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Research article

Recombinant YopE and LcrV vaccine candidates protect mice against plague and yersiniosis

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ABSTRACT

No licensed vaccine exists for the lethal plague and yersiniosis. Therefore, a combination of recombinant YopE and LcrV antigens of *Yersinia pestis* was evaluated for its vaccine potential in a mouse model. YopE and LcrV in formulation with alum imparted a robust humoral immune response, with isotyping profiles leaning towards the IgG1 and IgG2b subclasses. It was also observed that a significantly enhanced expression of IFN-γ, TNF-α, IL-6, IL-2, and IL-1β from the splenic cells of vaccinated mice, as well as YopE and LcrV-explicit IFN-γ eliciting T-cells. The cocktail of YopE + LcrV formulation conferred complete protection against 100 LD_{50} *Y. pestis* infection, while individually, LcrV and YopE provided 80 % and 60 % protection, respectively. Similarly, the YopE + LcrV vaccinated animal group had significantly lower colony forming unit (CFU) counts in the spleen and blood compared to the groups administered with YopE or LcrV alone when challenged with *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. Histopathologic evidence reinforces these results, indicating the YopE + LcrV formulation provided superior protection against acute lung injury as early as day 3 post-challenge. In conclusion, the alum-adjuvanted YopE + LcrV is a promising vaccine formulation, eliciting a robust antibody response including a milieu of pro-inflammatory cytokines and T-cell effector functions that contribute to the protective immunity against *Yersinia* infections. YopE and LcrV, conserved across all three human-pathogenic Yersinia species, provide cross-protection. Therefore, our current vaccine (YopE + LcrV) targets all three pathogens: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. However, the efficacy should be tested in other higher mammalian models.

1. Introduction

Yersinia pestis (Y. pestis), is designated as a tier-1 select agent to recognize its potential for use as a biological warfare agent by the Centers for Disease Control and Prevention (CDC), posing a grave threat to public health globally [1,2]. The devastating impact of the

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plague is reflected in the reported over 200 million fatalities across the globe during previous pandemics [3]. At present, human plague infections continue to occur in rural areas in the western United States, but significantly more cases occur in parts of Africa and Asia (www.cdc.gov/plague/) [4]. In 2017, the island of Madagascar experienced its largest plague outbreak in two decades, causing 2,417 cases and 209 fatalities [5].

In the past two decades, global plague rates have escalated steadily, and the emergence of drug-resistant plague strains has prompted World Health Organization (WHO) to classify it as a re-emerging infectious disease [6–8]. A licensed vaccine is not available for this pathogen, and extensive animal studies suggest that a vaccine should induce humoral and cell-mediated immunity to provide complete and long-lasting protection [9–11]. A live attenuated *Y. pestis* EV76 strain as a vaccine had been used by various countries [12]. Despite being effective against both bubonic and pneumonic plague, this vaccine was highly reactogenic for people suffering from hemochromatosis [13,14]. To develop subunit vaccines, a capsular antigen known as F1 and a LcrV (low calcium response V) antigen, secreted by type III secretion system (T3SS) were observed protective in rodents and cynomolgus macaques against the lethal challenge of aerosolized *Y. pestis* but confer inconsistent protection in African Green monkeys [15,16]. Despite robust antibody responses, the vaccine's consistent efficacy has been hindered by the inability to generate an effective Th1 immune response, which relies on TNF-α and IFN-γ cytokines. Trials of an alhydrogel-adjuvanted F1/LcrV vaccine in humans showed that while it induces humoral immunity, it fails to produce optimal cell-mediated immune response. These findings imply that inducing *Y. pestis*-specific memory T cells eliciting TNF-α and IFN-γ rather than only antigen-specific humoral immune responses could be a promising means of improving the vaccine's efficacy [17–20]. The rF1-V vaccine by DynPort received orphan drug classification from the FDA (https://globalbiodefense.com/2017/03/10/fda-grants-orphan-drug-designation-plague-vaccine/). Few reports have documented that there exist F1 deficient strains that are also virulent and lethal [21,22]. Therefore, it is needed to study such vaccine antigens (LcrV, a multi-factorial virulence factor) and Yersinia outer protein E (YopE, an anti-phagocytic factor) which may provide effective protection to both F1 antigen positive and F1 antigen deficient strains of *Y. pestis*. However, in this study, the vaccine antigens were evaluated against S1 strain (an Indian clinical isolate, F1 positive) of *Y. pestis*. The S1 strain was isolated from a human patient during a small outbreak of primary pneumonic plague which took place in Shimla District of Himachal Pradesh State in northern India during February 2002 [23].

Apart from *Y. pestis*, there are two other species in the genus *Yersinia* i.e., *Y. enterocolitica*, and *Y. pseudotuberculosis* (zoonotic foodborne pathogens), which are pathogenic to humans, causing the disease collectively known as yersiniosis [24]. While the infection usually presents as a self-limiting gastrointestinal illness that manifests as mesenteric lymphadenitis, *acute* enteritis (particularly in children), enterocolitis, and terminal ileitis, severe complications such as septicemia may develop, which could be fatal to immunocompromised individuals or those with an underlying condition [25]. *Y. enterocolitica* is the foremost cause of yersiniosis in the USA, causing almost 117,000 cases and 35 deaths each year [26]. In fact, it was the fourth most reported zoonotic infection in the European Union from 2015 to 2019 and is prevalent in numerous countries, including Japan, the United Kingdom, and Scandinavia [27]. *Despite this, little has been done to develop effective measures to combat yersiniosis.*

In this study, the immunogenicity of recombinant YopE, a T3SS protein conserved among all the pathogenic *Yersinia* species that is associated with the stimulation of YopE distinct $CD8^+$ T-cells was investigated [18], and recombinant LcrV, a multifunctional protein that modulates the host immune response and enables the pathogen's survival. Our objective was to assess the vaccine-induced humoral immunity by alum-adjuvanted YopE, LcrV, and a cocktail of YopE + LcrV vaccine candidates, their associated immune response, and the protective efficacy of these vaccine candidates against *Y. pestis* and yersiniosis. More significantly, YopE and LcrV are conserved across all three human-pathogenic *Yersinia* species as imparted cross protection, however, F1 is specific to *Y. pestis* alone: hence, our current vaccine formulation (YopE + LcrV) targets against all three human pathogens i.e., *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*.

2. Materials and methods

2.1. Bacterial strains and plasmids

All the pathogenic bacterial strains such as *Y. pestis* (S1 strain), *Y. enterocolitica* (O:8 serotype), and *Y. pseudotuberculosis* (A87 strain) were procured from the repository of DRDE, Gwalior, India**.** Both *Y. pestis* and *Y. pseudotuberculosis* were cultivated separately in Brain Heart Infusion (BHI) broth and incubated for 48 h at 28 °C. *Y. enterocolitica* was cultivated in BHI broth and incubated for 24 h at 37 °C. All the experiments handling live *Y. pestis* were conducted in a biosafety level-3 facility at DRDE, Gwalior.

2.2. Cloning, expression, and purification of recombinant proteins

The yopE and lcrV genes were cloned in the pET28a vector as previously described [28] and transformed in BL21 (DE3) cells. The positive colonies were selected on Luria-Bertani (LB) agar plates using kanamycin (50 μg/ml). The single colony of each construct was inoculated individually into 5 ml of LB broth and grown at 37 °C for overnight. Next day, 500 ml of LB broth was inoculated with 1 % of overnight grown culture and incubated at 37 °C. When the optical density (OD600) reached up to ~0.6, the cultures were induced with 1 mM isopropylthiogalactoside (IPTG) and grown for 4 h at 37 °C. The cultures were pelleted by centrifugation at 10,000×*g* for 10 min at 4 °C. The cells were lysed by dissolving the pellets in 1X SDS-PAGE buffer (31.5 mM Tris-HCl, pH 6.8 buffer 10 % glycerol 1 % SDS, 0.005 % bromophenol blue), boiled at 100 °C for 10 min in a dry bath and analyzed by SDS–PAGE for the expression.

