MANIPULATION OF THE CELLULAR MICROBICIDAL RESPONSE AND ENDOCYTIC DYNAMIC BY PATHOGENS MEMBRANE FACTORS

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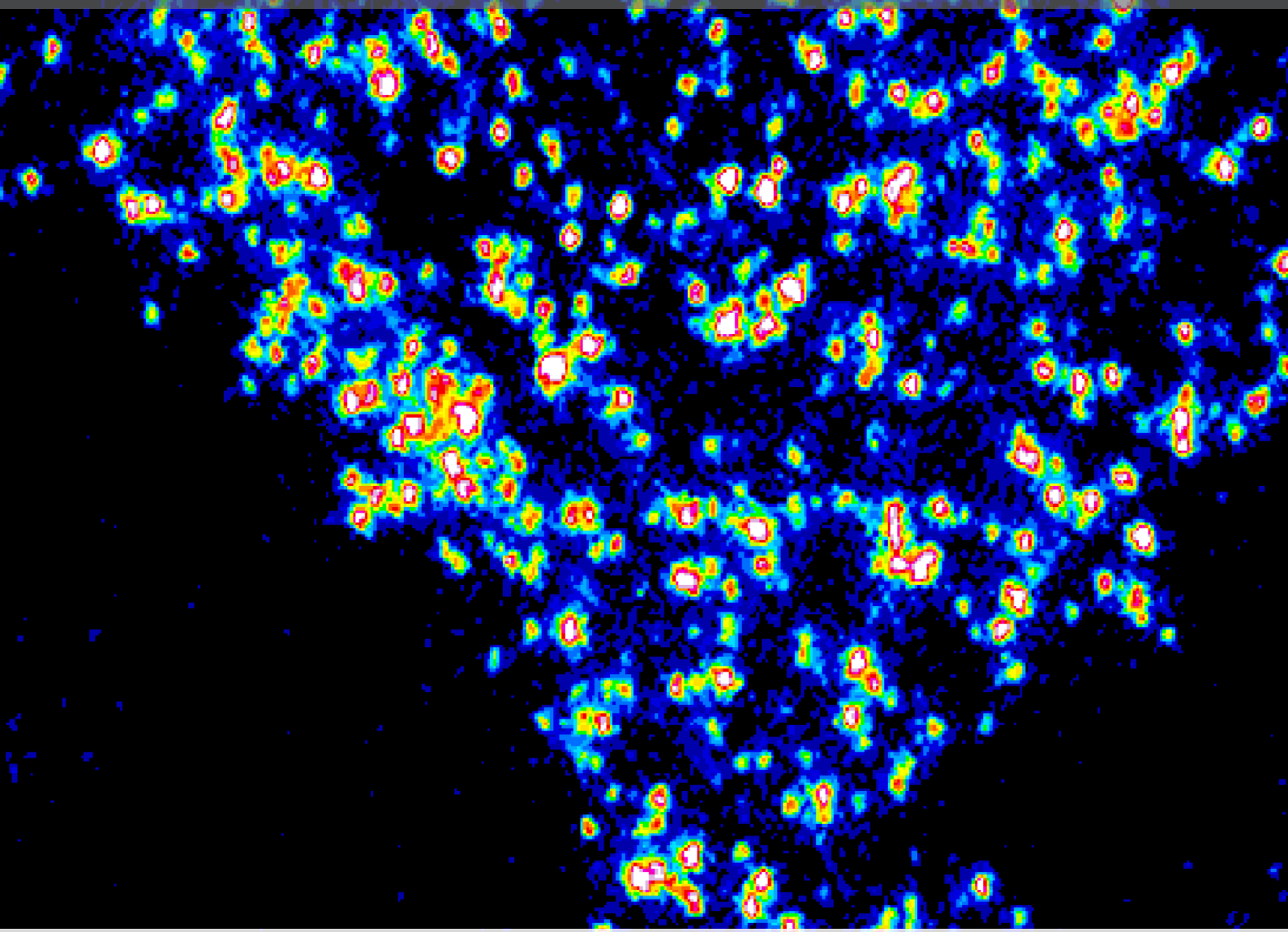

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MANIPULATION OF THE CELLULAR MICROBICIDAL RESPONSE AND ENDOCYTIC DYNAMIC BY PATHOGENS MEMBRANE FACTORS

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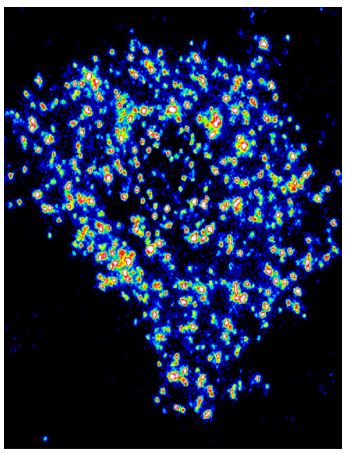

TLR-4 distribution at the BMDMs after *C. burnetii* LPSs stimulation.
BMDMs from wild type mice were challenged for 5 min with *C. burnetii* LPS (1 μg/ml) and the distribution of TLR-4 at the BMDMs surface was examined by confocal microscopy. TLR distribution at the cell surface analyses were performed using ImageJ software. PseudoColor indicate the level of distribution of TLR4. (adapted from Conti et al, in this issue).
Image taken from: Conti F, Boucherit N, Baldassarre V, Trouplin V, Toman R, Mottola G, Mege JL, Ghigo E. Coxiella burnetii lipopolysaccharide blocks p38α-MAPK activation through the disruption of TLR-2 and TLR-4 association. Front Cell Infect Microbiol. 2015 Jan 6;4:182.

Intracellular pathogens, such as bacteria and parasites, have evolved specialized mechanisms to survive and replicate in their host, leading to disorders and diseases. The principle of these mechanisms is to reprogram the microbicidal cell function in order to disable the host cells defence that aims to control and eliminate foreign invaders. Devoid of their defence, cells become permissive to pathogens invasion.

The aim of this Research Topic is to highlight and cover recent understanding of mechanisms and molecules used by pathogens to interfere with the microbicidal function of cells. This Research Topic will focus on the reprogramming of the cellular dynamics, the immune response, the phagolysosome biogenesis and the signal transduction pathways bypathogens. Special attention will be made on non-proteic virulence factors, however this Research Topic is not restricted to non-proteic virulence factors.

Topic Editor Dr. Peter J.K. Kuppen is the founder and owner of Antibodies for Research Applications BV. The other Topic Editors declare no competing interests with regard to the Research Topic subject.

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EDITORIAL
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Editorial: Manipulation of the cellular microbicidal response and endocytic dynamic by pathogens membrane factors

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Microbes such as bacteria, parasites and fungi, have evolved specialized mechanisms to survive and replicate in their host. While high pathogen reproduction is the main purpose to improve next generation growth, adaptation of pathogens to their hosts depends on factors affecting mostly their survival rate. The principle of these mechanisms is to hijack the microbicidal cell function in order to disable and destabilize the host cell defense that. controls and eliminates foreign invaders. Devoid of their defense, cells become permissive to pathogens invasion, a phenomenon that leads to disorders and diseases. To counterstrike the microbicidal functions of the host, microbes use a large arsenal of molecules, known as virulence factors, ranging from proteins and lipids to saccharides. Several evidences highlighted that pathogens use these molecules in order to interfere with the phagolysosome biogenesis, to reprogram signal transduction pathways and, therefore, create a replicative niche.

This special issue covers recent understanding of mechanisms and molecules used by bacterial pathogens such as *Coxiella burnetii* (LPS), *Mycobacterium tuberculosis* (LAM) and parasites such as Leishmania (LPG) to interfere with the microbicidal function of cells (e.g., Rab network, ubiquinilation, TLRs signaling). Attention is mainly focused on the reprogramming of the cellular dynamics (granulomas formation), immune response, phagolysosome biogenesis and signal transduction pathways by pathogens. Thus, Vergne and colleagues well summarize the scientific literature on Lipoarabinomannan, a major immunomodulatory lipoglycan found in the cell envelope of *Mycobacterium tuberculosis,* focusing their attention on its structure and its ability to manipulate the endocytic pathway as well as phagocyte functions (Vergne et al., 2015). In similar manner, the review of Astarie-Dequeker group, address exclusively the role played by phthiocerol dimycocerosates in the modulation of the resident macrophage response (Arbues et al., 2014). Similarly, the composition of the *Leishmania* lipophosphoglycan, its peculiar chemical structure and what is currently known about its effects favoring parasite virulence in the mammalian host, are the subject of a short perspective written by Forestier et al. (2015). The capacity for bacteria to used LPS to hijack molecular process is highlighted by Conti and colleagues which described how *C. burnetii* avoids macrophage activation by the disruption of the TLR-2 and TLR-4 association through the reorganization of the macrophage cytoskeleton by *C. burnetii* LPS (Conti et al., 2015); the same pathogen is the object of the work of Faugaret which have studied the molecular mechanisms of granuloma formation in response to *C. burnetii* and found that it is that it is associated with a core of transcriptional response based on inflammatory genes (Faugaret et al., 2014). Finally, Mottola comments and emphasizes the recent discoveries on bacterial pathogens

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that control the localization or function of the small GTPases Rab5 and Rab7, and therefore modify the maturation from early to late phagosomes (Mottola, 2014). Alomairi and colleagues recapitulate what is currently known about the normal functions of ubiquitination during host cell infection, and they highlight its hijacking to escape clearance and proliferate (Alomairi et al., 2015). It is also important to note that model organisms in the continuous effort to decipher the role of the molecular players involved, contribute strongly to the study of host pathogen interaction and to the discovery of new virulence factors or microbicidal mechanisms: in this special issue, three articles will discuss their invaluable characteristics, with a special attention to the unconventional animal models called also exotic models or ExoMod (Conti et al., 2014; Abnave et al., 2015; Coulaud et al., 2015).

The issue of drug resistance is as old as antibiotics themselves, but so far very few steps have been undertaken to reduce the impact of this threatening public health menace. Beyond the variety of novel approaches being utilized by biotech companies, fundamental research is essential to elucidate how microbes replicate in the host, how molecular players are involved in the host-parasite interactions and how intracellular pathogens finally could become resistant to drugs. Conversely, the capacity of pathogens to perturb the microbicial response can be used to define new therapeutic strategies, and as tools to investigate cells properties: Gorvel and colleagues exploited *C. burnetii* and *Brucella abortus* as tools to elucidate the role of a specific subpopulations of Dendritic cells, the decidual Dendritic cells (dDCs), in placental immune system (Gorvel et al., 2015).

References

Abnave, P., Conti, F., Torre, C., and Ghigo, E. (2015). What RNAi screens in model organisms revealed about microbicidal response in mammals? *Front. Cell. Infect. Microbiol.* 4:184. doi: 10.3389/fcimb.2014.00184

Alomairi, J., Bonacci, T., Ghigo, E., and Soubeyran, P. (2015). Alterations of host cell ubiquitination machinery by pathogenic bacteria. *Front. Cell. Infect. Microbiol.* 5:17. doi: 10.3389/fcimb.2015.00017

Arbues, A., Lugo-Villarino, G., Neyrolles, O., Guilhot, C., and Astarie-Dequeker, C. (2014). Playing hide-and-seek with host macrophages through the use of mycobacterial cell envelope phthiocerol dimycocerosates and phenolic glycolipids. *Front. Cell. Infection Microbiol.* 4:173. doi: 10.3389/fcimb.2014.00173

Conti, F., Abnave, P., and Ghigo, E. (2014). Unconventional animal models: a booster for new advances in host-pathogen interactions. *Front. Cell. Infection Microbiol.* 4:142. doi: 10.3389/fcimb.2014.00142

Conti, F., Boucherit, N., Baldassarre, V., Trouplin, V., Toman, R., Mottola, G., et al. (2015). *Coxiella burnetii* lipopolysaccharide blocks p38alpha-MAPK activation through the disruption of TLR-2 and TLR-4 association. *Front. Cell. Infection Microbiol.* 4:182. doi: 10.3389/fcimb.2014.00182

Coulaud, P. J., Lepolard, C., Bechah, Y., Berenger, J. M., Raoult, D., and Ghigo, E. (2015). Hemocytes from *Pediculus humanus humanus* are hosts for human bacterial pathogens. *Front. Cell. Infection Microbiol.* 4:183. doi: 10.3389/fcimb.2014.00183

Faugaret, D., Ben Amara, A., Alingrin, J., Daumas, A., Delaby, A., Lepolard, C., et al. (2014). Granulomatous response to *Coxiella burnetii*, the agent of Q fever: the lessons from gene expression analysis. *Front. Cell. Infection Microbiol.* 4:172. doi: 10.3389/fcimb.2014.00172

Forestier, C. L., Gao, Q., and Boons, G. J. (2015). Leishmania lipophosphoglycan: how to establish structure-activity relationships for this highly complex and multifunctional glycoconjugate? *Front. Cell. Infection Microbiol.* 4:193. doi: 10.3389/fcimb.2014.00193

Gorvel, L., Ben Amara, A., Ka, M. B., Textoris, J., Gorvel, J. P., and Mege, J. L. (2015). Myeloid decidual dendritic cells and immunoregulation of pregnancy: defective responsiveness to *Coxiella burnetii* and *Brucella abortus*. *Front. Cell. Infection Microbiol.* 4:179. doi: 10.3389/fcimb.2014.00179

Mottola, G. (2014). The complexity of Rab5 to Rab7 transition guarantees specificity of pathogen subversion mechanisms. *Front. Cell. Infection Microbiol.* 4:180. doi: 10.3389/fcimb.2014.00180

Vergne, I., Gilleron, M., and Nigou, J. (2015). Manipulation of the endocytic pathway and phagocyte functions by *Mycobacterium tuberculosis* lipoarabinomannan. *Front. Cell. Infection Microbiol.* 4:187. doi: 10.3389/fcimb.2014.00187

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OPINION ARTICLE
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Unconventional animal models: a booster for new advances in host—pathogen interactions

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ANIMALS AS TOOLS

Historically, biology has greatly benefited and is still benefiting from the study of animals. A quick search of the PubMed database for "animal models" yields some 41,000 studies, and many others will come. Once regarded as the subject of biological studies [Darwin's finches are a well-known example (Peterson, 2008)], animals have become tools to seize and solve the genetic secrets that underlie most of the biological aspects of life. Like in developmental or cancer biology, studies of bacterial infections and more general studies on the interactions involved in host-parasite relationships have been greatly boosted by the availability of animal models that can recapitulate such complex events.

Looking backward for the root of this transition, the work of the English scientist Edward Jenner can be observed as an important turning point. At the beginning of the 19th century, it was known that milkmaids were generally immune to smallpox. Thus, Jenner postulated that the pus that was discharged from the blisters that milkmaids received from cowpox (a less virulent disease) protected them from smallpox. A short time later, Jenner scratched some matter from fresh human cowpox and injected it into a child's arm in an attempt to make him ill with cowpox and, at the same time, to test its protective properties against smallpox. The child became mildly ill with cowpox, fully recovered a week later, and did not contract smallpox once Jenner deliberately inoculated the virus into his arm. The child was then immune, and Jenner went on to test his idea in other humans. Jenner had turned animals (in this case, the cow) into tools, similar to Pasteur, who discovered the vaccines for chicken cholera and rabies a few decades later.

Thereafter, the status of animals changed. They were no longer only subjects of study, they became tools in human hands and, like every tool, animals, such as mice, rabbits and flies, underwent several rounds of optimization. Although experimental immunology began with large animals, today, mouse models, and to a lesser extent *Drosophila* and *C. elegans*, dominate modern biological research. Too often however, scientists think of these models as perfect models of human biology, as if manipulating some genes could actually recapitulate physiology and diseases in different species. Experiment involving mice, genetically modified or not, are extremely helpful to mirror the pathophysiology of most of the diseases, but the comparison human equal mice can turn particularly dangerous. Furthermore, mice used in research are usually young, while many of the diseases that are studied by researchers (such as cancer and neurological diseases) are most common in old people.

Very recently, a large scale collaborative research project showed that inflammation response in human is not depicted by the corresponding mouse models as, the authors stated, "these results show that the genomic responses to different acute inflammatory stresses are highly similar in humans, but these responses are not reproduced in the current mouse models. New approaches need to be explored to improve the ways that human diseases are studied" (Seok et al., 2013).

Moreover, animal research focusing excessively on one laboratory species (mice) may lessen the chance of large scientific advances occurring in the next years.

Here, we will discuss how these chances may be improved greatly by evaluating the unique opportunities that are offered by other unconventional model organisms.

THE REVENGE OF THE FALLEN: DICTYOSTELIUM, ZEBRAFISH, AND LARGE ANIMALS

Living within the soil, *D. discoideum* (**Figure 1A**) phagocytose bacteria for nutritional purposes. In turn, bacteria have evolved several mechanisms to escape amoeboid phagocytosis, and this defense would add an effective advantage to the bacterium. On the other hand, *D. discoideum* is not without defense against infection by bacteria and has evolved mechanisms to efficiently detect and kill bacteria.

A recent study shows that *D. discoideum* cells can discriminate between Gram-negative and Gram-positive bacteria (Nasser et al., 2013; Snyder, 2013). It is still not clear whether this recognition plays a role in defense against potential pathogenic bacteria, and it is conceivable that metabolic alterations induced in pathways may act as triggers for other defense responses. The group of Adam Kuspa identified different sets of genes that are critical for the survival of *D. discoideum* during feeding on Gram-positive or Gram-negative bacteria. The group showed

FIGURE 1 | (A) The soil-living amoeba *Dictyostelium discoideum*. **(B)** The tropical freshwater fish *Danio rerio* (Zebrafish) **(C)** *Bos taurus*, also known as cow (credit: Wikipedia). **(D)** The freshwater flatworm *Schmidtea mediterranea*. **(A–C)** illustrations are under the Creative Common Attribution License (Credit: Wikipedia).

that the cell-surface glycol-protein gp130, among others, is required for growth on Gram-positive bacteria, whereas the putative AX4 family lysozyme-like protein AlyL is essential for sustained growth on Gram-negative bacteria. Moreover, the same group has shown that the metabolic flux of hexose monomers, from the catabolic breakdown of bacterial cell walls to the anabolic production of pentose monomers, is used by *D. discoideum* to tune the appropriate responses to Gram-positive bacteria (Nasser et al., 2013). Interestingly, a recent study links the burst of pentose phosphate to the activation of macrophages. The sedoheptulose kinase CARKL (Carbohydrate Kinase-Like protein) orchestrates the balance between pro- and anti-inflammatory immune responses through metabolic control. These examples highlight how the study of a very simple amoeba may shed light into the function of innate immune systems in a variety of different organisms (Haschemi et al., 2012).

The expansion of *Danio rerio* (zebrafish) (**Figure 1B**) organism in biomedical research is establishing it as a suitable disease model to study infection related pathologies such as tubercolosis (TB). In fact, *D. rerio* is naturally susceptible to TB caused by *Mycobacterium marinum* (Mm) and, similarly to mammals, both innate and adaptive immunity are involved in protection against TB infection. In 2002, Davis et al., thanks to the optical transparency of zebrafish embryos, performed a real time visualization of granuloma formation following *M. marinum* infection (Davis et al., 2002). In this study the authors showed that granuloma structures appeared surprisingly at the early step of the infection in a context of innate immunity. This result was remarkable since previous reports suggested that components of adaptive immunity, principally T lymphocytes, played a leading role in the recruitment and activation of macrophages to form Mycobacterium granulomas (Flynn and Chan, 2001).

The use of large animals as experimental models has provided important advances in an increasing number of developmental immunology studies, and swine, horses, cattle, sheep, and deer might be as good as or better than mice for studying several human pathologies such as influenza, tuberculosis, Crohn's disease, asthma, and viral diarrhea. Beyond the obvious advantages due to their size (sampling tissues or liquids and easier surgical intervention), it is important to note that large animals and humans have often developed as out-bred populations over millennia, so it is plausible that their immune systems have been modeled by exposure to a similar extent of infectious agents.

A classic example of convergent disease, even if still controversial, is Crohn's disease in humans, which shows some similarities with Johne's disease in large animals (**Figure 1C**) (Shanahan, 2002). Johne's disease is caused by *Mycobacterium avium* (*Spp. paratuberculosis* or *MAP*), and the main clinical signs, which are rarely evident until two or more years after the initial infection, are diarrhea and wasting. Several studies showed that a high percentage of people with Crohn's disease are infected with *M. avium* (*Spp. paratuberculosis*). Interestingly, recent studies have shown that IL-23 plays a central role in driving the inflammatory response in Crohn's disease; therefore, extending these studies to cattle at the clinical stage of infection might reveal that IL-23 also plays a central role in Johne's disease and that IL-23 might be one of the factors involved in the breakdown in protective immunity. Despite *M. paratuberculosis* began first proposed as an etiologic agent in Crohn's disease more than 25 years ago (Davis and Madsen-Bouterse, 2013), in some cases, there is no clear evidence indicating that *M. avium* is a causative agent or that its presence only represents an incidental association. More detailed studies in cattle may provide background information for comparisons to the immune response during the latent stage of MAP infection in healthy subjects and in patients with Crohn's disease.

FROM HERE TO ETERNITY: THE PLANARIANS EXPERIENCE

Planarians (**Figure 1D**) are non-parasitic flatworms that live in fresh waters. They are mainly known by the scientific community for their ability to almost limitlessly regenerate thanks to the high presence of neoblast throughout their tissues. Neoblast are pluripotent somatic stem cells present in the parenchyma and

they can give rise to all other 30–40 different cell types. Due to this property, planarians have been extensively used as an animal model: they are cheap, small and can multiply by simply cutting their body, which is a property that is mainly used for developmental biology studies. Finally, they do not rise any ethical concern (Newmark and Sánchez Alvarado, 2002; Sánchez Alvarado, 2007).

Lately, planarians have become one of the model references for studying stem cell biology, but these flatworms may also be useful for studying other biological issues. More than 20 years ago, M. Morita and T. Sakurai (Ishii and Sakurai, 1991; Morita, 1991; Morita and Collins, 1995) noted the peculiar role of certain cells called "reticular cells" that could mediate the early immune response. These high-degree mobility cells could recognize foreign material, such as bacteria, as early as 8 h after their introduction. In 2012, Zhou et al. characterized a serine protease whose expression was induced after ingestion of bacteria (a non-pathogenic laboratory strain of *E. coli DH5*α) in the flatworm *Dugesia japonica* (Zhou et al., 2012). As serine proteases may be mediators of immune responses in mosquito, the authors hypothesized that induction, which is specifically triggered when the worms were challenged with bacteria, could represent a first step of a wider and unknown host-pathogen relationship in planarian.

Finally, it has been shown (Abnave et al., 2014) that planarians are highly resistant to infections by bacteria that are highly pathogenic to humans, *C. elegans* and *D. melanogaster*, and planarian display a genuine immune response involving at least three genes with orthologs in humans, MORN2 (Membrane Occupation and Recognition Nexus-2 protein), DUSP19 (Dual-Specificity phosphatase enzyme), and PAQR3 (progestin/adipoQ receptor-3). Particularly, they demonstrated for the first time that MORN2 has a role in LC3-associated phagocytosis (LAP), and it is essential in eliminating bacterial pathogens by human macrophages as well as by flatworms. Thus, the dataset collected by P. Abnave and colleagues highlights, for the first time, a major interest in studying planarian defense mechanisms to identify conserved immune factors.

Last year, the discovery that *S. mansoni*, a parasitic tapeworm that is the cause of Schistosomiasis, one of the most prevalent human parasitic diseases, has its own neoblast population prompted researchers to hypothesize that some species of planarians (*Schmidtea mediterranea* and *D. japonica*) might be good models for studying the disease, whose cure currently relies on a single compound, praziquantel (Collins and Newmark, 2013; Collins et al., 2013).

THINK DIFFERENTLY

It is now widely accepted that mouse models, although still very precious, may show some limits in simulating human biological processes or diseases (Seok et al., 2013; Özdemir et al., 2014). Using non-human primates may be a straightforward solution because their physiologies are closer to that of humans, but economical and ethical issues are a barrier for most research institutes. Regarding host-parasite diseases, working on new model such zebrafish might open completely new routes of study. In the same way, using *S. mediterranea* or *D. japonica* flatworms as model organisms (actually absent) to study the physiology of the parasite *S. mansoni* might help us to find new drugs to counter Schistosomiasis, whose control currently relies on a single drug. On the other hand, some less-considered animals could effectively mirror the human immune response. Cow, for example, can be a suitable model to study intestinal and uterine infection because the production of lipopolysaccharides or pro-inflammatory cytokines is similar to that observed during human inflammation processes.

It is indisputable that mouse models are and will be for a long time the most suitable and convenient animal model in biological research, but it is now clear that mice do not represent the best biological model due to the intrinsic differences between rodents and humans. Hence, there is interest in developing and studying less regarded organisms that, as the few examples here have shown, may be greatly useful to the biological understanding of complex host-pathogen interactions.

REFERENCES

Abnave, P., Mottola, G., Gimenez, G., Boucherit, N., Trouplin, V., Torre, C., et al. (2014). Screening in planarians identifies MORN2 as a key component in LC3-associated phagocytosis and resistance to bacterial infection. *Cell Host Microbe* 16, 338–350. doi: 10.1016/j.chom.2014.08.002

Collins, J. J., and Newmark, P. A. (2013). It's no fluke: the planarian as a model for understanding schistosomes. *PLoS Pathog.* 9:e1003396. doi: 10.1371/journal.ppat.1003396

Collins, J. J., Wang, B., Lambrus, B. G., Tharp, M. E., Iyer, H., and Newmark, P. A. (2013). Adult somatic stem cells in the human parasite *Schistosoma mansoni*. *Nature* 494, 476–479. doi: 10.1038/nature11924

Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity* 17, 693–702. doi: 10.1016/S1074-7613(02)00475-2

Davis, W. C., and Madsen-Bouterse, S. A. (2013). Crohn's disease and *Mycobacterium avium* subsp.paratuberculosis: the need for a study is long overdue. *Vet. Immunol. Immunopathol.* 145, 1–6. doi: 10.1016/j.vetimm.2011.12.005

Flynn, J. L., and Chan, J. (2001). Immunology of tuberculosis. *Annu. Rev. Immunol.* 19, 93–129. doi: 10.1146/annurev.immunol.19.1.93

Haschemi, A., Kosma, P., Gille, L., Evans, C. R., Burant, C. F., Starkl, P., et al. (2012). The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* 15, 813–826. doi: 10.1016/j.cmet.2012.04.023

Ishii, S., and Sakurai, T. (1991). Food ingestion by planarian intestinal phagocytic cells? A study by scanning electron microscopy.*Hydrobiologia* 227, 179–185. doi: 10.1007/BF00027600

Morita, M. (1991). Phagocytic response of planarian reticular cells to heat-killed bacteria. *Hydrobiologia* 227, 193–199. doi: 10.1007/BF00027602

Morita, M., and Collins, F. (1995). Structure and function of the reticular cell in the planarian *Dugesia dorotocephala*. *Hydrobiologia* 305, 189–196.

Nasser, W., Santhanam, B., Miranda, E. R., Parikh, A., Juneja, K., Rot, G., et al. (2013). Bacterial discrimination by dictyostelid amoebae reveals the complexity of ancient interspecies interactions. *Curr. Biol.* 23, 862–872. doi: 10.1016/j.cub.2013.04.034

Newmark, P. A., and Sánchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. *Nat. Rev. Genet.* 3, 210–219. doi: 10.1038/nrg759

Özdemir, B. C., Pentcheva-Hoang, T., Carstens, J. L., Zheng, X., Wu, C.-C., Simpson, T. R., et al. (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell* 25, 719–734. doi: 10.1016/j.ccr.2014.04.005

Peterson, A. T. (2008). How and why species multiply: the radiation of Darwin's Finches. *Q. Rev. Biol.* 83, 304–305. doi: 10.1086/592628

Sánchez Alvarado, A. (2007). Stem cells and the planarian *Schmidtea mediterranea*. *C. R. Biol.* 330, 498–503. doi: 10.1016/j.crvi.2007.05.005

Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3507–3512. doi: 10.1073/pnas.12228 78110

Shanahan, F. (2002). Crohn's disease. *Lancet* 359, 62–69. doi: 10.1016/S0140-6736(02)07284-7

Snyder, M. L. D. (2013). Bacterial discrimination: dictyostelium's discerning taste. *Curr. Biol.* 23, R443–R446. doi: 10.1016/j.cub.2013.04.021

Zhou, L., Wu, S., Liu, D., Xu, B., Zhang, X., and Zhao, B. (2012). Characterization and expression analysis of a trypsin-like serine protease from planarian *Dugesia japonica*. *Mol. Biol. Rep.* 39, 7041–7047. doi: 10.1007/s11033-012-1535-x

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What RNAi screens in model organisms revealed about microbicidal response in mammals?

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The strategies evolved by pathogens to infect hosts and the mechanisms used by the host to eliminate intruders are highly complex. Because several biological pathways and processes are conserved across model organisms, these organisms have been used for many years to elucidate and understand the mechanisms of the host-pathogen relationship and particularly to unravel the molecular processes enacted by the host to kill pathogens. The emergence of RNA interference (RNAi) and the ability to apply it toward studies in model organisms have allowed a breakthrough in the elucidation of host-pathogen interactions. The aim of this mini-review is to highlight and describe recent breakthroughs in the field of host-pathogen interactions using RNAi screens of model organisms. We will focus specifically on the model organisms *Drosophila melanogaster, Caenorhabditis elegans,* and *Danio rerio*. Moreover, a recent study examining the immune system of planarian will be discussed.

Keywords: *D. melanogaster, C. elegans, D. rerio,* RNA interference, host-pathogen interaction, innate immunity, orthologs

INTRODUCTION

The innate immune system is the first line of defense against invading pathogens and is therefore considered to be of prime importance in host-pathogen interaction studies. Various model organisms have been studied for many years to understand the role of various components of the innate immune system pathways involved in host-pathogen interactions. Several strategies are available to identify the role of genes involved in various pathways. Gene knock-outs or mutations have been used routinely to identify the functions of genes. However, these strategies are limited by several constraints; for example, the number of genes that can be targeted, two or three at a time, is highly limited. The development of RNA interference, a post-transcriptional gene silencing technique in small model organisms, represents a major breakthrough in these types of studies. When the RNAi system was discovered in *Caenorhabditis elegans* by Fire and Mello in 1998, the scientific community soon realized the potential of this tool for research (Fire et al., 1998). Later, when it was confirmed that RNAi also operates efficiently in *Drosophila* (Hammond et al., 2000), large-scale gene silencing strategies were developed for *Drosophila* cells (Ramet et al., 2002; Kiger et al., 2003; Lum et al., 2003; Boutros et al., 2004). The RNAi technique is associated with several limitations and shortcomings such as, transient and incomplete inhibition and even if there is sufficient down regulation of gene the phenotype can differ from the genetic null phenotype. It has also been observed that RNAi may not elicit effective and specific inhibition in all situations and may have nonspecific and off-target effects. However, despite some important limitations such as the lack of cell-type specifity, RNAi has become one of the highly popular and favorite techniques of researchers for analyzing gene functions. The high-throughput screens carried out in various model organisms have enabled the discovery of several components involved in host-pathogen interactions and thus helped to identify and/or validate the functions of their orthologs present in the mammalian immune system.

In this mini review, we will highlight various components of innate immune pathways involved in multiple stages of host-pathogen interactions, which were discovered from RNAi screenings in *D. melanogaster, C. elegans, D. rerio* and their orthologs in mammals. Finally, a recent study confirming the existence of a genuine innate immune response in planarian will be discussed briefly.

DROSOPHILA MELANOGASTER

Several *Drosophila* immune response elicitors have been discovered so far (**Table 1**). Pattern recognition receptors (PRRs) are molecules involved in recognizing microbe specific patterns. They are proteins expressed by cells of the innate immune system (which includes macrophages and neutrophils in mammals) to identify pathogen-associated molecular patterns (PAMPs) that play a role during pathogen defense or cellular stress. They are also known as primitive PRRs, as they evolved before adaptive immunity (Buchmann, 2014). Usually, these molecules induce an antimicrobial signaling cascade in response to microorganisms. Peptidoglycan recognition molecules (PGRPs), which include CD14 (cluster of differentiation 14), TLR2 (Toll-like receptor 2), NOD1, and NOD2 (nucleotide-binding oligomerization domain 1 and 2), and peptidoglycan-lytic enzymes (lysozyme and amidases), are PRRs that are highly conserved in higher eukaryotes, from insects to mammals (Dziarski, 2003).

In *Drosophila,* PGRP-LC, a transmembrane protein required for the response to bacterial infection, was discovered by three separate groups in the year 2002 (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). One of these discoveries was a study

Table 1 | Discoveries in *D. melanogaster, C. elegans* and *D. rerio* using RNAi and the homology of the identified genes in mammals.

	Mammalian Orthologs	*D. melanogaster*	*C. elegans*	*D. rerio*	References
Recognition	Glycopeptide hormone receptors		FSHR1		Cho et al., 2007
	PGRPs	PGRP-LC		PGRP-SC1a PGRP-SC2	Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Li et al., 2007
	TOL receptors		TOL-1		Aballay et al., 2003
			Bus-2/2/12/8		Gravato-Nobre et al., 2011
	C-type lectins		C-type lectins		
Signaling	IAPs	Iap2 Dnr1			Foley and O'Farrell, 2004; Gesellchen et al., 2005
	PDGF/VEGF receptor	PVR			Ragab et al., 2011
	Myd88			Myd88 (TLR adaptor)	van der Sar et al., 2006
		Deaf-1			Kuttenkeuler et al., 2010
	CNOT4	Not4			Grönholm et al., 2012
	GRK5	Gprk2			Valanne et al., 2010
	Akirin1/2	Akirin			
	Insulin receptor		DAF-2 (Membrane receptor)		Murphy et al., 2003
	FOXO		DAF-16 (Transcription Factor)		Murphy et al., 2003
	P38 MAPK JNK		P38 MAPK JNK		Pukkila-Worley et al., 2012
	TGF-β		TGF-β		Irazoqui et al., 2010
Antimicrobial response	Transglutaminases	Transglutaminases			Shibata et al., 2013
	NADPH oxidase (NOX)			Duox	Flores et al., 2010
	SPP proteins		SPP proteins		Roeder et al., 2010
Cell migration	CXCR3/CXCR5			CXCR3.2 (Chemokine receptor)	Meijer and Spaink, 2011
	MMP9 (Metalloproteinase)			MMP9	Meijer and Spaink, 2011

by Ramet and colleagues. It involved the first large-scale RNAi screen in *Drosophila* S2 cells, and it demonstrated the potential of the RNAi screening approach. Rosetto and colleagues reported in 1995 that the Toll receptor acts as an immune activator in a *Drosophila* blood cell line, thereby demonstrating the role of the Toll pathway in immunity (Rosetto et al., 1995); moreover, the importance of the Toll receptor for antifungal resistance in *Drosophila* was confirmed the next year (Lemaitre et al., 1996). Unlike in other species, the *Drosophila* Toll receptor acts as a cytokine receptor and not as a PRRs (Rämet, 2012). Kuttenkeuler et al. (2010) performed a genome-wide RNAi screen to identify the transcriptional factors involved in the Toll-dependent immune response and demonstrated that Deformed Epidermal Autoregulatory Factor 1 (DEAF1) is required for the expression of the Toll target gene Drosomycin, both in cultured cells and *in vivo*. They also showed that DEAF1 is required to survive fungal, but not *E. coli*, infection (Kuttenkeuler et al., 2010). Another targeted screen by Huang and colleagues revealed that the endosomal proteins Myopic (MOP) and Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) are required to activate the Toll signaling pathway both in cultured cells and in flies (Huang et al., 2010). During the same year, Valanne et al. performed genome-wide RNAi screening to find components of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway and identified an evolutionarily conserved G-protein coupled receptor kinase 2 (GPRK2) that interacts with Cactus and regulates the Toll pathway.

The JAK/STAT (Janus kinase and Signal Transducer and Activator of Transcription) pathway is known to play an important role in the control of a wide variety of biological processes. To

investigate its mechanisms in the context of infection, two groups (Baeg et al., 2005; Muller et al., 2005) carried out a genome-wide RNAi screen in cultured *Drosophila* cells. Comparison between the screens by Baeg and Muller reveals that although both groups used essentially the same library of dsRNA molecules, they performed the studies under distinct conditions and used different cell lines and reporters, causing very little overlap (5-6%) in their discoveries. During the Muller screen, 73% of the genes identified are positive regulators of the pathway (regulators that enhance the Drosophila JAK/STAT activity), whereas 75% of the genes identified during the Baeg screen are putative negative regulators (regulators that negatively regulate the Drosophila JAK/STAT pathway). Negative regulators were also discovered during *in vivo* genome-wide RNAi screens (Kleino et al., 2005; Cronin et al., 2009) to discover genes implicated in susceptibility or resistance to infection with the bacterium *Serratia marcescens*. The group identified multiple genes involved in antibacterial defense and showed that the JAK-STAT signaling pathway regulates stem cell proliferation, highlighting an essential role of epithelial cell homeostasis in the gut during the immune response (Cronin et al., 2009). Several other screens allowed the identification of essential components of the immune deficiency (IMD) pathway. These screens identified Inhibitor of apoptosis 2 (IAP2) and TAK1-associated binding protein 2 (TAB2/TAB/CG7417) as essential components of the IMD pathway (Gesellchen et al., 2005; Kleino et al., 2005) and demonstrated that the proteolytic activity of DREDD is required to cleave IMD proteins (Paquette et al., 2010). Applying RNAi to target transglutaminases (TGs) in *Drosophila* has demonstrated that TG suppresses the expression of genes encoding IMD-controlled antimicrobial peptides, enabling immune tolerance against commensal microorganisms (Shibata et al., 2013).

Phagocytosis is a specific form of endocytosis involving the vesicular internalization of solids, such as bacteria. Utilizing this phagocytic capability of *Drosophila* S2 cells, several RNAi screenings have been performed to discover various components involved in phagocytosis. Ramet and colleagues identified 34 gene products involved in phagocytosis, including proteins that participate in vesicle transport, actin cytoskeleton regulation and a cell surface receptor, and they demonstrated the involvement of PGRP-LC in phagocytosing Gram-negative bacteria (Ramet et al., 2002). Using *Mycobacterium fortuitum,* factors required for general phagocytosis and infection in *Drosophila* S2 cells have been identified; they are mostly involved in the actin cytoskeleton and vesicle trafficking (Philips et al., 2005). These results have been confirmed by the results of several other studies, identifying host factors required for the pathogenesis of intracellular bacteria, such as *Listeria monocytogenes* (Agaisse et al., 2005), and yeast, such as *Candida albicans* (Stroschein-Stevenson et al., 2006). A comparison of the genes appearing in multiple screens revealed that most of the involved genes encode actin regulatory proteins and vesicle transport proteins, suggesting the importance of these processes for pathogen phagocytosis. Moreover, a recent screen targeting factors required for the phagocytosis of *Leishmania donovani* indicated the importance of the small GTPase RAB5, the RAC1-associated protein SRA1 and the actin cytoskeleton regulatory protein SCAR in parasite phagocytosis (Peltan et al., 2012). Thus different RNAi screenings performed in *Drosophila* as well as in *Drosophila* S2 cells have discovered several components of innate immune system such as pathogen recognition receptors (PGRP-LC), transcriptional factors (DEAF1) involved in immune response, components required for phagocytosis of pathogens, components of IMD and NF-κB pathway, molecules required to activate Toll signaling pathway (MOP, HRS) and also the modulators of JAK/STAT pathway.

CAENORHABDITIS ELEGANS

C. elegans exhibits a strong host defense response when challenged by different microorganisms (**Table 1**). For these worms, microorganisms are both a food source and potential pathogens. Although *C. elegans* does not have PGRPs, it nevertheless appears to discern microorganisms directly by binding pathogen-associated molecules. The underlying mechanism remains unknown (Schulenburg and Ewbank, 2007). The results of studies on *S. marcescens* avoidance implicate G protein-coupled chemoreceptors; other candidate pathogen recognition receptors include proteins with leucine-rich repeat domains (Schulenburg et al., 2004) or the large family (>500 members) of F-box domain proteins (Thomas, 2006).Very recently, the role of DCAR1 (dihydrocaffeic acid receptor 1), a G-coupled receptor, has been confirmed by Ewbank's group (Zugasti et al., 2014) in antimycotic response using a genome-wide RNA screen. Although the surface of the nematode *C. elegans* is poorly understood, it is critical for interactions with its surroundings and with pathogens. Recently, Hodgkin's group identified six genes (bus-2, bus-4, bus-12, srf-3, bus-8, and bus-17) encoding proteins predicted to act in surface glycosylation, thereby influencing disease susceptibility (Gravato-Nobre et al., 2011). Mutations in these genes induce resistance to *Microbacterium nematophilum* and perturb the adhesion and biofilm formation of *Yersinia* species, highlighting the importance of interactions with complex surface carbohydrates during infection and biofilm formation processes. A clear, recent example of the potential benefits of this type of study can be found in the work of Alper's group (De Arras et al., 2013). Focusing on host-pathogen interactions, they performed comparative RNA-interference screens in the nematode *C. elegans* and in mouse macrophages. Specifically, they analyzed molecular candidates necessary to recognize pathogens through the LPS (lipopolysaccharide) ligand. Using RNAi, they showed that nearly every gene in this network modulates the response to LPS in mouse macrophage cell lines.

In *C. elegans,* only one Toll homolog (TOL-1) has been identified until now. In 2008, Tenor and Aballay (Tenor and Aballay, 2008) showed that TOL-1 is necessary to avoid *Salmonella enterica* invasion through the pharynx, a first line of defense against pathogens in *C. elegans*. They also demonstrated that TOL-1 is required for the expression of a defensin-like molecule (ABF-2) and a heat-shock protein (HSP-16.41) that is a member of the HSP family proteins needed for *C. elegans* immunity. Thus, for the first time, TOL-1 has been shown to play a direct role in the defense of *C. elegans* against pathogens.

One notable pathway activated by the cell host during pathogen invasion is the mitogen-activated protein kinase signaling pathway. The MAPK signaling mediated innate immunity

in *C. elegans* during *S. enterica* infection (Aballay et al., 2003). The *C. elegans* homolog of P38 mitogen-activated protein kinase (MAPK), which is encoded by the pmk-1 gene, is a prerequisite for activation of the Salmonella-induced programmed cell death (PCD). Inactivation of pmk-1 using RNAi completely attenuated Salmonella-elicited PCD. The same group confirmed the importance of the P38 MAP kinase pathway in the *C. elegans* immune response (Pukkila-Worley et al., 2012).

One well-described mechanism that regulates aging in *C. elegans* is the DAF-2 mediated pathway; "the abnormal Dauer formation/insulin-like growth factor (DAF-2/IGF) pathway." DAF-2 activity shortens life span through its inhibition of DAF-16, a forkhead transcription factor. Using microarrays, Kenyon's group (Murphy et al., 2003) has shown that several DAF-16 targets are antimicrobial genes, as well as genes encoding saposins (related to NK-lysin) and thaumatins which also exhibit antimicrobial activity. Other DAF-16 targets are involved in detoxification and resistance to oxidative stress (e.g., glutathione-S-transferase, catalase and superoxide dismutase) or more general anti-stress mechanisms (Ookuma et al., 2003). Daf-2 mutant worms are resistant to infection, particularly by Gram-positive bacteria.

Intrinsic agents, such as antimicrobial peptides, are important to protect the worm against infection, and most of these peptides belong to the Signal Peptide Peptidase (SPP) protein family. In the intestine of *C. elegans*, SPP-5 exhibits a pore-forming effect on the bacterial membrane and thus kills the bacteria. This antimicrobial polypeptide is needed to deal with *Escherichia coli*, the food source of *C. elegans* in the laboratory, as worms lacking these molecular tools develop poorly due to the substantial number of bacteria that spread throughout their intestines. Certain genes, such as SPP-3, require a contact with particular bacteria to be expressed, whereas others, such as SPP-6, are expressed regardless of the bacteria they get along (Roeder et al., 2010).

C. elegans was found to produce reactive oxygen species (ROS) as a powerful defense against infection. Through a combination of studies employing RNA interference and mutants to examine this ROS production (Hoeven et al., 2011), Hoeven and colleagues proposed a theoretical model in which the ROS produced by Ce-Duox1/BLI-3 during infection form part of a protective immune response in the nematode, indicating that ROS production is a conserved, ancient defense mechanism. It is clear from the cited studies that the knowledge on *C. elegans* immune response greatly benefited from RNAi screening approach. The roles of some well-known key factors such as JNK-MAP kinases and DAF-2 protein have been better understood in the immunity context and the remarkable study of Melo and Ruvkun (Melo and Ruvkun, 2012) clearly filled the existing gap between cellular events and behavioral response, showing how molecular pathways coordinate aversion mechanism allowing animals to detect invading pathogens.

DANIO RERIO

Morpholino is the most efficient method for gene silencing in *D. rerio* (zebrafish). In zebrafish, four PGRPs have been identified, three of which have been cloned and named *pglyrp-2 pglyrp-5, (pgrp-sc)*, and *pglyrp-6* (Li et al., 2007); these genes encode 6 PGRPs (Chang et al., 2007). Zebrafish PGRPs share common features with mammals PGRPs (**Table 1**); they possess both amidase and broad-spectrum bactericidal activities. *In vitro*, zebrafish PGRPs exhibit bactericidal activity against both Gram-negative and Gram-positive bacteria. *In vivo*, pglyrp-5 has been identified using morpholino knockdown to be an essential component of the host defense against *Salmonella typhimurium* and *Bacillus subtilis* in the absence of adaptive immunity in the zebrafish embryo. The intracellular signaling pathway downstream of this receptor has been described, indicating that *pglyrp-5 (pgrp-sc)* is not only linked to the immune response but also to apoptosis and developmental processes (Chang et al., 2009).

The adaptor protein MyD88 plays a role in signal transduction downstream from the recognition of pathogens by TLR. In zebrafish, MyD88 morphants are more susceptible to a strain of avirulent *Salmonella typhimurium* (van der Sar et al., 2006). Similarly to mammals, the zebrafish MyD88 signaling pathway causes induction of *il1b* and interferon (*ifnphi1*) (Stockhammer et al., 2009). In terms of TLRs homologous to mammalian cell surface and endosomal TLRs, zebrafish has specific TLRs (*Tlr19, Tlr20a/b, Tlr20f, Tlr21, Tlr22*) that are present on the endosome (Matsuo et al., 2008; Keestra et al., 2010; Meijer and Spaink, 2011). Traf mediates signal transduction from members of the TNF receptor superfamily.

Unlike mammals, zebrafish possess two type II (γ) IFNs: Ifn-γ1 (*ifng1-1*) and Ifn-γ 2 (*ifng1-2*). Morpholino knockdown studies have shown a partially redundant function of *ifng1-1* and *ifng1-2* in mediating resistance of the zebrafish embryo to *Escherichia coli* and *Yersinia ruckeri* infections (Sieger et al., 2009). IFN-γ has been demonstrated to be the major inducer of ROS production in mice and humans. Furthermore, the role of ROS in pathogen killing was first suggested by the observation that patients with chronic granulomatous disease, who have increased susceptibility to infections, were found to produce little or no superoxide radicals (Curnutte and Babior, 1974). In the zebrafish, a tissue-scale gradient of H_2O_2 is formed following wound induction (Niethammer et al., 2009). Using morpholino knockdown, researchers have shown that H_2O_2 is necessary for the migration of leukocytes to the wound site, particularly due to its known antiseptic role. In this context, the function of ROS production has been shown in zebrafish larvae from knockdown of the NADPH oxidase family member dual oxidase (*duox*) (Flores et al., 2010); *duox* morphants are unable to control enteric *Salmonella typhimurium* infection. In 2010, Phennicie and colleagues reported that cystic fibrosis transmembrane conductance regulator (*cftr*) knockdown disturbs ROS production, which increases the bacterial burden during *Pseudomonas aeruginosa* infection of zebrafish embryos (Phennicie et al., 2010). The role of TNF receptor signaling in resistance to *Mycobacterium marinum* has been described using knockdown in a zebrafish embryo model (Clay et al., 2008). Disruption of TNF receptor expression increases bacterial growth and accelerates granuloma formation. Because granuloma has been shown to play a critical role in tissue dissemination of *Mycobacterium marinum* (Clay et al., 2007), it appears clear that TNF signaling is protective during early stages of infection in the absence of adaptive immunity.

