Cell
P R E S S

Preserving Immunogenicity of Lethally Irradiated Viral and Bacterial Vaccine Epitopes Using a Radio-Protective Mn^{2+}-Peptide Complex from *Deinococcus*

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SUMMARY

Although pathogen inactivation by γ-radiation is an attractive approach for whole-organism vaccine development, radiation doses required to ensure sterility also destroy immunogenic protein epitopes needed to mount protective immune responses. We demonstrate the use of a reconstituted manganous peptide complex from the radiation-resistant bacterium *Deinococcus radiodurans* to protect protein epitopes from radiation-induced damage and uncouple it from genome damage and organism killing. The Mn^{2+} complex preserved antigenic structures in aqueous preparations of bacteriophage lambda, Venezuelan equine encephalitis virus, and *Staphylococcus aureus* during supralethal irradiation (25–40 kGy). An irradiated vaccine elicited both antibody and Th17 responses, and induced B and T cell-dependent protection against methicillin-resistant *S. aureus* (MRSA) in mice. Structural integrity of viruses and bacteria are shown to be preserved at radiation doses far above those which abolish infectivity. This approach could expedite vaccine production for emerging and established pathogens for which no protective vaccines exist.

INTRODUCTION

Ionizing radiation has been reported as a vaccine development strategy since the early days of vaccinology (Moore and Kersten, 1936). The sterilizing effects of ionizing radiation (X-rays and γ-rays) on targeted organisms are usually ascribed to the

sum of two indiscriminately destructive processes. 'Direct action' refers to the unavoidable damaging effects of energy deposited by photons, damage which predominates in deeply frozen ($-80°C$) or dry preparations (Ward, 1988; Ito et al., 1993). In contrast, the overwhelming majority of cellular lesions in aqueous preparations are caused by the 'indirect action' of reactive oxygen species (ROS) formed from water (Ward, 1988; Ito et al., 1993; Daly, 2009). Nucleic acids, protein and viability of viruses and bacteria generally display near-exponential decay during irradiation (Daly et al., 2010; Sullivan et al., 1971; Krisch et al., 1991; Daly, 2012; Daly et al., 2004; Daly et al., 2007). For a given target, inactivation by ionizing radiation in aqueous preparations occurs at doses which are typically 4-5 times lower than when deeply frozen. Frozen or not, the major drawback of vaccine approaches which apply ionizing radiation has been the inability to uncouple genome damage from epitope damage. At doses needed to kill an organism, oxidative modifications of protein epitopes and consequent alteration or abolishment of their antibody binding specificity can render irradiated vaccines nonprotective (Hrusková, 1969; Reitman et al., 1970; Martin et al., 2010). Typically, as genome-size decreases, the dose of γ-rays needed to sterilize an organism increases, with viruses presenting the greatest challenge to preparing irradiated vaccines (Alsharifi and Müllbacher, 2010). In order to preserve the maximum number of epitopes, radiation exposure is usually limited to the minimum dose needed to inactivate or attenuate a pathogen. However, the greater immunogenic potency of vaccines prepared at lower doses is offset by inherent risks including host cell reactivation of damaged viral genomes (Eady et al., 1992) and directed evolution of bacterial radiation resistance (Harris et al., 2009). Ideally, an irradiated vaccine would be prepared at doses far greater than those needed to eliminate infectivity while maintaining epitope integrity needed to mount a protective immune response.

Direct damage by γ-rays accumulates at a rate proportional to dose and does not discriminate between DNA and proteins. However, it may be possible to selectively protect proteins from the far more damaging indirect effects of γ-rays in aqueous preparations, while leaving DNA open to ROS attack. This hypothesis is supported by recent findings for *Deinococcus radiodurans*, a bacterium which demonstrates extreme resistance to ionizing radiation (Daly, 2012; Slade and Radman, 2011). The amount of protein damage caused in *D. radiodurans* by a given dose of γ-rays is substantially less than in naturally radiosensitive organisms, but the amount of DNA damage is about the same (Daly, 2009; Daly, 2012; Kriško and Radman, 2010; Kriško et al., 2012). Protein-free cell extracts of *D. radiodurans* are highly enriched in Mn^{2+} complexes which specifically protect proteins from ROS damage (Daly et al., 2010). Based on the composition of *D. radiodurans* extracts, we reconstituted an extremely radioprotective complex consisting of Mn^{2+} and a decapeptide (DP; H-Asp-Glu-His-Gly-Thr-Ala-Val-Met-Leu-Lys-OH) in orthophosphate (Pi) buffer (Daly et al., 2010). Mn^{2+} and orthophosphate form complexes which catalytically remove superoxide ($O_2^{\cdot-}$) via a disproportionation mechanism (Barnese et al., 2008); and amino acids and peptides, which scavenge hydroxyl radicals ($OH^{\cdot}$) very efficiently (Daly et al., 2010), form complexes with Mn^{2+} which catalytically decompose hydrogen peroxide (H_2O_2) (Berlett et al., 1990). When combined in vitro, the constituents of Mn-DP-Pi (1 mM $MnCl_2$, 3 mM DP, 25 mM Pi) interacted synergistically in preventing the inactivation of enzymes during high-dose irradiation (Daly et al., 2010). In vitro at 50,000 Gy, Mn-DP-Pi preserved 50% activity of the dodecameric enzyme glutamine synthetase (622 kDa), which is normally inactivated by 150 Gy; however, Mn-DP-Pi did not significantly protect DNA from double strand breaks (DSBs), the most serious form of DNA damage (Daly et al., 2010; Daly, 2012). The protection of irradiated aqueous proteins by mixtures of Mn^{2+}, Pi, peptides and other small molecules is highly dependent on the presence and concentration of Mn^{2+}. We previously showed that radioprotection was lost when Mn^{2+} was substituted with Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}. The highest levels of protection against radiation-induced ROS were conferred by mixtures of 3 mM DP and 1 mM Mn^{2+} in 25 mM Pi, which represent physiological concentrations in *D. radiodurans* (Daly et al., 2010). Evidently, the quaternary structures of proteins and their functions can be preserved in aqueous solution by Mn-DP-Pi at doses of ionizing radiation which destroy similarly treated DNA (Daly et al., 2010). We now demonstrate that when applied exogenously, Mn-DP-Pi preserves immunogenic epitopes on intact viruses and bacteria exposed to doses of γ-rays which are far greater than needed to destroy their genomes.

