# PERSISTENT BACTERIAL INFECTIONS: THE INTERFACE OF THE PATHOGEN AND THE HOST IMMUNE SYSTEM

*Denise M. Monack, Anne Mueller and Stanley Falkow*

Abstract | Persistent bacterial infections involving *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhi (*S. typhi*) and *Helicobacter pylori* pose significant public-health problems. Multidrug-resistant strains of *M. tuberculosis* and *S. typhi* are on the increase, and *M. tuberculosis* and *S. typhi* infections are often associated with HIV infection. This review discusses the strategies used by these bacteria during persistent infections that allow them to colonize specific sites in the host and evade immune surveillance. The nature of the host immune response to this type of infection and the balance between clearance of the pathogen and avoidance of damage to host tissues are also discussed.

When a pathogenic microorganism first infects its host, there is usually a dramatic activation of the INNATE and ADAPTIVE immune responses, which can result in disease symptoms. If the pathogen and the host survive this initial interaction, the adaptive host immune system usually clears the invading offender. However, some pathogenic bacteria are capable of maintaining infections in mammalian hosts even in the presence of inflammation, specific antimicrobial mechanisms and a robust adaptive immune response, and can therefore be described as giving rise to persistent infection[1,2] (BOX 1; see TABLE 1 for a list of bacteria that cause persistent infections in humans). For example, *Helicobacter pylori* inhabits the human gastric mucosa and persistence of this bacterium in its host can be life-long[3]; *Mycobacterium tuberculosis* can establish long-term infections that can manifest as acute or chronic disease, or can be clinically asymptomatic with the potential to become reactivated later[4,5]; and *Salmonella enterica* serovar Typhi (*S. typhi*) causes systemic infection (typhoid fever) that involves colonization of the RETICULOENDOTHELIAL SYSTEM (RES). Some individuals who are infected with *S. typhi* become life-long carriers, periodically shedding large numbers of bacteria in their stools. Persistently infected carriers serve as the reservoir for these pathogens, and the carrier state is an essential feature that is required for survival of the bacteria within a restricted host population.

Persistent colonization with these bacterial pathogens is usually not clinically apparent. However, even in the absence of clinical symptoms, infection poses some risk to the host. Individuals who are infected with *M. tuberculosis* are at risk of reactivation of the pathogen to produce an active disease state that can be life-threatening. A significant proportion of people who are infected with *H. pylori* develop peptic or duodenal ulcers, or even gastric cancer[6]. In addition, individuals carrying *S. typhi* have an increased risk of developing hepatobiliary cancer[7]. The long-term residence of the bacteria in a privileged host niche — such as the MACROPHAGE vacuole or gastric mucosal layer — poses several fundamental biological questions. For example, what is the replicative and metabolic state of the bacteria during persistent asymptomatic infection, and how do these organisms manage to escape clearance for so long in the presence of the host immune response? We are only now beginning to understand the bacterial and host factors that are involved in the host–pathogen interaction during persistent infection, and the answers to these questions are likely to provide new and exciting directions for research in the fields of microbial pathogenesis and immunology.

INNATE IMMUNE RESPONSE
A cellular defence reaction that counteracts invading pathogens, such as bacteria and viruses. It uses interferon-dependent signalling and leads to the activation of genes that are responsible for bactericidal or antiviral responses.

ADAPTIVE IMMUNE RESPONSE
This involves specificity and immunological memory. It is mediated by T and B cells through the activation of cytotoxic $CD8^+$ T cells for pathogen killing or by interaction with $CD4^+$ T cells for antibody production.

*Department of Microbiology and Immunology, Stanford School of Medicine, Stanford University, Stanford, California 94305, USA.*
*Correspondence to D.M.M.*
*e-mail: dmonack@leland.stanford.edu*
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Box 1 | **Persistence versus commensalism**

When thinking about persistent bacterial infections, it is important to keep in mind the distinction between bacteria that are true commensals or part of our normal flora and those that can cause disease symptoms in certain circumstances. We believe that bacterial pathogens that are capable of persisting in a human host for long periods of time fall into at least two classes, both of which have characteristics that distinguish them from commensal species.

One class is defined by a group of organisms that, after causing an initial disease state, are kept in check by an adaptive immune response, but are not completely cleared from the host and persist in a privileged niche — perhaps inside host cells — for long periods of time. Examples of such species are *Helicobacter pylori*, *Salmonella enterica* serovar Typhi and *Mycobacterium tuberculosis*.

A second class of persistent bacterial pathogens are carried asymptomatically in the nasopharynx in most people among the commensal flora, although they still have the ability to cause life-threatening disease in seemingly immunocompetent individuals. *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* type B are perhaps the best-known members of this group. All of us at some time in our life are colonized, typically asymptomatically, by these species. The host and bacterial factors that contribute to the disease state are unknown, but the epidemiology of meningitis caused by *S. pneumoniae* and *N. meningitidis* shows that it is caused by the acquisition of a new serotype of the same species, rather than by superinfection with the strain that is already present[216].

How do the organisms in this second class of persistent bacteria colonize the human nasopharynx in an apparently silent manner? The virulence mechanisms of these organisms usually involve antiphagocytic capsules, immunoglobulin A (IgA) protease and antigenic variation of outer-membrane proteins[217]. However, an intriguing hypothesis that can be overlooked in this focus on disease is that these pathogenicity determinants have evolved to allow the bacteria to colonize deeper tissues in the nasopharynx, and not just the mucosal surfaces as organisms of the normal flora do. We propose that these organisms use virulence factors to penetrate the mucosal barrier and become resident in the nasopharyngeal-associated lymphatic tissue (NALT)[218] in the same way that *Salmonella* persists in the mesenteric lymph nodes adjacent to the Peyer's patches. In this way, the persistent bacteria in the NALT can re-seed the mucosal surface, which is constantly exposed to host cleansing mechanisms, such as those involving neutrophils and IgA. The trigger that causes colonization to go awry and develop into disease is presumably a combination of both host and pathogen physiological and genetic factors that shifts the delicate balance.

The ability to cause persistent infection is a fundamental aspect of the interaction between many diverse viral, bacterial and eukaryotic pathogens and their mammalian hosts. This review is not intended to address all or even a significant proportion of these pathogens. Rather, we will discuss several aspects of three persistent bacterial pathogens: *H. pylori*, a predominantly extracellular pathogen, and *M. tuberculosis* and *S. typhi*, which are both facultative intracellular pathogens. We believe that the information that is emerging from these studies will provide an insight into the general features shared by all microorganisms that have adapted to persist in the face of a highly evolved host immune system.

## Persistent mycobacterial infections

Pathogenic mycobacteria cause several long-term infections in their respective hosts. *M. tuberculosis* causes tuberculosis (TB), one of the oldest known human infectious diseases, and this bacterium is estimated to infect one-third of the global population[8]. Primary infection with *M. tuberculosis* involves replication of the organism at the initial pulmonary site of infection. This is followed by bacillaemia, in which small numbers of bacteria are disseminated to the extrapulmonary organs — such as the regional lymph nodes — as well as to uninfected portions of the lung, by a mechanism that may involve the migration of *M. tuberculosis* within DENDRITIC CELLS[9,10]. Adaptive immunity and restriction of bacterial growth occurs after this dissemination and is probably promoted by the arrival of bacteria in extrapulmonary lymphoid organs[4,11].

Most individuals resolve infection with *M. tuberculosis* soon after the onset of adaptive immunity[12]. However, in some infected individuals, the organisms are never completely cleared by the immune response[4]. Persistently infected individuals can harbour bacteria for many years, and even throughout their life. Infected individuals are at risk of experiencing the conversion of an asymptomatic infection into a highly contagious, clinically active and potentially deadly disease state that is known as reactivation TB. The risk of conversion from an asymptomatic infection to one that is clinically active is greatest soon after the initial infection, and this occurs most often in immunologically compromised individuals, such as newborns, the elderly and those who are infected with HIV[13,14].

## Mycobacterial survival at the immune interface

***Persistent mycobacteria reside in granulomas.*** Although the exact location of viable latent mycobacteria during persistent infections remains controversial, bacteria are often found inside macrophages within granulomas, which are formed in response to persistent intracellular pathogens[15] (FIG. 1). Tuberculous granulomas in humans and mice contain an organized collection of differentiated macrophages, T lymphocytes, some B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components[16,17]. Granulomas are thought to arise initially from aggregates of mononuclear phagocytes that surround individual infected macrophages. These macrophages become activated, and in many cases several macrophages fuse to form giant cells, which are also formed in response to other persistent

RETICULOENDOTHELIAL SYSTEM
A diffuse system of cells that helps the body fight infection and eliminate cellular debris through the action of phagocytic cells (such as macrophages), Kupffer cells in the liver and reticular cells of the spleen, bone marrow and lymph nodes.

MACROPHAGES
Cells of the mononuclear-phagocyte system that can phagocytose foreign particulate material. Macrophages are present in many tissues and are important for nonspecific immune reactions.

DENDRITIC CELLS
Professional antigen-presenting cells that take up proteins and present peptide antigens to T cells in conjunction with accessory molecules that stimulate T-cell activation. They are characterized by many long, thin processes extending from the cell body.

Table 1 | **Some persistent bacterial pathogens of humans**

| Pathogen | Disease conditions | Likely sites of persistence |
|---|---|---|
| *Mycobacterium tuberculosis* | Tuberculosis | Macrophages in various sites and in granulomas |
| *Salmonella enterica* serovar Typhi | Typhoid fever | Macrophages in bone marrow, the RES and possibly the gall bladder |
| *Chlamydia* spp. | *C. pneumonia* causes respiratory and cardiovascular disease; *C. trachomatis* causes trachoma, genital-tract infections and lymphogranuloma venereum | Epithelial and endothelial cells |
| *Helicobacter pylori* | Gastritis; ulcers; gastric cancer; MALT lymphoma | Extracellular; possibly also intracellular in the stomach |
| *Brucella* spp. | Brucellosis (this can be chronic, leading to lymphadenopathy and hepatosplenomegaly) | Macrophages in the RES |
| *Borrelia burgdorferi* | Lyme disease | Disseminated in various organs |
| *Bartonella henselae* | Cat-scratch disease; bacillary angiomatosis; bacillary peliosis hepatitis | Extracellular; in erythrocytes in blood |
| *Neisseria gonorrhoea* | Genital-tract infections, which can lead to epididymitis, pelvic inflammatory disease and infertility | Extracellular; intracellular at mucosal sites |
| *Neisseria meningitidis* | Invasive infection results in meningititis | Nasopharynx; NALT? |
| *Streptococcus pneumoniae* | Acute otitis media; bacteraemia; meningitis | Nasopharynx; NALT? |
| *Streptococcus pyogenes* | Acute pharyngotonsillitis; pneumonia; endocarditis; skin, soft tissue and bone infections (necrotizing fasciitis) | Nasopharynx; NALT? |
| *Haemophilus influenzae* type B | Pneumonia; meningitis; bactereamia | Nasopharynx; NALT? |

NALT, nasopharyngeal-associated lymphatic tissue; RES, reticuloendothelial system.

infections, particularly those caused by viruses[15]. T lymphocytes and other immune cells are recruited early during the process of granuloma formation[18]. The lesion that is formed is sealed off from surrounding tissue by epithelioid cells, which have tightly interdigitated cell membranes that form zipper-like arrays and link adjacent cells, and which can also be fibrotic and calcified. In the centre of granulomas there is usually an area of caseous necrosis — a region of cellular debris that has a distinct appearance.

