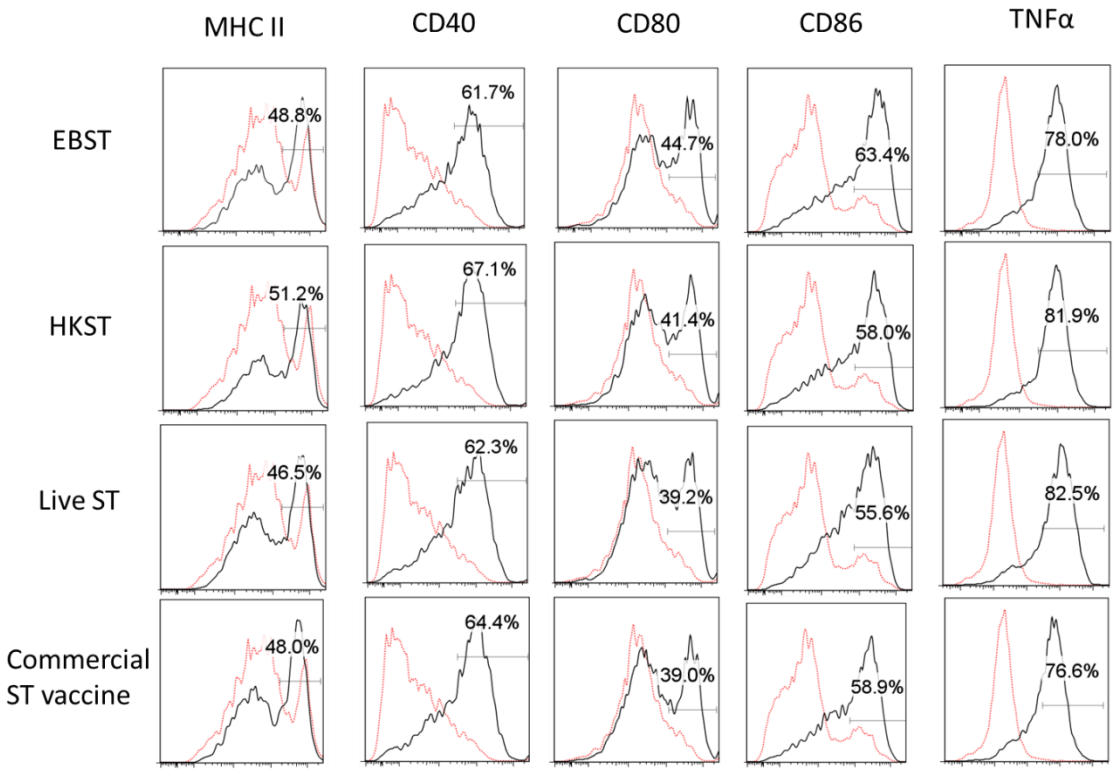


Figure 4-12. EBST induces efficient DC maturation and triggers proinflammatory cytokine production in BMDC.

BMDCs were coincubated with EBST, HKST, live *S*. Typhimurium and a commercially available live attenuated ST vaccine (MOI 1:10 for 24 h or 4 h) as indicated to the left side of each row of histograms. Each column of histogram represents expression level of surface markers MHC-II, CD40, CD80, CD86 and proinflammatory cytokine TNFα. Percentages in the gated region indicate the proportion of $CD11c^{+}$ DC expressing high levels of various surface markers and TNFα. Expression of levels of unstimulated DC (dotted red line) is compared to the antigen stimulated (thin black line) DC. Data are representative of three independent experiments.

A

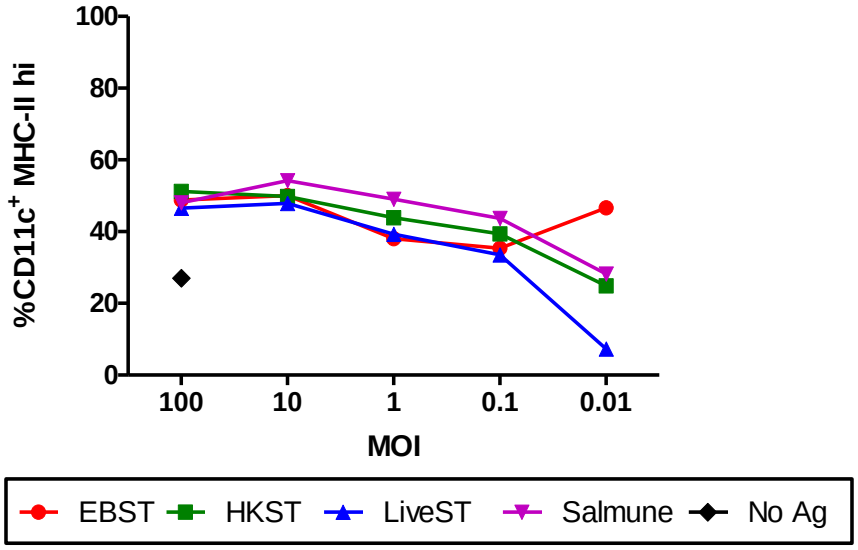
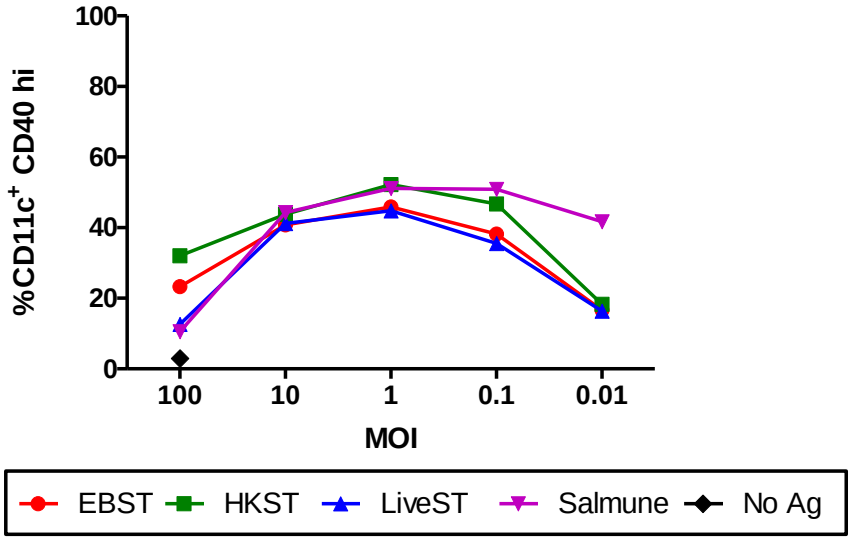


Figure 4-13. EBST induces maturation of BMDC and retains the agonist activity over wide range of concentration.

BMDC were co-incubated with EBST, HKST, live *S.* Typhimurium and a commercially available ST vaccine at different multiplicity of infection (MOI) – 100, 10, 1, 0.1, 0.01 for 24 h or 4 h. Line graphs represent the percentages of $CD11c^+$ DC expressing high levels of (A) MHC-II, (B) CD40, (C) CD80, (D) CD86 and (E) TNFα. Unstimulated controls (No Ag) were included to assess the basal level of expression of surface markers and proinflammatory cytokine by BMDC in the absence of any external stimulation.

B



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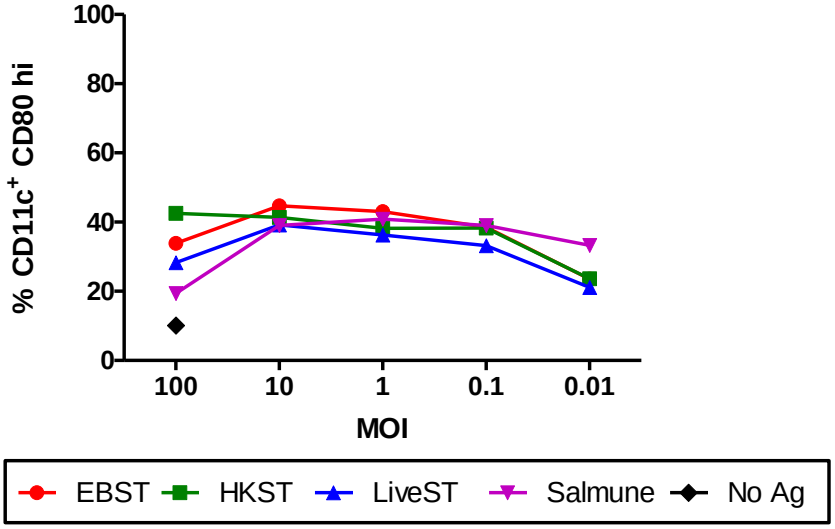

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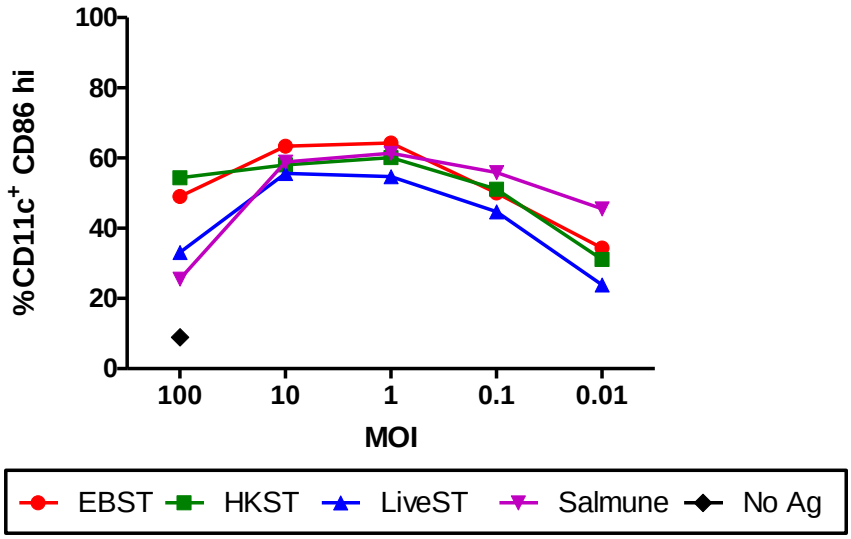

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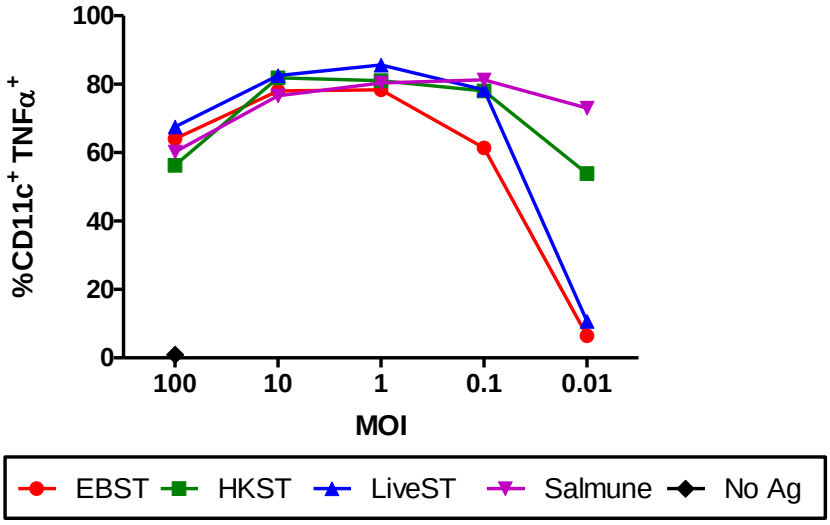


Figure 4-13 Continued.

Similar to the studies conducted in DC2.4 cells, BMDCs were also co-incubated with stimulants at multiple MOI, to investigate the influence of antigen concentration in inducing the DC maturation and activation. For this, DCs were infected with stimulants at different MOIs (100, 10, 1, 0,1, 0.01), and then cell surface expression of CD40, CD80, CD86 and MHC-II molecules was analyzed by flow cytometry at 24 h post stimulation, and proinflammatory cytokine TNFα production was measured after 4 h of co-incubation. A gradual reduction in the expression levels of surface markers were observed with decreasing MOI (Figure 4-13A-D). However, co-incubation of BMDC with stimulants at a high MOI of 100 impaired their ability to up-regulate co-stimulatory molecules and proinflammatory cytokine. It was found that MOI of 10 and 1 were most effective in inducing DC maturation for all the stimulants that were tested. Retention of agonist activity and TNFα production were low at lower MOI for EBST and live ST.