RESULTS

Uncoupling Genome Damage from Epitope Damage in Irradiated Viruses

Inactivation of viruses by ionizing radiation proceeds through damage in nucleic acids, but also by destruction of protein. Bacteriophage inactivation mediated by protein damage is caused by loss of adsorption, and by disruption of the phage head that leads to discharge of DNA into the medium (Watson,

1952; Luthjens and Blok, 1969). We tested the ability of Mn-DP-Pi to protect irradiated bacteriophage λ, which infects *Escherichia coli*. The phage particle tail recognizes and binds to its host, causing dsDNA (48.5 kbp) in the head of the phage to be ejected through the tail into the bacteria, ultimately causing cell lysis. For a given λ phage preparation exposed to 0–40 kGy in the presence or absence of Mn-DP-Pi, we determined virus inactivation, DNA and protein damage, and immunogenic epitope preservation (Figure 1). In Pi buffer alone, 10^9 λ phage were sterilized by 5 kGy (Figure 1A). When irradiated in Mn-DP-Pi, the dose required to sterilize the λ phage was increased 5 times, but DNA was only approximately 2 times more resistant to DSBs (Figures 1A and 1B). Thus, we attributed the increase in λ phage survival in Mn-DP-Pi mainly to protein protection. This was confirmed by analysis of phage proteins (Figure 1C). In λ phage exposed to 40 kGy in Pi buffer alone, there was loss of the two major phage proteins (head, 38 kDa; tail, 32 kDa). In contrast, there was no significant loss of these proteins in λ phage exposed to 40 kGy in Mn-DP-Pi (Figure 1C). Rabbit antibodies raised against nonirradiated λ phage were used to determine the preservation of epitopes of λ phage irradiated in the absence or presence of Mn-DP-Pi (Figure 1D, I). Mn-DP-Pi preserved the immunogenicity of head and tail proteins at 40 kGy. By comparison, antibody binding by λ phage irradiated in Pi buffer alone was diminished at 10 kGy and abolished at 30 kGy (Figure 1D, I). We then raised antibodies against λ phage exposed to 40 kGy in Mn-DP-Pi, an approach which conceivably could be used to develop inactivated irradiated vaccines for any purified virus. These antibodies bound λ phage proteins (Figure 1D, II) similarly to the antibodies raised against nonirradiated λ phage (Figure 1D, I). Thus, immunogenic epitope destruction was uncoupled from DNA damage and killing of λ phage during irradiation in Mn-DP-Pi.

As an irradiated viral vaccine ideally would be based on a fully inactivated virus that displays undamaged surface protein conformations, we examined the distinctive morphological and adsorption properties of λ phage after exposure to 40 kGy (Figure S1). Compared to nonirradiated λ phage (Figure S1A), λ phage particles irradiated in Pi buffer alone lost their tails by 40 kGy (Figure S1B, I) and the phage heads were rendered vulnerable to rupture (Figure S1B, II). These structural changes tracked with dose-dependent increases in tail and head protein damage, and loss in immunogenicity (Figures 1C and 1D). In contrast, head and tail morphologies were preserved in λ phage particles exposed to 40 kGy in Mn-DP-Pi (Figure S1B, I and II). Further, λ phage exposed to 40 kGy in Mn-DP-Pi retained their ability to adsorb to *E. coli* (Figure S1C, I and II). Thus, for λ phage particles exposed to supralethal doses of γ-rays (40 kGy) in Mn-DP-Pi, the structural integrity and binding capacities of the whole viruses were preserved (Figure S1), and their immunogenic properties were indistinguishable from nonirradiated λ phage (Figure 1D). As the genomes of λ phage (48.5 kb) exposed to 40 kGy in Mn-DP-Pi were obliterated (Figure 1B), this approach presents itself as an effective route to preparing highly immunogenic lethally-irradiated intact viral vaccines.

We then tested the Mn-DP-Pi irradiation approach on Venezuelan equine encephalitis virus (VEEV), an important human pathogen which causes encephalitis and for which a
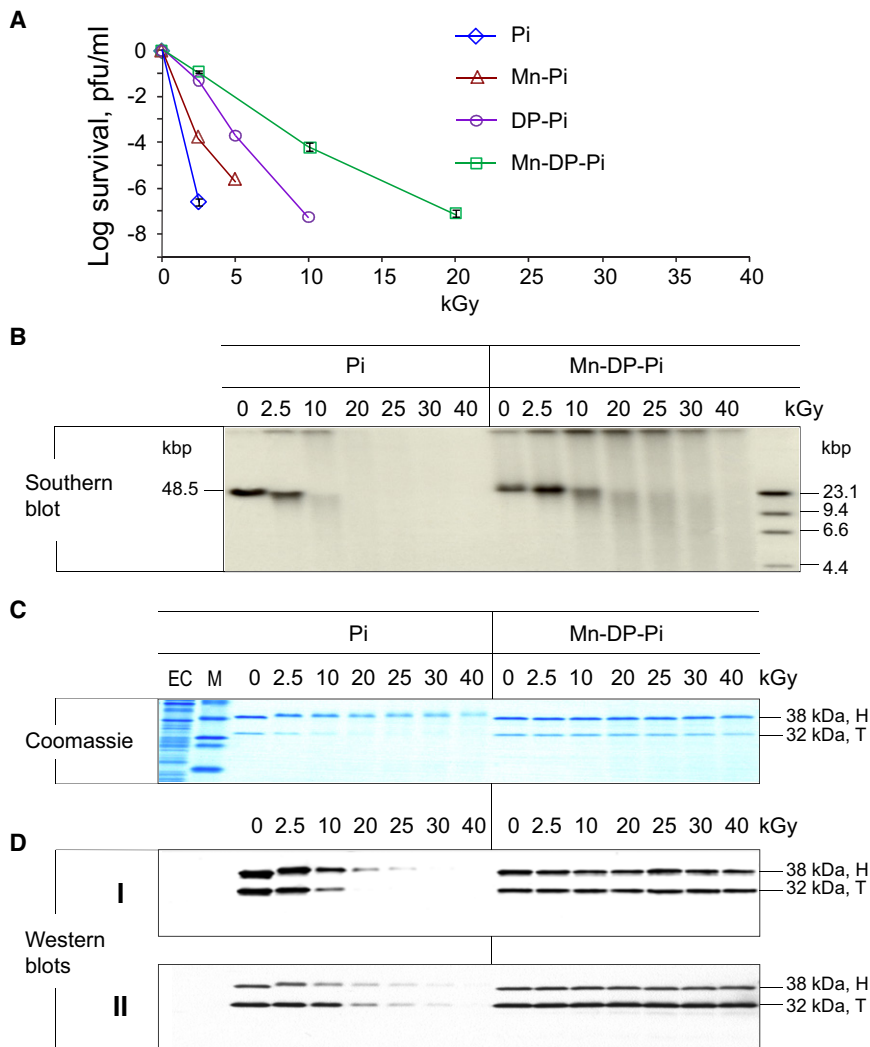
Figure 1. Uncoupling Genome Damage and Killing from Epitope Destruction in Viruses Irradiated in Mn-DP-Pi

