Antibodies protect against intracellular bacteria by Fc receptor-mediated lysosomal targeting

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The protective effect of antibodies (Abs) is generally attributed to neutralization or complement activation. Using *Legionella pneumophila* and *Mycobacterium bovis* bacillus Calmette–Guérin as a model, we discovered an additional mechanism of Ab-mediated protection effective against intracellular pathogens that normally evade lysosomal fusion. We show that Fc receptor (FcR) engagement by Abs, which can be temporally and spatially separated from bacterial infection, renders the host cell nonpermissive for bacterial replication and targets the pathogens to lysosomes. This process is strictly dependent on kinases involved in FcR signaling but not on host cell protein synthesis or protease activation. Based on these findings, we propose a mechanism whereby Abs and FcR engagement subverts the strategies by which intracellular bacterial pathogens evade lysosomal degradation.

immunoglobulins | opsonization | macrophage | intracellular trafficking | intracellular bacterial replication

Infections with intracellular bacterial pathogens cause a number of severe respiratory diseases, such as tuberculosis, or intestinal tract-associated medical conditions, such as listeriosis or salmonellosis. These intracellular bacteria survive and replicate inside phagocytes by effectively avoiding the host cell's antimicrobial processes. To achieve this common goal, they have evolved multiple strategies to interfere with phagosomal maturation. *Mycobacterium tuberculosis*, *Legionella pneumophila* (*Lpn*), and *Listeria monocytogenes*, for example, paralyze the innate defense mechanisms of their host cells by arresting or reprogramming phagosomal maturation or by escaping from the maturing phagosome, allowing them to replicate and cause disease (reviewed in ref. 1). *M. tuberculosis* survives in macrophages (MΦ) by arresting phagosomal maturation at an early stage through inhibition of cytosolic Ca^{2+} flux and PI(3)P hydrolysis, whose depletion from early phagosomes results in impaired maturation to the late phagolysosomal stage (2). Similarly, *Lpn* arrests phagosome maturation at an early stage by active modulation of the *Legionella*-containing vacuole (LCV), thereby avoiding contact with the lysosomal pathway (3).

We have used *Lpn*, the etiological agent of Legionnaires' disease, as a model to study the protective mechanism of antibodies (Abs) in infections with intracellular bacterial pathogens that are specialized to interfere with phagolysosomal maturation (4). Inhalation of contaminated aerosols allows *Lpn* to gain access to the lung, where they infect alveolar MΦ, replicate inside these cells, and cause disease. The interaction between *Lpn* and MΦ is governed by the bacterial Icm/Dot type IV secretion system (T4SS), through which the bacteria secrete effector proteins into the host cell cytoplasm, resulting in the formation of a replication-permissive LCV (5–7).

Although primary infection of *Lpn* is largely controlled by innate immune mechanisms, particularly, natural killer cell-derived IFN γ (8, 9), early reports also documented adaptive cell-mediated and humoral immune responses upon *Lpn* infection (10). We have previously analyzed the *Lpn*-specific humoral response in detail and documented that *Lpn*-specific Abs can mediate protection against *Lpn* challenge (11).

In this study, we dissect the mechanisms by which Abs mediate protection against intracellular bacterial pathogens that have

evolved strategies to evade phagolysosomal fusion. Using the vacuolar bacterial pathogen *Lpn*, we show that Abs protect by altering intracellular trafficking of this pathogenic bacterium into lysosomal compartments. This lysosomal targeting is independent of Fc receptor (FcR)-mediated phagocytosis but strictly dependent on FcR signaling, and it is operational in vitro and in vivo.

