Chapter 10
Immunology of *Yersinia pestis* Infection

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Abstract As a pathogen of plague, *Yersinia pestis* caused three massive pandemics in history that killed hundreds of millions of people. *Yersinia pestis* is highly invasive, causing severe septicemia which, if untreated, is usually fatal to its host. To survive in the host and maintain a persistent infection, *Yersinia pestis* uses several stratagems to evade the innate and the adaptive immune responses. For example, infections with this organism are biphasic, involving an initial "noninflammatory" phase where bacterial replication occurs initially with little inflammation and following by extensive phagocyte influx, inflammatory cytokine production, and considerable tissue destruction, which is called "proinflammatory" phase. In contrast, the host also utilizes its immune system to eliminate the invading bacteria. Neutrophil and macrophage are the first defense against *Yersinia pestis* invading through phagocytosis and killing. Other innate immune cells also play different roles, such as dendritic cells which help to generate more T helper cells. After several days post infection, the adaptive immune response begins to provide organism-specific protection and has a long-lasting immunological memory. Thus, with the cooperation and collaboration of innate and acquired immunity, the bacterium may be eliminated from the host. The research of *Yersinia pestis* and host immune systems provides an important topic to understand pathogen-host interaction and consequently develop effective countermeasures.

Keywords *Yersinia pestis* • Immunology • Inflammatory • Innate immunity • Acquired immunity

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10.1 Interactions of *Yersinia pestis* and Immune Cells

Most human plague cases present clinically as one of three primary forms: bubonic, septicemic, or pneumonic plague. The most common form is bubonic plague, which is transmitted from rodent reservoirs to humans via the biting of infected fleas. Patients with primary bubonic plague can develop secondary septic or pneumonic infections, the latter of which can then be spread from person to person via respiratory droplets generated from severe sneezing and coughing of patients. Pneumonic plague is nearly always fatal unless treated with effective antibiotics within 20 h post symptom onset. All types of infection will cause a response of the host immune system against the invading *Yersinia pestis*.

During the early stages of infection, *Y. pestis* can enter both macrophages and neutrophils through either active or passive entry mechanisms [1]. Although early researches showed *Y. pestis* is typically killed in neutrophils, whereas in macrophages, it can survive and acquire antiphagocytic capabilities, which enables its extracellular survival in vivo. Recent studies indicated that a small percent of *Y. pestis* can also survive and replicate in neutrophils and send a PS (a marker of early apoptosis) to macrophages [2, 3]. This progress may provide *Y. pestis* with a less inflammatory route of entry into macrophages, resulting in decreased proinflammatory signaling. To survive inside the host and maintain a persistent infection, *Y. pestis* uses a variety of different mechanisms to evade or overcome the host immune system, especially the innate immune system, as shown in Fig. 10.1.

10.2 *Yersinia pestis* Overcomes the Immune Response

Y. pestis uses many different virulence factors to resist host immune responses, facilitate cellular attachment or invasion, subvert endocytic trafficking, block phagocytosis, modulate apoptotic pathways, and manipulate innate immunity and host responses as part of the initial infection process.

10.2.1 Dampening of the Inflammatory Response

Many studies have demonstrated that infection by *Y. pestis* elicits a notably delayed inflammatory response [4–6]. Type III secretion system (T3SS), which is one of the pathogens' major virulence factors, plays a crucial role in dampening host inflammatory responses. Multiple signaling pathways are repressed when the host is infected by *Y. pestis* by various *Yersinia* outer proteins (Yops). YopH inactivates the phosphatidylinositol-3 kinase (PI3K)-AKT cascade in macrophages, which correlates with the downregulation of mRNA coding for monocyte chemoattractant protein 1 (MCP-1) [7]. YopJ/P suppresses the mitogen-activated protein kinase (MAPK)

