YopJ-Promoted Cytotoxicity and Systemic Colonization Are Associated with High Levels of Murine Interleukin-18, Gamma Interferon, and Neutrophils in a Live Vaccine Model of *Yersinia pseudotuberculosis* Infection[∇]

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Several *Yersinia* species have been utilized as live attenuated vaccines to prime protective immunity against yersiniae and other pathogens. A type III secretion system effector known as YopJ in *Y. pseudotuberculosis* and *Y. pestis* and YopP in *Y. enterocolitica* has been shown to regulate host immune responses to live *Yersinia* vaccines. YopJ/P kills macrophages and dendritic cells, reduces their production of tumor necrosis factor alpha (TNF- α) and interleukin-12 (IL-12), and promotes systemic colonization in mouse models of intestinal *Yersinia* infection. Furthermore, YopP activity decreases antigen presentation by dendritic cells, and a *yopP* mutant of a live *Y. enterocolitica* carrier vaccine elicited effective priming of CD8 T cells to a heterologous antigen in mice. These results suggest that YopJ/P activity suppresses both innate and adaptive immune responses to live *Yersinia* vaccines. Here, a sublethal intragastric mouse infection model using wild-type and catalytically inactive *yopJ* mutant strains of *Y. pseudotuberculosis* was developed to further investigate how YopJ action impacts innate and adaptive immune responses to a live vaccine. Surprisingly, YopJ-promoted cytotoxicity and systemic colonization were associated with significant increases in neutrophils in spleens and the proinflammatory cytokines IL-18 and gamma interferon (IFN- γ) in serum samples of mice vaccinated with *Y. pseudotuberculosis*. Secretion of IL-18 accompanied YopJ-mediated killing of macrophages infected *ex vivo* with *Y. pseudotuberculosis*, suggesting a mechanism by which this effector directly increases proinflammatory cytokine levels *in vivo*. Mice vaccinated with the wild-type strain or the *yopJ* mutant produced similar levels of antibodies to *Y. pseudotuberculosis* antigens and were equally resistant to lethal intravenous challenge with *Y. pestis*. The findings indicate that a proinflammatory, rather than anti-inflammatory, process accompanies YopJ-promoted cytotoxicity, leading to increased systemic colonization by *Y. pseudotuberculosis* and potentially enhancing adaptive immunity to a live vaccine.

Understanding how a host initiates an immune response against invading pathogens and how bacterial virulence factors counteract immunity can provide critical insights into pathogenesis as well as allow for the rational development of vaccines. As components of the body's first line of defense, neutrophils, monocytes, and macrophages are important for innate immunity against pathogenic microbes (23, 58). These cells secrete proinflammatory cytokines after detection of pathogen-associated molecular patterns (PAMPs) (68). In addition, they can kill invading bacterial pathogens after phagocytosis (23, 58), while macrophages and especially dendritic cells also serve to initiate an adaptive immune response through presentation of antigens (56). To evade, destroy, or diminish the activities of these cells is vital for a pathogen to establish infection.

The three human-pathogenic *Yersinia* species, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, have been extensively studied to identify bacterial virulence factors that function to counteract immunity (13, 47, 51, 71). *Y. pestis* is the causative

agent of bubonic, septicemic, and pneumonic plague and is commonly transmitted to humans by flea bites or air droplets (47). *Y. pseudotuberculosis*, which is closely related to *Y. pestis*, and the more distantly related *Y. enterocolitica* are transmitted by the fecal-oral route (71). These enteropathogens typically cause self-limiting gastrointestinal diseases in humans but can also cause fatal septicemias (71). An important virulence factor common to all three pathogenic *Yersinia* species is a type III secretion system (T3SS) and its secreted effectors encoded on a virulence plasmid, which is called pYV in *Y. pseudotuberculosis* and *Y. enterocolitica* or pCD1 in *Y. pestis* (15, 24, 50, 69). The T3SS secretes numerous key proteins, including LcrV and *Yersinia* outer proteins, or Yops (11, 69). Six effector Yops are YopE, YopH, YopM, YopT, YpkA, and YopJ (in *Y. enterocolitica*, the last two are named YopO and YopP, respectively). These Yops are translocated across the host cell plasma membrane in a process that requires LcrV, YopB, and YopD (15, 24, 69). LcrV localizes to the tip of the T3SS structure on the bacterial surface (10), is a well-characterized protective antigen (16, 64), and can exhibit direct immunosuppressive activity following its secretion (11, 16). The Yop effectors act through enzymatic activities and/or protein-protein interactions to antagonize phagocytosis (YopH, -E, -T, and YpkA/YopO) (22, 27, 30, 76), modulate cytokine production (YopH, -E, -T, -J/P) (45, 57, 69), activate cytoplasmic kinases (YopM) (38), or in-

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duce death of dendritic cells and macrophages (YopJ/P) (20, 39, 42, 53, 75, 77).

Vaccination experiments performed with attenuated strains or purified virulence factors have also been used to probe the basis of adaptive immunity to *Yersinia*; these studies reveal that both antibody and cellular immunity contribute to protection (3, 7, 16, 21, 61, 64). Interestingly, sufficient protection can be achieved in certain vaccination conditions in animals deficient either in B cells or for gamma interferon (IFN- γ) (29, 46). Furthermore, *Yersinia* mutants attenuated by loss of pYV or inactivation of genes encoding components of the T3SS have been successfully used as live vaccines (5, 12, 32, 55, 59, 65, 67).

In addition, the T3SS has been exploited for delivery of heterologous antigens by live attenuated *Yersinia* carrier vaccines (3, 32, 55, 62, 65, 67, 70). Evidence was obtained that YopP inhibited CD8 T-cell priming to a heterologous antigen in mice infected with a *Y. enterocolitica* carrier vaccine strain (32, 65, 67). A *yopP* mutant of a *Y. enterocolitica* carrier vaccine strain was shown to elicit effective CD8 T-cell priming and protective responses to a heterologous antigen in mice (32, 65, 67). These results suggest that YopJ/P can inhibit adaptive immune responses during *Yersinia* infection. However, it has not been evaluated if YopP inhibits adaptive immune responses to native *Yersinia* antigens during infection.

YopJ/P acetylates the Ser and/or Thr residue in the activation loops of MAP kinase kinases (MKKs) and the inhibitor κ B kinase β (IKK β) (40, 43) and thereby inhibits the activation of these kinases (40, 43). The inhibition of MAP kinase and NF- κ B pathways by YopJ/P results in lowered expression of cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-12 (IL-12) and surface molecules such as major histocompatibility complex class II (MHC-II) in macrophages or dendritic cells infected with *Yersinia* (8, 9, 20, 45). YopP was shown to inhibit antigen uptake by dendritic cells infected with *Y. enterocolitica*, and inhibition of MAP kinase signaling was implicated in this activity (4). In addition, reduced production of survival factors under the control of NF- κ B and MAP kinase pathways as a result of YopJ/P activity, combined with lipopolysaccharide (LPS) stimulation of TLR4 signaling, activates apoptosis in macrophages and dendritic cells infected with *Yersinia* (9, 52, 53, 75, 77). As a consequence of these actions, YopP inhibited heterologous antigen presentation to CD8 or CD4 T cells from dendritic cells infected *ex vivo* with *Y. enterocolitica* carrier vaccine strains (32, 65, 67).