Vaccination with EBST reduces virulent *Salmonella* colonization similar to live attenuated ST vaccine

Live attenuated ST vaccines are known to be effective in inducing protection against virulent ST challenge (Harrison et al., 1997; Hormaeche et al., 1991; Mastroeni et al., 1992). Therefore, the effect of EBST immunization was tested in comparison with AroA$^-$ ST, a widely studied live attenuated ST vaccine in mice model system (Harrison et al., 1997; Hormaeche et al., 1991; Killar and Eisenstein, 1985). *Salmonella* susceptible C57BL/6J mice were immunized with 2x 10^9 EBST and AroA$^-$ ST on day 0 by oral gavage. Two booster doses were given to EBST immunized mice, with 2 x 10^9

EBST on day 14 and 28. A Sham immune control group of mice was maintained, which received PBS. After 14 weeks of primary vaccination, EBST immune, AroA immune and Sham immune mice were challenged orally with 1 x 10^8 virulent Nalidixic acid resistant *Salmonella* Typhimurium. Survivors in all three groups of mice were monitored up to day 7 post challenge. It was observed that, 70% of the EBST immune mice survived the virulent ST challenge (Figure 4-14). AroA immune mice had relatively higher proportion i.e., 80% of survivors, when compared to only 60% survivors in case of sham immune mice. Mice were sacrificed 3 and 7 days post challenge, for assessing the bacterial bioburden on liver, spleen, mesenteric lymph nodes and cecum. A delayed onset of bacterial colonization was observed in case of AroA immune mice compared to EBST and sham immune mice (Figure 4-15 A, B). On day 7, an increase in organ colonization was observed in all three mice groups. But, Sham immune mice had significantly higher bacterial CFU counts compared to EBST and AroA immune mice in liver and cecum (Figure 4-15 A, D). The difference in organ colonization between EBST and AroA immune mice were found to be statistically non-significant ($p \geq 0.05$) indicating their similarity in controlling *in vivo* replication of bacteria. Overall, a relatively higher bacterial bio-burden was observed in all mice groups (Figure 4-15 A-D) which can be attributed to the high challenge titer of virulent ST.

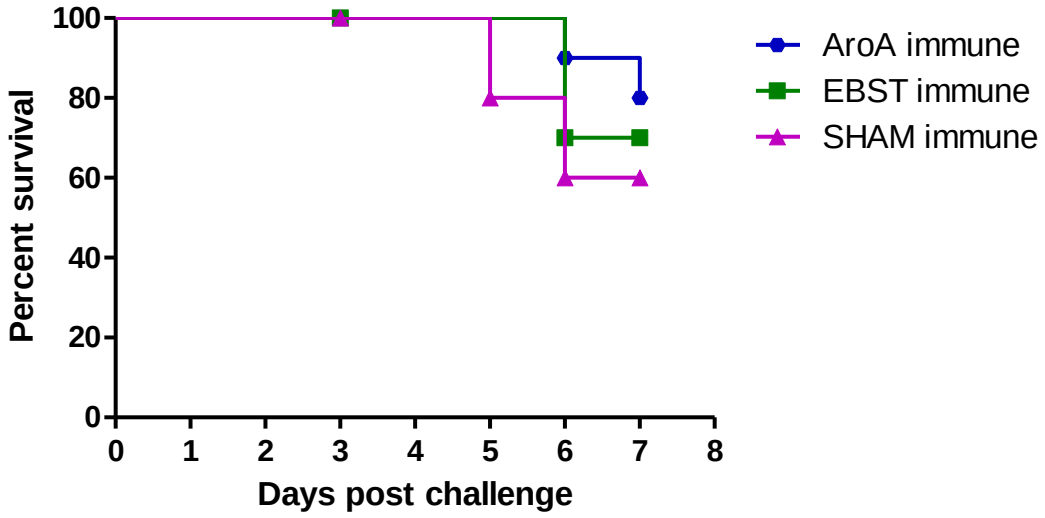
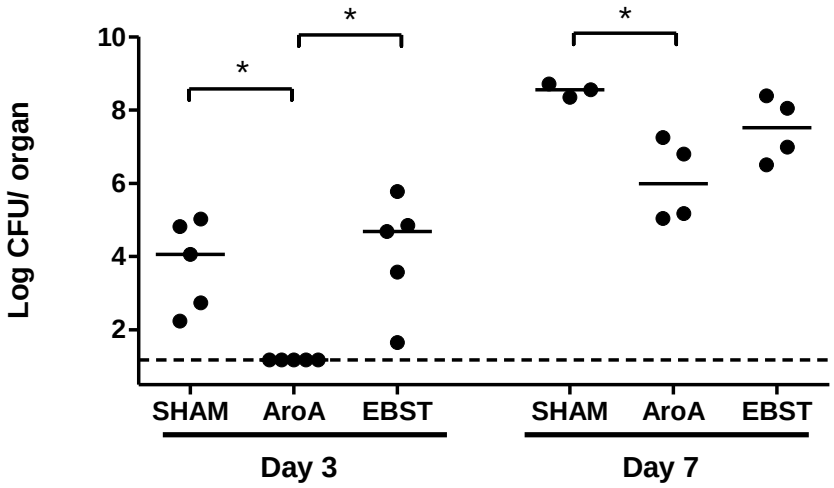


Figure 4-14. Survival of EBST immune, AroA ST immune and sham immune mice till 7 days post challenge with virulent S. Typhimurium.

C57BL/6 mice were unimmunized (Sham) or immunized with $2x10^9$ EBST, AroA ST on day 0 via oral gavage. EBST immune group was given two booster doses of $2x\ 10^9$ EBST on week 2 and 4. All mice groups were orally challenged with $1x\ 10^8$ virulent S. Typhimurium and observed for survival till day 7 post challenge. Data shown is a censored Kaplan Meier survival curve for Sham immune (n=10), EBST immune (n=15), AroA immune (n=15)

A



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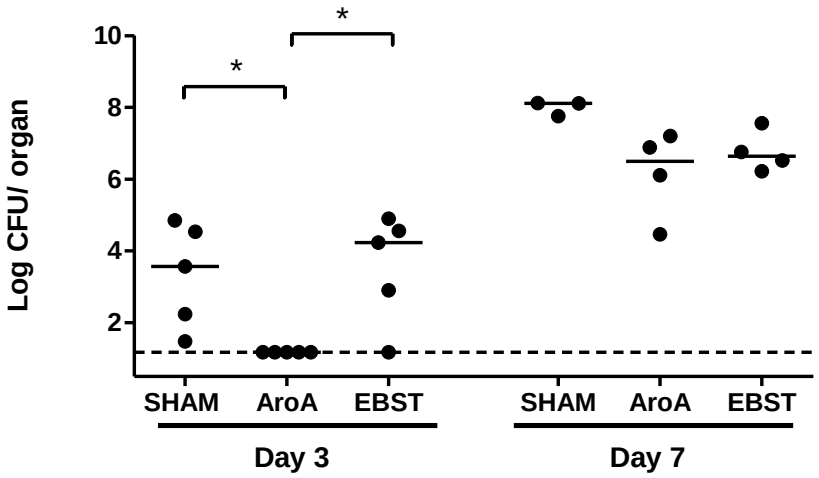
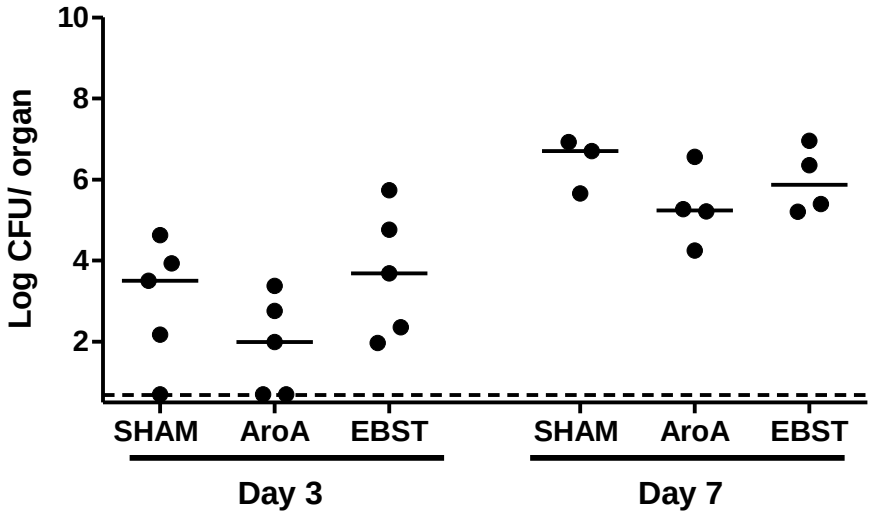


Figure 4-15. Bacterial bioburden on organs of EBST, AroA and sham immune mice.

EBST immune, AroA immune and sham immune mice were challenged with virulent ST, 14 weeks after primary vaccination. After 3 (n=5mice/group) and 7 (n=3-4 mice/group) days of challenge mice were sacrificed and organ bacterial burden in (A) liver (B) spleen (C) mesenteric lymph nodes (MLN) and (D) cecum were determined. X axis represents log values of CFU/organ; bar denotes median values of log CFU for each group of mice; dotted line represents the limit of detection (Liver, spleen, cecum = 1.17 log CFU; MLN = 0.69 log CFU). Kruskal wallis non parametric ANOVA was used for statistical analysis. *, median significantly different at $p \leq 0.05$.

C



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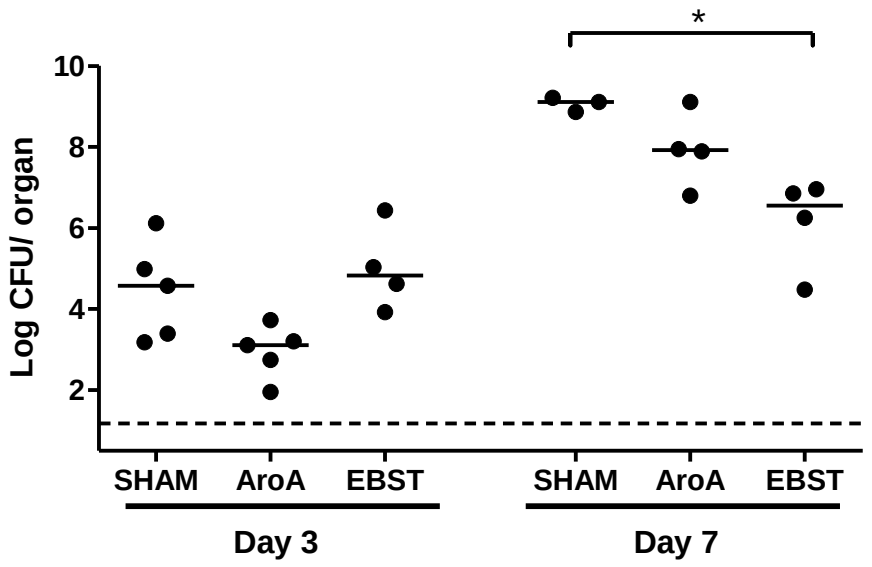


Figure 4-15 Continued.

EBST induces antigen specific cellular immune responses

Live attenuated ST vaccines are supposedly more effective than inactivated vaccines in mice models, due to their ability to induce strong cell-mediated immunity (Collins, 1974; Harrison et al., 1997; Hormaeche et al., 1991; Mastroeni et al., 1992). Therefore, the ability of EBST immunized mice to develop antigen specific cellular immune response during virulent *Salmonella* challenge was tested. Mice were orally immunized with EBST, AroA ST and PBS (Sham immune). EBST immune mice were boosted twice at 2 weeks intervals via same route. All groups of mice were challenged with virulent ST, 14 weeks after primary immunization and spleens were harvested at days 3 and 7 post challenge. Frequencies of $CD4^+$ and $CD8^+$ T cells that produced cytokines IFNγ and TNF were measured after *ex vivo* re-stimulation of splenocytes with antigens for 24 h. Electron beam irradiated *Salmonella* (EBST) and heat killed *Salmonella* (HKST) were used as *Salmonella* specific antigens, whereas anti-CD3 antibody was used as positive control for T cell stimulation.

Measuring the frequency of IFNγ producing T cells provides a direct indication of the Th1 mediated immune response (Alaniz et al., 2007). In case of naïve and sham immune mice, frequencies of $CD4^+$ T cell producing IFNγ or TNF spontaneously or in response to antigen stimulation, were found to be low on day 3 and day 7 post challenge (Figure 4-16, 4-17). However on day 7 post challenge, both EBST and AroA immune mice showed increased frequencies of $CD4^+IFN\gamma^+$ and $CD4^+TNF^+$ T cells that responded to antigens - EBST and HKST (Figure 4-18, 4-19).