Results

***Lpn*-Specific Abs Constitute the Dominant Protective Mechanism in Vivo.** To address whether Abs are the major component mediating protection on secondary *Lpn* infection in vivo, wild-type (wt) B6 and B cell-deficient JHT mice, intranasally (i.n.) immunized with *Lpn*, were challenged through the same route, and bacterial titers were determined 2 d later. Preimmunized (memory) wt mice showed a marked reduction (100-fold) in colony-forming units compared with the naive controls, whereas only a minor reduction could be observed in JHT memory mice (Fig. S1), indicating that Ab-mediated protection is the dominant protective mechanism in secondary *Lpn* infection. To determine which Ab isotype confers this protection, we challenged naive mice with *Lpn* opsonized with purified polyclonal IgG or IgA from the bronchio-alveolar lavage (BAL) of *Lpn* immune mice, and we found that both isotypes were equally protective and led to a reduction in bacterial titers comparable with memory mice (Fig. 1A).

Complement Is Not Involved in Protection Against *Lpn*. To investigate the mechanisms by which *Lpn*-specific Abs confer protection against this intracellular bacterium, we addressed the role of complement in this process, because Ab-induced complement activation can result in lysis of Gram-negative bacteria. Although *Lpn* was reported to be resistant to complement-mediated lysis in vitro (12), the role of complement in the protection from *Lpn* infection in vivo has not been investigated. We addressed this by either depleting memory mice from complement or infecting $\text{C3}^{-/-}$ mice with Ab-opsonized *Lpn*, and we show that complement does not play a role in protection from *Lpn* infection in vivo (Fig. S2). Furthermore, because IgA is unable to activate complement, our finding that *Lpn*-specific IgA is protective (Fig. 1A) further supports the conclusion that complement activation is dispensable for Ab-mediated protection against *Lpn* infection in vivo.

Opsonized *Lpn* Fails to Replicate in Mammalian Host Cells. We next addressed the question if opsonization with Abs has an effect on the ability of *Lpn* to replicate within their host cells. To test this in vitro, we infected MΦ with *Lpn* that had been pretreated with immune serum, naive serum, or medium and monitored bacterial

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Fig. 1. Antibodies protect from *Lpn* infection and inhibit *Lpn* growth by redirection into lysosomes in vitro. (A) B6 mice were vaccinated with *Lpn* (memory) or left naive. Colony-forming units (CFU) were determined in the lung and BAL 2 d after i.n. challenge with IgG- or IgA-opsonized *Lpn* (IgG and IgA) or untreated *Lpn* (naive; $n = 3$). (B–D) RAW MΦ were infected with *Lpn* pretreated with medium, naive serum, or *Lpn*-specific immune serum, and (B) intracellular growth of *Lpn* was measured. (C and D) LCVs were isolated from infected MΦ and stained for the T4SS substrate SidC (blue) and the lysosomal associated membrane protein 1 (LAMP-1) (red), and the percentage of *Lpn* (green) localizing in LAMP-1⁺ vacuoles was quantified. T4SS-deficient *Lpn* (ΔT), which are unable to evade lysosomal fusion, were used as positive controls for the localization in LAMP-1⁺ vacuoles. Dotted lines visualize LCVs. (Scale bar: 2 μm.) At least 40 LCVs were analyzed in each experimental condition. SDs from three independent experiments are shown. (E and F) Bone marrow-derived MΦ from B6 or *FcRγ*^{−/−} mice were infected with *Lpn* pretreated with medium, naive serum, *Lpn*-specific immune serum, or *Lpn*-specific F(ab')₂. LCVs were isolated from infected MΦ and stained for the lysosomal marker LAMP-1 (red), and the percentage of *Lpn* (green) localized in LAMP-1⁺ vacuoles was quantified. Dotted lines visualize LCVs. (Scale bar: 2 μm.) At least 40 LCVs were analyzed in each experimental condition, and SDs from three independent experiments are shown. Means ± SD are indicated. *P* values were calculated by Student *t* test (two-tailed, unpaired). ns, not significant.

growth. Interestingly, *Lpn* pretreated with immune serum were no longer able to replicate intracellularly (Fig. 1B).