In contrast to results of these *ex vivo* studies, the role of YopJ/P during systemic infection is less well defined. In some studies, YopJ/P promoted systemic colonization of mice infected orally with *Y. pseudotuberculosis* or *Y. enterocolitica* (9, 41, 66) and inhibited innate immunity during *Yersinia* infection *in vivo*, as shown by YopJ-dependent apoptosis of immune cells in murine lymph tissues (9, 33, 41) and YopJ-mediated reduction in levels of TNF- α in rat serum (33). However, a recent study reported that YopJ-promoted systemic colonization of mice by *Y. pseudotuberculosis* was associated with higher serum levels of cytokines, including TNF- α , IFN- γ , and IL-12 (9). In addition, systemic colonization of mice by wild-type *Y. pseudotuberculosis* was associated with marked signs of inflammation, including elevated levels of Gr1⁺ CD11b⁺ neutrophils in blood (36). Therefore, it remains unclear to what extent

Yersinia, and in particular YopJ, inhibits innate proinflammatory responses during systemic infection.

Amino acid differences exist between YopJ/P proteins from different *Yersinia* strains, and these polymorphisms are associated with significant differences in cytotoxic activity (9, 18, 35, 54, 72–74; S. Lilo, Y. Zheng, I. E. Brodsky, Y. Zhang, R. Medzhitov, K. B. Marcu, and J. B. Bliska, submitted for publication). The YopP from *Y. enterocolitica* serogroup O:8 and YopJ from *Y. pestis* KIM have high levels of cytotoxic activity toward macrophages and dendritic cells (9, 35, 54, 72–74). YopJ from *Y. pseudotuberculosis* is characterized as having intermediate cytotoxicity (9, 35; Lilo et al., submitted for publication), while the *Y. pestis* Kimberley YopJ effector has low cytotoxic activity (72–74). High cytotoxicity can trigger proinflammatory forms of cell death, as shown by the finding that dendritic cells infected with *Y. enterocolitica* O:8 undergo a YopP-dependent necrosis-like death (26). Furthermore, ectopic expression of YopP (O:8) in *Y. pseudotuberculosis* or *Y. pestis* Kimberley results in attenuation of these recombinant strains in mouse infection models (9, 73), suggesting the possibility that high cytotoxic activity on the part of this effector may elicit protective immune responses. However, the mechanism of such protection has not been elucidated.

Here we have characterized the impact of YopJ from *Y. pseudotuberculosis* on murine innate and adaptive immune responses to the bacterium in a sublethal infection model. This model allowed us to directly compare the innate and adaptive responses to *Yersinia* wild-type strains and *yopJ* mutants over an extended time course of infection. We could confirm that YopJ-promoted apoptosis was associated with sustained colonization of systemic sites in mice. Higher levels of neutrophils as well as proinflammatory cytokines IL-18 and IFN- γ were observed in mice infected with the wild-type strain than in those infected with the *yopJ* mutant. Macrophages infected *ex vivo* with *Y. pseudotuberculosis* died and released IL-18 in a YopJ-dependent process, showing that this effector can directly elicit proinflammatory cytokines. In addition, mice vaccinated with the *Y. pseudotuberculosis* wild-type strain or *yopJ* mutant had similar antibody responses and upon challenge with *Y. pestis* were protected equivalently. These results indicate that YopJ activity, by promoting sustained colonization and inflammation, is not detrimental in the context of a live *Y. pseudotuberculosis* 32777 vaccine and may be beneficial for eliciting adaptive immunity.

MATERIALS AND METHODS

Bacterial strains and infection conditions. The bacterial strains used were *Y. pseudotuberculosis* serogroup O:1 strain 32777 (previously known as IP2777) and its plasmid pYV-cured derivative 32777c (60). Generation of the catalytically inactive *yopJ*C1724 mutant strain (mJ) was described previously (76). The *Y. pestis* strain used was KIM D27 (48), which lacks the *pgm* locus and is exempt from select agent guidelines.

Macrophage cultures and infection. Bone marrow-derived macrophages (BMDMs) were prepared as previously described (14, 77). Twenty-four hours before infection, the cells were seeded in Dulbecco's modified Eagle medium (DMEM) containing 15% L-cell-conditioned medium, 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 1 mM pyruvate, and 2 mM glutamate at a density of 1.5×10^5 per well in 24-well plates. To prepare bacteria for infection, overnight cultures in Luria-Bertani medium (LB) were diluted in fresh LB containing 20 mM magnesium chloride and 20 mM sodium oxalate and were grown with shaking at 28°C for 1 h and then shifted to 37°C for 2 h. The bacteria were then washed once and resuspended in Hanks' balanced salt solution

(HBSS), mixed in 0.4 ml fresh tissue culture medium, and applied to the cells at a multiplicity of infection (MOI) of 10. To bring the bacteria into contact with the macrophages, the plates were centrifuged for 5 min at $200 \times g$. After the infection mixture was incubated at 37°C for 2 h, 0.1 ml fresh medium containing gentamicin (Gm) was added to each well to reach a final Gm concentration of $8 \mu\text{g/ml}$, and the wells were incubated for another 22 h. Supernatant was collected at this time point.

Infection of mice. For infection with *Y. pseudotuberculosis*, female C57BL/6J mice (Taconic or Jackson Laboratory) 9 to 10 weeks old were fasted for 16 h before orogastric inoculation with $200 \mu\text{l}$ of *Y. pseudotuberculosis* culture through a 20-gauge feeding needle. To prepare bacteria, overnight cultures grown in LB at 26°C were washed once and resuspended in phosphate-buffered saline (PBS) to the indicated concentrations. Mice were provided with food and water thereafter. For subcutaneous immunization, overnight cultures of KIM D27 grown in heart infusion broth (HI) (Difco Laboratories) at 26°C were washed and diluted in PBS to 1×10^7 CFU per ml and $100 \mu\text{l}$ was injected in the nape of the neck. For challenging, mice were injected with 1,000 CFU (~ 100 50% lethal doses [LD_{50}]) or 1×10^5 CFU of KIM D27 in $100 \mu\text{l}$ of PBS via the lateral tail vein. At the indicated time postinfection, or when death was imminent, mice were euthanized by CO_2 asphyxiation. When indicated, blood was collected through tail vein or cardiac puncture and separated into serum after centrifugation in Z-Gel Micro tubes (Sarstedt). Where indicated, mouse spleens were dissected aseptically, weighed, and homogenized in 5 ml of sterile PBS or dispersed to separate into single-cell suspensions in DMEM. Serial dilutions were plated on LB agar to determine bacterial colonization. All animal procedures were approved by the Stony Brook University institutional animal care and use committee.