To determine the solubility of the recombinant protein, the cell pellets corresponding to LcrV and YopE were re-suspended in cell lysis buffer of pH 8.0 (50 mM NaH2PO4, 250 mM NaCl; 10 mm imidazole; pH 8.0) individually. Then cells were lysed by sonication at

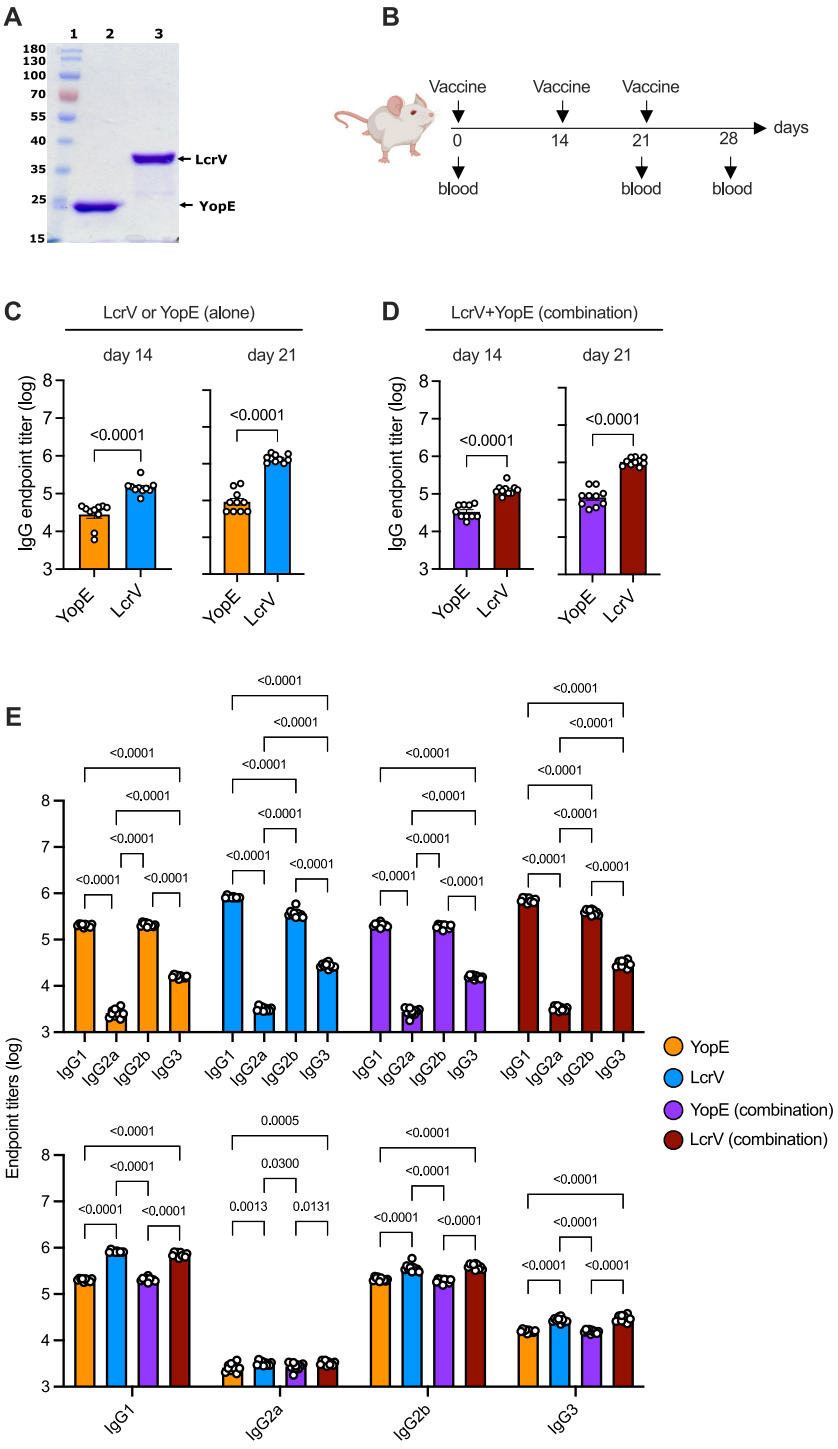

Fig. 1. Antibody responses after YopE, LcrV, and YopE + LcrV immunization (A–J). SDS-PAGE profile of purified recombinant YopE and LcrV proteins (A), Uncropped version of gel image for figure [1A] is available in supplementary material. Schematic description of vaccine administration and bleeding schedule for BALB/c mice (B). Serum samples of days 14 and 21 administered boosters were collected after 7 days following each booster, and anti-LcrV and anti-YopE IgG were assessed for mice immunized with individually formulated vaccine candidates (C) along with mice administered with the combination group (D). Mann-Whitney *U* test was conducted for the comparison of two groups. Serum IgG subclasses following second booster were analyzed including IgG1; IgG2a; IgG2b; and IgG3. The group comparisons for all four IgG isotypes (upper panel) and group comparisons for each isotype induced by different combinations (lower panel) (E). Two-way ANOVA test was conducted to interpret the data, and for multiple comparisons, Tukey's test was conducted. $n = 10$/group. Mean ± SEM is shown. $P < 0.05$ was considered statistically significant.

20 % output power for 10 min with 30 s pulse using a Sonicator (Ultrasonic processor XL, Heat System Inc.) on ice, centrifuged at 10,000×*g* for 30 min at 4 °C. The clear supernatant and remaining pellets were collected and analyzed by SDS-PAGE. LcrV was present in supernatant therefore it was purified under native conditions. To purify LcrV protein, Ni-NTA column was equilibrated with lysis buffer (15 ml). The supernatant was passed through the Ni-NTA column, then washed with 50 ml of wash buffer (50 mM NaH_2PO_4, pH-8.0; 250 mM NaCl; 30 mM imidazole). The LcrV protein was eluted using 15 ml of elution buffer (50 mM NaH_2PO_4, 250 mM NaCl; 200 mM imidazole; pH 8.0). YopE was present in pellet fraction as it made inclusion bodies (IB). The IB pellet was re-suspended in 20 ml of solubilization buffer of pH 8.0 (10 mM Tris HCl, 100 mM NaH_2PO_4, 100 mM NaCl, 8 M urea), stirred on a magnetic stirrer for overnight at 4 °C, centrifuged at 25,000×*g* for 30 min at 4 °C. To purify YopE protein, Ni-NTA column was equilibrated with solubilization buffer. The supernatant was passed through the Ni-NTA column, the column was washed with solubilization buffer at pH 6.0. The protein bound to the column was eluted by passing 15 ml solubilization buffer at pH 4.0. Protein concentrations were measured by BCA method. Limulus Amoebocyte Lysates (LAL) assay was performed to determine the endotoxin levels using Limulus Amoebocyte Lysates (LAL) QCL-1000 kit (Cambrex Biosciences, USA) in accordance with the kit protocol.