How does *M. tuberculosis* survive within these lesions for so many years? One hypothesis is that persistent bacteria are either in a non-replicative state or only have low levels of replication within the amorphous debris at the caseous centre of the granuloma[19]. Evidence in humans that the persisting bacteria are in a dormant state comes from the results of culturing and staining diseased tissues from patients who have undergone chemotherapy, which might have resulted in false-negative culturing results[19-21]. An alternative hypothesis as to how a constant bacterial load is maintained is that there is a balance between active bacterial replication and killing by the immune system. This is an active area of research and is discussed in more detail below.

***Survival within macrophages.*** Pathogenic mycobacteria initiate long-term infection by entering host macrophages[22], after which they cause extensive remodelling of the PHAGOSOMAL environment to prevent the normal maturation of this organelle into an acidic, hydrolytic compartment[23]. The ability of pathogenic mycobacteria to replicate and/or survive in macrophages has an essential role in persistence *in vivo*, and is a feature that distinguishes pathogenic from non-pathogenic strains[22].

As infected macrophages are the main reservoir of infection by pathogenic mycobacteria, studies of the biology and biogenesis of the mycobacteria-containing phagosome have generated information that is important for the understanding of the cell biology, immunology and microbiology of these pathogens[23]. Many groups have described the trafficking of mycobacteria within unactivated macrophages in tissue-culture experiments. In brief, mycobacteria interfere with phagosome maturation by blocking the fusion of nascent phagosomes with endosomal and lysosomal compartments and by causing alterations in membrane proteins that normally promote the formation of an acidic phagolysosome. The steps involved in this process are beyond the scope of this review, but are described in detail in REF. 24

In addition to the ability to block phagosome maturation in some circumstances, pathogenic mycobacteria have also evolved mechanisms that allow them to persist in macrophage phagolysosomes, within granulomas, in the presence of a host immune response. A recent study indicated that within frog granulomas ~60% of intact bacteria of the species *Mycobacterium marinum* — which is closely related to *M. tuberculosis* — resided in phagolysosomes, and the level of phagolysosomal fusion correlated with the level of macrophage activation[25]. Therefore, it is possible that mycobacteria have at least two mechanisms of adaptation to intramacrophage survival: restriction of phagolysosomal fusion early in infection and adaptation to phagolysosomal fusion within the activated macrophages of granulomas later

PHAGOSOME
A membrane-bound, cytoplasmic vacuole formed around particles that are ingested by phagocytosis.

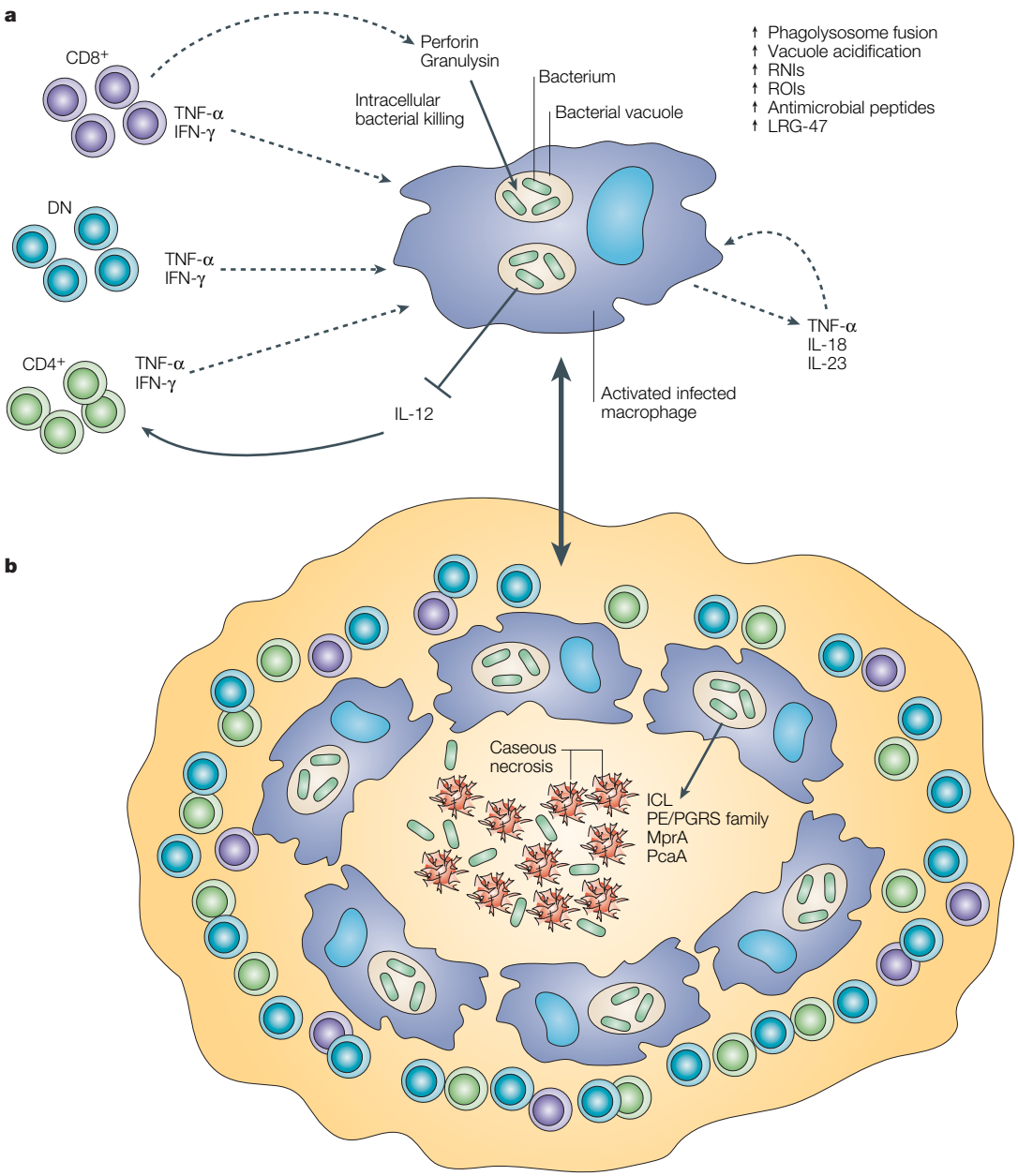


Figure 1 | **Persistent mycobacterial infection and the host immune response. a** | Pathogenic mycobacteria use several receptors to enter macrophages, where they reside in a unique vacuole. The amount of mycobacterial replication is controlled by many host immune factors. Effector T cells (including $CD4^+$ and $CD8^+$ T cells and double-negative (DN) T cells) and macrophages participate in the control of infection. Interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α) produced by T cells are important macrophage activators. Macrophage activation promotes phagosomal maturation, vacuole acidification and the production of antimicrobial molecules, such as reactive nitrogen intermediates (RNIs) by nitric oxide synthase 2 (NOS2), reactive oxygen intermediates (ROIs), antimicrobial peptides and the NOS2-independent 47-kDa guanosine triphosphatase protein LRG-47, which can block bacterial replication. The production of the proinflammatory cytokines TNF-α, interleukin (IL)-18 and IL-23 by activated macrophages also contributes to controlling the intracellular replication of mycobacteria. The ability of mycobacteria to inhibit the secretion of IL-12 by infected macrophages might contribute to bacterial survival, as this cytokine normally functions to induce the production of IFN-γ. **b** | In most persistent mycobacterial infections, the bacteria are initially contained in granulomas. Tuberculous granulomas are thought to arise from aggregates of phagocytic cells that surround individual infected macrophages. These structures contain many T and B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components (for simplicity, only T cells are shown here). Another striking feature of certain tuberculous granulomas is the presence of caseous necrosis in the centre of the granuloma. Some of the genes that are specifically expressed by mycobacteria in granulomas encode the following proteins: isocitrate lyase (ICL)[60], an enzyme essential for the metabolism of fatty acids; outer-membrane proteins of the PE/PGRS family that might have a role in antigenic variation; the transcriptional regulator MprA, which is involved in the regulation of unidentified genes during adaptations that are required for persistence[61]; and PcaA, which encodes a cyclopropane synthase. Mycobacteria that lack PcaA have reduced levels of persistence in the chronic mouse model[63].

**Box 2 | Insights into host–pathogen interactions gained from genetics**

Host genetic factors strongly determine the outcome of infectious disease. However, the molecular mechanisms of resistance and susceptibility in humans are only just beginning to be investigated. Mouse models of human infectious disease have been used to identify and map host loci that are involved in controlling the complex aspects of host–pathogen interactions (for recent reviews, see REFS 128,219). Three main approaches have been taken to identify these loci: production of mouse mutants by gene targeting; positional cloning of host-resistance genes in mutant mice; and mapping and characterization of quantitative trait loci (QTL) that control the complex aspects of host–pathogen interactions.