ELISA. Cytokine concentrations from serum or tissue culture medium were determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. Sera were routinely diluted 10-fold in the appropriate dilution buffer and adjusted accordingly to measure again if necessary to ensure that the value was within the range of the assay. Quantikine mouse TNF- α and IL-12 p70 immunoassay kits were from R&D Systems, Inc., and the mouse IL-18 ELISA kit was from Medical & Biological Laboratories Co., Ltd. The mouse IFN- γ ELISA Max deluxe kit was from BioLegend.

LDH release. Lactate dehydrogenase (LDH) content in the supernatant collected from infected wells or wells left uninfected was measured in triplicate with a CytoTox 96 nonradioactive cytotoxicity assay (Promega) following the manufacturer's instructions. Total LDH was determined from separate uninfected wells that had been lysed by a freeze-thaw cycle in the medium. The percentage of LDH released was calculated by using the formula $100 \times \text{LDH}_{\text{released}} / \text{LDH}_{\text{total}}$.

Flow cytometry. After a single-cell suspension was prepared, 1×10^6 cells were blocked using anti-mouse CD16/CD32 (Fc γ III/II receptor) clone 2.4G2 (BD Pharmingen), labeled with fluorophore-conjugated antibodies, and analyzed using a BD FACSCaliber. Data were processed with WinList software. Isotype-matched antibodies were used to control for nonspecific binding. The antibodies used were rat anti-mouse CD45R/B220-FITC (clone RA3-6B2; Southern-Biotech), anti-mouse CD3e-PerCP (clone 145-2C11; Pharmingen), Alexa-Fluor647 anti-mouse CD4 (clone GK1.5; BioLegend), AlexaFluor488 anti-mouse CD8a (53-6.7; BD, BioLegend), AlexaFluor488 or PerCP-CY5.5 rat anti-mouse CD11b (M1/70, BD), AlexaFluor488 or AlexaFluor647 rat anti-mouse F4/80 (AbD seroTec), AlexaFluor488 anti-mouse CD11c (clone N418; BioLegend), AlexaFluor488 rat anti-mouse Ly-6C (AbD seroTec), and phycoerythrin (PE)-labeled anti-mouse Ly6G (BD).

Cloning and purification of 32777 LcrV protein. The coding sequence of LcrV from 32777 was PCR amplified with primers 5'-AGGATCCCATATGATTAG AGCCTACGAACAAACC-3' and 5'-TAATGAATTCATCTAGCAGACGT GTCATCTAGC-3', digested with EcoRI and NdeI, ligated into the plasmid pET28a (Novagen), and used to transform Zappera competent cells (Novagen). Two independent clones were sequenced, and both contained the same sequences. The GenBank accession number for the LcrV sequence from 32777 is GU356638. The resulting plasmid, pET28a-HisLcrV, was then used to transform Tuner(DE3)pLacI cells (Novagen). IPTG (isopropyl- β -D-thiogalactopyranoside) was used at 0.1 mM to induce protein expression in bacterial cultures at 37°C for 3 h, and the 6 $\times$ His-tagged LcrV was partially purified with a HiTrap chelating HP column (Amersham).

Immunoblotting analysis. To prepare bacterial lysates and secreted Yop proteins, overnight cultures in LB were diluted into fresh LB containing 20 mM sodium oxalate and 20 mM MgCl_2 to an optical density at 600 nm of 0.1. The bacteria were then grown at 28°C for 1 h and 37°C for 4 h with shaking. Lysates of 32777c were prepared from culture grown at 26°C in LB for 4 h after subculture for logarithmic phase. Bacteria were pelleted after microcentrifugation, washed once in PBS, and resuspended in $1 \times$ Laemmli sample buffer. Yop proteins in culture supernatant were precipitated with 10% trichloroacetate, washed in cold acetone, dried, and resuspended in $1 \times$ Laemmli sample buffer.

Bacterial lysates and the Yop proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Mouse serum was used at a 1:1,000 dilution in 1% Casein Hammarsten in PBS and 0.05% Tween 20. Rabbit anti-LcrV antibody (provided by Matt Nilles, University of North Dakota) was used at a 1:50,000 dilution. The secondary antibody was IRDye800-conjugated anti-mouse IgG (Rockland) or anti-rabbit IgG conjugated with Alexa Fluor680 (Molecular Probes). The membranes were scanned with an Odyssey VI scanner (LI-COR Biosciences).

Antibody subclass determination by ELISA. Bacterial lysate from 32777c and secreted Yops, prepared as described in the previous section, were used to coat a 96-well Maxisorp Nunc-Immunoplate at $1.28 \mu\text{g/well}$. Serum samples were collected before infection and weekly postinfection from five pairs of mice that were infected with 32777 or mJ. Antibody subclasses were assessed with the ImmunoPure monoclonal antibody isotyping kit I [horseradish peroxidase-2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (HRP/ABTS)] from Pierce according to the manufacturer's instructions. Serum samples were used at dilutions of 1:100 or 1:1,000.

Statistical analysis. Statistical analysis was performed with Prism 4.0 (Graphpad) software. The tests used are as indicated in the figure legends or main text. *P* values of less than 0.05 were considered significant.

RESULTS

Development of a sublethal intragastric infection model that allowed characterization of the impact of YopJ on innate and adaptive immune responses to *Y. pseudotuberculosis* in mice. An intragastric mouse infection procedure was established in order to characterize the innate and adaptive immune responses to wild-type and *yopJ* mutant *Y. pseudotuberculosis*. Toward this goal, it was important to determine a challenge dose that would allow the majority of mice to survive infection with either strain. For these experiments, a wild-type *yopJ*⁺ *Y. pseudotuberculosis* serogroup O:1 strain (32777) and an isogenic catalytically inactive *yopJ*C172A mutant of 32777 (mJ) were used. Strain 32777 was shown previously to inhibit production of TNF- α and induce apoptosis in macrophages, and mJ was defective in these phenotypes (76). Intragastric infection with 32777 at 5×10^9 CFU per mouse resulted in 100% lethality, while 5×10^8 CFU per mouse resulted in 62.5% lethality and 5×10^7 CFU per mouse resulted in 20% lethality (Fig. 1A to C). At the three infective doses used, the survival curves for mice infected with mJ were not significantly different from those for mice infected with the wild-type strain (Fig. 1A to C). Therefore, in this infection procedure, 32777 and mJ were equally virulent. The sublethal infection dose of 5×10^7 CFU of *Y. pseudotuberculosis* per mouse was used in the remainder of the study.