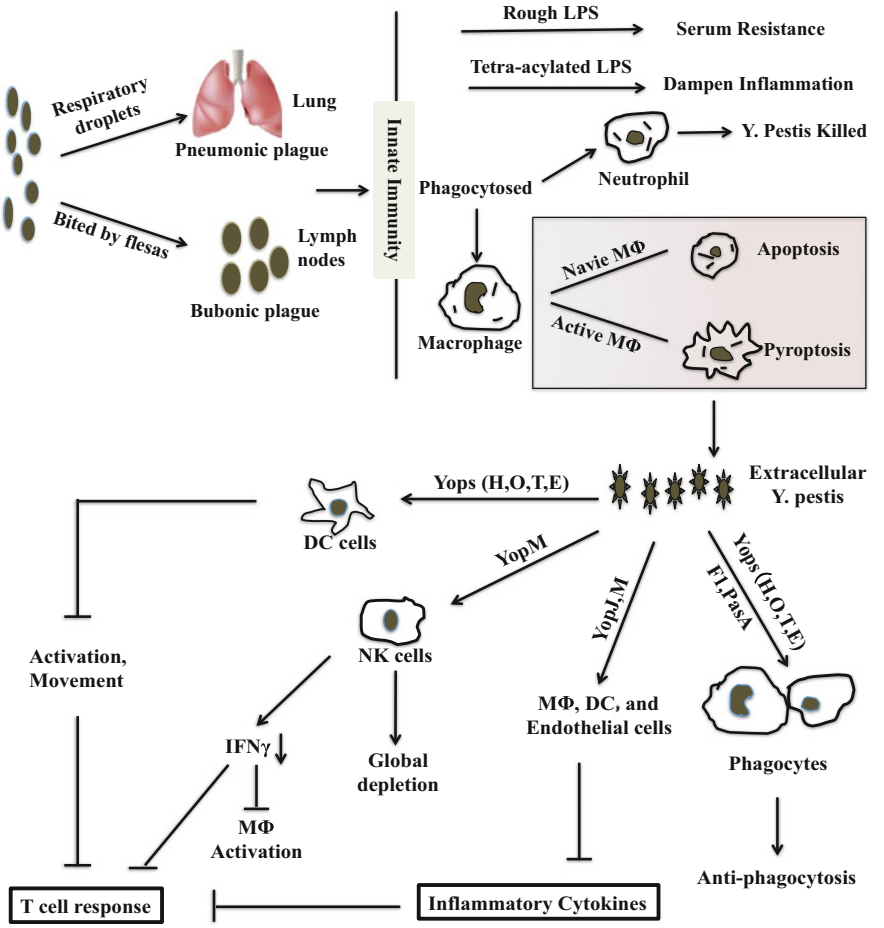

Fig. 10.1 Interactions between *Yersinia pestis* and the host immune system

signaling pathway by an unknown mechanism [8]. In vitro experiments indicated that YopE strongly inhibits nuclear factor-kappa B (NF-kB) activation and JNK and ERK activation, whereas YopT only moderately inhibits these responses [9]. YopK and YopM both inhibit inflammasomes [10, 11]. In addition, *Y. pestis* can suppress the production of cytokines and chemokines. LcrV, which is the needle tip protein that assembles into the needle tip complex, is associated with suppression of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) and the induction of immunosuppressive interleukin (IL)-10 in mice [12]. YopE can also prevent the production of IL-8 [9]. YopM interacts with protein kinase C-like 2 and ribosomal protein S6 kinase, which are also involved in proinflammatory signaling [13]. Recently, the N terminus of YscF was also found to decrease cytokine induction [14]. Inhibition of signaling pathways and suppression of cytokines not only reduces

the activation of natural killer (NK) cells and phagocytes but also destroys the inflammatory environment necessary for adaptive immunity.

Lipopolysaccharide (LPS) is a major component of the outer membrane in Gram-negative bacteria and a ligand for toll-like receptor 4 (TLR4). In different host-specific environments, the expression and formation of LPS in *Y. pestis* change accordingly [15, 16]. When the bacteria grow in the flea gut (21–26 °C), they produce a typical hexa-acylated LPS, which activates TLR4-mediated immune signaling to induce the expression of proinflammatory cytokines (TNFα, IL-1, IL-6, and IL-8). However, after the temperature transition from the flea (26 °C) to the mammalian host (37 °C), *Y. pestis* instantly begins to produce tetra-acylated LPS, which is nonstimulatory for TLR4 and an antagonist for the stimulatory hexa-acylated form of LPS. Importantly, a *Y. pestis* strain that produces hexa-acylated lipid A at 37 °C was found to be more than 100,000-fold attenuated in a mouse model of bubonic plague [17]. The attenuated phenotype is accompanied by increased production of TNFα and depends upon host expression of TLR4. It is thus clear that *Y. pestis* evades innate immunity, at least in part, by avoiding TLR-mediated activation of innate immunity.