(A) Bacteriophage lambda survival. For batches of 10^9 phage, complete inactivation (loss of all pfu) occurred at 5 kGy in Pi buffer, and 25 kGy in Mn-DP-Pi. 0 on *y* axis corresponds to 100% survival.

(B) Bacteriophage λ DNA damage assessed by Southern blotting using a ^{32}P-labeled λ DNA probe.

(C) λ protein damage assessed by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining. λ phage head protein (H) and tail protein (T).

(D) Protection of antibody binding epitopes of λ phage assessed by western blotting using duplicate gels of the one shown in panel (C). I, Antibodies raised against nonirradiated λ phage; II, Antibodies raised against λ phage exposed to 40 kGy in Mn-DP-Pi: Mn, 1 mM $MnCl_2$; DP, 3 mM decapeptide (DP; H-Asp-Glu-His-Gly-Thr-Ala-Val-Met-Leu-Lys-OH); Pi, 25 mM potassium phosphate buffer (pH 7.4). pfu, plaque forming units; EC, purified *E. coli* proteins (1.25 μg); M, protein size standards. Viability assays for phage irradiated in Pi or Mn-DP-Pi were in triplicate, with standard deviations shown. Also see Figure S1.

in Pi buffer and Mn-DP-Pi. However, in irradiated lambda phage particles, which contain dsDNA genomes, protein damage limits survival in Pi buffer at lower doses but DSBs become the critical inactivating events at higher doses in Mn-DP-Pi, when lambda proteins are protected (Figure 1A). Thus, Mn-DP-Pi uncoupled killing from the destruction of antigenic epitopes for both V3526 and lambda phage during gamma-irradiation of aqueous preparations at doses extending to at least 40 kGy.

Preparing a Mn-DP-Pi-Irradiated Bacterial Vaccine

The functional redundancy and larger number of proteins expressed by bacteria make them more likely to evade single subunit vaccines than viruses. Current bacterial vaccines almost exclusively target organisms that utilize single toxins or capsular polysaccharides as a dominant virulence strategy (Plotkin and Plotkin, 2008). Development of vaccines against bacteria that employ alternative or multiple virulence mechanisms remains a significant challenge as exemplified by *Staphylococcus aureus*. *S. aureus* is responsible for the majority of skin and soft tissue infections in humans. Complications arising from these infections are becoming increasingly problematic with the widespread emergence of antibiotic-resistant bacterial strains. Invasive methicillin-resistant *S. aureus* (MRSA) infections result in ~18,500 deaths annually in the United States, more than any other single infectious agent and exceeding the number of deaths associated with HIV/AIDS, viral hepatitis and influenza combined (Miller and Cho, 2011; Chambers and Deleo, 2009).
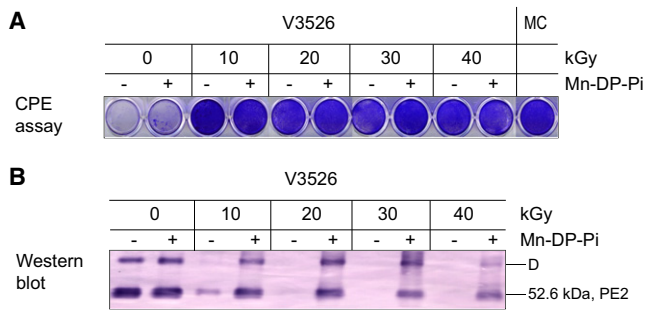
licensed protective killed vaccine does not exist (Paessler and Weaver, 2009). VEE viruses are a group of serologically related positive-stranded RNA viruses of the genus *Alphavirus* in the family *Togaviridae*. V3526 is a live attenuated strain derived from a full-length infectious clone of VEEV, and previous studies showed loss of antibody binding after inactivation by γ-rays on dry ice, which appeared to limit its efficacy as a vaccine (Reitman et al., 1970; Martin et al., 2010). We tested the infectivity and antibody binding capacity of aqueous V3526 preparations irradiated to 0-40 kGy in the presence or absence of Mn-DP-Pi. At 10 kGy in Pi buffer alone, loss of infectivity of V3526 (Figure 2A and Table S1) coincided with the initial marked decay in antibody binding capacity of PE2 (Figure 2B), a surface glycoprotein of V3526; the reactivity of PE2 in Pi buffer with the antibody was extinguished by 30 kGy. In stark contrast, PE2 of V3526 irradiated in Mn-DP-Pi retained its antibody binding capacity at 40 kGy (Figure 2B), but Mn-DP-Pi did not increase survival of this ssRNA virus (Figure 2A and Table S1). Single-strand breaks (SSBs) are lethal in ssRNA genomes but not in dsDNA genomes (Slade and Radman, 2011). This renders SSBs in irradiated V3526 the main cause of virus inactivation

A

B

Figure 2. Irradiated VEEV (Strain V3526): Virus Infectivity and Epitope Protection in the Presence or Absence of Mn-DP-Pi

(A) Infectivity following irradiation assessed in vitro by the cytopathic effect (CPE) assay after 5 successive passages in Vero cells (also see Table S1). Live cells are stained by crystal violet.