YopJ activity promotes apoptosis and sustained spleen colonization by *Y. pseudotuberculosis*. Groups of mice infected with 32777 or mJ were euthanized on days 4, 7, 10, and 14 postinfection. Spleens were removed, weighed, and processed for immunohistochemistry or CFU assay. Infection with 32777 but not mJ resulted in the production of apoptosis-positive cells, as shown by staining spleen sections with antibodies to active caspase-3 or *Yersinia*, followed by examination by fluorescence microscopy (Fig. 2A and B). The results of CFU assays to measure spleen colonization showed that the average values for 32777 and mJ were not significantly different at day 4 postinfection (Fig. 3). Average CFU values for 32777 remained at about the same level ($\sim 10^5$) from days 4 to 10 before decreasing to nearly undetectable on day 14 (Fig. 3). The average CFU value for mJ was decreased significantly compared to that for 32777 on day 7 ($10^{2.5}$ versus 10^5 CFU/

FIG. 1. Survival of C567B/L6 mice following intragastric infection with *Y. pseudotuberculosis* 32777 or mJ. The inoculation dosages of 32777 (circles) or mJ (triangles) were 5×10^9 CFU/mouse (A), 5×10^8 CFU/mouse (B), and 5×10^7 CFU/mouse (C). Mouse survival was monitored for 28 days. Results shown are the summary results of two independent experiments with four mice per group in each experiment.

spleen), remained low on day 10, and also decreased to a nearly undetectable level on day 14 (Fig. 3). These results showed that YopJ activity was not required for dissemination of *Y. pseudotuberculosis* to the spleen but promoted sustained colonization of this tissue between days 4 and 7 postinfection. By day 14, both 32777 and mJ were eliminated by the immune response from the majority of mice.

YopJ activity promotes increases in spleen weight. As shown in Fig. 4, in mice infected with 32777, the average spleen weight increased between days 4 and 10 postinfection, then decreased by day 14. Day 14 spleen weights were still heavier than the 0.08-g average for uninfected spleens (data not shown). Spleen weight changes over time in mice infected with mJ showed a trend similar to that for the 32777-infected spleens, although on average the weights were lower on days 7, 10, and 14, and on day 7 the difference was statistically significant (Fig. 4). Thus, spleen colonization by *Y. pseudotuberculosis* caused transient splenomegaly, possibly due to increased recruitment of immune cells. Furthermore, YopJ-promoted bacterial colonization of the spleen was associated with increased weight of this organ.

FIG. 2. Detection of active caspase-3-positive cells in mouse spleens infected with either wild-type 32777 (A) or mJ (B). Spleens were dissected from mice that had been infected 4 days before. After removing a small piece to determine colonization, the rest were fixed, cryoprotected, frozen, and sectioned with standard procedure. *Yersinia* (arrowhead) was labeled with anti-*Yersinia* antibody and secondary antibody conjugated with fluorescein isothiocyanate (FITC) (76). Activated caspase-3 (arrow) was detected with anti-cleaved caspase-3 antibody followed by secondary antibody conjugated with Alexa Fluor 594 (76). Green and red fluorescent pictures were taken sequentially with a Spot camera and assembled in Photoshop.

Increased numbers of CD11b⁺ Ly6C^{int} Ly6G⁺ neutrophils in spleens of infected mice are YopJ dependent.

The composition of the immune cell population in spleens infected with 32777 or mJ was investigated using flow cytometry. Different immune cell types present at days 4 or 7 postinfection were detected using antibodies to representative surface markers (CD11b for granulocytes, monocytes, some dendritic cells, and macrophages, F4/80 for mature macrophages, CD11c for dendritic cells, B220 for B cells, CD3 for total T cells, CD4 for CD4 T cells, and CD8 for CD8 T cells). At day 4, there was a significant increase in CD11b⁺ cells both in absolute numbers (Fig. 5A) and percentages (not shown) in spleens infected with 32777 or mJ compared to values for uninfected spleens. The level of F4/80⁺ mature macrophages also increased slightly on days 4 and 7 in the infected spleens, compared to that in uninfected spleens, although this difference did not reach statistical significance (Fig. 5A and B). The levels of CD11c⁺ dendritic cells, CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, and B220⁺ cells did not increase upon infection (Fig. 5A, C, and D).

There were greater numbers of CD11b⁺ cells in spleens infected with 32777 than in those infected with mJ on day 7 (Fig. 5B), although the difference was not statistically significant ($P = 0.31$). CD11b⁺ cells include neutrophils, monocytes,

FIG. 3. Spleen colonization by 32777 or mJ at different days postinfection. Mice were infected intragastrically with 5×10^7 CFU of 32777 (circles) or mJ (triangles). Spleens were isolated from euthanized mice and processed for CFU determination on the days indicated. Each symbol represents the value in $\log_{10}$ CFU per spleen from one mouse, and the bar indicates the geometric mean of the values for the group. The detection limit was 50 CFU/spleen (dashed line). Results shown are the summary results of five (day 4), four (day 7), three (day 10), or two (day 14) experiments. With a two-tailed Mann-Whitney test, a P value of less than 0.05 was indicated.

FIG. 4. Spleen weights of mice at different days after infection with 32777 or mJ. Mice were infected intragastrically with 5×10^7 CFU of 32777 (circles) or mJ (triangles). Spleens were isolated from euthanized mice at the days indicated and weighed. Each symbol represents the weight in grams of one spleen, and the bar indicates the geometric mean of the group. Results shown are the summary results of five (day 4), four (day 7), three (day 10), or two (day 14) experiments. With a two-tailed Mann-Whitney test, a *P* value of less than 0.05 was indicated.

some dendritic cells, and macrophages. To gain additional insight into the nature of the CD11b⁺ cells present in increased numbers in infected spleens, flow cytometry was used to characterize them with respect to expression of additional surface markers. Gr1⁺ cells, commonly detected using the RB6-8C5 antibody, correspond to granulocytes and inflammatory monocytes. Gr1 is comprised of two different surface molecules, Ly6C and Ly6G, which are differentially expressed by granulocytes and monocytes and can be differentiated using specific antibodies (17). CD11b⁺ Ly6C⁺ Ly6G⁻ cells are considered inflammatory monocytes, while CD11b⁺ Ly6C^{int}