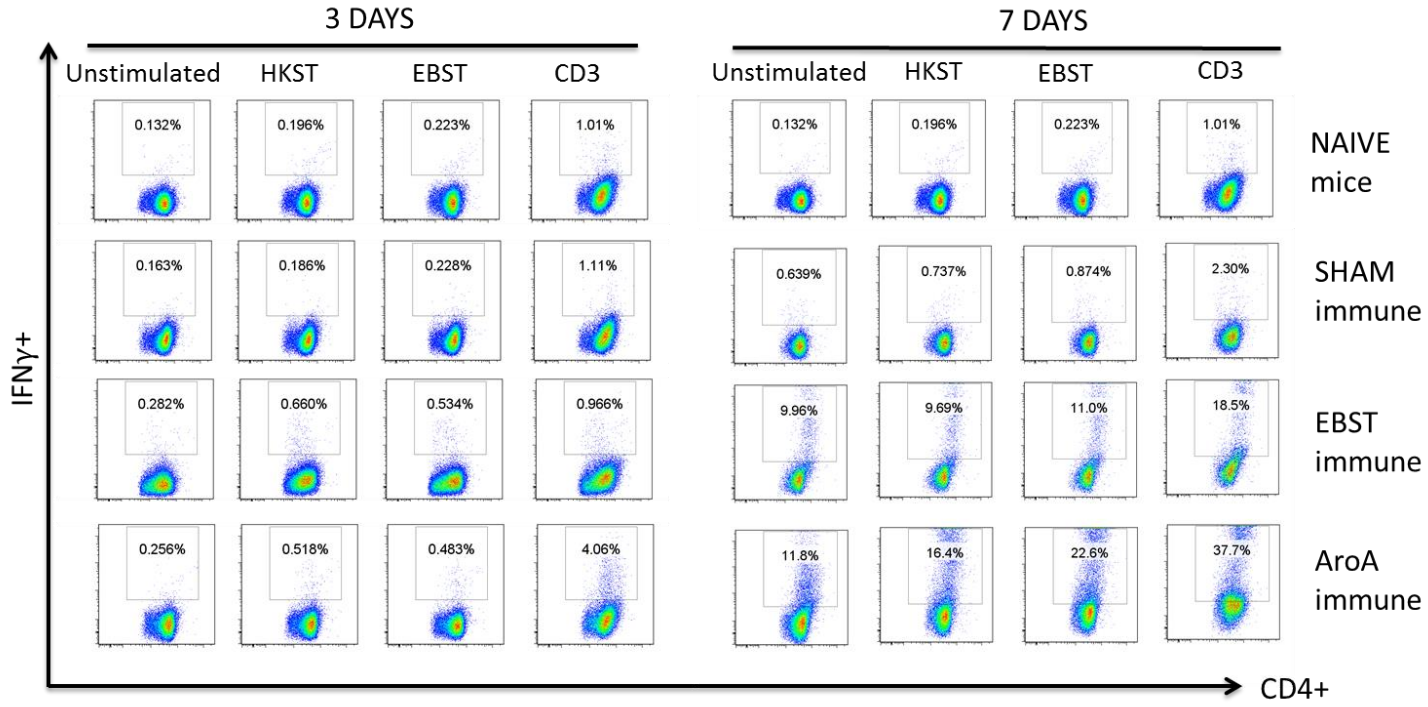


Figure 4-16. *Salmonella* specific T cell ($CD4^+IFN\gamma^+$) responses by EBST immune mice during virulent ST challenge.

Production of Th1 cytokine IFNγ by antigen specific CD4+ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 3 and 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2 $x10^7$ cells/ well) and anti-CD3 (CD3) antibody as positive control. Unstimulated splenocytes were included as control. Percentages in the gated region indicate the proportion of $CD4^+IFN\gamma^+$ of total $CD4^+$ splenocytes. Data are representative of three-five individual mice per group.

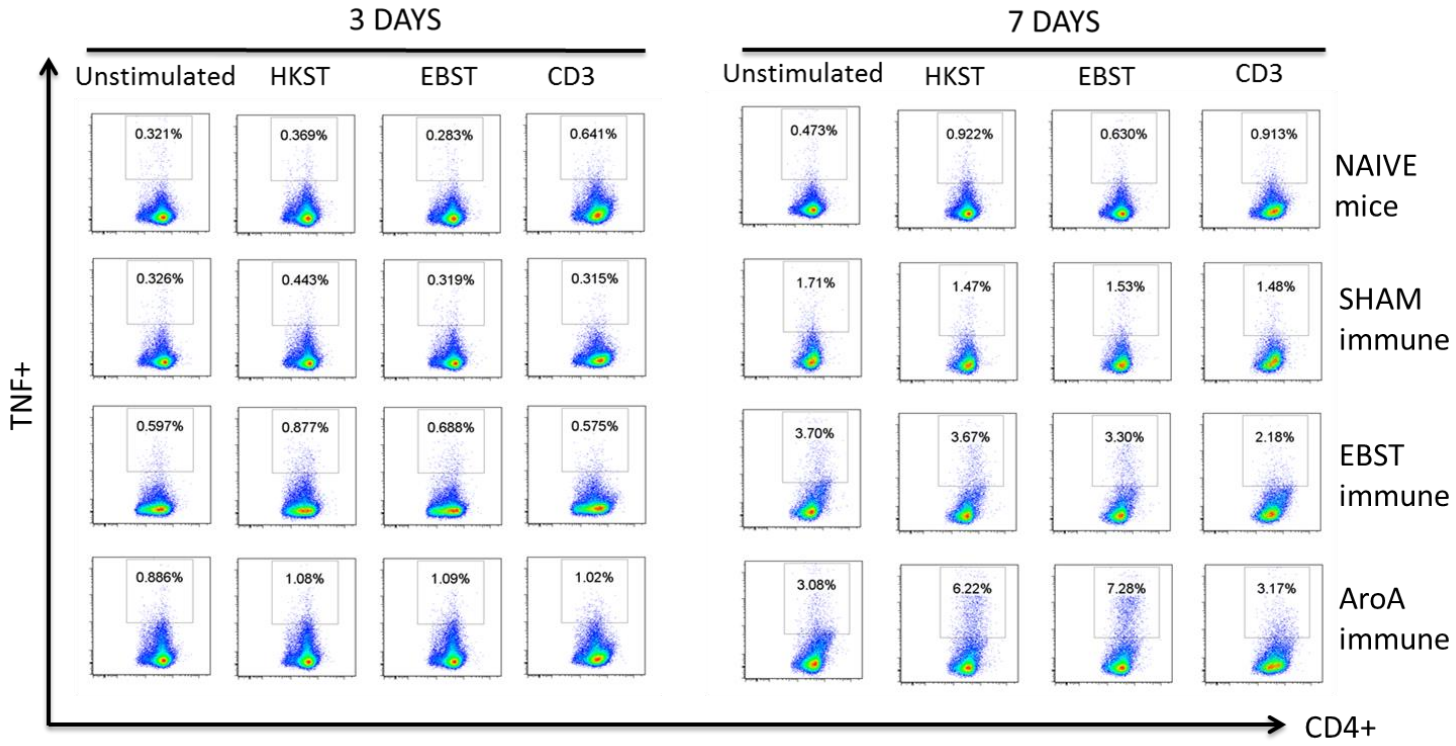
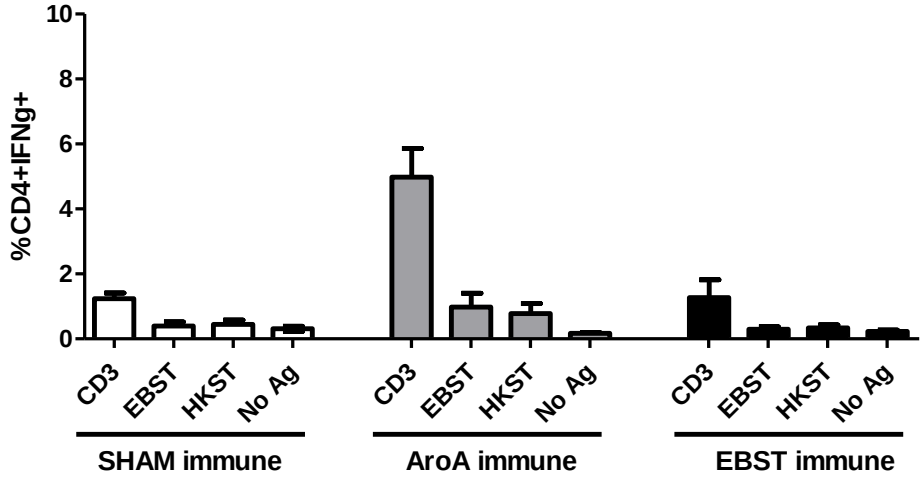


Figure 4-17. *Salmonella* specific T cell ($CD4^+$ TNF^+) responses by EBST immune mice during virulent ST challenge

Production of cytokine TNF by antigen specific $CD4^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 3 and 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2×10^7 cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Percentages in the gated region indicate the proportion of $CD4^+TNF^+$ of total $CD4^+$ splenocytes. Data are representative of three-five individual mice per group.

A



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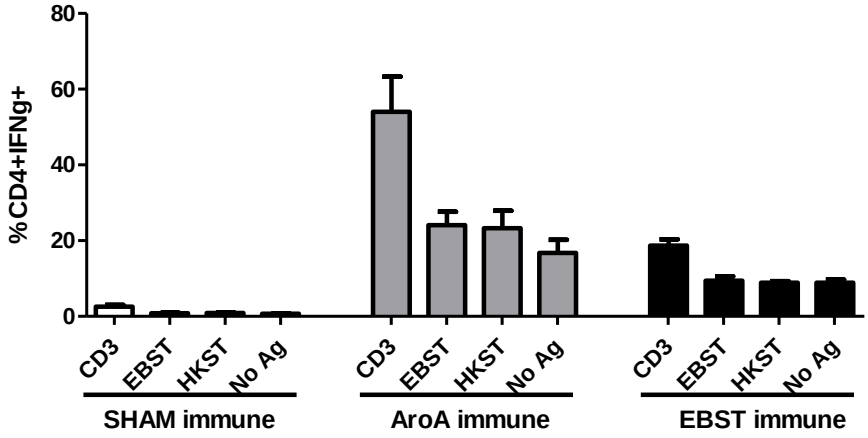
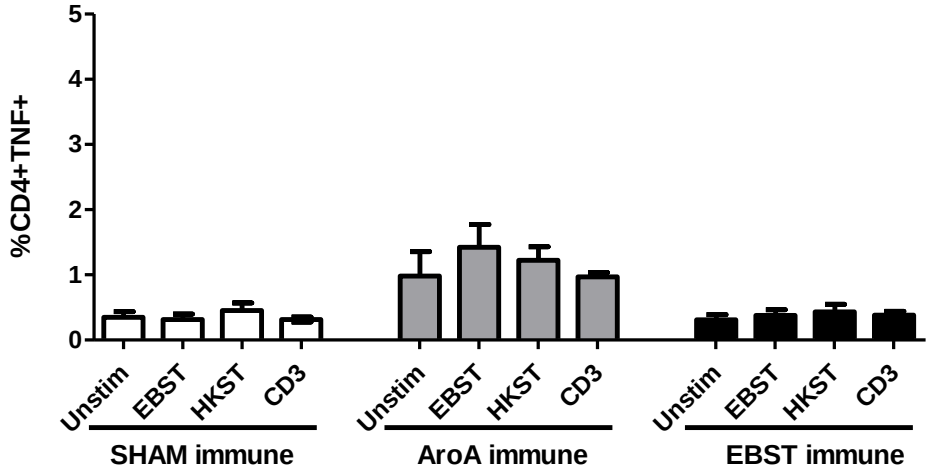


Figure 4-18. EBST immune mice exhibit strong *Salmonella* specific $CD4^+IFN\gamma^+$T cell responses during virulent ST challenge.

Production of cytokine IFNγ by antigen specific $CD4^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at (A) 3 and (B) 7 days post infection with virulent ST. Antigens used were EBST and HKST (2 $x10^7$ cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Data are represented as mean ± SEM of 3-5 mice per group. Y axis represents percentage of $CD4^+IFN\gamma^+$ of total $CD4^+$ splenocytes.