PLANARIAN

Over the last several years, planarians have become a favored model system for studying regeneration and development (Elliott and Sánchez Alvarado, 2012). Studies of these organisms have provided invaluable insights into the mechanisms of tissue growth and regeneration. Twenty-two years ago, a pioneering study suggested that planarians exhibit a phagocytic response to heat-killed bacteria (Morita, 1991). However, no further efforts were made to carry out a more in-depth molecular characterization of planarian immunity.

Quite recently, Abnave et al. (2014) examined and documented the extraordinary capacity of planarians to destroy a wide range of pathogenic bacteria. Using an RNAi screening technique on *Dugesia Japonica* species, they identified 18 antibacterial resistance factors and highlighted the gene *MORN2*, which is conserved in *Homo sapiens* but has been lost in the *C. elegans* and *D. melanogaster*. Functional analysis of the *MORN2* gene in macrophages demonstrated the role of human MORN2 (Hs-MORN2) in restricting bacterial intracellular growth through non-canonical phagocytosis. This work established planarians as a suitable model organism for identifying anti-bacterial immune factors. Out of 18 antibacterial resistance genes identified they found the human orthologs for half of the genes including MORN2. In planarians, MORN2 protected against all strains of bacteria tested. Combining genetic and functional screen in planarians, researchers ascribed the function of human MORN2 to phagocytosis-mediated restriction of *S. aureus*, *L. pneumophila* and *M. tuberculosis* growth in macrophages. Further analyses supported the importance of MORN2 expression in promoting LC3-associated phagocytosis of *M. tuberculosis* and the targeting of bacteria to cathepsin-D-positive phagolysosomes. Complementary to these findings, MORN2 co-immunoprecipitated with LC3 and promoted the lipidation of LC3-I in cells challenged by autophagy-inducing stressors: LPS, IFN-γ and starvation.

CONCLUSION

Genetic screening in model organisms have proved to be a powerful and valuable tool in researcher's hand, and several remarkable studies on model organism (Tobin et al., 2010, 2012) have helped to understand fundamental principles of vertebrates resistance to infection. Few methods have been as important in changing biology research as the RNAi technique, and it can truly be considered one of the most important technological breakthroughs in modern biology. Today, RNAi methods have greatly evolved, and high-throughput screening can be used to identify and functionally assess the thousands of genes within a genome that potentially participate and tune biological processes. Moreover, as the molecular machinery required for this RNAi technique is naturally present in the cells and is also highly conserved throughout different species, we can readily apply this technique on various animal models.

Study of the immune response process has greatly benefited from the application of RNAi screens, as this approach is most successful when it incorporates a focused search for predicted pathway-regulatory proteins; moreover, the ability to adapt the RNAi method to several species, broadening the spectrum of meaningful data, has allowed researchers to deepen knowledge of the immune system in a way that was never possible before.

REFERENCES

Aballay, A., Drenkard, E., Hilbun, L. R., and Ausubel, F. M. (2003). *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway. *Curr. Biol.* 13, 47–52. doi: 10.1016/S0960-9822(02)01396-9

Abnave, P., Mottola, G., Gimenez, G., Boucherit, N., Trouplin, V., Torre, C., et al. (2014). Screening in planarians identifies MORN2 as a key component in LC3-associated phagocytosis and resistance to bacterial infection. *Cell Host Microbe* 16, 338–350. doi: 10.1016/j.chom.2014.08.002

Agaisse, H., Burrack, L. S., Philips, J. A., Rubin, E. J., Perrimon, N., and Higgins, D. E. (2005). Genome-wide RNAi screen for host factors required for intracellular bacterial infection. *Science* 309, 1248–1251. doi: 10.1126/science.1116008

Baeg, G.-H., Zhou, R., and Perrimon, N. (2005). Genome-wide RNAi analysis of JAK/STAT signaling components in Drosophila. *Genes Dev.* 19, 1861–1870. doi: 10.1101/gad.1320705

Boutros, M., Kiger, A. A., Armknecht, S., Kerr, K., Hild, M., Koch, B., et al. (2004). Genome-wide RNAi analysis of growth and viability in Drosophila cells. *Science* 303, 832–835. doi: 10.1126/science.1091266

Buchmann, K. (2014). Evolution of innate immunity: clues from invertebrates via fish to mammals. *Front. Immunol.* 5:459. doi: 10.3389/fimmu.2014.00459

Chang, M. X., Nie, P., and Wei, L. L. (2007). Short and long peptidoglycan recognition proteins (PGRPs) in zebrafish, with findings of multiple PGRP homologs in teleost fish. *Mol. Immunol.* 44, 3005–3023. doi: 10.1016/j.molimm.2006.12.029

Chang, M. X., Wang, Y. P., and Nie, P. (2009). Zebrafish peptidoglycan recognition protein SC (zfPGRP-SC) mediates multiple intracellular signaling pathways. *Fish Shellfish Immunol.* 26, 264–274. doi: 10.1016/j.fsi.2008.11.007

Cho, S., Rogers, K. W., and Fay, D. S. (2007). The *C. elegans* glycopeptide hormone receptor ortholog, FSHR-1, regulates germline differentiation and survival. *Curr. Biol.* 17, 203–212. doi: 10.1016/j.cub.2006.12.027

Choe, K.-M., Werner, T., Stöven, S., Hultmark, D., and Anderson, K. V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. *Science* 296, 359–362. doi: 10.1126/science.1070216

Clay, H., Davis, J. M., Beery, D., Huttenlocher, A., Lyons, S. E., and Ramakrishnan, L. (2007). Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. *Cell Host Microbe* 2, 29–39. doi: 10.1016/j.chom.2007.06.004

Clay, H., Volkman, H. E., and Ramakrishnan, L. (2008). Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity* 29, 283–294. doi: 10.1016/j.immuni.2008.06.011

Cronin, S. J. F., Nehme, N. T., Limmer, S., Liegeois, S., Pospisilik, J. A., Schramek, D., et al. (2009). Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. *Science* 325, 340–343. doi: 10.1126/science.1173164

Curnutte, J. T., and Babior, B. M. (1974). Biological defense mechanisms. The effect of bacteria and serum on superoxide production by granulocytes. *J. Clin. Invest.* 53, 1662–1672. doi: 10.1172/JCI107717

De Arras, L., Seng, A., Lackford, B., Keikhaee, M. R., Bowerman, B., Freedman, J. H., et al. (2013). An evolutionarily conserved innate immunity protein interaction network. *J. Biol. Chem.* 288, 1967–1978. doi: 10.1074/jbc.M112.407205

Dziarski, R. (2003). Recognition of bacterial peptidoglycan by the innate immune system. *Cell. Mol. Life Sci.* 60, 1793–1804. doi: 10.1007/s00018-003-3019-6

Elliott, S. A., and Sánchez Alvarado, A. (2012). The history and enduring contributions of planarians to the study of animal regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 301–326. doi: 10.1002/wdev.82

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888

Flores, M. V., Crawford, K. C., Pullin, L. M., Hall, C. J., Crosier, K. E., and Crosier, P. S. (2010). Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties. *Biochem. Biophys. Res. Commun.* 400, 164–168. doi: 10.1016/j.bbrc.2010.08.037

Foley, E., and O'Farrell, P. H. (2004). Functional dissection of an innate immune response by a genome-wide RNAi screen. *PLoS Biol.* 2:e203. doi: 10.1371/journal.pbio.0020203

Gesellchen, V., Kuttenkeuler, D., Steckel, M., Pelte, N., and Boutros, M. (2005). An RNA interference screen identifies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in Drosophila. *EMBO Rep.* 6, 979–984. doi: 10.1038/sj.embor.7400530

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J., et al. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature* 416, 640–644. doi: 10.1038/nature734

Gravato-Nobre, M. J., Stroud, D., O'Rourke, D., Darby, C., and Hodgkin, J. (2011). Glycosylation genes expressed in seam cells determine complex surface properties and bacterial adhesion to the cuticle of *Caenorhabditis elegans*. *Genetics* 187, 141–155. doi: 10.1534/genetics.110.122002

Grönholm, J., Kaustio, M., Myllymäki, H., Kallio, J., Saarikettu, J., Kronhamn, J., et al. (2012). Not4 enhances JAK/STAT pathway-dependent gene expression in Drosophila and in human cells. *FASEB J.* 26, 1239–1250. doi: 10.1096/fj.11-195875

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404, 293–296. doi: 10.1038/35005107

Hoeven, R., McCallum, K. C., Cruz, M. R., and Garsin, D. A. (2011). Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*. *PLoS Pathog.* 7:e1002453. doi: 10.1371/journal.ppat.1002453

Huang, H.-R., Chen, Z. J., Kunes, S., Chang, G.-D., and Maniatis, T. (2010). Endocytic pathway is required for Drosophila Toll innate immune signaling. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8322–8327. doi: 10.1073/pnas.1004031107

Irazoqui, J. E., Urbach, J. M., and Ausubel, F. M. (2010). Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. *Nat. Rev. Immunol.* 10, 47–58. doi: 10.1038/nri2689

Keestra, A. M., de Zoete, M. R., Bouwman, L. I., and van Putten, J. P. M. (2010). Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. *J. Immunol.* 185, 460–467. doi: 10.4049/jimmunol.0901921

Kiger, A. A., Baum, B., Jones, S., Jones, M. R., Coulson, A., Echeverri, C., et al. (2003). A functional genomic analysis of cell morphology using RNA interference. *J. Biol.* 2:27. doi: 10.1186/1475-4924-2-27

Kleino, A., Valanne, S., Ulvila, J., Kallio, J., Myllymäki, H., Enwald, H., et al. (2005). Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. *EMBO J.* 24, 3423–3434. doi: 10.1038/sj.emboj.7600807

Kuttenkeuler, D., Pelte, N., Ragab, A., Gesellchen, V., Schneider, L., Blass, C., et al. (2010). A large-scale RNAi screen identifies Deaf1 as a regulator of innate immune responses in Drosophila. *J. Innate Immun.* 2, 181–194. doi: 10.1159/000248649

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86, 973–983. doi: 10.1016/S0092-8674(00)80172-5

Li, X., Wang, S., Qi, J., Echtenkamp, S. F., Chatterjee, R., Wang, M., et al. (2007). Zebrafish peptidoglycan recognition proteins are bactericidal amidases essential for defense against bacterial infections. *Immunity* 27, 518–529. doi: 10.1016/j.immuni.2007.07.020

Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., et al. (2003). Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* 299, 2039–2045. doi: 10.1126/science.1081403

Matsuo, A., Oshiumi, H., Tsujita, T., Mitani, H., Kasai, H., Yoshimizu, M., et al. (2008). Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *J. Immunol.* 181, 3474–3485. doi: 10.4049/jimmunol.181.5.3474

Meijer, A. H., and Spaink, H. P. (2011). Host-pathogen interactions made transparent with the zebrafish model. *Curr. Drug Targets* 12, 1000–1017. doi: 10.2174/138945011795677809

Melo, J. A., and Ruvkun, G. (2012). Inactivation of conserved *C. elegans* genes engages pathogen- and xenobiotic-associated defenses. *Cell* 149, 452–466. doi: 10.1016/j.cell.2012.02.050

Morita, M. (1991). Phagocytic response of planarian reticular cells to heat-killed bacteria. *Hydrobiologia* 227, 193–199. doi: 10.1007/BF00027602

Muller, P., Kuttenkeuler, D., Gesellchen, V., Zeidler, M. P., and Boutros, M. (2005). Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature* 436, 871–875. doi: 10.1038/nature03869

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., et al. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* 424, 277–283. doi: 10.1038/nature01789

Niethammer, P., Grabher, C., Look, A. T., and Mitchison, T. J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* 459, 996–999. doi: 10.1038/nature08119

Ookuma, S., Fukuda, M., and Nishida, E. (2003). Identification of a DAF-16 transcriptional target gene, scl-1, that regulates longevity and stress resistance in *Caenorhabditis elegans*. *Curr. Biol.* 13, 427–431. doi: 10.1016/S0960-9822(03)00108-8

Paquette, N., Broemer, M., Aggarwal, K., Chen, L., Husson, M., Ertürk-Hasdemir, D., et al. (2010). Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for Drosophila NF-kappaB signaling. *Mol. Cell* 37, 172–182. doi: 10.1016/j.molcel.2009.12.036

Peltan, A., Briggs, L., Matthews, G., Sweeney, S. T., and Smith, D. F. (2012). Identification of Drosophila gene products required for phagocytosis of Leishmania donovani. *PLoS ONE* 7:e51831. doi: 10.1371/journal.pone.0051831

Phennicie, R. T., Sullivan, M. J., Singer, J. T., Yoder, J. A., and Kim, C. H. (2010). Specific resistance to Pseudomonas aeruginosa infection in zebrafish is mediated by the cystic fibrosis transmembrane conductance regulator. *Infect. Immun.* 78, 4542–4550. doi: 10.1128/IAI.00302-10

Philips, J. A., Rubin, E. J., and Perrimon, N. (2005). Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. *Science* 309, 1251–1253. doi: 10.1126/science.1116006

Pukkila-Worley, R., Feinbaum, R., Kirienko, N. V., Larkins-Ford, J., Conery, A. L., and Ausubel, F. M. (2012). Stimulation of host immune defenses by a small molecule protects *C. elegans* from bacterial infection. *PLoS Genet.* 8:e1002733. doi: 10.1371/journal.pgen.1002733

Ragab, A., Buechling, T., Gesellchen, V., Spirohn, K., Boettcher, A.-L., and Boutros, M. (2011). Drosophila Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells. *EMBO J.* 30, 1123–1136. doi: 10.1038/emboj.2011.4

Rämet, M. (2012). The fruit fly Drosophila melanogaster unfolds the secrets of innate immunity. *Acta Paediatr.* 101, 900–905. doi: 10.1111/j.1651-2227.2012.02740.x

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R. A. B. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. *Nature* 416, 644–648. doi: 10.1038/nature735

Roeder, T., Stanisak, M., Gelhaus, C., Bruchhaus, I., Grötzinger, J., and Leippe, M. (2010). Caenopores are antimicrobial peptides in the nematode *Caenorhabditis elegans* instrumental in nutrition and immunity. *Dev. Comp. Immunol.* 34, 203–209. doi: 10.1016/j.dci.2009.09.010

Rosetto, M., Engström, Y., Baldari, C. T., Telford, J. L., and Hultmark, D. (1995). Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. *Biochem. Biophys. Res. Commun.* 209, 111–116. doi: 10.1006/bbrc.1995.1477

Schulenburg, H., and Ewbank, J. J. (2007). The genetics of pathogen avoidance in *Caenorhabditis elegans*. *Mol. Microbiol.* 66, 563–570. doi: 10.1111/j.1365-2958.2007.05946.x

Schulenburg, H., Leopold Kurz, C., and Ewbank, J. J. (2004). Evolution of the innate immune system: the worm perspective. *Immunol. Rev.* 198, 36–58. doi: 10.1111/j.0105-2896.2004.0125.x

Shibata, T., Sekihara, S., Fujikawa, T., Miyaji, R., Maki, K., Ishihara, T., et al. (2013). Transglutaminase-catalyzed protein-protein cross-linking suppresses the activity of the NF-κB-like transcription factor relish. *Sci. Signal.* 6, ra61. doi: 10.1126/scisignal.2003970

Sieger, D., Stein, C., Neifer, D., van der Sar, A. M., and Leptin, M. (2009). The role of gamma interferon in innate immunity in the zebrafish embryo. *Dis. Model. Mech.* 2, 571–581. doi: 10.1242/dmm.003509

Stockhammer, O. W., Zakrzewska, A., Hegedûs, Z., Spaink, H. P., and Meijer, A. H. (2009). Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to Salmonella infection. *J. Immunol.* 182, 5641–5653. doi: 10.4049/jimmunol.0900082

Stroschein-Stevenson, S. L., Foley, E., O'Farrell, P. H., and Johnson, A. D. (2006). Identification of Drosophila gene products required for phagocytosis of *Candida albicans*. *PLoS Biol.* 4:e4. doi: 10.1371/journal.pbio.0040004

Tenor, J. L., and Aballay, A. (2008). A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity. *EMBO Rep.* 9, 103–109. doi: 10.1038/sj.embor.7401104

Thomas, J. H. (2006). Adaptive evolution in two large families of ubiquitin-ligase adapters in nematodes and plants. *Genome Res.* 16, 1017–1030. doi: 10.1101/gr.5089806

Tobin, D. M., Roca, F. J., Oh, S. F., McFarland, R., Vickery, T. W., Ray, J. P., et al. (2012). Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell* 148, 434–446. doi: 10.1016/j.cell.2011.12.023

Tobin, D. M., Vary, J. C., Ray, J. P., Walsh, G. S., Dunstan, S. J., Bang, N. D., et al. (2010). The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell* 140, 717–730. doi: 10.1016/j.cell.2010.02.013

Valanne, S., Myllymäki, H., Kallio, J., Schmid, M. R., Kleino, A., Murumägi, A., et al. (2010). Genome-wide RNA interference in *Drosophila* cells identifies G protein-coupled receptor kinase 2 as a conserved regulator of NF-κB signaling. *J. Immunol.* 184, 6188–6198. doi: 10.4049/jimmunol.1000261

van der Sar, A. M., Stockhammer, O. W., van der Laan, C., Spaink, H. P., Bitter, W., and Meijer, A. H. (2006). MyD88 innate immune function in a zebrafish embryo infection model. *Infect. Immun.* 74, 2436–2441. doi: 10.1128/IAI.74.4.2436-2441.2006

Zugasti, O., Bose, N., Squiban, B., Belougne, J., Kurz, C. L., Schroeder, F. C., et al. (2014). Activation of a G protein-coupled receptor byits endogenous ligand triggers the innate immune response of *Caenorhabditis elegans*. *Nat. Immunol.* doi: 10.1038/ni.2957

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Hemocytes from *Pediculus humanus humanus* are hosts for human bacterial pathogens

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Pediculus humanus humanus is an human ectoparasite which represents a serious public health threat because it is vector for pathogenic bacteria. It is important to understand and identify where bacteria reside in human body lice to define new strategies to counterstroke the capacity of vectorization of the bacterial pathogens by body lice. It is known that phagocytes from vertebrates can be hosts or reservoirs for several microbes. Therefore, we wondered if *Pediculus humanus humanus* phagocytes could hide pathogens. In this study, we characterized the phagocytes from *Pediculus humanus humanus* and evaluated their contribution as hosts for human pathogens such as *Rickettsia prowazekii*, *Bartonella Quintana,* and *Acinetobacter baumannii*.

Keywords: phagocytes, body lice, typhus

INTRODUCTION

Pediculus humanus humanus is a strictly human ectoparasite with a worldwide distribution (Brouqui and Raoult, 2006) and represents a serious public health threat because it acts as a vector for pathogenic bacteria (Raoult and Roux, 1999). Human body lice may transmit epidemic typhus, which is caused by *Rickettsia prowazekii* (Bechah et al., 2008), the louse-borne relapsing fever, which is caused by *Borrelia recurrentis* (Houhamdi and Raoult, 2005), and trench fever, which is caused by *Bartonella quintana* (Badiaga and Brouqui, 2012). It has also been described that body lice can vectorize *Acinetobacter baumannii* (La Scola and Raoult, 2004). Because body lice are vectors of several human diseases, it is important to understand and identify the compartments (organs, tissue, cells) in which these bacteria reside to define new strategies to counterstroke the capacity of vectorization of the bacterial pathogens by body lice.

Whereas the immune systems of several invertebrates, such as mosquitos (Blandin and Levashina, 2007; Hillyer, 2009), shrimps (Tassanakajon et al., 2013), fruit flies (Kounatidis and Ligoxygakis, 2012), *Caenorhabditis elegans* (Pukkila-Worley and Ausubel, 2012), and more recently, *Mytilus galloprovincialis* (Koutsogiannaki et al., 2014), have been investigated, there is a crucial lack of knowledge concerning the immune system of body lice.

In 2012, evidence suggesting that the immune system of *Pediculus humanus humanus* relies on phagocytosis was reported (Kim et al., 2012), which implied the existence and function of phagocytic cells in these organisms. It is known that phagocytes from vertebrates can be hosts or reservoirs for several microbes. Therefore, we wondered if *Pediculus humanus humanus* phagocytes could hide pathogens.

In this study, we characterized the phagocytes from *Pediculus humanus humanus* and evaluated their contribution as hosts for human pathogens such as *Rickettsia prowazekii*, *Bartonella quintana* and *Acinetobacter baumannii*.

RESULTS

BODY LICE HEMOCYTE PREPARATION AND CULTURE

To purify hemolymph phagocytes, we took advantage of the phagocytes' adherence to coated dishes. Hemolymphs in the abdomen of the body louse (**Figure 1A**) was collected and incubated at either 28 or 37°C in different culture media (EMEM, RPMI, L-15, Schneider) in the presence or absence of CO_2 (**Figures 1B–D**), and the percentage of adherent cells was measured after 16 h of incubation. At 37°C or 28°C and in the presence of CO_2, approximately 10% of cells were adherent, independent of the type of culture medium used (**Figure 1B**). Similar results were obtained at 37°C in the absence of CO_2 (**Figure 1C**). At 28°C and in the absence of CO_2, approximately 70% of cells were adherent when grown in Schneider medium (**Figures 1C,D**), whereas less than 55% of cells were adherent in the other medium conditions (**Figures 1C,D**). Furthermore, 7-day-old hemocytes could be maintained in Schneider medium without extensive cell death. Indeed, after 4 days of culture, cell viability of 72% was observed (**Figure 1E**), and after 7 days of culture, the cell viability decreased to 46%. Beyond 7 days, the cell viability decreased more rapidly, reaching 6% on the 15th day in culture (**Figure 1E**). Therefore, the subsequent experiments were performed in Schneider medium for no more than 7 days.

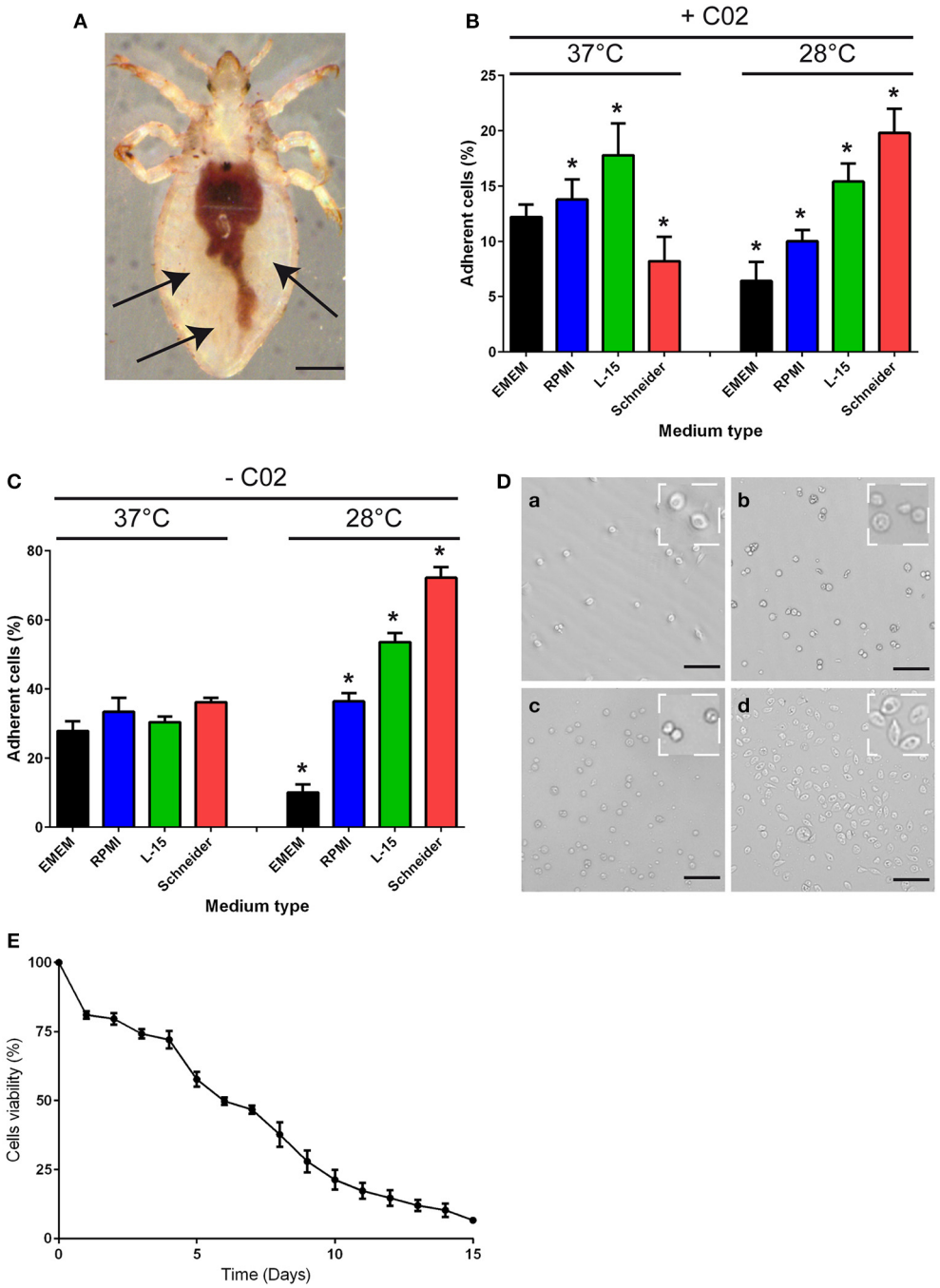

FIGURE 1 | Body lice hemocyte preparation and culture. (A) Hemolymph was collected from the abdomen of *Pediculus humanus humanus* (black arrow) using an insulin syringe equipped with a 29G needle. Scale bar, 400 μm. **(B,C)** The collected hemolymph was added to various culture media in the **(B)** presence or **(C)** absence of CO_2. After 16 h, the number of adherent cells in each condition was evaluated, and the percentage of adherent cells was calculated. The results are expressed as the means ± SDs ($n = 5$) (*$p < 0.05$). **(D)** Representative image of adherent cells observed under phase contrast microscopy after incubation at 28°C without CO_2 in (a) EMEM, (b) RPMI, (c) L-15 medium, or (d) Schneider medium. Scale bar, 25 μm. **(E)** Hemocytes from *Pediculus humanus humanus* were cultivated in Schneider medium at 28°C without CO_2 for 15 days, and their viability was evaluated each day by counting cells. The results are expressed as the mean percentages of viable cells ± SDs ($n = 3$).

CHARACTERIZATION OF THE PHAGOCYTIC PROPERTIES OF BODY LICE HEMOCYTES

We then analyzed the functional properties of the isolated adherent cells to define their phagocytic and microbicidal activities. Mammalian cells that are able to ingest particles, generate ROS and clear bacteria are often considered phagocytes (Aderem and Underhill, 1999; Underhill and Ozinsky, 2002; Puertollano et al., 2011; Underhill and Goodridge, 2012). First, the capacity of the

cells to phagocytose was evaluated (**Figures 2A,B**). The cells were incubated with latex beads at 28°C, and the number of beads captured per cell (**Figure 2A**) (phagocytosis index) was evaluated at various time points (**Figure 2B**). The adherent cells internalized latex beads in a time-dependent manner, and after 30 min, 17% of the cells had phagocytosed 2–3 latex beads; thus, the phagocytosis index was 44.2 ± 5.40 (**Figure 2B**). The percentage of cells that phagocytosed beads and the number of beads per cell increased over time. After 6 h, the phagocytosis index reached 401.6 ± 20.1, with 80% of cells having internalized at least 5 beads/cell (**Figures 2A,B**).

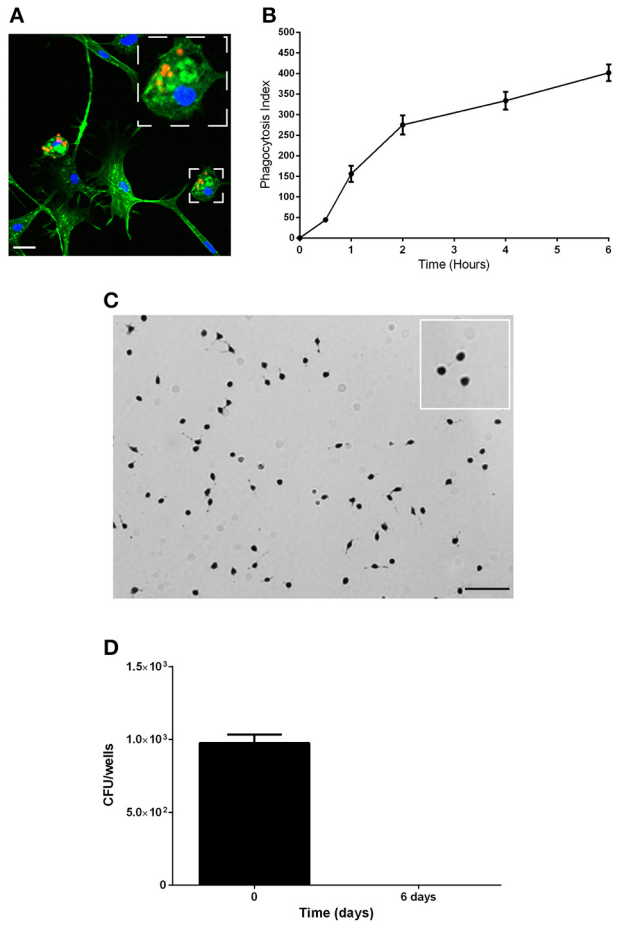

FIGURE 2 | Characterization of the phagocytic properties of the body lice hemocytes. (A,B) The phagocytic capacity of the hemocytes was assessed based on their capacity to internalize latex beads (1/5000 dilution) over time at 28°C. **(A)** Representative image of actin-labeled cells (green) that had internalized latex beads (red). Scale bar, 10 μM. **(B)** The number of beads per cell and the percentage of cells containing engulfed beads were evaluated by microscopy, and these results were used to calculate the phagocytosis index. The mean ± SD is shown ($n = 3$). **(C)** The production of reactive oxygen species was evaluated using a NBT test. Cells were incubated with latex beads in Schneider medium at 28°C to stimulate the production of ROS, and the cells were observed by microscopy. Nearly more than 85% of cells were blue, indicating that they all produced ROS. Images representative of 3 experiments are shown. **(D)** The microbicidal activity of the hemocytes was evaluated by measuring their capacity to eliminate the non-pathogenic bacterial strain *E. coli* K12. Replication was evaluated by cfu counting. The results are shown as the means ± SDs ($n = 2$).

Second, the capacity of the isolated adherent cells to possess microbicidal activities was assessed by analyzing the ability of the adherent cells to produce ROS and to eliminate non-pathogenic bacteria. To evaluate ROS production, we used Nitro blue tetrazolium assays (NBT). We observed the formation of formazan precipitates in more than 85% of cells, which demonstrated that adherent cells produce ROS (**Figure 2C**). To evaluate the microbicidal capacity of the isolated hemocytes, cells were incubated with the non-pathogenic bacterial strain *E. coli* K12, and the behaviors of the bacteria were followed by cfu counting (**Figure 2D**). We found that *E. coli* were phagocyted by the hemocytes and then eliminated. Indeed, after 4 h (day 0) of incubation 976 ± 58 cfu were detected, and after 6 days, bacteria were not detected (no cfu). Taken together, these data show that hemocytes are able to phagocytose and that they have microbicidal activities; therefore, we named these adherent cells from the body louse hemolymph as body louse phagocytes (BLPs).

BLPs ARE RESERVOIRS FOR HUMAN PATHOGENS

Next, we investigated whether BLPs may serve as hosts for bacterial pathogens. For that, we selected several microbes that are vectorized by *Pediculus humanus humanus,* including *R. prowazekii, B. quintana,* and *A. baumannii.* BLPs were infected with the set of selected microbes and cultivated for several days at 28°C in Schneider medium, and the bacterial behaviors and BLP viability were evaluated (**Figure 3**). After internalization, *R. prowazekii* survived and replicated in BLPs. Indeed, using real time PCR, we detected $1 \times 10^3 \pm 140$ copies of bacterial DNA after 4 h of infection (day 0); the number of copies of bacterial DNA increased at day 3 and then reached $1.5 \times 10^4 \pm 2 \times 10^3$ copies 6 days post-infection (**Figures 3A,B**). We observed that *R. prowazekii* replication dramatically affected the viability of the BLPs (**Figure 3E**), and thus bacterial replication led to BLP death and bacterial release into the culture medium. In a similar manner, we found that *B. quintana* was internalized by BLPs ($4 \times 10^3 \pm 1.20 \times 10^3$ *B. quintana* DNA copies) and that *B. quintana* replicated in the phagocytes ($1.8 \times 10^4 \pm 4 \times 10^3$ *B. quintana* DNA copies at day 6) (**Figures 3C,D**). As for *R. prowazekii,* BLPs infected with *B. quintana* exhibited decreased viability (**Figure 3E**). Surprisingly, we observed that *A. baumannii* was not internalized by BLPs, and this lack of internalization was independent of the infection time or the bacteria-to-cell ratio (**Table 1**).

To complete the analysis, we compared the behaviors of the bacteria in BLPs to their behaviors in human macrophages. Interestingly, we found that *R. prowazekii*, *B. quintana*, and *A. baumannii* were able to infect human macrophages (**Table 2**). *R. prowasekii* and *B. quintana* survived but did not replicate in human macrophages, whereas *A. baumannii* replicated and induced the death of human macrophages (**Table 2**). Taken together, these results revealed that BLPs were unable to eliminate *R. prowazekii* and *B. quintana* and allowed their replication.

DISCUSSION

Several experimental models of body louse infestation (Houhamdi et al., 2002) have shown that body lice acquire *R. prowazekii* after feeding from an infected host, thereby allowing *R. prowazekii* to infect the epithelial cells of the upper gut of

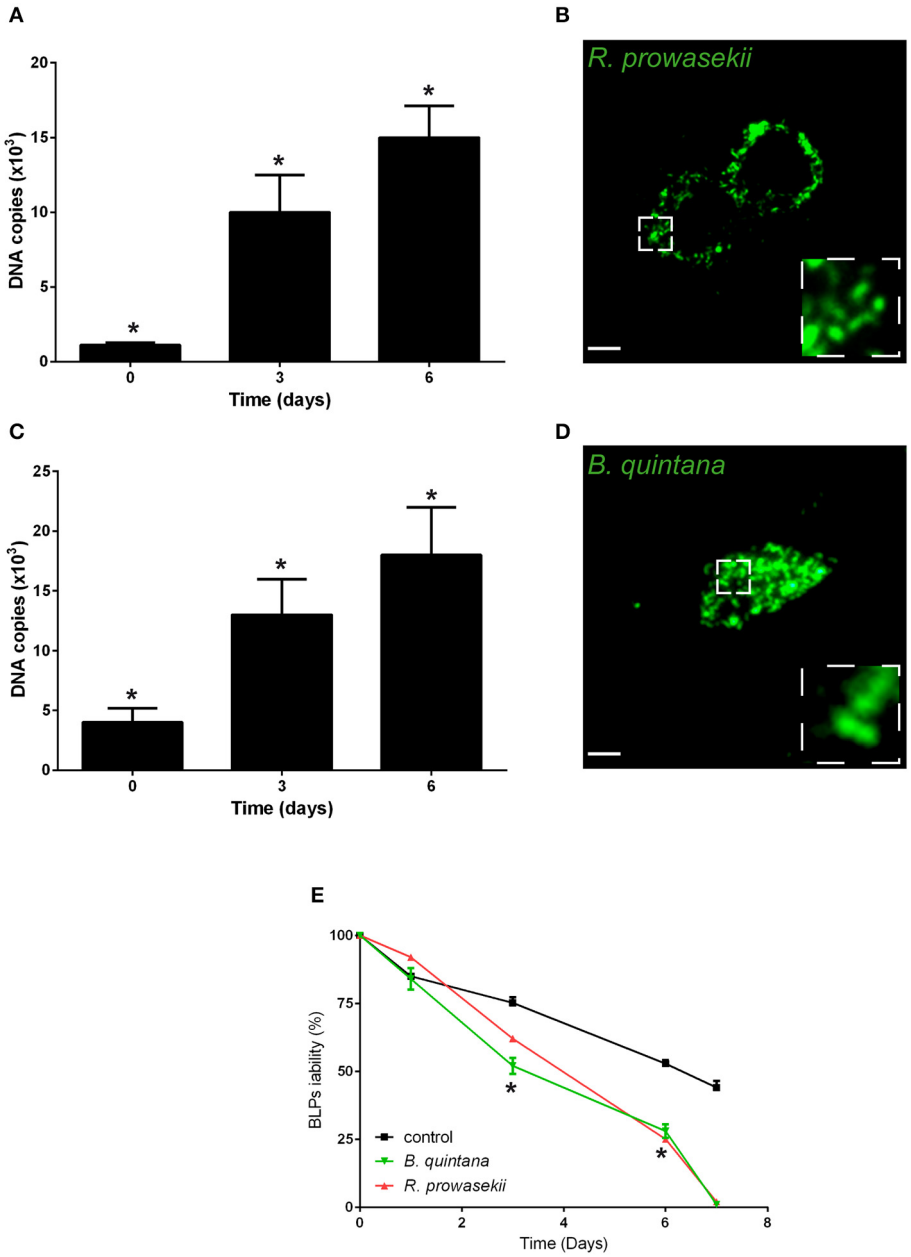

FIGURE 3 | BLPs are reservoirs for *R. prowazekii* and *B. quintana*. Body lice phagocytes were infected for 4 h (day 0) with *R. prowazekii* (100 bacteria-to-cell ratio) or *B. quintana* (100 bacteria-to-cell ratio), bacterial replication was then evaluated by real time PCR, and cell viability was evaluated. **(A)** *R. prowazekii* replicate in BLPs. The results are shown as the means ± SDs ($n = 4$, $^*p < 0.05$). **(B)** Representative epifluorescence microscopy image of body lice phagocytes infected with *R. prowazekii* (green) at 6 days post-infection. Scale bar, 25 μm. **(C)** *B. quintana* replicate in BLPs. The results are shown as the means ± SDs ($n = 3$, $^*p < 0.05$). **(D)** Representative epifluorescence microscopy image of body lice phagocytes infected with *B. quintana* (green) at 6 days post-infection. **(E)** Cell viability was evaluated by cell counting. The results are shown as the means ± SDs ($n = 3$, $^*p < 0.05$).

the lice (Houhamdi et al., 2002). While the immune systems of insects such as *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007) have been carefully investigated, few studies have focused on the immune systems of body lice (Pedra et al., 2003; Kim et al., 2012). Recently, it was reported that the immune system of body lice involves a humoral immune response that requires phagocytosis (Kim et al., 2012). However, the cells involved in this process were not characterized, and their contributions to disease vectorization by the body louse *Pediculus humanus humanus* remained unknown. We have isolated hemocytes from body louse hemolymph and have unraveled the capacity of these cells to produce ROS and to internalize and eliminate non-pathogenic bacteria, similar to mammalian phagocytes. Thus, we suggest that body lice hemocytes are phagocytic cells (BLPs) that are fully

equipped to have antimicrobicidal activity. Body lice, similar to other organisms, have an immune system containing phagocytes.

Next, we investigated the capacity of BLPs to be infected by human pathogens vectorized by *Pediculus humanus humanus.* BLPs were able to phagocytose pathogens such as *R. prowazekii* and *B. quintana*; however, despite their antimicrobial capacity, BLPs were unable to eliminate internalized *R. prowazekii*. In addition, we found that *R. prowazekii* and *B. quintana* replicated within BLPs. Interestingly, we observed that replication of *R. prowazekii* and *B. quintana* induced BLP lysis, and thus, the bacteria were released into the culture media. Surprisingly, *A. baumannii* is not internalized by BLPs, indicating that BLPs are not permissive to *A. baumannii*. We compared the behaviors of *R. prowazekii, B. quintana* and *A. baumannii* in BLPs to their behaviors in human macrophages. Interestingly, the becoming of *R. prowazekii* and *B. quintana* into human macrophages was different than in BLPs. Indeed, we observed survival of *R. prowazekii* and *B. quintana* in human macrophages; however, these bacteria replicated strongly in BLPs. This finding suggests that BLPs most likely do not have the microbicidal equipment to kill *R. prowazekii* and *B. quintana*, in contrast to human macrophages. Unlike *A. baumannii*, *R. prowazekii* and *B. quintana*, we found that *A. baumannii* were not internalized by BLPs, whereas there were internalized by macrophages. It is possible that receptors allowing *A. baumannii* uptake in mammalian phagocytes are not expressed by BLPs or are not conserved from mammalian phagocytes to BLPs.

Our results suggest that BLPs might host microbes and contribute to making *Pediculus humanus humanus* a vector for human diseases. In addition, our data provide new knowledge about the possible localization of human pathogens in body lice. It was known that *R. prowazekii* invades the gut cells (Houhamdi et al., 2002); here, we discover that hemocytes can also be hosts for this pathogenic bacterium. Moreover, the death of the BLPs during bacterial replication might contribute to the spreading of the bacteria into the body lice, and thus, this could be a method for bacterial contamination of the host. It is possible that some viruses and microbes that are responsible for human diseases have no identified vectors because there are hiding in hemocytes, which is small population of the cells of body lice (we scored ∼750 hemocytes/body lice), and thus, the microbes responsible for human diseases could not be detectable using the classical methods of investigation. We also suggest that, in the near future, it will be important to search for viruses and microbes that infect BLPs because as amoebas, BLPs could be reservoirs for unidentified pathogens. In conclusion, we have characterized phagocytes of body lice and unraveled their capacity to be vectors for human pathogens.

Table 1 | *A. baumannii* is not internalized by BLPs.

Infection time (hours)	*A. baumannii*-to-cell ratio				
	10	25	50	100	200
2	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
4	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
6	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
12	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
24	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu

BLPs were incubated for different periods of time with various concentrations of A. baumannii, and A. baumannii uptake was evaluated by CFU counting.

MATERIALS AND METHODS

MEDIA

PMI 1640, DMEM Leibovitz's 15 medium, and Schneider medium were obtained from Invitrogen and were supplemented with 10% fetal calf serum (Gibco-BRL) and 100 U/ml penicillin (100 U/ml), streptomycin (50 μg/ml), gentamycin (10 μg/ml), and vancomycin (5 μg/ml). Before the experiments, the antibiotics were removed by extensive washing.

BACTERIAL STRAINS

Rickettsia prowazekii (Rp22 strain) (Birg et al., 1999; Bechah et al., 2010), *Bartonella quintana* strain Oklahoma (ATCC 49793) (Kernif et al., 2014), and *Acinetobacter baumannii* homeless isolate (La Scola and Raoult, 2004) were grown as previously described.

BODY LICE STRAINS

Colonies of *Pediculus humanus humanus*, strain *Orlando*, were grown as previously described (Fournier et al., 2001).

HEMOLYMPH COLLECTION

Pediculus humanus humanus were starved for 48 h and then washed 3 times in each of four successive solutions: solution A, phosphate-buffered saline (PBS), pH 7, plus Tween 80 (0.1%); solution B, sterile water; solution C, 70% ethanol; solution D, sterile PBS, pH 7. The hemolymph was collected from the abdomens of body lice using an insulin syringe equipped with 29G needles. The collected hemolymph was added to the culture media.

Table 2 | *R. prowasekii, B. quintana* or *A. baumanii* behaviors in human macrophages.

	Day 0	Day 6	Cell viability (%) at day 6
R. prowasekii (DNA copy numbers)	$1.6 \times 10^5 \pm 1.2 \times 10^3$	$1.7 \times 10^5 \pm 1.45 \times 10^3$	83.6 ± 15.3
B. quintana (DNA copy numbers)	$2.5 \times 10^5 \pm 2.4 \times 10^4$	$3.4\ 10^5 \pm 2.8 \times 10^4$	88.2 ± 13.8
A. baumanii (CFU)	$1.0 \times 10^4 \pm 2.0 \times 10^2$	$5 \times 10^4 \pm 1.05 \times 10^3$	5.4 ± 3.2

Human macrophages were infected for 4 h (day 0) with R. prowazekii, B. quintana or A. baumannii, and bacterial replication was evaluated at day 6 by real time PCR or CFU counting.

CELL VIABILITY

The percentage of adherent cells was measured using a phase contrast microscope (Leica DMI 3000 B; Leica, France) as previously described (Prescott and Breed, 1910).

PHAGOCYTOSIS ASSAY

Cells were incubated at day 3 with latex beads (1 μm, Sigma) at 28°C, washed extensively to remove non-internalized beads and then fixed with 3% paraformaldehyde for 20 min. Using an epifluorescence microscope (Leica DMI 3000 B), the numbers of latex beads per cell and the numbers of cells containing latex beads were evaluated. The phagocytosis index is defined as (the average number of latex beads per cell in cells containing latex beads) × (the percentage of cells containing beads).

DETECTION OF REACTIVE OXYGEN SPECIES

The production of reactive oxygen species was evaluated using the NBT test, as previously described (Jozefowski and Marcinkiewicz, 2010). The cells were incubated with latex beads (1 μm, Sigma) for 2 h in Schneider medium at 28°C to induce the production of ROS.

BACTERIAL INFECTION

BLPs were infected with *R. prowazekii*, *B. Quintana,* or *A. baumannii* and then extensively washed to remove the free bacteria; the BLPs were then incubated further. In some experiments, the bacteria were visualized by immunofluorescence, as previously described (Bechah et al., 2010), and cellular F-actin was stained using Alexa 488-conjugated phallacidin. Infection was quantified by real time PCR or cfu counting.

STATISTICAL ANALYSIS

The results are expressed as the means ± SDs and were analyzed using the nonparametric Mann–Whitney *U*-test. Differences were considered significant at $p < 0.05$.

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REFERENCES

Aderem, A., and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 17, 593–623. doi: 10.1146/annurev.immunol.17.1.593

Badiaga, S., and Brouqui, P. (2012). Human louse-transmitted infectious diseases. *Clin. Microbiol. Infect.* 18, 332–337. doi: 10.1111/j.1469-0691.2012.03778.x

Bechah, Y., Capo, C., Mege, J. L., and Raoult, D. (2008). Epidemic typhus. *Lancet Infect. Dis.* 8, 417–426. doi: 10.1016/S1473-3099(08)70150-6

Bechah, Y., El Karkouri, K., Mediannikov, O., Leroy, Q., Pelletier, N., Robert, C., et al. (2010). Genomic, proteomic, and transcriptomic analysis of virulent and avirulent *Rickettsia prowazekii* reveals its adaptive mutation capabilities. *Genome Res.* 20, 655–663. doi: 10.1101/gr.103564.109

Birg, M. L., La Scola, B., Roux, V., Brouqui, P., and Raoult, D. (1999). Isolation of *Rickettsia prowazekii* from blood by shell vial cell culture. *J. Clin. Microbiol.* 37, 3722–3724.