(B) Protection of antibody binding epitopes assessed by western blotting using the monoclonal antibody 13D4-1, which binds the E3 domain of the PE2 glycoprotein of V3526. (+), V3526 irradiated in Mn-DP-Pi. (−), V3526 irradiated in Pi buffer alone. MC, no virus, medium control. D, dimer of PE2. Other abbreviations as in Figure 1.

There has been considerable interest in developing a vaccine against *S. aureus*, with notable clinical trial failures of vaccines directed against limited epitopes (Schaffer and Lee, 2009; Kobayashi and DeLeo, 2011; McKenna, 2012).

Multiple bacterial epitopes included in a whole-cell vaccine would improve the chance of protection against the manifold virulence strategies of MRSA. We therefore tested if the ability of Mn-DP-Pi to protect purified enzymes (Daly et al., 2010) and viral particles (Figures 1 and 2) could be extended to proteins contained within the relative structural complexity of bacteria. This possibility was supported by the finding that the native morphology of *E. coli* which was lethally-irradiated (25 kGy) in Mn-DP-Pi was maintained and the cells could adsorb λ phage (Figures S1D and S1E). In contrast, *E. coli* cells exposed to 25 kGy in Pi buffer alone were extremely damaged (Figure S1F). As λ phage/host binding is mediated by the LamB receptor, an integral outer membrane protein of *E. coli* (Gehring et al., 1987), Mn-DP-Pi was inferred to have protected *E. coli* surface proteins from oxidative damage at 25 kGy. We further tested this approach by preparing an irradiated bacterial vaccine using MRSA.

We first tested the effect of Mn-DP-Pi on MRSA (strain USA300) survival. The radiation resistance of MRSA in Mn-DP-Pi was only slightly increased over irradiation in Pi buffer, with all viable cells killed by 2 kGy under both conditions (Figure 3A). To assess if Mn-DP-Pi protected surface antigenic structures, we coated plates with the Wood 46 strain of *S. aureus* that had been irradiated in the absence or presence of Mn-DP-Pi, and then incubated the coated plates with immune serum from mice previously infected with MRSA. We used Wood 46 because it lacks protein A, a virulence factor that binds the Fc portion of IgG molecules; therefore, any serum antibody bound to Wood 46 should reflect specific binding of staphylococcal epitopes and not nonspecific binding by protein A that would occur with MRSA USA300. We found that Wood 46 exposed to 25 kGy in Mn-DP-Pi (MnDP-Wood 46) showed enhanced anti-MRSA IgG binding compared to Wood 46 irradiated in Pi buffer, confirming

preservation of antibody targets (Figure 3B). Irradiated preparations of MnDP-Wood 46 bound less IgG than nonirradiated preparations (Figure 3B), supporting that further optimization of protection against ionizing radiation is possible. Mn-DP-Pi similarly preserved antibody binding by irradiated MRSA USA300 (Figure 3B), although for this strain we cannot rule out that some of the detected antibody binding may be due to preserved functionality of protein A.

To test if epitope protection by Mn-DP-Pi translated to enhanced immune responses in vivo, we immunized mice with MRSA USA300 that had been exposed to 25 kGy in the absence (IRS, Irradiated *Staphylococcus*) or presence (MnDP-IRS) of Mn-DP-Pi. The preparations were pelleted and washed after irradiation to remove soluble Mn-DP-Pi, and then resuspended in complete Freund's adjuvant (CFA). Compared to mice immunized with IRS, mice immunized with MnDP-IRS generated higher anti-*S. aureus* serum IgG titers (Figure 3C). The higher levels of antibody in serum from mice immunized with MnDP-IRS (Figure 3C) compared to serum from previously infected mice (Figure 3B) further supported the immunogenicity of MnDP-IRS. However, serum from IRS- and MnDP-IRS-immunized mice showed equivalent ability to promote opsonophagocytosis-mediated killing of MRSA (Figure 3D).