Because *Lpn* depend on their T4SS to evade lysosomal fusion and generate a replication-permissive LCV, it is conceivable that *Lpn*-specific Abs block the T4SS leading to *Lpn* degradation in the lysosome, as seen with T4SS-deficient *Lpn* ΔT. To address this, we isolated LCVs from MΦ infected with *Lpn*, which had been pretreated with immune serum, and from controls and stained the vacuoles for the lysosomal associated membrane protein 1 (LAMP-1) as well as the T4SS substrate SidC (13) (Fig. 1C). Opsonized *Lpn* showed significantly increased lysosomal localization (LAMP-1⁺) compared with control infections (47% vs. 21%) (Fig. 1C and D). Further characterization of the compartment into which the opsonized *Lpn* are targeted revealed that it is positive for the late endosomal marker Rab7 (Fig. S3A) and associates with the acidic organelle label LysoTracker (Fig. S3B). The lysosomal localization of opsonized *Lpn* was not caused by an impaired function of the T4SS, because they were still able to secrete comparable amounts of the effector protein SidC (Fig. S4). Furthermore, persistence and replication of *Lpn* in the amoeba *Dictyostelium discoideum*, which requires a functional T4SS, were not affected by opsonization (Fig. S5), confirming that the T4SS of opsonized *Lpn* remained fully functional. Finally, lysosomal targeting of opsonized *Lpn* was also observed with human macrophages, indicating that the Ab-mediated lysosomal targeting of *Lpn* is conserved across species (Fig. S6).

FcR Engagement Targets Opsonized *Lpn* to Lysosomal Compartments.

The fact that opsonized *Lpn* are able to replicate in *D. discoideum* but not in MΦ indicates that a host cell component specific for mammalian cells is critical for growth restriction of opsonized *Lpn*. Potential candidates are FcRs, because they are absent in *D. discoideum*. Therefore, we hypothesized that FcR engagement is involved in redirecting *Lpn* to lysosomal compartments, resulting

in restriction of intracellular growth. To address this, we infected bone marrow-derived MΦ from wt or *FcRγ*^{−/−} mice, which lack all activating FcγRs, with *Lpn* that had been pretreated with immune or naive serum and stained LCVs for LAMP-1 (Fig. 1E). Although opsonized *Lpn* preferentially localized to lysosomes in wt MΦ, trafficking of opsonized *Lpn* was not affected in *FcRγ*^{−/−} MΦ, and the bacteria were preferentially found in LAMP-1-negative vacuoles (Fig. 1E and F). In agreement with a role of FcRs for altered trafficking of opsonized *Lpn*, bacteria opsonized with F(ab')₂ fragments derived from immune serum were able to evade phagolysosomal fusion in wt MΦ (Fig. 1E and F). These results show that the host FcRs and the Fc moiety of the Ab are essential to redirect *Lpn* to lysosomes, where they are no longer able to replicate.

To verify the importance of FcR in the protective mechanism against *Lpn* infection in vivo, we infected *FcRγ*^{−/−} mice with IgG-opsonized *Lpn*. In accordance with our in vitro results, IgG opsonization had no protective effect in these animals (Fig. 2A). Furthermore, opsonization of *Lpn* with F(ab')₂ fragments did not result in decreased bacterial titers compared with naive unopsonized controls (Fig. 2B). Thus, in agreement with our in vitro results, both the FcR and the Fc moiety of the Ab are essential to mediate in vivo protection against *Lpn* infection.

FcR Triggering Is Sufficient to Target *Lpn* to Lysosomal Compartments.