Blandin, S. A., and Levashina, E. A. (2007). Phagocytosis in mosquito immune responses. *Immunol. Rev.* 219, 8–16. doi: 10.1111/j.1600-065X.2007.00553.x

Brouqui, P., and Raoult, D. (2006). Arthropod-borne diseases in homeless. *Ann. N.Y. Acad. Sci.* 1078, 223–235. doi: 10.1196/annals.1374.041

Fournier, P. E., Minnick, M. F., Lepidi, H., Salvo, E., and Raoult, D. (2001). Experimental model of human body louse infection using green fluorescent protein-expressing *Bartonella quintana*. *Infect. Immun.* 69, 1876–1879. doi: 10.1128/IAI.69.3.1876-1879.2001

Hillyer, J. F. (2009). Transcription in mosquito hemocytes in response to pathogen exposure. *J. Biol.* 8:51. doi: 10.1186/jbiol151

Houhamdi, L., Fournier, P. E., Fang, R., Lepidi, H., and Raoult, D. (2002). An experimental model of human body louse infection with *Rickettsia prowazekii*. *J. Infect. Dis.* 186, 1639–1646. doi: 10.1086/345373

Houhamdi, L., and Raoult, D. (2005). Excretion of living *Borrelia recurrentis* in feces of infected human body lice. *J. Infect. Dis.* 191, 1898–1906. doi: 10.1086/429920

Jozefowski, S., and Marcinkiewicz, J. (2010). Aggregates of denatured proteins stimulate nitric oxide and superoxide production in macrophages. *Inflamm. Res.* 59, 277–289. doi: 10.1007/s00011-009-0096-5

Kernif, T., Leulmi, H., Socolovschi, C., Berenger, J. M., Lepidi, H., Bitam, I., et al. (2014). Acquisition and excretion of *Bartonella quintana* by the cat flea, Ctenocephalides felis felis. *Mol. Ecol.* 23, 1204–1212. doi: 10.1111/mec.12663

Kim, J. H., Min, J. S., Kang, J. S., Kwon, D. H., Yoon, K. S., Strycharz, J., et al. (2012). Comparison of the humoral and cellular immune responses between body and head lice following bacterial challenge. *Insect Biochem. Mol. Biol.* 41, 332–339. doi: 10.1016/j.ibmb.2011.01.011

Kounatidis, I., and Ligoxygakis, P. (2012). Drosophila as a model system to unravel the layers of innate immunity to infection. *Open Biol.* 2:120075. doi: 10.1098/rsob.120075

Koutsogiannaki, S., Franzellitti, S., Fabbri, E., and Kaloyianni, M. (2014). Oxidative stress parameters induced by exposure to either cadmium or 17beta-estradiol on *Mytilus galloprovincialis* hemocytes. The role of signaling molecules. *Aquat. Toxicol.* 146, 186–195. doi: 10.1016/j.aquatox.2013.11.005

La Scola, B., and Raoult, D. (2004). *Acinetobacter baumannii* in human body louse. *Emerg. Infect. Dis.* 10, 1671–1673. doi: 10.3201/eid1009.040242

Lemaitre, B., and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* 25, 697–743. doi: 10.1146/annurev.immunol.25.022106.141615

Pedra, J. H., Brandt, A., Li, H. M., Westerman, R., Romero-Severson, J., Pollack, R. J., et al. (2003). Transcriptome identification of putative genes involved in protein catabolism and innate immune response in human body louse (Pediculicidae: *Pediculus humanus*). *Insect Biochem. Mol. Biol.* 33, 1135–1143. doi: 10.1016/S0965-1748(03)00133-4

Prescott, S. C., and Breed, R. S. (1910). The determination of the number of body cells in milk by a direct method. *Am. J. Public Hygiene* 20, 663–664.

Puertollano, M. A., Puertollano, E., De Cienfuegos, G. A., and De Pablo, M. A. (2011). Dietary antioxidants: immunity and host defense. *Curr. Top Med. Chem.* 11, 1752–1766. doi: 10.2174/156802611796235107

Pukkila-Worley, R., and Ausubel, F. M. (2012). Immune defense mechanisms in the Caenorhabditis elegans intestinal epithelium. *Curr. Opin. Immunol.* 24, 3–9. doi: 10.1016/j.coi.2011.10.004

Raoult, D., and Roux, V. (1999). The body louse as a vector of reemerging human diseases. *Clin. Infect. Dis.* 29, 888–911. doi: 10.1086/520454

Tassanakajon, A., Somboonwiwat, K., Supungul, P., and Tang, S. (2013). Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish Shellfish Immunol.* 34, 954–967. doi: 10.1016/j.fsi.2012.09.021

Underhill, D. M., and Goodridge, H. S. (2012). Information processing during phagocytosis. *Nat. Rev. Immunol.* 12, 492–502. doi: 10.1038/nri3244

Underhill, D. M., and Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.* 20, 825–852. doi: 10.1146/annurev.immunol.20.103001.114744

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Alterations of host cell ubiquitination machinery by pathogenic bacteria

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Response of immune and non-immune cells to pathogens infections is a very dynamic process. It involves the activation/modulation of many pathways leading to actin remodeling, membrane engulfing, phagocytosis, vesicle trafficking, phagolysosome formation, aiming at the destruction of the intruder. These sophisticated and rapid mechanisms rely on post-translational modifications (PTMs) of key host cells' factors, and bacteria have developed various strategies to manipulate them to favor their survival. Among these important PTMs, ubiquitination has emerged as a major mediator/modulator/regulator of host cells response to infections that pathogens have also learned to use for their own benefit. In this mini-review, we summarize our current knowledge about the normal functions of ubiquitination during host cell infection, and we detail its hijacking by model pathogens to escape clearance and to proliferate.

Keywords: post-translational modifications, ubiquitin, intracellular bacterial pathogens, cell signaling, phagocytosis, xenophagy, immunological response

INTRODUCTION

Host invasion by bacteria initiates an immune response which relies on multiple cell populations and communications between them. This normally results in the clearance of the intruder. However, in the case of pathogenic bacteria, host defenses are challenged with specific attacks on their molecular machineries.

Several pathogenic bacteria use different types of apparatus (secretion systems), and various molecules (such as endotoxins and exotoxins) to modulate host cells processes and responses to infection. The pathogenicity of these bacteria is associated with their capacity to survive and replicate within a specialized vacuole or within the cytoplasm of host cells. This can be achieved by avoiding or surviving the phagolysosome formation, escaping the autophagy process of bacteria, a process also known as Xenophagy, and interfering with signaling pathways important for immune response, cell survival, and apoptosis.

Host cells response to invaders depends on the modulation of key cellular functions, from signals transduction to receptors and vesicles trafficking. This rapid tuning is only enabled by post-translational modifications (PTMs) of key proteins implicated in these processes (Broberg and Orth, 2010). These PTMs can be of different kinds, chemical such as protein phosphorylation or peptidic such as protein modification by ubiquitin (ubiquitination) and other ubiquitin-like proteins (Ubls) like SUMOs (Sumoylation) and Nedd8 (Neddylation).

Ubiquitination is considered as one of the most common PTM, and regulates virtually every intracellular functions as it is involved in essential eukaryotic cellular processes (Hochstrasser, 2009). Ubiquitin is a small 76 amino acids protein which is linked to a lysine residue of the target protein by its carboxyl terminal end to the amino group of the lysine, creating an isopeptide bond. Ubiquitin itself contains seven lysine residues which can be ubiquitinated. This results in the formation of seven different types of polyubiquitin chains, in addition to the linear ubiquitin chain type that consists in the conjugation of one ubiquitin to the N-terminus of another one. PTM by ubiquitin is a three step process requiring the successive action of an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) which gives target specificity (Pickart and Eddins, 2004) (**Figure 1**). Like any PTM, protein modification by ubiquitin can be reversed by the activity of specific deubiquitinating enzymes (DUBs) (Nijman et al., 2005).

The large variety of regulations mediated by ubiquitin conjugation is also due to this variety of modifications. Indeed, a protein can be mono-ubiquitinated (one ubiquitin on one lysine residue), multi-monoubiquitinated (several mono-ubiquitinated lysine residues), or polyubiquitinated with different kind of polyubiquitin chains (depending on the lysine residue of ubiquitin engaged in the chain). Hence, PTM of proteins by ubiquitin can result in a large variety of modulations, from activity to

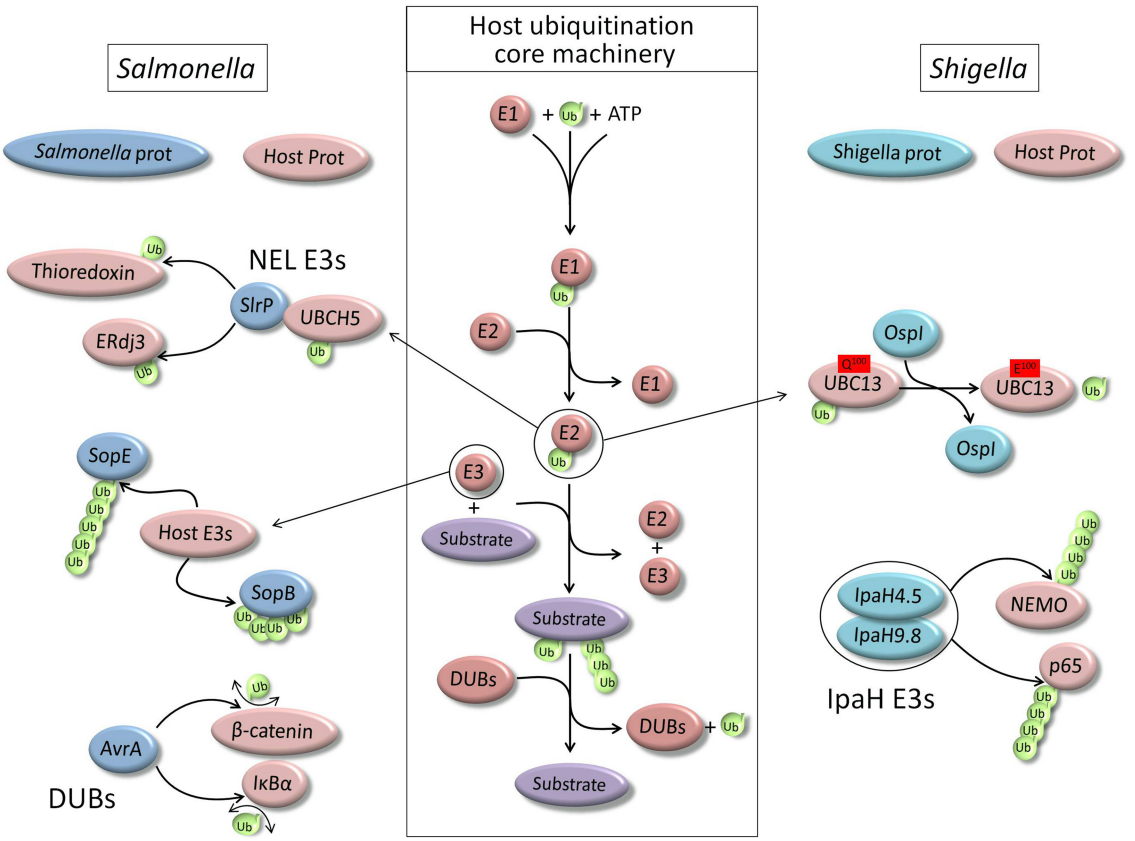

FIGURE 1 | Schematic representation of the core ubiquitination machinery of the host cell and main examples of its hijacking by two model pathogens, *Salmonella typhimurium* and *Shigella flexneri*.

stability, from interactions to sub-cellular localization. Hence, ubiquitination plays important roles in every crucial step of cellular response to pathogens. Therefore, these kinds of PTMs represent good targets for pathogens to impede host cells defense and to increase their virulence.

ROLE OF UBIQUITIN IN NORMAL HOST CELL RESPONSE TO NON-PATHOGENIC BACTERIA

When a bacterium is recognized by a host defense cell, such as a macrophage, it is rapidly phagocytosed with the aim to be destroyed. During this process, the bacterium is typically packed into a membrane, forming a phagosome which is addressed to the lysosome. There, membranes from both organelles fused to form the phagolysosome where the acidic pH and degradative enzymes rapidly digest the intruder (15–30 min). PTMs play important roles in every steps of this process and ubiquitination has a particularly important role at the cell signaling level (inflammatory signals) and at the membrane dynamic level (vesicle trafficking and membrane fusion).

UBIQUITIN IN INFECTION SIGNALING

The first necessity for host defense cells is to recognize invading bacteria as targets. This necessary step is endorsed by receptors of the Toll-like family, a type of pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs). When engaged and activated, these receptors initiate several signaling pathways, major one being the NF-kB pathway which is involved in cytokines production for immune response and cell survival. Interestingly, activation of this pathway is highly dependent on the proteolytic and non-proteolytic ubiquitination of key proteins (Chen, 2005).

NF-kB is a family of heterodimeric transcription factors that, in absence of stimulation, are bound to inhibitory proteins of kB family (IkB) and thereby sequestrated in the cytoplasm.

Bacteria derived molecules, PAMPs, are recognized by toll-like receptors (TLRs) which trigger signaling cascades inside host immune cells once activated (Kawai and Akira, 2010). Upon recognition of PAMPs by TLRs, the kinase IRAK1 (Interleukin-1 Receptor-Associated Kinase 1) is phosphorylated by IRAK4 kinase, and then associates with TRAF6 (TNF receptor associated factor 6), a member of a family of RING-domain E3 ubiquitin ligases (Deng et al., 2000).

TRAF6 then interacts with an E2 ubiquitin-conjugating complex to polymerize K63-linked polyubiquitin chains on itself and on NEMO (NF-kB essential modulator) (Deng et al., 2000; Chen, 2005). Ubiquitinated TRAF6 recruits TAB2 (via its ubiquitin binding domain) and activates the TAB2-associated kinase

TAK1 (Tat-associated kinase 1). TAK1 then phosphorylates the beta subunit of IKK complex, which further phosphorylates the inhibitory IkB component of the NF-kB complex. Ubiquitin-activated TAK1 also phosphorylates and activates MKK kinases, such as MKK6, which in turn activates the JNK and p38 kinases pathways (Wang et al., 2001). Phosphorylated IkB is then polyubiquitinated with K48-linked chains and targeted for proteasomal degradation, while releasing NF-kB to activate the transcription of cytokines and chemokines (Kawai and Akira, 2010).

UBIQUITIN AND XENOPHAGY

Autophagy is a mechanism by which cells can isolate part of their content in a double membrane structure to create autophagosomes in order to degrade it via its fusion with lysosome (Mizushima et al., 2011). This includes cytosol, old mitochondria, proteins aggregates, and also intruders such as bacteria. Like every kinds of autophagy, this class of autophagy, termed Xenophagy (digestion of foreign materials), is a process highly dependent on ubiquitin and ubiquitin-like conjugation (Kirkin et al., 2009). Since its first observation 30 years ago (Rikihisa, 1984), xenophagy appeared to be crucial for pathogens elimination (Gomes and Dikic, 2014). Pathogens targeting to autophagy for destruction has now been extensively studied and we currently know that this process depends on the core machinery of autophagy. Ubiquitin seems to correspond to a "eat-me" signal for autophagy pathways, and this is also true for xenophagy (Perrin et al., 2004; Kirkin et al., 2009).

Following internalization, some pathogens can actively modify their vacuolar compartment in order to block its maturation, or even escape from it and replicate within the cytosol. Host cells xenophagy can target pathogens at any steps of this process, whether they are in their intact or damaged vacuole or within the cytoplasm. Indeed, ubiquitination can take place on proteins of the damaged membrane (Birmingham et al., 2006) and/or directly on bacterial proteins (Perrin et al., 2004). This ubiquitination depends on the activation of PRRs as well as others danger receptors which can sense perturbations in host cell homeostasis caused by invading bacteria (Chen and Nunez, 2010).

This ubiquitination enables the recruitment of standard autophagy receptors which then initiate the formation of the phagophore (also termed isolation membrane), to which ATG (autophagy-related) proteins are recruited. These autophagy receptors include p62 (Zheng et al., 2009), nuclear domain 10 protein 52 (NDP52) (Thurston et al., 2009), and optineurin (OPTN) (Wild et al., 2011), neighbor of BRCA1 gene 1 (NBR1), or TANK binding kinase 1 (TBK1) (Watson et al., 2012).

HIJACKING OF HOST CELL UBIQUITINATION MACHINERY BY PATHOGENIC BACTERIA

Bacterial pathogens have developed multiple ways for manipulating host cell functions to avoid their elimination. As we could previously see, because ubiquitination is involved in major cell signaling responses to infection as well as xenophagy process, interfering with cell host ubiquitination machinery proved to be an efficient way for the survival of many pathogens. Indeed, protein ubiquitination plays a role in any of these processes and pathogens have learned to use it for their own benefit and there are many examples of pathogens interfering with ubiquitination of the host cell. Many pathogenic bacteria utilize specialized type III or type IV secretion systems (T3SS or T4SS) to deliver bacterial effectors proteins into host cells, to modify a variety of cellular processes. There are increasing numbers of effectors that infringe on the ubiquitin pathway, acting as substrates for host cell ubiquitination machinery or as ligases that target specific host and/or bacterial proteins (**Figure 1**).

LEGIONELLA PNEUMOPHILA

Legionella is a Gram-negative intracellular pathogen that is responsible for a severe pneumonia in humans called as Legionnaire's disease. It establishes a niche called the Legionella-Containing Vacuole (LCV), which is permissive for intracellular bacterial propagation. *Legionella* has a type IV secretion system injecting a cocktail of bacterial proteins targeting host cell processes to support bacterial growth, and numbers of these Icm/Dot effectors contain regions with sequence similarity to F-box or U-box domains contained in eukaryotic E3 ligases (Cazalet et al., 2004; de Felipe et al., 2005). Several of these effectors, such as LegAU13/AnkB, LegU1, and LicA, have been shown to interact with components of the Skp-Cullin-F-box (SCF) ubiquitin ligase complex (Price et al., 2009; Ensminger and Isberg, 2010; Lomma et al., 2010). Moreover, the ubiquitin ligase activity has been verified *in vitro* for LegU1, LegAU13/AnkB (Ensminger and Isberg, 2010) as well as for LubX (Legionella U-box protein) (Kubori et al., 2008). Some substrates for these different *Legionella's* ligases have been identified by using standard interactomic techniques such as yeast two hybrid. Hence, LubX was shown to polyubiquitinate the host cell kinase Clk1 (Kubori et al., 2008) and the *Legionella* effector SidH (Kubori et al., 2010). LegU1 was shown to mediate the ubiquitination of the host cell chaperone BAT3 (Ensminger and Isberg, 2010).

Recently, a unique family of ubiquitin ligases has been identified among *Legionella*'s effectors, SidC (substrate of Icm/Dot transporter C) (Hsu et al., 2014). This protein is anchored to the cytoplamic face of the LCV and recruits host endoplasmic reticulum (ER) proteins to this organelle. Structure analysis revealed the presence of a catalytic triad containing a cysteine, a histidine, and an aspartate residue. It has the capacity to catalyze the formation of high-molecular-weight polyubiquitin chains of different types. Its role is essential for phagosomal membrane remodeling by *Legionella* (Hsu et al., 2014).

SALMONELLA TYPHIMURIUM

Salmonella is a common cause of gastroenteritis in humans. It has the ability to invade non-phagocytic cells such as enterocytes of the intestinal epithelium. This capacity depends on a T3SS, known as T3SS1. A second T3SS, T3SS2, is required for post-invasion establishment of the replicative niche, a modified phagosome known as the *Salmonella*-containing vacuole (SCV) (Steele-Mortimer, 2008). Several *Salmonella* effectors, from both T3SS1 and T3SS2, alter host cell ubiquitin pathways.

Invasion of host cells by Salmonella depends on the sequential activity of SopE, a guanine nucleotide exchange factor (GEF)

which activates Cdc42 and Rac1 (Hardt et al., 1998), and of SptP, a GTPase-activating protein (GAP) which inactivates SopE (Fu and Galan, 1999). Actually, both proteins are targeted for ubiquitin-dependent degradation, but SopE is degraded more efficiently and therefore inactivated more rapidly than SptP (Kubori and Galan, 2003).

SopB, an inositol phosphate phosphatase that has several functions during invasion (Steele-Mortimer et al., 2000; Bakowski et al., 2010), is another essential effector for *Salmonella* virulence. Following delivery into host cells, SopB is monoubiquitinated on at least six lysine residues, via a mechanism that does not require any of the known *Salmonella* E3 ubiquitin ligases (Knodler et al., 2009). This ubiquitination down-regulates SopB activity at the plasma membrane but increases its retention on the SCV. Hence, depending on its ubiquitination status, SopB has several functions, ranging from actin-mediated bacterial internalization and Akt activation to vesicular trafficking and intracellular bacterial replication at the phagosome (Knodler et al., 2009).

Some *Salmonella* effectors are real ubiquitin enzymes acting as ligases or DUBs. Based on functional and structural data, SopA is a novel HECT-like E3 ligase, although it has little sequence similarity with any eukaryotic E3 ligase. SopA was shown to form an Ub-thioester intermediate and its crystal analysis revealed a C-terminal domain architecture that resembles the N- and C-lobe arrangement of HECT domains (Diao et al., 2008). It has been shown to interact with the host cell conjugating enzyme UbcH7 (Lin et al., 2012). But so far, no substrate has been identified. Interestingly, SopA can be targeted for degradation following ubiquitination by the endoplasmic reticulum (ER)-bound RING finger protein 5 (RNF5/RMA1) (Zhang et al., 2005), a protein part of the ER-anchored Ubiquitin ligase complex which processes malfolded proteins (Delaunay et al., 2008).

Three other effectors of *Salmonella*, SlrP, SspH1 and SspH2, are ligases of the NEL family (Novel E3 Ligase). The NEL domain contains a conserved catalytic cysteine residue involved in E2 binding and ubiquitination reaction (Quezada et al., 2009), as well as a leucine-rich repeat (LRR) of variable length supposedly involved in substrate-recognition (Quezada et al., 2009). Whereas SspH2 is injected into host cells only by T3SS2 (Miao et al., 1999), SlrP and SspH1 are translocated via both T3SS1 and T3SS2. Hence SspH2 has function in late infection whereas Slrp and SspH1 play a role during the early steps of infection. The NEL domain of these ligases has no equivalent among all known mammalian ligases but these ligases are very efficient in using the host cells ubiquitination machinery such as the conjugating enzyme UBCH5, and the negative regulation of their activity seems to be realized upon the binding of the LRR to a target protein (Quezada et al., 2009). Only few potential host substrates have been identified, such as PKN1 (protein kinase called protein kinase N 1) for SspH1 (Haraga and Miller, 2006), and Thioredoxin and ERdj3 for SlrP (Bernal-Bayard and Ramos-Morales, 2009; Bernal-Bayard et al., 2010). However, the biological outcome of these identifications still needs further investigation.

At least two *Salmonella* effectors are deubiquitinases, SseL and AvrA, and both were shown to be involved in down regulating immune signaling (Collier-Hyams et al., 2002; Le Negrate et al., 2008). AvrA was supposed to have anti-inflammatory effects because of its ability to deubiquitinate number of proteins, such as IκB-α and β-catenin, thereby regulating host inflammatory responses through NF-κB (Collier-Hyams et al., 2002) and β-catenin (Sun et al., 2004). However, it has also been shown that AvrA has no significant anti-inflammatory function when injected by *Salmonella* at endogenous levels (Du and Galan, 2009). Therefore, the real function of AvrA still needs to be fully determined. Similarly, recent reports showed that SseL has no effect, negative or positive, on the NFkB pathway (Mesquita et al., 2013), but its deubiquitinase activity was shown to reduce the autophagic flux in infected cells and to favor bacterial replication (Mesquita et al., 2012).

Finally, a recent study of the impact of *Salmonella* LPS stimulation on the ubiquitination profile of macrophages revealed a profound and global alteration of this PTM in the host cell (Nakayasu et al., 2013). This change negatively modulates the activity of DUBs, resulting most likely in the polyubiquitination and degradation of specific proteins such as DBC1 (deleted in breast cancer 1), a histone deacetylase (HDAC) inhibitor that controls chromatin remodeling during inflammatory response. This work is a unique example showing that bacterial membrane associated factors can also interfere with many ubiquitination pathways of the host cell.

SHIGELLA FLEXNERI

Shigella is a Gram-negative pathogenic bacterium which causes shigellosis in human by invading intestinal epithelial cells, after it has been ingested. *Shigella* delivers effectors into host cells via a type III secretion system in order to modulate cellular processes and to favor multiplication (Ashida et al., 2011). As usual, several targets of these effectors are signaling pathways important for host defense cell. The phosphothreonine lyase activity of OspF effector inhibits the MAPK signaling pathway by irreversibly dephosphorylating MAPKs, Li et al. (2007) and Zhu et al. (2007). IpaH9.8 and IpaH4.5, which belong to a new IpaH family of E3 ubiquitin ligases (Rohde et al., 2007), inhibit the NF-κB signaling pathway by mediating the ubiquitination of NEMO and of p65 (Ashida et al., 2010; Wang et al., 2013).

Moreover, the VirA effector of *Shigella* inactivates Rab1 with TBC-like GAP activity, inhibiting the host cell autophagy-mediated defense (Dong et al., 2012).

A recent study revealed that a newly identified *Shigella* effector, OspI, targets the host UBC13 by deamidating glutamine 100, producing a glutamate residue, and leading to the disruption of TRAF6-catalyzed polyubiquitination (Sanada et al., 2012). The disruption of TRAF6 polyubiquitination suppresses the diacylglycerol-CBM (CARD-BCL10-MALT1 complex)-TRAF6-NF-κB signaling pathway and significantly reduces the host inflammatory responses (Sanada et al., 2012). OspI targets UBC13 via extensive interactions and UBC13 binding remodels the structure of OspI for catalysis. The structural analysis of UBC13 in complex with OspI, TRAF6, CHIP, and OTUB1 revealed that OspI binds to the same surface region on UBC13 as the host proteins (Fu et al., 2013).

OspG is an effector kinase whose function during invasion is to suppress the host inflammatory response. OspG can interact with at least 10 distinct human ubiquitin-charged E2 conjugating

enzymes, and this binding strongly enhances the kinase activity of OspG (Pruneda et al., 2014).

LISTERIA MONOCYTOGENES

Listeria is the causative agent of listeriosis, a serious invasive disease that primarily affects pregnant women, newborns and immunocompromised individuals (Bonazzi et al., 2009). It can invade host cells through two different pathways, depending on which cell surface receptors is engaged, internalin A (InlA) which binds to E-cadherin of the host cell, or internalin B (InlB) which binds to c-Met (Braun et al., 1999; Lecuit et al., 1999). Both pathways involve PTMs of host cell proteins, such as ubiquitination, as well as actin remodeling (Cossart and Lecuit, 1998; Bonazzi et al., 2008).

The surface-bound protein InlA binds to E-cadherin, a cell to cell adhesion molecule that forms a physical link between the cell membranes of adjacent cells (Mengaud et al., 1996). In epithelial cells, E-cadherin complexes are endocytosed following activation of the tyrosine kinase Src, which induces tyrosine phosphorylation of E-cadherin thus enabling its subsequent phospho-dependent ubiquitination by the ubiquitin ligase Hakai (Fujita et al., 2002). Internalization of *Listeria* via InlA induces the same phospho-dependent ubiquitination of E-cadherin followed by clathrin-dependent endocytosis (Sousa et al., 2007).

InlB binds to the host cell receptor c-Met, a RTK (Receptor Tyrosine Kinase) (Shen et al., 2000) which is normally activated by HGF (Hepatocyte Growth Factor). InlB interacts with the first immunoglobulin-like domain and the Sema domain of c-Met thereby stabilizing the receptor that can initiate signaling (Niemann et al., 2007). Activation of c-Met receptor leads to its clathrin-dependent internalization and its down-regulation, a process that requires the ubiquitin ligase c-Cbl, which is recruited to c-Met in a phospho-dependent manner (Peschard et al., 2001). Binding of InlB to c-Met induces the c-Cbl-dependent ubiquitination and endocytosis of c-Met and so the internalization of the bacteria. Importantly, *Listeria* invasion is directly dependent on the c-Cbl mediated ubiquitination of the receptor (Veiga and Cossart, 2005).

This bacterium also uses the host cell ubiquitination machinery to target some of its own proteins. Listeriolysin O (LLO), a pore-forming toxin that is essential for *Listeria* to escape from the phagosome into the host cell cytoplasm, may also be deleterious for the pathogen if not tightly regulated. Hence LLO is normally ubiquitinated and degraded by host cell machinery, and stabilizing mutation or overexpression of LLO seriously decreases the virulence of this bacterium (Schnupf et al., 2007).

CONCLUDING REMARKS

Pathogenic bacteria have coevolved with their target organisms and therefore they have learned how to use and/or subvert their defense mechanisms. The cell response to bacterial invasion needs to be rapid and hence relies on PTMs of key proteins. Ubiquitination appeared to be one of these PTMs important for host cell defense that is targeted by pathogens. These last years, new tools have been developed to explore PTMs dynamics (Vertegaal, 2011; Bonacci et al., 2014) that will surely help to identify new important mechanisms enabling pathogens to survive and proliferate within host cells.

REFERENCES

Ashida, H., Kim, M., Schmidt-Supprian, M., Ma, A., Ogawa, M., and Sasakawa, C. (2010). A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKKgamma to dampen the host NF-kappaB-mediated inflammatory response. *Nat. Cell Biol.* 12, 66–73; sup pp 61–69. doi: 10.1038/ncb2006

Ashida, H., Ogawa, M., Mimuro, H., Kobayashi, T., Sanada, T., and Sasakawa, C. (2011). Shigella are versatile mucosal pathogens that circumvent the host innate immune system. *Curr. Opin. Immunol.* 23, 448–455. doi: 10.1016/j.coi.2011.06.001

Bakowski, M. A., Braun, V., Lam, G. Y., Yeung, T., Heo, W. D., Meyer, T., et al. (2010). The phosphoinositide phosphatase SopB manipulates membrane surface charge and trafficking of the Salmonella-containing vacuole. *Cell Host Microbe* 7, 453–462. doi: 10.1016/j.chom.2010.05.011

Bernal-Bayard, J., Cardenal-Munoz, E., and Ramos-Morales, F. (2010). The Salmonella type III secretion effector, salmonella leucine-rich repeat protein (SlrP), targets the human chaperone ERdj3. *J. Biol. Chem.* 285, 16360–16368. doi: 10.1074/jbc.M110.100669

Bernal-Bayard, J., and Ramos-Morales, F. (2009). Salmonella type III secretion effector SlrP is an E3 ubiquitin ligase for mammalian thioredoxin. *J. Biol. Chem.* 284, 27587–27595. doi: 10.1074/jbc.M109.010363

Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T., and Brumell, J. H. (2006). Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J. Biol. Chem.* 281, 11374–11383. doi: 10.1074/jbc.M509157200

Bonacci, T., Audebert, S., Camoin, L., Baudelet, E., Bidaut, G., Garcia, M., et al. (2014). Identification of new mechanisms of cellular response to chemotherapy by tracking changes in post-translational modifications by ubiquitin and ubiquitin-like proteins. *J. Proteome Res.* 13, 2478–2494. doi: 10.1021/pr401258d

Bonazzi, M., Lecuit, M., and Cossart, P. (2009). *Listeria monocytogenes* internalin and E-cadherin: from bench to bedside. *Cold Spring Harb. Perspect. Biol.* 1:a003087. doi: 10.1101/cshperspect.a003087

Bonazzi, M., Veiga, E., Pizarro-Cerda, J., and Cossart, P. (2008). Successive post-translational modifications of E-cadherin are required for InlA-mediated internalization of *Listeria monocytogenes*. *Cell. Microbiol.* 10, 2208–2222. doi: 10.1111/j.1462-5822.2008.01200.x

Braun, L., Nato, F., Payrastre, B., Mazie, J. C., and Cossart, P. (1999). The 213-amino-acid leucine-rich repeat region of the *Listeria monocytogenes* InlB protein is sufficient for entry into mammalian cells, stimulation of PI 3-kinase and membrane ruffling. *Mol. Microbiol.* 34, 10–23. doi: 10.1046/j.1365-2958.1999.01560.x

Broberg, C. A., and Orth, K. (2010). Tipping the balance by manipulating post-translational modifications. *Curr. Opin. Microbiol.* 13, 34–40. doi: 10.1016/j.mib.2009.12.004

Cazalet, C., Rusniok, C., Bruggemann, H., Zidane, N., Magnier, A., Ma, L., et al. (2004). Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat. Genet.* 36, 1165–1173. doi: 10.1038/ng1447

Chen, G. Y., and Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10, 826–837. doi: 10.1038/nri2873

Chen, Z. J. (2005). Ubiquitin signalling in the NF-kappaB pathway. *Nat. Cell Biol.* 7, 758–765. doi: 10.1038/ncb0805-758

Collier-Hyams, L. S., Zeng, H., Sun, J., Tomlinson, A. D., Bao, Z. Q., Chen, H., et al. (2002). Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. *J. Immunol.* 169, 2846–2850. doi: 10.4049/jimmunol.169.6.2846

Cossart, P., and Lecuit, M. (1998). Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. *EMBO J.* 17, 3797–3806. doi: 10.1093/emboj/17.14.3797

de Felipe, K. S., Pampou, S., Jovanovic, O. S., Pericone, C. D., Ye, S. F., Kalachikov, S., et al. (2005). Evidence for acquisition of Legionella type IV secretion substrates via interdomain horizontal gene transfer. *J. Bacteriol.* 187, 7716–7726. doi: 10.1128/JB.187.22.7716-7726.2005

Delaunay, A., Bromberg, K. D., Hayashi, Y., Mirabella, M., Burch, D., Kirkwood, B., et al. (2008). The ER-bound RING finger protein 5 (RNF5/RMA1) causes

degenerative myopathy in transgenic mice and is deregulated in inclusion body myositis. *PLoS ONE* 3:e1609. doi: 10.1371/journal.pone.0001609

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., et al. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103, 351–361. doi: 10.1016/S0092-8674(00)00126-4

Diao, J., Zhang, Y., Huibregtse, J. M., Zhou, D., and Chen, J. (2008). Crystal structure of SopA, a Salmonella effector protein mimicking a eukaryotic ubiquitin ligase. *Nat. Struct. Mol. Biol.* 15, 65–70. doi: 10.1038/nsmb1346

Dong, N., Zhu, Y., Lu, Q., Hu, L., Zheng, Y., and Shao, F. (2012). Structurally distinct bacterial TBC-like GAPs link Arf GTPase to Rab1 inactivation to counteract host defenses. *Cell* 150, 1029–1041. doi: 10.1016/j.cell.2012.06.050

Du, F., and Galan, J. E. (2009). Selective inhibition of type III secretion activated signaling by the Salmonella effector AvrA. *PLoS Pathog.* 5:e1000595. doi: 10.1371/journal.ppat.1000595

Ensminger, A. W., and Isberg, R. R. (2010). E3 ubiquitin ligase activity and targeting of BAT3 by multiple *Legionella pneumophila* translocated substrates. *Infect. Immun.* 78, 3905–3919. doi: 10.1128/IAI.00344-10

Fu, P., Zhang, X., Jin, M., Xu, L., Wang, C., Xia, Z., et al. (2013). Complex structure of OspI and Ubc13: the molecular basis of Ubc13 deamidation and convergence of bacterial and host E2 recognition. *PLoS Pathog.* 9:e1003322. doi: 10.1371/journal.ppat.1003322

Fu, Y., and Galan, J. E. (1999). A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* 401, 293–297. doi: 10.1038/45829

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H. E., Behrens, J., et al. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.* 4, 222–231. doi: 10.1038/ncb758

Gomes, L. C., and Dikic, I. (2014). Autophagy in antimicrobial immunity. *Mol. Cell* 54, 224–233. doi: 10.1016/j.molcel.2014.03.009

Haraga, A., and Miller, S. I. (2006). A Salmonella type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1. *Cell. Microbiol.* 8, 837–846. doi: 10.1111/j.1462-5822.2005.00670.x

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galan, J. E. (1998). *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* 93, 815–826. doi: 10.1016/S0092-8674(00)81442-7

Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* 458, 422–429. doi: 10.1038/nature07958

Hsu, F., Luo, X., Qiu, J., Teng, Y. B., Jin, J., Smolka, M. B., et al. (2014). The Legionella effector SidC defines a unique family of ubiquitin ligases important for bacterial phagosomal remodeling. *Proc. Natl. Acad. Sci. U.S.A.* 111, 10538–10543. doi: 10.1073/pnas.1402605111

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11, 373–384. doi: 10.1038/ni.1863

Kirkin, V., McEwan, D. G., Novak, I., and Dikic, I. (2009). A role for ubiquitin in selective autophagy. *Mol. Cell* 34, 259–269. doi: 10.1016/j.molcel.2009.04.026

Knodler, L. A., Winfree, S., Drecktrah, D., Ireland, R., and Steele-Mortimer, O. (2009). Ubiquitination of the bacterial inositol phosphatase, SopB, regulates its biological activity at the plasma membrane. *Cell. Microbiol.* 11, 1652–1670. doi: 10.1111/j.1462-5822.2009.01356.x

Kubori, T., and Galan, J. E. (2003). Temporal regulation of salmonella virulence effector function by proteasome-dependent protein degradation. *Cell* 115, 333–342. doi: 10.1016/S0092-8674(03)00849-3

Kubori, T., Hyakutake, A., and Nagai, H. (2008). Legionella translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Mol. Microbiol.* 67, 1307–1319. doi: 10.1111/j.1365-2958.2008.06124.x

Kubori, T., Shinzawa, N., Kanuka, H., and Nagai, H. (2010). Legionella metaeffector exploits host proteasome to temporally regulate cognate effector. *PLoS Pathog.* 6:e1001216. doi: 10.1371/journal.ppat.1001216

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B., and Cossart, P. (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO J.* 18, 3956–3963. doi: 10.1093/emboj/18.14.3956

Le Negrate, G., Faustin, B., Welsh, K., Loeffler, M., Krajewska, M., Hasegawa, P., et al. (2008). Salmonella secreted factor L deubiquitinase of *Salmonella typhimurium* inhibits NF-kappaB, suppresses IkappaBalpha ubiquitination and modulates innate immune responses. *J. Immunol.* 180, 5045–5056. doi: 10.4049/jimmunol.180.7.5045

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., et al. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. *Science* 315, 1000–1003. doi: 10.1126/science.1138960

Lin, D. Y., Diao, J., and Chen, J. (2012). Crystal structures of two bacterial HECT-like E3 ligases in complex with a human E2 reveal atomic details of pathogen-host interactions. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1925–1930. doi: 10.1073/pnas.1115025109

Lomma, M., Dervins-Ravault, D., Rolando, M., Nora, T., Newton, H. J., Sansom, F. M., et al. (2010). The *Legionella pneumophila* F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cell. Microbiol.* 12, 1272–1291. doi: 10.1111/j.1462-5822.2010.01467.x

Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M., and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* 84, 923–932. doi: 10.1016/S0092-8674(00)81070-3

Mesquita, F. S., Holden, D. W., and Rolhion, N. (2013). Lack of effect of the Salmonella deubiquitinase SseL on the NF-kappaB pathway. *PLoS ONE* 8:e53064. doi: 10.1371/journal.pone.0053064

Mesquita, F. S., Thomas, M., Sachse, M., Santos, A. J., Figueira, R., and Holden, D. W. (2012). The Salmonella deubiquitinase SseL inhibits selective autophagy of cytosolic aggregates. *PLoS Pathog.* 8:e1002743. doi: 10.1371/journal.ppat.1002743

Miao, E. A., Scherer, C. A., Tsolis, R. M., Kingsley, R. A., Adams, L. G., Baumler, A. J., et al. (1999). *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol. Microbiol.* 34, 850–864. doi: 10.1046/j.1365-2958.1999.01651.x

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. *Annu. Rev. Cell Dev. Biol.* 27, 107–132. doi: 10.1146/annurev-cellbio-092910-154005

Nakayasu, E. S., Brown, R. N., Ansong, C., Sydor, M. A., Imtiaz, S., Mihai, C., et al. (2013). Multi-omic data integration links deleted in breast cancer 1 (DBC1) degradation to chromatin remodeling in inflammatory response. *Mol. Cell. Proteomics* 12, 2136–2147. doi: 10.1074/mcp.M112.026138

Niemann, H. H., Jager, V., Butler, P. J., van den Heuvel, J., Schmidt, S., Ferraris, D., et al. (2007). Structure of the human receptor tyrosine kinase met in complex with the Listeria invasion protein InlB. *Cell* 130, 235–246. doi: 10.1016/j.cell.2007.05.037

Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., et al. (2005). A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123, 773–786. doi: 10.1016/j.cell.2005.11.007

Perrin, A. J., Jiang, X., Birmingham, C. L., So, N. S., and Brumell, J. H. (2004). Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. *Curr. Biol.* 14, 806–811. doi: 10.1016/j.cub.2004.04.033

Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., et al. (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol. Cell* 8, 995–1004. doi: 10.1016/S1097-2765(01)00378-1

Pickart, C. M., and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta* 1695, 55–72. doi: 10.1016/j.bbamcr.2004.09.019

Price, C. T., Al-Khodor, S., Al-Quadan, T., Santic, M., Habyarimana, F., Kalia, A., et al. (2009). Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathog.* 5:e1000704. doi: 10.1371/journal.ppat.1000704

Pruneda, J. N., Smith, F. D., Daurie, A., Swaney, D. L., Villen, J., Scott, J. D., et al. (2014). E2~Ub conjugates regulate the kinase activity of Shigella effector OspG during pathogenesis. *EMBO J.* 33, 437–449. doi: 10.1002/embj.201386386

Quezada, C. M., Hicks, S. W., Galan, J. E., and Stebbins, C. E. (2009). A family of Salmonella virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4864–4869. doi: 10.1073/pnas.0811058106

Rikihisa, Y. (1984). Glycogen autophagosomes in polymorphonuclear leukocytes induced by rickettsiae. *Anat. Rec.* 208, 319–327. doi: 10.1002/ar.1092080302

Rohde, J. R., Breitkreutz, A., Chenal, A., Sansonetti, P. J., and Parsot, C. (2007). Type III secretion effectors of the IpaH family are E3 ubiquitin ligases. *Cell Host Microbe* 1, 77–83. doi: 10.1016/j.chom.2007.02.002

Sanada, T., Kim, M., Mimuro, H., Suzuki, M., Ogawa, M., Oyama, A., et al. (2012). The *Shigella flexneri* effector OspI deamidates UBC13 to dampen the inflammatory response. *Nature* 483, 623–626. doi: 10.1038/nature10894

Schnupf, P., Zhou, J., Varshavsky, A., and Portnoy, D. A. (2007). Listeriolysin O secreted by *Listeria monocytogenes* into the host cell cytosol is degraded by the N-end rule pathway. *Infect. Immun.* 75, 5135–5147. doi: 10.1128/IAI.00164-07

Shen, Y., Naujokas, M., Park, M., and Ireton, K. (2000). InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* 103, 501–510. doi: 10.1016/S0092-8674(00)00141-0

Sousa, S., Cabanes, D., Bougneres, L., Lecuit, M., Sansonetti, P., Tran-Van-Nhieu, G., et al. (2007). Src, cortactin and Arp2/3 complex are required for E-cadherin-mediated internalization of Listeria into cells. *Cell. Microbiol.* 9, 2629–2643. doi: 10.1111/j.1462-5822.2007.00984.x

Steele-Mortimer, O. (2008). The Salmonella-containing vacuole: moving with the times. *Curr. Opin. Microbiol.* 11, 38–45. doi: 10.1016/j.mib.2008.01.002

Steele-Mortimer, O., Knodler, L. A., Marcus, S. L., Scheid, M. P., Goh, B., Pfeifer, C. G., et al. (2000). Activation of Akt/protein kinase B in epithelial cells by the *Salmonella typhimurium* effector sigD. *J. Biol. Chem.* 275, 37718–37724. doi: 10.1074/jbc.M008187200

Sun, J., Hobert, M. E., Rao, A. S., Neish, A. S., and Madara, J. L. (2004). Bacterial activation of beta-catenin signaling in human epithelia. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G220–G227. doi: 10.1152/ajpgi.00498.2003

Thurston, T. L., Ryzhakov, G., Bloor, S., von Muhlinen, N., and Randow, F. (2009). The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat. Immunol.* 10, 1215–1221. doi: 10.1038/ni.1800

Veiga, E., and Cossart, P. (2005). Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat. Cell Biol.* 7, 894–900. doi: 10.1038/ncb1292

Vertegaal, A. C. (2011). Uncovering ubiquitin and ubiquitin-like signaling networks. *Chem. Rev.* 111, 7923–7940. doi: 10.1021/cr200187e

Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412, 346–351. doi: 10.1038/35085597

Wang, F., Jiang, Z., Li, Y., He, X., Zhao, J., Yang, X., et al. (2013). *Shigella flexneri* T3SS effector IpaH4.5 modulates the host inflammatory response via interaction with NF-kappaB p65 protein. *Cell. Microbiol.* 15, 474–485. doi: 10.1111/cmi.12052

Watson, R. O., Manzanillo, P. S., and Cox, J. S. (2012). Extracellular *M. tuberculosis* DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* 150, 803–815. doi: 10.1016/j.cell.2012.06.040

Wild, P., Farhan, H., McEwan, D. G., Wagner, S., Rogov, V. V., Brady, N. R., et al. (2011). Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. *Science* 333, 228–233. doi: 10.1126/science.1205405

Zhang, Y., Higashide, W., Dai, S., Sherman, D. M., and Zhou, D. (2005). Recognition and ubiquitination of Salmonella type III effector SopA by a ubiquitin E3 ligase, HsRMA1. *J. Biol. Chem.* 280, 38682–38688. doi: 10.1074/jbc.M506309200

Zheng, Y. T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J. H. (2009). The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. *J. Immunol.* 183, 5909–5916. doi: 10.4049/jimmunol.0900441

Zhu, Y., Li, H., Long, C., Hu, L., Xu, H., Liu, L., et al. (2007). Structural insights into the enzymatic mechanism of the pathogenic MAPK phosphothreonine lyase. *Mol. Cell* 28, 899–913. doi: 10.1016/j.molcel.2007.11.011

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The complexity of Rab5 to Rab7 transition guarantees specificity of pathogen subversion mechanisms

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Non-pathogenic bacteria are commonly eliminated by the host. Professional phagocytic cells of the immune system, such as macrophages and dendritic cells, recognize and by phagocytosis internalize microbes in specialized endocytic compartments called phagosomes. By fusing with endosomes, phagosomes mature, changing from an early to late state, and early and late endosomes differ in their external and internal biochemical composition. Then, fusion of late phagosomes with lysosomes leads to the formation of an acidic and degradative compartment, the phagolysosome, where bacteria are ultimately eliminated (Fairn and Grinstein, 2012). Bacterial pathogens have evolved several mechanisms to subvert the process of phagosome maturation and to survive and replicate in an intracellular niche that is protected from the immune response. Remarkably, distinct bacterial pathogens can be localized in similar endocytic compartments, suggesting they have a role in the control of the same endocytic steps. The biological machineries controlling endocytosis involve a variety of regulatory events in each step of intracellular membrane trafficking. Here, I would like to summarize and comment on all the discoveries on bacterial pathogens that control the localization or function of the small GTPases Rab5 and Rab7, and therefore modify the maturation from early to late phagosomes, because I believe such a transition is the best way to highlight how bacterial pathogens exploit the complexity of membrane trafficking to establish specific subversion mechanisms.