10.2.2 Resistance to Phagocytosis

Because *Y. pestis* proliferates extracellularly, it is essential to block phagocytosis after contact with host cells. At least four Yop effectors (YopH, YopE, YopT, and YopO) are involved in inhibiting the phagocytosis of yersiniae; however, their mechanisms are all different. YopH is a tyrosine phosphatase, which acts on several cytoskeletal proteins including p130Cas [18–20]. Dephosphorylation of p130Cas disrupts its interaction with Crk and subsequent Rac activation required for *Yersinia* internalization [21]. YopE, which acts as a GTPase-activating protein for the RhoA family of GTPases (RhoA, Rac and Cdc42) [22], inhibits Rac-dependent actin polymerization either directly or through inactivation of the upstream RhoG [23, 24]. YopT is a protease that specifically cleaves RhoA, Rac, and Cdc42 near their carboxyl termini [25], which irreversibly inactivates host cell proteins. YopO interferes with the host cell regulation of Rho GTPases by actin [26–30]. Using various models, several different cellular activities have been attributed to YopJ, including deubiquination of Ikβ and TNF receptor-associated factors (TRAFs), as well as acetylation of MAPK kinases. However, precisely how YopJ exerts its activity during infection is presently unclear. In summary, the translocated Yop effectors are exotoxins that disable the phagocytic machinery by (1) disrupting the host cell cytoskeleton, (2) suppressing cytokine production, and (3) interfering with cell signal pathways.

Another crucial antiphagocytic factor is the F1 capsule protein (Caf1), which is solely produced by *Y. pestis*. However, the mechanism of resistance to phagocytosis induced by this capsule protein is different from that of T3SS and presumably by preventing interactions with receptors that could potentially recognize and result in

the uptake of pathogens [31]. PsaA was also shown to inhibit phagocytosis, most likely by binding lipoproteins that prevent recognition by host cells [32, 33].

10.2.3 Resistance to Complement-Dependent Bacteriolysis

Complement-dependent bacterial killing is one of the first lines of innate immunity against pathogens. *Y. pestis* must survive in blood to cause disease and be transmitted from host to host by fleas. Thus, resistance to serum complement is an essential phenotype for bacterial survival in blood. Unlike the enteropathogenic yersiniae (*Y. enterocolitica* and *Y. pseudotuberculosis*), which are fully resistant to complement when they are grown at 37 °C, but not when they are grown at 26 °C [5, 30], *Y. pestis* is constitutively resistant to complement at both 26 and 37 °C. In *Y. enterocolitica,* resistance to complement has been shown to involve YadA, Ail, OmpR, and LPS O antigen [34]. By binding to the complement regulatory proteins factor H and the C4b-binding protein, Ail and YadA provide resistance against complement-mediated killing [35, 36]. OmpR might alter the susceptibility of *Y. enterocolitica* to complement-mediated killing through remodeling of the outer membrane [37]. However, *Y. pestis* does not express YadA; therefore, *Y. pestis* LPS may mediate serum resistance [38], although there is no evidence of a direct role for LPS in serum resistance. Thus, Ail was found to play the crucial role in the inhibition of the bactericidal properties of complement by *Y. pestis*. Notably, there are four Ail/OmpX homologues encoded by the *Y. pestis* genome, but only *y1324* (in KIM strain, corresponding to YPO2506 in CO92, *ompX*) confers resistance to human serum. Deletion of this gene results in a rapid, essentially 100 % loss of serum resistance [39, 40]. This loss is attributed to the action of complement because heat-inactivated serum does not have lethal properties. Although currently the exact mechanisms of resistance to complement-mediated killing conferred by *Y. pestis* Ail are still unknown, based on the studies from two other yersiniae (*Y. enterocolitica* and *Y. pseudotuberculosis),* it is likely that the protein binds to negative regulators of alternative (factor H) and classical and lectin [C4b-binding protein (C4BP)] complement pathways, thus preventing complement-mediated attack of the pathogen [36, 41–43].