To address whether the process of FcR-dependent phagocytosis itself or alternatively, downstream signaling events triggered by FcR engagement would be responsible for lysosomal targeting of *Lpn*, we pretreated MΦ with IgG-coupled latex beads (IgG-beads) or mouse serum albumin-coupled beads (MSA-beads) as a control (Fig. 3A). In this setup, only MΦ that were in contact with beads decorated with IgG received an activating signal through their FcRs. The MΦ were then washed and infected with wt *Lpn*, and intracellular replication was quantified. Although

Fig. 2. Host FcR and the Fc portion of Abs are essential for protection from *Lpn* in vivo. B6 mice were vaccinated with *Lpn* (memory) or were left naive. (A) B6 or FcR $\gamma^{-/-}$ mice were challenged i.n. with IgG- or (B) F(ab')₂-opsonized *Lpn* (IgG, F(ab')₂) or untreated *Lpn* (naive, memory), and CFUs were determined in the lung and BAL 2 d later. Means $\pm$ SD are indicated ($n = 3$). P values were calculated by Student t test (two-tailed, unpaired). ns, not significant.

M Φ that were treated with control beads readily supported *Lpn* growth, M Φ that were pretreated with IgG-beads were no longer permissive for *Lpn* replication, despite unaltered bacterial uptake (Fig. 3A). Comparable results were obtained when opsonized replication-deficient *Lpn* ΔT were used to stimulate FcRs before infection with untreated wt *Lpn* (Fig. S7). In line with our previous findings, pretreatment with IgG-beads led to significantly increased *Lpn* localization in LAMP-1⁺ compartments (35% vs. 16% in controls) (Fig. 3B and C). These results show that FcR engagement, even if it is temporally and spatially separated from

the uptake of the bacteria, renders M Φ nonpermissive for *Lpn* by targeting the bacteria to lysosomes.

To analyze whether downstream FcR signaling events were required for redirecting *Lpn* into lysosomes, we pharmacologically inhibited kinases involved in FcR signaling and analyzed the lysosomal targeting of *Lpn* (Fig. 3C and D). We targeted kinases that are involved in proximal FcR signaling (phosphoinositide 3-kinases and Syk) as well as kinases involved in signaling further downstream of FcRs (ERK2, Raf, p38 MAPK, and PKC α/β). Targeting of wt *Lpn* into LAMP-1⁺ compartments on FcR stimulation was completely abolished in the presence of any tested inhibitor relevant for FcR signaling, whereas the lysosomal targeting of replication-incompetent *Lpn* ΔT was not affected. In contrast to kinases involved in FcR signaling, blocking of PKC δ/θ , which is not implicated in FcR signaling, had no effect on *Lpn* localization (Fig. 3D). FcR-dependent signaling, involving all major downstream protein kinases in a nonredundant manner, is, therefore, essential for redirecting *Lpn* into lysosomes.

Although the short time period in which the opsonized bacteria are targeted to lysosomes suggests that involvement of de novo host cell protein synthesis in the lysosomal targeting is unlikely, we nevertheless tested if inhibition of protein synthesis affected the intracellular localization of the bacteria. As expected, the preferential localization of opsonized wt *Lpn* in LAMP-1⁺ compartments was not altered in the presence of the inhibitor (Fig. S8), confirming that de novo host cell protein synthesis is not required for FcR-dependent lysosomal targeting of intracellular bacteria.

Host cell proteases, such as the M Φ elastase, can exhibit bactericidal activity against Gram-positive and Gram-negative bacteria (14). To address whether FcR signaling ultimately induces activation and mobilization of host proteases to the LCV, we pretreated the host cells with cell-permeable protease inhibitors before infecting them with opsonized *Lpn*. These targeted elastase-like serine proteases, matrix metalloproteinases, trypsin-like proteases, cysteine proteases, aspartic proteases, serine proteases, peptidyl arginine aldehyde proteases, or the proteasome, re-