2.3. Mice immunization

Naive female BALB/c mice aged 7–8 week old, were procured from the DRDE's animal facility. The animals were divided into five batches and each batch contained 4 groups i.e., (I) the control group (PBS immunized); (II) alum-adjuvanted YopE group; (III) alum-adjuvanted LcrV group; and (IV) a cocktail of alum-adjuvanted YopE + LcrV group. Batch-I was used to study humoral immunity (level of IgG and its subclasses) and protection efficacy against *Y. pestis* ($n = 10$ animals/group); Batch-II was used to investigate cell-mediated immunity, including cytokine profiling and FACS analysis of IFN-γ secreting T-cells ($n = 6$ animals/group). Histopathological analysis was performed by using Batch-III ($n = 10$ animals/group) and Batch-IV and Batch V were used to assess protective efficacy against *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively ($n = 10$ animals/group for each batch). A prime-boost strategy was adopted for the immunization regimen, wherein the alum-adjuvanted vaccine groups were administered with 15 μg/antigen/mouse assigned to their respective vaccine candidates on day 0 subcutaneously, accompanied by two boosters on the 14th and 21st days, respectively, as illustrated in Fig. 1B. The control group was sham immunized with PBS. On days 0, 21, and 28, blood samples were drawn through the orbital vein by a sterile hematocrit tube, and the serum was isolated and refrigerated until further investigation at −20 °C.

2.4. Measurement of antigen-specific serum IgG and its subclasses

The total serum IgG levels elicited by recombinant antigens on days 21 (booster I) and 28 (booster II) were assessed by an indirect enzyme-linked immunosorbent assay (ELISA). In brief, the vaccine-elicited anti-YopE and anti-LcrV antibodies in the sample of each mouse serum were measured by constituting 1 μg/ml of recombinant protein individually in 0.05 M CO_3^{2-}/HCO_3^-, pH 9.6 buffer separately. First, 100 μl of the solution was dispensed into each well of 96-well flat-bottom microtiter ELISA plates and incubated at 4 °C overnight. Next, ELISA plates were rinsed once using PBS-T (0.05 % Tween 20 in PBS, 200 μl) solution, blocked with 250 μl/well of 5 % skim milk-PBS solution, and incubated at 37 °C for 1 h. Following incubation, plates were rinsed with PBS-T (5×), and test sera (two-fold serial dilutions) were added in triplicate wells (100 μl/well), proceeded by 1 h of incubation at 37 °C. After, 5× PBS-T rinses, wells were probed at 37 °C with a dilution of 1:15000 rabbit anti-mouse IgG-HRP (Sigma, USA) for 1 h.

For IgG isotype determination, goat anti-mouse HRP-conjugated isotypes such as IgG1, IgG2a, IgG2b, and IgG3 were added to the wells at a dilution of 1:2000.

Plates were again rinsed using PBS-T (5×) before initiating the colorimetric reaction with the ortho-phenylenediamine dihydrochloride (OPD) substrate. The reaction was halted after 10 min at 37 °C with 2 N H_2SO_4 (50 μl/well). A multimode reader was used to detect absorbance at 492 nm. The IgG titers were computed as $\log_{10}$ titers of the OD_{490} value of the highest serial dilution with an average value higher than the cut-off value (2* mean value) of pre-immune sera.

2.5. Splenocyte culturing, stimulation, and cytokine measurement

Single cell suspension of splenocytes was prepared by maceration of spleens in complete RPMI medium (RPMI 1640 medium with 10 % FBS), with a 3.0 mL syringe plunger followed by passing through a 70 μM cell strainer. Splenocyte viability was determined using a hemocytometer and 0.4 % trypan blue. In a 24-well cell culture plate, splenocytes were seeded at 1×10^6 cells/well (in triplicate) and incubated in RPMI media alone (control; negative control) or stimulated with the corresponding vaccine antigen (5 μg/ml), i.e., YopE, LcrV, and the cocktail of YopE + LcrV. For positive control, Concanavalin-A (5 μg/well) stimulated splenocytes were used. After 48 h of incubation with 5 % CO_2 at 37 °C, culture supernatant was collected, and cytokines were determined following the manufacturer's instructions of the BD OptEIA kit. In brief, high-affinity microtiter plates were coated with 100 μl/well of capture antibody, followed by overnight incubation at 4 °C. The next day, plates were rinsed thrice with PBS-T (0.05 % Tween-20 in PBS) then blocked with 200 μl/well assay diluent to limit non-specific binding and incubated for 1 h at room temperature (RT). Next, the plates were rinsed again as noted above, and two-fold dilution of cytokine standards (in assay diluent) was added in duplicate (100 μl/well), while non-diluted culture supernatants were added in triplicate, and incubated at RT for 2 h. Following incubation, the plates were rinsed 5× and incubated with 100 μl/well of streptavidin-HRP conjugated antibody diluted in the assay diluent for 1 h at RT. After seven washes, the colorimetric reaction was developed by adding TMB substrate to the wells, incubating for 30 min at RT in the dark, and the intensity of the color was measured at a final wavelength of 450 nm with a multi-mode plate reader after halting the reaction with 2 N H_2SO_4 (50

μl/well).

2.6. Cell proliferation assay

To assess the function of exogenously administered stimuli, *i.e.,* recombinant antigens, on splenocytes cell proliferation assay was conducted. In brief, Splenocytes (2 × 106 cells/well) from each subject group were incubated in triplicate with complete RPMI media and 5 μg Ag/well designated to their group for 72 h at 37 °C with 5 % CO_2 in a 96-well cell culture plate. Concanavalin-A (5 μg/well) stimulated splenocytes were treated as positive controls, while unstimulated cells served as negative controls. Alamar blue was added (100 μl/well) and cells were further incubated at 37 °C with 5 % CO2 for 16 h. The mean absorbance was calculated by subtracting the 560 nm absorbance value from the absorbance value at 600 nm.

2.7. Flow cytometric analysis

To analyze the kinetics of IFN-γ eliciting T-cells namely $CD4^+$ and $CD8^+$ cells, flow cytometric analysis was utilized. In brief, splenic cells from three mice per immunized group were randomly selected to prepare a single-cell suspension and stimulated with YopE, LcrV, and YopE + LcrV antigens (5 μg/ml each) while negative control cells were unstimulated. A positive control was induced with leukocyte activation cocktail (LAC) at a concentration of 5 μg/ml/well. To co-stimulate T cells, cells were treated with CD28 anti-mouse antibody and brefeldin A (1.0 μg/well, Golgi stop, BD Biosciences) to inhibit cytokine secretion. After 6 h incubation, to lyse the red blood cells (RBCs), FACS lysing solution (BD Biosciences) was added to the cells, splenocytes were washed with FACS staining buffer (BD Biosciences). Cells were stained with FITC-conjugated anti-mouse CD4 and CD8a monoclonal antibodies, (BD Biosciences) at 1:200 dilutions in FACS staining buffer, incubated for 30 min at room temperature and washed with cold 1XPBS. Cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4 °C. For intra cellular staining, PE-conjugated anti-mouse IFN-γ antibody (BD Biosciences) was added to the cells (1:200 dilution) for 30 min at room temperature in the dark, washed with cold PBS. Cells were acquired using Becton Dickinson FACS Calibur Flow Cytometer. A total of 10,000 live events, according to forward and side-scatter parameters were accumulated for each sample and analyzed using CellQuest Pro software.

2.8. Yersinia infections and determination of protective efficacy

All the inoculated animal groups, i.e., the control, the YopE, the LcrV, and the cocktail of YopE + LcrV were exposed to *Y. pestis* with a dosage of 1×10^5 colony forming unit (CFU)/mouse (LD_{50}) on day 60 post-prime immunization via subcutaneous (*s.c.)* route. Animals were monitored post-challenge for 1 month to assess the protective efficacy of vaccine candidates (Fig. 3A). After 30 days, all the remaining mice were euthanized.

Similarly, in the case of yersiniosis, one batch of vaccine groups of animals was challenged via *i.p.* route with *Y. pseudotuberculosis* (10^9 CFU/mouse, A:87 strain) and another batch of vaccine groups with *Y. enterocolitica* (10^8 CFU/mouse, O:8 serotype). These animals were euthanized from 1 to 5 d.p.i. to determine the microbial burden in blood and spleen tissues (Fig. 4A).