In some cases, the results of knockout-mouse studies have provided important information about the genetic basis of susceptibility to bacterial infections in humans (TABLE 2). For example, mice with null mutations in the genes encoding interferon-γ (IFN-γ) and either of the subunits of the IFN-γ receptor have been shown to be susceptible to infection with pathogens such as *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and *Mycobacterium tuberculosis*, among others[219,220]. The investigation of a paediatric syndrome — known as idiopathic mycobacterial infection or Mendelian susceptibility to mycobacterial infection — led to the identification of various human mutations in the genes encoding the IFN-γ receptor, interleukin-12 (IL-12) and the IL-12 receptor. These mutations lead to deficiencies that abrogate IFN-γ-mediated and IL-12-mediated immunity. The phenotype of patients with this syndrome is an increase in the occurrence and severity of infections with mycobacteria that are usually poorly pathogenic, such as the bacille Calmette–Guérin strain, and non-typhoidal salmonella, such as *Salmonella enteritidis*. IFN-γ and IL-12 are therefore indispensable for bactericidal granuloma formation and protective immunity against mycobacteria and salmonella in mice and humans. By contrast, *Helicobacter pylori* does not seem to cause invasive or more severe disease in immunocompromised or very young individuals. For example, IFN-γ-knockout mice are colonized with higher numbers of *H. pylori* during the first 4 weeks post-infection, but the mice survive. Indeed, persistent *H. pylori* infection of IFN-γ-knockout mice resulted in less gastric inflammation even in the presence of high levels of bacteria. So, IFN-γ and other pro-inflammatory cytokines (TABLE 2), which are ordinarily necessary for controlling bacterial infections, might actually contribute to the histological changes that are associated with *H. pylori* infection, such as atrophic gastritis, intestinal metaplasia and dysplasia — conditions that can lead to the development of *H. pylori*-induced gastric cancer.

in infection. It is still unclear how the bacteria sense these different intramacrophage environments; the identification of the bacterial effector proteins that are involved in this should provide considerable insight into this crucial stage of mycobacterial pathogenesis.

In further support of the proposal that mycobacteria use different strategies according to the circumstances, recent publications indicate that mycobacteria have temporal and immune-response-triggered differences in gene expression both in activated macrophages *in vitro* and in macrophages isolated from infected tissue[26–31]. In addition, a recent study showed differential mycobacterial survival in type 1 (interleukin-23 (IL-23)-producing) and type 2 (IL-10-producing) macrophages[32]. The immune status of the macrophage therefore has an important role in bacterial persistence. Indeed, the control of bacterial growth in murine models of latency requires interferon-γ (IFN-γ), tumour-necrosis factor-α (TNF-α) and nitric oxide (NO)[16], all of which can alter the environment of the bacteria-containing phagosome and lead to killing of the pathogen.

IFN-γ is a crucial component of immunity to TB as it activates infected host macrophages, which directly inhibit the replication of *M. tuberculosis*. The importance of this molecule in the control of mycobacterial infections is highlighted by the discovery of IFN-γ-related genetic mutations that predispose affected individuals to active TB, as well as to other infections that are caused by intracellular bacterial species, such as *Salmonella* spp.[33] (BOX 2; TABLE 2). IFN-γ induces the expression of NO synthase 2 (NOS2) and of the newly identified, NOS2-independent, 47-kDa guanosine triphosphatase protein LRG-47, and both pathways are important in controlling intracellular *M. tuberculosis* replication[34–37]. However, a proportion of the bacteria are clearly still able to survive in macrophages, perhaps by a mechanism that involves inhibiting STAT1-mediated IFN-γ transcriptional responses[38] and/or suppressing the secretion of IL-12 — a proinflammatory CYTOKINE that acts to amplify IFN-γ production[39,40] — which may in part be mediated by the *M. tuberculosis* Snm secretion pathway[41]. It is also likely that the ability to resist killing by antimicrobial peptides contributes to mycobacterial survival in macrophages[42].

## Animal models of mycobacterial persistence

From both a therapeutic and an epidemiological viewpoint, the study of persistent mycobacterial infections in animal models is very important given the difficulties of studying latent TB in humans. Some research has been done using guinea pigs, which are able to arrest the initial acute phase of bacterial replication during *M. tuberculosis* infection[43]. However, although the resulting pathology resembles that seen in human disease, these animals succumb to the pathological consequences of infection[43]. Persistent infection can also occur after intratracheal infection of cynomolgus monkeys[44,45]. Although it is likely that this closely mimics human infections, high costs limit the widespread use of this non-human primate model. Therefore, many research groups commonly use mouse models of *M. tuberculosis* infection or frog and fish models of *M. marinum* infection. Although much information can be gained from these models, care should be taken in extrapolating the results obtained with these models directly to human TB.

CYTOKINES
Low-molecular-weight proteins that are important for immunity, inflammation and development, and which contribute to the pathophysiology of acute and chronic infections.

Table 2 | **Genes involved in susceptibility to *M. tuberculosis*, *Salmonella* serotypes and *H. pylori***

| Gene product | Phenotype in knockout mice* | Phenotype in humans | References |
|---|---|---|---|
| ***Mycobacterium tuberculosis*** | | | |
| TNF-α p55 receptor | High susceptibility; increased granuloma necrosis | U | 75 |
| TNF-α | High susceptibility; increased granuloma necrosis | U | 75 |
| IFN-γ | High susceptibility; increased granuloma necrosis | I | 128,221,222 |
| IL-12 (p40) | High susceptibility; defective granuloma formation | I | 128,223,224 |
| IL-12Rb1 | High susceptibility | I | 225–227 |
| IL1α/β | Moderate susceptibility; larger granulomas, but no necrosis | U | 228 |
| IL-18 | Moderate susceptibility; larger granulomas in lungs | U | 229 |
| NOS2 | High susceptibility; necrotic granulomatous pneumonitis | U | 35 |
| NRAMP1 | High susceptibility associated with mice homozygous for the Nramp1D169/D169 allele | I | 94 |
| TRL3 | Increased resistance | U | 230 |
| TRL4 | Increased resistance | U | 230 |
| ***Salmonella* serotypes** | | | |
| NF-IL6 | High susceptibility; impaired macrophage killing of bacteria | U | 231 |
| TNF-α p55 receptor | Higher susceptibility; reduced clearance of bacteria from spleen and liver | U | 232 |
| IFN-γ | High susceptibility | I | 233 |
| IL-12 (p40) | – | I | 128 |
| IL-12Rb1 | – | I | 128 |
| LPS-binding protein | High susceptibility | U | 234 |
| NOS2 | High susceptibility; increased bacterial replication in early phase of infection | U | 235 |
| NRAMP1 | High susceptibility associated with mice homozygous for the Nramp1D169/D169 allele | U | 94 |
| ***Helicobacter pylori*** | | | |
| IFN-γ | Higher numbers of bacteria early in infection; no inflammation in persistently infected mice | U | 236 |
| IL-1β | – | Cancer‡ | 237 |
| IL-1β receptor | – | Cancer‡ | 238 |
| TNF-α | – | Cancer‡ | 239 |
| IL-10 | More severe gastritis followed by bacterial clearance | Cancer§ | 116, 239 |

*The phenotype listed for mice is for experimental infections with virulent bacteria. ‡Human polymorphisms that are associated with higher levels of expression have been linked to an increased risk of gastric cancer and its precursors. §Human polymorphisms that reduce expression of the anti-inflammatory cytokine IL-10 are associated with increased risk of distal gastric cancer. I, proven susceptibility in humans where normal individuals do not become infected with *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium bovis* bacille Calmette–Guérin, *Mycobacterium smegmatis*, *Mycobacterium intracellulare*, other non-pathogenic mycobacterial strains, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis*, *Salmonella enterica* serovar Paratyphi, and group B *Salmonella*. U, unknown or no proven susceptibility in humans. IFN-γ, interferon-γ; IL, interleukin; IL-12Rβ1, IL-12 receptor β1; LPS, lipopolysaccharide; NF-IL6, nuclear protein IL6; NOS2, nitric oxide synthase 2; TRL, tuberculosis-resistance locus; TNF-α, tumour-necrosis factor-α.

***Mouse models of* M. tuberculosis *infection.*** Several mouse models of TB latency have been established[46–48]. The Cornell mouse model (also known as the drug-induced model) involves partial clearance of *M. tuberculosis* infection by incomplete chemotherapy; the infection is reduced to a point at which no bacterial colonies are recovered[46]. Drug intervention is needed to induce the latent state, which is not necessary in human disease. The low-dose mouse model of latent TB (also known as the chronic or plateau model) involves aerosol infection or infection by intravenous routes. This results in an initial acute phase of bacterial replication that is controlled by the onset of an adaptive immune response, followed by a stable maintenance at high levels in the lung over many months. The mice seem healthy until the disease reactivates, which can take place as much as 18 months later[48]. This chronic model resembles latency in humans in that it depends on the host immune response to contain the infection. However, unlike latent TB in humans, this model results in large numbers of bacteria, which leads to pulmonary damage that steadily accumulates in the lungs of chronically infected animals[49].

Although these mouse models have limitations, several groups have used them to address the metabolic state of persistent mycobacteria. Rees and Hart

compared total microscopic counts of bacteria in lungs from infected mice with counts of viable bacteria over a period of several months[50]. The number of bacteria obtained by both techniques remained constant. One interpretation of these findings is that the bacteria were replicating either very slowly or not at all. However, it is possible that any difference between the total number of bacteria visualized and the number of viable bacteria was not within the limits of detection of the methods used.

More recently, other groups have used mouse models to compare bacterial gene expression under *in vitro* conditions that induce a non-replicating or dormant bacterial state (that is, conditions of nutrient deprivation[51] and oxygen depletion[52]) with *in vivo* bacterial gene expression. These studies used either quantitative real-time PCR (qRT-PCR) or cDNA microarray techniques[26,27,30]. One such study of *M. tuberculosis* gene expression in infected mice showed that α-CRYSTALLIN and genes of the DosR regulon are highly expressed in response to host immunity mediated by type 1 HELPER T CELLS ($T_H1$ cells)[27]. DosR seems to have a crucial role in mediating the expression of a series of hypoxia-induced and NO-induced *M. tuberculosis* genes[53–55] — a transcription pattern that is characteristic of the non-replicating persistence that is associated with the adaptation of tubercle bacilli to hypoxia *in vitro*[52]. It was inferred from this study that host immunity induces the arrest of bacterial growth[27].

More recently, qRT-PCR has been used to measure the levels of selected *M. tuberculosis* mRNAs during laboratory culture, *in vivo* in the lungs of mice and in lung tissue from four chronically infected humans[26]. In culture, the differential expression of *M. tuberculosis* mRNAs that are associated with iron limitation, alternative carbon metabolism and cellular hypoxia — conditions that are thought to occur in granulomatous lesions associated with TB — correlated with those that were seen in bacteria isolated from wild-type mice. However, in bacteria isolated from human TB lung specimens, this set of mRNAs did not show the same expression patterns as those seen in mice. This might reflect host-specific differences or the fact that the human lung tissue was not microdissected, resulting in mixed bacterial populations from different environments. The latter hypothesis is supported by a recent study that used DNA microarrays to examine genome-wide expression profiles of *M. tuberculosis* isolated from human lung tissue that was surgically removed from patients who had not been treated with antibiotics, but were infected with high levels of bacteria. The gene-expression profiles of *M. tuberculosis* were shown to be characteristic of the site of infection (caseous centres of granulomas, pericavities or distant lung), indicating that *M. tuberculosis* actively senses and responds to its microenvironment (H. Rachman and S. H. E. Kaufmann, personal communication).