10.2.4 Other Impacts on Host Immunity

Many bacteria have evolved means to convert host plasminogen to plasmin, a protease that degrades extracellular matrix. In *Y. pestis* plasminogen activator (Pla), encoded by the *Y. pestis*-specific plasmid pPCP1, plays an important role. In comparison with the wild type, *Y. pestis* lacking Pla has been reported to have greatly reduced virulence when introduced via the intradermal and subcutaneous routes but produced equivalent or nearly equivalent virulence when introduced by the

intravenous route [5, 44, 45].These results partly different from early studies, which indicated even lack of plasmid pPst (also call pPCP1), did not lead to an increase in LD50 with either by subcutaneously or by respiratory route challenge [46, 47]. In the pneumonic plague model, the dissemination of Pla-deficient *Y. pestis* to the circulatory system was found to be unaffected, whereas bacterial growth in the lung was greatly reduced [44, 48]. Further studies are required to discern the specific mechanisms by which Pla impacts *Y. pestis* virulence. In addition, Pla has been reported to be a ligand for a macrophage and DC surface receptor, DEC-205, which is related to antigen presentation [49].

The 2-component regulatory system (2CS) OmpR-EnvZ is required to resist innate immunity during the early and late stages of the plague [50]. Different from *Y. pestis*' 23 other 2CSs, OmpR-EnvZ is the only one required for production of bubonic, septicemic, and pneumonic plague. In in vitro studies, OmpR-EnvZ was required to counter serum complement and leukocytes but was not required for the secretion of antiphagocytic exotoxins. In contrast, in in vivo studies, *Y. pestis* lacking OmpR-EnvZ did not induce an early immune response in the skin and was fully virulent in neutropenic mice.

10.3 Innate Immunity in Plague

Normally, once a bacterium infects the host, the innate immune response provides immediate protection and 4–5 days post infection, the T or B cell-mediated adaptive immune responses begins to provide organism-specific protection. In 2005, using Yop beta-lactamase hybrids and fluorescent staining of live cells from plague-infected animals; Marketon et al. found that *Y. pestis* selected immune cells for injection. Further research in vivo showed that macrophages, dendritic cells, and granulocytes/neutrophils are early targets for injection, whereas B and T lymphocytes were rarely selected. Thus, it appears that *Y. pestis* appears to destroy cells with innate immune functions that represent the first line of defense [51]. A similar study identified the pulmonary cells targeted by *Y. pestis* during primary pneumonic plague using a FRET-based probe to quantitate injection of effector proteins by the *Y. pestis* T3SS [52]. They found that these bacteria target alveolar macrophages early during infection of mice, followed by a switch in host cell preference to neutrophils. In addition to mouse models, the Gunnison's prairie dog, an important natural host of plague, was investigated [53]. This study highlights the importance of innate immunity against plague in wild Gunnison's prairie dogs.

10.3.1 Macrophages

Y. pestis has long been considered to be a facultative intracellular pathogen [54]. Macrophages are often regarded as permissive sites for survival and replication of *Y. pestis* at early stages of infection. Bubonic plague is typically initiated as an intradermal infection following the bite of an infected flea. Then, *Y. pestis* may invade the host directly through the skin and encounter phagocytes such as neutrophils and macrophages at the site of invasion. Most *Y. pestis* bacilli initially present are likely killed by neutrophils; however, the bacteria phagocytosed by macrophages can survive. *Y. pestis* preferentially infects host macrophages, probably via recognition of specific surface-associated CCR5 molecules [55]. This intracellular growth is essential for the pathogenesis of *Y. pestis* in three ways. First, macrophages provide a niche, allowing bacteria to proliferate and acquire the ability to evade phagocytosis. Second, intracellular growth in macrophages provides a protected environment for the bacteria to avoid contact with other components of the host immune system such as complement. Third, *Y. pestis* in macrophages can express various virulence determinants [56, 57]. Furthermore, macrophages may provide the bacterium with a vehicle for transport from the initial site of infection to deeper lymph tissues [58, 59]. The late stage of *Y. pestis* infection is characterized by a rapid increase in the number of bacteria within the spleen and escape of bacteria from macrophages into the extracellular compartment of the spleen. The cause of this escape is likely to be related to the macrophage necrosis or apoptosis observed during in vitro studies [57, 60]. Once *Y. pestis* replicates and expresses various virulence determinants in macrophages, they can be released into the extracellular compartment and spread systemically with the acquisition of phagocytosis resistance. Although *Y. pestis* can survive and replicate in macrophages during the early stage of infection, this macrophage compliance can be overcome in vitro by stimulation with a combination of IFNγ and TNFα [1].