Fig. 3. FcR activation and downstream signaling are essential for directing *Lpn* into lysosomes in vitro. (A) RAW M Φ were incubated with IgG- (red ●) or MSA-beads (green ○) or were left untreated (squares). Cells were then infected with wt (■, red ●, and green ○) or ΔT *Lpn* (□), and intracellular growth was measured. (B and C) RAW M Φ expressing LAMP-1-GFP were pretreated with FcR signaling inhibitors or left untreated, and they were subsequently incubated with IgG- or MSA-beads or media. The cells were then infected with DsRed-expressing wt or ΔT *Lpn*, and the percentage of bacteria localizing in LAMP-1⁺ vacuoles was quantified. (Scale bar: B, 5 μ m.) (C) One hundred LCVs were analyzed in each experimental condition, and SDs from two to four independent experiments are shown. (D) RAW M Φ expressing LAMP-1-GFP were pretreated with PKC inhibitors or left untreated and then were infected with DsRed-expressing wt or ΔT *Lpn* pretreated with medium, naive serum, or *Lpn*-specific immune serum; the percentage of bacteria localizing in LAMP-1⁺ vacuoles was quantified. One hundred LCVs were analyzed in each experimental condition, and SDs from three to five independent experiments are shown. P values were calculated by Student t test (two-tailed, unpaired). ns, not significant.

spectively. The lysosomal targeting of opsonized wt *Lpn* in MΦ was affected neither by any of the inhibitors used nor by a combination thereof (Fig. S8). Thus, neither host cell proteases nor host cell protein synthesis is involved in lysosomal targeting of *Lpn* on FcR activation.

FcR Triggering Is Sufficient to Target *Mycobacterium bovis* bacillus Calmette–Guérin into Lysosomal Compartments. Because evasion of phagolysosomal fusion represents a pivotal step in establishing infection for many intracellular pathogens, we next tested whether the lysosomal targeting on FcR stimulation was restricted to *Lpn* or whether other intracellular bacteria were similarly affected. To this end, we infected MΦ that had been pretreated with IgG- or MSA- beads with *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) and analyzed its intracellular localization. In line with our findings for *Lpn*, FcR stimulation readily targeted the bacteria into LAMP-1⁺ compartments (62% vs. 35% in controls) (Fig. S9), indicating that this pathway is more generally used to protect mammalian cells from infections with intracellular vacuolar bacteria.

***Lpn*-Specific Abs Target *Lpn* to Lysosomal Compartments in Vivo.** To confirm that this mechanism was also effective in vivo, we infected naive and memory mice with wt *Lpn* and analyzed their intracellular localization in the lung. In line with our in vitro observations, untreated bacteria did not colocalize with lysosomal markers within alveolar MΦ in naive mice (Fig. 4). In contrast, in memory mice and mice infected with opsonized *Lpn*, the majority of the bacteria colocalized with LAMP-1. Furthermore, F(ab')₂-opsonized *Lpn* did not colocalize with the lysosomal marker, confirming the central role of FcRs in lysosomal targeting of the bacteria in vivo.

Discussion

Antibodies mediate protection against infections by many different intracellular pathogens, and the mechanisms by which they protect the organism from secondary challenges are as manifold as the strategies used by the pathogens to evade destruction by the host. Nevertheless, the protective function of Abs can be attributed to several general mechanisms, including (i) neutralization, which is an important protective mechanism against viral infections (15) and extra- and intracellular bacterial toxins (16, 17), (ii) antibody-dependent cellular cytotoxicity (ADCC), which plays a role in responses against multicellular parasites as well as in eliminating infected host cells (18–20), and (iii) complement activation, which can result in lysis of Gram-negative bacteria (21). With the results presented in this study, we add lysosomal targeting to this list, a mechanism in which Abs engage FcRs and the subsequent downstream signaling renders the host cell non-permissive for intracellular replication of the pathogen, resulting in its lysosomal degradation.