***Frog and fish models of* M. marinum *infection.*** Other animal models have been developed more recently that use *M. marinum*, which causes a TB-like disease in ectothermic hosts such as frogs and fish and is a useful model system to study mycobacterial pathogenesis[22]. Experiments using the *M. marinum* model have indicated that persistent mycobacteria are in a metabolically and replicatively active state. Bouley *et al.* have shown by transmission electron microscopy in conjunction with immunohistochemistry and acid phosphatase cytochemistry that even long-term, single granulomas are surprisingly dynamic environments, within which bacterial replication and phagocytic killing occur simultaneously[25]. In support of this, using the attenuated BACILLE CALMETTE–GUÉRIN strain in rabbits, Dannenberg and colleagues arrived at a similar conclusion[56]. These data indicate that mycobacteria are not in an inert or 'spore-like' state, and that relatively constant bacterial numbers are maintained in the presence of an active immune response. Furthermore, studies using DIFFERENTIAL FLUORESCENCE INDUCTION to analyse *M. marinum* gene expression in granulomas showed that most of the promoters that were found to be induced drive the expression of genes that encode proteins with metabolic and synthetic functions and that are expressed during logarithmic-phase growth in laboratory media[57]. These results indicate that persistent mycobacteria are metabolically active; however, they do not prove that they are actively dividing. Furthermore, these studies were performed using the relatively stable reporter green fluorescent protein (GFP), so that downregulation of bacterial gene expression within granulomas might not have been detected. It is therefore possible that persisting bacteria exist as a mixed population, in which some are actively replicating and others are in an inactive state.

## Mycobacterial persistence factors

In recent years, our understanding of mycobacterial pathogenesis has advanced rapidly. Many genes that are important for pathogenesis have been identified in virulence expression screens and mutant screens, and this work has been reviewed recently[22]. Sasseti *et al.* have combined mutagenesis using the mariner transposon with microarray technology to determine the genes that are required for mycobacterial growth under certain *in vitro* conditions (a method called TraSH, for transposon site hybridization)[58]. When this method was applied to a mouse model of infection, several genes were found to be required at different stages after infection[59]. One class of mutants — known as persistence mutants — are able to establish an infection to the same level as wild-type bacteria, but are unable to maintain levels of bacteria in the lungs to the same extent as wild-type strains.

The further characterization of these persistence mutants awaits further studies. However, a number of *M. tuberculosis* genes have previously been indicated to be important for persistent infection in the chronic mouse model on the basis of gene-expression studies and experiments using bacteria carrying knockout mutations (reviewed in REF. 5). Most notably, the results of McKinney *et al.* show that persistence in mice is facilitated by isocitrate lyase (ICL)[60], an enzyme that is essential for the metabolism of fatty acids. Disruption of the *icl* gene attenuated bacterial persistence and virulence in immunocompetent mice, without affecting bacterial

α-CRYSTALLIN
The expression of this chaperonin protein is upregulated *in vitro* by hypoxia.

HELPER T CELLS
A subpopulation of activated $CD4^+$ T cells that secrete characteristic cytokines and function primarily in cell-mediated responses by promoting the activation of cytotoxic T cells and macrophages.

BACILLE CALMETTE–GUÉRIN
The attenuated *Mycobacterium bovis* live vaccine.

DIFFERENTIAL FLUORESCENCE INDUCTION
A selection strategy used to identify bacterial genes that are preferentially expressed when a bacterium is in a particular environment. By inserting random pieces of bacterial DNA in front of a promoterless green fluorescent protein (GFP) gene, flow cytometry can be used to screen for genes expressed in specific environments.

growth during the acute phase of infection. These data indicate that during late stages of infection, *M. tuberculosis* cells might convert lipids into carbohydrates through the GLYOXYLATE-SHUNT PATHWAY[60] and that latent bacteria might reside in an environment such as lung granulomas, in which carbohydrates are limited but lipids are available.

Several other persistence mutants have been identified. One of these affects the transcriptional regulator MprA, which is involved in the regulation of unidentified genes during adaptive responses that are required for persistence[61]. In addition, several mutants have been identified that have alterations in their cell walls[62]. For example, the *M. tuberculosis pcaA* mutant (*pcaA* encodes a cyclopropane synthase) shows reduced levels of persistence in the chronic mouse model[63]. The roles of these bacterial genes in persistent infections and their functions in immune modulation await further studies.

## Immune responses to persistent mycobacteria

Substantial progress has been made in understanding the immune-system mechanisms that are involved in the containment of the initial phase of *M. tuberculosis* infection (reviewed recently in REFS 4,5,16). However, less is known about the immune mechanisms that are involved during persistent mycobacterial infections. In addition to reactivation, recent work using molecular-fingerprinting techniques has documented the reinfection of immunocompetent individuals with new strains of *M. tuberculosis*[22]. These data show that immunity to TB can be incomplete, and indicate that reinfection, at least in areas where TB is prevalent, probably has a greater role than was previously appreciated.

Indeed, the dynamic nature of mycobacteria and their interactions with granulomas during persistent infection is highlighted by the recent findings of Cosma *et al.* This group showed that exogenously infecting *M. marinum* in zebrafish rapidly enter pre-existing granulomas by specific mycobacteria-mediated mechanisms that direct infected macrophages into granulomas[64] (FIG. 1). To probe the cellular dynamics of mycobacterial reinfection *in vivo*, Cosma and colleagues followed the route of superinfecting *M. marinum* or an unrelated *Salmonella* strain — which were labelled with macrophage-inducible GFP reporter constructs — in the context of a previously established infection. The superinfecting *M. marinum* — and not the *Salmonella* strain — trafficked into pre-existing granulomas, rapidly expressed granuloma-specific promoters and remained in these lesions for up to 2 months[64]. These findings indicate that mycobacteria rapidly adapt to the mature granuloma environment and provide new insights into the interactions of these bacteria with the adaptive immune system.

The role of the adaptive immune response in persistent mycobacterial infections is an area of great interest. There is some evidence that $CD8^+$ T cells secrete most of the IFN-γ that is produced during persistent infection in the chronic mouse model. This is in contrast to the acute phase of disease, during which $CD4^+$ T cells produce most of the IFN-γ[65,66], indicating a differential activation of T-cell subsets in these two phases of infection[4]. Whether or not this is the case, $CD4^+$ T cells have an important but as yet undefined role in the control of persistent infection[67]. Although other activities of $CD8^+$ T cells, in addition to IFN-γ production, might also be important for the control of persistent infections[68-70], further studies are needed to determine the exact roles of these cells.

The production of immunosuppressive cytokines — such as IL-10 and transforming growth factor-β (TGF-β) — has been documented in humans with active TB[71-73], and IL-10 production is increased in the lungs of mice that show chronic mycobacterial infection. This indicates that the ability of IL-10 to downregulate the immune response might contribute to the reactivation of chronic *M. tuberculosis* infection[74]. In animal models, the proinflammatory cytokine TNF-α has a key role in host responses against TB[75,76], including granuloma formation and the containment of disease[18,77]. Furthermore, treatments with antibodies that neutralize TNF-α cause reactivation of TB in a mouse model of latent infection[78] and in human latent infection, as shown by the clinical observation of reactivation of TB during the treatment of autoimmune disease[79]. TNF-α therefore has a significant role in the control of persistent *M. tuberculosis* infections and, as discussed below, is equally important in persistent *Salmonella* infections.

## Persistent *Salmonella* infections

*Salmonella* serovars are responsible for human diseases ranging from gastroenteritis to systemic infections. Systemic *Salmonella* infection is usually host-dependent, and *S. typhi* causes only systemic infection — typhoid fever — in humans. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) infection of mice and *S. typhi* infection of humans is characterized by inflammation at the site of bacterial entry, which is typically at the PEYER'S PATCHES[80]. After *Salmonella* spp. penetrate the epithelial barrier, they preferentially infect phagocytes within the lamina propria (FIG. 2). In *Salmonella* gastroenteritis, the infection is usually self-limiting and does not proceed beyond the lamina propria. However, in host-adapted salmonellosis, such as typhoid fever, *Salmonella*-infected phagocytes gain access to the lymphatics and bloodstream, allowing the bacteria to spread to the liver and the spleen[81], and can persist in the gall bladder and bone marrow[82,83].

*S. typhi* and *Salmonella enterica* serovar Paratyphi *(S. paratyphi)* serovars are important human pathogens of immense concern to public health and with considerable economic impact. They are endemic in regions of the world where drinking-water quality and sewage-treatment facilities are poor[1,84] and infections remain difficult to treat by antibiotic therapy due to the increasing frequency of resistant bacteria[85]. A significant percentage (1–6%) of typhoid patients become chronic carriers of *S. typhi*, as do many people who have never had a clinical history of typhoid fever[86–88]. These individuals shed bacteria in their stools and urine for periods of time that range from a year to a lifetime, without any apparent signs of disease[89]. Typhoid carriers are of

GLYOXYLATE-SHUNT PATHWAY
A biochemical pathway that is used by plants and microorganisms to metabolize acetate or long-chain fatty acids as a source of energy.

PEYER'S PATCHES
Lymphoid nodules located in the small intestine that trap antigens from the gastrointestinal tract and provide sites where immune cells — such as B and T lymphocytes, macrophages and dendritic cells — can interact with antigen.