Although macrophages provide a niche for *Y. pestis* survival and replication in the early stages of infection, *Y. pestis* can also cause macrophage death. Two distinct processes, corresponding to the inflammatory crescendo, are observed in vivo. *Y. pestis* causes apoptosis in naïve macrophages, but cell death in activated macrophages by inflammatory pyroptosis [61]. During macrophage apoptosis, *Y. pestis* YopJ is necessary. When the bacterial LPS contacts host macrophages, proapoptotic signaling is activated [62, 63]. LPS also upregulates cell survival genes and inflammatory cytokine production controlled by MAPK and NF-kB [62, 64, 65]; however, YopJ inhibits their activation [66, 67], and, therefore, apoptotic signaling predominates [62, 68]. Apoptotic cells are cleared by phagocytes, and this encounter triggers production of the anti-inflammatory cytokines TGFα and IL-10 [69, 70], making the process noninflammatory. Pyroptosis results from the activation of caspase-1, which is functionally distinct from the structurally related apoptotic caspases [36]. *Y. pestis*-induced pyroptosis requires plasmid-encoded T3SS, but not YopJ or any of the other known effector molecules [61]. Caspase-1 stimulates maturation and secretion of multiple inflammatory cytokines such as IL-1 and IL-18

[71]. Thus, pyroptosis causes inflammation. Why are two kinds of macrophage death necessary in *Y. pestis* infection? In bubonic plague, the infections are obviously biphasic: bacteria initially replicate without a measurable host response for periods up to 36–48 h, with a noticeable lack of inflammation, but, eventually, phagocyte influx into infected tissues and lymph nodes results in inflammation, cytokine production, and tissue necrosis [6, 72–74]. The change in mode of macrophage death is partly explained by observation of the host responses to *Y. pestis* infection. Apoptosis (noninflammatory death) [75–77] of naïve macrophages is consistent with initial bacterial growth in the relative absence of inflammation. Pyroptosis (inflammatory death) [61, 78] in activated macrophages corresponds to later stages of infection, accompanied by enhanced cytokine production and tissue damage. Induction of both apoptosis and pyroptosis in macrophages may be a mechanism by which pathogens preferentially trigger immune cell death, resulting in bacterial dissemination and disruption of host innate immune signaling.

The available data strongly suggest that *Y. pestis* growth within macrophages plays an important, perhaps critical, pathogenic role during plague [58]. During bubonic plague, macrophages may provide a protected intracellular niche that allows time for flea-transmitted *Y. pestis* bacilli to adjust to growth within mammals, in part by upregulating expression of capsular F1 protein, LcrV, and Yops, thus enabling subsequent growth as extracellular, phagocyte-resistant bacteria. However, in pneumonic plague the role of macrophages seems different from that in bubonic plague. Dr. Goldman's group found that depletion of roughly 92 % of alveolar macrophages had little or no effect on bacterial burden in the lungs [52]. They gave two possible reasons: macrophages are not involved in limiting yersiniae survival in the lungs or that *Y. pestis* is able to neutralize the antibacterial effects of sentinel alveolar macrophages, presumably as a result of expressing antiphagocytic/anti-inflammatory factors including the Yops, F1 capsular protein, and pH 6 antigen.

10.3.2 Neutrophils

Histological, flow cytometric, and laser confocal microscopy evidence indicates that *Y. pestis* phagocytosed by neutrophils are killed [1, 54]. However, *Y. pestis* primarily targets neutrophils early after inoculation in the lung, presumably to limit host innate immune mechanisms aimed at bacterial killing and clearance. Thus, it seems that the interaction between *Y. pestis* and host neutrophils may have a strong bearing on the outcome of infection [79].

Researchers often assess bubonic plague through needle-inoculated *Y. pestis*. However, Shannon et al. found that the innate cellular host responses to flea-transmitted *Y. pestis* differed from and were more variable than responses to needle-inoculated bacteria [80]. They therefore developed new tools allowing for intravital microscopy of *Y. pestis* in the dermis of an infected mouse after transmission by its natural route of infection, the bite of an infected flea. They found that uninfected flea bites typically induced minimal neutrophil recruitment. The magnitude of

neutrophil response to flea-transmitted *Y. pestis* varied considerably and appeared to correspond to the number of bacteria deposited at the bite site.