Phagosome maturation is highly dependent on the endocytic pathway and requires several regulators of this pathway. Rab proteins make up a large family of small GTPases that specifically control various steps in the endosomal and phagosomal transport process (Gutierrez, 2013; Sherwood and Roy, 2013). Each Rab protein is associated with an organelle and a specific step in intracellular trafficking and controls several factors, such as protein and lipid composition of an organelle membrane, fusion between distinct compartments, vesicle motility along microtubules, and interaction with the cytoskeleton. Each compartment has a specific and precise set of Rab proteins, which confers organelle identity. The complexity of Rab protein function is reflected in their life cycle. All Rabs alternate between an active (GTP-bound) state and an inactive (GDP-bound) state. This molecular switch is strictly regulated; one or more guanine nucleotide exchange factors (GEFs) catalyze the release of GDP in exchange for GTP. In the GTP-bound form, Rabs are targeted to their specific organelle membrane by prenylation and recruit a large number of downstream effectors. In the GDP-bound form, they associate with a soluble factor, the guanine dissociation inhibitor (GDI), which stabilizes the inactive species in the cytosol and precludes access to the GEFs. The hydrolysis of GTP to GDP by GTPase-activating proteins (GAPs) terminates the activity of the Rab protein until another activation cycle is initiated.

Rab5 and Rab7 are the best-characterized Rab proteins in the endocytic process and, especially by analogy, in phagosome maturation (Fairn and Grinstein, 2012). Rab5 is mainly associated with early endosomes and early phagosomes and controls the identity and functionality of these compartments. Rab7 defines late endosomes and late phagosomes, and it has been implicated in the transport through these compartments. Therefore, when internalized, bacteria are first localized in Rab5-positive phagosomes and then in Rab7-positive phagosomes. There is much debate about the mechanisms whereby an early Rab5-positive endosome or a phagosome becomes a late Rab7-positive endosome or phagosome and whereby Rab5 is consecutively replaced by Rab7 and much effort has been given to understanding the regulation of Rab5 and Rab7 function. The picture so far shows Rabs as complex, highly regulated molecular machineries. A large number of effector proteins interacting with each Rab and of the GEFs and GAPs that regulate their function have been described. In the case of Rab5, for example, more than 60 effectors have been found and several remain to be characterized, and at least 4 GEFs of Rab5 have been characterized so far (Horiuchi et al., 1997; Christoforidis and Zerial, 2000; Kajiho et al., 2003; Otomo et al., 2003; Olchowik and Miaczynska, 2009; Balaji et al., 2012). These discoveries led to a more detailed investigation of the compartment where pathogens reside, which is not simply Rab5- or Rab7-positive, and to a larger comprehension of the molecular mechanisms that bacterial pathogens have evolved. In fact host activities and

molecules that pathogens modify the Rab5 to Rab7 transition are different and specific for each pathogen (**Figure 1**). For example, *Mycobacterium tuberculosis* and *Listeria monocytogenes* have been localized in modified Rab5-positive endocytic compartment. Nonetheless, at molecular level they distinctly affect the Rab5 machinery. *M. tuberculosis*, responsible for the human disease tuberculosis, enters alveolar macrophages and modifies the formation of phosphatidylinositol 3-phosphate [PI(3)P] at the early phagosome membranes (Fratti et al., 2001). The mannose-capped lipoarabinomannan (man-LAM) in the bacterial membrane is released into the phagosomal membrane and inactivates Vps34, the PI(3)P kinase that regenerates PI(3)P (Fratti et al., 2003). At the same time, an *M. tuberculosis* lipid phosphatase, SapM, consumes PI(3)P and arrests phagosome maturation (Saikolappan et al., 2012; Puri et al., 2013). PI(3)P is specifically enriched in early endosome/phagosome membranes and stabilizes Rab5 and all its effectors. Its absence in *M. tuberculosis* infection interferes with the recruitment of the Rab5 effectors and therefore with phagosome maturation to the Rab7 state (Purdy et al., 2005). Interestingly, the *M. tuberculosis*-containing phagosome is also enriched for Rab22a, which inhibits Rab7 acquisition and arrests phagosomal maturation (Roberts et al., 2006).

A GEF Rabex5-Rabaptin5 complex is recruited by the active GTP-bound form of Rab5 and through a positive feedback loop regulates Rab5 recruitment to the early endosomes (Lippe et al., 2001). Recent evidence suggests that Rab5 activation is also regulated by RIN1, a RAS effector and a Rab5-GEF (Jiwani et al., 2012; Balaji et al., 2014). *Listeria monocytogenes* is a gram-positive food-borne pathogen that causes severe infection with symptoms ranging from gastroenteritis to bacterial meningitis and has a mortality rate of about 30% (Chen et al., 2013). *L. monocytogenes* invades intestinal epithelial cells and survives in a Rab5-positive phagosome until it is prepared to lyse the phagosomal membrane and escape into the cytosol (Farber and Peterkin, 1991). For this purpose, *L. monocytogenes* acts at the level of Rab5 localization and functions in two steps. First, its attachment to the host cell triggers activation of RIN1, which activates Rab5 for efficient internalization by receptor-mediated phagocytosis and transport to early phagosomes (Jiwani et al., 2012; Balaji et al., 2014). Bacteria then need to block Rab5 activity to avoid maturation of early phagosomes into late phagosomes. Thus, the *L. monocytogenes* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein ADP-ribosylates Rab5, rendering this GTPase unresponsive to activation by GEFs, and thereby blocks maturation into Rab7-positive phagosomes (Prada-Delgado et al., 2005).

Tropheryma whipplei is a nice example of blockade of endocytic trafficking in an intermediate state of the Rab5 and

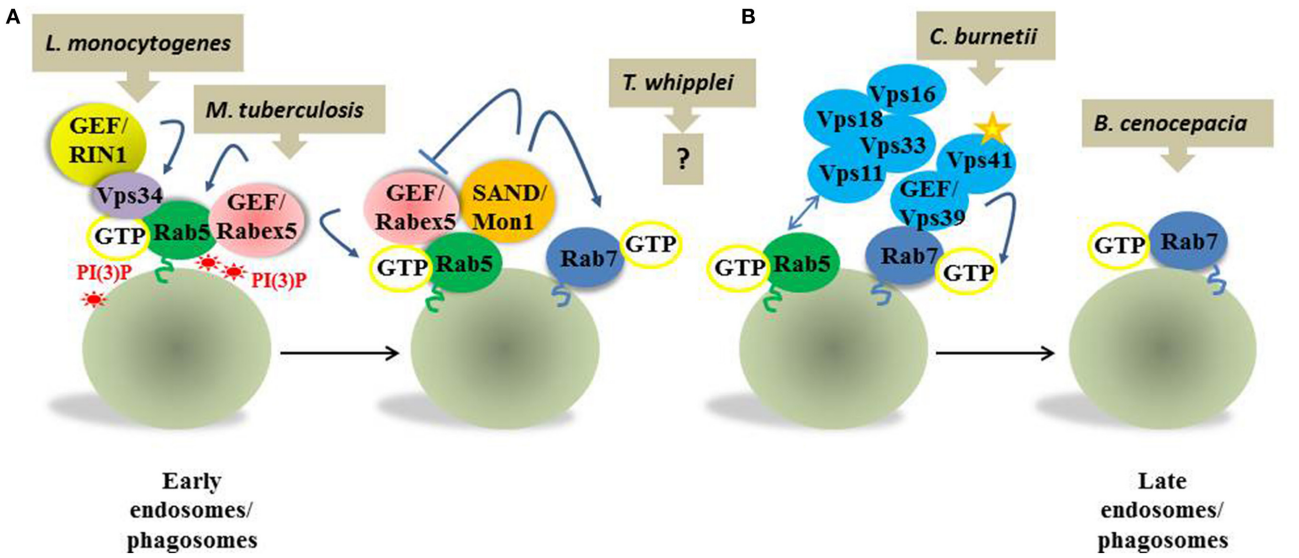

FIGURE 1 | Simplified view of the molecular mechanisms involved in Rab5 to Rab7 transition exploited by bacterial pathogens. Rab5 in its active form recruits on the early compartment a GEF Rabex5, which stabilizes Rab5 recruitments to the membrane, and Vps34, the PI(3)P kinase that regenerates PI(3)P. Rab5 activity at the early endosomes/phagosomes is also regulated by GEF RIN1. Two events take place on the early endosomes, which implicate simultaneous recruitment of Rab7 and maturation toward the late endosomes/phagosomes. **(A)** SAND1/Mon1 binds Rabex5 and displaces it from early endosome, inactivating the Rab5 recruitment loop. Additionally, SAND1/Mon1 interacts with a Rab7 GEF, the Vps39 subunit of the HOPS complex (blue). **(B)** The HOPS complex Vps11 subunit interacts with Rab5-GTP, probably stabilizing the Rab5-Rab7 transition. Interestingly p38α-MAPK dependent phosphorylation of the HOPS complex Vps41 subunit also seems important for Rab7 recruitment. Upon Rab7 recruitment and activation, Rab5 is released and early endosomes/phagosomes mature in late endosomes/phagosomes. For each of the described steps a distinct subversion mechanism has been evolved by bacterial pathogens. *L. monocytogenes* engages RIN1 to promote accumulation in a Rab5-positive compartment. *M. tuberculosis* inactivates Vps34 and consumes PI(3)P, interfering with Rab5 recruitment. *T. whipplei* blocks the transition in a Rab5- and Rab7 positive state by an unknown mechanism. *C. burnetii* interferes with Vps41 phosphorylation and Rab7 recruitment. *B. cenocepacia* affects Rab7 activation on the membranes.

Rab7 transition. This pathogen is responsible for a multi-systemic infection called Whipple's disease, which is fatal without antibiotic treatment (Schneider et al., 2008). *T. whipplei* resides and replicates in both macrophages and non-microbicidal cells in a phagosome that does not become a phagolysosome (Ghigo et al., 2002, 2010). Recently, the purification and characterization of the intracellular compartment where *T. whipplei* localizes revealed that *T. whipplei*-containing compartments are the first example of Rab5- and Rab7-positive phagosomes containing bacteria (Mottola et al., 2014). How is the pathogen establishing this intermediate state? Actually, the transition from a Rab5-positive to Rab7-positive endosome requires both Rabs, and Rab7 is already present on early endosomes together with Rab5 (Poteryaev et al., 2007). Such transition involves two multimeric complexes. The SAND1/Mon1 and ccz1 complex binds Rabex5 and displaces it from the early endosome, inactivating the Rab5 recruitment loop (**Figure 1** and Poteryaev et al., 2007). At the same time, the SAND1/Mon1–Ccz1 complex is also a Rab7 GEF (Nordmann et al., 2010; Cabrera et al., 2014). Intriguingly, Rab5 and Rab7 both bind to the hexameric tethering complex HOPS ("homotypic fusion and protein sorting"). Rab5 binds to subunit Vps11 (Rink et al., 2005). Rab7 interacts with subunits Vps39 and Vps41, but SAND1/Mon1 also interacts with Vps39, which in yeast is a Rab7 GEF (Peralta et al., 2010; Plemel et al., 2011). By a hitherto undescribed mechanism *T. whipplei* therefore might affect either the function of the SAND1/mon1-ccz1 complex or of the HOPS complex and block endosomes at an intermediate Rab5- and Rab7-positive state. Further investigation of the mechanism responsible for the presence of both Rab5 and Rab7 on *T. whipplei* phagosomes will help us better understand both the *T. whipplei* infectious process and the regulatory mechanism of the Rab5-to-Rab7 switch.

The investigation of *Coxiella burnetii* subversion mechanisms has also revealed insights on pathogen specificity. *C. burnetii*, the causative agent of the zoonosis Q fever, is also responsible for lethal endocarditis (Raoult et al., 2005). In macrophages, virulent *C. burnetii* bacteria reside and replicate in compartments known as "phagolysosome-like vacuoles" that have properties of both late endosomes and lysosomes. These compartments do not harbor lysosomal enzymes or Rab7, but they have acidic properties and are positive for lysosomal-associated membrane protein-1 (LAMP-1) (Ghigo et al., 2009, 2012). Recently, by using an avirulent form of this pathogen, Barry A.O. et al. discovered that p38α-MAPK-dependent phosphorylation of HOPS complex Vps41 subunit is crucial for Rab7 recruitment to endosomal membranes (Barry et al., 2012). Remarkably, the lipopolysaccharide of virulent *C. burnetii* (LPS), a bacterial outer membrane component, is responsible for this subversion mechanism (Barry et al., 2012). Indeed, it interferes with the activation of p38α-MAPK and therefore with Vps41 phosphorylation. Consequently, *C. burnetii*-containing phagosomes become positive for Rab5, lose Rab5, but do not recruit Rab7.

Burkholderia cenocepacia has evolved a distinct mechanism to interfere with early to late phagosome transition. *B. cenocepacia* is an opportunistic pathogen that infects patients with cystic fibrosis (Drevinek and Mahenthiralingam, 2010). It can survive within macrophages because it arrests the fusion of phagosomes with lysosomes by acting at the level of Rab7 function (Lamothe et al., 2007; Lamothe and Valvano, 2008). Vacuoles containing *B. cenocepacia* transiently recruit Rab5 and synthesize PI(3)P. Vacuoles can also acquire the late phagosomal markers CD63 and Rab7, but activation of Rab7 is impaired by the bacteria (Huynh et al., 2010). Findings have indicated that the type III secretion system is not necessary for maturation arrest (Lamothe et al., 2007), and *B. cenocepacia* also expresses type IV and type VI secretion mechanisms (Aubert et al., 2008; Sajjan et al., 2008), but the identities of the secreted effectors and their mode of action on Rab7 remain unclear.

In conclusion, I have described five distinct pathogens that distinctly exploit the complexity of Rab5 and Rab7 regulation in order to survive and replicate in the host environment. Why is that important? First, this example highlights the coevolution of the two systems. Mammalian cells have evolved a complicated regulation of Rab5 and Rab7 transition in order to guarantee redundancy and therefore "resistance" to any possible dangerous genetic or acquired alteration. Also, pathogens more specifically explore transport steps to establish their own pathogen-specific subversion mechanism. Indeed, such specificity guarantees longer survival and evolution before the host immune system becomes able to find and positively select an adequate immune response. Moreover, this example underlines the strong need for multidisciplinary approaches in the study of infectious diseases. To understand pathogen behavior, membrane trafficking in these pathological contests must be investigated biologically and biochemically. This will be determinant in the development of specific prognostic, diagnostic, and therapeutic tools against an infectious pathology.

REFERENCES

Aubert, D. F., Flannagan, R. S., and Valvano, M. A. (2008). A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. *Infect. Immun.* 76, 1979–1991. doi: 10.1128/IAI.01338-07

Balaji, K., French, C. T., Miller, J. F., and Colicelli, J. (2014). The RAB5-GEF function of RIN1 regulates multiple steps during *Listeria monocytogenes* infection. *Traffic* 15, 1206–1218. doi: 10.1111/tra.12204

Balaji, K., Mooser, C., Janson, C. M., Bliss, J. M., Hojjat, H., and Colicelli, J. (2012). RIN1 orchestrates the activation of RAB5 GTPases and ABL tyrosine kinases to determine the fate of EGFR. *J. Cell Sci.* 125, 5887–5896. doi: 10.1242/jcs.113688

Barry, A. O., Boucherit, N., Mottola, G., Vadovic, P., Trouplin, V., Soubeyran, P., et al. (2012). Impaired stimulation of p38alpha-MAPK/Vps41-HOPS by LPS from pathogenic *Coxiella burnetii* prevents trafficking to microbicidal phagolysosomes. *Cell Host Microbe* 12, 751–763. doi: 10.1016/j.chom.2012.10.015

Cabrera, M., Nordmann, M., Perz, A., Schmedt, D., Gerondopoulos, A., Barr, F., et al. (2014). The Mon1-Ccz1 GEF activates the Rab7 GTPase Ypt7 via a longin-fold-Rab interface and association with PI3P-positive membranes. *J. Cell Sci.* 127, 1043–1051. doi: 10.1242/jcs.140921

Chen, Y., Dennis, S. B., Hartnett, E., Paoli, G., Pouillot, R., Ruthman, T., et al. (2013). FDA-iRISK–a comparative risk assessment system for evaluating and ranking food-hazard pairs: case studies on microbial hazards. *J. Food Prot.* 76, 376–385. doi: 10.4315/0362-028X.JFP-12-372

Christoforidis, S., and Zerial, M. (2000). Purification and identification of novel Rab effectors using affinity chromatography. *Methods* 20, 403–410. doi: 10.1006/meth.2000.0953

Drevinek, P., and Mahenthiralingam, E. (2010). *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clin. Microbiol. Infect.* 16, 821–830. doi: 10.1111/j.1469-0691.2010.03237.x

Fairn, G. D., and Grinstein, S. (2012). How nascent phagosomes mature to become phagolysosomes. *Trends Immunol.* 33, 397–405. doi: 10.1016/j.it.2012.03.003

Farber, J. M., and Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55, 476–511.

Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S., and Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J. Cell Biol.* 154, 631–644. doi: 10.1083/jcb.200106049

Fratti, R. A., Chua, J., Vergne, I., and Deretic, V. (2003). *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5437–5442. doi: 10.1073/pnas.0737613100

Ghigo, E., Barry, A. O., Pretat, L., AL Moussawi, K., Desnues, B., Capo, C.. et al. (2010). IL-16 promotes *T. whipplei* replication by inhibiting phagosome conversion and modulating macrophage activation. *PLoS ONE* 5:e13561. doi: 10.1371/journal.pone.0013561

Ghigo, E., Capo, C., Aurouze, M., Tung, C. H., Gorvel, J. P., Raoult, D., et al. (2002). Survival of *Tropheryma whipplei*, the agent of Whipple's disease, requires phagosome acidification. *Infect. Immun.* 70, 1501–1506. doi: 10.1128/IAI.70.3.1501-1506.2002

Ghigo, E., Colombo, M. I., and Heinzen, R. A. (2012). The *Coxiella burnetii* parasitophorous vacuole. *Adv. Exp. Med. Biol.* 984, 141–169. doi: 10.1007/978-94-007-4315-1_8

Ghigo, E., Pretat, L., Desnues, B., Capo, C., Raoult, D., and Mege, J. L. (2009). Intracellular life of *Coxiella burnetii* in macrophages. *Ann. N.Y. Acad. Sci.* 1166, 55–66. doi: 10.1111/j.1749-6632.2009.04515.x

Gutierrez, M. G. (2013). Functional role(s) of phagosomal Rab GTPases. *Small GTPases* 4, 148–158. doi: 10.4161/sgtp.25604

Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., et al. (1997). A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell* 90, 1149–1159. doi: 10.1016/S0092-8674(00)80380-3

Huynh, K. K., Plumb, J. D., Downey, G. P., Valvano, M. A., and Grinstein, S. (2010). Inactivation of macrophage Rab7 by *Burkholderia cenocepacia*. *J. Innate Immun.* 2, 522–533. doi: 10.1159/000319864

Jiwani, S., Wang, Y., Dowd, G. C., Gianfelice, A., Pichestapong, P., Gavicherla, B., et al. (2012). Identification of components of the host type IA phosphoinositide 3-kinase pathway that promote internalization of *Listeria monocytogenes*. *Infect. Immun.* 80, 1252–1266. doi: 10.1128/IAI.06082-11

Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H., et al. (2003). RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway. *J. Cell Sci.* 116, 4159–4168. doi: 10.1242/jcs.00718

Lamothe, J., Huynh, K. K., Grinstein, S., and Valvano, M. A. (2007). Intracellular survival of *Burkholderia cenocepacia* in macrophages is associated with a delay in the maturation of bacteria-containing vacuoles. *Cell. Microbiol.* 9, 40–53. doi: 10.1111/j.1462-5822.2006.00766.x

Lamothe, J., and Valvano, M. A. (2008). *Burkholderia cenocepacia*-induced delay of acidification and phagolysosomal fusion in cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages. *Microbiology* 154, 3825–3834. doi: 10.1099/mic.0.2008/023200-0

Lippe, R., Miaczynska, M., Rybin, V., Runge, A., and Zerial, M. (2001). Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. *Mol. Biol. Cell* 12, 2219–2228. doi: 10.1091/mbc.12.7.2219

Mottola, G., Boucherit, N., Trouplin, V., Oury Barry, A., Soubeyran, P., Mege, J. L., et al. (2014). *Tropheryma whipplei*, the agent of Whipple's disease, affects the early to late phagosome transition and survives in a Rab5- and Rab7-positive compartment. *PLoS ONE* 9:e89367. doi: 10.1371/journal.pone.0089367

Nordmann, M., Cabrera, M., Perz, A., Brocker, C., Ostrowicz, C., Engelbrecht-Vandre, S., et al. (2010). The Mon1-Ccz1 complex is the GEF of the late endosomal Rab7 homolog Ypt7. *Curr. Biol.* 20, 1654–1659. doi: 10.1016/j.cub.2010.08.002

Olchowik, M., and Miaczyńska, M. (2009). [Effectors of GTPase Rab5 in endocytosis and signal transduction]. *Postepy Biochem.* 55, 171–180.

Otomo, A., Hadano, S., Okada, T., Mizumura, H., Kunita, R., Nishijima, H., et al. (2003). ALS2, a novel guanine nucleotide exchange factor for the small GTPase Rab5, is implicated in endosomal dynamics. *Hum. Mol. Genet.* 12, 1671–1687. doi: 10.1093/hmg/ddg184

Peralta, E. R., Martin, B. C., and Edinger, A. L. (2010). Differential effects of TBC1D15 and mammalian Vps39 on Rab7 activation state, lysosomal morphology, and growth factor dependence. *J. Biol. Chem.* 285, 16814–16821. doi: 10.1074/jbc.M110.111633

Plemel, R. L., Lobingier, B. T., Brett, C. L., Angers, C. G., Nickerson, D. P., Paulsel, A., et al. (2011). Subunit organization and Rab interactions of Vps-C protein complexes that control endolysosomal membrane traffic. *Mol. Biol. Cell* 22, 1353–1363. doi: 10.1091/mbc.E10-03-0260

Poteryaev, D., Fares, H., Bowerman, B., and Spang, A. (2007). Caenorhabditis elegans SAND-1 is essential for RAB-7 function in endosomal traffic. *EMBO J.* 26, 301–312. doi: 10.1038/sj.emboj.7601498

Prada-Delgado, A., Carrasco-Marin, E., Pena-Macarro, C., Del Cerro-Vadillo, E., Fresno-Escudero, M., Leyva-Cobian, F., et al. (2005). Inhibition of Rab5a exchange activity is a key step for *Listeria monocytogenes* survival. *Traffic* 6, 252–265. doi: 10.1111/j.1600-0854.2005.00265.x

Purdy, G. E., Owens, R. M., Bennett, L., Russell, D. G., and Butcher, B. A. (2005). Kinetics of phosphatidylinositol-3-phosphate acquisition differ between IgG bead-containing phagosomes and *Mycobacterium tuberculosis*-containing phagosomes. *Cell. Microbiol.* 7, 1627–1634. doi: 10.1111/j.1462-5822.2005.00580.x

Puri, R. V., Reddy, P. V., and Tyagi, A. K. (2013). Secreted acid phosphatase (SapM) of *Mycobacterium tuberculosis* is indispensable for arresting phagosomal maturation and growth of the pathogen in guinea pig tissues. *PLoS ONE* 8:e70514. doi: 10.1371/journal.pone.0070514

Raoult, D., Marrie, T., and Mege, J. (2005). Natural history and pathophysiology of Q fever. *Lancet Infect. Dis.* 5, 219–226. doi: 10.1016/S1473-3099(05)70052-9

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 122, 735–749. doi: 10.1016/j.cell.2005.06.043

Roberts, E. A., Chua, J., Kyei, G. B., and Deretic, V. (2006). Higher order Rab programming in phagolysosome biogenesis. *J. Cell Biol.* 174, 923–929. doi: 10.1083/jcb.200603026

Saikolappan, S., Estrella, J., Sasindran, S. J., Khan, A., Armitige, L. Y., Jagannath, C., et al. (2012). The fbpA/sapM double knock out strain of *Mycobacterium tuberculosis* is highly attenuated and immunogenic in macrophages. *PLoS ONE* 7:e36198. doi: 10.1371/journal.pone.0036198

Sajjan, S. U., Carmody, L. A., Gonzalez, C. F., and Lipuma, J. J. (2008). A type IV secretion system contributes to intracellular survival and replication of *Burkholderia cenocepacia*. *Infect. Immun.* 76, 5447–5455. doi: 10.1128/IAI.00451-08

Schneider, T., Moos, V., Loddenkemper, C., Marth, T., Fenollar, F., and Raoult, D. (2008). Whipple's disease: new aspects of pathogenesis and treatment. *Lancet Infect. Dis.* 8, 179–190. doi: 10.1016/S1473-3099(08)70042-2

Sherwood, R. K., and Roy, C. R. (2013). A Rab-centric perspective of bacterial pathogen-occupied vacuoles. *Cell Host Microbe* 14, 256–268. doi: 10.1016/j.chom.2013.08.010

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Playing hide-and-seek with host macrophages through the use of mycobacterial cell envelope phthiocerol dimycocerosates and phenolic glycolipids

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Mycobacterial pathogens, including *Mycobacterium tuberculosis*, the etiological agent of tuberculosis (TB), have evolved a remarkable ability to evade the immune system in order to survive and to colonize the host. Among the most important evasion strategies is the capacity of these bacilli to parasitize host macrophages, since these are major effector cells against intracellular pathogens that can be used as long-term cellular reservoirs. Mycobacterial pathogens employ an array of virulence factors that manipulate macrophage function to survive and establish infection. Until recently, however, the role of mycobacterial cell envelope lipids as virulence factors in macrophage subversion has remained elusive. Here, we will address exclusively the proposed role for phthiocerol dimycocerosates (DIM) in the modulation of the resident macrophage response and that of phenolic glycolipids (PGL) in the regulation of the recruitment and phenotype of incoming macrophage precursors to the site of infection. We will provide a unique perspective of potential additional functions for these lipids, and highlight obstacles and opportunities to further understand their role in the pathogenesis of TB and other mycobacterial diseases.

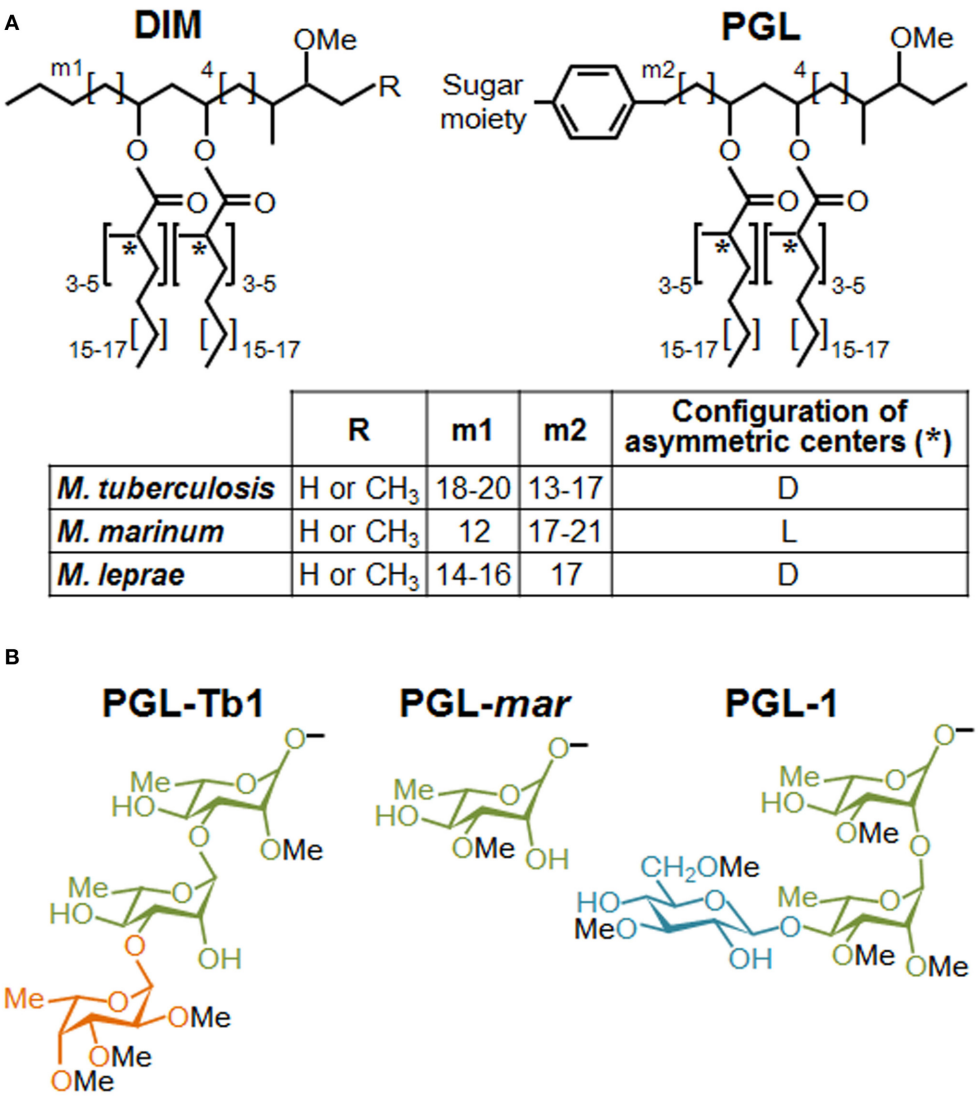
Keywords: mycobacteria, pathogens, virulence, lipids, macrophages, immune responses

INTRODUCTION

Research progress has identified key players for mycobacterial pathogenicity including the major lipid virulence factor, phthiocerol dimycocerosates (DIM), and its structurally-related compound, phenolic glycolipids (PGL). In an elegant study, Cambier et al. proposed an exciting mechanism for their role in immune evasion strategies evolved by *Mycobacterium tuberculosis* (*Mtb*), and its close pathogenic relative *M. marinum* (Cambier et al., 2014). The objective of this perspective is to present a brief view of the literature concerning the immunomodulatory functions of DIM and PGL at the initial site of infection, and to propose a scenario for their molecular mechanism of action. In particular, we will address their impact on resident macrophages and recruitment of incoming precursors to the site of infection. Considering that very little is known about the actual interaction of these lipid virulence factors with the host immune system, a better understanding about their role in immune evasion may contribute to novel therapeutic strategies for mycobacterial diseases, such as tuberculosis (TB).

The virulence of mycobacterial pathogens is a multifaceted process that grants the means to circumvent a dedicated immune response and to thrive in host cells, including macrophages. In spite of recent progress demonstrating the contribution of mycobacterial cell envelope lipids to pathogenicity (for review see Guenin-Mace et al., 2009; Neyrolles and Guilhot, 2011), relatively little is known about their mechanism of action, and more specifically, how these lipids interact with the host at the molecular level. Most biological effects caused by lipids loosely associated to mycobacterial envelope derived from specific interactions with pattern recognition receptors (PRRs) on innate immune cells. Among the different PRR families, carbohydrate-recognition receptors (e.g., calcium-dependent (C)-type lectin receptors, CLR) have generated great interest because they recognize specific sugar moieties on glycolipids, referred to as PAMPs (pathogen associated molecular patterns). This, in turn, can trigger the internalization of mycobacteria and eventually a downstream signaling cascade that can generate opposite effects on host immune responses. For example, the macrophage inducible CLR Mincle recognizes the mycobacterial trehalose-6,6 dimycocerosate (TDM), likely through its trehalose motif, and triggers a pro-inflammatory cytokine production and Th1 and Th17 cell responses (Ishikawa et al., 2009; Schoenen et al., 2010). By contrast, the mannosylated moieties from the mycobacterial lipoglycans, ManLAM, mediate entry of the bacillus into macrophages through mannose receptor and DC-SIGN, whose signaling cascades engage an anti-inflammatory effect that enables *Mtb* to evade immune surveillance (for review see Mishra et al., 2011). Interestingly, the critical motifs of ManLAM for its recognition by DC-SIGN have been shown to be the mannose caps as well as the fatty acids (Maeda et al., 2003; Riviere et al., 2004). Moreover, data indicated that fatty acids are involved in a supramolecular organization of ManLAM, associated with an

increased avidity for their receptors (Riviere et al., 2004).These findings strongly suggest that the lipid moiety of glycolipids interferes with their macromolecular organization, which may be necessary for efficient recognition of the terminal mannosyl epitopes. Lipids may also physically interfere with host membranes and thereby impair immune response-related signaling pathways, as proposed by Laneelle and Daffe (1991). Indeed, insertion of TDM in model or natural membranes can affect their biophysical properties (Laneelle and Tocanne, 1980; Sut et al., 1990; Almog and Mannella, 1996), decreasing notably membrane fluidity by ordering the surrounding phospholipids and cross-linking the leaflets (Laneelle and Tocanne, 1980; Almog and Mannella, 1996). Taken together, these data highlight the importance to consider the specific structural features of lipids in exploring their functional role. This is especially true for the highly hydrophobic DIM, and even more essential for their glycosylated version, PGL. DIM and PGL exhibit a common lipid backbone which is well conserved, with minor structural variations, among the few mycobacterial species synthesizing these molecules (**Figure 1**). In contrast, the saccharide domain of PGL is species-specific (**Figure 1**). Of note, all the major mycobacterial pathogens produce both substances, but in the particular case of *Mtb* only a subset of clinical isolates belonging to Beijing family is capable to synthesize PGL.



	R	m1	m2	Configuration of asymmetric centers (*)
M. tuberculosis	H or CH_3	18-20	13-17	D
M. marinum	H or CH_3	12	17-21	L
M. leprae	H or CH_3	14-16	17	D

FIGURE 1 | Structures of DIM and PGL produced by various mycobacterial species. (A) Structure of the major DIM and PGL lipid moieties. The lipid core is composed of a long-chain β-diol (phthiocerol and phenolphthiocerol), showing slight length variations (see m1 and m2 values in embedded table), esterified by polymethyl-branched fatty acids. In most cases, the configuration of the asymmetric centers bearing the methyl branches (asterisks) are of the D series, mycocerosic acids, but in a limited number of mycobacterial species, they belong to the L series and are then called phthioceranic acids (see table). Minor structural variants of the β-diol can contain a keto group in place of the methoxy group. **(B)** Structure of the species-specific sugar moiety of the major forms of PGL produced by *Mtb* (PGL-Tb1), *M. marinum* (PGL-*mar*) and *M. leprae* (PGL-1). Rhamnose is represented in green, fucose in orange and glucose in blue.

NOW YOU SEE ME, NOW YOU DON'T: THE HIDING EFFECT OF DIM AND MORE

The role of DIM in virulence was first suggested by two independent studies using signature-tagged transposon mutagenesis (Camacho et al., 1999; Cox et al., 1999), and thereafter supported by a large body of evidence (for review, see Neyrolles and Guilhot, 2011). Historically, the only well-established function for DIM was their structural support to the mycobacterial cell wall as protective permeability barrier (Camacho et al., 2001). For example, DIM deficient mutants are sensitive to reactive nitrogen species (RNS) generated in murine macrophages pretreated with IFN-γ and TNF-α (Rousseau et al., 2004). However, it is now apparent that other roles exist for DIM beyond the physical protection against host microbicidal factors, as mutants incapable of transporting DIM or spontaneous DIM deficient variants are more attenuated in mice lacking inducible nitric oxide synthase (iNOS) compared to control counterparts (Murry et al., 2009; Kirksey et al., 2011). Indeed, recent progress indicate that DIM play a dominant role in the modulation of protective host immune responses, specifically during the early steps of infection, when the bacilli encounter host macrophages (Rousseau et al., 2004; Astarie-Dequeker et al., 2009; Cambier et al., 2014; Passemar et al., 2014).

Two different but likely complementary mechanisms have emerged from the latest set of studies addressing this subject. On the one hand, Cambier et al. postulated that DIM mask physically mycobacterial PAMPs, and thus prevent their recognition by toll-like receptors (TLR) and the subsequent recruitment of microbicidal macrophages (Cambier et al., 2014). Using zebrafish to model *in vivo* the earliest interactions between bacteria and host macrophages, these authors reported that a *M. marinum* mutant lacking DIM on its surface was unable to prevent the recruitment of iNOS-expressing macrophages, which were effective in killing this mutant through RNS production. Further analysis indicated that DIM act by preventing TLR signaling via the common TLR adaptor MyD88 (Cambier et al., 2014). Supporting this notion, Rousseau et al. reported that infection of murine macrophages with a DIM-deficient *Mtb* mutant induced a high secretion of TNF-α and IL-6, two pro-inflammatory cytokines produced downstream of TLR- and MyD88-dependent signaling pathway (Rousseau et al., 2004). The authors also mentioned that the greater amount of TNF-α and IL-6 at the site of infection could overstimulate the recruitment of macrophages. The exact mechanism of how DIM are able to mask the mycobacterial PAMPs, and which TLR-containing immune cell is involved, remain to be described. On the other hand, our group proposed that DIM target lipid organization in the membrane of host macrophages, thereby modifying its biophysical properties and subsequently the activity of membrane effectors (Astarie-Dequeker et al., 2009). We observed that DIM-deficient mutants are poorly efficient to infect human macrophages, and accumulate in acidified phagosomes at early time post-infection. Importantly, prevention of phagosomal acidification by the proton-ATPase inhibitor, bafilomycine, rescued the growth defect of a DIM deficient mutant (Passemar et al., 2014). This suggests that DIM contribute to the intracellular growth of *Mtb* by excluding somehow the proton-ATPase from the phagosomal membrane. At late post-infection stages, DIM-deficiency affects the capacity of *Mtb* to induce cell death and to disseminate into bystander macrophages (Passemar et al., 2014). While these results should be interpreted with caution, they suggest that DIM participate in control of the outcome of infected macrophages and cell-to-cell spread of *Mtb*. At the root of these observations, we noticed that *Mtb* interacts with macrophages in a manner that induces changes in membrane fluidity along with the requirement of DIM at the bacterial surface (Astarie-Dequeker et al., 2009). The ability of DIM to induce changes in membrane fluidity is consistent with their hydrophobic properties that would facilitate their insertion into membranes. As non-covalently bound molecules to the outer cell wall layer of mycobacteria, DIM could thus insert into membranes of macrophages to induce biophysical changes that would modulate the activity of membrane-associated effectors. At the level of the plasma membrane, this would increase macrophage infection by enhancing the activity of phagocytic receptors (e.g., complement receptor 3, CR3), and subsequent bacterial replication through inhibition of phagosome acidification (Astarie-Dequeker et al., 2009; Passemar et al., 2014). Moreover, DIM could also collaborate with the secreted *Mtb* protein, ESAT-6 by modulating its membrane lytic activity. Indeed, DIM and ESAT-6 share the capacity to induce cell death and cell-to-cell spread of *Mtb* (Aguilo et al., 2013; Passemar et al., 2014), two events associated to membrane damages.

Collectively, these results clearly established that DIM play an active role at modulating the recognition of mycobacterial PAMPs and avoiding the activation of pro-inflammatory macrophages, and at the same time, altering macrophage responses to its favor possibly by provoking changes in the membrane fluidity that ultimately affect the microbicidal functions.

SEEKING THE RIGHT PARTNER TO PLAY: THE SIGNIFICANCE OF PGL STRUCTURAL VARIABILITY

PGL possess a common lipid core closely related to DIM that, consequently, may share the properties just described for these molecules. However, PGL also exhibit species-specific saccharide domains (**Figure 1**) and this variation in sugar content may have a strong impact on the PRRs involved in their recognition, and therefore, on their biological activities.

Most of *Mtb* clinical isolates, as well as the reference laboratory strains, are unable to produce PGL (Constant et al., 2002). Yet, some *Mtb* strains are able to synthesize a specific form of PGL named PGL-Tb1. This strongly suggests that PGL are not essential for TB pathogenesis. However, their presence may have an impact on the interaction with the host and increases *Mtb* virulence. Indeed, Reed et al. established that the production of PGL-Tb1 in a Beijing clinical isolate is associated with a hypervirulent phenotype in animal models (Reed et al., 2004). It was also reported that treatment of mouse macrophages with purified PGL-Tb1 inhibits the production of the pro-inflammatory cytokines TNF-α, IL-6 and CCL2, and that this effect was dependent on its saccharide domain. The activity of PGL-Tb1 on the modulation of the immune response was also supported by independent results showing that the production of this lipid in the laboratory strain H37Rv, usually unable to synthesize it, modifies cytokine secretion by infected macrophages (Sinsimer et al., 2008). However,

in that series of experiments, PGL-Tb1-producing H37Rv was not found to be more virulent than the parental strain in mice. Pointing to the relevance of this molecule, PGL-deficient *Mtb* strains were found to release *p*-hydroxybenzoic acid derivatives (*p*-HBAD), a truncated form of PGL containing just the saccharide domain and the phenol ring, which may also have biological activities (Constant et al., 2002). Mutants unable to produce PGL-Tb1 glycosylated-phenol moiety (*p*-HBAD II) induced increased secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12p40 (Stadthagen et al., 2006).

PGL are also produced by the fish pathogen *M. marinum,* used in several laboratories to model host-pathogen interactions in the context of human TB. PGL-*mar* (**Figure 1**) was found to be a key factor in the phagosomal maturation arrest induced by *M. marinum* (Robinson et al., 2008). In addition, the ability of purified PGL-*mar* to abrogate the secretion of pro-inflammatory cytokines TNF-α and IL-12p40 by human macrophages was also described. Little more was known about the biological activities of this molecule until recently, when it was demonstrated that PGL-*mar* is involved in the chemokine (C-C motif) receptor 2 (CCR2)-dependent recruitment of macrophages by inducing the expression of the chemokine CCL2 through a molecular mechanism still unknown (Cambier et al., 2014). Indeed, by selectively recruiting pathogen-permissive macrophages, Cambier et al. demonstrated that PGL-*mar*, in a proposed coordinated manner with the masking effect of DIM, increases *M. marinum* fitness in the host.

Finally, PGL-1 from *M. leprae* has retained special attention among the different PGL species since its discovery in the early 1980s, mainly due to the high immunogenicity of its sugar moiety (for a review see Spencer and Brennan, 2011). Like PGL-Tb1, PGL-1 contains a trisaccharide domain but the structure is different (**Figure 1**). Mainly due to the inability to grow the leprosy bacillus *in vitro*, there is a large body of literature using isolated PGL-1 to study its role in the modulation of host immune (Mehra et al., 1984; Schlesinger et al., 1994). Among others, PGL-1 was found to inhibit the pro-inflammatory cytokine secretion by human monocytes (Silva et al., 1993). In addition, *M. leprae* induces a poor activation and maturation of dendritic cells, and dampens their ability to induce T-cell responses (Hashimoto et al., 2002). This inhibition was partially relieved by treatment of *M. leprae*-infected cells with anti-PGL-1 antibodies. Together, those studies suggest that PGL-1, through its terminal trisaccharide motif, is the factor responsible for the immunosuppression observed in lepromatous leprosy, one of the most characteristic forms of the disease. Using an original genetic engineering strategy, our team has demonstrated that PGL-1 expression in *M. bovis* BCG enables this recombinant strain to exploit CR3 to promote bacterial uptake and to inhibit TNF-α secretion by human macrophages (Tabouret et al., 2010). In line with this finding, a recent study showed that synthetic PGL-1 saccharide moiety reduces the production of cytokines (TNF-α, IL-6, IL-1β and CCL2) induced by TLR-2 (Elsaidi et al., 2013).

These results indicate that mycobacteria make use of PGL to modulate host innate immune response, and that this activity is primarily mediated by their saccharide domain through an unknown molecular mechanism. Our data also support the notion that some PGL forms, such as PGL-1, could enable mycobacteria to take advantage of CR3 (Tabouret et al., 2010) to invade macrophages and exert anti-inflammatory properties by interfering with the TLR-dependent pro-inflammatory responses, as previously described for other pathogens (Wang et al., 2007; Hajishengallis and Lambris, 2011; Dai et al., 2013). However, we cannot rule out the possibility of a direct inhibition of TLR-dependent cytokine secretion, or the direct interaction with a "PGL receptor" on epithelial cells, is involved in the induction of CCL2 and subsequent macrophage recruitment, as hypothesized by Cambier et al. for *M. marinum* (Cambier et al., 2014). Finally, it is important to keep in mind that the structure of the saccharide domain of PGL is highly variable and species-specific (**Figure 1**). Therefore, it remains to be seen whether the strategy involved in immunomodulation is the same or varies for each PGL variant, and whether a PGL variant is more active than others.

CONCLUSION: PLAYING HIDE-AND-SEEK WITH HOST MACROPHAGES

From these data, it emerges that pathogenic mycobacteria utilize DIM, on their own or in conjunction with PGL (depending on mycobacterial strains) for diverting macrophages from their natural function and for establishing infection. Most of the research has focused on the macrophage because its dialog with mycobacteria is thought to be the seminal step of the immune response. The study of Cambier et al. marks an important step in understanding the functions of lipids, as it takes into account the full repertoire of the innate immune system (Cambier et al., 2014). However, several questions remain pending: for example, what is the population of recruited macrophages? If we refer to the model illustrating changes in macrophage polarization throughout the infectious process (Lugo-Villarino et al., 2011), lipids might mediate evasion of microbicidal functions of M1 macrophages in the early stage of infection, whereas they might favor recruitment of M2 macrophages with weak microbicidal competences later on during infection. It also remains to be determined how lipids behave within macrophages with distinct phenotype/polarization in order to assess whether, by virtue of their pleiotropic effect, they have an intracellular impact throughout the disease.

We anticipate that the identification of the molecular mechanisms involved in *Mtb* lipid effects will lead to the characterization of signaling pathways that are modulated for the benefit or detriment of mycobacteria survival in the host. Therefore, we would like to provide a framework to study the molecular mechanisms governing mycobacterial DIM and PGL activity at the direct interface with macrophages (**Figure 2**). We propose that DIM exert more than a masking effect of cell wall exposed *Mtb* PAMPs (Cambier et al., 2014) and play an active role through their hydrophobic part to reshape macrophage activity (**Figure 2A**). We also propose that PGL, on their part, act through their saccharide domains as potential ligands for carbohydrate-recognizing receptors expressed at the surface of macrophages (**Figure 2B**).