Ly6G⁺ cells are neutrophils. Analysis of single-cell suspensions of infected spleens for forward- and side-scatter characteristics by flow cytometry showed increased numbers of leukocytes in spleens infected with 32777 compared to those in spleens infected with mJ on day 7 (compare Fig. 6A and D). When spleen cells from day 7 were stained with antibodies to CD11b, Ly6C, and Ly6G and analyzed by flow cytometry, two distinct populations of CD11b⁺ cells (gated as R2 in Fig. 6B and E) were detected. In 32777-infected spleens, 27% of the CD11b⁺ cells expressed high levels of Ly6C but were negative for the Ly6G epitope (gated as R6 in Fig. 6C). Furthermore, these cells were also F4/80 positive (data not shown) and therefore could be classified as inflammatory monocytes (CD11b⁺ Ly6C⁺ Ly6G⁻). A large percentage (73%) of the CD11b⁺ cells expressed high levels of the Ly6G epitope but intermediate levels of Ly6C, and the majority of these cells were F4/80 negative (data not shown) and therefore were neutrophils (CD11b⁺ Ly6C^{int} Ly6G⁺; gated as R4 in Fig. 6C). Interestingly, when these results were compared to results of the same analysis performed on spleens infected with mJ, a significantly smaller number of cells of the CD11b⁺ Ly6C^{int} Ly6G⁺ neutrophil phenotype were detected (Fig. 6, compare panels C and F and see summary in panel H). Furthermore, the Ly6C levels of these cells were lower than those of cells infected with the wild type. In contrast, the numbers of CD11b⁺ Ly6C⁺ Ly6G⁻ inflammatory monocyte cells present in spleens infected with 32777 and mJ were not statistically different (Fig. 6C and F and summarized in panel G). Thus, on day 7, at which time there was a higher colonization level of spleen by 32777 than by mJ

FIG. 5. Determination by flow cytometry of immune cell populations in mouse spleens infected with 32777 or mJ. Mice were infected intragastrically with 5×10^7 CFU of 32777 (circles) or mJ (triangles) or left uninfected (UI; squares). Spleens were collected on day 4 (A and C) or day 7 (B and D) from euthanized mice. Single-cell suspensions generated from the spleens were stained with antibodies against the indicated surface markers. Each symbol represents the cell number ($\times 10^6$) calculated from the total numbers of splenocytes obtained from one spleen and the percentage of cells measured by flow cytometry that were positive for the respective marker. The bar represents the geometric mean. Results shown were from two (A to C) or three (D) independent experiments. *P* values were determined with a nonparametric test (Kruskal-Wallis test) followed by Dunn's multiple comparison test.

FIG. 6. Determination by flow cytometry of numbers of Ly6C- and Ly6G-positive cells in mouse spleens infected with 32777 or mJ. Spleens were collected on 4 or 7 days postinfection from euthanized mice, and single-cell suspensions were analyzed by flow cytometry by staining with antibodies against the corresponding surface markers. Representative flow cytometry analysis obtained for 32777-infected (A to C) or mJ-infected (D to F) mice for 7 days is shown. (A and D) Forward and side scatter of the splenocytes; (B and E) presence of CD11b-positive cells; (C and F) presence of Ly6G and/or Ly6C on the CD11b-positive cells (within R2 in panel B or E). Numbers of CD11b⁺ Ly6C⁺ Ly6G⁻ cells (lower right quadrants, or R6 in panels C and F) and CD11b⁺ Ly6C^{int} Ly6G⁺ cells (upper right quadrants, or R4 in panels C and F) were calculated from the total number of splenocytes and the percentages of these cells obtained from one spleen and are plotted in panels G and H, respectively. UI, samples were collected from mice left uninfected. Bars represent geometric means. Bacterial colonization levels in the spleens of these mice were plotted in panel I. Arrows point to the two samples shown in panels A to F. Data are the summary results of one (day 4) or two (day 7) independent experiments. *P* values were determined with the Mann-Whitney test.

(Fig. 3 and 6I), there was also a significantly larger population of CD11b⁺ Ly6C^{int} Ly6G⁺ neutrophils.

YopJ activity is associated with increased levels of cytokines in the sera of mice. ELISA was used to measure IFN- γ , TNF- α , IL-12(p70), and IL-18 concentrations in sera of mice infected with 32777 or mJ to determine how YopJ activity impacted proinflammatory cytokine levels. The average serum levels of IFN- γ were significantly higher in 32777-infected mice than in mJ-infected mice on days 4 and 7 (Fig. 7A) and day 10 (data not shown). In addition, serum IL-18 levels were significantly higher in 32777-infected mice than in mJ-infected animals on day 7 (Fig. 7C). Average serum levels of TNF- α and IL-12(p70) were not significantly different on day 4 or 7 in the

32777-infected and mJ-infected mice (Fig. 7B and D). Thus, the presence of active YopJ during infection of mice by *Y. pseudotuberculosis* was associated with the production of larger amounts of the proinflammatory cytokines IFN- γ and IL-18.

YopJ promotes secretion of IL-18 from macrophages *ex vivo*. Because IL-18 stimulates IFN- γ production (44), ELISA was used to measure levels of IL-18 secreted from macrophages infected *ex vivo* with 32777 or mJ. As a control, we determined the amounts of IL-18 secreted from uninfected macrophages dying from staurosporine-induced apoptosis, and in parallel, death of these macrophages was determined by measuring the amounts of released LDH. As shown in Fig. 8A, higher levels of IL-18 were secreted from macrophages infected with 32777

FIG. 7. Cytokine levels in sera of mice infected with 32777 or mJ. Mice were infected intragastrically with 5×10^7 CFU of 32777 (squares) or mJ (triangles). Serum samples collected on the indicated days were analyzed to measure IFN- γ (A), TNF- α (B), IL-18 (C), or IL-12 p70 (D) concentrations. Results shown are compiled from three to five independent experiments. The *P* values were calculated with a two-tailed Mann-Whitney test.

than from those infected with mJ at 24 h postinfection. We have previously shown that 32777 and mJ are internalized and survive equally in macrophages (76), ruling out differences in pathogen load as a cause of differential IL-18 secretion. Pretreatment of macrophages with IFN- γ increased levels of IL-18 secreted from infected macrophages overall, but the amounts of cytokine released continued to be larger after infection with 32777 than after infection with mJ (Fig. 8A). Low levels of IL-18 were secreted from macrophages undergoing staurospo-

FIG. 8. Determination of IL-18 secretion (A) and LDH release (B) in macrophages infected with 32777 or mJ or treated with staurosporine. Bone marrow-derived macrophages (BMDMs) were infected at an MOI of 10 with either 32777 or mJ and treated with 2 μ M staurosporine (STS) or left uninfected but otherwise treated similarly (UI). Where indicated, IFN- γ was included at 100 U/ml (20 ng/ml) 17 h before and during infection. Bacteria were induced to maximally express Yops before infection (see Materials and Methods). Two hours postinfection, gentamicin was added with fresh medium to reach a final concentration of 8 μ g/ml. Supernatants were collected 24 h postinfection, LDH release was quantified by CytoTox 96 nonradioactive cytotoxicity assay, and concentrations of IL-18 were determined with ELISA. Results shown are the means and standard deviations from results of three independent experiments. *P* values were determined with one-way analysis of variance followed by Bonferroni's multiple comparison test. Furthermore, the values of the percentage of LDH released were first logarithmically transformed to ensure normal distribution. Data were divided into two groups—either with or without IFN- γ treatment. All comparisons were within the group, and values were compared to the values obtained from cells infected with 32777. Only *P* values less than 0.05 were indicated.