We next used a mouse model of subcutaneous MRSA infection to evaluate the ability of MnDP-IRS immunization to elicit protective immunity. In this model, mice infected subcutaneously with MRSA USA300 develop abscesses that peak in size between 3-5 days after infection and then resolve between days 10-14. Consistent with human disease, a prior infection does not protect mice from subsequent challenge with the same organism (Figure S2A). Previously infected mice did not show evidence of a protective adaptive immune response, developing only low titer serum antibody (Figure S2B). Moreover, compared to mice encountering a primary infection, infected tissue upon a second challenge of previously infected mice did not show increased induction of interleukin-17 (IL-17), a cytokine recently identified as contributing to anti-staphylococcal immunity (Lin et al., 2009; Cho et al., 2010; Narita et al., 2010; Puel et al., 2011) (Figure S2C). However, mice immunized with MnDP-IRS+CFA showed decreased abscess formation upon challenge two weeks after the last immunization (Figure 4A). Compared to mice immunized with IRS (either in phosphate buffered saline (PBS) or CFA), mice immunized with MnDP-IRS+CFA had significantly decreased abscess size (Figure 4B) and skin MRSA bacterial burden (Figure 4C). MnDP-IRS immunization in PBS without adjuvant showed a lesser degree of protection (Figures 4B and 4C). Importantly, Mn-DP-Pi did not confer any antigen-independent protection when administered without MRSA (Figure S3A), and immunization with MnDP-IRS was also protective when used with alum, an adjuvant approved for clinical use (Figure S3B). To address if the vaccine-induced adaptive immune response was dependent on B cell antibody production or effector T cells, we immunized B cell-deficient (μMT) mice, or depleted CD4 T cells in wild-type mice prior to challenge. B cell-deficiency did not reduce vaccine protection. CD4 T cell-depletion diminished protection partially, and loss of both B cells and CD4 T cells abrogated protection (Figures 4D and 4E). Of note, B cell deficiency alone, or administration of isotype control antibody, showed a protective trend that was statistically
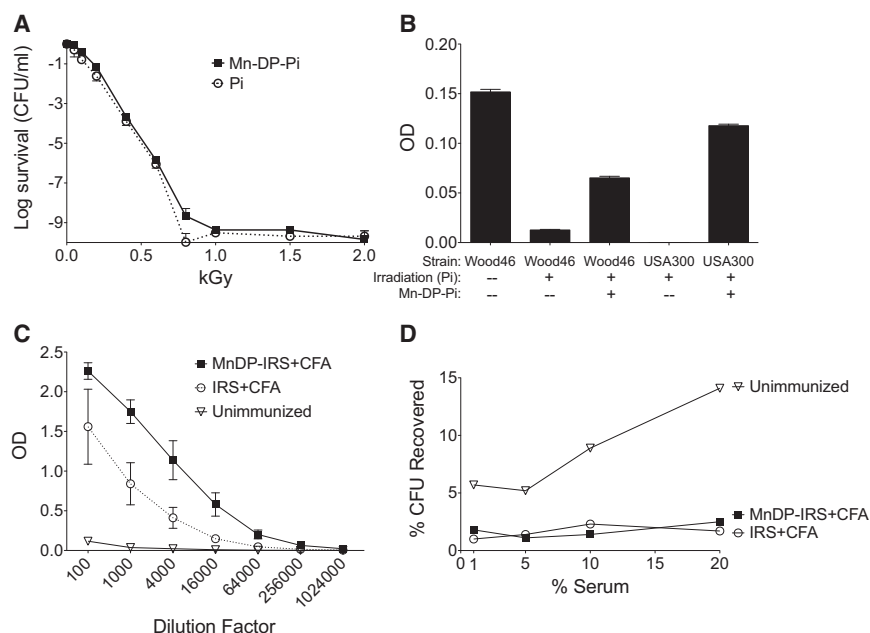
Figure 3. Mn-DP-Pi Protects Staphylococcal Epitopes from Radiation Damage but Does Not Increase Survival

(A) Viability of *Staphylococcus aureus* (MRSA USA300 strain) after exposure to ionizing radiation in the absence or presence of Mn-DP-Pi.

(B) Binding of anti-MRSA IgG in immune serum (1:5000 dilution) to protein A-deficient Wood 46 strain of *S. aureus* or MRSA USA300 that had been irradiated with 25 kGy in the absence or presence of Mn-DP-Pi.

(C) Anti-*S. aureus* IgG titers in serum from mice immunized in complete Freund's adjuvant (CFA) with MRSA USA300 irradiated in the absence (IRS) or presence (MnDP-IRS) of Mn-DP-Pi.

(D) Recovery of MRSA USA300 after opsonophagocytosis-mediated killing by RAW 264.7 macrophages in the presence of titrated serum pooled from mice immunized as indicated.

Data are representative of 2–3 independent experiments and show mean values from triplicate samples (panels A and B) or n = 4 mice (panels C and D). Error bars reflect SEM (standard error of the mean). Also see Figure S2.

nonsignificant. MnDP-IRS immunization induced MRSA-specific IL-17 production from splenic CD4 T cells (Th17 cells) that was enhanced compared to unimmunized or IRS-immunized mice (Figure 4F). There was no difference between groups in production of MRSA-specific interferon-γ, IL-4, IL-5, IL-10, or IL-13 (data not shown). There was a correspondingly greater influx of Th17 cells into skin abscesses in MnDP-IRS-immunized mice, and the increased IL-17 response seen in the abscess tissue depended on CD4 T cells (Figures S3C and S3D). Together, these data suggest that MnDP-IRS immunization elicits antibody- and Th17 cell-mediated immunity that combine to protect against staphylococcal skin infection.

DISCUSSION

Recent work suggests that pathogens killed by ionizing radiation are more immunogenic than those killed by heat or chemicals (Datta et al., 2006). The use of Mn-DP-Pi during aqueous ionizing irradiations to further preserve immunogenicity while eliminating infectivity provides an effective approach for the production of whole-microbe killed vaccines. As the Mn-DP-Pi complex works as an extremely efficient ROS scavenger, it would not significantly protect epitopes in deeply frozen or dried irradiated preparations (Daly, 2012; Ward, 1988; Ito et al., 1993). Importantly, Mn-DP-Pi does not rescue organisms from supralethal (>25 kGy) doses of radiation (Figures 1–3). Thus, whole-microbe vaccines could prospectively be developed against any class of cultivatable infectious organism, including viruses, bacteria, fungi, protozoa, and multicellular parasites. Eliminating the need for identification and production of recombinant protein targets, and utilizing relatively easy access to irradiation facilities, make this approach rapid, safe, cost-effective, and readily scalable. With Mn-DP-Pi, we preserved viral epitopes (Figures 1D and 2B) at γ-ray doses far above those needed to ensure sterilization of the aqueous preparations. Similarly, we

preserved the immunogenicity of MRSA (Figure 3) at γ-ray doses far above those needed to sterilize even the most radiation-resistant bacteria (Daly, 2009; Daly, 2012; Slade and Radman, 2011). This underscores the advantages of using Mn-DP-Pi to uncouple epitope destruction from killing, thereby ensuring the opportunity to generate vaccines which are absolutely noninfective yet highly immunogenic and protective. The rapidity of vaccine development that could be achieved by killing of whole isolated pathogens also makes this an attractive approach against bioterror threats (Hayden, 2011) and emerging infections caused by poorly characterized new or rapidly mutating agents, such as pandemic influenza and HIV (Alsharifi and Müllbacher, 2010).