2.8.1. Determination of bacterial load

The bacterial enumeration in blood and spleen was conducted by collecting blood via a retro-orbital route and cultured on BHI agar plates in duplicates prior to being euthanized on days 1–5 following the challenge. Spleens were collected, weighed under aseptic conditions, homogenized, serially diluted in sterile PBS, and cultured on BHI agar plates. Y. pseudotuberculosis was enumerated by counting colony-forming units (CFU) on agar plates after 48 h of incubation at 28 °C, whereas *Y. enterocolitica* was counted after 24 h of incubation at 37 °C. Data were reported as the mean log CFU ± SEM for each group. The unit of protection was expressed as the delta log differences between the control and inoculated groups.

2.9. Histopathological study

To conduct the histopathological studies, mice of all the inoculated groups, *i.e.,* YopE, LcrV, YopE + LcrV, and the control group, were challenged subcutaneously with *Y. pestis* (100 LD_{50}). On day 3 post infection (p.i.), lungs from three mice/groups were excised, pruned, and perfused for 24 h at RT with a 10 % neutral phosphate formalin buffered solution. Lung tissue specimens were dehydrated with a gradient of ethanol solutions (70–100 %), embedded in paraffin wax with multiple 4–5 μm thick sections, trimmed, mounted on a glass slide, and air-dried for 24 h. The sections were processed as specified [29], including deparaffinization, hematoxylin and eosin staining in an auto-stainer, and cover-slipping using an automated cover-slipper. The slides were digitized by a Brightfield Inverted Microscope at 20× magnification. Lung histopathology was blindly scored, and the severity of the pathological changes was computed by a grading system called the Acute Lung Injury (ALI) scoring scheme for small animal models using the recommendations of the American Thoracic Society [30].

2.10. Statistics and reproducibility

Data for IgG titers and subclasses were log-transformed and analyzed using Two-way ANOVA test to interpret the data followed by multiple comparisons, Tukey's test was conducted. For the comparison of two groups, an unpaired nonparametric *t*-test followed by the Mann–Whitney *U* test was used. Data for cytokine response, bacterial burden (log-transformed data), lung pathology, One-way

ANOVA test followed by multiple comparisons, Tukey's test was conducted. GraphPad Prism 8.0 was used to generate the survival curve using Kaplan-Meier method and the Log-rank (Mantel-Cox) test. The experiments in this study were conducted at least twice independently. Shapiro-Wilk test was performed to evaluate normality and outliers. Figures depicted the significance level of p values. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and purification of recombinant YopE and LcrV proteins

Heterologous expression of YopE and LcrV was achieved with 1 mM IPTG induction of bacterial cell cultures. Following 4 h of induction, the cells were isolated, and their protein expression levels were determined by SDS-PAGE analysis (Figs. S1A and B). Cells corresponding to YopE expressed signatory protein in the sonicated pellet, while LcrV expression analysis revealed protein in the soluble extract. These proteins, i.e., YopE and LcrV, were subsequently purified to near homogeneity using denatured and native conditions, respectively (Figs. S1C and D), and the eluted fractions were pooled and dialyzed (Fig. 1A). The endotoxin levels for each purified protein were <5 endotoxin units/15 μg/protein.

3.2. Alum-adjuvanted YopE and LcrV elicited robust humoral responses

The efficacy of YopE and LcrV, both separately and in combination, as immunogens was verified by detecting antigen-specific IgG antibodies in individual serum samples of BALB/c mice following first (day 14) and second (day 21) booster shots administered after post-prime vaccinations. A comparative analysis of the first and second boosters of both recombinant antigens alone or in combination revealed that the serum level of IgG in mice vaccinated individually with YopE was significantly higher (~4.2-fold, $p < 0.001$), while for individually vaccinated LcrV mice sera, a ~6.5-fold increase was observed with a $p < 0.0001$ significance level (Fig. 1C). Further, a ~5.8-fold rise ($p < 0.001$) and an ~7.3-fold rise ($p < 0.0001$) was noted in anti-YopE and anti-LcrV IgG titers in mice sera co-

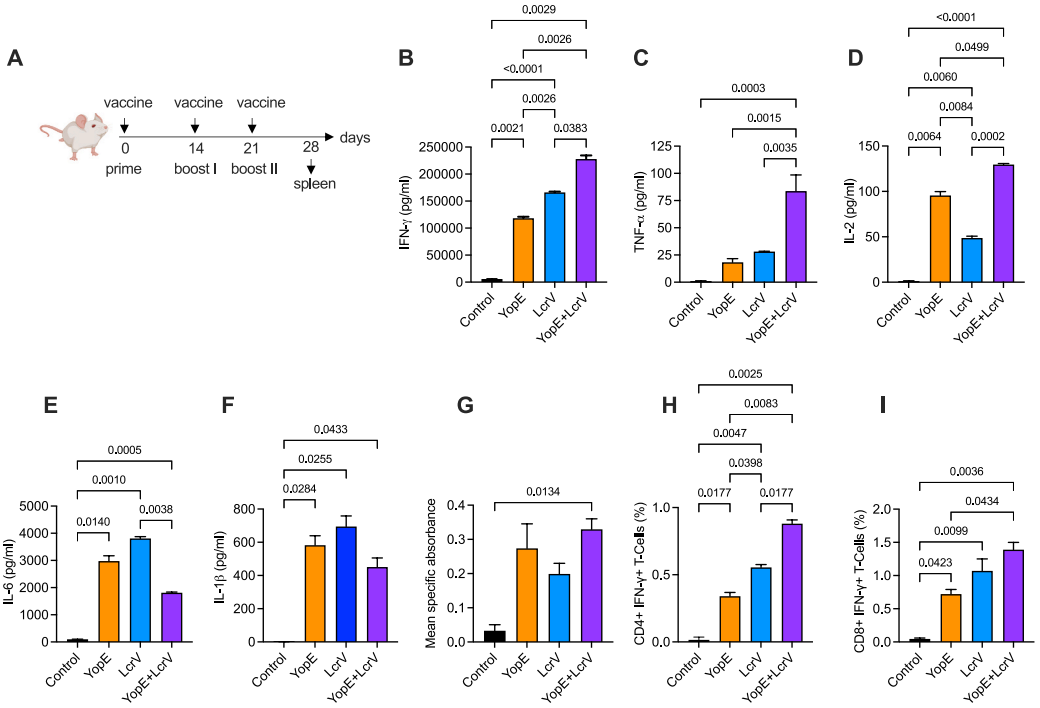

Fig. 2. Cell-mediated immune responses (A–K). Schematic description of the schedule for vaccine administration, bleeding, and spleen harvesting from BALB/c mice (A). Splenocytes were collected one week after the final immunization and stimulated with the corresponding vaccine antigens. After 48 h, supernatants were removed, and cytokine quantities were quantified using ELISA. Concentrations of IFN-γ (B); TNF-α (C); IL-2 (D); IL-1β (E); and IL-6 (F) cytokines were reported as pg/ml. Simultaneously, the proliferative response of the splenocyte was assessed by re-incubating it with Alamar blue dye for 16 h, and optical density was observed at 570 nm and a reference wavelength of 600 nm (G). Bar graph representing the % incidence of $CD4^+$ IFN-γ^+ T-cells in control, YopE, LcrV, and the combination of YopE + LcrV group (H). Bar graph representing the % frequency of $CD8^+$ IFN-γ^+ T cells in control, YopE, LcrV, and the combination of YopE + LcrV group (I). Brown-Forsythe and Welch ANOVA tests were conducted on log-transformed data, followed by Dunnett T3 tests for multiple comparisons. $n = 6$/group. Mean ± SEM is shown. $P < 0.05$ was considered statistically significant.

immunized with YopE + LcrV (Fig. 1D). The results indicated that alum-adjuvanted YopE and LcrV elicited robust humoral responses with a notable disparity between YopE and LcrV antibody titers administered alone or in the form of a cocktail.