A



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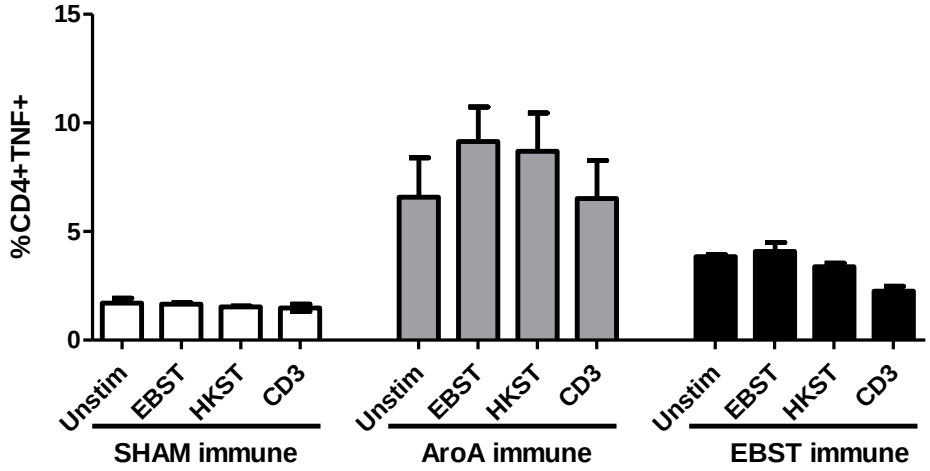

Figure 4-19. EBST immune mice exhibit strong *Salmonella* specific $CD4^+TNF^+$ T cell responses during virulent ST challenge.

Production of cytokine TNF by antigen specific $CD4^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at (A) 3 and (B) 7 days post infection with virulent ST. Antigens used were EBST and HKST (2 $x10^7$ cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Data are represented as mean ± SEM of 3-5 mice per group. Y axis represents percentage of $CD4^+TNF^+$ of total $CD4^+$ splenocytes.

Compared to sham immune mice, EBST immune mice splenocytes had significantly high frequency of *Salmonella* specific $CD4^+IFN\gamma^+$ ($p = 0.016$) and $CD4^+TNF^+$ ($p= 0.0043$) T cells. Both AroA and EBST immune mice showed an increase in antigen specific $CD4^+IFN\gamma^+$ and $CD4^+TNF^+$ T cells from day 3 to day 7 post challenge. $CD4^+$Tcells responding to positive control anti-CD3 antibody indicates the presence of previously primed memory T cells in EBST and AroA immune mice splenocytes, that rapidly responded to the CD3 stimulation to produce cytokines IFNγ and TNF.

Production of IFNγ and TNF by $CD8^+$T cells in response to *Salmonella* specific antigen re-stimulation was also investigated. Frequency of antigen specific $CD8^+$ T cells producing IFNγ was far lower compared to $CD4^+$ T cells (Figure 4-20, 4-22). Antigen specific $CD8^+$ $IFN\gamma^+$T cells were higher in AroA immune mice splenocytes compared to EBST and sham immune mice (Figure 4-20, 4-22). $CD8^+TNF^+$ T cells were found to be present in similar proportion in sham immune, AroA immune and EBST immune mice (Figure 4-21, 4-23). These results demonstrate the involvement of $CD4^+$T cells rather than CD8+ T cells, as key player in cell mediated immune response of EBST immunized mice, triggering the production of Th1 cytokine IFNγ.

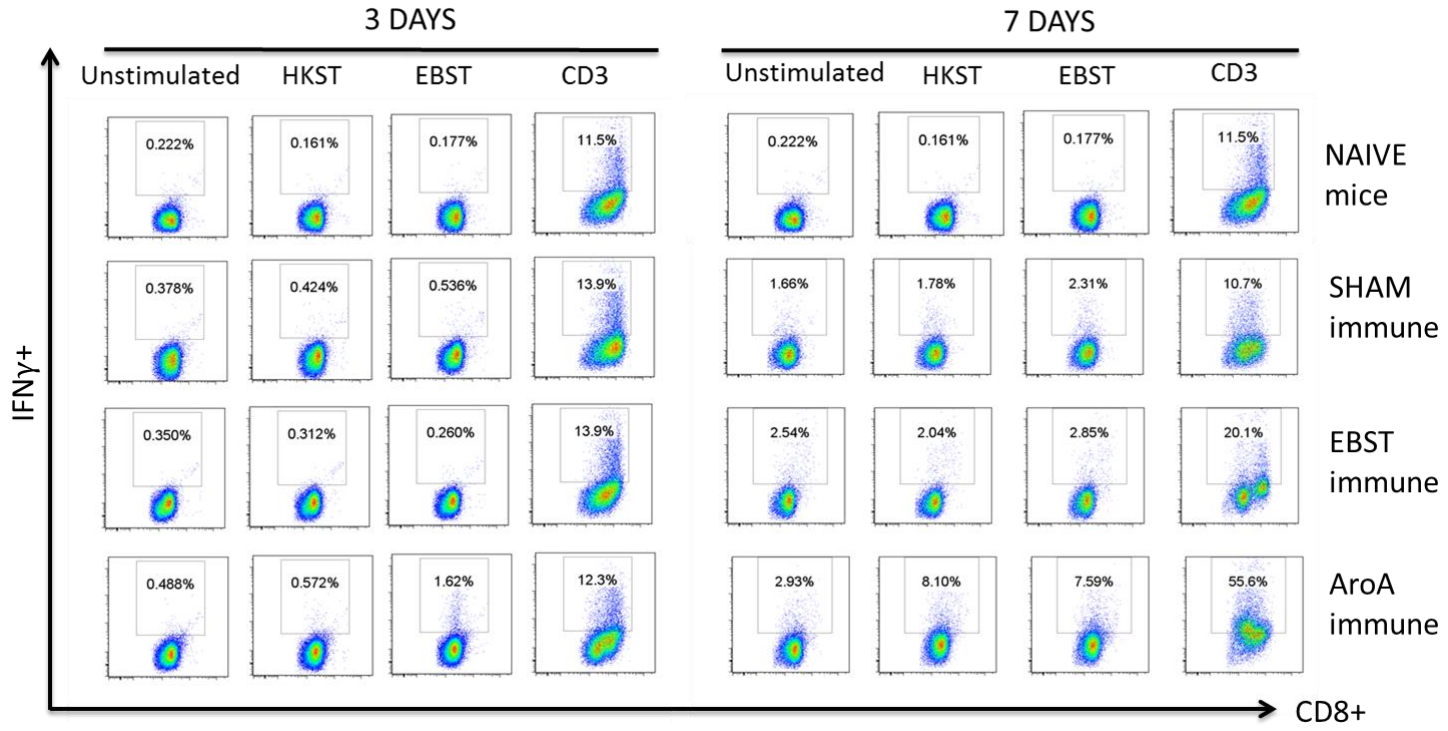

Figure 4-20. *Salmonella* specific T cell ($CD8^+IFN\gamma^+$) responses by EBST immune mice during virulent ST challenge.

Production of cytokine IFNγ by antigen specific $CD8^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 3 and 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2×10^7 cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Percentages in the gated region indicate the proportion of $CD8^+IFN\gamma^+$ of total $CD8^+$ splenocytes. Data are representative of three-five individual mice per group

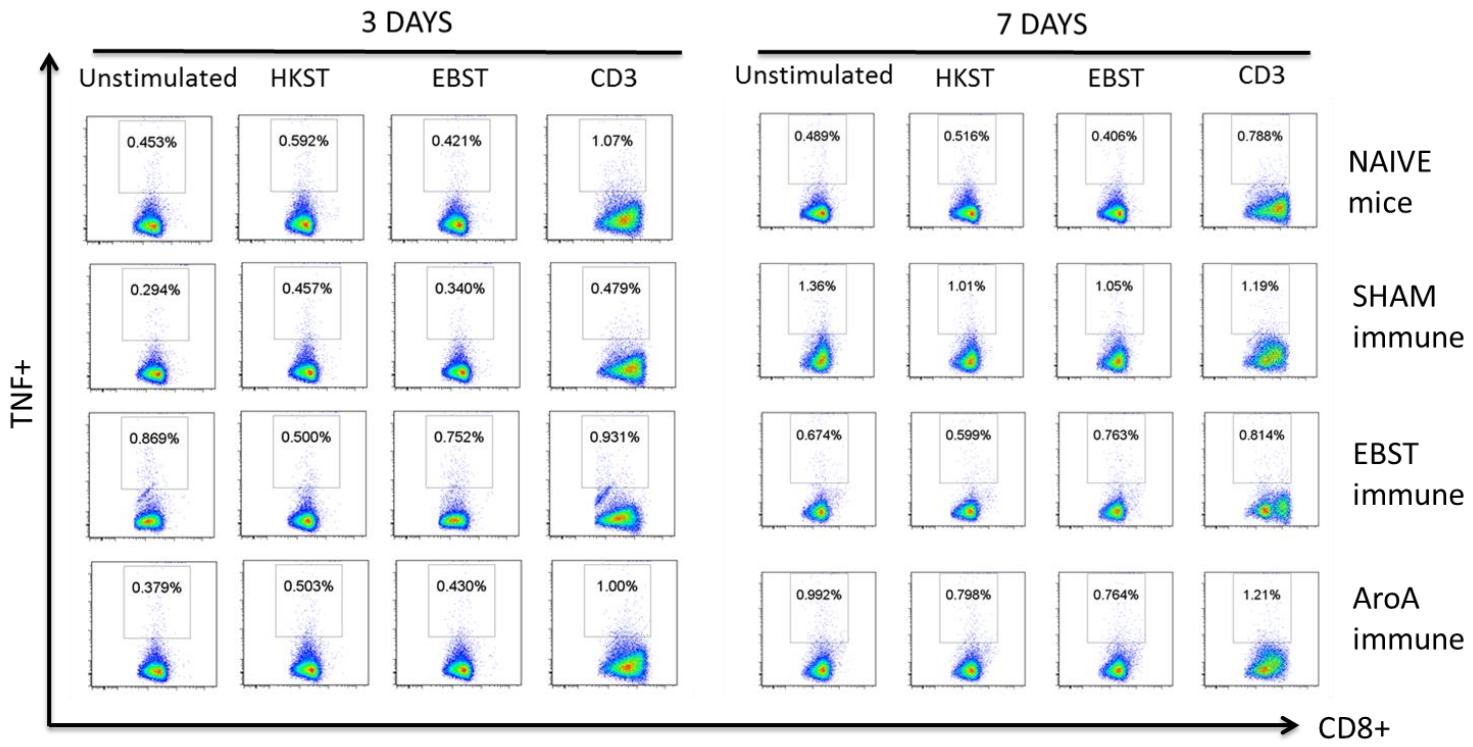

Figure 4-21. *Salmonella* specific T cell ($CD8^+$ TNF^+) responses by EBST immune mice during virulent ST challenge.

Production of cytokine TNF by antigen specific $CD8^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 3 and 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2×10^7 cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Percentages in the gated region indicate the proportion of $CD8^+TNF^+$ of total $CD8^+$ splenocytes. Data are representative of three-five individual mice per group

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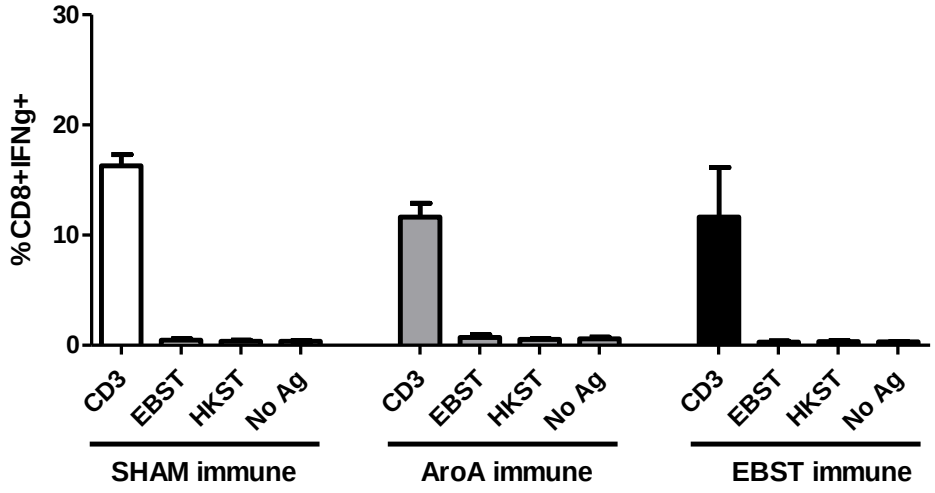

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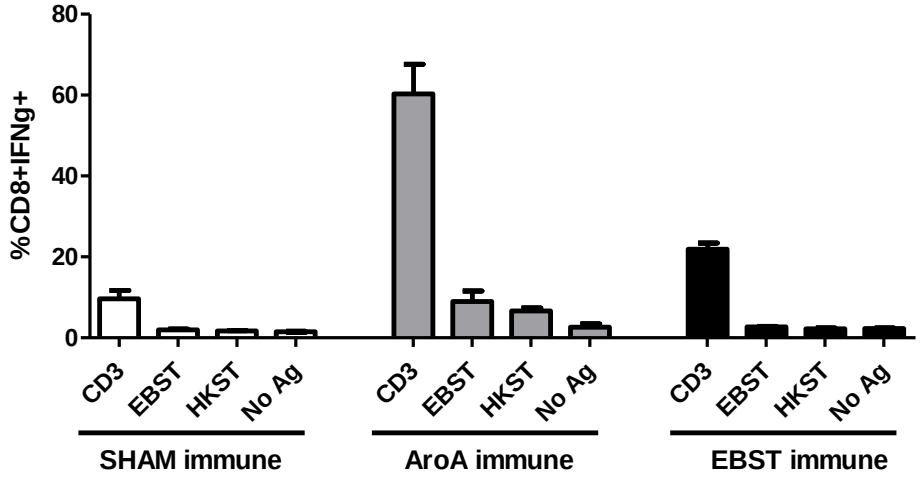

Figure 4-22. *Salmonella* specific $CD8^+IFN\gamma^+$ T cell responses.