Dr. Goldman's group first identified the initial host cell targets of fully virulent *Y. pestis* during pulmonary infection. Using flow cytometry to monitor injection of a YopE-TEM fusion protein by the T3SS, they showed that *Y. pestis* initially targets CD11c high alveolar macrophages and neutrophils in the lungs. However, in contrast to the bubonic plague, during the first 24 h after pulmonary infection with a fully virulent *Y. pestis* strain, no significant changes were observed in the lungs in the levels of neutrophil infiltrate, the expression of adhesion molecules, or the expression of the major neutrophil chemoattractant CXCL1 (also known as keratinocyte cell-derived chemokine, KC) [4]. These results indicate that *Y. pestis* can slow the rate of neutrophil influx to the lungs by delaying the onset of chemokine and cytokine release. Moreover, neutrophils were observed to be "tightly packed" within spaces 72 h post infection during pneumonic plague [6]. However, in mice infected with an avirulent *Y. pestis* strain, early induction of chemokines, rapid neutrophil infiltration, and reduced bacterial burden were observed in the lungs of mice [81]. These results indicate that strain virulence may determine the host immune responses. Moreover, it seems that prevention of the early influx of neutrophils to the lungs is of major importance for *Y. pestis* virulence.

A growing volume of literature highlights interactions between *Y. pestis* and neutrophils, but the deletion of neutrophils during pneumonic plague shows significantly different results in various studies. Dr. Goldman's group showed that depletion of neutrophils had little to no effect on bacterial burden in the lungs [52]. This is in contrast to others studies, such as by Laws et al. who reported a modest increase in bacterial titers in lungs of infected mice early after neutrophil depletion [79]. Additionally, our group found neutrophil deletion with anti-Ly6G antibodies decreased survival in an intranasal *Y. pestis* mouse model [82]. Notably, a fully virulent *Yersinia pestis* strain, CO92, was used in Dr. Goldman's research; however, our study used a *Y. pestis* strain (strain 201) virulent to mice but nonvirulent to humans. Thus, the disparity in findings may come from differences in mouse lines and *Y. pestis* strains.

The mechanisms used by *Y. pestis* for targeting neutrophils have also been described. The *Y. pestis* adhesin Ail is required for efficient targeting of neutrophils in vivo [83]. The Ail protein was previously reported to inhibit the innate immune response, in particular the recruitment of a protective polymorphonuclear leukocyte (PMN) responses to the infected lymph node [84]. To identify factors conferring specificity to neutrophil targeting, a study investigated the role of serum [83]. They found that neutrophil targeting is mediated by complement receptor 3 (CR3) and, to a lesser extent, CD14. However, the exact nature of the receptor-ligand interactions and their contributions toward target cell selection remain unknown.

10.3.3 Other Innate Immune Cells

10.3.3.1 Dendritic Cells

Dendritic cells (DCs) are potent and specialized antigen-presenting cells, which help to generate more effective T helper cells. Therefore, an effective immune evasion strategy by a pathogen would be to target DCs and induce them to become tolerogenic, thus priming a regulatory IL-10 response that blocks inflammation and allows the pathogen to multiply without restraint. Furthermore, impairing DC maturation and promoting apoptosis of DCs can also help pathogens to disarm host defenses. During *Y. pestis* infection, DCs are one of the early targets of T3SS effectors [51]. Shannon et al. observed minimal interaction between *Y. pestis* and DCs; however, DCs consistently migrate toward flea-bitten sites containing *Y. pestis* [80]. The most pronounced effect of *Y. pestis* on DCs appears to be the paralysis of DC movement by impairing the cytoskeleton rearrangement function, attenuating the presentation of *Y. pestis* antigens by DCs [85]. Richard et al. found that the ability of *Y. pestis* to initiate DC activation is determined by its lipid A structure and depends on the pattern recognition receptor TLR4 [86]. In the bubonic plague model, IL-10 and TLR6 deficient mice are protected from plague infection, the mechanism of which was that TLR6 drove differentiation of tolerogenic DC and contributed to LcrV-mediated plague pathogenesis [87]. In plague vaccine studies, the interaction between vaccine and DCs also plays an important role. The protective mechanisms induced by rF1 + rV probably involve the activation of DCs, which initiate a primary immune response in naïve T cells [88]. Further study proved that LcrV targeting of DCs elicits combined humoral and cellular immunity and induces protection in a mouse model of pneumonic plague [89].

10.3.3.2 Natural Killer Cell

NK cells are a subset of lymphocytes that arrive at inflammatory sites and directly kill pathogen-infected cells without the recognition of antigenic peptides. Notably, *Y. pestis* can cause a global depletion of NK cells and decrease the secretion of IFNγ, resulting in reduced production of reactive nitrogen intermediates by macrophages. The cause of these anti-NK effects is the effector YopM, possibly by affecting the expression of IL-15 and its receptor IL-15Rα [90]. To elucidate whether $NK1.1^+$ cells were critical for the virulence effect of YopM, Ye et al. continued to test the effects of NK cell depletion on bacterial growth and found no effect of ablation on viable bacterial numbers of either the wild type or the YopM mutant strain in either the liver or spleen [91]. Thus, they concluded that NK cells are redundant for YopM's pathogenic mechanism.