rine-induced apoptosis (Fig. 8A). Results of LDH release assays showed that the highest levels of cell death occurred in macrophages infected with 32777 in the presence or absence of IFN- γ or treated with staurosporine (Fig. 8B). Lower levels of cytotoxicity were seen in macrophages infected with mJ in the presence or absence of IFN- γ . Thus, YopJ-induced cytotoxicity is associated with IL-18 secretion from macrophages infected with *Y. pseudotuberculosis*, suggesting a source of the increased IL-18 detected in sera of mice. Furthermore, because IL-18 is able to stimulate T cells to secrete IFN- γ (44), this also provided an explanation for the elevated serum levels of IFN- γ observed 7 days postinfection.

YopJ activity is not associated with diminished antibody or protective immune responses in mice infected with *Y. pseudotuberculosis*. Murine antibody responses to *Y. pseudotuberculosis* antigens were measured on days 7, 14, 21, and 28 postinfection. Results of ELISA using antigens in whole bacterial cell

lysates showed that mice infected with 32777 or mJ produced similar levels of the different antibody subclasses over time (Fig. 9A to D). The exception to this was increased levels of IgG2a and IgM at day 7 in mice infected with 32777 compared to those infected with mJ (Fig. 9A). The predominant subclasses at 28 days postinfection were IgG1 and IgG2b (Fig. 9D). Similar results were obtained by using preparations of secreted Yops as antigens (Fig. 9E to H). IgG antibody responses to *Y. pseudotuberculosis* antigens in mice infected with 32777 or mJ were subsequently characterized by immunoblotting. Analysis of serum samples isolated on day 7 postinfection showed that low levels of IgG antibodies recognizing several secreted Yops as well as multiple antigens in whole-cell lysates of 32777 were present (Fig. 10A). Higher levels of these antibodies were present in serum isolated on day 14 and thereafter, and in general there were no reproducible differences in the magnitudes or specificities of the IgGs from mice infected with 32777 or mJ (Fig. 10B). The Yops most prominently recognized by these antibodies were YopM, YopB, YopD, and YopE (Fig. 10B and C). Pooled sera from 32777-infected mice were used to probe immunoblots to further characterize the antigens recognized by these antibodies. Figure 10C shows that a number of antigens detected in whole bacterial lysates did not correspond to Yops, since they were present in lysates of a 32777 strain lacking pYV (32777c; lane 3). Curiously, none of the 18 mice infected in five independent experiments produced serum IgGs during the first 4 weeks of infection that distinctively recognized LcrV (Fig. 10D, compare lanes 1 and 7, and data not shown). To further evaluate this phenomenon, LcrV encoded by 32777 was expressed as a 6×His-tagged fusion protein in *Escherichia coli* and partially purified (Fig. 10D, lanes 2 and 3). A rabbit polyclonal antibody against LcrV recognized the 32777 LcrV among the secreted Yop proteins and the partially purified LcrV (Fig. 10D, lanes 4 to 6); however, a mixture of sera from infected mice did not contain detectable anti-LcrV IgG, even though the large amount of LcrV formed a shadow on the blot and a bacterial contaminant was recognized nonspecifically (Fig. 10D, lanes 8 and 9). The results suggest that 32777 and mJ infections elicit similar IgG antibody responses against Yops and chromosomally encoded *Y. pseudotuberculosis* antigens.

To determine if infections with 32777 or mJ elicit different levels of protective immunity, the ability of mice vaccinated with *Y. pseudotuberculosis* to withstand secondary lethal intravenous challenge with *Y. pestis* KIM D27 was tested. As a negative control, one group of mice was left uninfected, and as a positive control, one group of mice was immunized by subcutaneous infection with $\sim 1 \times 10^6$ CFU of KIM D27. Twenty-eight days after immunization with a sublethal dose of 32777, mJ, or KIM D27, surviving mice were infected with $\sim 1,000$ CFU (100 LD_{50}) of KIM D27 intravenously. All mice immunized with *Y. pseudotuberculosis* or KIM D27 survived the challenge without losing weight, while all nonimmunized mice died (Fig. 11A and data not shown). The experiment was then repeated with a higher challenge dose of KIM D27 ($\sim 100,000$ CFU; $10,000 \text{ LD}_{50}$). All mice immunized with 32777 survived the infection, 70% of mice immunized with mJ survived, and none of the control mice survived (Fig. 11B). These results indicated that the presence of active YopJ during immuniza-

tion with *Y. pseudotuberculosis* does not result in diminished protective immunity.

DISCUSSION

In this study, we established an intragastric murine infection model to characterize the impact of YopJ activity on host immune responses to *Y. pseudotuberculosis*. The dose of bacteria chosen, 5×10^7 CFU, was low enough to allow the majority of infected mice to survive ($\sim 80\%$) but high enough to allow uniform colonization of systemic sites and development of a strong adaptive immune response. It is acknowledged that an 80% survival rate would be unacceptable for the application of a live vaccine in a clinical setting. However, the results obtained with this model allowed a fair comparison of the immune responses generated, since groups of mice infected with wild-type and *yopJ* mutant *Y. pseudotuberculosis* had equivalent survival rates. Furthermore, this model will allow for future studies to delineate the protective components of the adaptive immune responses. Using this model, we confirmed that YopJ activity induced host cell apoptosis *in vivo* and promoted sustained colonization of systemic tissues (9, 42, 66). YopJ activity was not required for virulence in terms of lethality in this model, similar to what was reported by Galyov et al. (25) but different from reports of Monack et al. (41) and Trulzsch et al. (66). The last two studies found that strains of *Y. pseudotuberculosis* (41) or *Y. enterocolitica* (66) carrying a *yopJ* or *yopP* mutation were significantly attenuated for virulence in oral infections of mice. Thus, although YopJ/P plays a reproducible role in systemic colonization following bacterial spread from the intestinal tract, its role in virulence remains variable and likely will depend heavily on the conditions of the experiment, including, most critically, the strains of *Yersinia* and mice used. On the other hand, evidence is accumulating that YopJ activity is not required for virulence or tissue colonization by *Y. pestis* in bubonic, pneumonic, or septicemic plague models (33, 63, 73, 74) or *Y. pseudotuberculosis* when mice are infected by a route (e.g., intraperitoneal or intravenous) that bypasses the intestinal phase (2, 37). Why YopJ/P specifically promotes systemic colonization following intestinal *Yersinia* infection is unclear. Enteropathogenic *Yersinia* can infect systemic sites by spreading directly from a replicating pool of bacteria in the intestine, bypassing mesenteric lymph node colonization (6). The similar colonization levels initially argue against the possibility that YopJ activity is important for optimal dissemination from the intestinal tract, although it cannot be ruled out yet that sustained colonization of spleen by YopJ⁺ *Y. pseudotuberculosis* results from higher rates of continuous bacterial seeding of this organ. Alternatively, YopJ activity may be important for optimal bacterial survival in the spleen following initial dissemination from the intestinal tract.