Our data from the MRSA skin infection model suggests there are immunological advantages with a whole-cell killed bacterial vaccine. The ability to induce both antibody- and CD4 T cell-mediated protective responses is likely enhanced by the presentation of multiple epitopes. Antibody-mediated protection is the main focus of most current vaccine development, but our data suggests T cell secretion of IL-17 may play an important role. Immune responses that protect skin and mucosal barriers have been particularly difficult to achieve with vaccines. Thus, the ability to elicit protection against MRSA at the skin holds particular promise for the ongoing epidemic of cutaneous MRSA skin infection that affects healthy individuals as well as individuals with predisposing conditions such as atopic dermatitis. Natural infection in these individuals does not induce protective immunity against subsequent infections, so the efficacy of the MnDP-IRS vaccine promises to further elucidate the requirements for anti-staphylococcal skin immunity. Prevention of hospital-acquired invasive MRSA infections that occur after iatrogenic breaches of the skin barrier may involve different immune mechanisms that will need to be tested in appropriate models. Optimization of strain, culture conditions, growth stage-dependent epitope presentation, irradiation conditions,
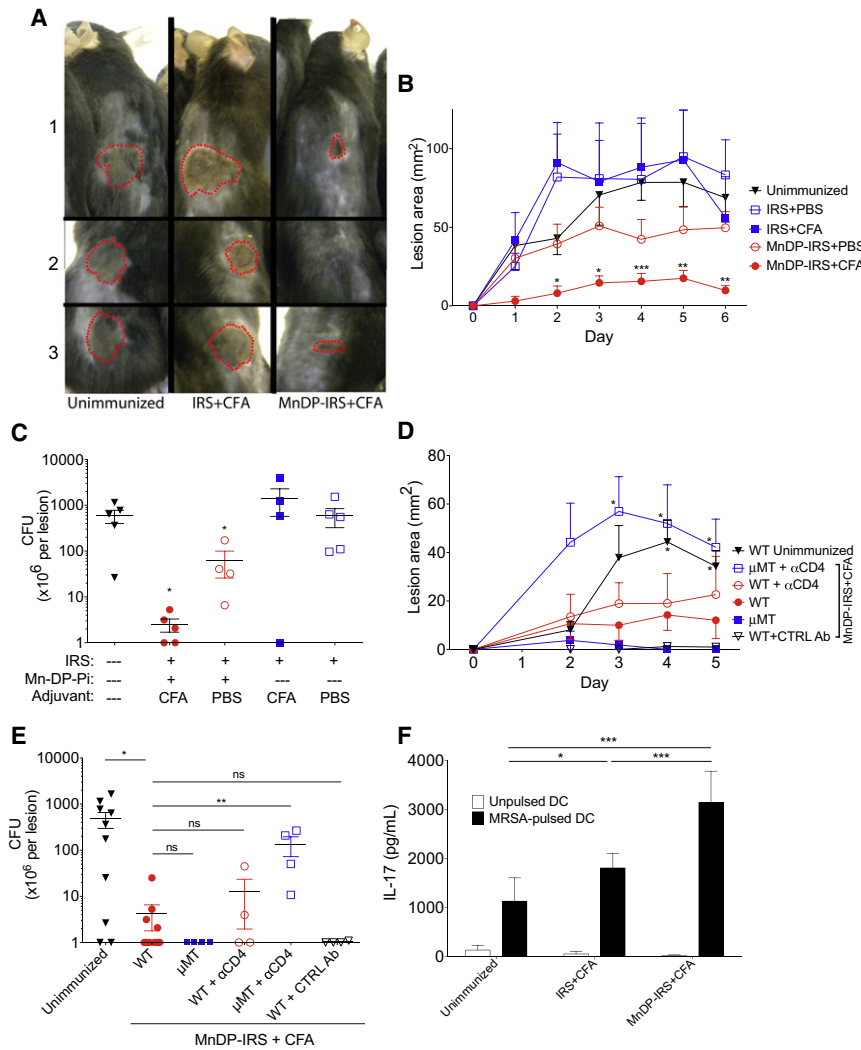
Figure 4. Immunization with a Mn-DP-Pi-Based Irradiated MRSA Vaccine Protects against Staphylococcal Skin Infection

(A) Skin lesions in wild-type (WT) mice. Three representative mice/group are shown with lesions demarcated in red. Lesions were photographed 6 days after challenge, which occurred 14 days after the last immunization.

(B) Daily lesion area in WT mice.

(C) Skin MRSA burden in WT mice. Colony forming units (CFU) were determined 6 days after challenge.

(D–F) WT or B cell-deficient (μMT) mice were immunized and challenged as in panels (A)–(C). Lesion area (panel D). Skin CFU (panel E). Supernatant IL-17 from splenic CD4 T cells restimulated with unpulsed or MRSA-pulsed dendritic cells (DC) (panel F).

In all studies, mice were immunized with IRS or MnDP-IRS in PBS or CFA on day 0 and day 14. On day 28, mice were challenged subcutaneously with 10^7 CFU of live MRSA. Control antibody (CTRL Ab) or depleting antibody against CD4 T cells (αCD4) was administered to the indicated groups 24 hr prior to challenge. Data are representative of 2–3 independent experiments. n = 4–10 mice/group. Error bars reflect SEM. Asterisks indicate p value compared to unimmunized (panels B and C), immunized WT (panel D) or indicated (panels E and F) group (*p < 0.05, **p < 0.01, ***p < 0.001, ns = nonsignificant). Also see Figure S3.

EXPERIMENTAL PROCEDURES

(Also see Supplemental Experimental Procedures.)