10.4 Adaptive Immunity in Plague

In addition to innate immunity, *Y. pestis* infection can induce adaptive immunity. Adaptive immunity is characterized by the expansion, differentiation, and persistence of antigen-specific B and T cells. The primary function of B cells is to produce antibodies, thereby facilitating humoral defense, while the primary function of T cells is to produce phagocyte-activating cytokines, thereby facilitating cellular defense. However, during *Y. pestis* infection, humoral immunity and cellular immunity are not separated, and indeed they cooperate and collaborate with each other against the plague. For example, antibodies can protect T cell-deficient mice [92], and conversely T cells can protect antibody-deficient mice [93]. Therefore, characterization of *Y. pestis*-specific adaptive immune response in the host will provide a wealth of information for illustrating bacterial virulence and promoting the development of specific vaccines.

10.4.1 T Cell-Mediated Immune Responses to Yersinia pestis

A growing body of evidence demonstrates the crucial roles of T cell-mediated immunity against *Y. pestis*. A function of expanded pathogen-specific T cells is to secrete IFNγ and TNFα, and depletion of these proinflammatory cytokines prior to the passive transfer of the LcrV antibody into mice completely abrogated the protective effect observed in undepleted mice [94]. This indicates that a cellular proinflammatory response provides critical protective functions during humoral defense against lethal pulmonary *Y. pestis* infection. Direct evidence for the importance of the cellular response in protection against the plague came from studies in μMT mice, which are functional B cell-deficient mice and cannot produce antibodies. Transfer of *Y. pestis*-primed T cells into naïve μMT mice protected them against lethal intranasal *Y. pestis* challenge [93]. In another study, anti-F1 IgG transferred passively into T cell receptor knockout mice ($TCR^{-/-}$) at 24 h after infection was unable to rescue them, whereas it fully protected μMT mice, indicating that T cells play a critical role in the protection mediated by antibody to F1-antigen[95]. Thus, identification of *Y. pestis* antigens that stimulate a protective T cell response is one of the major goals for vaccine development. Using silico computer analysis and an in vitro IFNγ assay, we identified potential T cell antigens. In all, 34 individual proteins that stimulated a strong IFNγ response from splenocytes of mice immunized with *Y. pestis* live attenuated vaccine EV76 have been identified. Furthermore, in addition to LcrV, nine proteins may provide partial protection against challenge with a low dose of *Y. pestis* [96].

$CD4^+$ T helper (Th) cells include different subtypes based on their cytokine and transcription factor signatures [97]: Th1 cells produce IFNγ as their "signature" cytokine; Th2 cells produce IL-4, IL-5, and IL-13; Th17 cells produce IL-17; and regulatory T cells (Tregs) express the transcription factor FoxP3. Different T cell

lineages play different roles in *Y. pestis* infection. Signal transducer and activator of transcription (STAT)-4 knockout mice, which have a reduced Th1 response when immunized with F1/V, were poorly protected against *Y. pestis* challenge. However, STAT-6 knockout mice with intact Th1 responses and diminished Th2 responses were fully protected [98]. These results indicate that Th1-mediated immune mechanisms, activated following Stat 4 phosphorylation, are essential in protection against the plague. Therefore, one potential way to improve the efficacy of plague vaccines would be to increase the Th1 response. Dinc et al. assessed the efficacy of the novel SA-4-1BBL costimulatory molecule as a Th1 adjuvant to improve cellular responses generated by the rF1-V vaccine [99]. They proved that addition of SA-4-1BBL improves the efficacy of the subunit vaccine by generating a strong Th1 cellular immune response without significant impact on the generation of Ab responses. Additionally, Smiley's group demonstrated that vaccination with live attenuated *Y. pestis* induces Th17 cells [100]. IL-17 was found to contribute to defense against pulmonary *Y. pestis* challenge [82], although this IL-17-mediated protection does not appear to result entirely from enhanced bacterial clearance. These results indicate that plague vaccines aiming to induce mixed Th1 and Th17 cellular responses would provide more powerful and comprehensive protection.