3.3. IgG subclass analysis revealed a highly skewed immune response towards the IgG1/IgG2b isotype

Specific IgG1, IgG2b and IgG3 isotypes were detectable on day 21 and substantially increased by day 28 following vaccination for each group. In the co-immunized group following the second booster, serum IgG1 levels specific to recombinant YopE and LcrV differed ~3.4-fold, ($p < 0.0001$), and by ~4.0-fold, ($p < 0.0001$) from groups vaccinated with each vaccine antigen separately. In addition, it was observed that serum IgG2b titers increased substantially (~1.7-fold, $p < 0.0001$) between groups vaccinated separately with YopE and LcrV, and a ~2.0-fold, ($p < 0.0001$), for the co-immunized group following the second booster. Similar IgG3 titers differences were observed between YopE and LcrV antigens for the co-immunized group (~1.9-fold, $p < 0.0001$) whereas a ~1.7-fold, ($p < 0.0001$), differences were noted between YopE and LcrV in the groups vaccinated alone. On the contrary, relatively low levels of serum IgG2a titers were observed only after the second booster for each vaccine group (Fig. 2E).

3.4. Recombinant antigens elicited pro-inflammatory cytokines that accounted for T and B-cell activation

The results showed that comparative to the control groups, vaccinated mice had significantly heightened expression of IFN-γ, TNF-α, IL-1β, IL-2, and IL-6 cytokines. In contrast, IL-4 and IL-10 cytokines were not detected in the culture supernatants. The levels of IFN-γ in YopE + LcrV immunized mice were ~1.92 and ~1.37-fold higher than in YopE and LcrV vaccinated mice, respectively ($p < 0.0001$) (Fig. 2B). However, YopE + LcrV immunized mice expressed TNF-α at levels ~4.5-fold and ~2.9-fold higher than YopE and LcrV vaccinated mice ($p < 0.01$), respectively (Fig. 2C). In addition, IL-2 expression levels in YopE + LcrV immunized mice were found to be ~1.3-fold and ~2.6-fold higher than in YopE and LcrV vaccinated mice ($p < 0.0001$) (Fig. 2D). Interestingly, IL-1β and IL-6 pro-inflammatory cytokines were also evaluated and noted that the co-immunized group had a ~1.29-fold, $p =$ ns, and ~1.54-fold, $p < 0.05$ lower elicitation of IL-1β compared to the YopE and LcrV groups (Fig. 2E). Also, IL-6 expression was noted to be ~1.64-fold, $p =$ ns, and ~2.09-fold, $p < 0.01$ lower in magnitude in the co-immunized group compared to the YopE and LcrV groups respectively (Fig. 2F). Additionally, the antigen-specific proliferation of splenocytes from each vaccinated group was assessed. The mean specific absorbance of 0.382 (~11.58-fold, $p < 0.001$), 0.244 (~7.40-fold, $p =$ ns), and 0.238 (~7.23-fold, $p =$ ns) was noted for the co-immunized group, YopE, and rLcrV groups, respectively, in comparison to the specific absorbance (0.033) of the placebo group (Fig. 2G).

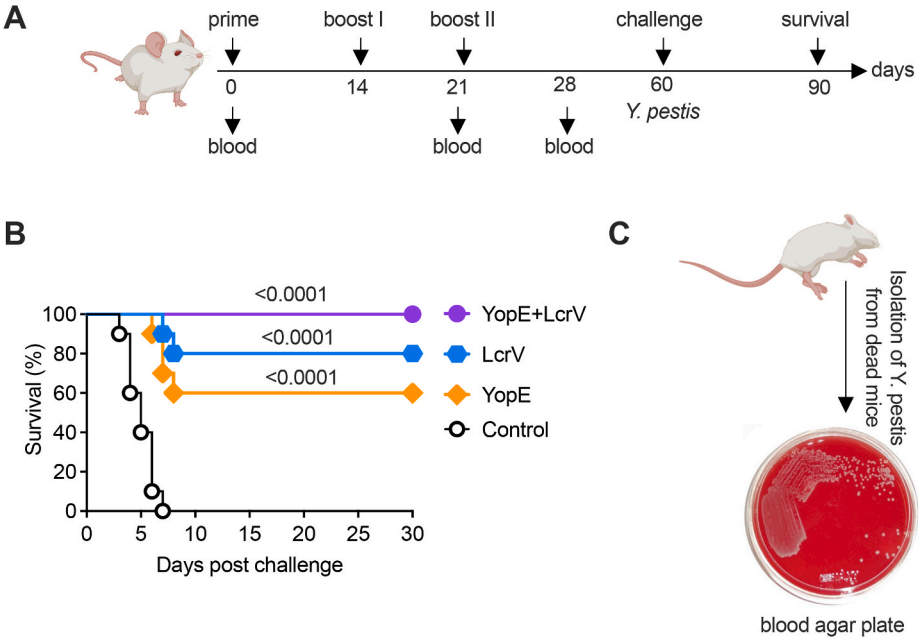

Fig. 3. Protection studies against *Y. pestis* challenge (A–C). Schematic description of the naive female BALB/c mice vaccine administration, bleeding, and challenge study schedule (A). Kaplan-Meier analysis and the magnitude of protection imparted by alum-adjuvanted YopE, LcrV, and the combination of YopE + LcrV. The percentage of BALB/c mice that survived was plotted against the number of days animals survived post-*Y. pestis* (LD_{50}) challenge. To ascertain survivability, animals were observed for one-month post-bacterial exposure. P values (<0.0001) represent comparisons to the control group; log-rank (Mantel-Cox) test. $P < 0.05$ was considered statistically significant (B). *Y. pestis* was isolated from the carcass following the challenge and was grown on a blood agar plate (C).