Production of cytokine IFNγ by antigen specific $CD8^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at (A) 3 and (B) 7 days post infection with virulent ST. Antigens used were EBST and HKST (2 $x10^7$ cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Data are represented as mean ± SEM of 3-5 mice per group. Y axis represents percentage of $CD8^+IFN\gamma^+$ of total $CD8^+$ splenocytes.

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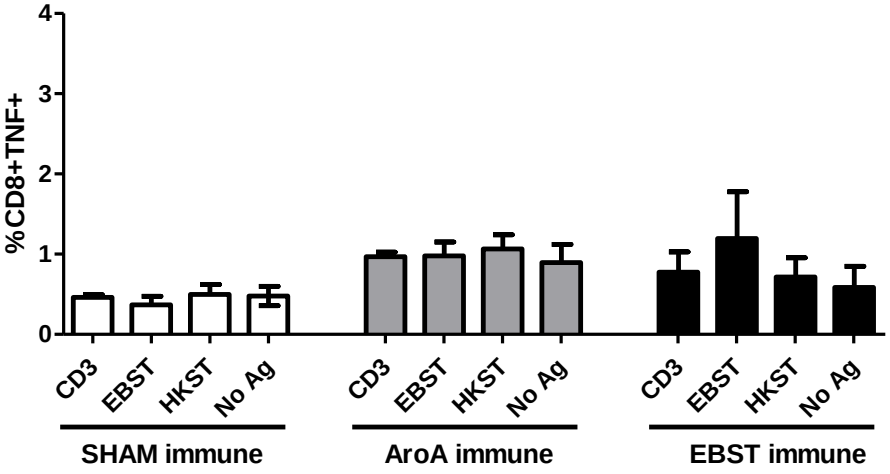

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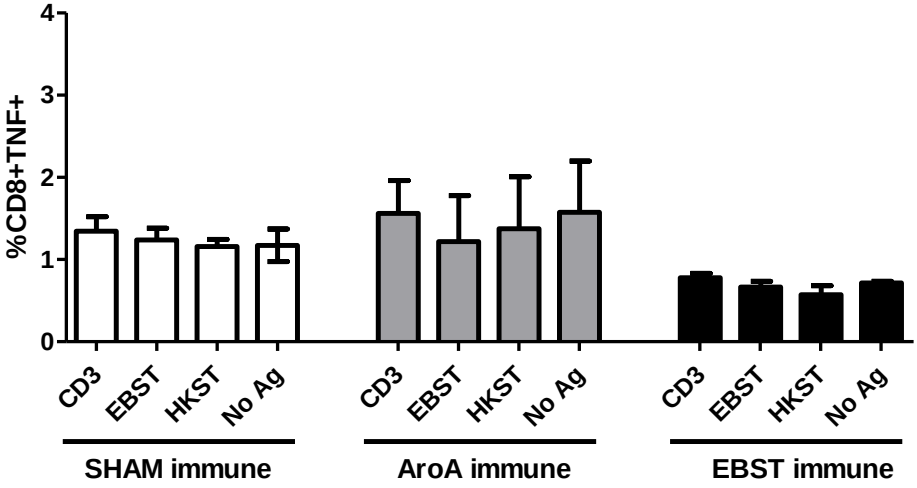


Figure 4-23. *Salmonella* specific $CD8^+TNF^+$ T cell responses.

Production of cytokine TNF by antigen specific $CD8^+$ T cells was measured using intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at (A) 3 and (B) 7 days post infection with virulent ST. Antigens used were EBST and HKST (2 $x10^7$ cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Data are represented as mean ± SEM of 3-5 mice per group. Y axis represents percentage of $CD8^+TNF^+$ of total $CD8^+$ splenocytes.

EBST vaccination induces generation of antigen specific multifunctional $CD4^+$ T cells

Presence of multifunctional Th1 cells which can simultaneously secrete multiple cytokines such as IFNγ, TNF and IL-2 are found to be better correlates of vaccine induced protection compared to single cytokine secreting T cells (Darrah et al., 2007). The presence of $CD4^+IFN\gamma^+TNF^+$ T cells in splenocytes from EBST, AroA and sham immune mice after *in vitro* restimulation with *Salmonella* specific antigens was tested (Figure 4-24). It was observed that both EBST and AroA immune mice splenocytes had higher frequencies of $CD4^+IFN\gamma^+TNF^+$ T cells that responded to total *Salmonella* antigens. Sham immune mice on the other hand, did not trigger stimulation of multifunctional T cells (Figure 4-24, 4-25). EBST immune mice had significantly higher ($p= 0.003$) frequency of multifunctional CD4+ T cells compared to sham immune mice. Multifunctional $CD4^+$ T cells were less stimulated in response to anti-CD3 antibody control (Figure 4-24). Thus, it was evident that immunization with EBST vaccine triggers production of multifunctional $CD4^+$ T cells simultaneously secreting cytokines IFNγ and TNF.

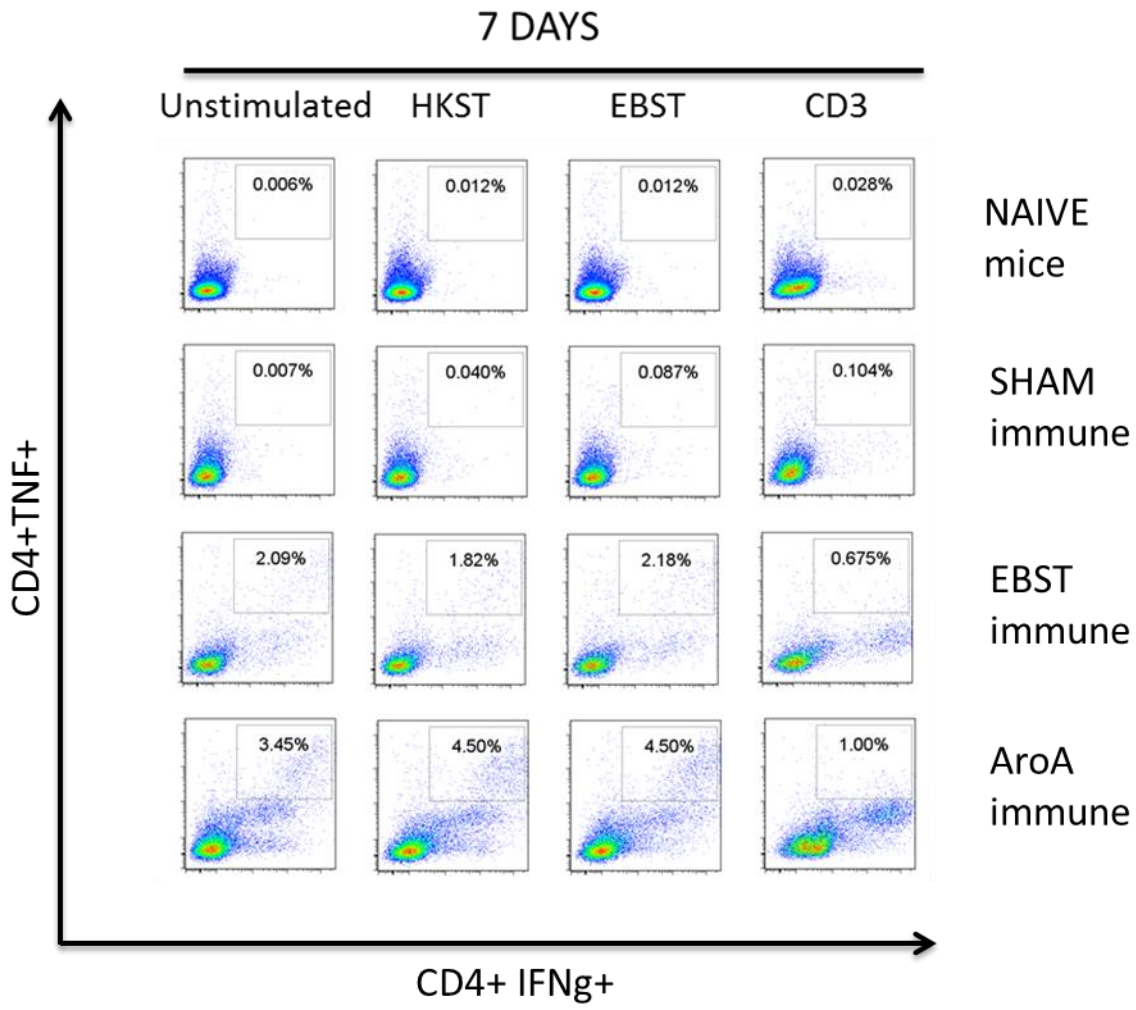


Figure 4-24. *Salmonella* specific multifunctional $CD4^+$ T cell responses by EBST immune mice during virulent *Salmonella* challenge.

Simultaneous production of cytokine IFNγ and TNF by antigen specific $CD4^+$ T cells was measured using multiparameter flow cytometry by intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2×10^7 cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. Percentages in the gated region indicate the proportion of $CD4^+IFN\gamma^+TNF^+$ of total $CD4^+$ splenocytes. Data are representative of three individual mice per group.

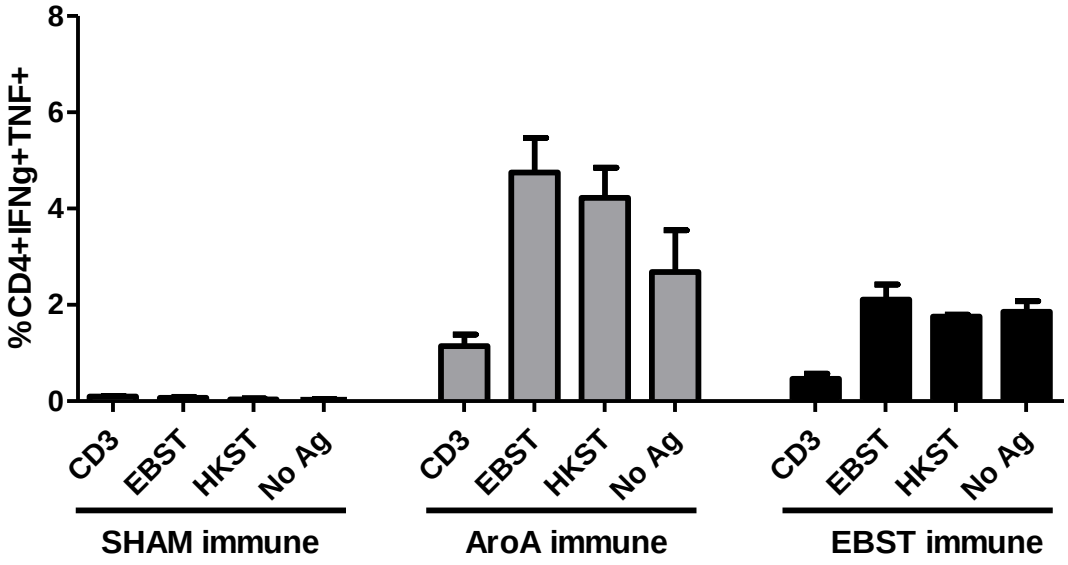


Figure 4-25. EBST immune mice exhibit strong *Salmonella* specific multifunctional $CD4^+$ T cells during virulent *Salmonella* challenge.

Simultaneous production of cytokine IFNγ and TNF by antigen specific $CD4^+$ T cells was measured using multiparameter flow cytometry by intracellular cytokine staining (ICS) from individual EBST immune, AroA immune and Sham immune mice splenocytes at 7 days post infection with virulent ST. For comparison, naïve mice splenocytes were included. Antigens used were EBST and HKST (2×10^7 cells/ well) and anti-CD3 antibody as positive control. Unstimulated splenocytes were included as control. . Data are represented as mean ± SEM of 3 mice per group. Y axis represents percentage of $CD4^+IFN\gamma^+TNF+$ of total $CD4^+$ splenocytes.