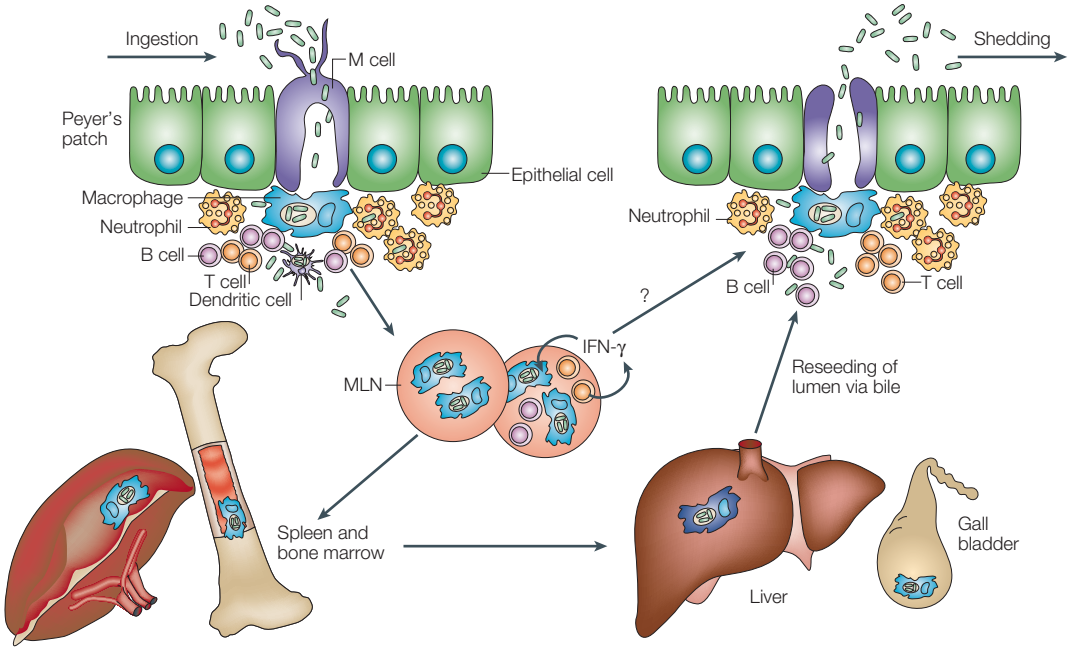


Figure 2 | **Persistent *Salmonella* infection.** Schematic representation of persistent infection with *Salmonella enterica* serovar Typhi in humans. Bacteria enter the Peyer's patches of the intestinal tract mucosal surface by invading M cells — specialized epithelial cells that take up and transcytose luminal antigens for uptake by phagocytic immune cells. This is followed by inflammation and phagocytosis of bacteria by neutrophils and macrophages and recruitment of T and B cells. In systemic salmonellosis, such as typhoid fever, *Salmonella* may target specific types of host cells, such as dendritic cells and/or macrophages that favour dissemination through the lymphatics and blood stream to the mesenteric lymph nodes (MLNs) and to deeper tissues. This then leads to transport to the spleen, bone marrow, liver and gall bladder. Bacteria can persist in the MLNs, bone marrow and gall bladder for life, and periodic reseeding of the mucosal surface via the bile ducts and/or the MLNs of the small intestine occur, and shedding can take place from the mucosal surface. Interferon-γ (IFN-γ), which can be secreted by T cells, has a role in maintaining persistence by controlling intracellular *Salmonella* replication. Interleukin (IL)-12 — which can increase IFN-γ production — and the proinflammatory cytokine tumour-necrosis factor-α (TNF-α) also contribute to the control of persistent *Salmonella* (not shown).

special concern from a public-health viewpoint as they are the reservoirs for the spread of infection and disease. From the bacterial perspective, persistent infection is essential for microbial survival in nature. *S. typhi* is carried for years — even in the presence of an immune response — and chronic carriers of *S. typhi* have high levels of circulating serum antibodies to the Vi antigen and to flagellar antigens[84,90]. Investigating the chronic carrier state in salmonellosis should provide an insight into bacterial survival strategies, as well as information that could be used to develop new approaches for the treatment of typhoid and other persistent microbial infections.

## Mouse models of *Salmonella* persistence

Mouse typhoid is similar to human typhoid in a number of ways, although different strains of mice have varying levels of susceptibility to *Salmonella* infection[91]. In mice, a significant component of innate resistance or susceptibility to infection with *S. typhimurium* is controlled by the gene *Nramp1* (also known as *Slc11a1*), which encodes a proton/divalent-cation antiporter that regulates susceptibility to infectious disease[92]. *Nramp1* expression is restricted to cells of the monocyte/macrophage lineage, and because it localizes to the vacuolar membrane it affects the capacity of the host to control intracellular replication of *Salmonella* bacteria — as well as *Mycobacterium* spp. and *Leishmania* spp. — presumably by depriving the bacteria of divalent cations[93]. *Nramp1* is therefore involved in the control of the exponential growth of *S. typhimurium* in the reticuloendothelial organs during the early phase (first week) of infection in mice[94]. Consequently, mice carrying two copies of the mutant *Nramp1*$^{Asp169}$ allele are significantly less resistant to lethal *S. typhimurium* infections than mice that harbour the wild-type *Nramp1*$^{Gly169}$ allele[94].

*Salmonella* clearance during the late phase of infection (3–4 weeks post-infection) seems to be influenced by various host loci through effects on the acquired immune response. The mouse MAJOR HISTOCOMPATIBILITY COMPLEX (MHC; also known as H2) has an important role in the clearance of *Salmonella*[91,95]. Similarly, in humans, a genetic link between specific class II and class III MHC haplotypes and relative resistance to *S. typhi* has been shown[96]. Several studies have also shown a requirement for both CD4$^+$ and CD8$^+$ T lymphocytes for clearance of *Salmonella* infections[97–100], and the humoral response is also required for this[100–102].

Historically, *Salmonella* pathogenesis has been investigated in *Nramp1*$^{Asp169}$ mice, which are highly sensitive to *Salmonella* infection. This reflects, in part, the fact that death is an experimental endpoint that is readily measured, so this model is useful for assessing the relative contributions of *Salmonella* virulence factors and host

MAJOR HISTOCOMPATIBILITY COMPLEX
A complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells.

immune responses in an acute infection. Although acute *S. typhimurium* infections have been well characterized using this model, it is not suitable for studies of long-term carriage, as the mice either die rapidly from relatively low doses of *Salmonella* or attain sterilizing immunity. Previous studies using specific *S. typhimurium* mutant strains — such as an attenuated strain that is unable to synthesize aromatic amino acids *de novo* (*aroA*⁻ strain) or a mutant in polynucleotide phosphorylase (PNPase) that has altered virulence gene expression — in *Nramp1*-deficient strains of mice have shown that these bacterial mutants can colonize mice for as long as 2 months[103–105]. Although these models are useful for understanding the development of protective immunity to *Salmonella*, they have not added a great deal to the understanding of the biology and pathogenesis of natural persistent *Salmonella* infections with wild-type bacteria.

Persistent *Salmonella* infection can be effectively studied using the 129sv mouse strain — which carries a wild-type *Nramp1* allele — and wild-type *S. typhimurium*. Oral infection of 129sv mice results in systemic infection that, in most cases, does not lead to death of the host. Persistent infection in this model is characterized by sporadic excretion of bacteria in stools and long-term carriage of *S. typhimurium* in low numbers within classical granulomatous lesions, which arise in the spleen, liver, gall bladder and mesenteric lymph nodes (MLNs)[106]. The data obtained from studies using persistently infected mice indicate that the most common site of chronic carriage of *S. typhimurium* is the MLNs[106]. Indeed, this is often the only site from which viable *Salmonella* can be recovered.

Chronic infections with *S. typhi* and *Salmonella enterica* serovar Dublin are classically associated with long-term excretion of bacteria and localization in the gall bladder[107–109]. Although humans that carry *Salmonella* chronically often have BILIARY-TRACT disease, this condition is not an absolute requirement for development of the carrier state[88,110]. A previous study showed that *S. typhi* was carried exclusively in MLNs 50 days after oral infection of chimpanzees[111]. In the case of *Salmonella enterica* serovar Pullorum, it was recently shown that bacteria are carried in the spleen and reproductive tract, specifically in the ovaries and oviducts of hens[112]. In a recent study from our own laboratory, we found the main site of chronic carriage of *S. typhimurium* in mice to be the MLNs, and not the gall bladder[106]. These studies indicate that the true reservoir of persistent bacterial carriage might change in response to the host immune status and the underlying disease, a situation that might also apply to human infections.

## Persistent *Salmonella* in macrophages

The ability of *Salmonella* to survive in macrophages is required for systemic colonization of the host. Indeed, chronically infected humans and mice harbour *Salmonella* within the reticuloendothelial system for long periods of time, and our group has shown that the persistent bacteria reside in low numbers within MOMA2⁺ MACROPHAGES residing in the MLNs[106]. The intracellular trafficking of the *Salmonella* phagosome has been analysed in unactivated tissue-culture macrophages and it has been concluded that most *Salmonella*-containing vacuoles do not interact extensively with late endosomes and lysosomes[113]. Studies of intracellular *Salmonella* gene expression in unactivated macrophages have shown that numerous virulence and SOS-response genes show significant changes in expression in response to the vacuolar environment[114]. However, the trafficking and gene expression patterns of persistent intracellular *Salmonella* have not yet been investigated. Furthermore, the fate of macrophages that are persistently infected with *Salmonella* is not known, nor is it clear how the bacteria infect new host cells over time. It is possible that bacteria persist within macrophages for the lifetime of the host cell and then infect a new macrophage. However, *S. typhimurium* is able to induce host-cell death *in vivo*[115,116], providing a potential mechanism by which *Salmonella* can escape from an infected cell to infect neighbouring cells. *S. typhimurium* mediates macrophage death by at least two mechanisms. One mechanism involves rapid macrophage death that requires the type III secretion system (TTSS) that is encoded by the *Salmonella* PATHOGENICITY ISLAND SPI1 (REF. 117). The potential role of SPI1 and SPI1-mediated macrophage cytotoxicity in persistent *S. typhimurium* infections is under investigation.

*S. typhimurium* can also induce macrophage death that occurs approximately 18 hours after infection. This delayed macrophage death requires another TTSS that is encoded by a second pathogenicity island, SPI2, and is used inside host cells[118,119]. It is possible that dead or dying macrophages containing *S. typhimurium* are phagocytosed by other macrophages that are recruited to the site of infection, which then serve as a safe haven in which *Salmonella* can survive while avoiding extracellular host defences. It is also possible that the SPI2-mediated mechanism of cell death is not active during persistent infection of macrophages. Indeed, differential expression of *SPI* genes could be a strategy used by persistent *Salmonella*[120]. It is clear that SPI2 is required to avoid the effects of PHAGOCYTIC OXIDASE (PHOX) during infection of macrophages[121] and to initiate systemic infection[122,123], but its role and the role of individual SPI2-secreted effector molecules in the continuing presence of persistent of *S. typhimurium* is not yet known.