For the purpose of conciseness, we focused the current perspective on DIM and PGL, but we believe that in the near future there will be a need for a broad assessment of mycobacterial lipids and their overall effects in the immune system. Novel strategies need to be devised in order to deal with the redundant role as

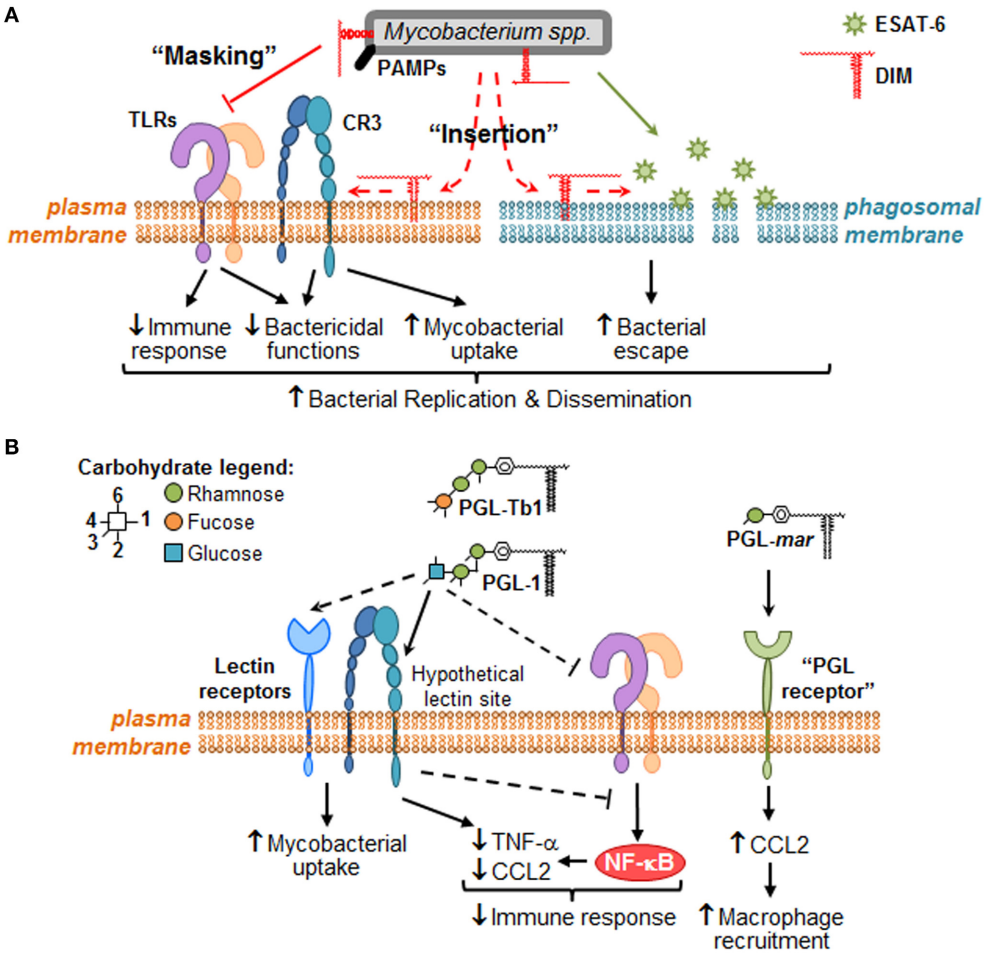


FIGURE 2 | Putative molecular mechanisms through which DIM and PGL remodel macrophage activity during the early steps of infection. When mycobacteria encounter macrophages, they use cell surface exposed PAMPs to recognize numerous plasma membrane PRRs, such as TLR (e.g., TLR-2) and carbohydrate-recognition receptors (e.g., CR3 or other lectin receptors). **(A)** During bacterial recognition, DIM exert a masking effect on PAMPs, thereby preventing TLR-2 detection and triggering of subsequent bactericidal and immune responses. DIM could also insert into plasma membrane, changing its biophysical properties in a manner that increases CR3-mediated bacteria uptake, and decreases bactericidal functions. Likewise, DIM could insert within intracellular membranes (e.g., phagosomal membranes) where they collaborate with ESAT-6 to increase its membrane lytic activity, thereby inducing membrane damage and consequently allowing mycobacteria to escape into the cytosol. Altogether, DIM-mediated effects may lead to mycobacteria replication and innate immune evasion. **(B)** Some PGL species, such *M. leprae* PGL-1 and potentially *Mtb* PGL-Tb1, are able to decrease host immune response either by direct inhibition of TLRs or by taking advantage of lectin receptor (e.g., CR3) capacity to interfere with TLR-triggered pro-inflammatory cytokine secretion (e.g., TNF-α). They may also exploit these lectin receptors to promote mycobacterial uptake by macrophages. In the case of PGL-*mar*, a putative "PGL receptor" at the surface of epithelial cells has been proposed to be responsible for the induction of CCL2, and the subsequent macrophage recruitment.

virulence or immunomodulatory factor among mycobacteria cell envelope lipids. In this aspect, our group has undertaken the construction of single and multiple lipid mutants to assess the role of trehalose-derived lipids, sulfolipids, diacyltrehaloses and polyacyltrehaloses, and their respective contribution to the virulence together with DIM (Passemar et al., 2014). Similarly, there is a well-known redundancy among host PRRs that may hamper the characterization of signaling pathways triggered by mycobacterial lipids; our group has also made headways to develop techniques for the simultaneous double-gene silencing in primary mononuclear phagocytes (Troegeler et al., 2014). Finally, PGL variants merit careful consideration, as there is a strong potential for variation at the functional level according to species. This is particularly true for the assessment of permissive macrophage recruitment toward the site of infection for both PGL-Tb1 and PGL-1. Unlike the inducing effect by PGL-*mar* in the context of zebrafish infection (Cambier et al., 2014), both PGL-Tb1 and PGL-1 inhibit the CCL2 secretion by macrophages (Reed et al., 2004; Elsaidi et al., 2013). This divergence could be due to a differential effect of PGL depending on cellular context, or more interestingly, through a distinctive recognition by the involved receptor according to the saccharide composition. To address this type of question, the strategy developed by our team based on the use of *M. bovis* BCG as surrogate might be a valuable tool to

enable the direct comparison of the effects of the PGL variants in the context of a relevant mycobacterial envelope and within the same genetic background (Tabouret et al., 2010).

In conclusion, we believe that the development of microbiological tools and adequate research models, in combination with multidisciplinary strategies, should open up new venues to achieve a better understanding of the ever-evolving relationship between host and pathogen. In many ways, the lipid at the mycobacteria wall is just a starting point!

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REFERENCES

Aguilo, N., Marinova, D., Martin, C., and Pardo, J. (2013). ESX-1-induced apoptosis during mycobacterial infection: to be or not to be, that is the question. *Front. Cell. Infect. Microbiol.* 3:88. doi: 10.3389/fcimb.2013.00088

Almog, R., and Mannella, C. A. (1996). Molecular packing of cord factor and its interaction with phosphatidylinositol in mixed monolayers. *Biophys. J.* 71, 3311–3319. doi: 10.1016/S0006-3495(96)79523-1

Astarie-Dequeker, C., Le Guyader, L., Malaga, W., Seaphanh, F. K., Chalut, C., Lopez, A., et al. (2009). Phthiocerol dimycocerosates of *M. tuberculosis* participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. *PLoS Pathog.* 5:e1000289. doi: 10.1371/journal.ppat.1000289

Camacho, L. R., Constant, P., Raynaud, C., Laneelle, M. A., Triccas, J. A., Gicquel, B., et al. (2001). Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. *J. Biol. Chem.* 276, 19845–19854. doi: 10.1074/jbc.M100662200

Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B., and Guilhot, C. (1999). Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol. Microbiol.* 34, 257–267. doi: 10.1046/j.1365-2958.1999.01593.x

Cambier, C. J., Takaki, K. K., Larson, R. P., Hernandez, R. E., Tobin, D. M., Urdahl, K. B., et al. (2014). Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature* 505, 218–222. doi: 10.1038/nature12799

Constant, P., Perez, E., Malaga, W., Laneelle, M. A., Saurel, O., Daffe, M., et al. (2002). Role of the pks15/1 gene in the biosynthesis of phenolglycolipids in the *Mycobacterium tuberculosis* complex. Evidence that all strains synthesize glycosylated p-hydroxybenzoic methyl esters and that strains devoid of phenolglycolipids harbor a frameshift mutation in the pks15/1 gene. *J. Biol. Chem.* 277, 38148–38158. doi: 10.1074/jbc.M206538200

Cox, J. S., Chen, B., McNeil, M., and Jacobs, W. R. Jr. (1999). Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* 402, 79–83.

Dai, S., Rajaram, M. V., Curry, H. M., Leander, R., and Schlesinger, L. S. (2013). Fine tuning inflammation at the front door: macrophage complement receptor 3-mediates phagocytosis and immune suppression for Francisella tularensis. *PLoS Pathog.* 9:e1003114. doi: 10.1371/journal.ppat.1003114

Elsaidi, H. R., Barreda, D. R., Cairo, C. W., and Lowary, T. L. (2013). Mycobacterial phenolic glycolipids with a simplified lipid aglycone modulate cytokine levels through Toll-like receptor 2. *Chembiochem* 14, 2153–2159. doi: 10.1002/cbic.201300505

Guenin-Mace, L., Simeone, R., and Demangel, C. (2009). Lipids of pathogenic Mycobacteria: contributions to virulence and host immune suppression. *Transbound. Emerg. Dis.* 56, 255–268. doi: 10.1111/j.1865-1682.2009.01072.x

Hajishengallis, G., and Lambris, J. D. (2011). Microbial manipulation of receptor crosstalk in innate immunity. *Nat. Rev. Immunol.* 11, 187–200. doi: 10.1038/nri2918

Hashimoto, K., Maeda, Y., Kimura, H., Suzuki, K., Masuda, A., Matsuoka, M., et al. (2002). *Mycobacterium leprae* infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. *Infect. Immun.* 70, 5167–5176. doi: 10.1128/IAI.70.9.5167-5176.2002

Ishikawa, E., Ishikawa, T., Morita, Y. S., Toyonaga, K., Yamada, H., Takeuchi, O., et al. (2009). Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J. Exp. Med.* 206, 2879–2888. doi: 10.1084/jem.20091750

Kirksey, M. A., Tischler, A. D., Simeone, R., Hisert, K. B., Uplekar, S., Guilhot, C., et al. (2011). Spontaneous phthiocerol dimycocerosate-deficient variants of *Mycobacterium tuberculosis* are susceptible to gamma interferon-mediated immunity. *Infect. Immun.* 79, 2829–2838. doi: 10.1128/IAI.00097-11

Laneelle, G., and Daffe, M. (1991). Mycobacterial cell wall and pathogenicity: a lipodologist's view. *Res. Microbiol.* 142, 433–437. doi: 10.1016/0923-2508(91)90116-R

Laneelle, G., and Tocanne, J. F. (1980). Evidence for penetration in liposomes and in mitochondrial membranes of a fluorescent analogue of cord factor. *Eur. J. Biochem.* 109, 177–182. doi: 10.1111/j.1432-1033.1980.tb04782.x

Lugo-Villarino, G., Verollet, C., Maridonneau-Parini, I., and Neyrolles, O. (2011). Macrophage polarization: convergence point targeted by *Mycobacterium tuberculosis* and HIV. *Front. Immunol.* 2:43. doi: 10.3389/fimmu.2011.00043

Maeda, N., Nigou, J., Herrmann, J. L., Jackson, M., Amara, A., Lagrange, P. H., et al. (2003). The cell surface receptor DC-SIGN discriminates between Mycobacterium species through selective recognition of the mannose caps on lipoarabinomannan. *J. Biol. Chem.* 278, 5513–5516. doi: 10.1074/jbc.C200586200

Mehra, V., Brennan, P. J., Rada, E., Convit, J., and Bloom, B. R. (1984). Lymphocyte suppression in leprosy induced by unique *M. leprae* glycolipid. *Nature* 308, 194–196. doi: 10.1038/308194a0

Mishra, A. K., Driessen, N. N., Appelmelk, B. J., and Besra, G. S. (2011). Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. *FEMS Microbiol. Rev.* 35, 1126–1157. doi: 10.1111/j.1574-6976.2011.00276.x

Murry, J. P., Pandey, A. K., Sassetti, C. M., and Rubin, E. J. (2009). Phthiocerol dimycocerosate transport is required for resisting interferon-gamma-independent immunity. *J. Infect. Dis.* 200, 774–782. doi: 10.1086/605128

Neyrolles, O., and Guilhot, C. (2011). Recent advances in deciphering the contribution of *Mycobacterium tuberculosis* lipids to pathogenesis. *Tuberculosis (Edinb.)* 91, 187–195. doi: 10.1016/j.tube.2011.01.002

Passemar, C., Arbues, A., Malaga, W., Mercier, I., Moreau, F., Lepourry, L., et al. (2014). Multiple deletions in the polyketide synthase gene repertoire of *Mycobacterium tuberculosis* reveal functional overlap of cell envelope lipids in host-pathogen interactions. *Cell. Microbiol.* 16, 195–213. doi: 10.1111/cmi.12214

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., et al. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* 431, 84–87. doi: 10.1038/nature02837

Riviere, M., Moisand, A., Lopez, A., and Puzo, G. (2004). Highly ordered supramolecular organization of the mycobacterial lipoarabinomannans in solution. Evidence of a relationship between supra-molecular organization and biological activity. *J. Mol. Biol.* 344, 907–918. doi: 10.1016/j.jmb.2004.09.092

Robinson, N., Kolter, T., Wolke, M., Rybniker, J., Hartmann, P., and Plum, G. (2008). Mycobacterial phenolic glycolipid inhibits phagosome maturation and subverts the pro-inflammatory cytokine response. *Traffic* 9, 1936–1947. doi: 10.1111/j.1600-0854.2008.00804.x

Rousseau, C., Winter, N., Pivert, E., Bordat, Y., Neyrolles, O., Ave, P., et al. (2004). Production of phthiocerol dimycocerosates protects *Mycobacterium tuberculosis* from the cidal activity of reactive nitrogen intermediates produced by macrophages and modulates the early immune response to infection. *Cell. Microbiol.* 6, 277–287. doi: 10.1046/j.1462-5822.2004.00368.x

Schlesinger, L. S., Hull, S. R., and Kaufman, T. M. (1994). Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *J. Immunol.* 152, 4070–4079.

Schoenen, H., Bodendorfer, B., Hitchens, K., Manzanero, S., Werninghaus, K., Nimmerjahn, F., et al. (2010). Cutting edge: mincle is essential for recognition

and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J. Immunol.* 184, 2756–2760. doi: 10.4049/jimmunol.0904013

Silva, C. L., Faccioli, L. H., and Foss, N. T. (1993). Suppression of human monocyte cytokine release by phenolic glycolipid-I of *Mycobacterium leprae*. *Int. J. Lepr. Other Mycobact. Dis.* 61, 107–108.

Sinsimer, D., Huet, G., Manca, C., Tsenova, L., Koo, M. S., Kurepina, N., et al. (2008). The phenolic glycolipid of *Mycobacterium tuberculosis* differentially modulates the early host cytokine response but does not in itself confer hypervirulence. *Infect. Immun.* 76, 3027–3036. doi: 10.1128/IAI.01663-07

Spencer, J. S., and Brennan, P. J. (2011). The role of *Mycobacterium leprae* phenolic glycolipid I (PGL-I) in serodiagnosis and in the pathogenesis of leprosy. *Lepr. Rev.* 82, 344–357.

Stadthagen, G., Jackson, M., Charles, P., Boudou, F., Barilone, N., Huerre, M., et al. (2006). Comparative investigation of the pathogenicity of three *Mycobacterium tuberculosis* mutants defective in the synthesis of p-hydroxybenzoic acid derivatives. *Microbes Infect.* 8, 2245–2253. doi: 10.1016/j.micinf.2006.04.008

Sut, A., Sirugue, S., Sixou, S., Lakhdar-Ghazal, F., Tocanne, J. F., and Laneelle, G. (1990). Mycobacteria glycolipids as potential pathogenicity effectors: alteration of model and natural membranes. *Biochemistry* 29, 8498–8502. doi: 10.1021/bi00488a042

Tabouret, G., Astarie-Dequeker, C., Demangel, C., Malaga, W., Constant, P., Ray, A., et al. (2010). *Mycobacterium leprae* phenolglycolipid-1 expressed by engineered *M. bovis* BCG modulates early interaction with human phagocytes. *PLoS Pathog.* 6:e1001159. doi: 10.1371/journal.ppat.1001159

Troegeler, A., Lastrucci, C., Duval, C., Tanne, A., Cougoule, C., Maridonneau-Parini, I., et al. (2014). An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells and macrophages. *Immunol. Cell Biol.* 92, 699–708. doi: 10.1038/icb.2014.39

Wang, M., Shakhatreh, M. A., James, D., Liang, S., Nishiyama, S., Yoshimura, F., et al. (2007). Fimbrial proteins of porphyromonas gingivalis mediate *in vivo* virulence and exploit TLR2 and complement receptor 3 to persist in macrophages. *J. Immunol.* 179, 2349–2358 doi: 10.4049/jimmunol.179.4.2349

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Manipulation of the endocytic pathway and phagocyte functions by *Mycobacterium tuberculosis* lipoarabinomannan

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Lipoarabinomannan is a major immunomodulatory lipoglycan found in the cell envelope of *Mycobacterium tuberculosis* and related human pathogens. It reproduces several salient properties of *M. tuberculosis* in phagocytic cells, including inhibition of pro-inflammatory cytokine production, inhibition of phagolysosome biogenesis, and inhibition of apoptosis as well as autophagy. In this review, we present our current knowledge on lipoarabinomannan structure and ability to manipulate the endocytic pathway as well as phagocyte functions. A special focus is put on the molecular mechanisms employed and the signaling pathways hijacked. Available information is discussed in the context of *M. tuberculosis* pathogenesis.

Keywords: lipoarabinomannan, *Mycobacterium*, cytokine, phagosome, apoptosis, autophagy

INTRODUCTION

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis, is one the most effective human pathogens. Its virulence is multifactorial but initially relies on its ability to parasite and manipulate phagocytic cells in the lung. Mannose-capped lipoarabinomannan (ManLAM), a macroamphiphilic lipoglycan exposed at the surface of *M.tb* cell envelope (Nigou et al., 2003; Pitarque et al., 2008), is a key factor allowing the bacilli to manipulate phagocyte functions (Chatterjee and Khoo, 1998; Gilleron et al., 2008). Indeed, it reproduces several salient properties of *M.tb* in phagocytic cells, including inhibition of pro-inflammatory cytokines production, inhibition of phagosome maturation, inhibition of macrophage apoptosis, and inhibition of autophagy. ManLAM is a Pathogen-Associated Molecular Pattern recognized by several receptors of the innate immune system, including the C-type lectins Mannose Receptor (MR), DC-SIGN and Dectin-2, as well as TLR2 (Gilleron et al., 2008; Ray et al., 2013). It is a potential ligand for the entry of *M.tb* into macrophages *via* the MR (Schlesinger et al., 1994) and into dendritic cells (DCs) *via* DC-SIGN (Maeda et al., 2003; Tailleux et al., 2003). ManLAM inhibitory properties mainly rely on its ability to bind these two lectins. ManLAM can be a ligand of these receptors not only at the surface of *M.tb* bacilli but also as a soluble molecule. Indeed, it is delivered from infected macrophages, through exosomes or apoptotic vesicles, to non-infected bystander phagocytic cells (Beatty et al., 2000; Schaible et al., 2003). This pathway is thought to be critical for shaping immune response but might also be used by the pathogen as a way to disseminate immunomodulatory molecules such as ManLAM.

LIPOARABINOMANNAN STRUCTURE AND PHYSIOLOGICAL ROLE

Lipoarabinomannan (LAM) is ubiquitously found in mycobacterial species (Nigou et al., 2003; Briken et al., 2004; Gilleron et al., 2008; Mishra et al., 2011; Angala et al., 2014). It presents a tripartite structure including a lipid anchor, namely Mannosyl-Phosphatidyl-*myo*-Inositol (MPI), a polysaccharide backbone composed of D-Mannan and D-Arabinan, and finally caps (**Figure 1A**). MPI anchor is based on a *sn*-glycerol-3-phospho-(1-D-*myo*-inositol) unit with one α-D-Mannopyranosyl (α-D-Man*p*) unit linked at *O*-2 of the *myo*-inositol. Four potential sites of acylation are present on the anchor: positions 1 and 2 of the glycerol unit, position 6 of the Man*p* unit and position 3 of the *myo*-inositol (Nigou et al., 1999; Gilleron et al., 2000) (**Figure 1A**). LAM and its biosynthetic precursors, phosphatidyl-*myo*-inositol-mannosides (PIMs) and lipomannan (LM), are predominantly tri- and tetra-acylated by palmitic and tuberculostearic (10-methyl-octadecanoic) acids (Khoo et al., 1995; Gilleron et al., 1999). Position *O*-6 of *myo*-inositol is glycosylated by the mannan core. PIMs comprise different glyco-forms, containing one to six α-D-Man*p* units (PIM_1 to PIM_6), PIM_2 and PIM_6 being the most abundant ones. The D-mannan core of LAM and LM is composed of an (α1→6)-Man*p* backbone substituted at some *O*-2 by a single α-D-Man*p* unit. The D-arabinan portion of LAM contains about 60 arabinofuranosyl (Ara*f*) units which are present as a single arabinan chain attached through an (α1→2) linkage near the middle of the D-mannan core (Kaur et al., 2014). The innermost region is made of a linear (α1→5)-Ara*f* backbone and is followed by a branched region. The non-reducing termini consist of branched hexa-arabinofuranosides

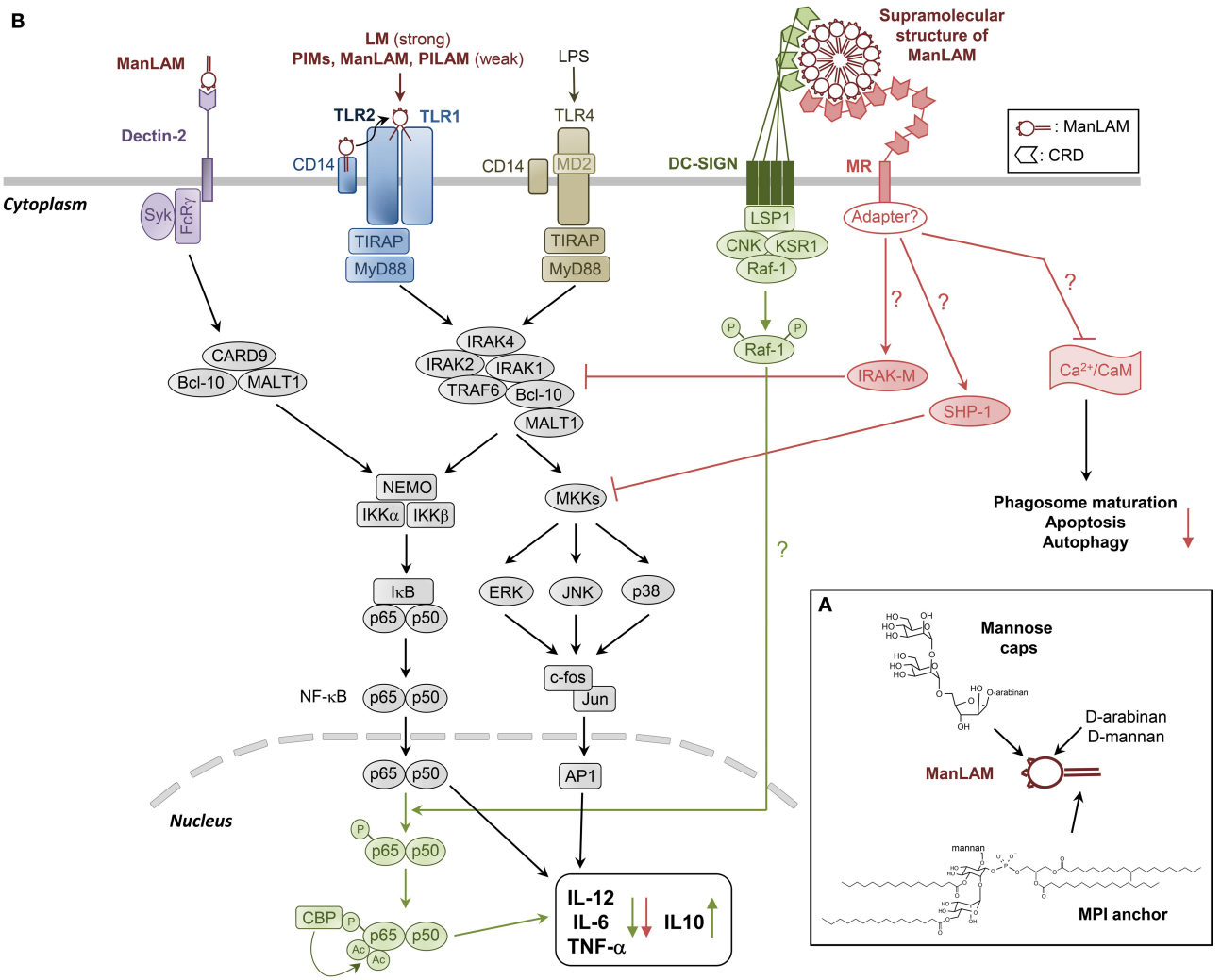

FIGURE 1 | Structural model of and cell signaling pathways triggered by ManLAM and its biosynthetic precursors LM and PIMs. (A) ManLAM is a 17 kDa heterogenous macromolecule exhibiting a tripartite structure: (i) a MPI anchor, which can be mono- to tetra-acylated, (ii) a polysaccharide backbone composed of D-Mannan and D-Arabinan, and (iii) mannose caps, which are mono-, (α1→2)-di- and (α1→2)-tri-mannoside units. PIMs and LM are biosynthetic precursors of LAM. Their structure is based on the MPI anchor, glycosylated by one to six mannose units (PIMs) or the full mannan domain (LM). To our present knowledge, the MPI anchor and the Mannose caps are the main structural determinants of ManLAM biological properties. **(B)** LM, and to a much lesser extent PIMs, PILAM and ManLAM, induce pro-inflammatory cytokines production in DCs and macrophages *via* the recognition of tri- or tetra-acylated MPI anchor by TLR2/TLR1 heterodimer. ManLAM elicits cytokines in bone marrow-derived DCs *via* mannose caps binding to Dectin-2. But it also inhibits the production of pro-inflammatory cytokines IL-12, TNF-α, and IL-6, and induces IL-10 by LPS-stimulated human DCs through DC-SIGN ligation. The signaling pathway involves activation of Raf-1, which results in the phosphorylation of the p65 subunit of NF-κB at Ser276, leading to the acetylation of p65 by two histone acetyltransferases. Translocation of NF-κB in response to TLR activation, and initially dedicated to the transcription of the pro-inflammatory cytokine-coding genes, is reoriented on anti-inflammatory promoter targets, resulting in the decrease of these cytokines to the benefit of IL-10. ManLAM also inhibits IL-12 and TNF-α in macrophages independently of IL-10 production, by directly acting on the TLR4 signaling cascade through induced expression of IRAK-M, which can compete with IRAK1 for binding to TRAF6 and thus inhibit NF-κB activation. ManLAM also promotes tyrosine dephosphorylation of multiple proteins including MAPK, an effect that might be explained by an increased activity of tyrosine phosphatase SHP-1. MR is likely to mediate ManLAM immunosuppressive activities in macrophages, although it has no signaling motif in its cytoplasmic domain, raising the intriguing question as to whether it associates with adapter molecules to transduce signals. The ability of ManLAM to bind MR might in part determine its other inhibitory properties as detailed in **Figure 2**.

and linear tetra-arabinofuranosides, which end with an Ara*f*-(β1→2)-Ara*f*-(α1→ motif. Some β-Ara*f* units are substituted at *O*-5 by capping motifs. The caps differ according to the mycobacterial species. LAM from slow-growing mycobacteria, including the pathogenic species *M.tb*, *Mycobacterium leprae* and *Mycobacterium ulcerans*, are capped with mono-, (α1→2)-di- and (α1→2)-tri-mannoside units (mannose-capped LAM is referred to as ManLAM) (Chatterjee et al., 1992) (**Figure 1A**). In contrast, LAM from fast-growing species is either capped by phospho-*myo*-inositol units (PILAM), such as in the non-pathogenic model

organism *Mycobacterium smegmatis*, or not capped (AraLAM). LAM in any strain further displays considerable structural micro-heterogeneity, with various acyl-forms and glyco-forms. In addition, ManLAM may be substituted by discrete motifs, such as succinyl residues on the arabinan chain or (α1→4)-linked methyl-thio-D-xylose (MTX) residues on some terminal Man*p* units of the mannose caps or the mannan core.

LAM is not restricted to mycobacteria. Indeed, LAM-like molecules are also produced by phylogenetically close relatives of bacteria of the suborders *Corynebacterineae* and *Pseudonocardineae*, including *Corynebacterium* (Tatituri et al., 2007), *Rhodococcus* (Garton et al., 2002; Gibson et al., 2003b), *Tsukamurella* (Gibson et al., 2004), *Turicella* (Gilleron et al., 2005), *Amycolatopsis* (Gibson et al., 2003a), or *Saccharothrix* (Gibson et al., 2005) genera. In these bacteria, lipoglycans are thought to functionally replace lipoteichoic acid otherwise produced by low G+C Gram-positive bacteria. These macroamphiphiles play a fundamental role in the physiology of bacteria, although yet not fully understood (Ray et al., 2013). Defective or deficient lipoglycans synthesis is associated with lethality or growth defects (Gilleron et al., 2008) and changes in lipoglycan structures have a significant impact on the cell wall integrity of mycobacteria (Fukuda et al., 2013). For example, structural defects in LM and LAM in *M. smegmatis* result in loss of acid-fast staining, increased sensitivity to β-lactam antibiotics, and faster killing by macrophages (Fukuda et al., 2013). Accordingly, mycobacterial D-arabinan biosynthesis is the target of ethambutol (Deng et al., 1995), a first-line drug in the treatment of tuberculosis, as well as of benzothiazinones (Makarov et al., 2009), which are new antituberculous drug candidates in preclinical development. The elucidation of the complete biosynthetic pathways of these important molecules is therefore expected to afford novel therapeutic targets (Angala et al., 2014).

In the context of host-pathogen interaction, to our present knowledge, the MPI anchor and the mannose caps are the main structural determinants of ManLAM biological properties, the role of the discrete motifs remaining elusive. The MPI anchor is recognized by TLR2/TLR1 heterodimer, whereas the mannose caps allow the binding to C-type lectins (Gilleron et al., 2008; Ray et al., 2013).

MANIPULATION OF PHAGOCYTES RESPONSES

The success of *M.tb* as an intracellular pathogen relies on its extraordinary capacity to disarm phagocyte antibacterial defenses whereby turning hostile phagocytes into safe havens for replication. Beyond their impact on innate immune responses such manipulations can also be detrimental in development of an efficient adaptive immunity. Interestingly, several of these manipulations can be mirrored by purified ManLAM.

PRO-INFLAMMATORY CYTOKINES PRODUCTION

The inflammatory response is crucial to control *M.tb* infection through macrophage activation and granuloma formation. LM, and to a much lesser extent PIMs, PILAM, and ManLAM, induce pro-inflammatory cytokines production *via* the recognition of tri- or tetra-acylated MPI anchor by TLR2/TLR1 (Gilleron et al., 2003, 2006; Vignal et al., 2003; Quesniaux et al., 2004; Nigou et al., 2008; Ray et al., 2013) (**Figure 1B**). However, a prolonged stimulation of TLR2 has been shown to result in inhibition of MHC class II transactivator expression, MHC class II molecule expression and antigen presentation (Gehring et al., 2004). *M.tb* might have subverted this general mechanism of negative-feedback regulation that prevents excessive T cell-mediated inflammation to evade recognition by $CD4^+$ T cells (Harding and Boom, 2010).

ManLAM was recently shown to elicit TNF-α, IL-6, and IL-10 in bone marrow-derived DCs *via* mannose caps binding to Dectin-2 (Yonekawa et al., 2014) (**Figure 1B**). However, we previously found that *M.tb* ManLAM can also inhibit the production of pro-inflammatory cytokines IL-12 and TNF-α by LPS-stimulated human DCs (Nigou et al., 2001, 2002) (**Figure 1B**). We initially proposed the C-type lectin MR to mediate ManLAM inhibitory activity because the latter (i) relied on the presence of both the mannose caps and the fatty acids which are also required for ManLAM binding to MR and (ii) could be mimicked by an agonist anti-MR monoclonal antibody. However, it was later shown that ManLAM binding to DCs is not inhibited by anti-MR but rather by anti-DC-SIGN antibodies and that a blocking anti-DC-SIGN antibody inhibits ManLAM-induced IL-10 production by LPS-stimulated DCs (Geijtenbeek et al., 2003). Why ManLAM only binds DC-SIGN on DCs, although MR is expressed on these cells, remains unclear (Blattes et al., 2013). ManLAM also inhibits IL-12 and TNF-α in human THP-1 (Knutson et al., 1998) and murine RAW 264.7 (Pathak et al., 2005) macrophage cell lines although DC-SIGN is absent. MR is likely to mediate ManLAM effect in these cells, as the ability of MR to trigger an anti-inflammatory signal was confirmed by other independent studies (Chieppa et al., 2003; Zhang et al., 2005).

How DC-SIGN or MR signal into the cells and interfere with LPS-induced TLR4 signaling is not yet completely understood (**Figure 1B**). DC-SIGN displays intracellular motifs that are able to constitutively recruit the lymphocyte-specific adaptor protein LSP1 which associates the complex KSR1-CNK-Raf-1 (Gringhuis et al., 2009) (**Figure 1B**). Upon ligand binding, activation of Raf-1 results in the phosphorylation of the p65 subunit of NF-κB at Ser276, leading to the acetylation of p65 (Gringhuis et al., 2007). NF-κB activity is then prolonged and increases the transcription rate at the IL-10 anti-inflammatory cytokine promoter. However, Raf-1 signaling alone does not induce cytokine expression. Translocation of NF-κB in response to TLR activation and initially dedicated to the transcription of the pro-inflammatory IL-12p35, IL-12p40, IL-6, and TNF-α cytokine-coding genes is reoriented on anti-inflammatory promoter targets, resulting in the decrease of these cytokines to the benefit of IL-10 (Gringhuis et al., 2007) (**Figure 1B**). Gringhuis et al. (2009) proposed that DC-SIGN may discriminate among mannosylated and fucosylated ligands and modulate the TLR signaling into a pro- or anti-inflammatory response respectively. However, this appears to be in contradiction with the set of data showing that ManLAM or synthetic mannosylated analogs engaging DC-SIGN inhibit pro-inflammatory cytokines production (Nigou et al., 2001; Geijtenbeek et al., 2003; Blattes et al., 2013). MR has no signaling motif in its cytoplasmic domain, raising the intriguing question as to whether it associates with adapter molecules to transduce

signals. Pathak et al. (2005) demonstrated that ManLAM dampens IL-12 in RAW 264.7 macrophages independently of IL-10 production, by directly acting on the TLR4 signaling cascade through induced expression of IRAK-M, which can compete with IRAK1 for binding to TRAF6 and thus inhibit NF-κB activation (**Figure 1B**).

ManLAM anti-inflammatory activity relies on its ability to bind DC-SIGN or MR and both the mannose caps and the fatty acids are required for efficient binding (Nigou et al., 2001, 2002). Indeed, fatty acids induce a supramolecular organization of ManLAM in aqueous solution, resulting in the formation of a 30 nm spherical structure (**Figure 1B**), composed of approximately 450 molecules with the mannose caps exposed at the surface (Riviere et al., 2004). This multivalent supramolecular structure allows multipoint attachment of ManLAM, *via* mannose caps, to the Carbohydrate Recognition Domains (CRD) of multimeric DC-SIGN receptors (Feinberg et al., 2001; Mitchell et al., 2001), thereby ensuring high affinity binding (Nigou et al., 2001; Riviere et al., 2004) (**Figure 1B**). Following this rationale, we were able to design fully synthetic compounds mimicking the bioactive supramolecular structure of ManLAM, i.e., mannodendrimers, that display potent anti-inflammatory activity both *in vitro* and *in vivo* and that could be of therapeutic use (Blattes et al., 2013).

The ability of ManLAM to bind MR might in part determine its other inhibitory properties as described below (**Figure 1B**) (Gilleron et al., 2008).

PHAGOSOME MATURATION

One main function of professional phagocytes is the uptake of microorganisms through phagocytosis. This event results in formation of a vacuole called phagosome which then matures into a phagolysosome through a series of fusion reactions with the endocytic and secretory pathways and ultimately fusion with lysosomes (Flannagan et al., 2012). Maturation endows phagosome with new bactericidal properties predominantly hydrolase activities, acidic pH and antimicrobial peptides. Therefore, phagosome maturation process is crucial for killing of captured microbes as well as their antigen presentation to T lymphocytes. Inhibition of phagosome maturation by *M.tb* was reported more than 40 years ago (Armstrong and Hart, 1971). Since then, numerous mycobacterial factors have been identified and characterized as disruptors of phagosome maturation (Russell, 2011), including ManLAM and PIMs (Fratti et al., 2001; Vergne et al., 2004). Importantly, the ability of *M.tb* to block phagosome maturation is shared by other pathogenic mycobacteria such as *Mycobacterium avium* and *Mycobacterium marinum* which produce ManLAM but not by non-pathogenic *M. smegmatis* which produces PILAM (Anes et al., 2006; Appelmelk et al., 2008; de Chastellier et al., 2009).

Early work by Deretic and colleagues showed that mycobacteria block phagosome maturation between stages orchestrated by Rab5 and Rab7, two small GTPases involved in membrane trafficking and present on early and late endosomes, respectively (Via et al., 1997). Later on, they pinpointed this block to impairment in recruitment of EEA1, a tethering protein and Rab5 effector, essential for phagosome maturation (Fratti et al., 2001). EEA1 recruitment is instrumental in delivering hydrolases such as Cathepsin D and H^+-ATPase subunit Vo from Trans-Golgi-Network (TGN) to the phagosome (Fratti et al., 2003). EEA1 is recruited to the phagosomal membrane via Rab5 and phosphatidylinositol 3-phosphatase (PI3P) which is synthesized by type III PI3Kinase, hVPS34 (Fratti et al., 2001). In the same report, authors showed that ManLAM-coated beads, in contrast to control beads, prevent EEA1 recruitment to the phagosomal membrane, delivery of Cathepsin D, and phagosome acidification (Fratti et al., 2001, 2003). Inhibition of phagosome maturation by ManLAM was later confirmed by several groups (Hmama et al., 2004; Kang et al., 2005; Welin et al., 2008).

Another important player in phagosome maturation is Ca^{2+} signaling. Phagocytosis of dead *M.tb* but not of live *M.tb* triggers an increase of cytosolic Ca^{2+} that results in activation of calmodulin-dependent kinase II (CaMKII) (Malik et al., 2000, 2001). Inhibition of Ca^{2+}, Calmodulin (CaM) and CaMKII prevents phagosome containing dead *M.tb* to fuse with lysosomes. Vergne et al. showed that Ca^{2+} signaling is central for PI3P synthesis on phagosomal membrane, consequently for EEA1 recruitment (Vergne et al., 2003). CaM and CaMKII seem to play a role in hVPS34 recruitment and/or activation. Notably, in contrast to PILAM, ManLAM limits Ca^{2+} influx in cytosol, thus explaining its effect on EEA1 recruitment and phagosome maturation (**Figure 2**). Interestingly, PIMs can also inhibit phagosome acidification, not by preventing EEA1 recruitment, but by promoting fusion between phagosome and early endosomes (Vergne et al., 2004). How PIMs trigger early endosome fusion remains to be elucidated but it might involve Rab14, a small GTPase specifically recruited by live mycobacteria to favor phagosome-early endosomes fusion and block phagosome acidification (Kyei et al., 2006) (**Figure 2**).

What are ManLAM molecular targets, upstream of Ca^{2+} signaling, responsible for phagosome maturation arrest? Two main mechanisms, non-mutually exclusive, have been uncovered. Schlesinger's group has demonstrated that ManLAM limits phagosome maturation by binding to MR (Kang et al., 2005). Interestingly, ManLAM acyl chains are important to maintain this blockade beyond 1 h, suggesting a possible additional mechanism for ManLAM action. ManLAM can insert into lipid microdomains, called rafts, *via* the MPI anchor, resulting in membrane disorganization and inhibition of membrane fusion (Hayakawa et al., 2007; Welin et al., 2008). However, it is still unclear whether rafts disruption and/or MR are responsible of Ca^{2+} signaling inhibition or are completely independent mechanisms.

APOPTOSIS

The role(s) of apoptosis and other cell-death pathways in Tuberculosis remain(s) a matter of intense debate. Several reports suggest that inhibition of excessive apoptosis may be beneficial for the pathogen during early stage of infection for maintaining its replicative niche and limiting cross-presentation and cross-priming of $CD8^+$ T-cells through phagocytosis of apoptotic bodies by DCs. *M.tb* seems to be able to block both extrinsic pathway of host cell apoptosis which relies on activation of death receptors (Fas/CD95, TNFR1) and the intrinsic pathway triggered

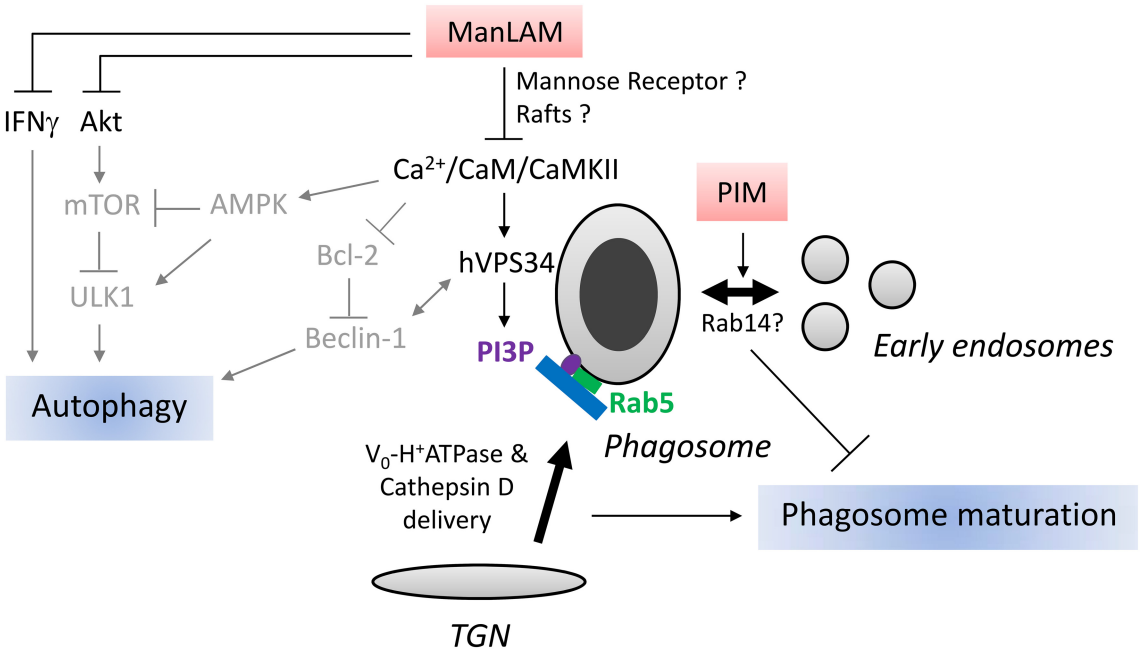

FIGURE 2 | Schematic representation of ManLAM and PIM action on phagosome maturation and autophagy. Right part: After phagocytosis, mycobacteria reside in a vacuole, called phagosome. Phagosome maturation consists in a series of fusion events with exocytic and endocytic pathways. One key step is the delivery of Cathepsin D and H^+-ATPase subunit Vo from Trans-Golgi-Network (TGN) to the phagosome. This step is mediated by tethering protein EEA1 which is recruited to the phagosome by small GTPase Rab5 and phosphatidylinositol 3-phosphate (PI3P). ManLAM blocks phagosome maturation through inhibition of Ca^{2+}/CaM/CaMKII signaling pathway involved in PI3P production by type III PI3Kinase hVPS34. ManLAM can also block phagosome maturation by engaging mannose receptor and disrupting membrane microdomains, rafts, however, the link with Ca^{2+}/CaM signaling has not been studied. PIMs, ManLAM precursor, impair phagosome maturation by stimulating fusion between phagosome and early endosomes. *Mycobacterium tuberculosis* recruits Rab14 to phagosome to promote early endosome fusion thus impairs phagosome maturation. It remains to be established whether PIMs promotes early endosome fusion through Rab14 recruitment. **Left part:** Mammalian target of rapamycin (mTOR) kinase, activated by Ser/Thr kinase Akt and inhibited by AMP-activated Protein Kinase (AMPK), is a master repressor of autophagy. Beclin-1, an autophagy-related protein in complex with hVPS34, is essential for autophagy. Beclin-1/hVPS34 complex is activated by AMPK and repressed by Bcl-2. ULK1, another important autophagy-related protein, is activated by AMPK and inhibited by mTOR. Ca^{2+} influx has been shown to activate AMPK, hVPS34, and represses Bcl-2 expression. IFNγ induces autophagy. Based on known effects of ManLAM on Ca^{2+} influx, Bcl-2, Akt and IFNγ signaling, we postulate that ManLAM might inhibit autophagy by targeting Beclin-1/hVPS34 complex, Akt/mTOR or IFNγ pathways. The relationship between effects of ManLAM on these different signaling pathways and autophagy awaits investigation. Arrows and characters are represented in gray to indicate that the molecular mechanisms of LAM action on autophagy are hypothetical.

by mitochondrial outer membrane permeabilization (Briken and Miller, 2008). Although *M.tb* genes involved in these inhibitions are just beginning to be unveiled, ManLAM was one of the first mycobacterial product identified as an inhibitor of apoptosis (Rojas et al., 2000; Briken and Miller, 2008).

The mechanisms of apoptosis inhibition by ManLAM seem to be multiple. As for phagosome maturation, ManLAM inhibition of Ca^{2+} signaling appears to be an important step in blocking infection-induced apoptosis (Rojas et al., 2000). Numerous Ca^{2+}-associated events are known to play a role in apoptosis, among them alteration of mitochondrial permeability transition and down-regulation of anti-apoptotic protein Bcl2 have been shown to be repressed by ManLAM (Rojas et al., 2000). Besides Ca^{2+} signaling, ManLAM can prevent intrinsic apoptosis pathway through activation of Ser/Thr kinase Akt and phosphorylation of the pro-apoptotic protein Bad (Maiti et al., 2001). More recently, one report indicates that ManLAM can promote extracellular release of soluble TNF-α receptor. Thus, ManLAM might also interfere with the extrinsic apoptosis pathway by neutralizing TNF-α (Richmond et al., 2012).

AUTOPHAGY

Autophagy is a highly conserved eukaryotic intracellular process that carries out lysosomal degradation of damaged, superfluous or toxic cytoplasmic components (Levine et al., 2011; Rubinsztein et al., 2012). In addition to its housekeeping role, autophagy plays major immunological functions, especially, in host antibacterial defenses (Levine et al., 2011; Deretic et al., 2013). These functions range from effector of pattern recognition receptors and inflammation regulation to antigen presentation and direct elimination of microbial agents. Specifically, autophagy is a key immune effector involved in intracellular clearance of important bacterial pathogens such as *M.tb*.

Autophagy is orchestrated by more than 30 dedicated proteins, called autophagy-related proteins (Atg) (Marino et al., 2014). The autophagic process begins with formation of an

isolation membrane initiated by Ser/Thr kinase Ulk1 (Atg1), which phosphorylates Beclin-1 (Atg6) in complex with hVPS34 to promote its activation (Russell et al., 2013). The isolation membrane is then expanded through action of two ubiquitin-like conjugation systems, the covalent linkage of Atg12 with Atg5 and of LC3 (Atg8) with phosphatidylethanolamine, which lead to engulfment of intracellular components inside a double-membrane bound organelle called autophagosome. LC3, along with entrapped cytosolic content, is then degraded after fusion of autophagosome with lysosomes. In the context of phagocytosis, a non-canonical autophagy pathway, called LC3-associated phagocytosis (LAP), has been described which involves direct LC3 lipidation on the phagosomal membrane (Mehta et al., 2014). This alternative pathway, triggered by some Pattern Recognition Receptors, such as TLR2, appears to be ULK1 independent and important in modulating innate immune response (Mehta et al., 2014). However, the detailed molecular mechanisms and the functional role(s) of LAP still remain to be fully elucidated.