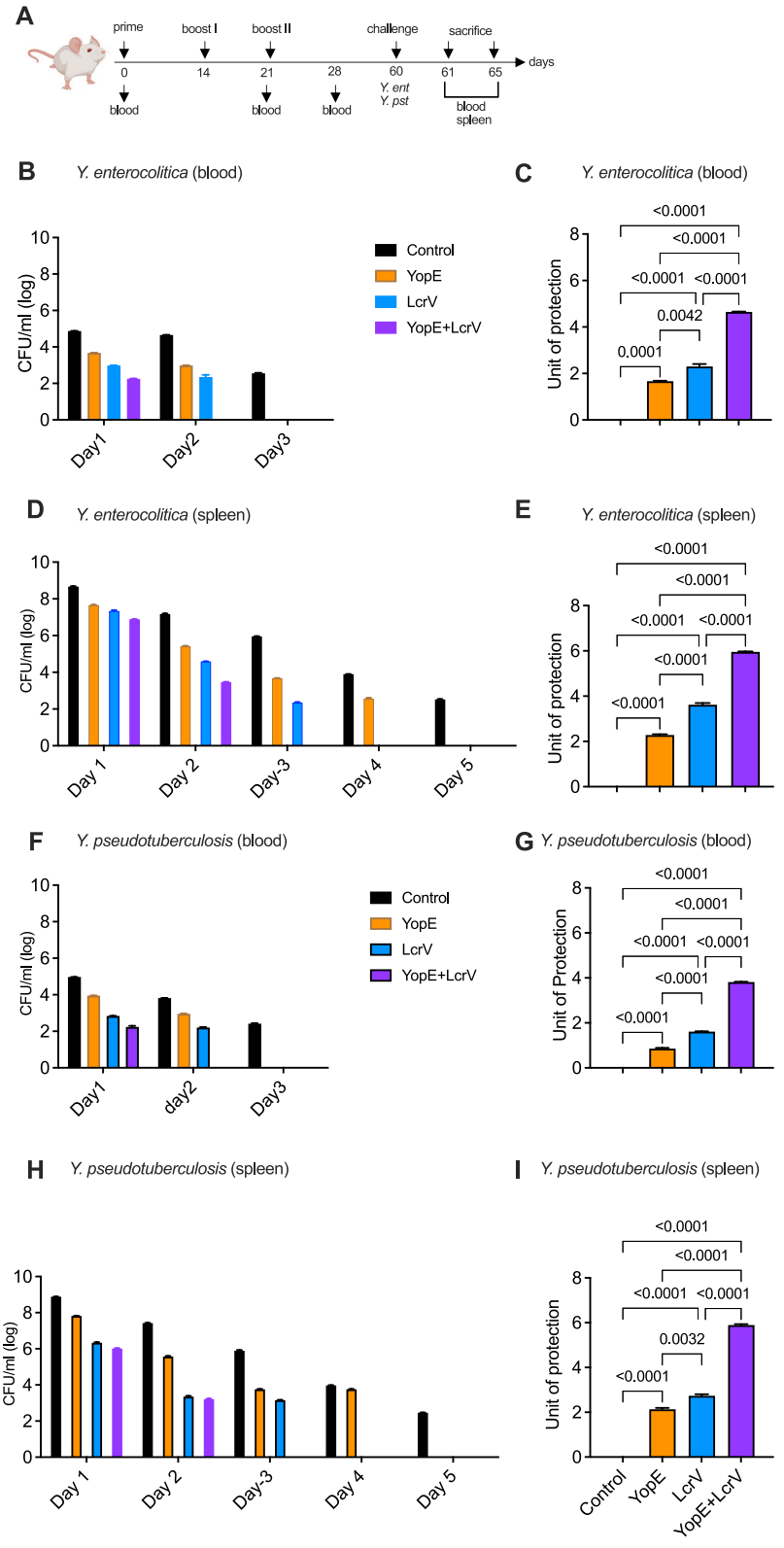

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Fig. 4. Protection studies against *Y. enterocolitica* and *Y. pseudotuberculosis* (A–I). Schematic description of vaccine administration, bleeding, and challenge schedule (A). Graphical representation of CFU count at days 1–3 post-*Y. enterocolitica* challenge from the blood of BALB/c mice (B). Graphical representation of the unit of protection imparted by vaccine candidates on day 2 in the blood (C). Graphical representation of CFU count at days 1–5 post-*Y. enterocolitica* challenge from the spleen of BALB/c mice (D). Graphical representation of the unit of protection imparted by vaccine candidates on day 3 in the spleen (E). Graphical representation of CFU count at days 1–3 post-*Y. pseudotuberculosis* challenge from the blood of BALB/c mice (F). Graphical representation of the unit of protection imparted by vaccine candidates on day 2 in the blood (G). Graphical representation of CFU count at days 1–5 post-*Y. pseudotuberculosis* challenge from the spleen of BALB/c mice (H). Graphical representation of the unit of protection imparted by vaccine candidates on day 3 in the spleen (I). $n = 10$/group. Mean ± SEM. One-way ANOVA test was conducted on log-transformed data, for multiple comparisons, Tukey's test was conducted. $P < 0.05$ was considered statistically significant.

3.5. Recombinant vaccine candidates evoked antigen-distinct IFN-γ eliciting $CD4^+$ & $CD8^+$ T-cell immunological responses

$CD4^+$ and $CD8^+$ T-cells population (%) eliciting IFN-γ from the splenocytes of all immunized mice groups were ascertained using flow cytometric analysis. On day 28th post-immunization, cells were re-stimulated with the appropriate antigen/s designated for their corresponding group. It was observed that when antigen-stimulated lymphocytes from the YopE + LcrV combination were compared to YopE, LcrV, and the control group, the co-immunized group elicited a substantially higher $CD4^+$ T-cells population eliciting IFN-γ (88-fold increment, $p < 0.0001$) (Fig. 2H). Additionally, a substantial increment was observed for $CD8^+$ T-cell populations eliciting IFN-γ from YopE + LcrV stimulated splenocytes (30.8-fold increment, $p < 0.01$) in comparison to the control group, whereas a substantial change of 16-fold increment, $p < 0.05$ and 23.7-fold increment, $p < 0.01$ was observed for IFN-γ production as compared to those stimulated with either YopE or LcrV alone (Fig. 2I).

3.6. Recombinant YopE and LcrV imparted protection against the lethal challenge of Y. pestis and reduced co-morbidity against yersiniosis

BALB/c mice inoculated with recombinant antigens were challenged with LD_{50} of *Y. pestis* to assess the protective effect of vaccination formulations. Both alum-adjuvanted recombinant antigens, alone and in combination, protected inoculated mice, albeit to

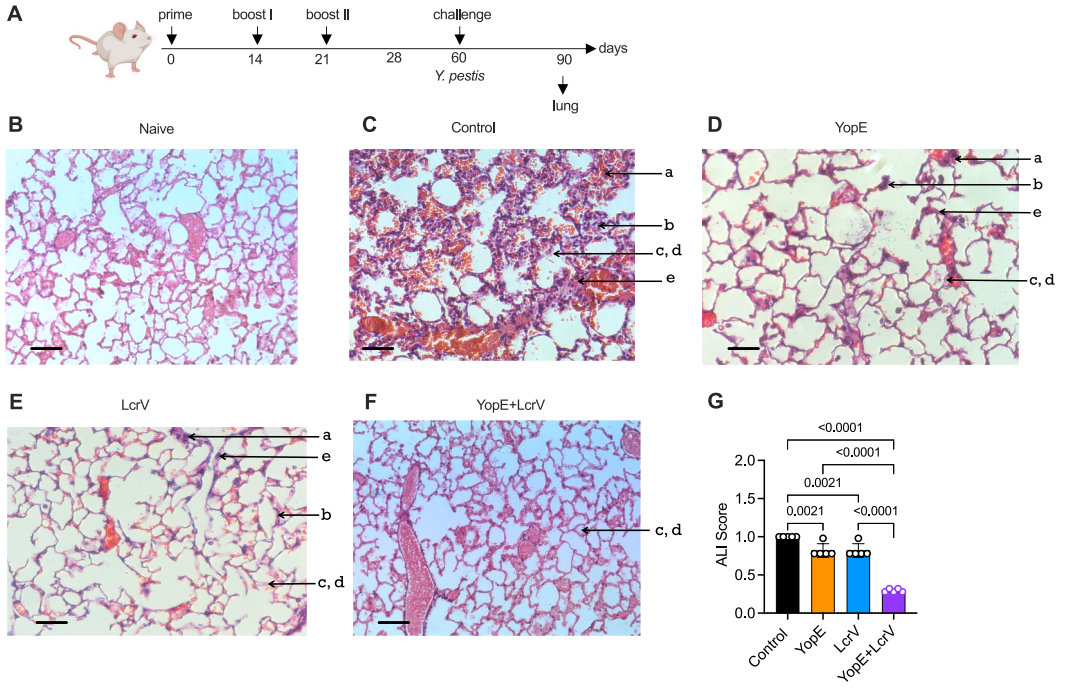

Fig. 5. Histopathological analysis of lungs of *Y. pestis* infected mice (A–G). Schematic description of schedule for vaccine administration, *Y. pestis,* and lung harvesting from BALB/c mice (A). Photomicrographs of naive and *Y. pestis* pestis-challenged lung injuries, day 3 post-challenge after immunization with YopE, LcrV, and YopE + LcrV vaccine candidates. Tissue section of all inoculated groups at day 3 p.i. Naive mice (B), Infected mice (C), YopE-treated (D), LcrV-treated (E), YopE + LcrV-treated mice (F). Blinded histopathological evaluation of lung damage using the American Thoracic Society (ATS) acute lung injury (ALI) scoring systems was done. $n = 3$ per group. Mean ± SEM. Data were analyzed and interpreted using a one-way ANOVA, for multiple comparisons, Tukey's test was conducted (G). Light microscopy was used to analyze tissue sections from three mice in each group stained with hematoxylin and eosin. 20× magnification; 50 μm scale bar. In the images (a) represents neutrophil's count (alveolar space); (b) represents neutrophil's count (interstitial space); (c) represents formation of hyaline membrane; (d) represents air spaces consist of proteinaceous debris and (e) represents the thickness of the alveolar septae. $P < 0.05$ was considered statistically significant.

varying degrees. For YopE + LcrV vaccinated mice, complete protection (100 %) was observed while LcrV and YopE imparted 80 % and 60 % protection, respectively. Control group animals succumbed to the infection by day 7 post-challenge (Fig. 3B).