## Persistent *Salmonella* and the immune response

Mice that are persistently infected with *S. typhimurium* have high anti-*Salmonella* antibody titres[106]. This might represent a deliberate infection-associated shift from a $T_H1$ to a $T_H2$ response, which might be involved in keeping the numbers of bacteria inside each macrophage lower in the persistent *S. typhimurium* model than those reported in previous studies of acute infections in *Nramp1*-susceptible mice in which the mice died[124]. However, the adaptive immune response also provides positive feedback to the innate immune system through the synthesis of cytokines that either increase effector-cell numbers or activate these cells to produce an increased antibacterial response.

BILIARY TRACT
Includes the gall bladder and bile ducts, which make and transport bile. Bile contains salts or detergents that disrupt bacterial membranes; it also activates autolysins that digest peptidoglycan.

MOMA2⁺ MACROPHAGES
MOMA2 is expressed in the cytoplasm of monocytes and macrophages. MOMA2⁺ macrophages can be found in the splenic red pulp, in the cortex of the thymus, in the subcapsule and medullary regions of lymph nodes and in sites of acute and chronic inflammation.

PATHOGENICITY ISLANDS
Large (10–50-kb) insertions in the bacterial chromosome that encode virulence determinants. They are thought to be acquired by horizontal transfer.

PHAGOCYTIC OXIDASE (PHOX)
Production of reactive oxygen intermediates, which can kill bacteria directly or after reacting with chlorine, is mediated by the NADPH oxidase system located in the membrane of the macrophage and includes the PHOX enzyme.

Table 3 | ***Helicobacter pylori* virulence determinants**

| Virulence determinant | Description/potential role in pathogenesis | References |
|---|---|---|
| VacA | 95-kDa secreted vacuolating toxin; induces apoptosis; involved in immunomodulation and colonization of mouse stomach | 160–163,240 |
| Cag-PAI | 37-kb genomic fragment; contains 29 genes that encode a type IV secretion apparatus | 241 |
| CagA | 120-kD protein; translocated into host cell by type IV secretion apparatus encoded on Cag-PAI; phosporylated in host cell and binds SHP-2 tyrosine phosphatase; disrupts tight junctions; epidemiologic link to cancer | 242–245 |
| BabA | 78-kDa outer membrane protein; binds to fucosylated Lewis B blood group antigen; mediates adhesion to epithelial cells and possibly stomach epithelium | 188,246 |
| Urease | Resists acidic conditions in the stomach; activates innate immune responses during early steps of infection | 247 |
| Flagella | Involved in motility; essential for colonization | 248 |

PAI, pathogenicity island.

Once T cells are activated during an infection, they produce the macrophage-activating factor IFN-γ, which has a role in the acute *Salmonella* mouse model in controlling the early phase of bacterial replication[125–127]. It was recently shown that IFN-γ has an important role in maintaining and controlling the level of bacterial replication in persistently infected animals, perhaps by stimulating infected macrophages to suppress bacterial replication[106]. Furthermore, people who lack the IL-12 receptor — in whom the $T_H1$ response and the production of IFN-γ are defective — are more susceptible to infections with *Salmonella* spp.[128] In addition to IFN-γ and IL-12, TNF-α might also have a role in maintaining and controlling the level of bacterial replication in persistently infected hosts. Similar to the results seen with *M. tuberculosis*, patients who were treated with anti-TNF-α antibodies developed *Salmonella* septicaemia[129]. HIV-positive individuals develop chronic bacteraemia caused by species of *Salmonella* that do not normally pass beyond the MLNs in healthy individuals[1] — further indicating that an intact adaptive immune system has a role in immunity to *Salmonella*.

*Salmonella* bacteria are probably not passive bystanders in terms of maintaining the balance between clearance and persistence. In this regard, *Salmonella* might have an active role in modulating or even directly manipulating host responses, thereby preventing clearance of intracellular bacteria. Many studies have shown that *S. typhimurium* may limit the *in vivo* proliferation of $CD4^+$ and $CD8^+$ T cells, despite their activated phenotype[130]. In addition, it has been shown that active *S. typhimurium* infection leads to immunosuppression in mice and causes the production of large amounts of IL-10, which has immunosuppressive activities, and nitric oxide (NO), which has both immunosuppressive and direct antibacterial activities[126,131–133]. Indeed, *S. typhimurium* mutants have been identified that are unable to persist in mice, indicating that they lack genes that are specifically required for persistence. *S. typhimurium* strains that are deficient for *mig-14*, *virK* and *somA* are able to replicate in unactivated macrophages and establish infections in mice; however, these mutants begin to be cleared in BALB/c mice between 7 and 10 days post-infection[134,135]. The exact functions of the proteins encoded by these genes are not known, but they contribute both to resistance to antimicrobial peptides, which are produced in activated macrophages[136], and to replication in these cells (I. Bradshaw, D.M.M. and S.F., unpublished observations). The identification of other *S. typhimurium* mutants that are unable to persist in mice will increase our understanding of bacterial mechanisms of persistence.

## Persistent *Helicobacter pylori* infections

During the past 20 years, *H. pylori* has emerged as an important example of a persistent bacterial pathogen. Not only does this bacterium successfully colonize the hostile environment of the human stomach, but the infection regularly persists for the lifetime of the host in the face of a constant, vigorous innate and adaptive immune response. In most infected people, *H. pylori* infection causes superficial chronic gastritis, which is usually clinically asymptomatic, although histologically apparent. However, a significant subset of infected individuals are at risk of the subsequent development of duodenal and peptic ulcers, and 1% of those that are infected will develop adenocarcinoma or lymphoma of the stomach[137].

Most basic research in the *Helicobacter* field has focused on the study of bacterial virulence determinants (reviewed in REFS 6,138,139; TABLE 3), particularly in the context of their association with severe gastrointestinal sequelae of infection. Although the experimental focus on the association of *H. pylori* with disease is understandable, it is important to point out that the ecological niche for *H. pylori* is progressively lost with the development of ATROPHIC GASTRITIS[140], and bacteria can only rarely be cultured from seropositive patients with adenocarcinoma. So, from the microbial standpoint, the progression of asymptomatic gastritis to more serious tissue destruction can be viewed as contrary to the best interests of the bacteria in terms of evolutionary success. Therefore, for the purpose of this review, we will shift the focus from the minority of colonized individuals who develop overt disease (~20%) to the majority who remain relatively symptom-free and are likely to represent the reservoir for human infection. We focus here on the bacterial and host characteristics that enable *H. pylori* to colonize its host persistently in the face of a normal immune response.

Most of the studies we will refer to here used animal models. Some *H. pylori* isolates establish long-term infections in rodents or have been adapted to do so by repeated passaging (reviewed in REF. 141). Indeed, the limiting factor for the development of animal models of persistent *H. pylori* infection seems to be host specificity rather than lack of persistence. The models that have been used so far vary markedly with respect to disease

ATROPHIC GASTRITIS
Chronic inflammation of the stomach with degeneration of the mucosa.

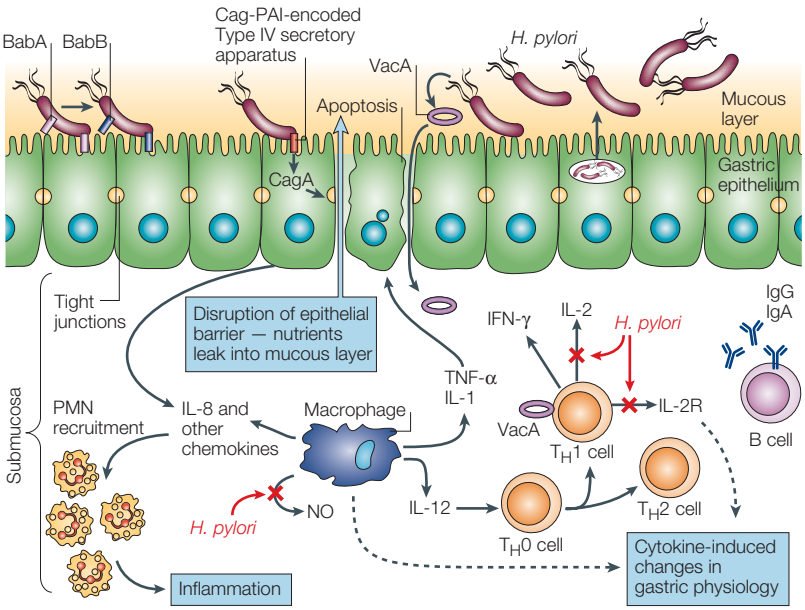


Figure 3 | **Persistent *Helicobacter pylori* infection.** Interplay between *H. pylori* factors and the host response leads to chronic gastritis and persistent colonization. *H. pylori* binds to gastric epithelial cells through BabA and other adhesins[249]. In strains that carry the Cag pathogenicity island (Cag-PAI), a type IV secretory apparatus allows translocation of effector molecules such as CagA into the host cell, resulting in the production of interleukin (IL)-8 and other chemokines by epithelial cells. The secreted chemokines lead to the recruitment of polymorphonuclear cells (PMNs), resulting in inflammation. Injected CagA also associates with tight junctions and targets *H. pylori* to them. In the long term, CagA might cause disruption of the epithelial barrier and dysplastic alterations in epithelial-cell morphology. Disruption of junctions by CagA might also cause leakage of nutrients into the mucous layer[245] and entry of bacterial VacA into the submucosa. VacA induces apoptosis in epithelial cells by reducing the mitochondrial transmembrane potential and inducing cytochrome *c* release, which might also contribute to the disruption of the epithelial barrier. Tumour-necrosis factor-α (TNF-α)-mediated apoptosis may also lead to disruption of the epithelial barrier. The chronic phase of *H. pylori* gastritis links an adaptive lymphocyte response with the initial innate response. Cytokines produced by macrophages, particularly IL-12, activate recruited cells — such as helper T cells ($T_H0$, $T_H1$ and $T_H2$), which respond with a biased $T_H1$ response, and B cells. Cytokines also alter the secretion of mucus, which contributes to *H. pylori*-induced disruption of the mucous layer, as they induce changes in gastric-acid secretion and homeostasis. *H. pylori* inhibits the host immune response by blocking the production of nitric oxide (NO) by macrophages and through the ability of VacA to interfere with the IL-2 signalling pathway in T cells (and therefore T-cell activation) by blocking transcription of the genes encoding IL-2 and its receptor, IL-2R (see main text for details). An intracellular pool of *H. pylori* may repopulate the mucous layer after cycles of extracellular clearance. Ig, immunoglobulin.

outcomes, which range from atrophic gastritis, intestinal metaplasia and gastric adenocarcinoma to mucosa-associated lymphoid tissue (MALT) lymphoma, depending on the species used and the genetic background of the host[141]. The identification of bacterial genes that are associated with colonization and persistence of *H. pylori* has been limited by the lack of animal models that support infection by strains for which the genomes have been completely sequenced. This obstacle has recently been overcome by the development of an IL-12-deficient mouse model that is susceptible to infection by the sequenced strain KE26695, which should facilitate whole-genome-based studies of virulence and persistence[142].