We show that, in the presence of specific Abs, the *Lpn*-mediated subversion of host cell trafficking no longer takes place, because *Lpn* are targeted to lysosomes and can no longer replicate intracellularly. Thus, opsonized *Lpn* are targeted into degradative pathways, indicating that specific Abs can effectively oppose the downstream events initiated by the T4SS. The opsonization of *Lpn* with specific Abs does not interfere with the function of the T4SS itself, because opsonized bacteria are still able to secrete effector proteins and replicate in *D. discoideum*—a process that is dependent on a functional T4SS. The lysosomal targeting of *Lpn* is, however, dependent on functional engagement of activating FcRs on the surface of MΦ. *Lpn* opsonization with F(ab')₂ fragments has no protective effect against intracellular multiplication within MΦ in vitro or in in vivo protection experiments, clearly showing the importance of the Fc moiety of the Abs for the altered intracellular trafficking into lysosomal compartments. The importance of FcR triggering for this protective effect is shown in vitro by the finding that opsonized *Lpn* are not targeted into lysosomal compartments in FcRγ^{−/−} MΦ, which lack activating FcRs. More importantly, *Lpn*-specific Abs do not mediate protection in FcRγ^{−/−}

Fig. 4. Abs redirect *Lpn* to lysosomes in vivo. B6 mice were vaccinated with *Lpn* (memory) or were left naive. The mice were challenged i.n. with IgG- or F(ab')₂-opsonized (IgG, F(ab')₂) or untreated *Lpn* GFP (naive, memory), and the localization of the bacteria was analyzed 16–20 h after infection. Lungs were stained for the lysosomal marker LAMP-1 (red), actin (blue), and nuclei (white), and the percentage of *Lpn* (green) localizing in LAMP-1⁺ compartments was quantified. (Scale bar: A, 20 μm.) (B) *n* = 20 each; means ± SD are depicted. *P* values were calculated by Student *t* test (two-tailed, unpaired). ns, not significant.

mice in vivo. Furthermore, the lysosomal targeting of *Lpn* is not merely a consequence of Ab-mediated phagocytosis, because the signal delivered through the FcR can be temporally and spatially separated from the infection. In addition, FcR-dependent lysosomal targeting is not restricted to *Lpn* infection but is also effective in BCG infection of MΦ.

The fact that MΦ, which had received an FcR trigger before infection, effectively targeted nonopsonized bacteria to lysosomal compartments indicates that FcR cross-linking induces a signaling cascade that counteracts the modulation of host cell trafficking by *Lpn* or BCG effectors and redirects these vacuolar pathogens to lysosomes. Indeed, pharmacological inhibition of protein kinases involved in FcR signaling abolished the Ab-induced lysosomal targeting of *Lpn*. Accordingly, all major FcR downstream signaling pathways are involved in the altered bacterial trafficking in the host cell in a nonredundant manner. In contrast, lysosomal targeting is dependent on neither de novo host cell protein synthesis nor protease activation resulting from FcR activation.

FcR engagement by inert particles was shown to accelerate phagolysosome fusion and maturation (22), and in case of opsonized bacteria, it has been shown to lead to increased bacterial antigen presentation (23, 24). Our results indicate another function of FcR signaling, namely to counteract the subversive strategies used by *Lpn* and BCG within their host cells, even if the Abs are not directly bound to the pathogen.

It is conceivable that this Ab-mediated intracellular re-routing is also relevant in infections by other prokaryotic and eukaryotic intracellular pathogens, such as *Toxoplasma gondii* (25, 26), *Encephalitozoon cuniculi* (27), *Rickettsia conorii* (28), and *Salmonella enterica* serovar Typhimurium (23, 24, 29). Like *Lpn* and BCG, *Salmonella* also actively induces a replication-permissive vacuole within host cells (30). The presence of *Salmonella*-specific Abs leads to enhanced antigen presentation by DCs and thereby, augments T-cell responses in an FcγRIII-dependent manner (23, 24).

It is, therefore, very likely that Ab-coated *Salmonella* are targeted to lysosomes by the same mechanism described in this study and that this not only modulates T-cell responses but also has a direct protective effect. This is supported by the fact that passive immunization protects mice from lethal challenge with *Salmonella* within a time frame that would not allow the generation of effective T-cell responses (31). We therefore propose that specific Abs mediate protection against *Salmonella* by targeting the bacteria into lysosomes, where they are degraded.