Induction of *Salmonella* specific IgG by EBST

In order to check the levels of *Salmonella* specific IgG in the sera of EBST immunized mice indirect ELISA was performed. Mice were orally immunized with EBST, AroA ST and PBS (Sham immune). EBST immune mice were boosted twice at 2 weeks intervals via same route. All groups of mice were challenged with virulent ST, 14 weeks after primary immunization and serum was harvested from individual mouse on days 3 and 7 post challenge. Reactivity of mice sera to total *Salmonella* antigen – EBST, was tested using EBST coated ELISA plates. Results showed that both AroA and EBST immune mice had high serum IgG levels compared to sham immune mice (Figure 4-26). Although the serum IgG levels of EBST immune mice were lower than AroA immune mice ($p = 0.0016$), it was significantly higher compared to sham immune mice ($p = 0.0095$). AroA immune mice had very high titers of serum IgG on both day 3 and day 7 post-challenge which was significantly greater than sham immune mice ($p \leq 0.0005$). Hence, it was observed that AroA immune mice developed strong *Salmonella* specific B cell response. Though the B cell response of EBST immune mice was slightly reduced, it was significantly greater than sham immune mice.

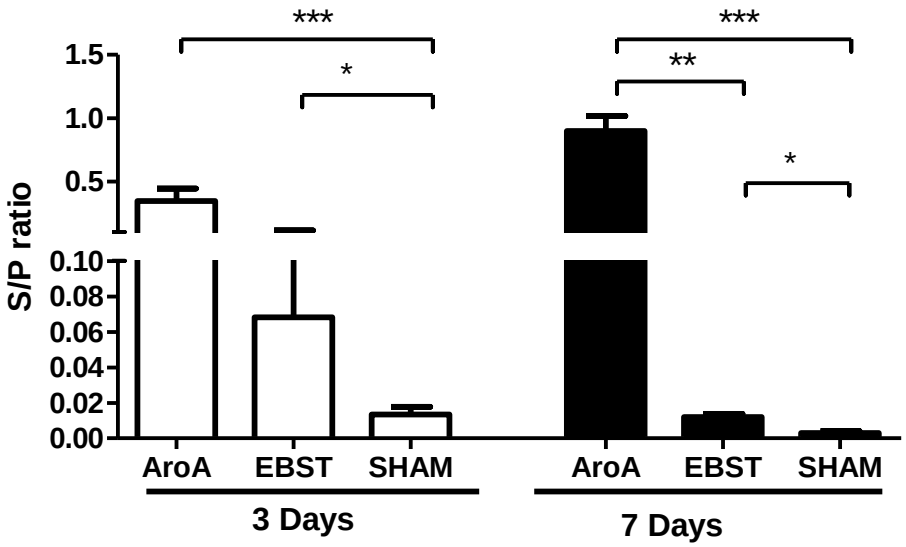


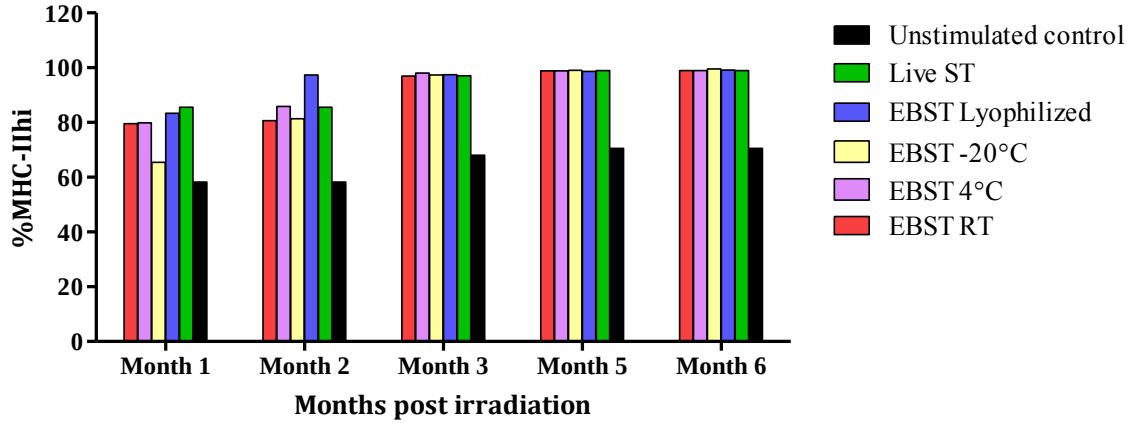
Figure 4-26. Mice immunized with EBST induced moderate *Salmonella* specifc IgG during virulent ST challenge.

ELISA detection of *Salmonella* specific IgG in the sera of EBST immune, AroA immune and Sham immune mice were tested for reactivity to total *Salmonella* antigen (EBST) after 3 and 7 days after challenge with virulent S. Typhimurium. Serum from individual mice were diluted 1 in 500 and run in triplicates. Data are represented as mean ± SEM of 3 mice per group. Y axis represents S/P ratio (Sample OD_{450}/ positive control OD_{450}). Stastics analysis using Student's unpaired t test was carried out to compare between groups. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

EBST maintains immunogenic and proinflammatory properties during storage at room temperature for extended time period

The ability of EBST vaccine to retain the immunogenic properties during storage at different temperature conditions was tested as a measure to assess the vaccine potency. Here, DC2.4 cell line was used as the *in vitro* model system to assess the DC maturation ability of EBST vaccine. EBST vaccines were stored up to 6 months at 4°C, -20°C, and room temperature and as lyophilized preparations. These vaccines were analyzed on a monthly basis for their ability to activate DC. A freshly prepared live ST culture was also used to stimulate DC2.4 cells, which served as a positive control for DC maturation. Expression of MHC-II and co-stimulatory molecules was measured using flow cytometry. The EBST vaccine preparation was found to be immunogenically stable evident by their consistent DC maturation capability up to 6 months of storage at various temperatures (Figure 4- 27A-D). Expression levels of DC maturation indicators such as CD40, MHC-II, CD80 and CD86 were up-regulated and were on par with the level of live ST stimulation (Figure 4- 27A-D). These findings provide evidence of EBST vaccine to be immunogenic when stored at room temperature for 6 months. Stability of EBST at room temperature for extended periods of time demonstrates the significant cost saving potential of such vaccines.

A



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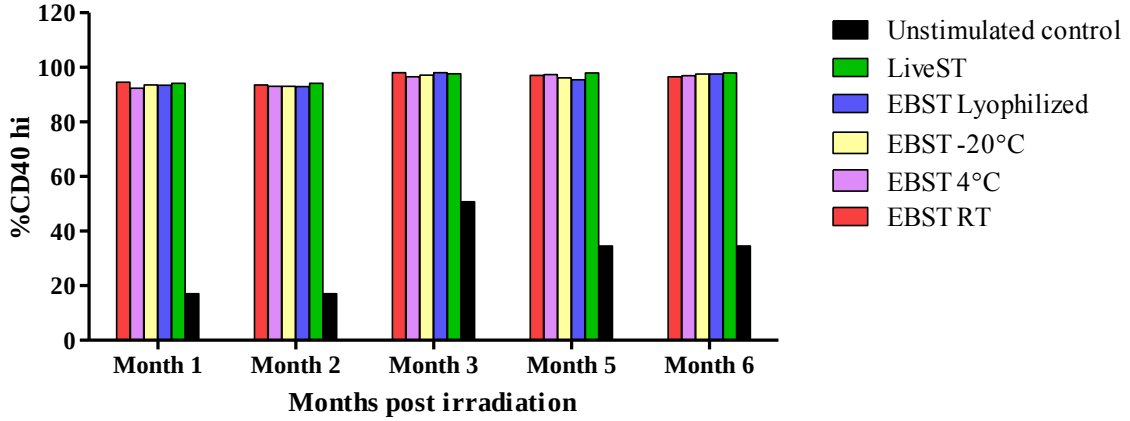
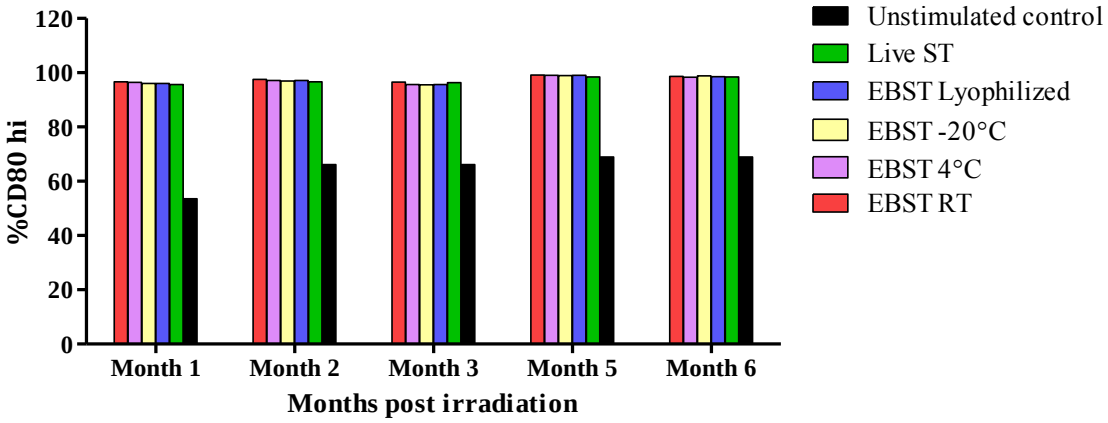


Figure 4-27. EBST remains immunogenically stable during storage at room temperature for extended time period.

EBST vaccine stored at different temperature conditions such as room temperature (RT), 4°C, -20°C and after lyophilization induced stable maturation of DC2.4 cells indicated by the upregulation of (A) MHC-II (B) CD40 (C) CD80 and (D) CD86 for up to 6 months of storage. Freshly prepared live *S.* Typhimurium was included as a positive control to stimulate DC2.4 cells, unstimulated DC2.4 cell provided base level expression of different surface markers on DC2.4 cells. Data represents mean values of proportion of cells positive for indicated surface marker.



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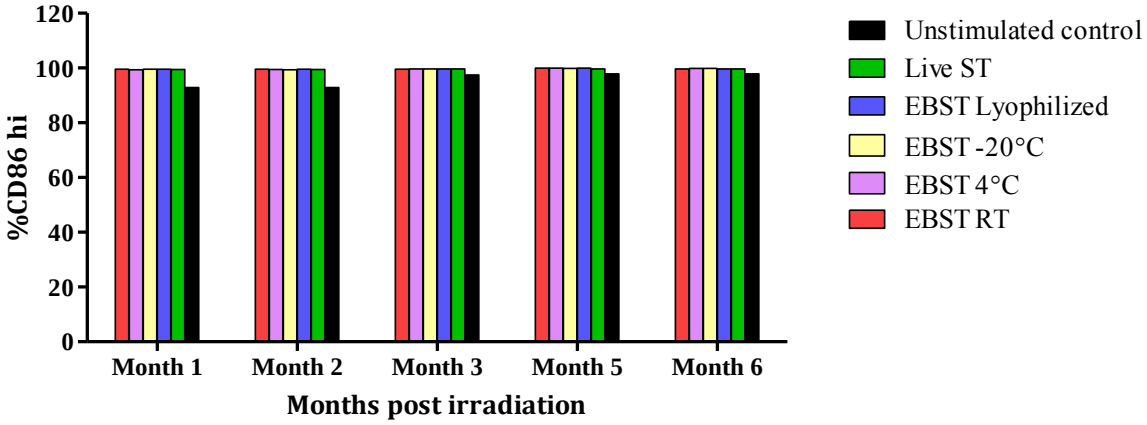

Figure 4-27. Continued.

CHAPTER V

DISCUSSION

The general rule behind vaccine development is - the more similar a vaccine is to the disease causing form of the organism, the better the immune response to the vaccine (CDC, 2012). This formed the basis for developing newer vaccine platforms that combine better immune response of live attenuated vaccines and safety profile of inactivated vaccines. The current study involves the use of electron beam (eBeam), as a next generation vaccine platform. Microbiological and immunological characterization of eBeam based vaccine against a model organism - *Salmonella* Typhimurium, was carried out. The eBeam *S*. Typhimurium (EBST) vaccine was developed by inactivating live *S.* Typhimurium (ST) using high energy electron beam. Bacterial inactivation by ionizing radiation occurs primarily due to direct/ indirect DNA damage (Ginoza, 1967; Sparrow et al., 1967), whereas damage to proteins or lipids contribute only minimally to the bacterial lethality (Kim and Thayer, 1995; Miller, 2005; Tahergorabi et al., 2012).