Mn-DP-Pi and Assembly of Viral and Bacterial Vaccine Preparations prior to Irradiation

The synthetic decapeptide (DP) H-Asp-Glu-His-Gly-Thr-Ala-Val-Met-Leu-Lys-OH (Daly et al., 2010) was custom-synthesized by American Peptide Co. Inc., Sunnyvale, CA. Stock solutions of 30 mM DP (in H_2O) were authenticated for sequence and concentration by HPLC-MS, and stored at −80°C. Prior to irradiation, purified bacteriophage lambda particles (10^{12} plaque forming units (pfu)/ml) (Supplemental Experimental Procedures) were adjusted to 1 mM $MnCl_2$, 3 mM DP, 25 mM potassium phosphate (Pi) buffer (pH7.4) (Mn-DP-Pi) using stock solutions: 100 mM $MnCl_2$ (Sigma Chemical Company, St. Louis, MO), 30 mM DP (American Peptide Co. Inc.), and Pi buffer (250 mM) (pH 7.4) (KH_2PO_4 and K_2HPO_4, Sigma) - prepared in H_2O from Barnstead Nanopure ultrapure water purification system, Thermo Scientific, Dubuque, IA. The final concentration of bacteriophage in Mn-DP-Pi was 1.25×10^{11} pfu/ml. Similarly, V3526 particles (Supplemental Experimental Procedures) were prepared at 1.12×10^{11} pfu/ml Mn-DP-Pi. MRSA cells (OD_{600} 0.6-0.8) were washed in 25 mM Pi buffer and resuspended at 10^9 colony forming units (CFU)/ml Mn-DP-Pi. Mn-DP-Pi suspensions of viruses and bacteria were prepared immediately prior to irradiation and held on ice prior to and during irradiation.

^{60}Co Irradiations and Survival Curves

All irradiations were performed in air on wet ice with ^{60}Co at 3 kGy/h in a Gammacell irradiation unit model 109-68, J. L. Shepherd and Associates, San Fernando, CA. Mn-DP-Pi preparations containing bacteriophage lambda or V3526 particles were irradiated up to 40 kGy. MRSA cells were irradiated up to 25 kGy. After each irradiation, 10^8 MRSA were plated on blood agar plates after serial dilutions to quantify CFU. No viable MRSA were ever detected at

and adjuvant may further enhance the immunogenicity and efficacy of a Mn-DP-Pi-irradiated vaccine.

The simplicity and economy of killing a whole organism that will elicit immunity against multiple epitopes is appealing compared to the costs and time required to molecularly characterize pathogens and develop recombinant vaccines. Our studies provide a proof-of-concept that preservation of protein epitopes by Mn-DP-Pi enhances the immunogenicity of whole viruses and bacteria killed by supralethal irradiation in aqueous preparations. The doses of γ-rays used here (25-40 kGy) far exceed the outer limits for survival of the vast majority of bacteria, viruses, simple eukaryotes and animals (Daly, 2009), making this inactivation approach applicable to any pathogen. For radiation-sensitive organisms, including MRSA (Figure 3A), sterilization doses could be reduced significantly, which would be expected to further reduce damage to epitopes needed to develop a protective immune response while still maintaining an adequate margin of safety to ensure complete inactivation. Thus, our Mn-DP-Pi irradiated vaccine approach represents a safe and rapidly adaptable vaccine strategy against any emerging or existing infectious disease.

irradiation doses greater than 2 kGy. For bacteriophage lambda survival, 6.25×10^8 particles, irradiated or not, were subjected to tenfold serial dilutions in TNT broth (10 g/L tryptone, 5g/L NaCl, 1 mg/L thiamine-HCl) (pH 7.3) and mixed with 100 μl log-phase ($OD_{600} = 0.3$) recipient *E. coli* (C600) culture (~5 × 10^6 cells). Incubation for adsorption was for 10 min at 37°C, followed by adding 3 ml of molten (46°C) TNT 0.5% agar, which were transferred to TNT 1.5% agar plates. Plaques were counted after overnight incubation at 37°C. V3526 survival was determined in liquid suspension by 50% tissue culture infectivity dose ($TCID_{50}$), as measured by visible cytopathic effect (CPE) following five serial passages in Vero cells (Supplemental Experimental Procedures, and Table S1).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.05.011.

REFERENCES

Alsharifi, M., and Müllbacher, A. (2010). The γ-irradiated influenza vaccine and the prospect of producing safe vaccines in general. Immunol. Cell Biol. *88*, 103–104.

Barnese, K., Gralla, E.B., Cabelli, D.E., and Valentine, J.S. (2008). Manganous phosphate acts as a superoxide dismutase. J. Am. Chem. Soc. *130*, 4604–4606.

Berlett, B.S., Chock, P.B., Yim, M.B., and Stadtman, E.R. (1990). Manganese(II) catalyzes the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-facilitated dismutation of hydrogen peroxide. Proc. Natl. Acad. Sci. USA *87*, 389–393.

Chambers, H.F., and Deleo, F.R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. *7*, 629–641.

Cho, J.S., Pietras, E.M., Garcia, N.C., Ramos, R.I., Farzam, D.M., Monroe, H.R., Magorien, J.E., Blauvelt, A., Kolls, J.K., Cheung, A.L., et al. (2010). IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. J. Clin. Invest. *120*, 1762–1773.

Daly, M.J. (2009). A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nat. Rev. Microbiol. *7*, 237–245.

Daly, M.J. (2012). Death by protein damage in irradiated cells. DNA Repair (Amst.) *11*, 12–21.

Daly, M.J., Gaidamakova, E.K., Matrosova, V.Y., Vasilenko, A., Zhai, M., Venkateswaran, A., Hess, M., Omelchenko, M.V., Kostandarithes, H.M., Makarova, K.S., et al. (2004). Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science *306*, 1025–1028.

Daly, M.J., Gaidamakova, E.K., Matrosova, V.Y., Vasilenko, A., Zhai, M., Leapman, R.D., Lai, B., Ravel, B., Li, S.M., Kemner, K.M., and Fredrickson, J.K. (2007). Protein oxidation implicated as the primary determinant of bacterial radioresistance. PLoS Biol. *5*, e92.