Results from studies with the intracellular parasite *T. gondii* suggest that protection through FcR-dependent lysosomal targeting is not restricted to intracellular bacteria but is also effective against eukaryotic intracellular pathogens. *Toxoplasma* build up a modified phagocytic vacuole in which the parasite resides and replicates (26, 32). In contrast to live *Toxoplasma*, dead or opsonized parasites are primarily located in lysosomes (25, 26); after *Toxoplasma* is located in the lysosomal compartment, MΦ are able to kill the parasites, and replication can no longer take place (33). Studies using μMT mice showed that Abs also play a crucial role in mediating resistance to *Toxoplasma* in vivo. It is, therefore, likely that, as in *Lpn* infection, Abs are able to activate MΦ through FcRs and convert them into a nonpermissive state for *Toxoplasma* replication (34). However, Ab-mediated enhanced lysosomal fusion of bacteria that normally evade phagolysosomal fusion might not always lead to control of intracellular bacterial replication, because it seems controversial in case of *M. tuberculosis*/BCG (35–37). These differences in Ab-mediated growth restriction might well relate to differential susceptibility to lysosomal degradation of the respective bacteria (36).

In conclusion, we propose a mechanism of Ab-mediated host cell protection against intracellular pathogens, which is effective against vacuolar pathogens that evade phagolysosomal fusion (Fig. 5A). In the presence of specific Abs, FcRs are engaged, resulting in a signaling cascade that activates the host cell and thereby renders the bacterial subversion of the phagolysosomal pathway ineffective (Fig. 5B). Moreover, FcR engagement and bacterial uptake can be temporally and spatially separated in this process (Fig. 5C). Through this pathway, the host cell becomes nonpermissive for intracellular replication, and the pathogens are targeted to the lysosome for degradation.

Materials and Methods

Bacteria, Mice, and Immunizations. *Lpn* strains used in this study were JR32 (wt Philadelphia-1) (38), G53011 (*icmT* deletion mutant lacking a functional Icm/Dot T4SS; ΔT) (39), thymidine-auxotroph derivatives of *Lpn* ΔT , and Corby (wt or *flaA* deletion mutant). Where indicated, *Lpn* constitutively expressed GFP from plasmid pMMB207-Km14-GFPc (8) or DsRed from plasmid pSW001 (40). Before infections, *Lpn* was grown for 3 d at 37 °C on charcoal yeast-extract agar (CYE) plates. Liquid cultures were inoculated in ACES-buffered yeast extract medium at an OD₆₀₀ of 0.1 and grown for 21 h at 37 °C. To maintain plasmids, chloramphenicol (5 μ g/mL) was added.

C57BL/6 (B6), B6.129P2-Igh-*J*^{m1Cgn} (JHT) (41), C3^{-/-} (42), and *Fcer1g*^{m1Rav} (FcRγ^{-/-}) (43) mice were bred at the Eidgenössische Technische Hochschule Zurich or Biosupport (Zurich-Schlieren, Switzerland) and used at 7–16 wk of age (sex- and age-matched within the experiments). All animal experiments were performed in accordance with institutional policies and have been reviewed by the cantonal veterinary office.

In. immunizations of mice were performed on days -64 and -32 before challenge with 5×10^6 CFU *Lpn* Corby as described (11). For i.n. challenge experiments, *Lpn* were opsonized by incubation for 1 h at room temperature with neat BAL from secondarily infected mice containing *Lpn*-specific IgG and IgA, purified *Lpn*-specific IgG or IgA from BAL, serum of secondarily infected mice, *Lpn*-specific F(ab)², or PBS as control. Mice were challenged i.n. with 5,000 CFU *Lpn flaA* or *Lpn flaA*-GFP.