In the case of yersiniosis, animal groups immunized with YopE, LcrV, and the combination of YopE + LcrV were challenged on day 60 post-prime with 1×10^8 CFU/mouse with *Y. enterocolitica* and 1×10^9 CFU/mouse with *Y. pseudotuberculosis* separately. The bacterial load in the spleen and blood from day 1–5 post-challenge was determined to establish the severity of the infection (Fig. 4B–D, F, H). In the spleen, a significant reduction, *i.e.,* 3.67 and 2.33 $\log_{10}$ CFU load on day 3 post-challenge by YopE + LcrV combination ($p < 0.0001$) with respect to YopE and LcrV, immunized groups for *Y. enterocolitica*, and a 3.75 and 3.15 $\log_{10}$ CFU load reduction for *Y. pseudotuberculosis* ($p < 0.0001$) was observed (Figs. S2A and C). In addition, a significant reduction, i.e., 2.97 and 2.34 $\log_{10}$ CFU load on day 2nd post-challenge by YopE + LcrV combination ($p < 0.0001$) with respect to YopE and LcrV, immunized groups for *Y. enterocolitica*, and a 2.95 and 2.20 $\log_{10}$ CFU load reduction for *Y. pseudotuberculosis* ($p < 0.0001$) was observed in the blood (Figs. S2B and D). The control group of both *Y. enterocolitica* and *Y. pseudotuberculosis* had the highest count of bacterial load. The unit of protection imparted by the vaccine candidate against yersiniosis was calculated and found that the combination of YopE + LcrV provides significantly higher protection units ($p < 0.0001$) when compared to the co-immunized groups (Fig. 4C–E, G, I).

3.7. Recombinant YopE + LcrV vaccine formulation protected against lung immunopathology

Histopathologic features were examined in the lung tissues of naive mice, infected mice, YopE-vaccinated mice, LcrV-vaccinated mice, and YopE + LcrV-vaccinated mice on day 3 p.i. of *Y. pestis*. Histopathologic features of the mice lungs challenged with *Y. pestis* and protection by proposed vaccine candidates are shown (Fig. 5A–F). The histopathological attributes of a typical lung are characterized by thin alveolar septae, clear alveolar spaces with occasional intra-alveolar macrophages, and intact pulmonary arteries and alveolar septa as seen in naïve mice (Fig. 5B). Following infection, the lung of mice displayed a severe case of pulmonary necrosis characterized by a marked increase in intra-alveolar infiltration of inflammatory cells such as neutrophils and macrophages; thickened alveolar septa; pulmonary hemorrhagic areas filled with neutrophils; and alveolar spaces filled with proteinaceous debris (analogous to the formation of hyaline membrane). Inflammation was prevalent throughout the tissue, and the animals suffered from diffuse interstitial pneumonia (Fig. 5C). The tissue injuries to the YopE and LcrV immunized mice were similar to those seen in the PBS control, including neutrophil and macrophage infiltration into the alveolar space; thickened alveolar septa; proteinaceous debris in the alveolar space; pulmonary hemorrhage, but to a lesser extent (Fig. 5D and E). While the lung sections of YopE + LcrV immunized mice exhibited intact pulmonary arteries, normal alveolar septa including thin alveolar walls, and clear alveolar spaces with occasional intra-alveolar macrophages. However, these animals did exhibit proteinaceous debris-filled alveolar space and mild pulmonary edema (Fig. 5F). Lungs from control mice demonstrated multifocal inflammatory infiltrates (Fig. 5C), whereas inoculated mice with the combination of YopE + LcrV showed dramatically decreased lung damage (Fig. 5F), more akin to uninfected mice (Fig. 5B). The YopE + LcrV vaccine formulation significantly decreased lung damage and prevented *Y. pestis*-induced lung injury in mice, resulting in a significantly lowered ALI score (Fig. 5G).

4. Discussion

No licensed vaccine exists for plague and yersiniosis. Previously developed, killed and live attenuated whole-cell *Yersinia pestis* vaccines have limited efficacy and significant adverse effects [14]. As a result, alternative vaccine strategies such as subunit vaccines are being investigated to provide better protection with fewer side effects. Two such vaccine antigens, namely F1 (*capsular antigen, fraction 1)* and LcrV of *Y. pestis*, have been extensively studied either alone or in a cocktail/genetically fused format. The combination of F1 and LcrV provides complete protection in various animal models and non-human primates but provided inconsistent efficacy in African green monkeys [15] due to inadequate cellular immunity [16]. Moreover, the presence of *Y. pestis* strains lacking the F1 antigen may be virulent [22] and has raised the concern about F1 as vaccine candidate. In our earlier studies, a combination of LcrV and HSP70 (heat shock protein 70) was used against plague and yersiniosis [31]. Here, to develop a more efficacious vaccine against plague and yersiniosis, LcrV and YopE with dominant $CD8^+$ T cell epitopes were examined [18]. The results indicated that the IgG antibody titers elicited by LcrV were superior to YopE in inducing humoral immunity alone and in combination; however, the cocktail formulation evoked significantly higher anti-YopE and anti-LcrV IgG titres compared to their individual homologs, suggesting a synergistic effect. This finding aligns with a prior investigation [32], indicating that bivalently fused YopE and LcrV epitopes elicit higher antibody titres compared to their solitary counterparts.

Analyzing the IgG subclass, LcrV immunization led to a IgG1 to IgG2a ratio of 2.4 and 2.3 in individual and cocktail formulation, respectively aligning with previous research indicating that LcrV elicits a Th2-mediated immune response [32,33]; however; unlike prior research showing Th1-type response, YopE immunization resulted in a mixed Th1/Th2 response with a 1.9 IgG1:IgG2a ratio in both individual and cocktail-formulation [32]. Overall, the high titers of IgG1 and IgG2b determine the mixed Th1 and Th2 type of immune response. Th1 immunity is a defensive response led by Th1 cells, promoting proliferation of pathogen-specific T and B cells that release cytokines interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) which in turns activate macrophages and cytotoxic T lymphocytes (CTLs), aiding clearance of intracellular pathogens [34]. *Y. pestis* reduces Th1 immune response [35], indicating that a vaccine enhancing Th1 immunity may be an effective strategy to protect against plague.

Analyzing the cellular response, it was observed that YopE and LcrV formulations significantly boosted IFN-γ, TNF-α, and IL-2 levels, while the cocktail formulation showed even higher cytokine levels, suggesting a potential synergistic effect as previously reported [32]. Furthermore, IL-1β and IL-6 levels demonstrated a notable increment, while IL-4 and IL-10 remained undetectable. These observations contradict the IgG isotype profile favoring Th2 immunity, possibly due to vaccine formulations producing IL-6. IL-6 could

modulate the overall cytokine expression profile, causing a Th2 antibody shift and skewing IgG1:IgG2a ratio [36]; however, this disparity raises intriguing inquiry that require further investigation. Overall, the expression of IFN-γ, TNF-α, and IL-2 indicates a Th1-biased immune response while IL-1β and IL-6, which are pro-inflammatory cytokines with Th2 bias immune responses contribute to imparting protection.