Y. pseudotuberculosis infection of mice was associated with splenomegaly, which appeared to result from recruitment of CD11b⁺ cells to these organs. Spleens infected with the wild-type strain had increased splenomegaly and increased numbers of neutrophils (CD11b⁺ Ly6C^{int} Ly6G⁺) on day 7 postinfection compared to the same organs infected with the *yopJ* mutant. The numbers of CD11b⁺ cells characterized as inflammatory monocytes (Ly6C⁺ Ly6G⁻) for spleens infected with wild-type strains and those infected with strains carrying the

FIG. 9. Measurement of serum antibody responses to *Y. pseudotuberculosis* antigens following infection with 32777 (open bars) or mJ (striped bars). Mice were infected intragastrically with 5×10^7 CFU. The relative presence of different subclasses of antibodies was determined by ELISA with serum collected 1, 2, 3, or 4 weeks postinfection as indicated. In either the 32777- or the mJ-infected group, the same five mice were used throughout. Results for these 10 mice were derived from three independent experiments. Bacterial lysate prepared from 32777c (A to D) or secreted Yops (E to H) were used as antigen. After incubation with diluted mouse serum, subclass-specific anti-mouse immunoglobulins were applied. Normal rabbit serum serves as a negative control (rabbit). Color reaction was developed using horseradish peroxidase-conjugated goat anti-rabbit IgG. Arbitrary unit (AU) represents the OD reading when serum was diluted at 1:100 or 10-fold OD reading when serum was diluted at 1:1,000. Specifically, all of the AU values for panels A and E and AU values of IgG3, IgA, and IgM for panels B to D and F to H were determined from 1:100 dilutions, and AU values of IgG2a and IgG2b for panels B to D and F to H were determined from 1:1,000 dilutions. Alternatively, 10-fold OD readings were used when serum was diluted at 1:1,000 (IgG2a and IgG2b in panels B to H). AU values of IgG1 were calculated from 1:1,000 dilutions unless the OD values from 1:100 dilutions were smaller than 1. *, $P < 0.001$ as determined by two-way repeated-measure analysis of variance (ANOVA) followed by Bonferroni post hoc test to compare replicate means.

FIG. 10. Detection of antigens recognized by IgG antibodies from mice infected with 32777 or mJ. Sera were collected from mice infected as indicated in the legend to Fig. 9. (A and B) Time course of IgG antibody responses to *Y. pseudotuberculosis* antigens in individual mice infected with either wild-type 32777 (mice i to iii) or mJ (mice iv to vi). Secreted Yops (antigen 1) or whole bacterial lysate (antigen 2) from 32777 was separated by SDS-PAGE, transferred to nitrocellulose membranes, and processed for immunoblotting using sera from individual mice (mice i to vi) and secondary antibody to IgG. Sera were collected 7 days postinfection (A) or 21 days postinfection (B). The

FIG. 11. Intra-gastric vaccination of mice with 32777 or mJ elicits a protective response against intravenous challenge with *Y. pestis*. Groups of mice were left unimmunized or vaccinated by intra-gastric infection with 5×10^7 CFU of 32777 or mJ. (A) Control mice were vaccinated by subcutaneous injection of 1×10^6 CFU of KIM D27. After 28 days, the surviving mice were infected intravenously with 1,000 (A) or 100,000 (B) CFU of KIM D27. Mouse survival was monitored for another 20 days. Results shown are the summary results of two independent experiments with total mouse numbers indicated in parentheses. By log rank test, the differences between the unvaccinated group and vaccinated groups are significant: $P < 0.0001$ in panel A and $P = 0.0082$ in panel B. However, the difference between 32777- and mJ-immunized groups in panel B is not significant ($P = 0.1385$).

yopJ mutation were not significantly different. One possible explanation for this finding is that in spleens infected with the wild-type strain there is enhanced recruitment of both neutrophils and inflammatory monocytes, but YopJ activity results in selective death of inflammatory monocytes. Cells characterized as CD11b⁺ macrophages/monocytes were previously shown to undergo YopJ-dependent apoptosis in *Y. pseudotuberculosis*-infected murine lymphoid tissues (9, 41). We favor the idea that YopJ-mediated killing of inflammatory monocytes allows for optimal bacterial survival and is the underlying cause of the

positions of YopM, -B, -D, and -E are indicated on the left of panel B. (C) Comparison of antibody responses to Yops or antigens encoded on the *Y. pseudotuberculosis* chromosome. Secreted Yops (lane 1) or whole bacterial lysates (lanes 2 and 3) prepared from 32777 (lane 2) or 32777c (lane 3) were subjected to immunoblotting analysis with pooled sera obtained from mice ii and vi. (D) Lack of IgG antibody responses to LcrV. Secreted Yops (lanes 1, 4, and 7) or 10 μ g (lanes 2, 5, and 8) or 1 μ g (lanes 3, 6, and 9) of partially purified LcrV was separated with SDS-PAGE and stained with GelCode Blue (left panel) or transferred to a membrane and subjected to immunoblot analysis with a rabbit polyclonal anti-LcrV antibody (middle panel) or pooled sera from mice infected with 32777 (right panel). The positions of LcrV and YopD are indicated on the left.

sustained spleen colonization by the wild-type strain. Although it is presently unclear why YopJ-mediated selective killing of inflammatory monocytes compared to neutrophils would enhance survival of *Y. pseudotuberculosis*, inflammatory monocytes have been shown to be critical for host defense against numerous bacterial pathogens in murine infection models (58). Murine inflammatory monocytes are known to abundantly produce reactive nitrogen intermediates (RNI) as a major bactericidal mechanism, especially after exposure to lipopolysaccharide and IFN- γ (58). It is conceivable that YopJ-mediated killing of inflammatory monocytes reduces RNI production, while other type III effectors, such as YopH and YopE, can protect *Y. pseudotuberculosis* against phagocytic defense mechanisms of inflammatory monocytes and neutrophils that are upregulated by IFN- γ (36, 37a).