*H. pylori* has developed a number of unique features and strategies that enable it to persist in its host (FIG. 3). These include escape from and neutralization of the innate and adaptive immune responses; avoidance of a strong proinflammatory response; extensive genetic intrastrain and interstrain diversity; and a partially intracellular lifestyle. In the following sections, we discuss each of these strategies in turn.

### *H. pylori* evasion of host immune responses

***Evasion of innate responses.*** NO is a key component of the innate immune system and an effective antimicrobial agent[143]. It is produced by activated macrophages through the action of NOS2, which uses L-arginine as a substrate and is highly expressed both in macrophages infected with *H. pylori*[144] and infected gastric tissues[145]. In a series of elegant experiments using both cultured and peritoneal macrophages, Gobert *et al.* showed that *H. pylori* prevents NO production by host cells by producing the enzyme arginase[146]. Encoded by the gene *rocF*, arginase — which is associated with the bacterial cell envelope — competes with NOS2 for the L-arginine substrate and converts it to urea and L-ornithine, rather than NO. Mutation of the *rocF* gene results in efficient killing of the bacteria in an NO-dependent manner, whereas wild-type bacteria survive under these conditions. Furthermore, the *rocF* mutant is mildly attenuated in its ability to colonize mice[147], indicating that arginase expression might indeed be important for survival and persistence *in vivo*.

Other bactericidal functions of macrophages also seem to be impaired in the presence of *H. pylori*, and two possible mechanisms for this have been suggested. In one study, Allen *et al.* showed delayed uptake of bacteria into macrophages followed by the formation of megasomes as a result of phagosome fusion. These megasomes protect intracellular bacteria from efficient killing[148]. In a second study using human blood monocytes and polymorphonuclear cells, Ramarao *et al.* showed that *H. pylori* can actively block its own uptake, as well as the uptake of co-cultured bacteria of other species and latex beads[149,150]. Both of these phenotypes depended on the presence of the Cag pathogenicity island (Cag-PAI), which is a 37–40-kb stretch of DNA that encodes a type IV secretion system (TFSS) and which epidemiological studies have linked to more severe disease outcomes[151–153].

***Evasion of adaptive responses.*** *H. pylori* has evolved to subvert not only the innate, but also the adaptive immune response, which is based on MHC-class-II-restricted — and to a lesser degree MHC-class-I-restricted — T cells[154]. Antigen-dependent proliferation of T cells is blocked specifically by *H. pylori*[155] — an effect that is mediated by the virulence factor vacuolating cytotoxin A (VacA)[156,157]. VacA is a 95-kDa, secreted protein that, among other functions, induces cellular vacuolization in epithelial cells[158,159]. VacA has been shown to act as an immunomodulator by interfering with the IL-2 signalling pathway in T cells by blocking $Ca^{2+}$ mobilization and the activity of the $Ca^{2+}$/calmodulin-dependent phosphatase calcineurin[156,157]. The secretion of IL-2 and the surface localization of the high-affinity IL-2 receptor (IL-2R) are necessary for

efficient T-cell proliferation and activation. In normal T cells, calcineurin dephosphorylates the transcription factor NFAT (for nuclear factor of activated T cells), which then translocates into the nucleus and activates the transcription of several genes that are involved in the immune response. Among these are the genes encoding IL-2 and IL-2Rα. In T cells that are infected with VacA$^+$ *H. pylori*, however, nuclear translocation of NFAT is blocked, as dephosphorylation is prevented and the downstream genes are not expressed.

Another possible function of VacA in subverting the adaptive immune response is its ability to interfere with antigen presentation mediated by MHC class II[160]. After it inserts into the plasma membrane, VacA is internalized by endocytosis and reaches the late-endosomal compartment. This compartment is then converted into large acidic vacuoles by the anion-selective channel activity of VacA[161,162]. In antigen-presenting cells, the processing of proteins into peptide epitopes, which takes place in the endocytic compartment, is greatly reduced owing to VacA activity, indicating that antigen presentation is abrogated in these cells[160].

The importance of VacA in establishing an infection has been corroborated by experiments in mice, which have shown that a null mutation of *vacA* compromises the ability of *H. pylori* to colonize the murine stomach in the presence of the corresponding parental strain[163]. However, the precise effect of VacA on cellular and epithelial physiology that facilitates *H. pylori* colonization in the murine stomach is unknown.

### *H. pylori* suppresses inflammatory responses

Several lines of evidence indicate that, to allow long-term colonization, there has been selective pressure on *H. pylori* to avoid triggering an intense inflammatory reaction[164]. It has been proposed that high levels of inflammation may lead to loss of gastric glandular structure and function[165] and that *H. pylori* disappears from stomachs that have developed atrophic gastritis[140]. Furthermore, an increased inflammatory reaction — as seen in *IL-10*-knockout mice — is associated with clearance of the bacteria from the stomach within 8 days of the infection[166]. Similarly, increased inflammation due to deletion of the gene encoding PHOX results in a marked reduction in bacterial numbers[167]. All of these findings indicate that, at least in animal models, a strong inflammatory reaction is necessary for the elimination of *H. pylori* and seems to be actively repressed by this bacterium.

But how does *H. pylori* modulate the host inflammatory response? Bacterial lipopolysaccharide (LPS) is the main mediator of inflammation during infections with most Gram-negative bacteria because it activates phagocytic cells, endothelial and epithelial cells and lymphocytes. *H. pylori* LPS, however, has very low biological activity when compared with *Escherichia coli* LPS, at least as measured by its ability to activate macrophages[165]. In fact, the minimum concentration of purified LPS that is required to achieve similar responses was several thousand times greater for *H. pylori* compared with *E. coli* LPS[165].

The effect of *H. pylori* LPS on gastric epithelial cells — the other main source of proinflammatory cytokines in the stomach besides macrophages — is still unclear. One study has shown that *H. pylori* LPS produced by strains harbouring the Cag-PAI, but not from Cag-PAI mutants, activates Toll-like receptor 4 (TLR4) on gastric-pit cells, thereby stimulating the innate immune responses of the gastric mucosa[168]. By contrast, Smith *et al.* reported that TLR2 mediates the proinflammatory effects of *H. pylori* on epithelial cells and show that *H. pylori* LPS is a TLR2 agonist[169]. These authors suggest that the low level of pathogenicity of *H. pylori* LPS does indeed result from its failure to activate TLR4, the receptor that mediates LPS signalling of most other Gram-negative species[170].

A recent study has reported similar findings for *H. pylori* flagellins. The two *H. pylori* flagellins — FlaA and FlaB — were shown to have a markedly reduced potential to activate TLR5 compared with flagellins of other Gram-negative bacteria, such as FliC of *S. typhimurium*[171]. The evolution of these unique flagellins, which share extensive amino-acid homology with flagellins from other species that do stimulate TLR5, has been proposed to preserve the essential function of the flagella during chronic colonization while avoiding the activation of the innate immune system[171].

In a recent study investigating a link between the Cag-PAI and the induction of proinflammatory responses, Philpott *et al.* showed that clinical isolates of *H. pylori* were more likely to colonize mice if they did nor harbour the Cag-PAI and were therefore unable to induce such responses in cultured cells[172]. Mouse-adapted variants that lacked the Cag-PAI infected mice at higher levels and had a reduced capacity to induce inflammatory responses *in vitro* compared with the respective parental strains. Taken together, these findings imply that there may be a profound *in vivo* selection against *H. pylori* strains and variants that induce a strong host inflammatory response[172], at least in the mouse model.

The relative *in vivo* advantages for *H. pylori* with Cag$^+$ versus Cag$^-$ phenotypes in human hosts are unclear[173,174]. One study of an individual infected with multiple strains showed recombination between strains resulting in excision of the Cag-PAI and subsequent positive selection of a Cag$^-$ strain[175]. It is therefore unclear as to how the Cag-PAI benefits the bacteria during a persistent infection; it may be that it is important for a specific stage of colonization, but is dispensable at other stages.

### Genetic diversity in *H. pylori*

Extensive recombination and a PANMICTIC overall population structure result in substantial genetic diversity among *H. pylori* strains[176–179]. This has been proposed to allow the bacteria to adapt rapidly to changing conditions in their current host as well as to facilitate the colonization of new hosts[180–182]. Although *H. pylori* populations in individuals and even families seem to be clonal, as determined by conventional procedures — such as RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) PCR and

PANMIXIS
Mating without regard to the genetic constitution of the mate.

DNA sequence analysis[179] — more sensitive approaches have indicated that extensive changes can and do occur in a single host over time[183]. For example, fortuitously, additional *H. pylori* isolates were obtained from one patient six years after an isolate of the sequenced strain J99 was first obtained from this individual. These new clinical isolates were subjected to extensive molecular comparisons with the original J99 strain. RAPD PCR and sequencing of several unlinked loci indicated that these isolates were undoubtedly related to the original strain; however, microarray analysis showed differences in genetic content that reflected both acquisitions and losses of genomic DNA[183]. Approximately 3% of J99 loci showed variation between isolates obtained from this individual, compared with 22% of loci when isolates from different individuals were compared[184]. Although most of the open reading frames that were affected represented 'hypothetical genes', one was predicted to encode a protein belonging to the TraG family, other members of which have been shown to be involved in genetic transfer[185,186]. A putative similar function of TraG implies that acquisition of this gene might confer an evolutionary advantage by providing another mechanism through which DNA exchange could occur.

In a recent similar study, three output strains from an experimental infection of Rhesus macaques were compared with the strain that was used for inoculation[187]. All three of the output strains had lost expression of the *babA* gene, which encodes an adhesin that binds to the LEWIS BLOOD GROUP $Le^b$ antigen[188]. Loss of *babA* expression occurred by two different mechanisms in different isolates. In some cases, *babA* was replaced by the closely related *babB* gene, leading to loss of *babA* and duplication of *babB*. In others, a change in the number of CT repeats in the 5′ coding region of *babA* resulted in a frameshift and subsequent loss of $Le^b$ adhesion. *H. pylori* therefore uses both antigenic variation and phase variation to regulate *babA* expression (and possibly the expression of other outer-membrane proteins) *in vivo*; however, the significance of this observation in the context of the host immune response remains to be shown.