The choice of an optimal eBeam dose for vaccine generation involves 2 major considerations: (1) should be high enough to ensure complete bacterial inactivation that prevents the ability of bacteria to repair the radiation induced damages and (2) should be low enough to preserve the integrity of immunological epitopes present on bacteria. Based on the inactivation kinetics analysis, D_{10} value of *S*. Typhimurium in PBS was calculated as 0.19 kGy, which is in agreement with previous studies reporting a D_{10}

value of 0.2 kGy for S. Typhimurium in phosphate buffer (Thornley et al., 1963; Hansen and Shaffer, 2001). The ability of microorganism either to reproduce significantly in nutrient broth or to produce a colony forming unit on a recovery medium after exposure to radiation were monitored as parameters for microbial survival (Hansen and Shaffer, 2001). Results from the recovery studies showed that *S*. Typhimurium completely losses its ability to multiply in TSB after subjecting to an eBeam dose of 7 kGy (Table 4-1). *In vivo* studies using *Salmonella* susceptible mice confirmed the inability of eBeam irradiated *S*. Typhimurium to multiply inside host system (Table 4-2). The eBeam irradiated *S*. Typhimurium (EBST) cannot be re-isolated from the host and grown to pure culture. It is also unable to cause any disease symptoms or mortality in susceptible host, thus rendering EBST to be non-pathogenic as per Koch's postulate (Koch, 1876). Based on the results from the bacterial inactivation studies and regrowth assays, EBST vaccine was generated by irradiating ~ $1x10^9$ CFU/ml of ST at 7 (SD ±0.38) kGy (Figure 4-1).

Membrane integrity analysis using Baclight assay and electron microscopy revealed that EBST maintained an intact cell membrane similar to non -irradiated live ST (Figure 4-2, Figure 4-3). Ultrastructural analysis using TEM showed the presence of similar cell surface structures in both live ST and EBST (Figure 4-5). Studies conducted by Moustafa et al. (2011) and Sanakkayala et al. (2005), support these findings as they detected the presence of intact cell membrane in gamma irradiated *Brucella* sp. Similarly, the study on gamma irradiated *Toxoxplasma gondii* showed that irradiated cells had same morphology as non-irradiated cells with no evidence of clumped

chromatin or cytoplasmic damage (Hiramoto et al., 2002). In contrast to eBeam irradiation, cell membrane damage was clearly observed in ST subjected to heat inactivation. The difference between live ST, EBST and HKST was investigated immunologically by generating an immunoreactivity profile of *Salmonella* specific antigens in respective bacterial preparations using immune serum from mice previously immunized with live *Salmonella* (Figure 4-7). Results showed that HKST lacked several antigenic proteins, whereas EBST had a very similar immunoreactivity profile as live ST. Thus, it can be inferred that eBeam irradiation of *S*. Typhimurium at a dose of 7 kGy preserved the integrity of immunological epitopes present on bacteria.

Although eBeam irradiation abrogated the reproductive ability of *S*. Typhimurium, EBST remained metabolically active, as shown by the results of Alamar blue assays and the biochemical assays (Figures 4-8 and 4-9). In the Alamar blue assay, increased fluorescence due to reduction of redox indicator resazurin to resorufin was observed in case of EBST and live ST cells. This indicates the presence of reduced environment in the irradiated cells that signifies the maintenance of metabolic activity by EBST. Positive results for the catalase assay indicates the presence of an intact catalase enzyme in the irradiated cell. The EBST was also found to be capable of fermenting sugar to produce acid, which requires coordinated action of multiple enzymes in the carbohydrate fermentation pathway. Detection of such enzymatic activities indicates that eBeam irradiation did not degrade enzymes present in the bacteria. These findings are comparable to the previous reports of maintenance of metabolic activity by gamma irradiated *Brucella* sp. (Magnani et al., 2009; Moustafa et al., 2011; Sanakkayala et al.,

2005) and *Toxoplasma gondii* (Hiramoto et al ., 2002). As per the studies by Hiramoto et al. (2002) and Magnani et al. (2009), irradiation does not cause significant interference to cellular functions. They utilized various assays such mitochondrial respiratory assay, luminescent promoter assay, ^{3}H-proline and ^{3}H-hypoxanthine incorporation assays, to prove that gamma irradiated cells maintained oxidative function and preserved the ability for protein and nucleic acid synthesis. Studies have shown that, despite impairing microbial replication by DNA fragmentation, ionizing radiation leaves considerable portions of genome amplifiable in the irradiated cells (Trampuz et al., 2006; Magnani et al., 2009). Thus, irradiated cells remain metabolically active and replication incompetent (Trampuz et al., 2006). The results from Alamar blue assay show that EBST retained metabolic activity for more than 10 days, with almost 2 fold increased activity over live ST (Figure 4-8). The increased activity might probably be due to DNA repair mechanisms. Although increased metabolic activity have been previously reported in gamma irradiated *Toxoplasma gondii* (Hiramoto et al., 2002), the exact reason has not yet been studied conclusively. Based on the results from metabolic activity and biochemical assay and also considering the similarity with previous studies on gamma irradiated cells (Magnani et al., 2009; Moustafa et al., 2011; Sanakkayala et al., 2005; Hiramoto et al., 2002), we can infer that eBeam irradiation rendered *S.* Typhimurium metabolically active yet non culturable (MAyNC). Further studies are required to investigate the ability of EBST for *de novo* protein synthesis.

Motility is considered as an important property of bacterial cells that helps in obtaining nutrients from the environment and to survive in detrimental physicochemical

conditions (Martinez- Garcia et al., 2014). Flagella dependent motions help the bacterial cells to swim in liquid media and swarm on wet surfaces. Bacterial flagella also helps in adhesion, biofilm formation, and host invasion (Kirov et al., 2003). But, the role of bacterial motility in *in vivo* colonization is still unsettled as there were many conflicting reports on the requirement of flagella for virulence (Olsen et al., 2013; Lockman and Curtiss, 1990; Carsiotis et al., 1984; La Ragione et al., 2003). Bacterial motility assessment showed that EBST lacked motility. However, the lack of motility cannot be attributed to the detachment of flagella during eBeam irradiation, as it was observed using TEM that EBST preserved intact flagella (Figure 4-6). Several reports suggest that lack of bacterial motility occurs in bacteria undergoing stress, for the purpose of metabolic trade-off (Martnez-Garcia et al., 2014; Lu et al., 2013). The lack of flagellar motility might confer surplus energy (ATP) and reducing power (NADPH), that could be allocated to other cellular functions, which will help the bacteria to cope up with the stress better than wild type bacteria (Martinez- Garcia et al., 2014). These reports provide clues for the ability of EBST to be increasingly metabolically active in spite of being non-motile. Presence of intact flagella in EBST is important, as the flagella is considered as a key pathogen associated molecular pattern (PAMP) capable of triggering host immune response (Kawai and Akira, 2011; Olsen et al., 2013).

In order to initiate a specific immune response against bacterial pathogens, bacterial antigens need to be captured, processed, and presented by specialized cells called antigen presenting cells (APC) (Banchereau and Steinman, 1998; Steinman, 1991). The phagocytic cells such as macrophages and dendritic cells are the most widely

studied APC (Svensson et al., 2000; Yrlid et al., 2000). Even though, both macrophage and dendritic cell (DC) share many functional similarities, DC is considered more potent APC, capable of stimulating naïve T cells to initiate *Salmonella* specific immune response (Svensson et al., 2000; Yrlid et al., 2000). The ability of DC to interact with naïve T cells following antigen uptake, determines the specificity and polarization of the T cell mediated immunity (Surendran et al., 2010; Yrlid et al., 2001). Hence, the DC maturation potential of EBST was studied as the first step of immunological characterization. When encountered with appropriate inflammatory stimuli, immature DC undergoes process of maturation to develop as a fully competent APC (Sundquist et al., 2004; Yrlid et al., 2000). A mature DC is characterized by increased and stable surface expression of MHC molecules, upregulation of co-stimulatory molecule surface expression, as well as enhanced cytokine secretion (Pierre et al., 1997; Svensson et al., 2000; Winzler et al., 1997). The results from *in vitro* DC stimulation assays using DC2.4 cell line and BMDC showed that EBST provided optimal DC maturation and activation (Figures 4-10, 4-12). Upregulation of MHC-II, CD40, CD80, CD86 and TNFα production by DC was observed, when stimulated with EBST (Figure 4-10, 4-12). Interestingly, the level of expression of surface markers and cytokine secretion induced by EBST were on par with that induced by viable live ST. These results are consistent with previous studies conducted using gamma irradiated *Brucella* sp. and *Listeria monocytogenes*, which showed similar DC activation by irradiated, heat killed and live bacterial preparations (Sanakkayala et al., 2005; Datta et al., 2006). Conflicting reports have been published in the past with respect to the requirement of bacterial viability and

longer incubation time for DC maturation by *Salmonella* (Yrlid et al., 2001, Norimatsu et al., 2004; Svensson et al., 2000; Kalupahana et al., 2005). Studies by Yrlid et al. (2001) and Norimatsu et al. (2004) showed differential upregulation of CD40 and CD86 surface molecules by live and heat killed *Salmonella*. By contrast, study by Svensson et al. (2000) showed no difference in expression levels of CD40, CD86 and MHC-II between live and heat killed *Salmonella*. Whereas, bacterial viability and internalization was found to be an important factor for CD80 expression levels. Findings by Kalupahana et al (2005) sheds light on the requirement of prolonged contact of DC with inactivated/ heat killed *Salmonella* to induce DC activation similar to live *Salmonella*. The findings by Kalupahana et al (2005) are in agreement with the present study as higher expression levels of MHC-II and co-stimulatory markers were observed after 24 h co-incubation with EBST and HKST (Figure 4-12). A commercial live attenuated ST vaccine was used as a control for BMDC maturation studies. For an appropriate comparison, DCs were stimulated with same MOI of EBST, HKST, live ST and commercial vaccine. The results suggested that EBST without any additional adjuvant, induced DC maturation similar to the commercial ST vaccine formulation (Figure 4-12). Besides activating DC, EBST also retained the agonist activity over wider antigen concentration (Figures 4-11, 4- 13). In conclusion, these findings demonstrate that, EBST can potently stimulate innate pro inflammatory response (TNFα) from DC, and can efficiently stimulate DC maturation (MHC-II, CD40, CD80 and CD86).

Through the DC activation studies, it was observed that besides EBST and live ST, HKST served as an important inflammatory stimulus for triggering DC maturation

markers (Figures 4-10, 4- 12). But, unlike eBeam irradiation, heat inactivation resulted in disrupted cell membrane, altered cell surface morphology and disintegrated cellular contents in HKST (Figures 4-2, 4-3, 4-4, 4-5). HKST also suffered from loss of certain immunogenic *Salmonella* antigens (Figure 4-7), which remained intact in EBST and live ST. Together, these results suggest that EBST serves as a superior inflammatory stimulus to effect antigen specific immune response.

Several studies have demonstrated higher level of protective immunity in mice by live *Salmonella* vaccines, compared to killed/ inactivated vaccines (Harrison et al., 1997; Hormaeche et al., 1991; Killar and Eisenstein, 1985; Mastroeni et al., 1993; Lehmann et al., 2006; Thatte et al., 1993; Yrlid et al., 2001). Reports have shown that oral immunization with live attenuated AroA$^-$ ST vaccine generated Th1 type biased cell mediated immune response, hallmarked by the increased production of Th1 cytokine IFNγ (Killar and Eisenstein, 1985; Harrison et al., 1997; Lehman et al., 2006). Whereas, immunization with inactivated vaccines such as heat killed or purified bacterial components, generated an IL-4 dominated Th2- type response (Galdiero et al., 1998; Thatte et al., 1993). Therefore, the ability of EBST (metabolically active yet non culturable cells) to induce cellular immune response, in particular Th1 mediated immune response, was studied in comparison to a live attenuated *Salmonella* vaccine (AroA$^-$ ST). The ability of EBST vaccine to enable the host to rapidly produce Th1 derived cytokines - IFNγ and TNFα, in response to the virulent bacterial infection was monitored (Lehmann et al., 2006; Alaniz et al., 2007). The results showed activation of antigen specific IFNγ and TNF producing CD4$^+$ T cells in both EBST immune and AroA

immune mice splenocytes (Figures 4-16). Even though we observed activation of antigen specific $CD4^+TNF^+$ T cells in both EBST and AroA immune mice, the frequency of TNFα producing cells were lower compared to IFNγ producing $CD4^+$ T cells (Figures 4-17 and 19). TNFα production by $CD4^+$ T cells in response to antigen stimulation occurs around 4 h of antigen co-incubation (Kalupahana et al., 2005). In the present study, intracellular cytokine staining was employed followed by multi-parameter flow cytometry to simultaneously analyze $IFN\gamma^+$ and TNF^+ T cells, in which BrefeldinA was added to plug Glogi bodies only after 18 h of co-incubation. So, the delay in addition of the Golgi plug™ might be responsible for the lower frequency of $CD4^+TNF^+$ T cells, as the cells might have already secreted considerable amount of TNF to the supernatant which was not detected with ICS. Interestingly, IFNγ and TNFα producing $CD4^+$ T cells were also detected in un-stimulated EBST immune and AroA immune splenocytes, but not in case of sham immune mice(Figures 4-16 and 17). Based on the bacterial colonization studies, it was observed that the spleens of EBST immune, AroA immune and sham immune mice were colonized heavily with challenge strain of *Salmonella* (Figure 4-15B). Hence, the un-stimulated splenocytes from EBST and AroA immune mice harbored challenge strain of *Salmonella*, which could have provided the trigger for secretion of IFNγ by $CD4^+$ T cells. Even though, sham immune mice splenocytes also were colonized with *Salmonella*, very low frequencies of $CD4^+IFNg^+$ and $CD4^+TNF^+$T cells were found, which signifies the antigen specific immune response triggered by EBST and AroA immunization (Mastroeni et al., 2001; Dougan et al., 2011; Mastroeni and Menager, 2003).