By comparing the host innate immune response to infection with *Y. pseudotuberculosis* wild-type and *yopJ* mutant strains, we found that the wild type induced significantly higher serum concentrations of the proinflammatory cytokines IFN- γ and IL-18. These results seemed at first paradoxical, since YopJ activity has traditionally been associated with suppression of proinflammatory cytokine production by macrophages and dendritic cells infected with *Yersinia* (8, 20, 45). One explanation for this result is that by promoting increased bacterial colonization, YopJ activity results in higher levels of PAMPs being presented to immune cells, and higher levels of cytokines are produced as a consequence. However, several observations suggest that YopJ activity directly elicits a proinflammatory response. First, higher levels of serum IFN- γ could be detected in mice infected with the wild-type strain than in those infected with the strain carrying the *yopJ* mutation even when spleen colonization levels were equivalent (compare Fig. 3 and Fig. 7A, day 4). Second, in mice infected with the wild-type strain there was a selective increase in levels of IFN- γ and IL-18 compared to those of TNF- α and IL-12(p70) on day 7 postinfection, which would not be expected if a higher load of PAMPs was the major cause of inflammatory cytokine production. Finally, we could show that YopJ activity leads to secretion of IL-18 from macrophages infected with *Y. pseudotuberculosis ex vivo*. Therefore, our data suggest the following scenario. During infection of systemic sites such as the spleen, macrophages, dendritic cells, and inflammatory monocytes infected with *Y. pseudotuberculosis* undergo YopJ-dependent cell death, leading to activation of caspase-1 (35) and secretion of IL-18. In turn, IL-18 stimulates NK cells and/or T cells to secrete IFN- γ .

Ectopic expression of YopP (O:8), which has a high level of cytotoxic activity, in *Y. pseudotuberculosis* decreases virulence and colonization of systemic sites following oral infection of mice (9). Similarly, ectopic expression of YopP (O:8) in *Y. pestis* decreases virulence and colonization of systemic tissues in mice infected by the subcutaneous route (73, 74). Several hypotheses have been forwarded to explain why high levels of cytotoxicity attenuate *Y. pseudotuberculosis* and *Y. pestis* (9, 73, 74). One suggestion is that macrophages or dendritic cells may be responsible for transporting *Y. pseudotuberculosis* from the intestinal tract to systemic sites and excessive cytotoxicity may decrease the efficiency of dissemination (9). Another suggestion is that YopP-induced cytotoxicity results in a rapid innate immune response that is protective against *Y. pestis* (73). Our

results show that a *Y. pseudotuberculosis* strain producing a YopJ protein of intermediate cytotoxic activity can elicit increased levels of proinflammatory cytokines IFN- γ and IL-18 in infected mice. Regarding IFN- γ , a host-protective cytokine during *Yersinia* infection, Brodsky and Medzhitov (9) found that serum levels of IFN- γ were lower in mice infected with YopP-expressing *Y. pseudotuberculosis* than in mice infected with the same strain expressing YopJ. So high IFN- γ levels are not associated with increased cytotoxicity of YopP. Therefore, if YopP action is attenuating *Yersinia* due to elicitation of a protective innate immune response, it is unlikely to be due to production of higher levels of IFN- γ . On the other hand, IL-18 levels could be directly linked to the cytotoxicity of YopJ, and IL-18 regulates both Th1 and Th2 responses (44). In addition, IL-18 plays an important protective role in mice during *Y. enterocolitica* infection (28). Whether IL-18 is the critical component of host innate immune response elicited by YopP action during infection deserves further investigation.

Results of previous studies have suggested that YopJ/P activity can inhibit the development of adaptive immune responses in mice infected with *Yersinia* (32, 37, 65, 67). However, a caveat of these previous studies is that immune responses in mice infected with *yopJ* or *yopP* mutant strains were compared to those of mice infected with other attenuated *Yersinia* strains but not to those infected with wild-type strains (32, 37, 65, 67). By establishing a sublethal infection model, we overcame the problem encountered in previous studies, namely, that doses used for infection of mice would be lethal for mice infected with wild-type strains. Comparison of the humoral immune responses of mice vaccinated with *Y. pseudotuberculosis* wild-type and *yopJ* mutant strains revealed similar antibody responses to bacterial antigens. Thus, our results are different from those obtained by Maia et al. (37), who found evidence of a role for YopJ in inhibiting production of immunoglobulins by splenic B cells in mice infected with *Y. pseudotuberculosis*. In our study, serum IgGs from wild-type- and *yopJ* mutant-immunized mice recognized a subset of Yops (YopM, -B, -D, and -E) as well as antigens encoded on the *Y. pseudotuberculosis* chromosome. Interestingly, we found that serum anti-LcrV IgG antibodies from both types of immunized mice were below the limit of detection by immunoblotting. It has been demonstrated recently that sera recovered from patients with *Y. pestis* Orientalis infection also lacked significant levels of IgG specific for LcrV in a protein microarray assay (34) and that mice vaccinated subcutaneously with *Y. pestis* KIM D27 also had extremely low levels of serum anti-LcrV IgG as determined by ELISA (49). These results suggest that in some infection conditions LcrV may not be an immunodominant antigen. In addition, our results indicated a slightly faster increase in the presence of serum IgG2a at 7 days after infection with 32777 (Fig. 9A). IL-18 has been shown before to promote the production of IgG2a, especially together with IL-12 (44). However, in our study this effect was transient, which may be related to the low serum IL-12 levels observed (Fig. 7).

Comparison of protective immune responses in mice vaccinated with wild-type and *yopJ* mutant *Y. pseudotuberculosis* was determined by challenge with lethal doses (100 or 100,000 LD₅₀) of *Y. pestis* KIM D27 in a septicemic plague model. Results showed that mice vaccinated with the wild-type strain did not have diminished protection, compared to mice immu-

nized with the *yopJ* mutant. In fact, there was a trend toward increased protection in mice vaccinated with the wild-type strain. These results argue that YopJ activity is not significantly impairing development of adaptive immunity during the infection process. It is conceivable that YopJ activity does inhibit priming of CD8 T cells to *Y. pseudotuberculosis* antigens, as was suggested from experiments with YopP, in which a *Y. enterocolitica* live carrier vaccine was used to vaccinate mice (32, 65), but in our experiments, if there is YopJ-mediated reduction in antigen-specific CD8 T cells, this process does not cause a measurable decrease in protection against *Y. pestis* infection. On the other hand, YopJ-induced production of IL-18 would be expected to enhance the differentiation of T helper 1-type CD4⁺ T cells, as well as the production of IFN- γ (1, 19), potentially enhancing adaptive immunity. A recent study obtained evidence that YopP activity could enhance the ability of *Y. pestis* to protect mice against lethal *Y. pestis* infection (73), although the basis of the enhanced adaptive immune response was not determined. Future studies to better understand how different YopJ/P isoforms regulate innate and adaptive immune response to *Yersinia* will likely lead to improved versions of live vaccines that can be used to vaccinate against yersiniae as well as other pathogens.

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