Vaccine-induced T cell responses to generate IFN-γ and TNF-α could be a promising strategy for safeguarding against *Y. pestis*, given the protective roles of these cytokines [9], along with recent findings highlighting the role of $CD8^+$ T cells in offering substantial defense against pneumonic plague and $CD4^+$ T cells amplifying the protective effect offered by $CD8^+$ T cells [37], a promising vaccine would be one that not only generates high antibody titers (LcrV) but also provides $CD8^+$ T cell epitopes (YopE) to defend against *Y. pestis*. Flowcytometry analysis revealed that immunization with YopE and LcrV led to a significant increase in the CD4 and CD8 subsets for individual and even higher for cocktail formulation suggesting synergy, consistent with a previous study [18]. However, for the $CD8^+$ T subset, YopE formulation does not led to higher T-cell population then its individual counterpart that could be indicative of YopE's ability to provide unique $CD8^+$ T epitopes to the cocktail formulation to defend against *Y. pestis* infection.

To further analysis the vaccine evoked protective immunity histopathological analysis was conducted. Lung tissue from PBS-immunized mice revealed significant pathology, including macrophage and neutrophil infiltration, thickened alveolar septum, pulmonary hemorrhage, proteinaceous debris from necrotic cells, and inflammation that led to diffuse interstitial pneumonia 72 h post-challenge consistent with the previous studies [38,39]. Mice immunized with YopE and LcrV shows comparable injuries, but to a noticeably lesser degree, while the combination group displayed intact lung architecture with normal alveolar septa, clear alveolar spaces with occasional intra-alveolar macrophages indicating a synergistic effect of YopE and LcrV antigens and highlighting the potential benefits of YopE + LcrV immunization in safeguarding against the pathology observed during *Y. pestis* infection. Studies on protective efficacy have indicated that the combination group displayed a 100 % survival rate against *Y. pestis* infection with prompt clearance of Yersiniosis causing bacteria thus proving to be a promising vaccine candidate; however, further validation through studies on higher animal models is required, the YopE + LcrV vaccine has potential as a precursor to an effective plague and yersiniosis vaccine.

What's the likely way the YopE and LcrV vaccine combination provides protection? In mice without prior exposure, plague pathogenesis and lethality were linked with a systemically higher bacterial load, which led to the rapid development of bronchopneumonia and the occurrence of a severe inflammatory cytokine response. Whereas in the case of mice immunized with the cocktail of YopE + LcrV formulation, the pathogenesis, the inflammatory response, and lethality did not develop upon infection which could be described by antigen-specific stimulation of antibodies and its subtypes titers and more importantly stimulation of $CD4^+$ and $CD8^+$ T cell immune responses which aided in providing 100 % survival protection when challenged with 100 times LD_{50} dose of *Y. pestis* (Fig. 6).

In conclusion, the combined administration of LcrV and YopE vaccine formulation results in imparting high IgG and its isotypes

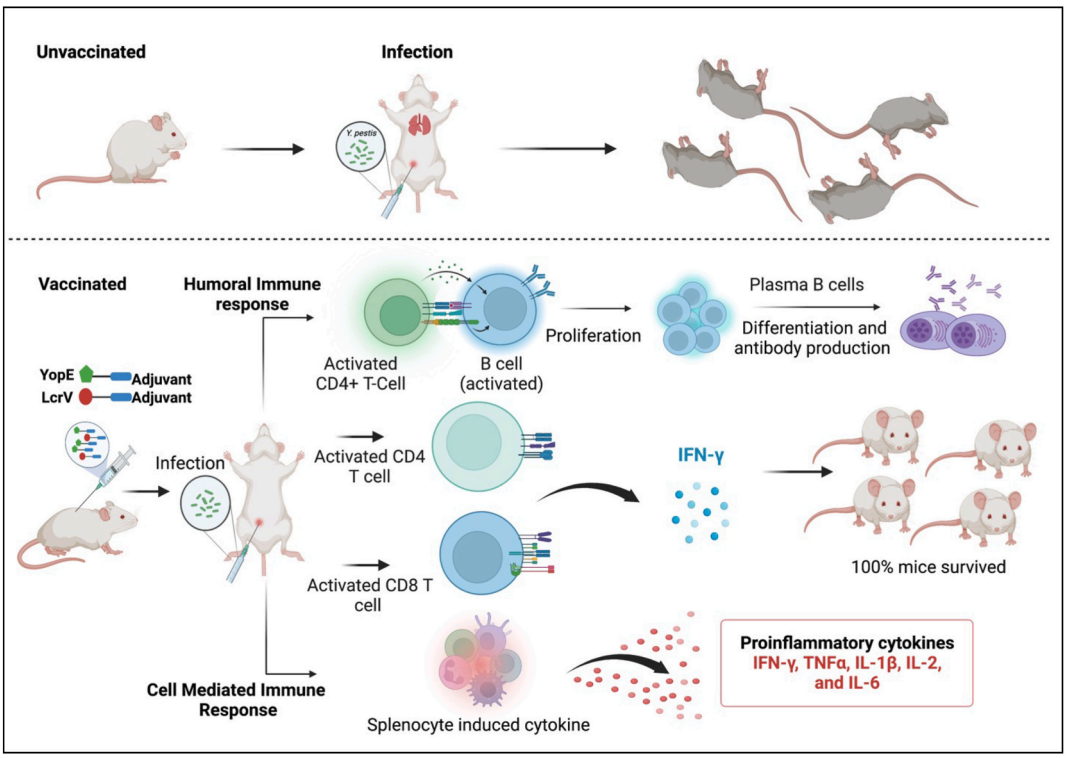

Fig. 6. YopE+LcrV Vaccine Induced protection against plague and Yersiniosis

antibody titers, particularly IgG1 and IgG2b, indicative of a mixed Th1 and Th2 immune response. The response is characterized by increased expression of IFN-γ TNF-α, and IL-2, suggesting a Th1 bias response, along with IL-1beta and IL-6, indicating a Th2 bias. Furthermore, this combination induces a significant increase in IFN-γ secreting $CD4^+$ and $CD8^+$ T cells. Overall, the synergistic effect of YopE and LcrV antigens demonstrates robust protection all three human-pathogenic *Yersinia* species i.e., *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, highlighting the potential of this immunization strategy in mitigating pathology and achieving 100 % protection. Therefore, our current vaccine (YopE + LcrV) targets all three pathogens.

5. Limitation and future incite

One limitation of this study is the absence of an evaluation of the protective efficacy of YopE + LcrV vaccine formulation against lethal inhalation challenge. Although the study assesses vaccine efficacy against systemic *Y. pestis* infection, it does not specifically investigate protection against inhalation exposure, a common route for the development of primary pneumonic plague. Another limitation is the lack of histopathology and cytokine measurements from bone marrow, lymph nodes, and intestinal samples, as the authors solely focused on lung tissue. However, authors plan to investigate these organs in future studies.

Ethics statement

The Institutional Animal Ethics Committee (IAEC) of the Defense Research and Development Establishment (DRDE), Gwalior, India, authorized the protocol (MB-44/57/SKV) that was used for animal research. To conduct this investigation, guidelines specified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, were followed. Survival studies of inoculated mice against *Y. pestis* challenges were carried out in a biosafety level-3 facility.

Data and materials availability

All data are available in the main text or the supplementary materials.

CRediT authorship contribution statement

Ankit Gupta: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Pooja Mahajan:** Writing – original draft, Methodology. **Sameer S. Bhagyawant:** Writing – review & editing. **Nandita Saxena:** Writing – review & editing, Methodology. **Atul Kumar Johri:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Subodh Kumar:** Writing – review & editing. **Shailendra Kumar Verma:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of competing interest

All the authors declare that we did not receive any financial and personal relationships with other people or organizations that could inappropriately influence (bias) our work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31446.

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