Daly, M.J., Gaidamakova, E.K., Matrosova, V.Y., Kiang, J.G., Fukumoto, R., Lee, D.Y., Wehr, N.B., Viteri, G.A., Berlett, B.S., and Levine, R.L. (2010).

Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*. PLoS ONE *5*, e12570.

Datta, S.K., Okamoto, S., Hayashi, T., Shin, S.S., Mihajlov, I., Fermin, A., Guiney, D.G., Fierer, J., and Raz, E. (2006). Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity *25*, 143–152.

Eady, J.J., Peacock, J.H., and McMillan, T.J. (1992). Host cell reactivation of gamma-irradiated adenovirus 5 in human cell lines of varying radiosensitivity. Br. J. Cancer *66*, 113–118.

Gehring, K., Charbit, A., Brissaud, E., and Hofnung, M. (1987). Bacteriophage lambda receptor site on the *Escherichia coli* K-12 LamB protein. J. Bacteriol. *169*, 2103–2106.

Harris, D.R., Pollock, S.V., Wood, E.A., Goiffon, R.J., Klingele, A.J., Cabot, E.L., Schackwitz, W., Martin, J., Eggington, J., Durfee, T.J., et al. (2009). Directed evolution of ionizing radiation resistance in *Escherichia coli*. J. Bacteriol. *191*, 5240–5252.

Hayden, E.C. (2011). Pentagon rethinks bioterror effort. Nature *477*, 380–381.

Hrusková, J. (1969). Effects of gamma radiation on Western equine encephalomyelitis and tick-borne encephalitis viruses. Acta Virol. *13*, 187–192.

Ito, T., Baker, S.C., Stickley, C.D., Peak, J.G., and Peak, M.J. (1993). Dependence of the yield of strand breaks induced by gamma-rays in DNA on the physical conditions of exposure: water content and temperature. Int. J. Radiat. Biol. *63*, 289–296.

Kobayashi, S.D., and DeLeo, F.R. (2011). A MRSA-terious enemy among us: boosting MRSA vaccines. Nat. Med. *17*, 168–169.

Krisch, R.E., Flick, M.B., and Trumbore, C.N. (1991). Radiation chemical mechanisms of single- and double-strand break formation in irradiated SV40 DNA. Radiat. Res. *126*, 251–259.

Kriško, A., and Radman, M. (2010). Protein damage and death by radiation in *Escherichia coli* and *Deinococcus radiodurans*. Proc. Natl. Acad. Sci. USA *107*, 14373–14377.

Kriško, A., Leroy, M., Radman, M., and Meselson, M. (2012). Extreme antioxidant protection against ionizing radiation in bdelloid rotifers. Proc. Natl. Acad. Sci. USA *109*, 2354–2357.

Lin, L., Ibrahim, A.S., Xu, X., Farber, J.M., Avanesian, V., Baquir, B., Fu, Y., French, S.W., Edwards, J.E., Jr., and Spellberg, B. (2009). Th1-Th17 cells mediate protective adaptive immunity against *Staphylococcus aureus* and *Candida albicans* infection in mice. PLoS Pathog. *5*, e1000703.

Luthjens, L.H., and Blok, J. (1969). The indirect action of gamma-rays on the coat protein of bacteriophage particles. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. *16*, 101–111.

Martin, S.S., Bakken, R.R., Lind, C.M., Garcia, P., Jenkins, E., Glass, P.J., Parker, M.D., Hart, M.K., and Fine, D.L. (2010). Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB. Vaccine *28*, 1031–1040.

McKenna, M. (2012). Vaccine development: man vs MRSA. Nature *482*, 23–25.

Miller, L.S., and Cho, J.S. (2011). Immunity against *Staphylococcus aureus* cutaneous infections. Nat. Rev. Immunol. *11*, 505–518.

Moore, H.N., and Kersten, H. (1936). Preliminary Note on the Preparation of Non-toxic Shiga Dysentery Vaccines by Irradiation with Soft X-rays. J. Bacteriol. *31*, 581–584.

Narita, K., Hu, D.L., Mori, F., Wakabayashi, K., Iwakura, Y., and Nakane, A. (2010). Role of interleukin-17A in cell-mediated protection against *Staphylococcus aureus* infection in mice immunized with the fibrinogen-binding domain of clumping factor A. Infect. Immun. *78*, 4234–4242.

Paessler, S., and Weaver, S.C. (2009). Vaccines for Venezuelan equine encephalitis. Vaccine *27* (*Suppl 4*), D80–D85.

Plotkin, S.L., and Plotkin, S.A. (2008). A short history of vaccination. In Vaccines, Fifth Edition, S.A. Plotkin, W.A. Orenstein, and P.A. Offit, eds. (Philadelphia: Saunders), pp. 1–16.

Puel, A., Cypowyj, S., Bustamante, J., Wright, J.F., Liu, L., Lim, H.K., Migaud, M., Israel, L., Chrabieh, M., Audry, M., et al. (2011). Chronic mucocutaneous

candidiasis in humans with inborn errors of interleukin-17 immunity. Science *332*, 65–68.

Reitman, M., Tribble, H.R., Jr., and Green, L. (1970). Gamma-irradiated Venezuelan equine encephalitis vaccines. Appl. Microbiol. *19*, 763–767.

Schaffer, A.C., and Lee, J.C. (2009). Staphylococcal vaccines and immunotherapies. Infect. Dis. Clin. North Am. *23*, 153–171.

Slade, D., and Radman, M. (2011). Oxidative stress resistance in *Deinococcus radiodurans*. Microbiol. Mol. Biol. Rev. *75*, 133–191.

Sullivan, R., Fassolitis, A.C., Larkin, E.P., Read, R.B., Jr., and Peeler, J.T. (1971). Inactivation of thirty viruses by gamma radiation. Appl. Microbiol. *22*, 61–65.

Ward, J.F. (1988). DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. Prog. Nucleic Acid Res. Mol. Biol. *35*, 95–125.

Watson, J.D. (1952). The properties of x-ray inactivated bacteriophage. J. Bacteriol. *63*, 473–485.