M.tb, like other intracellular intracellular pathogens, has developed mechanisms to manipulate autophagic pathway (Huang and Brumell, 2014). Interestingly, Shui et al. showed that phagosomes containing ManLAM-coated beads display less LC3 than those containing PILAM-coated beads (Shui et al., 2011). Likewise, macrophage treatment with ManLAM for 24 h results in diminution of autophagy as seen by LC3 immunoblotting (personal observation). Autophagy-related proteins play major roles in mediating IFNγ-induced host defenses (Levine et al., 2011; Deretic et al., 2013). Since ManLAM can repress IFNγ responses, it is tempting to speculate that ManLAM might also interfere with autophagy in this context (Sibley et al., 1988; Chan et al., 1991). The action mechanism of ManLAM on autophagy has not been revealed yet, but based on its effect on hVPS34 in phagosome maturation one can postulate that it might inhibit autophagy by modulating hVPS34 in complex with Beclin-1 (**Figure 2**). In addition, Bcl-2 interacts with Beclin-1 to block autophagy, thus ManLAM might impair autophagy via upregulation of Bcl-2 expression (Rojas et al., 2000; Pattingre et al., 2005). ManLAM inhibition of Ca^{2+} influx could also affect the Ca^{2+}/AMP-activated protein kinase (AMPK) signaling pathway involved in mammalian target of rapamycin (mTOR) kinase- and ULK1-dependent autophagy (Vergne et al., 2003; Alers et al., 2012). Alternatively, ManLAM might repress autophagy through activation of type I PI3Kinase and Akt (Maiti et al., 2001; Ravikumar et al., 2010). Further investigations are definitely required to better understand how ManLAM interferes with autophagy, whether it represses canonical autophagy and/or LAP and what is the significance of this inhibition in terms of phagosome trafficking and phagocyte survival.

CONCLUSION

ManLAM, as a purified molecule, reproduces several salient inhibitory properties of *M.tb* in phagocytic cells. However, the role played by ManLAM in the context on an infection by *M.tb* remains unclear. ManLAM immunosuppressive activities rely on the presence of the mannose caps. But, an *M.tb* mutant lacking the mannose caps on LAM was not affected for its virulence in mice nor for its interaction with phagocytic cells *in vitro* (Appelmelk et al., 2008; Afonso-Barroso et al., 2012). In contrast, an aptamer against ManLAM was found to inhibit *M.tb* infection in mice and Rhesus monkeys (Pan et al., 2014). Moreover, protein LprG, which binds ManLAM and determines its cell surface localization, was found to be essential for virulence of *M.tb* and to control phagolysosomal fusion (Gaur et al., 2014; Shukla et al., 2014). These data are not necessarily contradictory. Indeed, the envelope of mycobacteria is exceptionally rich in mannoconjugates bearing (α1→2)-oligomannosides, including LM, PIM_6, arabinomannan or mannoproteins that are able to bind C-type lectins (Pitarque et al., 2005; Torrelles and Schlesinger, 2010). Of note, LprG has been shown to bind LM and PIMs, in addition to ManLAM (Drage et al., 2010), suggesting that the role of LprG might not be attributable to ManLAM inhibitory activities only. Altogether, data converge to indicate that DC-SIGN/MR ligands are most probably redundant at the *M.tb* cell surface, possibly because targeting these receptors is mandatory for the pathogen to manipulate and survive inside the infected host.

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REFERENCES

Afonso-Barroso, A., Clark, S. O., Williams, A., Rosa, G. T., Nobrega, C., Silva-Gomes, S., et al. (2012). Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on *in vitro* inflammatory responses. *Cell. Microbiol.* 15, 660–674. doi: 10.1111/cmi.12065

Alers, S., Loffler, A. S., Wesselborg, S., and Stork, B. (2012). Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol. Cell. Biol.* 32, 2–11. doi: 10.1128/MCB.06159-11

Anes, E., Peyron, P., Staali, L., Jordao, L., Gutierrez, M. G., Kress, H., et al. (2006). Dynamic life and death interactions between *Mycobacterium smegmatis* and J774 macrophages. *Cell. Microbiol.* 8, 939–960. doi: 10.1111/j.1462-5822.2005.00675.x

Angala, S. K., Belardinelli, J. M., Huc-Claustre, E., Wheat, W. H., and Jackson, M. (2014). The cell envelope glycoconjugates of *Mycobacterium tuberculosis*. *Crit. Rev. Biochem. Mol. Biol.* 49, 361–399. doi: 10.3109/10409238.2014.925420

Appelmelk, B. J., den Dunnen, J., Driessen, N. N., Ummels, R., Pak, M., Nigou, J., et al. (2008). The mannose cap of mycobacterial lipoarabinomannan does not dominate the Mycobacterium-host interaction. *Cell. Microbiol.* 10, 930–944. doi: 10.1111/j.1462-5822.2007.01097.x

Armstrong, J. A., and Hart, P. D. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* 134, 713–740. doi: 10.1084/jem.134.3.713

Beatty, W. L., Rhoades, E. R., Ullrich, H. J., Chatterjee, D., Heuser, J. E., and Russell, D. G. (2000). Trafficking and release of mycobacterial lipids from infected macrophages. *Traffic* 1, 235–247. doi: 10.1034/j.1600-0854.2000.010306.x

Blattes, E., Vercellone, A., Eutamene, H., Turrin, C. O., Theodorou, V., Majoral, J. P., et al. (2013). Mannodendrimers prevent acute lung inflammation by inhibiting neutrophil recruitment. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8795–8800. doi: 10.1073/pnas.1221708110

Briken, V., and Miller, J. L. (2008). Living on the edge: inhibition of host cell apoptosis by *Mycobacterium tuberculosis*. *Future Microbiol.* 3, 415–422. doi: 10.2217/17460913.3.4.415

Briken, V., Porcelli, S. A., Besra, G. S., and Kremer, L. (2004). Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol. Microbiol.* 53, 391–403. doi: 10.1111/j.1365-2958.2004.04183.x

Chan, J., Fan, X. D., Hunter, S. W., Brennan, P. J., and Bloom, B. R. (1991). Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect. Immun.* 59, 1755–1761.

Chatterjee, D., and Khoo, K. H. (1998). Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* 8, 113–120. doi: 10.1093/glycob/8.2.113

Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M. R., and Brennan, P. J. (1992). Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. *J. Biol. Chem.* 267, 6234–6239

Chieppa, M., Bianchi, G., Doni, A., Del Prete, A., Sironi, M., Laskarin, G., et al. (2003). Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J. Immunol.* 171, 4552–4560. doi: 10.4049/jimmunol.171.9.4552

de Chastellier, C., Forquet, F., Gordon, A., and Thilo, L. (2009). Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes. *Cell. Microbiol.* 11, 1190–1207. doi: 10.1111/j.1462-5822.2009.01324.x

Deng, L., Mikusova, K., Robuck, K. G., Scherman, M., Brennan, P. J., and McNeil, M. R. (1995). Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. *Antimicrob. Agents Chemother.* 39, 694–701. doi: 10.1128/AAC.39.3.694

Deretic, V., Saitoh, T., and Akira, S. (2013). Autophagy in infection, inflammation and immunity. *Nat. Rev. Immunol.* 13, 722–737. doi: 10.1038/nri3532

Drage, M. G., Tsai, H. C., Pecora, N. D., Cheng, T. Y., Arida, A. R., Shukla, S., et al. (2010). *Mycobacterium tuberculosis* lipoprotein LprG (Rv1411c) binds triacylated glycolipid agonists of toll-like receptor 2. *Nat. struct. Mol. Biol.* 17, 1088–1095. doi: 10.1038/nsmb.1869

Feinberg, H., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2001). Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science* 294, 2163–2166. doi: 10.1126/science.1066371

Flannagan, R. S., Jaumouille, V., and Grinstein, S. (2012). The cell biology of phagocytosis. *Annu. Rev. Pathol.* 7, 61–98. doi: 10.1146/annurev-pathol-011811-132445

Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S., and Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J. Cell Biol.* 154, 631–644. doi: 10.1083/jcb.200106049

Fratti, R. A., Chua, J., Vergne, I., and Deretic, V. (2003). *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5437–5442. doi: 10.1073/pnas.0737613100

Fukuda, T., Matsumura, T., Ato, M., Hamasaki, M., Nishiuchi, Y., Murakami, Y., et al. (2013). Critical roles for lipomannan and lipoarabinomannan in cell wall integrity of mycobacteria and pathogenesis of tuberculosis. *mBio* 4, e00472–e00412. doi: 10.1128/mBio.00472-12

Garton, N. J., Gilleron, M., Brando, T., Dan, H. H., Giguere, S., Puzo, G., et al. (2002). A novel lipoarabinomannan from the equine pathogen *Rhodococcus equi*. Structure and effect on macrophage cytokine production. *J. Biol. Chem.* 277, 31722–31733. doi: 10.1074/jbc.M203008200

Gaur, R. L., Ren, K., Blumenthal, A., Bhamidi, S., Gibbs, S., Jackson, M., et al. (2014). LprG-mediated surface expression of lipoarabinomannan is essential for virulence of *Mycobacterium tuberculosis*. *PLoS Pathog.* 10:e1004376. doi: 10.1371/journal.ppat.1004376

Gehring, A. J., Dobos, K. M., Belisle, J. T., Harding, C. V., and Boom, W. H. (2004). *Mycobacterium tuberculosis* LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J. Immunol.* 173, 2660–2668. doi: 10.4049/jimmunol.173.4.2660

Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B., et al. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197, 7–17. doi: 10.1084/jem.20021229

Gibson, K. J., Gilleron, M., Constant, P., Brando, T., Puzo, G., Besra, G. S., et al. (2004). *Tsukamurella paurometabola* lipoglycan, a new lipoarabinomannan variant with pro-inflammatory activity. *J. Biol. Chem.* 279, 22973–22982. doi: 10.1074/jbc.M310906200

Gibson, K. J., Gilleron, M., Constant, P., Puzo, G., Nigou, J., and Besra, G. S. (2003a). Identification of a novel mannose-capped lipoarabinomannan from *Amycolatopsis sulphurea*. *Biochem. J.* 372, 821–829. doi: 10.1042/BJ20030197

Gibson, K. J., Gilleron, M., Constant, P., Puzo, G., Nigou, J., and Besra, G. S. (2003b). Structural and functional features of *Rhodococcus ruber* lipoarabinomannan. *Microbiology* 149, 1437–1445. doi: 10.1099/mic.0.26161-0

Gibson, K. J., Gilleron, M., Constant, P., Sichi, B., Puzo, G., Besra, G. S., et al. (2005). A lipomannan variant with strong TLR-2-dependent pro-inflammatory activity in *Saccharothrix aerocolonigenes*. *J. Biol. Chem.* 280, 28347–28356. doi: 10.1074/jbc.M505498200

Gilleron, M., Bala, L., Brando, T., Vercellone, A., and Puzo, G. (2000). *Mycobacterium tuberculosis* H37Rv parietal and cellular lipoarabinomannans. Characterization of the acyl- and glyco-forms. *J. Biol. Chem.* 275, 677–684. doi: 10.1074/jbc.275.1.677

Gilleron, M., Garton, N. J., Nigou, J., Brando, T., Puzo, G., and Sutcliffe, I. C. (2005). Characterization of a truncated lipoarabinomannan from the Actinomycete *Turicella otitidis*. *J. Bacteriol.* 187, 854–861. doi: 10.1128/JB.187.3.854-861.2005

Gilleron, M., Jackson, M., Nigou, J., and Puzo, G. (2008). "Structure, biosynthesis, and activities of the phosphatidyl-myo-inositol-based lipoglycans," in *The Mycobacterial Cell Envelope*, eds M. Daffé, J. M. Reyrat (Washington, DC: ASM Press), 75–105.

Gilleron, M., Nigou, J., Cahuzac, B., and Puzo, G. (1999). Structural study of the lipomannans from *Mycobacterium bovis* BCG: characterisation of multiacylated forms of the phosphatidyl-myo-inositol anchor. *J. Mol. Biol.* 285, 2147–2160. doi: 10.1006/jmbi.1998.2438

Gilleron, M., Nigou, J., Nicolle, D., Quesniaux, V., and Puzo, G. (2006). The acylation state of mycobacterial lipomannans modulates innate immunity response through toll-like receptor 2. *Chem. Biol.* 13, 39–47. doi: 10.1016/j.chembiol.2005.10.013

Gilleron, M., Quesniaux, V. F., and Puzo, G. (2003). Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guerin *and Mycobacterium tuberculosis* H37Rv and its implication in Toll-like receptor response. *J. Biol. Chem.* 278, 29880–29889. doi: 10.1074/jbc.M303446200

Gringhuis, S. I., den Dunnen, J., Litjens, M., van der Vlist, M., and Geijtenbeek, T. B. (2009). Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and Helicobacter pylori. *Nat. Immunol.* 10, 1081–1088. doi: 10.1038/ni.1778

Gringhuis, S. I., den Dunnen, J., Litjens, M., van Het Hof, B., van Kooyk, Y., and Geijtenbeek, T. B. (2007). C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity* 26, 605–616. doi: 10.1016/j.immuni.2007.03.012

Harding, C. V., and Boom, W. H. (2010). Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nat. Rev. Microbiol.* 8, 296–307. doi: 10.1038/nrmicro2321

Hayakawa, E., Tokumasu, F., Nardone, G. A., Jin, A. J., Hackley, V. A., and Dvorak, J. A. (2007). A *Mycobacterium tuberculosis*-derived lipid inhibits membrane fusion by modulating lipid membrane domains. *Biophys. J.* 93, 4018–4030. doi: 10.1529/biophysj.107.104075

Hmama, Z., Sendide, K., Talal, A., Garcia, R., Dobos, K., and Reiner, N. E. (2004). Quantitative analysis of phagolysosome fusion in intact cells: inhibition by mycobacterial lipoarabinomannan and rescue by an 1alpha,25-dihydroxyvitamin D3-phosphoinositide 3-kinase pathway. *J. Cell Sci.* 117, 2131–2140. doi: 10.1242/jcs.01072

Huang, J., and Brumell, J. H. (2014). Bacteria-autophagy interplay: a battle for survival. *Nat. Rev. Microbiol.* 12, 101–114. doi: 10.1038/nrmicro3160

Kang, P. B., Azad, A. K., Torrelles, J. B., Kaufman, T. M., Beharka, A., Tibesar, E., et al. (2005). The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. *J. Exp. Med.* 202, 987–999. doi: 10.1084/jem.20051239

Kaur, D., Angala, S. K., Wu, S. W., Khoo, K. H., Chatterjee, D., Brennan, P. J., et al. (2014). A single Arabinan chain is attached to the phosphatidylinositol mannosyl core of the major immunomodulatory mycobacterial cell envelope

Glycoconjugate, Lipoarabinomannan. *J. Biol. Chem.* 289, 30249–30256. doi: 10.1074/jbc.M114.599415

Khoo, K. H., Dell, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995). Structural definition of acylated phosphatidylinositol mannosides from *Mycobacterium tuberculosis*: definition of a common anchor for lipomannan and lipoarabinomannan. *Glycobiology* 5, 117–127. doi: 10.1093/glycob/5.1.117

Knutson, K. L., Hmama, Z., Herrera-Velit, P., Rochford, R., and Reiner, N. E. (1998). Lipoarabinomannan of *Mycobacterium tuberculosis* promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1. *J. Biol. Chem.* 273, 645–652. doi: 10.1074/jbc.273.1.645

Kyei, G. B., Vergne, I., Chua, J., Roberts, E., Harris, J., Junutula, J. R., et al. (2006). Rab14 is critical for maintenance of *Mycobacterium tuberculosis* phagosome maturation arrest. *EMBO J.* 25, 5250–5259. doi: 10.1038/sj.emboj.7601407

Levine, B., Mizushima, N., and Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature* 469, 323–335. doi: 10.1038/nature09782

Maeda, N., Nigou, J., Herrmann, J. L., Jackson, M., Amara, A., Lagrange, P. H., et al. (2003). The cell surface receptor DC-SIGN discriminates between Mycobacterium species through selective recognition of the mannose caps on lipoarabinomannan. *J. Biol. Chem.* 278, 5513–5516. doi: 10.1074/jbc.C200586200

Maiti, D., Bhattacharyya, A., and Basu, J. (2001). Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating bad through a phosphatidylinositol 3-kinase/Akt pathway. *J. Biol. Chem.* 276, 329–333. doi: 10.1074/jbc.M002650200

Makarov, V., Manina, G., Mikusova, K., Mollmann, U., Ryabova, O., Saint-Joanis, B., et al. (2009). Benzothiazinones kill *Mycobacterium tuberculosis* by blocking arabinan synthesis. *Science* 324, 801–804. doi: 10.1126/science.1171583

Malik, Z. A., Denning, G. M., and Kusner, D. J. (2000). Inhibition of Ca(2+) signaling by *Mycobacterium tuberculosis* is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. *J. Exp. Med.* 191, 287–302. doi: 10.1084/jem.191.2.287

Malik, Z. A., Iyer, S. S., and Kusner, D. J. (2001). *Mycobacterium tuberculosis* phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. *J. Immunol.* 166, 3392–3401. doi: 10.4049/jimmunol.166.5.3392

Marino, G., Niso-Santano, M., Baehrecke, E. H., and Kroemer, G. (2014). Self-consumption: the interplay of autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 15, 81–94. doi: 10.1038/nrm3735

Mehta, P., Henault, J., Kolbeck, R., and Sanjuan, M. A. (2014). Noncanonical autophagy: one small step for LC3, one giant leap for immunity. *Curr. Opin. Immunol.* 26, 69–75. doi: 10.1016/j.coi.2013.10.012

Mishra, A. K., Driessen, N. N., Appelmelk, B. J., and Besra, G. S. (2011). Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. *FEMS Microbiol. Rev.* 35, 1126–1157. doi: 10.1111/j.1574-6976.2011.00276.x

Mitchell, D. A., Fadden, A. J., and Drickamer, K. (2001). A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. *J. Biol. Chem.* 276, 28939–28945. doi: 10.1074/jbc.M104565200

Nigou, J., Gilleron, M., and Puzo, G. (1999). Lipoarabinomannans: characterization of the multiacylated forms of the phosphatidyl-myo-inositol anchor by NMR spectroscopy. *Biochem. J.* 337(pt 3), 453–460. doi: 10.1042/0264-6021:3370453

Nigou, J., Gilleron, M., and Puzo, G. (2003). Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 85, 153–166. doi: 10.1016/S0300-9084(03)00048-8

Nigou, J., Gilleron, M., Rojas, M., Garcia, L. F., Thurnher, M., and Puzo, G. (2002). Mycobacterial lipoarabinomannans: modulators of dendritic cell function and the apoptotic response. *Microbes Infect.* 4, 945–953. doi: 10.1016/S1286-4579(02)01621-0

Nigou, J., Vasselon, T., Ray, A., Constant, P., Gilleron, M., Besra, G. S., et al. (2008). Mannan chain length controls lipoglycans signaling via and binding to TLR2. *J. Immunol.* 180, 6696–6702. doi: 10.4049/jimmunol.180.10.6696

Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., and Puzo, G. (2001). Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J. Immunol.* 166, 7477–7485. doi: 10.4049/jimmunol.166.12.7477

Pan, Q., Wang, Q., Sun, X., Xia, X., Wu, S., Luo, F., et al. (2014). Aptamer against mannose-capped lipoarabinomannan inhibits virulent *Mycobacterium tuberculosis* infection in mice and rhesus monkeys. *Mol. Ther.* 22, 940–951. doi: 10.1038/mt.2014.31

Pathak, S. K., Basu, S., Bhattacharyya, A., Pathak, S., Kundu, M., and Basu, J. (2005). *Mycobacterium tuberculosis* lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *J. Biol. Chem.* 280, 42794–42800. doi: 10.1074/jbc.M506471200

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., et al. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939. doi: 10.1016/j.cell.2005.07.002

Pitarque, S., Herrmann, J. L., Duteyrat, J. L., Jackson, M., Stewart, G. R., Lecointe, F., et al. (2005). Deciphering the molecular bases of *Mycobacterium tuberculosis* binding to the lectin DC-SIGN reveals an underestimated complexity. *Biochem. J.* 392, 615–624. doi: 10.1042/BJ20050709

Pitarque, S., Larrouy-Maumus, G., Payre, B., Jackson, M., Puzo, G., and Nigou, J. (2008). The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. *Tuberculosis (Edinb.)* 88, 560–565. doi: 10.1016/j.tube.2008.04.002

Quesniaux, V. J., Nicolle, D. M., Torres, D., Kremer, L., Guerardel, Y., Nigou, J., et al. (2004). Toll-like receptor 2 (TLR2)-dependent-positive and TLR2-independent-negative regulation of proinflammatory cytokines by mycobacterial lipomannans. *J. Immunol.* 172, 4425–4434. doi: 10.4049/jimmunol.172.7.4425

Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z. W., et al. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol. Rev.* 90, 1383–1435. doi: 10.1152/physrev.00030.2009

Ray, A., Cot, M., Puzo, G., Gilleron, M., and Nigou, J. (2013). Bacterial cell wall macroamphiphiles: pathogen-/microbe-associated molecular patterns detected by mammalian innate immune system. *Biochimie* 95, 33–42. doi: 10.1016/j.biochi.2012.06.007

Richmond, J. M., Duffy, E. R., Lee, J., Kaboli, K., Kim, Y. S., Remick, D. G., et al. (2012). Mannose-capped Lipoarabinomannan from *Mycobacterium tuberculosis* induces soluble tumor necrosis factor receptor production through tumor necrosis factor alpha-converting enzyme activation. *Infect. Immun.* 80, 3858–3868. doi: 10.1128/IAI.00060-12

Riviere, M., Moisand, A., Lopez, A., and Puzo, G. (2004). Highly ordered supramolecular organization of the mycobacterial lipoarabinomannans in solution. Evidence of a relationship between supra-molecular organization and biological activity. *J. Mol. Biol.* 344, 907–918. doi: 10.1016/j.jmb.2004.09.092

Rojas, M., Garcia, L. F., Nigou, J., Puzo, G., and Olivier, M. (2000). Mannosylated lipoarabinomannan antagonizes *Mycobacterium tuberculosis*-induced macrophage apoptosis by altering Ca+2-dependent cell signaling. *J. Infect. Dis.* 182, 240–251. doi: 10.1086/315676

Rubinsztein, D. C., Codogno, P., and Levine, B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat. Rev. Drug Discov.* 11, 709–730. doi: 10.1038/nrd3802

Russell, D. G. (2011). *Mycobacterium tuberculosis* and the intimate discourse of a chronic infection. *Immunol. Rev.* 240, 252–268. doi: 10.1111/j.1600-065X.2010.00984.x

Russell, R. C., Tian, Y., Yuan, H., Park, H. W., Chang, Y. Y., Kim, J., et al. (2013). ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat. Cell Biol.* 15, 741–750. doi: 10.1038/ncb2757

Schaible, U. E., Winau, F., Sieling, P. A., Fischer, K., Collins, H. L., Hagens, K., et al. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat. Med.* 9, 1039–1046. doi: 10.1038/nm906

Schlesinger, L. S., Hull, S. R., and Kaufman, T. M. (1994). Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *J. Immunol.* 152, 4070–4079

Shui, W., Petzold, C. J., Redding, A., Liu, J., Pitcher, A., Sheu, L., et al. (2011). Organelle membrane proteomics reveals differential influence of mycobacterial lipoglycans on macrophage phagosome maturation and autophagosome accumulation. *J. Proteome Res.* 10, 339–348. doi: 10.1021/pr100688h

Shukla, S., Richardson, E. T., Athman, J. J., Shi, L., Wearsch, P. A., McDonald, D., et al. (2014). *Mycobacterium tuberculosis* lipoprotein LprG binds lipoarabinomannan and determines its cell envelope localization to control phagolysosomal fusion. *PLoS Pathog.* 10:e1004471. doi: 10.1371/journal.ppat.1004471

Sibley, L. D., Hunter, S. W., Brennan, P. J., and Krahenbuhl, J. L. (1988). Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. *Infect. Immun.* 56, 1232–1236

Tailleux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., et al. (2003). DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* 197, 121–127. doi: 10.1084/jem.20021468

Tatituri, R. V., Illarionov, P. A., Dover, L. G., Nigou, J., Gilleron, M., Hitchen, P., et al. (2007). Inactivation of *Corynebacterium glutamicum* NCgl0452 and the role of MgtA in the biosynthesis of a novel mannosylated glycolipid involved in lipomannan biosynthesis. *J. Biol. Chem.* 282, 4561–4572. doi: 10.1074/jbc.M608695200

Torrelles, J. B., and Schlesinger, L. S. (2010). Diversity in *Mycobacterium tuberculosis* mannosylated cell wall determinants impacts adaptation to the host. *Tuberculosis (Edinb.)* 90, 84–93. doi: 10.1016/j.tube.2010.02.003

Vergne, I., Chua, J., and Deretic, V. (2003). Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. *J. Exp. Med.* 198, 653–659. doi: 10.1084/jem.20030527

Vergne, I., Fratti, R. A., Hill, P. J., Chua, J., Belisle, J., and Deretic, V. (2004). *Mycobacterium tuberculosis* phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Mol. Biol. Cell* 15, 751–760. doi: 10.1091/mbc.E03-05-0307

Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A., and Deretic, V. (1997). Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J. Biol. Chem.* 272, 13326–13331. doi: 10.1074/jbc.272.20.13326

Vignal, C., Guerardel, Y., Kremer, L., Masson, M., Legrand, D., Mazurier, J., et al. (2003). Lipomannans, but not lipoarabinomannans, purified from *Mycobacterium chelonae* and *Mycobacterium kansasii* induce TNF-alpha and IL-8 secretion by a CD14-toll-like receptor 2-dependent mechanism. *J. Immunol.* 171, 2014–2023. doi: 10.4049/jimmunol.171.4.2014

Welin, A., Winberg, M. E., Abdalla, H., Sarndahl, E., Rasmusson, B., Stendahl, O., et al. (2008). Incorporation of *Mycobacterium tuberculosis* lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infect. Immun.* 76, 2882–2887. doi: 10.1128/IAI.01549-07

Yonekawa, A., Saijo, S., Hoshino, Y., Miyake, Y., Ishikawa, E., Suzukawa, M., et al. (2014). Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity* 41, 402–413. doi: 10.1016/j.immuni.2014.08.005

Zhang, J., Tachado, S. D., Patel, N., Zhu, J., Imrich, A., Manfruelli, P., et al. (2005). Negative regulatory role of mannose receptors on human alveolar macrophage proinflammatory cytokine release *in vitro*. *J. Leukoc. Biol.* 78, 665–674. doi: 10.1189/jlb.1204699

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Coxiella burnetii lipopolysaccharide blocks p38α-MAPK activation through the disruption of TLR-2 and TLR-4 association

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To survive in macrophages, *Coxiella burnetii* hijacks the activation pathway of macrophages. Recently, we have demonstrated that *C. burnetii*, via its lipopolysaccharide (LPS), avoids the activation of p38α-MAPK through an antagonistic engagement of Toll-like receptor (TLR)-4. We investigated the fine-tuned mechanism leading to the absence of activation of the p38α-MAPK despite TLR-4 engagement. In macrophages challenged with LPS from the avirulent variants of *C. burnetii*, TLR-4 and TLR-2 co-immunoprecipitated. This association was absent in cells challenged by the LPS of pathogenic *C. burnetii*. The disruption makes TLRs unable to signal during the recognition of the LPS of pathogenic *C. burnetii*. The disruption of TLR-2 and TLR-4 was induced by the re-organization of the macrophage cytoskeleton by *C. burnetii* LPS. Interestingly, blocking the actin cytoskeleton re-organization relieved the disruption of the association TLR-2/TLR-4 by pathogenic *C. burnetii* and rescued the p38α-MAPK activation by *C. burnetii*. We elucidated an unexpected mechanism allowing pathogenic *C. burnetii* to avoid macrophage activation by the disruption of the TLR-2 and TLR-4 association.

Keywords: TLR-2, TLR-4, cytoskeleton, *Coxiella burnetii*, macrophages

INTRODUCTION

Coxiella burnetii is an intracellular bacteria responsible of the Q fever zoonosis and is a potential bio warfare and bioterrorism agent (Regis, 1999; Madariaga et al., 2003). Q fever is characterized by a lethal endocarditis (Raoult et al., 2005). It has been shown that molecular variations in *C. burnetii* lipopolysaccharide (LPS) between LPS from virulent and avirulent *C. burnetii* (vLPS and avLPS, respectively) determine the pathogenic properties of *C. burnetii* (Lukacova et al., 2008; Toman et al., 2009; Toman and Vadovič, 2011; Barry et al., 2012).

To survive in macrophages, *C. burnetii* inhibits phagolysosome biogenesis (Ghigo et al., 2002; Barry et al., 2012) and induces cytoskeleton rearrangement of macrophages (Meconi et al., 1998; Honstettre et al., 2004). It has been demonstrated that LPS is the principal actor of the survival mechanism of *C. burnetii* (Meconi et al., 1998; Honstettre et al., 2004; Barry et al., 2012). vLPS stimulates morphologic changes characterized by an intense and transient membrane rearrangement of F-actin leading to protrusions and polarized projections, whereas avLPS does not induce any modification of the cell cytoskeleton morphology (Meconi et al., 1998; Honstettre et al., 2004). In addition, *C. burnetii* targeting to degradative compartments also involves an antagonistic engagement of Toll-like receptor (TLR)-4 by vLPS, lack of p38α-MAPK-driven phosphorylation, and block in recruitment of the HOPS (homotypic fusion and protein-sorting complex) component Vps41 to vLPS-containing vesicles (Barry et al., 2012).

In response to LPS stimulation, TLR-signaling initiates distinct innate immune defensive programs, such as the maturation of phagosomes (Blander and Medzhitov, 2004, 2006). This process involves crosstalk between mitogen-activated protein kinase (MAPK) signaling and components of the vesicular trafficking machinery (Blander and Medzhitov, 2006; Symons et al., 2006; Fontana and Vance, 2011). TLR-4 is involved in the recognition of Gram-negative bacteria such as *E. coli* through recognition of prototypic LPS. TLR-2 interacts with Gram-positive bacteria following interaction with lipoproteins, proteoglycans or lipopeptides. However, several studies have highlighted that LPS recognition is not restricted to TLR-4. Indeed, TLR-2 is able to recognize the LPS from *Porphyromonas gingivalis* (Medzhitov, 2001; Underhill, 2004). Recent studies have highlighted that TLR-2 is required along with TLR-4 for the response to bacterial LPS (Good et al., 2012); this response involves a physical interaction between TLR-2 and TLR-4 (Lee et al., 2004; Good et al., 2012). Much remains to be learned regarding the molecular basis underlying the crosstalk between the LPS variants and TLRs.

In this study, we investigated the mechanism leading to the absence of activation of the p38α-MAPK despite TLR-4 engagement by *C. burnetii* vLPS. We found that the association between

TLR-2 and TLR-4 is required to activate p38α-MAPK and was disrupted by the vLPS. The disruption of TLR-2 and TLR-4 association by vLPS was induced by the re-organization of the macrophage cytoskeleton. Interestingly, the block of the actin cytoskeleton re-organization inhibited the disruption of association TLR-2/TLR-4 by pathogenic *C. burnetii* and allowed the p38α-MAPK activation by *C. burnetii* LPS. We elucidated an unexpected mechanism allowing pathogenic *C. burnetii* to avoid activating macrophages.

RESULTS

vLPS DISRUPTS TLR-4 AND TLR-2 ASSOCIATION AT THE BMDMs MEMBRANE SURFACE

In wild-type BMDMs, vLPS was unable to induce the activation of p38α MAPK (<0 RFUs), in contrast to the avLPS (13.5 RFUs at 30 min) (**Figure 1**) as previously described (Barry et al., 2012). These data confirm the previous finding that the recognition of vLPS by TLRs is required to block p38α MAPK activation (Barry et al., 2012). We decided to deepen our analysis by investigating the distribution of TLR-4 and TLR-2 at the membrane surface of BMDMs challenged with *C. burnetii* LPSs (**Figure 2**). In control BMDMs, we observed a large number of TLR-2 and TLR-4 fluorescent small dots (129.2 ± 12.3 and 109.5 ± 18 a.u, respectively) with a dispersed distribution at the macrophage surface (**Figures 2A–C**) with a reduced area (5 ± 2 and 9 ± 5 a.u, respectively) (**Figures 2A,B,D**). In macrophages treated with the avLPS, we found a significant decrease in TLR-2 (4.5-fold) and TLR-4 (2.8-fold) dots compared to the control (**Figures 2A–C**) at the macrophage surface. The decreased dot number is associated with an increase in dot area (**Figures 2A,B,D**). Indeed the TLR-2 and TLR-4 dot area increased significantly 7.6-fold and 4.4 fold, respectively, compared to the control (**Figures 2A,B,D**). vLPS induced a significant a decrease of 3.6-fold of the small dots numbers present at cell membrane (**Figures 2A–C**) associated with a significant reorganization of TLR-2 small dots in large patch compared to control (23 ± 3 a.u vs. 5 ± 2) (**Figures 2A,B,D**). Interestingly, vLPS does not affect the distribution or the size of TLR-4 fluorescent dots at the BMDMs membrane surface. Then, we assessed the co-localization of TLR-4 with TLR-2 in BMDMs treated with *C. burnetii* LPSs (**Figures 2E**). In macrophages challenged with avLPS we find a strong co-localization of TLR-4 with TLR-2 (Pearson's coefficient 0.72 ± 0.12) whereas in presence of vLPS we found that TLR2 did not co-localize with TLR-4 (Pearson's coefficient 0.1 ± 0.02). Next, we investigated, by co-immunoprecipitation, the association of TLR-2 with TLR-4 in BMDMs either challenged or not challenged with vLPS or avLPS (**Figure 2F**). We observed that TLR-2 and TLR-4 co-immunopreciptated in the BMDMs control as well in BMDMs challenged with avLPS. In contrast, TLR-2 and TLR-4 did not co-immunoprecipitate in macrophages treated with vLPS. Taken together this data suggests that vLPS disrupts the membrane distribution of TLR-2 and TLR-4.

CYTOSKELETON RE-ORGANIZATION INDUCED BY vLPS DISRUPTS P38α MAPK ACTIVATION THROUGH TLRs

vLPS is known to induce cytoskeleton reorganization (Meconi et al., 1998; Honstettre et al., 2004). We postulated that this reorganization could influence the TLR-2 and TLR-4 distribution observed in BMDMs challenged with vLPS. We have evaluated the capacity of *C. burnetii* LPS to induce cytoskeleton reorganization. We observed, as previously described, that vLPS induced a dramatic reorganization of the BMDMs cytoskeleton (**Figure 3A**), whereas avLPS did not (**Figure 3B**) (Meconi et al., 1998; Honstettre et al., 2004). vLPS induced macrophage spreading and the formation of polarized filopodia and lamellipodia. F-actin was concentrated beneath filopodia and lamellipodia and as spots in cytoplasmic areas (**Figure 3A**). In contrast, avLPS had a slight effect on F-actin organization (**Figure 3B**). After 10 min of stimulation with vLPS, 81 ± 4% of macrophages exhibited filopodia, and the percentage of macrophages with filopodia decreased thereafter (**Figure 3C**). Only 35% of cells treated with avLPS exhibited filopodia (**Figure 3C**). Next, we have investigated if the block of the cytoskeleton re-organization could rescue the association between TLR-4 and TLR-2. We inhibited cytoskeleton re-organization using cytochalasin-D (**Figure 3C**). We revealed that in presence of cytochalasin-D, TLR-2 and TLR-4 co-immunoprecipitate in contrast to the experimental condition without the inhibitor (**Figure 3D**). Finally, we found that the inhibition of the cytoskeleton reorganization by cytochalasin-D recovers activation of p38α MAPK via vLPS (14.1 ± 0.56 RFUs after 90 min), whereas in the absence of cytochalasin-D, p38α MAPK is not activated (**Figure 3E**).

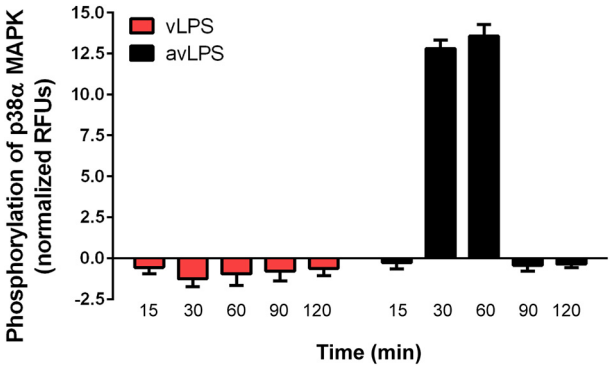

FIGURE 1 | Activation of p38α MAPKs. BMDMs from wild type mice, were challenged with *C. burnetii* vLPS and avLPS (1 μg/ml) for different periods (up to 120 min). The phosphorylation of p38α MAPK was determined using phospho-p38α MAPK cell-based ELISA. The results are expressed as normalized RFU and represent the mean ± SD ($n = 3$).

DISCUSSION

C. burnetii, the bacteria that causes Q fever, has evolved several strategies to survive in macrophages. One of these strategies is to avoid being targeted to the degradative compartments of immune cells. To do that *C. burnetii*, through its vLPS, blurs its own recognition by TLR receptors in order to interfere with the transduction of the signal (Barry et al., 2012). The consequences of such strategies are a deficiency of p38α-MAPK-driven phosphorylation and a block in recruitment of the homotypic fusion. In addition, several years ago it was shown that *C. burnetii* interferes with the cytoskeleton, and this inference is crucial

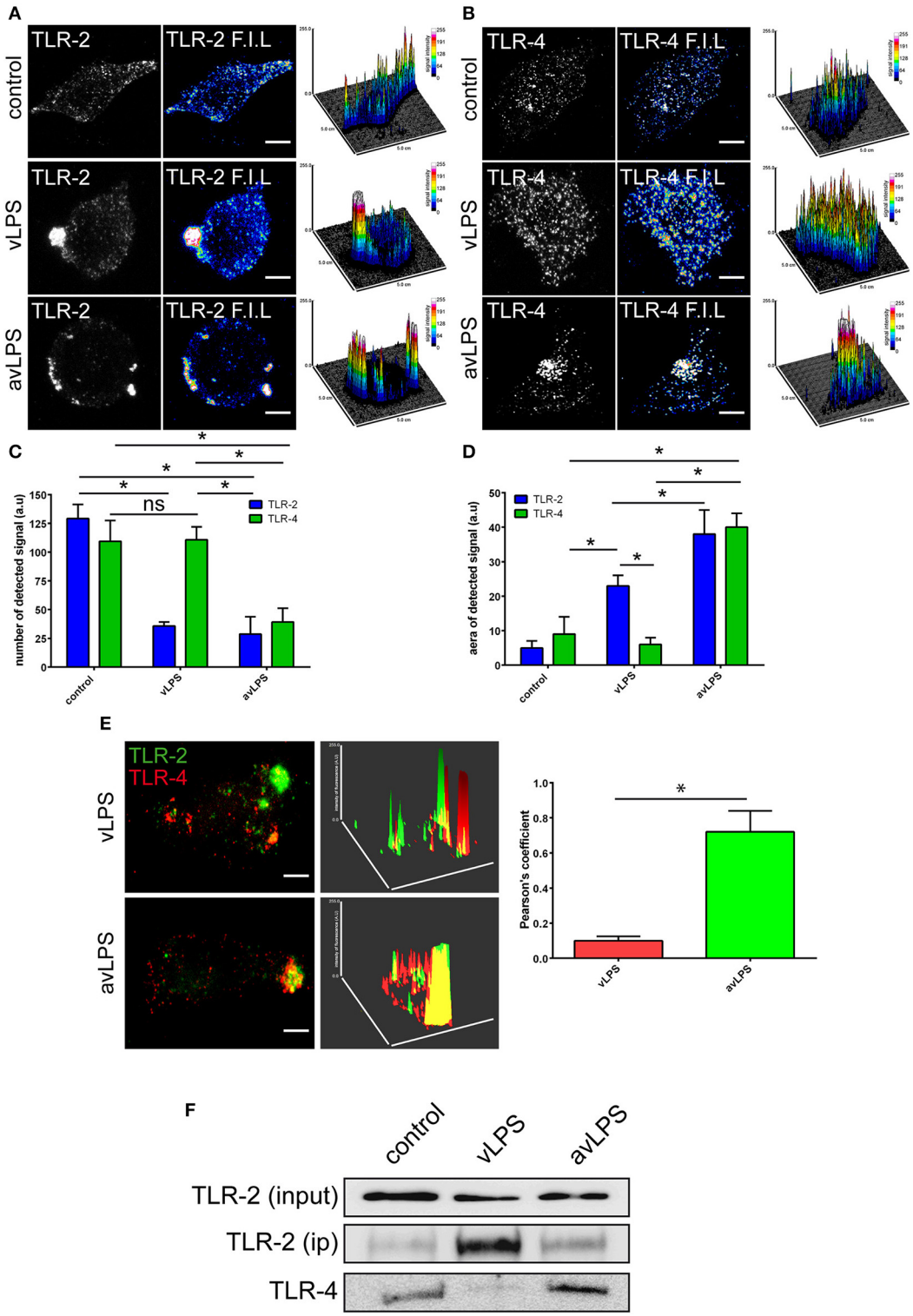

FIGURE 2 | TLR-2 and TLR-4 distribution and colocalization. BMDMs from wild type mice were challenged for 5 min with *C. burnetii* LPS (1 μg/ml). The distribution of **(A)** TLR-2 and **(B)** TLR-4 at the BMDMs surface was determined by confocal microscopy. The scale bar indicates 5 μm. The number of TLRs signal detected **(C)** and the area **(D)** were quantified using ImageJ software. The results are expressed as the mean ± SD ($n = 3$, $^*p < 0.05$). **(E)** The colocalization of TLR-2 with TLR-4 was determined using confocal microscopy. The colocalization of TLR-2 with TLR-4 was quantified using ImageJ software. The results are expressed as the mean ± SD ($n = 3$, $^*p < 0.05$). The scale bar indicates 5 μm. **(F)** BMDMs in non-starved conditions were either left untreated or treated with vLPS or avLPS (1 μg/ml) for 5 min, then TLR-2 was immunoprecipitated and coimmunoprecipitated with TLR-4 was visualized by immunoblotting. The blot shown is representative of three experiments.

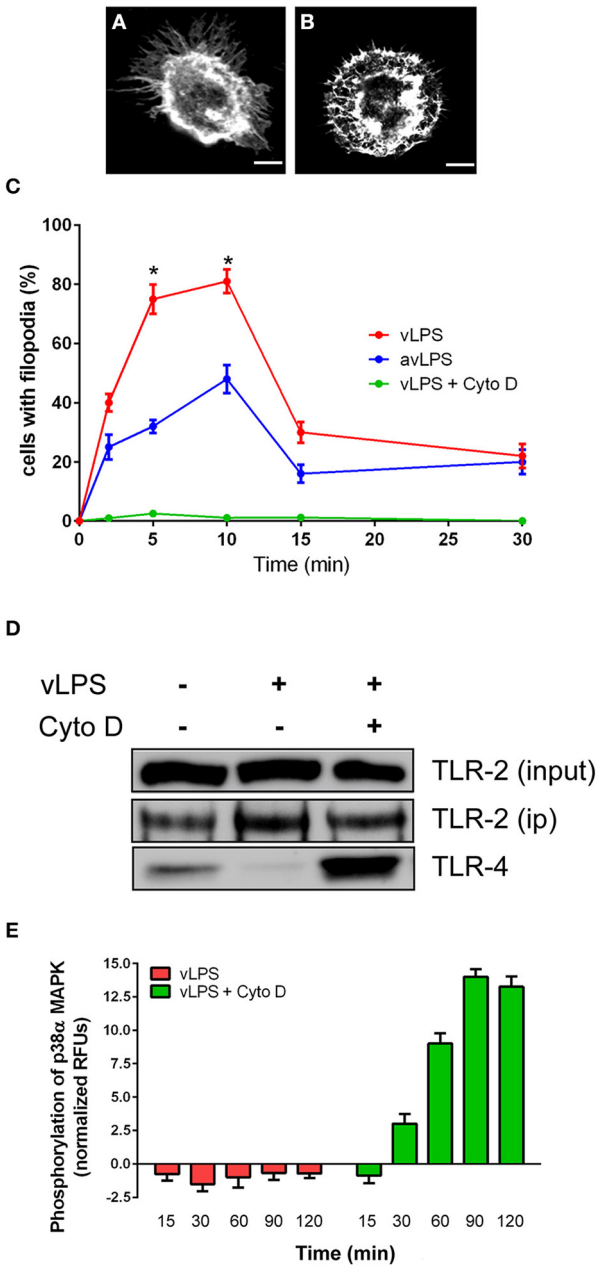

FIGURE 3 | Cytoskeleton remodeling induced by *C. burnetii* LPS impairs TLRs signaling. BMDMs were challenged with **(A)** vLPSs or **(B)** avLPS at 1 μg/ml for 5 min, then F-actin was labeled with phalloidin alexa-488. Macrophages were examined by confocal microscopy. Representative cells are shown, the scale bar indicates 5 μm. **(C)** The percentage of BMDMs showing filopodia was evaluated. For some experiments macrophages were treated with cytochalasin-D. The results are expressed as the mean ± SD ($n = 3$ *$p < 0.05$). **(D)** BMDMs in non-starved conditions were either left untreated or treated with vLPS (1 μg/ml) for 5 min in presence or not of cytochalasin-D, then TLR-2 was immunoprecipitated and coimmunoprecipitated with TLR-4 was visualized by immunoblotting. The blot shown is representative of three experiments. **(E)** BMDMs from wild type mice were challenged with vLPS (1 μg/ml) for different periods (min) in presence or not of cytochalasin-D, and the phosphorylation of p38α MAPK was determined using phospho-p38α MAPK cell-based ELISA. The results are expressed as normalized RFU and represent the mean ± SD ($n = 3$).

for its survival in macrophages (Meconi et al., 1998; Honstettre et al., 2004). We have investigated if the dramatic cytoskeleton re-organization induced by *C. burnetii* could explain the decrease of p38a MAPK signaling. In macrophages challenged with *C. burnetii* avLPS we observed a phosphorylation of the p38α MAPK. In contrast, p38α MAPK is not activated in macrophages challenged with vLPS has previously described (Barry et al., 2012). Because it is known that TLR-2 is required along with TLR-4 for the response to bacterial LPS (Good et al., 2012), through a physical interaction between TLR-2 and TLR-4 (Lee et al., 2004; Good et al., 2012) we have analyzed the distribution of TLR-2 and TLR-4 at the surface of macrophages via confocal microscopy. We have observed that C. *burnetii* vLPS induces a strong reorganization of the TLR-2 and TLR4 at the membrane. This redistribution hampers the colocalization between TLR-2 and TLR-4, in contrast to what is observed in macrophages challenged by avLPS. In addition, the co-immunopreciptation experiments highlight a physical link between TLRs in cells challenged with avLPS, whereas in cells treated with vLPS this is not found. Finally, we postulated that the TLR distribution was linked to the cytoskeleton re-organization induced by *C. burnetii* vLPS. Interestingly, the inhibition of the cytoskeleton reorganization by cytochalasin D allowed for the activation of p38α MAPK by vLPS. We observed also that in this condition TLR-4 co-immunoprecipitated with TLR-2. A possible mechanism to explain the role of the vLPS in the default of activation of p38α MAPK is that vLPS through the induction of the cytoskeleton remodeling, induces a relative dispersion and redistribution of TLR-2, TLR-4 receptors at outer membrane level, in such a way that TLR-2 and TLR-4 are not able to signal via p38a MAPK. Moreover, it has been already reported that a crosstalk between TLRs signaling and G-protein coupled receptors, such as Rho-GTPase and Rnd proteins, exists and could lead to a dramatic cytoskeleton rearrangement (Ruse and Knaus, 2006). Previously, we have demonstrated that *C. burnetii* vLPS interferes with phagosome maturation by inhibiting the activation of p38α MAPK (Barry et al., 2012), here we deepened the previous study by demonstrating that *C. burnetii*, through it vLPS blurs the TLR-2 and TLR-4 signaling through dramatic cytoskeleton reorganization and redistribution of TLR-2 and TLR-4 at the macrophage cells surface.