Intracellular Growth of *Lpn* in Macrophages and Immunofluorescence Microscopy. Bone marrow-derived MΦ were differentiated by culturing in medium supplemented with 20% L929 cell supernatant for 1 wk. RAW 264.7 MΦ and RAW MΦ expressing LAMP-1–GFP (44) or monomeric red fluorescent protein 1 (mRFP1)–Rab7 (45) were grown in Roswell Park Memorial Institute (RPMI) medium with 10% heat-inactivated FCS, which does not support extracellular *Lpn* growth. MΦ were seeded at 5×10^4 cells/well in 96-well plates

Fig. 5. The relevance of the FcR trigger and its downstream signaling for the intracellular fate of *Lpn* in host cells. Schematic view of *Lpn* taken up by a MΦ. (A) Using their T4SS, the bacteria actively avoid fusion with lysosomes but acquire vesicles trafficking between the endoplasmic reticulum (ER) and Golgi, and finally, they replicate in a cellular compartment studded with ribosomes. (B) Opsonized bacteria activate FcRs and are subsequently targeted to lysosomal compartments. (C) Temporal and spatial separation of the FcR activation and FcR-independent uptake of *Lpn* into the MΦ also result in lysosomal targeting of the bacteria.

(growth curves) or at 2.5×10^5 cells/well on sterile cover glasses in 24-well plates (microscopy) 1 d before the experiment. On day 0, *Lpn* from liquid cultures were opsonized with serum from naive or *Lpn*-infected mice, *Lpn*-specific F(ab')₂, or medium for 1 h at room temperature. For intracellular replication assays, MΦ were infected at a multiplicity of infection (MOI) of 0.1 (immune serum-opsonized *Lpn*) or 1 (all others). Infection was synchronized by centrifugation, and the infected MΦ were incubated at 37 °C. Where indicated, the MΦ were pretreated 1 h before infection with opsonized thymidine-auxotroph *Lpn* ΔT or latex beads coated with IgG or MSA. At the time points indicated, MΦ were lysed and the lysate was combined with supernatant from the same well and plated on CYE plates. Colonies were enumerated after 3 d of incubation at 37 °C.

For immunofluorescence microscopy, *Lpn* expressing GFP or DsRed was used. MΦ were infected at an MOI of 10–100, and the infection was synchronized by centrifugation. After 1 h incubation at 37 °C, the cells were washed with PBS, and vacuoles were isolated and stained as described previously (46) or were fixed as intact cells. Where indicated, the MΦ were pretreated 2 h before infection with the PI3K inhibitor LY294002 (Calbiochem), a Syk inhibitor, an ERK2 inhibitor, a Raf inhibitor, or a p38 MAPK inhibitor (Novartis Pharma AG) and 1 h before infection with IgG- or MSA-beads. In separate experiments, MΦ were pretreated with the PKC inhibitors Rottlerin and Gö6976, the host protein synthesis inhibitor cycloheximide, or selected host cell-permeable protease inhibitors, all at concentrations recommended by the manufacturers, or with DMSO as control. The MΦ were

then infected with wt or ΔT *Lpn* pretreated with medium, naive serum, or *Lpn*-specific immune serum.

For the preparation of lungs for microscopy, lungs were perfused with PBS and fixed by infusion of formalin, PBS, 20% sucrose, and finally, optimum cutting temperature (O.C.T.) compound (Sakura) into the lung lumen. The organs were embedded in O.C.T. compound and snap-frozen; 60 μ m cryosections were air-dried, permeabilized using 0.1% Triton X-100 (Sigma-Aldrich) in PBS, blocked (10% goat serum in PBS), and then stained and mounted with Vectashield (Vector Laboratories). Detailed information on antibodies, fluorescent dyes, and sample analysis is in *SI Materials and Methods*.

Determination of Bacterial Titers. Organs were excised and homogenized using a TissueLyser (Qiagen), and serial dilutions were plated on CYE plates.

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