Another *H. pylori* structure that undergoes phase variation is its LPS, more specifically the Lewis-blood-group determinant of the LPS O-antigen. The on/off statuses of at least five glycosyltransferase genes determine which LPS phase variant is expressed (reviewed in REF. 189). It was initially assumed that mimicry of the Lewis blood group antigens of the host by *H. pylori* would provide a mechanism of immune evasion and adaptation to the host, a concept that was supported by reports describing a link between the Lewis-blood-group phenotype of isolates with the phenotype of the individuals they were derived from (reviewed in REF. 190). However, this concept has been challenged by the isolation of both $Le^X$- and $Le^Y$-expressing strains from the same host and the fact that even high levels of anti-LPS antibodies do not eradicate the organism. It is now assumed that the production of Lewis antigens by *H. pylori* facilitates the colonization of the host by mediating adhesion to gastric epithelial cells[191,192].

The high degree of diversity that is seen in *H. pylori* is probably facilitated by its natural competence for DNA transformation. In contrast to other bacteria, natural competence in *H. pylori* is not mediated by type IV pili or type IV pilin-like proteins, but by a TFSS that is encoded by the *comB* operon[193,194]. The ComB8, ComB9 and ComB10 proteins correspond to the *Agrobacterium tumefaciens* VirB8, VirB9 and VirB10 proteins and constitute the basic components of a TFSS[193]. *H. pylori* therefore possesses two functionally independent TFSSs — one for protein secretion and translocation into the host cell that is encoded by the Cag-PAI, and one for DNA uptake. In addition, a third cluster of type IV secretion genes was recently discovered that seems to be present in only a subset of strains[195]. The functional significance of this cluster is unknown at present.

The identification of CRYPTIC PLASMIDS in approximately half of all *H. pylori* strains[196] has given rise to speculation that conjugative transfer of novel sequences carried on plasmids could be another means of horizontal gene transfer — and therefore strain diversity — in *H. pylori*. In support of this hypothesis, Hofreuter *et al.* have recently presented evidence that some of these plasmids contain hot spots for site-specific recombination, encode elements from CHROMOSOMAL PLASTICITY ZONES and might be mobilizable[197]. This indicates that exchange of genetic material between these plasmids and the chromosome can occur, and that these plasmids might be mobilized and spread rapidly in the population. So, genetic diversity, which in turn leads to antigenic diversity, might be an important strategy used by *H. pylori* to evade immune surveillance.

### Repopulation of the stomach by *H. pylori*

The gastric mucosa is normally well-protected against bacterial colonization due to the acidic pH of the lumen, the production of mucus and rapid epithelial-cell turnover. It is therefore tempting to speculate that the adoption of a partially intracellular lifestyle by some members of the bacterial population might allow *H. pylori* to achieve long-term persistence. Although there is little evidence to indicate that the bacterium is predominantly an intracellular pathogen, numerous experimental and clinical observations of biopsy specimens support the notion that a subpopulation of *H. pylori* is able to invade epithelial cells both *in vitro*[198,199] and *in vivo*[200–202].

Using time-lapse video microscopy and gentamicin-protection assays in a cell-culture system, Amieva *et al.* have provided evidence that *H. pylori* residing in multivesicular bodies survive in the intracellular milieu for at least 24 hours, remain motile and retain the ability to emerge from the cells to repopulate the extracellular space[199]. Up to several dozen bacteria can be found in one vesicle, indicating that intracellular replication does occur, at least sporadically. These findings indicate that the intracellular niche can potentially function as a 'hideout' and sustain the renewal of the population under the unfavourable conditions that are found in the mammalian stomach. Nevertheless, the vast majority of

RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) PCR
A molecular technique used for the classification and comparison of different isolates of the same species. This method uses a randomly chosen oligonucleotide to prime DNA synthesis and results in strain-specific patterns of DNA products.

LEWIS BLOOD GROUP
Antigens of red blood cells, saliva and other body fluids that are specified by the Le gene and react with the antibodies designated anti-$Le^a$ and anti-$Le^b$.

CRYPTIC PLASMIDS
Small, mobilizable genetic elements that can encode virulence factors.

CHROMOSOMAL-PLASTICITY ZONES
Segments of the chromosome that are characterized by their different G+C content, which is a hallmark of horizontally acquired sequences.

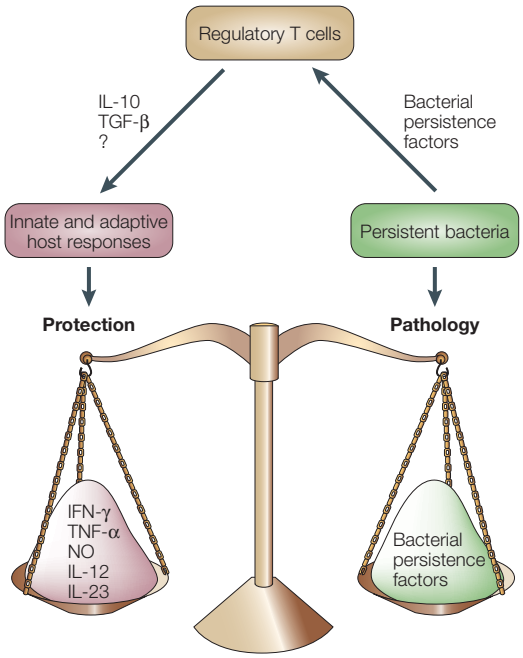


Figure 4 | **Balancing protective immunity and immunopathology during persistent bacterial infections.** The balance between the immune response and infection during persistent infections is important for both the host and the pathogen, with the host switching off the immune response when it becomes more harmful than the presence of the pathogen. This is depicted by the horizontal axis between protection and pathology on either side of the balance. The illustration shows how complex interactions between the pathogen and host lymphocytes and antigen-presenting cells may drive immune responses, with regulatory T cells having an important role in the balance between protection and immunopathology. Host factors involved in the immune response — such as interferon-γ (IFN-γ), tumour-necrosis factor-α (TNF-α), nitric oxide (NO) and the interleukins IL-12 and IL-23 — contribute to both immunopathology and suppression of bacterial persistence. Bacterial persistence is mediated by specific bacterial persistence factors and potentially dysregulated immune responses that could facilitate persistence by contributing to chronic inflammation and low-level tissue injury that facilitates bacterial survival. We postulate that bacterial factors that are important for persistence might have an as-yet-undetermined role in directing this balance by influencing regulatory T cells. TGF-β, transforming growth factor-β.

*H. pylori* in the stomach are extracellular, highly motile organisms that reside in the mucus overlying the gastric mucosa. How the organism survives here in the presence of high levels of antibody is one of the most poorly understood issues in *H. pylori* pathogenesis. It is equally remarkable that individuals who are cured of infection by antibiotic therapy after decades of colonization are susceptible to re-infection, although at a slightly lower rate[203].

## Conclusions

Many hypotheses can be proposed for the survival of a population of microorganisms in the presence of immune responses. The organisms could 'hide' inside macrophages within granulomas — as is the case for *M. tuberculosis* and *S. typhi* — where they are effectively screened from active immune surveillance. The ability of persistent species such as *M. tuberculosis*, *S. typhi*, *Chlamydia* spp. and *Brucella* spp. to modify the intravacuolar environment is a common feature of these bacteria that could clearly favour persistence and evasion of immune responses through reduced surface-antigen presentation or the control of apoptotic pathways[204–208]. Some persistent pathogens might seek an intracellular location at specific times during infection. Indeed, the ability of the mucosal-surface colonizer *H. pylori* to cycle between extracellular and intracellular locations highlights a strategy that may be crucial for some persistent pathogens. Localized subversion of the immune response is also an important feature of persistent bacteria — for example, by interference with cytokine signalling, as described for *M. tuberculosis*, or with innate immune signalling, as described for *H. pylori*.

Finally, the pathological damage that results from continued macrophage activation will at some stage outweigh the immediate risk that is posed by the residual bacteria, and the immune response might turn itself off, allowing bacterial persistence. Regulatory T cells co-expressing CD4 and CD25 markers have been shown to exert this type of control during *Leishmania major* infection[209]. Although there are diverse phenotypes of regulatory T cells, functionally they share the ability to downregulate immune responses. One way that this is achieved is by the secretion of cytokines such as IL-10 and TGF-β, which inhibit both $T_H1$ and $T_H2$ responses *in vivo* and have a role in controlling T-cell responses that are directed against self-antigens[210,211]. Belkaid *et al.* have shown that during persistent infection by *L. major* in the skin, $CD4^+CD25^+$ T cells accumulate in the dermis, where they suppress the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite from this site through both IL-10-dependent and IL-10-independent mechanisms[209]. Furthermore, the sterilizing immunity that is achieved in mice with impaired IL-10 activity is followed by loss of immunity to reinfection. Therefore, it is possible that the equilibrium that is established between effector and regulatory T cells in sites of chronic infection might reflect both pathogen and host survival strategies (FIG. 4). Whether regulatory T cells have a role in the mechanisms that are used in persistent *Helicobacter*, *Mycobacteria* and *Salmonella* infections is unknown. However, a high proportion of $CD4^+$ T cells that are able to release IL-10 can be found in chronic mycobacterial infections[212], and *S. typhimurium* induces macrophage and splenic IL-10 expression[213], possibly indicating the presence of regulatory T cells. It was recently shown that regulatory T cells reduce *H. pylori*-induced gastritis in mice, while allowing the bacterium to colonize the mucosa at higher densities[214], and that *H. pylori*-specific $CD4^+CD25^+$ regulatory T cells suppress memory T-cell responses to *H. pylori* in infected humans[215]. In line with this observation, *H. pylori* infection of *IL-10*-knockout mice resulted in more severe gastritis and bacterial clearance after 8 days[166].

Although we describe some possible mechanisms of pathogen persistence in this review, we actually know very little about how these microorganisms survive for long periods of time in the host in the presence of immunosurveillance. Future applications of genome-based techniques — including array-based analysis of libraries of bacterial mutants and host expression profiling — as well as laser microdissection, will allow further investigation of the fundamental genetics of bacterial persistence and host immune responses. These findings will hopefully lead to improvements in therapeutic approaches and, perhaps, the elimination of these unwanted companions.


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## Competing interests statement

The authors declare no competing financial interests.

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