MATERIALS AND METHODS

ETHICS STATEMENT

All animal experiments were conducted according to the Guiding Principles of Animal Care and Use defined by the Ethics Committee for Animal Experimentation (N°14 designated by the National Study Committee on the Ethics of Animal Experimentation) according to the rules of Decree N°87-848 as of October 19, 1987. All of the animal experiments conducted in this study were also approved by the Ethics Committee for Animal Experimentation (N°14 from the National Study Committee on the Ethics of Animal Experimentation) where the experiments were performed (Faculty of Medicine, Marseille, experimentation permit number to Eric Ghigo 10-300122013).

ANTIBODIES AND FLUORESCENT COMPOUNDS

Antibodies specific for TLR-2 and TLR-4 were purchased from the BD Bioscience. Cytochalasin D was purchased from

Sigma-Aldrich. Secondary antibodies and phalloidin alexa-448 were purchased from Invitrogen

LPS PREPARATIONS

LPS from virulent (vLPS) and avirulent (avLPS) *C. burnetii* (Barry et al., 2012) were isolated from *C. burnetii* RSA 493 (clone 7) and RSA 439 (clone 4), as previously described (Skultety et al., 1996; Toman and Skultety, 1996). The quality of the LPS preparation was confirmed using silver staining and compositional GC-MS (Toman et al., 2009).

CELL CULTURE

Bone marrow-derived macrophages (BMDMs) were generated from 6- to 8-week-old C57BL/6 mice, as previously described (Ren et al., 2005; Cook et al., 2007; Trouplin et al., 2013). BMDM were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. For some experiments macrophages were challenged with 1 μg/ml of *C. burnetii* LPS.

CONFOCAL MICROSCOPY

Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS pH 7.4) and prepared for immunofluorescence labeling, as previously described (Forestier et al., 1999; Chu and Ng, 2004; Ghigo et al., 2010). Coverslips were mounted in Mowiol, and the cells were imaged using an inverted Leica TCS SPE confocal laser-scanning microscope (Leica, Heidelberg, Germany). Image acquisition was performed using the Leica Confocal software. The collected images were processed using Adobe Photoshop CS5 software. The cells were evaluated as follows: twenty-five fields containing at least three cells per field were examined for each experimental condition; in total, approximately 100 cells were examined per experimental condition, as described elsewhere (Barry et al., 2012). TLR distribution at the cell surface and colocalization analyses were performed using ImageJ software (http://rsb.info.nih.gov/ij) (Bolte and Cordelieres, 2006; Barr et al., 2008). In certain experiments, morphological changes in BMDMs challenged or not challenged with *C. burnetii* LPS (1 μg/ml) were evaluated as previously described (Honstettre et al., 2004).

P38α MAPK PHOSPHORYLATION ASSAY

The phosphorylation of p38 was assessed using phospho-p38 MAPK cell-based ELISA (R&D Systems) (Boucherit et al., 2012) following the manufacturer recommendations.

IMMUNOPRECIPITATION

BMDMs were treated with or without LPSs (1 μg/ml) for 30 min and then lysed with 1% Triton X-100 in a buffer consisting of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1 mM EDTA pH 8.0. TLR-4 (bdbiosciences) was immunoprecipitated via overnight incubation of the total protein with the anti-TLR-2 antibody (bdbiosciences) followed by incubation with protein ASepharose beads (Roche). The immunoprecipitated pellets were washed and analyzed via immunoblotting on 6% polyacrylamide gels using anti-TLR-2 and anti-TLR-4 antibodies. The detection of TLR-2 from the input sample was performed using 50 μg of protein. The immunoblots were visualized using an LAS 4000 camera system (GE Healthcare) or an Amersham Biosciences revelator. In some experiments, macrophages were treated with 1 μg/ml of cytochalasin D (Sigma–Aldrich) as previously described (Meconi et al., 1998; Honstettre et al., 2004).

STATISTICAL ANALYSIS

The results are expressed as means ± SD and were analyzed using the non-parametric Mann–Whitney *U*-test. Differences were considered significant at $p < 0.05$.

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REFERENCES

Barr, D. J., Ostermeyer-Fay, A. G., Matundan, R. A., and Brown, D. A. (2008). Clathrin-independent endocytosis of ErbB2 in geldanamycin-treated human breast cancer cells. *J. Cell Sci.* 121(Pt 19), 3155–3166. doi: 10.1242/jcs.020404

Barry, A. O., Boucherit, N., Mottola, G., Vadovic, P., Trouplin, V., Soubeyran, P., et al. (2012). Impaired stimulation of p38alpha-MAPK/Vps41-HOPS by LPS from pathogenic *Coxiella burnetii* prevents trafficking to microbicidal phagolysosomes. *Cell Host Microbe* 12, 751–763. doi: 10.1016/j.chom.2012.10.015

Blander, J. M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. *Science* 304, 1014–1018. doi: 10.1126/science.1096158

Blander, J. M., and Medzhitov, R. (2006). On regulation of phagosome maturation and antigen presentation. *Nat. Immunol.* 7, 1029–1035. doi: 10.1038/ni1006-1029

Bolte, S., and Cordelieres, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* 224(Pt 3), 213–232. doi: 10.1111/j.1365-2818.2006.01706.x

Boucherit, N., Barry, A. O., Mottola, G., Trouplin, V., Capo, C., Mege, J. L., et al. (2012). Effects of *Coxiella burnetii* on MAPKinases phosphorylation. *FEMS Immunol. Med. Microbiol.* 64, 101–103. doi: 10.1111/j.1574-695X.2011.00852.x

Chu, J. J., and Ng, M. L. (2004). Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J. Virol.* 78, 10543–10555. doi: 10.1128/JVI.78.19.10543-10555.2004

Cook, P., Totemeyer, S., Stevenson, C., Fitzgerald, K. A., Yamamoto, M., Akira, S., et al. (2007). Salmonella-induced SipB-independent cell death requires Toll-like receptor-4 signalling via the adapter proteins Tram and Trif. *Immunology* 122, 222–229. doi: 10.1111/j.1365-2567.2007.02631.x

Fontana, M. F., and Vance, R. E. (2011). Two signal models in innate immunity. *Immunol. Rev.* 243, 26–39. doi: 10.1111/j.1600-065X.2011.01037.x

Forestier, C., Moreno, E., Pizarro-Cerda, J., and Gorvel, J. P. (1999). Lysosomal accumulation and recycling of lipopolysaccharide to the cell surface of murine macrophages, an *in vitro* and *in vivo* study. *J. Immunol.* 162, 6784–6791.

Ghigo, E., Barry, A. O., Pretat, L., Al Moussawi, K., Desnues, B., Capo, C., et al. (2010). IL-16 promotes T. whipplei replication by inhibiting phagosome conversion and modulating macrophage activation. *PLoS ONE* 5:e13561. doi: 10.1371/journal.pone.0013561

Ghigo, E., Capo, C., Tung, C. H., Raoult, D., Gorvel, J. P., and Mege, J. L. (2002). *Coxiella burnetii* survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN-gamma mediates its restoration and bacterial killing. *J. Immunol.* 169, 4488–4495. doi: 10.4049/jimmunol.169.8.4488

Good, D. W., George, T., Watts, B. A. III. (2012). Toll-like receptor 2 is required for LPS-induced Toll-like receptor 4 signaling and inhibition of ion transport in renal thick ascending limb. *J. Biol. Chem.* 287, 20208–20220. doi: 10.1074/jbc.M111.336255

Honstettre, A., Ghigo, E., Moynault, A., Capo, C., Toman, R., Akira, S., et al. (2004). Lipopolysaccharide from *Coxiella burnetii* is involved in bacterial phagocytosis, filamentous actin reorganization, and inflammatory responses through Toll-like receptor 4. *J. Immunol.* 172, 3695–3703. doi: 10.4049/jimmunol.172.6.3695

Lee, H. K., Dunzendorfer, S., and Tobias, P. S. (2004). Cytoplasmic domain-mediated dimerizations of toll-like receptor 4 observed by beta-lactamase enzyme fragment complementation. *J. Biol. Chem.* 279, 10564–10574. doi: 10.1074/jbc.M311564200

Lukacova, M., Barak, I., and Kazar, J. (2008). Role of structural variations of polysaccharide antigens in the pathogenicity of Gram-negative bacteria. *Clin. Microbiol. Infect.* 14, 200–206. doi: 10.1111/j.1469-0691.2007.01876.x

Madariaga, M. G., Rezai, K., Trenholme, G. M., and Weinstein, R. A. (2003). Q fever: a biological weapon in your backyard. *Lancet Infect. Dis.* 3, 709–721. doi: 10.1016/S1473-3099(03)00804-1

Meconi, S., Jacomo, V., Boquet, P., Raoult, D., Mege, J. L., and Capo, C. (1998). *Coxiella burnetii* induces reorganization of the actin cytoskeleton in human monocytes. *Infect. Immun.* 66, 5527–5533.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1, 135–145. doi: 10.1038/35100529

Raoult, D., Marrie, T., and Mege, J. (2005). Natural history and pathophysiology of Q fever. *Lancet Infect. Dis.* 5, 219–226. doi: 10.1016/S1473-3099(05)70052-9

Regis, E. (1999). *The Biology of Doom: The History of America's Secret Germ Warfare Project*. New York, NY: Holt.

Ren, F., Zhan, X., Martens, G., Lee, J., Center, D., Hanson, S. K., et al. (2005). Pro-IL-16 regulation in activated murine CD4+ lymphocytes. *J. Immunol.* 174, 2738–2745. doi: 10.4049/jimmunol.174.5.2738

Ruse, M., and Knaus, U. G. (2006). New players in TLR-mediated innate immunity: PI3K and small Rho GTPases. *Immunol. Res.* 34, 33–48. doi: 10.1385/IR:34:1:33

Skultety, L., Toman, R., and Patoprsty, V. (1996). A comparative study of lipopolysaccharides from two *Coxiella burnetii* strains considered to be associated with acute and chronic Q fever. *Polymers* 35, 189–194.

Symons, A., Beinke, S., and Ley, S. C. (2006). MAP kinase kinase kinases and innate immunity. *Trends Immunol.* 27, 40–48. doi: 10.1016/j.it.2005.11.007

Toman, R., and Skultety, L. (1996). Structural study on a lipopolysaccharide from *Coxiella burnetii* strain Nine Mile in avirulent phase II. *Carbohydr. Res.* 283, 175–185. doi: 10.1016/0008-6215(96)87610-5

Toman, R., Skultety, L., and Ihnatko, R. (2009). *Coxiella burnetii* glycomics and proteomics–tools for linking structure to function. *Ann. N.Y. Acad. Sci.* 1166, 67–78. doi: 10.1111/j.1749-6632.2009.04512.x

Toman, R., and Vadovič, P. (2011). "Lipopolysaccharides of *Coxiella Burnetii*: chemical composition and structure, and their role in diagnosis of Q fever," in *BSL3 and BSL4 Agents: Proteomics, Glycomics, and Antigenicity*, eds J. Stulik, R. Toman, P. Butaye, and R. G. Ulrich (Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA), 115–123. doi: 10.1002/9783527638192.ch10

Trouplin, V., Boucherit, N., Gorvel, L., Conti, F., Mottola, G., and Ghigo, E. (2013). Bone marrow-derived macrophage production. *J. Vis. Exp.* 81:e50966. doi: 10.3791/50966

Underhill, D. M. (2004). Toll-like receptors and microbes take aim at each other. *Curr. Opin. Immunol.* 16, 483–487. doi: 10.1016/j.coi.2004.05.012

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Leishmania lipophosphoglycan: how to establish structure-activity relationships for this highly complex and multifunctional glycoconjugate?

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A key feature of many pathogenic microorganisms is the presence of a dense glycocalyx at their surface, composed of lipid-anchored glycoproteins and non-protein-bound polysaccharides. These surface glycolipids are important virulence factors for bacterial, fungal and protozoan pathogens. The highly complex glycoconjugate lipophosphoglycan (LPG) is one of the dominant surface macromolecules of the promastigote stage of all *Leishmania* parasitic species. LPG plays critical pleiotropic roles in parasite survival and infectivity in both the sandfly vector and the mammalian host. Here, we review the composition of the *Leishmania* glycocalyx, the chemical structure of LPG and what is currently known about its effects in the mammalian host, specifically. We will then discuss the current approaches employed to elucidate LPG functions. Finally, we will provide a viewpoint on future directions that this area of investigation could take to unravel in detail the biological activity of the specific molecular elements composing the structurally complex LPG.

Keywords: *Leishmania* glycoconjugates, lipophosphoglycan, LPG structure, LPG function, chemical synthesis, LPG structure-activity relationships

THE *LEISHMANIA* SURFACE COAT

Like all the parasites of the Trypanosomatid family, *Leishmania* is characterized by the presence of a glycocalyx covering the entire parasite surface (Ferguson, 1999). The surface coats of these different trypanosomatid parasites exhibit a significant diversity in composition. However, all of the surface-bound molecules of this family share a common structural feature, which is that they all contain a highly conserved glycosylphosphatidylinositol (GPI)-anchor motif. Notably, this type of GPI-lipid anchor is unusual and structurally very different from those found in mammalian cells (Mcconville and Ferguson, 1993).

Unlike other trypanosomatids in which the glycocalyx is primarily composed of GPI-anchored glycoproteins, the glycocalyx of the *Leishmania* promastigote stage is dominated by GPI-anchored phosphoglycosylated glycans. Lipophosphoglycan (LPG) represents one of the most abundant promastigote-specific surface glycoconjugates, with approximately 5×10^6 copies/cell (Turco and Descoteaux, 1992). The glycosylinositol phospholipids (GIPLs) also termed free GPI, constitute a complex family of abundant low-molecular-weight molecules, with approximately 10^7 copies/cell. Three different types of GIPL molecules have been described based on the nature of their glycan moiety (Mcconville et al., 1993). In the GIPL of type 1, the glycan part is structurally similar to that of the LPG glycan core, whereas in the GIPL of type 2, the glycan part is related to that of the GPI-anchored glycoprotein. The GIPLs of type 3 exhibit features of type 1 and 2. The membrane-bound proteophosphoglycans (mPPGs) represent a distinct family of GPI-anchored protein-linked glycans that express a phosphoglycan domain structurally similar to LPG. The mPPGs are significantly expressed at the promastigote parasite surface but to a lesser proportion than LPG and GIPLs (Ilg, 2000). Last, one of the major GPI-anchored glycosylated proteins present at the promastigote plasma membrane is GP63, with around 5×10^5 copies/cell. Importantly, the composition of the *Leishmania* surface glycocalyx changes dynamically during the life cycle of the parasite. When infective, promastigote parasites differentiate into obligate intracellular amastigotes in the infected mammalian host cell, the expression of LPG is drastically downregulated. In contrast, GIPLs and PPGs remain highly expressed throughout the parasite life cycle (Turco and Sacks, 1991). Notably, the PPGs continue to be produced in amastigotes, but as free macromolecules rather than membrane-associated ones (Bahr et al., 1993).

The glycoconjugates of the *Leishmania* promastigote membrane are evenly distributed over the entire parasite surface. They form a highly hydrophilic barrier easily detected as an electron-dense material using electron microscopy. Its thickness can reach up to 15 nm due to the length of the LPG polysaccharide chain and potentially up to several hundred nanometers due to the lengths of the mPPGs (Ilg, 2000). Because of their abundance, structural uniqueness and specific distributions, the *Leishmania* membrane glycoconjugates are believed to play important functions in the mammalian host. Among these compounds, LPG has attracted considerable attention because its clear implication in multiple activities that favor parasite virulence.

LEISHMANIA LPG STRUCTURE

Leishmania LPG, is a highly complex macromolecule composed of four distinct domains: a GPI anchor, a glycan core, a linear phosphoglycan chain (PG) and a terminating oligosaccharide cap (**Figure 1**) (Turco and Descoteaux, 1992). The GPI anchor domain consists of an alkyl phosphatidylinositol having a single saturated C_{24-26} aliphatic chain (Ferguson, 1999). The LPG glycan core is a heptasaccharide comprising two galactopyranosides, a galactofuranoside (Gal_f), two mannosides and a glucosamine residue attached to inositol. The glycan core is linked to a linear PG that consists of 15–40 phosphodisaccharide (Galβ1,4Manα1-PO4) units. Finally, LPG is terminated by a di-, tri- or tetrasaccharide consisting of galactose and mannose assembled as Manα1,2Manα1 or as Galβ1,4(Manα1,2)Manα1 depending on the *Leishmania* species.

The lipid anchor, glycan core and the linear PG moieties that constitute the LPG are identical in all *Leishmania* species (Descoteaux and Turco, 1999). Despite conservation of these domains, however, LPG exhibits substantial heterogeneity, with important parasite stage- and species-modifications found in the oligosaccharide cap and in the substituents groups branched on the linear PG (Turco et al., 2001; De Assis et al., 2012). Stage-specific variations are observed throughout the parasite life cycle such that LPG undergoes considerable structural modifications in the PG and the terminating cap during parasite metacyclogenesis (Sacks et al., 1990, 1995). In the PG domain, the number of repeating units increases such that the metacyclic promastigote LPG is significantly longer than the procyclic promastigote LPG. In the oligosaccharide cap, change is made with the replacement of the galactoside residue by an arabinopyranoside residue. Species-specific variations occurring in the PG domain of the LPG are one of the main features of this virulent factor. Three types of LPG have been described depending on the nature of the side chain residues and on the site of substitution occupied by these residues in the PG domain. The LPG of *L. donovani* has no side substitution in the PG and therefore remains linear (Sacks et al., 1995). The LPGs of *L. major*, *L. mexicana*, *L. infantum*, and *L. tropica* are glycosylated at the C3 position of the galactose in the linear PG (Soares et al., 2002) and the LPGs of *L. aethiopica* are frequently mannosylated at position C2 of the mannose. Additionally, the variability in the sugar residues that branch on the PG domain increases significantly

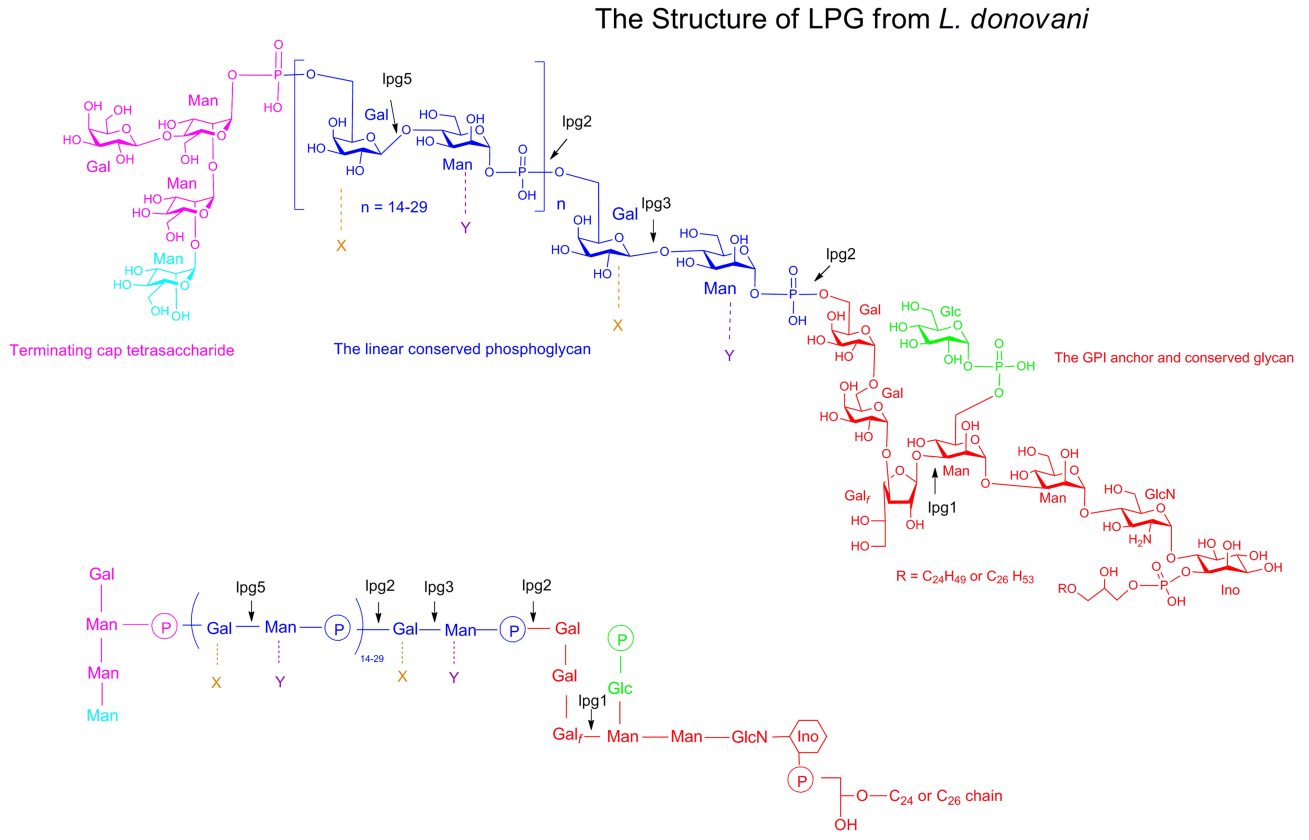

FIGURE 1 | Structure of the *Leishmania* LPG. Top and **bottom panels** show two different representations of the LPG structure of *Leishmania* parasite. LPG is constituted of four key domains. The GPI anchor and the glycan core are shown in red. The linear conserved phosphoglycan chain is in blue. The terminating oligosaccharide cap is shown in pink. In each domains, the residues that are not conserved among the LPG of the different Leishmania species are represented in a different color (Man, X, Y and Glc). The Glucose phosphate branched on the mannose residue of the glycan core (in green) is present in the LPG of *L. donovani*, *L.mexicana* and some subspecies of *L. major* but absent in some other subspecies. The linear phosphoglycan chain of *L. donovani* can be substituted with different residues. X represents the substitutent group express in the LPG *L. mexicana*, *L. major* and *L. tropica*. Y is a substitutent present in the LPG of *L. aethopica*. Gal, galactose; Man, Mannose; Glc, glucose; Galf, galactofuranose; GlcN, glucosamine; Ino, inositol.

the level of LPG complexity. Finally, intraspecific LPG variability has been observed among similar *Leishmania* species obtained from different field isolates (Coelho-Finamore et al., 2011). Such stage-specific polymorphisms and intra- and interspecies variations have been involved in the survival of *Leishmania* inside the sand fly, more precisely in the selectivity, permissivity, and competence of a given sand fly vector for particular *Leishmania* strains (Dobson et al., 2006, 2010; Volf et al., 2014). However, the role and the biological relevance of LPG polymorphism in the mammalian host has been poorly understood.

Given the structural complexity and heterogeneity of LPG molecules, the identification of the molecular elements responsible for its biological activity have only partially been resolved.

ROLE OF LPG IN *LEISHMANIA* INTERACTIONS WITH THE IMMUNE SYSTEM

The LPG-enriched glycocalyx of *Leishmania* constitutes the primary interface of the host-parasite interactions that take place in the dermis of the mammalian host immediately after parasite inoculation by the sandfly vector. Consequently, LPG is the first target for immune detection and at the same time a barrier protecting the parasite from the attack of the host immune system.

LPG has been shown to circumvent the lysis of the parasite by the host complement system. It acts either by sterically preventing the attachment of complement molecules or by directly inactivating the assembly of a functional complement complex at the promastigote surface (Puentes et al., 1989, 1990).

LPG has been shown to favor intracellular parasite survival by interfering with the pro-inflammatory host cell responses via binding of Toll-like receptor (TLR) 2 and 4 on macrophages and NK cells (Becker et al., 2003; De Veer et al., 2003; Kavoosi et al., 2009; Rojas-Bernabe et al., 2014). LPG-TLR interactions induce ERK phosphorylation while suppressing p38 MAP kinase phosphorylation, modulate the production of reactive oxygen species and nitric oxide and inhibit pro-inflammatory cytokine secretion (Chandra and Naik, 2008; De Assis et al., 2012). These studies showed that the integrity of the lipid anchor as well as the length of the PG domain of LPG are involved in the magnitude of LPG-mediated host cell activation via TLR2, as procyclic promastigotes are weaker stimulators than metacyclic promastigotes. The level of LPG expression is also been considered as a critical parameter of this specific host cell stimulation pathway (Srivastava et al., 2013). Given that the GPI anchor of *trypanozoma cruzi* has been implicated in TLR2-mediated activation, it is conceivable than the analogous site in *Leishmania* LPG play a similar function (Campos et al., 2001). Finally, by comparing the LPG from *L. braziliensis* and *L. infantum* a recent study reveals that interspecies LPG structural polymorphism has a significant impact on host cell stimulation via TLR (De Assis et al., 2012; Ibraim et al., 2013) Despite these advances, it remains to be determined which LPG motifs are exactly implicated in TLR binding and further immune cell stimulation.

The role of LPG during parasite internalization and multiplication within the host cell has been extensively studied but they have yielded contradictory results. For instance LPG has been shown to delay the maturation of the parasite-containing phagosome by preventing its fusion with lysosomes while some groups have demonstrated that such phenomenon does not occur (Desjardins and Descoteaux, 1997; Forestier et al., 2011). In parallel, LPG has been found to block the assembly of NADPH oxidase and prevent recruitment of proton ATPases at the phagosomal membrane (Lodge and Descoteaux, 2005; Vinet et al., 2009). This function has been attributed to the localization of LPG at the membrane of the *Leishmania*-containing phagosome (Dermine et al., 2005; Winberg et al., 2009) (**Figure 2**). Therefore investigating a potential correlation between the intracellular localization of LPG inside the host cells and a particular biological function would require to be explored. Since the first observation of the presence of the PG disaccharide repeat units of the LPG at the surface of infected host cells (Tolson et al., 1990), a phenomenon that was later confirmed by our group (Forestier, 2013), the fate and pattern of trafficking of LPG during the infection process remain elusive. Given this lack of knowledge, monitoring LPG trafficking in the host cell and in the host organism during the infection process will be key to better understand LPG functions in its mammalian host. Furthermore, investigating whether chemical modifications of LPG occur during the infection process will help unraveling the importance of the distinct LPG structural motifs on its biological functions.

LPG AS A PROMISING VACCINE CANDIDATE

Despite its lack of strong immunogenicity but because of its unique structure, dense distribution and accessibility, *Leishmania* LPG has been considered as an attractive vaccine target (Goel et al., 1999). Early vaccine studies indicated that purified LPG provides protection in mice against challenge with virulent parasites. These studies have demonstrated that LPG-mediated protection can be obtained with LPG alone and that its efficacy depended on the integrity of the LPG molecule and could be modulated by the use of adjuvants (Handman and Mitchell, 1985; Russell and Alexander, 1988; Mcconville and Ferguson, 1993;

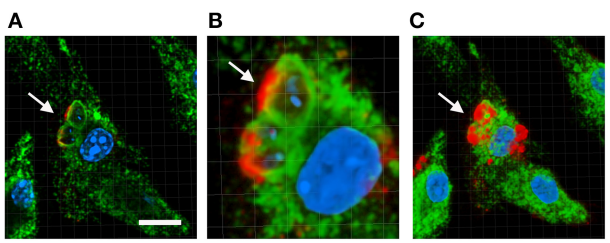

FIGURE 2 | Localization of LPG at the membrane of the *Leishmania*-containing phagosome. Bone marrow macrophages were infected with *L.donovani* promastigote for 1 h at 37°C then macrophages were washed to remove extracellular parasites and incubated in new medium for 24 h. Infected cells were processed for immunofluorescence staining. The LPG molecules were stained using the anti-PG antibody CA7AE (Red), the lysosome and phagosome compartments were stained using the anti-LAMP-1 antibody (Green), the host cell and the parasite nuclei were stained using the Hoechst dye (Blue). Images are analyzed using the Imaris software. **(A)** Represents a single Z section of 0.7 μm. **(B)** Represents a zoomed image area of **(A)**. Panel **(C)** is a 3D reconstruction of the entire Z stack. Arrow points to two Leishmania-containing vacuoles showing LPG at the phagosomal membranes. Scale bar, 10 μm.

Karanja et al., 2011). Contradictory results have arisen in more recent studies, in both protective and disease-promoting effects associated with LPG vaccination have been observed. These studies have revealed the importance of the immunization route in the vaccine outcome, with subcutaneous LPG injection failing to protect mice against *L. amazonensis* but intranasal administration of LPG showing to be protective (Pinheiro et al., 2005, 2007). Although the mechanisms of LPG-mediated immunization are yet not known, LPG has been shown to activate T cells and favor a Th1 immune response thus mediating protection against the intracellular stage of *Leishmania* (Handman and Mitchell, 1985; Moll et al., 1989; Moll and Rollinghoff, 1991; Tonui et al., 2003; Amprey et al., 2004). Paradoxically, a recent study showed that LPG vaccination, depending on the dose of LPG, induces the expression of the inhibitory receptors PD-1 and PD-L2 on T cells and macrophages respectively, therefore preventing proper protection against leishmaniasis (Martinez Salazar et al., 2014). Given the structural complexity of LPG, it remains unknown which feature of this glycoconjugate, independently or as a part of the whole macromolecule, is involved in the effective immunization process. Therefore, it is important to identify the functionally relevant molecular elements of LPG with the goal of developing artificial and well-designed LPG-based vaccines (Routier et al., 1999; Hewitt and Seeberger, 2001; Astronomo and Burton, 2010; Topuzogullari et al., 2013).

APPROACHES TO ASSESS LPG FUNCTIONS

The involvement of *Leishmania* LPG in virulence has been confirmed in non-physiological conditions, using purified LPG molecules tested on macrophages *in vitro* and, in a more biologically relevant context, using parasites defective in specific steps of the LPG biosynthesis pathway.

Although the use of purified LPG has been very valuable to unravel LPG functions, it also has several drawbacks. Among these is the complicated purification procedures that it requires and along with it the difficulty of obtaining pure LPG preparations devoid of various contaminants. More problematic is the possibility that LPG preparation may become contaminated with trace amount of endotoxin, a problem commonly faced with the purification of molecules and that will greatly bias the host cell immune response. Finally, the use of purified LPG does not reflect the physiological conditions experienced in host cell-parasite interaction, and the dose of LPG used in such artificial functional assays may not replicate that encountered in actual physiological conditions.

Genetic approaches rely on the identification of genes encoding for enzymes that are involved in LPG biosynthesis, on the disruption of these target genes in *Leishmania* and on the analysis of the phenotypes and functions of such null mutants (Beverley and Turco, 1998). This type of studies has led to the generation of parasites displaying LPG molecules that are truncated at different levels of their polysaccharide moieties. *Leishmania* parasites were generated with mutations in the LPG1, LPG2, LPG5, and LPG3 genes that, respectively, encodes a galactofuranosyltransferase involved in the synthesis of the LPG glycan core specifically (Ryan et al., 1993), a Golgi GDP-mannose transporter required for the synthesis of the PG domain common to LPG and mPPG (Ma et al., 1997), a Golgi UDP-Gal transporter critical for the synthesis of the PG (Capul et al., 2007a,b) and the *Leishmania* homolog of a mammalian endoplasmic reticulum chaperone required for complete PG synthesis (Descoteaux et al., 2002). As a result, lpg1$^-$ parasites express intact GIPLs and mPPG molecules, whereas LPG molecules have a truncated glycan core and no PG domain neither terminal oligosaccharide cap (Spath et al., 2000). In contrast, lpg2$^-$ and lpg5$^-$ parasites express normal GIPLs but these parasites lack all PG domains including those of LPG and mPPG; however LPG molecules have a normal glycan core (Spath et al., 2003; Liu et al., 2009). Finally, lpg3$^-$ parasites express LPG molecules truncated after the first mannoside residue of the first disaccharide unit composing the PG domain (Descoteaux et al., 2002).

These null-mutant parasites and others recent LPG-mutants (Phillips and Turco, 2014) provide powerful tools for identifying the functions of the LPG in a context that closely mimics the natural course of infection, including a physiological concentration of LPG interacting with the host. Importantly, analyses of these mutants have been proven to discriminate efficiently between the roles of LPG and those of other related glycoconjugates, including mPPG, that express similar polysaccharide domains. Nevertheless, the nature of the lpg1, lpg2, lpg3, and lpg5 mutants offers the possibility of assessing the biological activity of only the LPG polysaccharide domains including the last three sugar residues of the glycan core, the phosphoglycan disaccharide repeating units and the oligosaccharide cap. To the best of our knowledge, the functional impact of the species-specific substituents of the PG domain and the nature and structure of the GPI anchor has not been yet investigated. Most likely, this reflects the difficulty inherent in engineering parasites deficient in such specific and essential structural motifs. Indeed, such an approach requires first the identification of the specific genes involved in the addition of the substituent to the linear PG and involved in GPI-anchor biosynthesis and then the mutation of these genes, to obtain viable parasites expressing modified glycoconjugates. Previous attempts to generate GPI-null *Leishmania* have demonstrated that this domain is critical for parasite viability and infectivity (Ilgoutz et al., 1999; Garami et al., 2001). In contrast, parasites deficient exclusively in the assembly of the GPI-anchored glycoproteins but not in the expression of LPG or GIPLs retain their capacity to grow and remain virulent (Mensa-Wilmot et al., 1994; Hilley et al., 2000; Zufferey et al., 2003). However, these studies were not able to discriminate between the GPI-anchor of LPG and other related structures carried by the other glycoconjugates featuring the *Leishmania* surface. To achieve a complete map of LPG structure-function relationships, it is critically required to identify genes involved specifically in the different steps of LPG biosynthesis, so that new LPG mutants may be generated.

Nevertheless, despite the valuable information gained by these studies, the main limitation of such genetic approaches is the limited opportunity they afford to investigate in detail and independently the relative roles of the different structural motifs of highly complex LPG molecules (oligosaccharide cap, phosphopolysaccharide chain, glycan core, GPI anchor and fatty acid chain). Although LPGs display high levels of heterogeneity, whether the

molecular composition of the LPG motifs plays a distinct role in LPG function has never been determined. Therefore, there is an urgent need to explore the relative implications and contributions of the different LPG molecular elements to its biological activity.

FUTURE APPROACHES

In our point of view, one of the priorities for future research into the functions of LPG is to elucidate the structure-activity relationship of this membrane-bound glycoconjugate. The ultimate goal is to identify which LPG motifs are associated with specific effects in the mammalian host. Such informations are expected to bring key new insights into the mechanism of action of this macromolecule.

To overcome some aspects of the limitations linked to the genetic approaches, alternative methods that will aim to dissect the LPG functional groups at the molecular level will need to be developed. Chemical synthesis of such structurally complex glycoconjugates may be one of the promising experimental approaches for investigating the distinct functions of each of the structural elements that compose the multifunctional LPG molecules. With technological advances in chemistry, new synthetic strategies and methods for the chemical synthesis of highly complex glycoconjugates are currently developed which will make conceivably the synthesis of LPG feasible (Astronomo and Burton, 2010). These chemical synthesis methods will allow the design and production of panels of synthetic LPG variants having independent molecular variations within its four distinct domains. Such synthetic LPG compounds will be crucial tools for investigating the functional relevance of the molecular elements of LPG. Using chemical synthesis one could expect to address and elucidate the importance of (i) the composition of the fatty acid chain of the GPI-anchor; (ii) the glycan core; and (iii) the repetitive units in the PG domain. Comparisons among the chemically well-defined collection of LPG variants will allow us to assign a molecular motif to a biological function. Such chemical synthesis methodology-based research could be extended to the study of all the others GPI-anchored glyconconjugates expressed at the cell surface of *Leishmania* and others related parasites. This will include GIPLs that unlike LPG, is expressed both at the promastigote and amastigote parasite stages and for which information about its role in the mammalian host remains very limited.

This type of approach will significantly advance our knowledge of the structurally complex LPG. Significantly, it will help to dissect the various LPG domains and attribute a precise function to each specific LPG elements, thereby establishing a causal structure-activity relationship. Ultimately, such chemistry-based strategy will open new venue to the development of LPG-based therapeutics agents against Leishmaniasis using synthetic and biologically relevant molecular elements of LPG.

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REFERENCES

Amprey, J. L., Im, J. S., Turco, S. J., Murray, H. W., Illarionov, P. A., Besra, G. S., et al. (2004). A subset of liver NK T cells is activated during *Leishmania donovani* infection by CD1d-bound lipophosphoglycan. *J. Exp. Med.* 200, 895–904. doi: 10.1084/jem.20040704

Astronomo, R. D., and Burton, D. R. (2010). Carbohydrate vaccines: developing sweet solutions to sticky situations? *Nat. Rev. Drug Discov.* 9, 308–324. doi: 10.1038/nrd3012

Bahr, V., Stierhof, Y. D., Ilg, T., Demar, M., Quinten, M., and Overath, P. (1993). Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmania mexicana*. *Mol. Biochem. Parasitol.* 58, 107–121. doi: 10.1016/0166-6851(93)90095-F

Becker, I., Salaiza, N., Aguirre, M., Delgado, J., Carrillo-Carrasco, N., Kobeh, L. G., et al. (2003). Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Mol. Biochem. Parasitol.* 130, 65–74. doi: 10.1016/S0166-6851(03)00160-9

Beverley, S. M., and Turco, S. J. (1998). Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite Leishmania. *Trends Microbiol.* 6, 35–40. doi: 10.1016/S0966-842X(97)01180-3

Campos, M. A., Almeida, I. C., Takeuchi, O., Akira, S., Valente, E. P., Procopio, D. O., et al. (2001). Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J. Immunol.* 167, 416–423. doi: 10.4049/jimmunol.167.1.416

Capul, A. A., Barron, T., Dobson, D. E., Turco, S. J., and Beverley, S. M. (2007a). Two functionally divergent UDP-Gal nucleotide sugar transporters participate in phosphoglycan synthesis in Leishmania major. *J. Biol. Chem.* 282, 14006–14017. doi: 10.1074/jbc.M610869200

Capul, A. A., Hickerson, S., Barron, T., Turco, S. J., and Beverley, S. M. (2007b). Comparisons of mutants lacking the Golgi UDP-galactose or GDP-mannose transporters establish that phosphoglycans are important for promastigote but not amastigote virulence in Leishmania major. *Infect. Immun.* 75, 4629–4637. doi: 10.1128/IAI.00735-07

Chandra, D., and Naik, S. (2008). *Leishmania donovani* infection down-regulates TLR2-stimulated IL-12p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism. *Clin. Exp. Immunol.* 154, 224–234. doi: 10.1111/j.1365-2249.2008.03741.x

Coelho-Finamore, J. M., Freitas, V. C., Assis, R. R., Melo, M. N., Novozhilova, N., Secundino, N. F., et al. (2011). Leishmania infantum: Lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts. *Int. J. Parasitol.* 41, 333–342. doi: 10.1016/j.ijpara.2010.10.004

De Assis, R. R., Ibraim, I. C., Nogueira, P. M., Soares, R. P., and Turco, S. J. (2012). Glycoconjugates in New World species of Leishmania: polymorphisms in lipophosphoglycan and glycoinositolphospholipids and interaction with hosts. *Biochim. Biophys. Acta* 1820, 1354–1365. doi: 10.1016/j.bbagen.2011.11.001

De Veer, M. J., Curtis, J. M., Baldwin, T. M., Didonato, J. A., Sexton, A., Mcconville, M. J., et al. (2003). MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur. J. Immunol.* 33, 2822–2831. doi: 10.1002/eji.200324128

Dermine, J. F., Goyette, G., Houde, M., Turco, S. J., and Desjardins, M. (2005). *Leishmania donovani* lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. *Cell Microbiol.* 7, 1263–1270. doi: 10.1111/j.1462-5822.2005.00550.x

Descoteaux, A., Avila, H. A., Zhang, K., Turco, S. J., and Beverley, S. M. (2002). Leishmania LPG3 encodes a GRP94 homolog required for phosphoglycan synthesis implicated in parasite virulence but not viability. *EMBO J.* 21, 4458–4469. doi: 10.1093/emboj/cdf447

Descoteaux, A., and Turco, S. J. (1999). Glycoconjugates in Leishmania infectivity. *Biochim. Biophys. Acta* 1455, 341–352. doi: 10.1016/S0925-4439(99)00065-4

Desjardins, M., and Descoteaux, A. (1997). Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *J. Exp. Med.* 185, 2061–2068. doi: 10.1084/jem.185.12.2061

Dobson, D. E., Kamhawi, S., Lawyer, P., Turco, S. J., Beverley, S. M., and Sacks, D. L. (2010). Leishmania major survival in selective *Phlebotomus papatasi* sand

fly vector requires a specific SCG-encoded lipophosphoglycan galactosylation pattern. *PLoS Pathog.* 6:e1001185. doi: 10.1371/journal.ppat.1001185

Dobson, D. E., Scholtes, L. D., Myler, P. J., Turco, S. J., and Beverley, S. M. (2006). Genomic organization and expression of the expanded SCG/L/R gene family of Leishmania major: internal clusters and telomeric localization of SCGs mediating species-specific LPG modifications. *Mol. Biochem. Parasitol.* 146, 231–241. doi: 10.1016/j.molbiopara.2005.12.012

Ferguson, M. A. (1999). The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell Sci.* 112(Pt 17), 2799–2809.

Forestier, C. L. (2013). Imaging host-Leishmania interactions: significance in visceral leishmaniasis. *Parasite Immunol.* 35, 256–266. doi: 10.1111/pim.12044

Forestier, C. L., Machu, C., Loussert, C., Pescher, P., and Spath, G. F. (2011). Imaging host cell-Leishmania interaction dynamics implicates parasite motility, lysosome recruitment, and host cell wounding in the infection process. *Cell Host Microbe* 9, 319–330. doi: 10.1016/j.chom.2011.03.011

Garami, A., Mehlert, A., and Ilg, T. (2001). Glycosylation defects and virulence phenotypes of *Leishmania mexicana* phosphomannomutase and dolicholphosphate-mannose synthase gene deletion mutants. *Mol. Cell Biol.* 21, 8168–8183. doi: 10.1128/MCB.21.23.8168-8183.2001

Goel, A., Vohra, H., and Varshney, G. C. (1999). Strain-specific recognition of live *Leishmania donovani* promastigotes by homologous antiserum raised against a crude membrane fraction of infected macrophages. *Parasitol. Res.* 85, 19–24. doi: 10.1007/s004360050501

Handman, E., and Mitchell, G. F. (1985). Immunization with Leishmania receptor for macrophages protects mice against cutaneous leishmaniasis. *Proc. Natl. Acad. Sci. U.S.A.* 82, 5910–5914. doi: 10.1073/pnas.82.17.5910

Hewitt, M. C., and Seeberger, P. H. (2001). Solution and solid-support synthesis of a potential leishmaniasis carbohydrate vaccine. *J. Org. Chem.* 66, 4233–4243. doi: 10.1021/jo015521z

Hilley, J. D., Zawadzki, J. L., Mcconville, M. J., Coombs, G. H., and Mottram, J. C. (2000). *Leishmania mexicana* mutants lacking glycosylphosphatidylinositol (GPI):protein transamidase provide insights into the biosynthesis and functions of GPI-anchored proteins. *Mol. Biol. Cell* 11, 1183–1195. doi: 10.1091/mbc.11.4.1183

Ibraim, I. C., De Assis, R. R., Pessoa, N. L., Campos, M. A., Melo, M. N., Turco, S. J., et al. (2013). Two biochemically distinct lipophosphoglycans from Leishmania braziliensis and Leishmania infantum trigger different innate immune responses in murine macrophages. *Parasit. Vectors* 6:54. doi: 10.1186/1756-3305-6-54

Ilg, T. (2000). Proteophosphoglycans of Leishmania. *Parasitol. Today* 16, 489–497. doi: 10.1016/S0169-4758(00)01791-9

Ilgoutz, S. C., Zawadzki, J. L., Ralton, J. E., and Mcconville, M. J. (1999). Evidence that free GPI glycolipids are essential for growth of *Leishmania mexicana*. *EMBO J.* 18, 2746–2755. doi: 10.1093/emboj/18.10.2746

Karanja, R., Ingonga, J., Mwangi, M., Mwala, D., Lugalia, R., Magambo, J., et al. (2011). Immunization with a combination of Leishmania major lipophosphoglycan (LPG) and Phlebotomus duboscqui salivary gland lysates (SGLs) abrogates protective effect of LPG against L. major in BALB/C mice. *Afr. J. Health Sci.* 18, 1–5.

Kavoosi, G., Ardestani, S. K., and Kariminia, A. (2009). The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to Leishmania major phosphoglycans (PGs). *Parasitology* 136, 1193–1199. doi: 10.1017/S0031182009990473

Liu, D., Kebaier, C., Pakpour, N., Capul, A. A., Beverley, S. M., Scott, P., et al. (2009). Leishmania major phosphoglycans influence the host early immune response by modulating dendritic cell functions. *Infect. Immun.* 77, 3272–3283. doi: 10.1128/IAI.01447-08

Lodge, R., and Descoteaux, A. (2005). *Leishmania donovani* promastigotes induce periphagosomal F-actin accumulation through retention of the GTPase Cdc42. *Cell Microbiol.* 7, 1647–1658. doi: 10.1111/j.1462-5822.2005.00582.x

Ma, D., Russell, D. G., Beverley, S. M., and Turco, S. J. (1997). Golgi GDP-mannose uptake requires Leishmania LPG2. A member of a eukaryotic family of putative nucleotide-sugar transporters. *J. Biol. Chem.* 272, 3799–3805. doi: 10.1074/jbc.272.6.3799

Martinez Salazar, M. B., Delgado Dominguez, J., Silva Estrada, J., Gonzalez Bonilla, C., and Becker, I. (2014). Vaccination with *Leishmania mexicana* LPG induces PD-1 in CD8(+) and PD-L2 in macrophages thereby suppressing the immune response: a model to assess vaccine efficacy. *Vaccine* 32, 1259–1265. doi: 10.1016/j.vaccine.2014.01.016

Mcconville, M. J., Collidge, T. A., Ferguson, M. A., and Schneider, P. (1993). The glycoinositol phospholipids of *Leishmania mexicana* promastigotes. Evidence for the presence of three distinct pathways of glycolipid biosynthesis. *J. Biol. Chem.* 268, 15595–15604.

Mcconville, M. J., and Ferguson, M. A. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* 294(Pt 2), 305–324.

Mensa-Wilmot, K., Lebowitz, J. H., Chang, K. P., Al-Qahtani, A., Mcgwire, B. S., Tucker, S., et al. (1994). A glycosylphosphatidylinositol (GPI)-negative phenotype produced in Leishmania major by GPI phospholipase C from *Trypanosoma brucei*: topography of two GPI pathways. *J. Cell. Biol.* 124, 935–947. doi: 10.1083/jcb.124.6.935

Moll, H., Mitchell, G. F., Mcconville, M. J., and Handman, E. (1989). Evidence of T-cell recognition in mice of a purified lipophosphoglycan from Leishmania major. *Infect. Immun.* 57, 3349–3356.

Moll, H., and Rollinghoff, M. (1991). T-cell reactivity to purified lipophosphoglycan from Leishmania major: a model for analysis of the cellular immune response to microbial carbohydrates. *Behring Inst. Mitt.* 88, 161–169.

Phillips, M. R., and Turco, S. J. (2014). Characterization of a ricin-resistant mutant of *Leishmania donovani* that expresses Lipophosphoglycan. *Glycobiology*. doi: 10.1093/glycob/cwu130. [Epub ahead of print].