Although we observed higher polyclonal stimulation of $CD8^+IFN\gamma^+$ T cells in both EBST and AroA immune mice, significant levels of antigen specific IFNγ producing $CD8^+$ T cells were present only in case of AroA immune mice. In contrast to $CD4^+$ T cells, $CD8^+$ T cells are mostly restricted to MHC class-I molecules that present endogenous antigens processed by proteasome in cytosol of APC (Yrlid et al., 2000; Dougan et al., 2011). Consistent with the results of Yrlid et al. (2001), we observed that in AroA immunized mice; contribution of IFNγ production by *Salmonella* specific $CD8^+$ T cells appeared to be minor compared to $CD4^+$ T cells (Figure 4-20). Based on these findings, we can infer that EBST is capable of inducing Th1 type cellular immune response similar to live attenuated *Salmonella* vaccines. Therefore, unlike killed or inactivated vaccines (Thatte et al., 1993; Harrison et al., 1997), mice immunized with EBST develops robust *Salmonella* specific T cell response. This result is similar to what was reported with gamma irradiation with Listeris sp. (Datta et al?)

Although, production of IFNγ or effector cytokine TNF by $CD4^+$ T cells is considered necessary to mediate protection, using it as a single immune parameter to predict protection may not be sufficient (Darrah et al., 2007). As IFNγ and TNF act synergistically to mediate pathogen control, use of multifunctional $CD4^+$ T cells capable of simultaneously secreting IFNγ and TNF is considered a better correlate of protection (Darrah et al., 2007). Therefore, multi-parameter flow cytometry was used to assess the frequency of multifunctional $CD4^+$ T cells simultaneously producing IFNγ and TNF (Figure 4-24). Increased *Salmonella* specific multifunctional $CD4^+IFN\gamma^+TNF^+$ T cells were observed in EBST immune and AroA immunized mice (Figures 4-24 and 4-25).

Notably, nonspecific polyclonal stimulation of multifunctional $CD4^+$ T cells were not observed in both EBST and AroA immune mice, which signifies the specificity of $CD4^+IFN\gamma^+TNF^+$ T cells towards *Salmonella* antigens. EBST immune mice had significantly higher frequency of multifunctional $CD4^+$ T cells compared to sham immune mice, but were relatively lower than AroA immune mice (Figure 4-25), which is consistent with the difference observed in bacterial colonization between the immunized mice groups (Figure 4-15).

Preliminary survival analysis conducted on EBST, AroA and sham immune mice revealed no obvious deficiency in survival of EBST immune mice (Figure 4-14). But these results provide only an estimate of survival of immunized mice post virulent challenge, as large number of animals in the study was used for determining other correlates of protection. Future studies are needed with a larger number of immunized mice dedicated for survival analysis. In order to determine the level of protective immunity in EBST, AroA and sham immune mice post virulent ST challenge, organ CFU was determined from liver, spleen, mesenteric lymph nodes and cecum. Results suggest that the extent of reduction of virulent *Salmonella* colonization in EBST immune mice were on par with that by AroA immune mice, whereas sham immune mice had high level of virulent *Salmonella* colonization (Figure 4-15). An increase in organ colonization was observed in EBST, AroA and sham immune mice from day 3 to day 7 post challenge. Among all three groups of mice, sham immune mice exhibited substantial increase in level of organ colonization between day 3 and 7 post virulent ST challenge (Figure 4-15). The higher bacterial colonization observed in AroA immune

mice on day 7 post challenge might be because of the very high titer of (~ 2x 10^8 CFU) virulent *Salmonella* used for challenging the mice. EBST immune mice also had similar (not statistically significant) level of bacterial colonization as AroA immune mice. In future, more defined challenge studies need to be conducted with multiple vaccine dose regimes and challenge titers to definitively evaluate the protective immunity induced by EBST immune mice.

The titers of *Salmonella* specific IgG antibody in AroA immune mice on day 3 and day 7 post challenge, was found to be higher compared to EBST and sham immune mice. Abundant antigen specific IgG indicates the development of strong *Salmonella* specific B cell response in AroA immune mice (Figure 4-26). Though the B cell response of EBST immune mice was slightly reduced, it was significantly greater than sham immune mice. These findings are consistent with previous studies which demonstrated the requirement of concerted action of cellular and humoral immune response to induce superior protection by live attenuated vaccine (Harrison et al., 1997; Thatte et al., 1993). Inactivated *Salmonella* vaccines such as heat killed ST have been shown to induce *Salmonella* specific IgG antibodies, when immunized via intra-peritoneal or subcutaneous routes (Harrison et al., 1997; Thatte et al., 1993). Intra-peritoneal administration of gamma irradiated *Brucella* elicited high titers of IgG, IgG1 and IgG2a (Moustafa et al., 2011; Sanakkayala et al., 2005). Studies using eBeam irradiated *S*. Enteritidis intramuscularly administered to chicken, exhibited high *Salmonella* specific IgG after virulent *Salmonella* challenge (Jesudhasan et al., 2010). Results from these studies provides clue that EBST is capable of inducing B cell

response. But the IgG titer observed in the present study by the orally immunized EBST mice were lower compared to AroA immune mice, which could be improved by further optimization of immunization route and doses.

Appropriate storage and handling of vaccines plays a key role in the success of immunization. Vaccines exposed to temperatures outside the recommended range can have reduced potency and protection (CDC, 2012). Hence it is important to maintain a proper cold chain, ie., temperature controlled supply chain for vaccine, which increases the cost of vaccination. The eBeam based vaccines may provide a rather inexpensive vaccine platform, especially with respect to storage and transportation without the requirement of cold chain. The ability of EBST vaccine to retain the immunogenic properties during storage at different temperature conditions was used as a measure to assess the vaccine potency. The vaccine potency of EBST over extended storage period was determined using *in vitro* DC2.4 activation model system. EBST retained stable immunogenic properties such as increased surface expression of DC maturation markers and production of proinflammatory cytokine, for several months at room temperature, 4°C, -20°C and also after lyophilization (Figure 4-27). These properties are considered to be of high value in any vaccine formulation (Ulmer et al., 2006). The EBST provided consistent and stable upregulation of DC maturation markers similar to live ST. The EBST vaccine stability at room temperature or as lyophilized powder for extended storage period demonstrates immense cost saving potential of the vaccine.

Use of eBeam irradiation for vaccine production is a relatively novel field of research. Much work has been done by various research groups across the globe with regard to the development of gamma irradiated vaccines (Sanakkayala et al., 2005; Datta et al. 2006; Alsharifi and Mullbacher 2010; Furuya et al., 2010; Moustafa et al., 2011; Seder et al., 2013). Although the basic mechanism by which eBeam and gamma irradiation inactivate microorganisms is similar (Miller, 2005), there are fundamental differences between eBeam and gamma irradiation with respect to irradiation source, energy and dose-rate. Radioactive isotopes such as ^{137}Cs and ^{60}Co emit gamma radiation, whereas, electron beam is machine generated (linear accelerator), which does not involve any radioactive sources. The energy of gamma rays in ^{60}Co is approximately 1 MeV, while for high energy eBeam, it is around 10 MeV. The dose rate of gamma rays from ^{60}Co is often in the range of hundreds of grays per minute, while in the case of eBeam the dose rate is in the range of tens of millions of grays per minute (Miller, 2005; Praveen et al., 2013). Thus, the high dose rate of high energy eBeam can compensate for the relatively limited penetration ability of eBeam. During eBeam irradiation, microbial pathogens experience ionizing radiation at significantly higher dose rate (usually in seconds) conditions compared to gamma irradiation, hence eBeam has a very short processing time compared to gamma. The eBeam is also ideal for integrating into a production line, hence suitable for commercial vaccine manufacturing. Additionally, eBeam technology serves as an environment friendly alternative to the radio nuclide based gamma irradiation.

Overall, it has been demonstrated that eBeam inactivation preserved the potent proinflammatory and immunogenic properties of *S*. Typhimurium. EBST remained metabolically active yet unable to multiply *in vitro* and *in vivo*. Increasing numbers of immunocompromised population who suffer from congenital immunodeficiency, immunosuppressive therapies, HIV patients are at risk of vaccine induced diseases after immunization with replicating vaccines (McFarland, 1999; Wolfe and Bhatt, 2005). Being a non-replicating vaccine, provides added advantage to eBeam based vaccines to be a potential immunization strategy for such immunocompromised individuals. Historically, non-replicating inactivated vaccines fail to induce adequate immune response, especially against pathogen requiring cell mediated immune response (Harrison et al., 1997; Robertsson et al., 1983; Thatte et al., 1993; Ulmer et al., 2006). But, metabolically active EBST induce substantial *Salmonella* specific T cell responses, thus playing a meaningful role in host response during infection. The eBeam based vaccine serves as a promising platform for commercial vaccine development as the immunogenic properties of EBST remained stable during storage at room temperature or after lyophilzation for extended time period.

CHAPTER VI

SUMMARY

1. The eBeam irradiation irreversibly inactivated *S*. Typhimurium. These cells were unable to multiply even under the most favorable *in vitro* and *in vivo* conditions. , Thus, eBeam irradiated *S*. Typhimurium has a better safety profile with reduced risk of reversion similar to killed/ inactivated vaccines.

2. The eBeam irradiated *S*. Typhimurium maintained intact cell membrane, cell surface morphology and immunoreactivity profile similar to live ST. Thus it can be inferred that eBeam irradiated *S*. Typhimurium preserved the integrity of immunological epitopes present on bacteria.

3. The eBeam irradiated *S*. Typhimurium maintained a reduced cellular environment similar to live bacterial cells, had functional enzymes and was capable of fermenting test carbohydrates. However, they were incompetent in terms of multiplication or in culture medium or a model animal host (mice). Thus, eBeam irradiated *S*. Typhimurium remained metabolically active yet non culturable (MAyNC).

4. The eBeam irradiated *S*. Typhimurium served as a superior inflammatory stimulus comparable to live ST or commercial vaccine - Salmune®. The eBeam irradiated *S*. Typhimurium potently stimulated innate pro inflammatory response

by increased TNFα production from DC, and efficiently stimulated DC maturation by up-regulation of cell surface markers MHC-II, CD40, CD80 and CD86.

5. In eBeam irradiated *S*. Typhimurium immunized mice, *Salmonella* specific $CD4^{+}$T cells served as key player in cell mediated immune response, triggering the production of Th1 cytokine IFNγ. Increased *Salmonella* specific multifunctional $CD4^{+}IFN\gamma^{+}TNF^{+}$ T cells simultaneously secreting IFNγ and TNF were observed in EBST immunized mice splenocytes. Therefore, it can be inferred that eBeam irradiated *S*. Typhimurium is capable of inducing Th1 type cellular immune response similar to live attenuated *Salmonella* vaccines.
6. The eBeam irradiated *S*. Typhimurium immune mice were capable of reducing virulent *Salmonella* colonization in liver, spleen and cecum on par with that of AroA immune mice, whereas sham immune mice had higher levels of organ colonization. Hence, eBeam irradiated *S*. Typhimurium immunization can impart protective immunity to mice against virulent *Salmonella* challenge.
7. The eBeam irradiated *S*. Typhimurium retained stable immunogenic properties such as increased surface expression of DC maturation markers and production of proinflammatory cytokine, for up to 6 months at room temperature, 4°C, -20°C and also after lyophilization. The wide range of temperature tolerance by eBeam irradiated *S*. Typhimurium indicates the significant cost saving potential of such vaccines by evading cold chain storage.

8. The eBeam based vaccine platform utilizes a green technology without the use of any chemicals and hence environmentally safe. The eBeam dose required for various level of inactivation can be delivered with high precision process control. The eBeam technology can also be customized to fit in to the vaccine manufacturing environment. Thus, eBeam technology serves as a promising next generation vaccine platform for commercial vaccine development.

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