In addition to $CD4^+$ T cells, specific $CD8^+$ T cell responses also contribute to defense against pulmonary *Y. pestis* infection, although alone they may be insufficient to combat fully virulent *Y. pestis* strains. YopE of *Y. pestis* was found to contain a dominant CD8 T cell epitope, which can be recognized by nearly 20 % of pulmonary CD8 T cells. Moreover, immunizing mice with a single peptide, $YopE_{69–77}$, suffices to confer protection from lethal pulmonary challenge [101]. A further study investigated the effector functions of $YopE_{69–77}$-specific CD8 T cells during pulmonary *Y. pestis* infection [102]. They concluded that specific CD8 T cell-mediated protection against pneumonic plague is dependent on TNFα and IFNγ, but not on perforin. In addition, $CD4^+$ and $CD8^+$ T cells were found to synergistically protect against pneumonic plague in this mouse model [103].

10.4.2 Antibody-Mediated Defense Against Yersinia pestis

Because of the complex antigen structure of *Y. pestis*, a number of antibodies are found in *Y. pestis*-infected patients and model animals. Serum samples collected from plague convalescent patients can transfer passive protection to naïve mice, indicating that antibody-mediated defense plays an important role against *Y. pestis* challenge. In previous studies, F1 and LcrV were proven to provide a high degree of protection, and the corresponding vaccines also showed efficacy in small animal models [104–107]. Using an antigen microarray, which contained more than 140 *Y. pestis* virulence-associated proteins, we screened the antibody responses of plague patients [108]. Apart from F1, YopD, YopE, and pH6 antigens, which have been described previously as immunogens, ten other novel immunogenic proteins were found. Antibody titers and persistence are correlated with vaccine efficacy. Previous

studies confirmed that the F1 antibody could persist for 1–4 years in humans [109]. Further studies in our lab explored the antibody profile from 65 plague patients who were in remission for more than 10 years using a protein microarray [110]. Results showed that antibody to F1 can persist in recovered patients for more than 10 years, while antibodies to LcrV and YopD were present for an even longer period of time. As artificial passive immunization has been demonstrated to be effective against *Y. pestis* infection in animals, how about maternal antibodies? We carried out a study evaluating the kinetics, protective efficacy, and transmission modes of maternal antibodies, using mice immunized with plague subunit vaccine [111]. The results indicated that maternal antibodies induced by the plague subunit vaccine in mother mice can be transferred to newborn mice via both the placenta and lactation were sustained for more than 10 weeks and provide early protection against plague for newborn mice.

Long-lived plasma cells and memory B cells are responsible for the long-term humoral immunity elicited by vaccination. Memory B cells are in charge of driving the rapid anamnestic antibody response that occurs after re-exposure to antigen. The serum antibody level is maintained by long-lived plasma cells. Thus, studies in our lab investigated the kinetics of memory B cell and plasma cell response in mice immunized with plague subunit vaccine F1 or the live attenuated vaccine EV76 [112]. The number of memory B cells in the spleens was significantly higher than that in the bone marrow, which is consistent with a previous study that found that the majority of memory B cells are present in the spleen [113]. We also found that the boost of antibody titer after revaccination may be dependent on the existence of memory B cells and an excess of antigen.

Although passive transfer of specific antibody can provide protection in rodents against pneumonic plague, in nonhuman primates vaccinated with F1/LcrV, high-titer specific antibody at the time of challenge cannot protect primates against pneumonic plague [114, 115]. These observations strongly suggest that antibody titer alone, at least as measured by standard ELISA, do not suffice in predicting the efficacy of pneumonic plague vaccines. Therefore, which factors are involved in antibody-mediated protection? First, neutrophils were found to contribute to antibody-mediated defense against pneumonic plague because neutrophil depletion abrogates serotherapy-mediated protection from pneumonic plague in a mouse model. Similar results were reported in a mouse model of septicemic plague, where neutrophil depletion abrogates protection mediated by polyclonal anti-LcrV [116]. Second, cytokines from phagocytes enhances antibody-mediated protection. Whether genetic deficiency or antibody neutralization of IFNγ and TNFα, cytokine depletion significantly impairs serotherapy-mediated protection [98, 117]. In addition, passive protection with antibody to F1 in infected mice was reduced in $C3^{-/-}$ mice that lack a functional complement system, indicating the contribution of Fc-effector mechanisms to antibody-mediated protection [95].

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