Pinheiro, R. O., Pinto, E. F., De Matos Guedes, H. L., Filho, O. A., De Mattos, K. A., Saraiva, E. M., et al. (2007). Protection against cutaneous leishmaniasis by intranasal vaccination with lipophosphoglycan. *Vaccine* 25, 2716–2722. doi: 10.1016/j.vaccine.2006.05.093

Pinheiro, R. O., Pinto, E. F., Lopes, J. R., Guedes, H. L., Fentanes, R. F., and Rossi-Bergmann, B. (2005). TGF-beta-associated enhanced susceptibility to leishmaniasis following intramuscular vaccination of mice with Leishmania amazonensis antigens. *Microbes Infect.* 7, 1317–1323. doi: 10.1016/j.micinf.2005.04.016

Puentes, S. M., Da Silva, R. P., Sacks, D. L., Hammer, C. H., and Joiner, K. A. (1990). Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. *J. Immunol.* 145, 4311–4316.

Puentes, S. M., Dwyer, D. M., Bates, P. A., and Joiner, K. A. (1989). Binding and release of C3 from *Leishmania donovani* promastigotes during incubation in normal human serum. *J. Immunol.* 143, 3743–3749.

Rojas-Bernabe, A., Garcia-Hernandez, O., Maldonado-Bernal, C., Delegado-Dominguez, J., Ortega, E., Gutierrez-Kobeh, L., et al. (2014). *Leishmania mexicana* lipophosphoglycan activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4. *Parasitology* 141, 788–800. doi: 10.1017/S0031182013002187

Routier, F. H., Nikolaev, A. V., and Ferguson, M. A. (1999). The preparation of neoglycoconjugates containing inter-saccharide phosphodiester linkages as potential anti-Leishmania vaccines. *Glycoconj. J.* 16, 773–780. doi: 10.1023/A:1007171613195

Russell, D. G., and Alexander, J. (1988). Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *J. Immunol.* 140, 1274–1279.

Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J., and Beverley, S. M. (1993). Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of Leishmania. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8609–8613. doi: 10.1073/pnas.90.18.8609

Sacks, D. L., Brodin, T. N., and Turco, S. J. (1990). Developmental modification of the lipophosphoglycan from Leishmania major promastigotes during metacyclogenesis. *Mol. Biochem. Parasitol.* 42, 225–233. doi: 10.1016/0166-6851(90)90165-I

Sacks, D. L., Pimenta, P. F., Mcconville, M. J., Schneider, P., and Turco, S. J. (1995). Stage-specific binding of *Leishmania donovani* to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. *J. Exp. Med.* 181, 685–697. doi: 10.1084/jem.181.2.685

Soares, R. P., Macedo, M. E., Ropert, C., Gontijo, N. F., Almeida, I. C., Gazzinelli, R. T., et al. (2002). Leishmania chagasi: lipophosphoglycan characterization and binding to the midgut of the sand fly vector Lutzomyia longipalpis. *Mol. Biochem. Parasitol.* 121, 213–224. doi: 10.1016/S0166-6851(02)00033-6

Spath, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., et al. (2000). Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite Leishmania major. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9258–9263. doi: 10.1073/pnas.160257897

Spath, G. F., Garraway, L. A., Turco, S. J., and Beverley, S. M. (2003). The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9536–9541. doi: 10.1073/pnas.1530604100

Srivastava, S., Pandey, S. P., Jha, M. K., Chandel, H. S., and Saha, B. (2013). Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. *Clin. Exp. Immunol.* 172, 403–409. doi: 10.1111/cei.12074

Tolson, D. L., Turco, S. J., and Pearson, T. W. (1990). Expression of a repeating phosphorylated disaccharide lipophosphoglycan epitope on the surface of macrophages infected with *Leishmania donovani*. *Infect. Immun.* 58, 3500–3507.

Tonui, W. K., Mpoke, S. S., Orago, A. S., Turco, S. J., Mbati, P. A., and Mkoji, G. M. (2003). *Leishmania donovani*-derived lipophosphoglycan plus BCG induces a Th1 type immune response but does not protect Syrian golden hamsters (*Mesocricetus auratus*) and BALB/c mice against *Leishmania donovani*. *Onderstepoort J. Vet. Res.* 70, 255–263. doi: 10.4102/ojvr.v70i4.290

Topuzogullari, M., Cakir Koc, R., Dincer Isoglu, S., Bagirova, M., Akdeste, Z., Elcicek, S., et al. (2013). Conjugation, characterization and toxicity of lipophosphoglycan-polyacrylic acid conjugate for vaccination against leishmaniasis. *J. Biomed. Sci.* 20, 35. doi: 10.1186/1423-0127-20-35

Turco, S. J., and Descoteaux, A. (1992). The lipophosphoglycan of Leishmania parasites. *Annu. Rev. Microbiol.* 46, 65–94. doi: 10.1146/annurev.mi.46.100192.000433

Turco, S. J., and Sacks, D. L. (1991). Expression of a stage-specific lipophosphoglycan in Leishmania major amastigotes. *Mol. Biochem. Parasitol.* 45, 91–99. doi: 10.1016/0166-6851(91)90030-A

Turco, S. J., Spath, G. F., and Beverley, S. M. (2001). Is lipophosphoglycan a virulence factor? A surprising diversity between Leishmania species. *Trends Parasitol.* 17, 223–226. doi: 10.1016/S1471-4922(01)01895-5

Vinet, A. F., Fukuda, M., Turco, S. J., and Descoteaux, A. (2009). The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. *PLoS Pathog.* 5:e1000628. doi: 10.1371/journal.ppat.1000628

Volf, P., Nogueira, P. M., Myskova, J., Turco, S. J., and Soares, R. P. (2014). Structural comparison of lipophosphoglycan from Leishmania turanica and L. major, two species transmitted by *Phlebotomus papatasi*. *Parasitol. Int.* 63, 683–686. doi: 10.1016/j.parint.2014.05.004

Winberg, M. E., Holm, A., Sarndahl, E., Vinet, A. F., Descoteaux, A., Magnusson, K. E., et al. (2009). *Leishmania donovani* lipophosphoglycan inhibits phagosomal maturation via action on membrane rafts. *Microbes Infect.* 11, 215–222. doi: 10.1016/j.micinf.2008.11.007

Zufferey, R., Allen, S., Barron, T., Sullivan, D. R., Denny, P. W., Almeida, I. C., et al. (2003). Ether phospholipids and glycosylinositolphospholipids are not required for amastigote virulence or for inhibition of macrophage activation by Leishmania major. *J. Biol. Chem.* 278, 44708–44718. doi: 10.1074/jbc.M308063200

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Granulomatous response to *Coxiella burnetii*, the agent of Q fever: the lessons from gene expression analysis

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The formation of granulomas is associated with the resolution of Q fever, a zoonosis due to *Coxiella burnetii*; however the molecular mechanisms of granuloma formation remain poorly understood. We generated human granulomas with peripheral blood mononuclear cells (PBMCs) and beads coated with *C. burnetii*, using BCG extracts as controls. A microarray analysis showed dramatic changes in gene expression in granuloma cells of which more than 50% were commonly modulated genes in response to *C. burnetii* and BCG. They included M1-related genes and genes related to chemotaxis. The inhibition of the chemokines, CCL2 and CCL5, directly interfered with granuloma formation. *C. burnetii* granulomas also expressed a specific transcriptional profile that was essentially enriched in genes associated with type I interferon response. Our results showed that granuloma formation is associated with a core of transcriptional response based on inflammatory genes. The specific granulomatous response to *C. burnetii* is characterized by the activation of type 1 interferon pathway.

Keywords: granuloma, Q fever, *Coxiella burnetii*, BCG, transcriptome, type 1 interferon pathway

INTRODUCTION

Q fever is a worldwide zoonosis caused by *Coxiella burnetii* (Mege et al., 1997). The primary *C. burnetii* infection leads to isolated fever, pneumonia, or hepatitis in 40% of exposed individuals. *C. burnetii* infection may become chronic in patients with valvular lesions, pregnant women, or immuno-compromised patients. In contrast with acute Q fever where the outcome is usually favorable, chronic Q fever is characterized by a long-term drug treatment and persistent risk of relapses. Interestingly, tissue granulomas are present in patients with acute Q fever. In chronic Q fever, granulomas are absent, replaced by lymphocyte infiltrates (Raoult et al., 2005), suggesting that granulomas play an important role in the resolution of Q fever.

Granulomas, defined as tissue collections of macrophages, are generated in response to various microorganisms (Zumla and James, 1996). Their organization varies according to the type of microorganism. In humans, *C. burnetii*-induced granulomas, which are paucibacillary, are composed of a lipid vacuole surrounded by a fibrinoid ring, the "doughnut granuloma" (Srigley et al., 1985; Travis et al., 1986). In contrast, tuberculous granulomas, which are multibacillary, consist of a necrotic core containing bacilli, enclosed by macrophages and surrounded by lymphocytes (Ulrichs and Kaufmann, 2006).

Granulomas are not static organizations but are characterized by continual remodeling and interactions between cell partners (Ramakrishnan, 2012; Shaler et al., 2013). After initial uptake of microorganisms by resident macrophages, the granuloma formation is initiated by recruiting macrophages and blood-derived myeloid cells. The recruitment of activated T-cells by these nascent granulomas completes granuloma formation, and renders them functional (Egen et al., 2008). The main function of granulomas is to contain infectious agents within a limited area, thus restricting the spread of pathogens. Once the infection is contained, the granuloma cells participate in the destruction of infectious agents. Indeed, wild type mice clear mycobacterial infection through granuloma formation whereas mycobacteria disseminate and granulomas are absent in mice that do not express interferon-γ (Cooper et al., 1993). In the majority of patients with tuberculosis, the presence of calcified granulomatous lesions is associated with a controlled infection (Ulrichs and Kaufmann, 2006). However, in others, mycobacteria induce the necrosis of infected macrophages, resulting in a caseum at the center of granulomas. This accumulation of caseum leads to collapsing granulomas and the spread of bacteria (Russell et al., 2009).

Studying granuloma formation in mice requires invasive methods that are not appropriate for human studies. A method was recently developed to generate human granulomas *in vitro* using peripheral blood mononuclear cells (PBMCs) co-cultured with beads coated with BCG (Puissegur et al., 2004; Delaby et al., 2010) or *C. burnetii* extracts (Delaby et al., 2010). This method enables to follow the initial events of granuloma formation and to investigate the molecular mechanisms of granulomas (Egen et al., 2008; Delaby et al., 2012). In this study, we used a

high throughput transcriptomic approach to characterize human granulomas induced *in vitro* by *C. burnetii* and to compare them with those induced by BCG. We found that numerous modulated genes were shared by *C. burnetii*- and BCG-induced granulomas, including chemotaxis-associated genes and M1 genes. *C. burnetii* induced a specific repertoire of upmodulated and downmodulated genes that included the activation of interferon-stimulated genes (ISGs), which confers a new role for this pathway in host response to *C. burnetii*.

MATERIALS AND METHODS

PATIENTS WITH Q FEVER

The study was approved by the Ethics Committee of the Aix-Marseille University. Written informed consent was obtained from each subject. Four patients with acute Q fever and 5 patients with Q fever endocarditis were selected. The diagnostic of acute Q fever was based on serological determination of anti-phase II *C. burnetii* antibodies (Abs). The suspicion of Q fever endocarditis was based on standardized questionnaire that included pathological evidence of endocarditis, positive echocardiograms, positive blood cultures, high titers of IgG specific for phase I *C. burnetii* (Raoult, 2012). The average age of patients with acute Q fever was 43 years old (ranging from 30 to 57 years old). The average age of patients with Q fever endocarditis was 54 years old (ranging from 40 to 74 years old). Six healthy individuals (with a mean age of 37 years, ranging from 28 to 56 years old) were used as controls.

PREPARATION OF CIRCULATING CELLS

PBMCs were prepared from leukopacks (Etablissement Français du Sang) or blood collected in ethylene-diamine-tetraacetic acid (EDTA) tubes from donors and patients after centrifugation through a Ficoll density cushion. Monocytes were isolated from PBMCs by CD14 positive selection using magnetic beads coated with anti-CD14 antibodies (Miltenyi Biotec). $CD14^+$ monocytes were differentiated into macrophages by cell culture (Ghigo et al., 2010). To obtain M1 macrophages, macrophages were stimulated with 20 ng/mL recombinant human IFN-γ (Tebu-bio) for 18 h. M2 macrophages were obtained by incubating macrophages with 10 ng/mL IL-10 or 20 ng/mL IL-4 (R&D Systems) for 18 h.

IN VITRO GENERATION OF GRANULOMAS

Granulomas were induced by using two procedures. First, sepharose beads were coated with bacterial extracts from phase I *C. burnetii* or BCG as previously described (Delaby et al., 2010). PBMCs recovered from leukopacks (2×10^6 per assay) were cultured with 800 coated beads for 8–12 days in the presence of mAbs against CCL2 and CCL5 or control isotypes (R&D Systems). Individual granulomas were then collected by micromanipulation and incubated in 2 mM EDTA, allowing cells to dissociate (Delaby et al., 2012). Second, the granuloma formation in patients with Q fever and healthy donors was determined by incubating PBMCs with *C. burnetii* (Puissegur et al., 2004). PBMCs (2×10^6 cells per assay) were cultured with 2×10^7 heat-killed bacteria (100°C, 1 h) in RPMI 1640 supplemented with fetal calf serum, L-glutamine and antibiotics in 6-well culture plates at 37°C. Cell aggregation was observed every 2 days under light microscopy, and cells were recovered after 8–10 days when the size of aggregates was the highest.

RNA EXTRACTION AND MICROARRAY

Total RNA was extracted from granuloma cells using the RNeasy Mini kit (Qiagen) and DNAse treatment. The granuloma cell gene expression was analyzed using 45,000 probes microarray chips (4×44K whole human genome G4112F, Agilent Technologies) and One-color Microarray Based Gene Expression Analysis kit, as previously described (Ben Amara et al., 2010). Three samples per experimental conditions were included in the analysis. Following array scans, image analysis and correction of intra-array signals were performed with Feature Extraction Software A.10.5.1.1 (Agilent Technologies) using default parameters. Minimum Information About a Microarray Experiment-compliant data are provided in the Gene Expression Omnibus (GEO) (Moal et al., 2013) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/), and can be accessed with the GEO series accession number (GSE37666).

MICROARRAY ANALYSIS

Raw signal data were normalized with a False Discovery Rate below 0.1 and an absolute fold change (FC) value of 3.0. All analyses were performed using R software (version 3) with the bioconductor libraries (Gentleman et al., 2004). Functional annotation was performed using ClueGO plug-in (Bindea et al., 2009) and selecting terms belonging to GO Biological (Moal et al., 2013) in Cytoscape software (Smoot et al., 2011). An enrichment/depletion test, along with the Benjamin-Hochberg correction method, was performed for statistic analysis. GO terms from levels 6 to 8 in GO tree were selected, with a kappa score above 0.44, to create functional annotation network. Each node represents a GO term and contains at least 3 genes. The leading group term of a functional group was defined as the group containing the largest number of genes. Identification of biological groups depicted in the pie chart was established by manual selection of articles from Pubmed database, filtered to show those describing gene functions in immune cells. The identification of M1 and M2 signatures in granuloma cells was performed using the gene profiles of macrophages stimulated with IL-4, IL-10, and IFN-γ referenced in Gene Expression Omnibus database (GSE36537).

REAL-TIME QUANTITATIVE RT-PCR (qRT-PCR)

Reverse transcription of 100 ng of total RNA was performed as previously described (Ben Amara et al., 2010). Primers were designs using Primer3 (Moal et al., 2013) and their sequences were listed in Table 1. Quantitative PCR was performed with Light Cycler Fast Start DNA master^{PLUS} SYBR Green I (Roche applied Science). The results were normalized with the housekeeping gene β-actin. The FC of target genes relative to β-actin was computed using the formula $FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct_{Target} - Ct_{Actin})_{stimulated} - (Ct_{Target} - Ct_{Actin})_{unstimulated}]$ (Moal et al., 2013). The agreement between qRT-PCR and microarray data was assessed by Pearson correlation coefficient.

STATISTICAL ANALYSIS

Comparisons between two groups were performed using the Mann-Whitney *U*-test.

RESULTS

GLOBAL GENE PROGRAM OF GRANULOMA CELLS

We previously reported that PBMCs are capable to form granulomas when they are incubated with beads coated with bacterial extracts (Delaby et al., 2012). We wondered if granuloma formation was associated with specific changes in gene expression programs. We compared the transcriptional profiles of granuloma cells with that of naïve PBMCs using whole genome microarrays. Correspondence analysis was conducted to assess the reproducibility of data. The first axis of variance showed large differences between PBMCs and granuloma cells, while the second axis of variance revealed smaller differences between granuloma cells generated in response to *C. burnetii* and BCG (**Figure 1A**). Hierarchical clustering also showed that the transcriptional responses of granuloma cells were different from that of PBMCs (**Figure 1B**). Most probes that were upmodulated (1337) and downmodulated (1183) were common in *C. burnetii* and BCG challenges. The probes that were specifically modulated by *C. burnetii* included 524 upmodulated probes and 530 downmodulated probes. Conversely, 345 and 247 probes were upmodulated and downmodulated, respectively, by BCG. The expression of only one probe, the *CD163* gene, increased after *C. burnetii* challenge but decreased after BCG challenge (**Figure 1C**). These data showed that about 60% of modulated genes were shared by granuloma cells but that the granulomatous responses to *C. burnetii* and BCG were, in part, specific.

M1/M2 POLARIZATION OF GRANULOMA CELLS

The macrophages are known to be polarized into M1 or M2 cells, which is associated with microbicidal response or permissive response for intracellular bacteria respectively (Benoit et al., 2008). We wondered whether granuloma macrophages, which represent about 40% of granuloma cells (Delaby et al., 2012), were polarized. Nine M1- and 9 M2-related genes (corresponding to 12 and 17 probes, respectively) were selected according to published data (Martinez et al., 2006). Hierarchical clustering analysis showed that granuloma cells clustered with IFN-γ-stimulated macrophages (M1 macrophages) but not with IL-4- or IL-10-stimulated macrophages (M2 macrophages) (**Figure 2A**), demonstrating that granuloma cells exhibited inflammatory/microbicidal phenotype. This finding was confirmed by the measurement of inflammatory cytokines, TNF and IFN-γ, which were released after 9 days (IFN-γ: 204 ± 53 pg/ml; TNF: 1250 ± 250 pg/ml). However, the granulomatous responses to *C. burnetii* and BCG included some features of M2 macrophages. Indeed, *C. burnetii* upmodulated the expression of *CCL23* and *CCL13* genes, and BCG that of *FN1* and *SLCA47* genes. Other differences were evident: *C. burnetii* caused a slight increase in the expression of *TNF* and *EDN1* genes, but increased greatly the expression of *HESX1* and *CXCL9* genes, in comparison with BCG. To confirm microarray results, qRT-PCR was performed on M1 genes (**Figure 2B**). The profiles of each gene were significantly correlated in qRT-PCR and microarray ($R^2 = 0.64$, $p < 0.008$). In addition, the expression of *HESX1* and *TNFSF10* genes was greater in *C. burnetii*-induced granulomas than in BCG-induced granulomas. Conversely, BCG induced a higher increase in the expression of *IDO1* and *TNF* genes

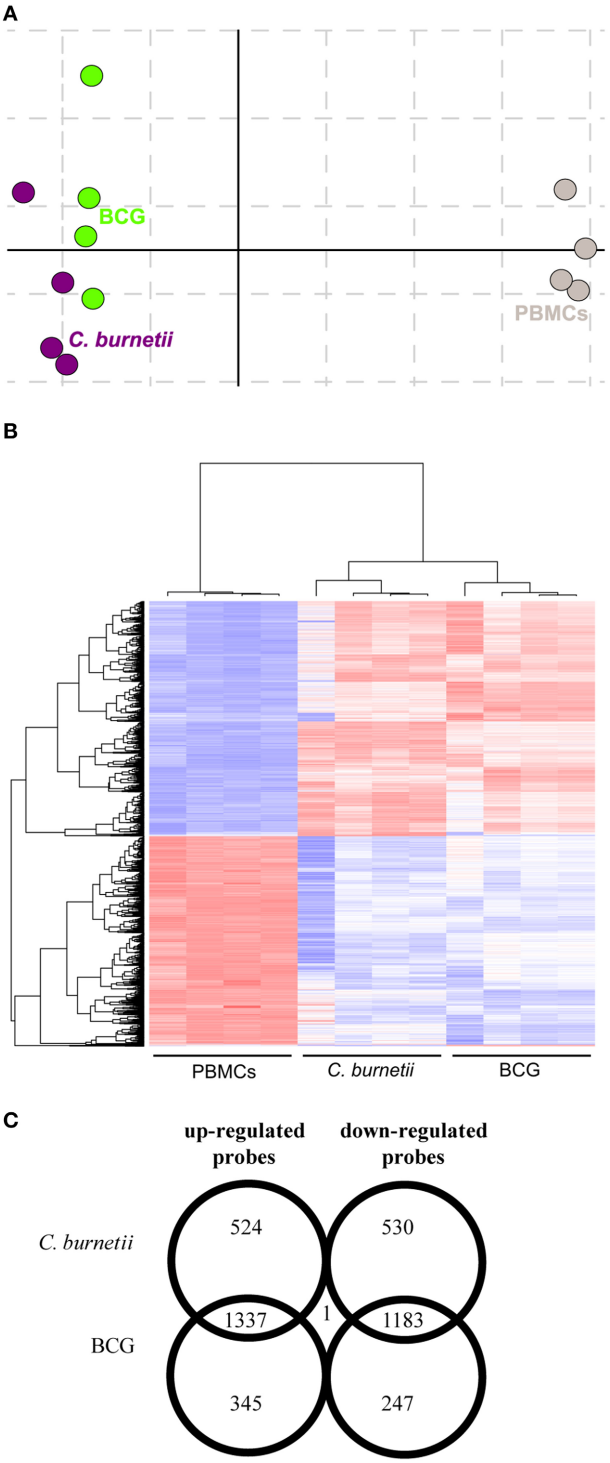

FIGURE 1 | Transcriptional responses of granuloma cells. Granuloma cells and PBMCs were analyzed by microarray. **(A)** The correspondence analysis of the probeset signature revealed that *C. burnetii* and BCG-induced granulomas were clearly separated from PBMCs. **(B)** The hierarchical clustering analysis showed specific clusters of upmodulated and downmodulated probes in granuloma cells compared to PBMCs. Gene expression level is color-coded from blue (downregulation) to red (upregulation). **(C)** The Venn diagram represents the expression of

(Continued)

FIGURE 1 | Continued
upmodulated and downmodulated probes in *C. burnetii* and BCG granuloma compared with PBMCs. The numbers in overlapping regions indicate the number of probes commonly modulated in granuloma cells obtained in response to *C. burnetii* and BCG. The numbers in non-overlapping regions indicate the number of probes specifically modulated in response to *C. burnetii* or BCG.

than *C. burnetii*. Taken together, these results suggest that granuloma macrophages were rather polarized into M1 macrophages, with subtle differences between *C. burnetii*- and BCG-induced granulomas.

FUNCTIONAL ANNOTATION OF *C. BURNETII*-SPECIFIC TRANSCRIPTIONAL PROGRAM

The gene expression program specifically induced by *C. burnetii* was analyzed by retaining only genes differentially modulated between *C. burnetii* and BCG (absolute $FC > 3.0$). We found that 206 genes were specifically modulated, and their roles were studied using GO Biological Process annotation and according to immune function. Nearly 50% of them were related to inflammatory mediators (17%), microbicidal activity (12%), anti-inflammatory mediators (9%) and pathogen recognition (8%). Other cell functions, such as chemotaxis, cell death, and metabolism were also differentially modulated (**Figure 3A**). Taken together, the genes that were specifically modulated by *C. burnetii* were organized into functional networks, suggesting a role in granuloma function.

Next, we investigated how these 206 genes were differently modulated in response to BCG or *C. burnetii* relative to PBMCs. A number of genes involved in chemotaxis were similarly modulated in granuloma cells in response to BCG or *C. burnetii* (**Figure S1**). The genes involved in chemotaxis play a critical role in granuloma formation. This is illustrated by the inhibition of granuloma formation when *C. burnetii*-coated beads were incubated with PBMCs in the presence of mAb directed against CLL2 and CCL5 but not with control isotypes. The mAb directed against CCR5 did not affect granuloma formation (**Figure 3B**). The functional groups associated with inflammation were differently modulated in granulomas induced by *C. burnetii* or BCG. Many genes related to inflammation were downmodulated by *C. burnetii* and upmodulated by BCG. The genes belonging to anti-inflammatory mediators were weakly upmodulated in response to *C. burnetii* but were strongly downmodulated by BCG. Nevertheless, *C. burnetii*-induced granulomas were not only characterized by anti-inflammatory program; indeed, *C. burnetii* did induce several genes related to inflammation such as *TNFSF13*, *CH25H*, and *IRF7* genes. The expression of genes related to cell death was also decreased in *C. burnetii*-generated granulomas, but increased in BCG-induced granulomas. Finally, *C. burnetii* strongly upmodulated the expression of genes involved in microbicidal response and, especially, ISGs including *MX1*, *MX2*, *IFI44*, *IFI6*, *IFIT1*, *IFITM2*, *IFITM3*, *ISG15*, *OAS1*, *OAS2*, *OAS3*, and *HERC5* genes, whereas BCG had little effect on these genes. The transcriptional differences between granulomas induced by *C. burnetii* and BCG compared with PBMCs were confirmed by qRT-PCR performed on several genes. Indeed, the expression of genes encoding inflammatory mediators such as *EPHB2* gene and *EDN1* gene was highly increased in response to *C. burnetii* and BCG, respectively. The expression of genes involved in cell death, such as *FASLG* and *GNLY*, was increased

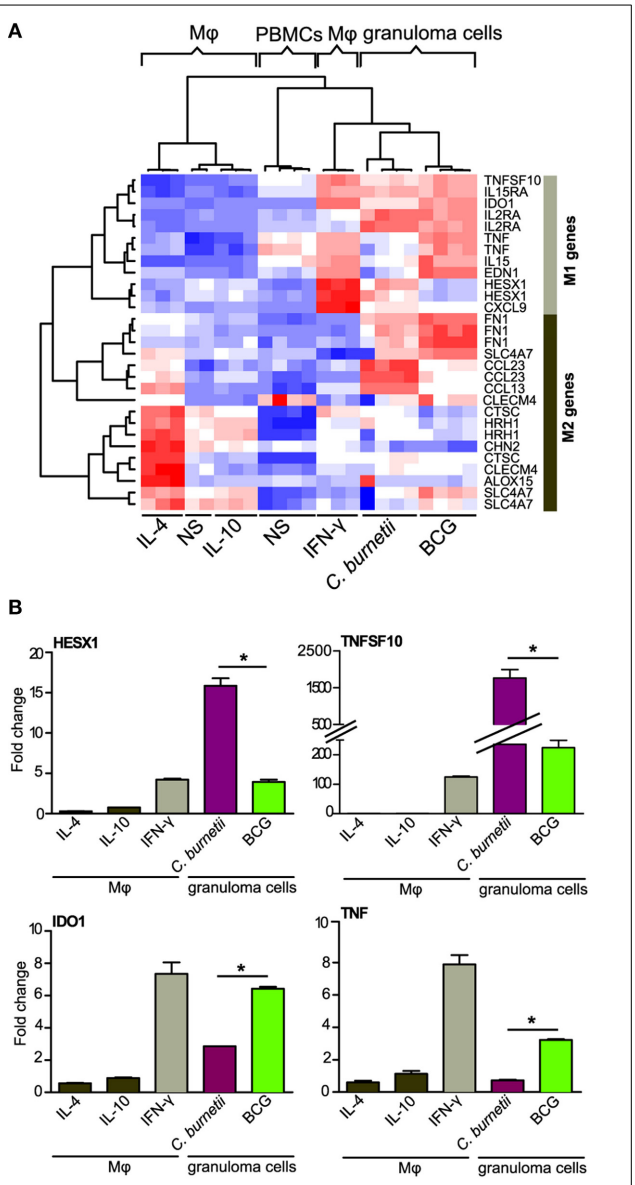

FIGURE 2 | Atypical M1 polarization of granuloma cells. The M1/M2 polarization of granuloma cells was studied using macrophages stimulated with IFN-γ (M1), IL-10, IL-4 (M2) as controls of M1 and M2 polarization. Microarrays **(A)** and qRT-PCR **(B)** were performed on M1/M2 macrophages and granuloma cells. **(A)** The hierarchical clustering of specific markers of M1 or M2 polarization indicates that granuloma cells induced by *C. burnetii* and BCG were located within a unique cluster near M1 macrophages. Note that M2 genes were modulated in granuloma cells as well. Gene expression level is color-coded from blue (downregulation) to red (upregulation). **(B)** The expression of *HESX1, TNFSF10, IDO1,* and *TNF* (M1 genes) was increased in *C. burnetii*- and BCG-induced granulomas, but their expression was differentially modulated in both types of granulomas. $^*p < 0.05$ for the comparison between *C. burnetii* and BCG using Mann-Whitney *U*-test. Mφ, Macrophages; NS, unstimulated.

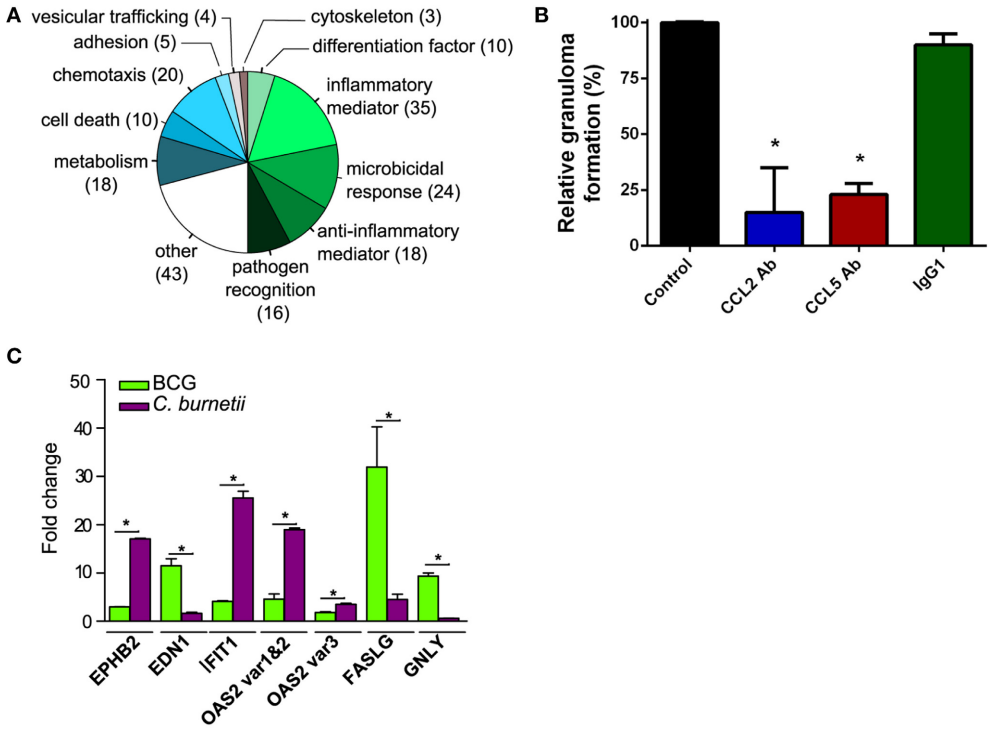

FIGURE 3 | Genes related to immune response in granuloma cells. (A) The genes that were modulated in granulomas induced by *C. burnetii* compared with BCG-induced granulomas were manually classified according to functions previously reported in immune response. The pie chart represents the distribution of modulated genes in each biological group. **(B)** PBMCs were incubated with *C. burnetii*-coated beads in the presence of CCL2 Ab, CCL5 Ab, and IgG1 or in their absence (Control) and the formation of granulomas was measured. Results were expressed as relative granuloma formation. $*p < 0.05$ for the comparison of conditions with and without antibodies. **(C)** The expression of *EPHB2, EDN1, IFIT1, OAS2, FASLG,* and *GNLY* genes in granuloma cells was confirmed by qRT-PCR. $*p < 0.05$ for the comparison between *C. burnetii* and BCG using Mann-Whitney *U*-test.

in response to BCG. In contrast, the expression of ISGs (*IFIT1* and *OAS2*) was highly upmodulated in granulomas induced by *C. burnetii* (**Figure 3C**). Taken together, these results showed that the granulomatous response shared common features, but also included specific characteristics, according to the nature of the pathogen. They also showed that the granulomas induced by *C. burnetii* were characterized by the activation of type 1 IFN genes.

TRANSCRIPTIONAL RESPONSE OF GRANULOMAS IN Q FEVER PATIENTS

We finally inquired whether the granulomatous response of patients with Q fever was associated with alteration in type 1 IFN pathway. First, we developed a simple method to obtain granulomas by incubating control PBMCs with *C. burnetii* to simplify the recovery of isolated granulomas using *C. burnetii*-coated beads. Cell aggregates were observed after a few days. Their size progressively increased and was greatest at 8–10 days, demonstrating that the time course of granuloma formation was similar when granulomas were generated *in vitro* using beads coated with bacterial extracts (Delaby et al., 2012) or heat-killed bacteria. We investigated the expression of *IFIT1* and *OAS2*, two genes belonging to type 1 IFN pathway which were specifically modulated in *C. burnetii*-induced granulomas. The expression of *IFIT1* (**Figure 4A**) and *OAS2* (**Figure 4B**) genes was strongly upmodulated in stimulated PBMCs, but not in unstimulated PBMCs. We noted that the levels of expression of *IFIT1* and *OAS2* genes were similar to those obtained with isolated granuloma cells (see **Figure 3C**). The expression of *IFIT1* genes (**Figure 4C**) and *OAS2* genes (**Figure 4D**) was similar in *C. burnetii*-induced granulomas from patients with Q fever and healthy controls. These results demonstrated that type 1 IFN pathway was not altered in Q fever granulomas.

DISCUSSION

The favorable outcome of Q fever is associated with the presence of granulomas (Raoult et al., 2005), but the real functions of granulomas during *C. burnetii* infection remain unknown. Therefore, we employed a technique that generated *in vitro* human granulomas (Delaby et al., 2010), then performed whole genome transcriptional profile of *C. burnetii*-induced granulomas and we compared it to that induced by BCG.

More than 50% of genes that were modulated in granulomas, were commonly modulated by *C. burnetii* and BCG. First, they included genes involved in chemotaxis, especially those related to the recruitment of monocytes and lymphocytes, such as *CCL2* (Loetscher et al., 1994), *CCL8* (Loetscher et al., 1994), *CCL13* (Garcia-Zepeda et al., 1996), *CCL17* (Cronshaw et al., 2006), and *CCL18* (Adema et al., 1997) genes. This finding is consistent with the critical role of the recruitment of monocytes and lymphocytes

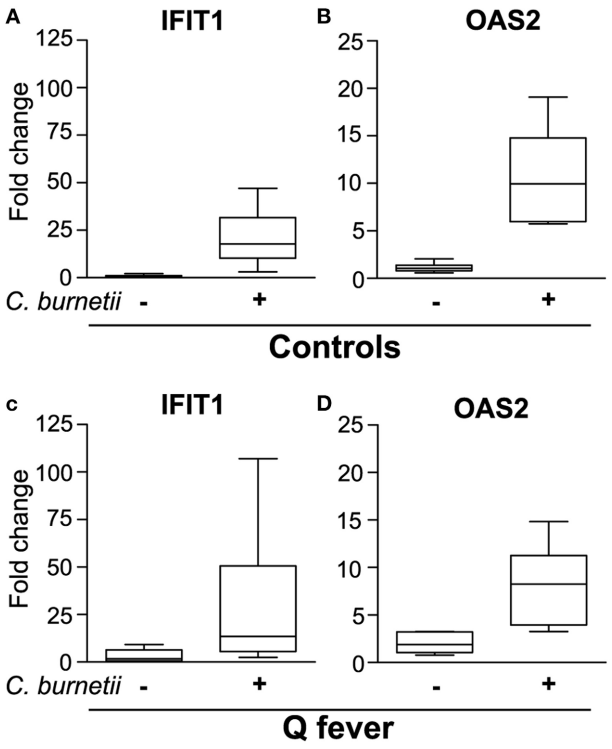

FIGURE 4 | Expression of ISGs in granulomas generated in Q fever patients. PBMCs from healthy donors **(A,B)** and patients with Q fever **(C,D)** were stimulated with heat-killed *C. burnetii* for 9 days and analyzed for the expression of IFIT1 **(A,C)** and OAS2 **(B,D)** by qRT-PCR.

in *in vitro*-generated granulomas (Delaby et al., 2012). Our data extend to human granulomas the role of chemokines initially described in animal models (Chensue, 2013). Hence, in mice deficient for CCR2, a receptor for CCL2 and CCL8, the number and size of granulomas, as well as monocyte recruitment at site of infection, are decreased (Jinnouchi et al., 2003). We provided evidence that the neutralization of CCL2 was sufficient to prevent the formation of granulomas in response to *C. burnetii* and BCG. As reported above in CCR2 deficient mice, it is likely that CCL2 is involved in the initial stages of granuloma formation. Similar results were obtained with the neutralization of CCL5. This result may be related to the model of CCL5 ko mice in which granuloma function is transiently impaired (Vesosky et al., 2010). In addition, granuloma cells in intestinal tissues from patients with Crohn's disease express CCL5 (Oki et al., 2005). It has been suggested that CCL5 via its interaction with CCR5 augments type 2 granuloma formation (Chensue, 2013). The role of CCL2 and CCL5 in granuloma formation in response to *C. burnetii* is likely not redundant. We hypothesize that a temporal regulation of chemokines is necessary for granuloma formation. Second, granulomas are essentially composed of macrophages, which were similarly polarized in response to *C. burnetii* and BCG. Indeed, granuloma cells expressed M1 profile with some features of M2 cells. This finding is markedly distinct from isolated macrophages infected with *C. burnetii* that express an atypical M2 profile (Benoit et al., 2008). This difference between isolated macrophages and macrophages involved in a functional unit such as granulomas has been reported in mice infected with *Mycobacterium tuberculosis*. Indeed, granuloma-associated macrophages are polarized into M1 cells whereas macrophages surrounding granulomas shift from an M1 to an M2 profile (Redente et al., 2010). In addition, the loss of M2 macrophages during *Leishmania major* infection delays disease progression and increases resistance to pathogens (Hölscher et al., 2006). We hypothesize that the microenvironment of granulomas sustains M1 polarization to eradicate pathogens.

The transcriptional response of *C. burnetii*-generated granulomas was, in part, specific. First, inflammatory genes were differentially modulated. Indeed, *C. burnetii* downmodulated the expression of *TNF*, *NCR3*, *EDN1* and genes related to the Th1 profile, such as *IFNG, TBX21*, which encodes the transcription factor tbet, *IL18RAP*, *IL26*, and *HOPX* genes. Murine macrophages infected with *Leishmania major*, a granuloma-inducing pathogen, are unable to produce EDN1 (Wahl et al., 2005). A dramatic decrease in inflammatory cytokines (IFN-γ, IL12-p40) was observed in granulomas from BCG-vaccinated guinea pigs induced by *M. tuberculosis* (Ly et al., 2007). Second, *C. burnetii* specifically induced the expression of *CH25H*, *TNFSF13*, and *IRF7* genes, known to be related to type 1 IFN pathway (Park and Scott, 2010; Tezuka et al., 2011) and likely involved in microbicidal response of granuloma cells. For instance, CH25H is an enzyme involved in production of oxysterol, 25-hydroxycholesterol. Oxysterols are known to bind liver X receptor (LXR) and its deficiency is associated with loss of microbicidal competence and apoptosis (Joseph et al., 2004). TNFSF13 likely plays a role in Th1 polarization and TNFSF13-deficient mice exhibit defective bacterial clearance (Xiao et al., 2008). Taken together, these results suggest that the activation of inflammatory genes may limit *C. burnetii* infection in specific granulomas directly or indirectly via type 1 IFN pathway.

The granuloma cells induced by *C. burnetii* exhibited upmodulated expression of numerous genes belonging to type 1 IFN pathway; most of these genes were ISGs. Although ISGs have been associated initially with antiviral response (Schoggins et al., 2011), many recent studies report their upregulation in response to bacteria and bacterial components, such as lipopolysaccharide (Textoris et al., 2012). Type 1 IFN signaling has also been implicated in efficient clearance of group B *Streptococcus*, *S. pneumoniae*, and *Escherichia coli* (Textoris et al., 2012). Indeed, injection of *C. burnetii* components in mice induces a transient IFN-α/β production, and a steady synthesis of OAS for several days (Zvilich et al., 1995). This finding is potentially important for the defense against *C. burnetii* since type 1 IFN was either not detected or its pathway was defective in macrophages or dendritic cells stimulated by *C. burnetii*, respectively (Gorvel et al., 2014). This may be related to previous reports, which state that a BCG mucoprotein is unable to induce the expression of interferon-inducible antiviral proteins (Ishii et al., 2005). The stimulation of type 1 IFN pathway (*OAS2* and *IFIT1* genes) was similar in granulomas from healthy controls or patients with Q fever. ISGs may therefore play an important role in the anti-infectious activity of *C. burnetii* granulomas.

In conclusion, the transcriptional response of *C. burnetii* granulomas includes a common part shared with other infectious granulomas and a part specific for *C. burnetii*. The specific response to *C. burnetii* involved the activation of genes involved in inflammation including type 1 IFN related genes, microbicidal activity, and apoptosis. This transcriptional program may account for the granuloma-mediated elimination of *C. burnetii*, in accordance with the natural history of Q fever where granulomas are associated with a protective immune response and resolution of the disease, whereas the absence of granulomas is associated with the chronic evolution of the disease.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00172/abstract

Figure S1 | Functional annotation of *C. burnetii*- and BCG-modulated genes. The expression of the modulated genes in *C. burnetii*- and BCG-induced granulomas was compared to their expression in PBMCs. The functional groups that contain the largest number of distinctly modulated genes were selected, and genes were depicted. The direction of gene regulation is color-coded, with blue indicating downregulation, and red indicating upregulation.

REFERENCES

Adema, G. J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., et al. (1997). A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 387, 713–717. doi: 10.1038/42716

Ben Amara, A., Ghigo, E., Le Priol, Y., Lépolard, C., Salcedo, S. P., Lemichez, E., et al. (2010). *Coxiella burnetii*, the agent of Q fever, replicates within trophoblasts and induces a unique transcriptional response. *PLoS ONE* 5:e15315. doi: 10.1371/journal.pone.0015315

Benoit, M., Barbarat, B., Bernard, A., Olive, D., and Mege, J.-L. (2008). *Coxiella burnetii*, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur. J. Immunol.* 38, 1065–1070. doi: 10.1002/eji.200738067

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., et al. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25, 1091–1093. doi: 10.1093/bioinformatics/btp101

Chensue, S. W. (2013). Chemokines in innate and adaptive granuloma formation. *Front. Immunol.* 4:43. doi: 10.3389/fimmu.2013.00043

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., and Orme, I. M. (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* 178:2243. doi: 10.1084/jem.178.6.2243

Cronshaw, D. G., Kouroumalis, A., Parry, R., Webb, A., Brown, Z., and Ward, S. G. (2006). Evidence that phospholipase-C dependent, calcium-independent mechanisms are required for directional migration of T-lymphocytes in response to the CCR4 ligands CCL17 and CCL22. *J. Leukoc. Biol.* 79, 1369–1370.

Delaby, A., Espinosa, L., Lépolard, C., Capo, C., and Mège, J.-L. (2010). 3D reconstruction of granulomas from transmitted light images implemented for long-time microscope applications. *J. Immunol. Methods* 360, 10–19. doi: 10.1016/j.jim.2010.06.008

Delaby, A., Gorvel, L., Espinosa, L., Lépolard, C., Raoult, D., Ghigo, E., et al. (2012). Defective monocyte dynamics in Q fever granuloma deficiency. *J. Infect. Dis.* 205, 1086–1094. doi: 10.1093/infdis/jis013

Egen, J. G., Rothfuchs, A. G., Feng, C. G., Winter, N., Sher, A., and Germain, R. N. (2008). Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity* 28, 271–284. doi: 10.1016/j.immuni.2007.12.010

Garcia-Zepeda, E. A., Combadiere, C., Rothenberg, M. E., Sarafi, M. N., Lavigne, F., Hamid, Q., et al. (1996). Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *J. Immunol.* 157, 5613–5626.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80. doi: 10.1186/gb-2004-5-10-r80

Ghigo, E., Barry, A. O., Pretat, L., Al Moussawi, K., Desnues, B., Capo, C., et al. (2010). IL-16 promotes *T. whipplei* replication by inhibiting phagosome conversion and modulating macrophage activation. *PLoS ONE* 5:e13561. doi: 10.1371/journal.pone.0013561

Gorvel, L., Textoris, J., Banchereau, R., Ben Amara, A., Tantibhedhyangkul, W., von Bargen, K., et al. (2014). Intracellular bacteria interfere with dendritic cell functions: role of the type I interferon pathway. *PLoS ONE* 9:e99420. doi: 10.1371/journal.pone.0099420

Hölscher, C., Arendse, B., Schwegmann, A., Myburgh, E., and Brombacher, F. (2006). Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice. *J. Immunol.* 176, 1115–1121. doi: 10.4049/jimmunol.176.2.1115

Ishii, K., Kurita-Taniguchi, M., Aoki, M., Kimura, T., Kashiwazaki, Y., Matsumoto, M., et al. (2005). Gene-inducing program of human dendritic cells in response to BCG cell-wall skeleton (CWS), which reflects adjuvancy required for tumor immunotherapy. *Immunol. Lett.* 98, 280–290. doi: 10.1016/j.imlet.2004.12.002

Jinnouchi, K., Terasaki, Y., Fujiyama, S., Tomita, K., Kuziel, W. A., Maeda, N., et al. (2003). Impaired hepatic granuloma formation in mice deficient in C-C chemokine receptor 2. *J. Pathol.* 200, 406–416. doi: 10.1002/path.1362

Joseph, S. B., Bradley, M. N., Castrillo, A., Bruhn, K. W., Mak, P. A., Pei, L., et al. (2004). LXR-dependent gene expression is important for macrophage survival and the innate immune response. *Cell* 119, 299–309. doi: 10.1016/j.cell.2004.09.032

Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M., and Moser, B. (1994). Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human $CD4^+$ and $CD8^+$ T lymphocytes. *FASEB J.* 8, 1055–1060.

Ly, L. H., Russell, M. I., and McMurray, D. N. (2007). Microdissection of the cytokine milieu of pulmonary granulomas from tuberculous guinea pigs. *Cell. Microbiol.* 9, 1127–1136. doi: 10.1111/j.1462-5822.2006.00854.x

Martinez, F. O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* 177, 7303–7311. doi: 10.4049/jimmunol.177.10.7303

Mege, J. L., Maurin, M., Capo, C., and Raoult, D. (1997). *Coxiella burnetii*: the "query" fever bacterium. A model of immune subversion by a strictly intracellular microorganism. *FEMS Microbiol. Rev.* 19, 209–217. doi: 10.1016/S0168-6445(96)00030-7

Moal, V., Textoris, J., Ben Amara, A., Mehraj, V., Berland, Y., Colson, P., et al. (2013). Chronic hepatitis E virus infection is specifically associated with an interferon-related transcriptional program. *J. Infect. Dis.* 207, 125–132. doi: 10.1093/infdis/jis632

Oki, M., Ohtani, H., Kinouchi, Y., Sato, E., Nakamura, S., Matsumoto, T., et al. (2005). Accumulation of $CCR5^+$ T cells around $RANTES^+$ granulomas in Crohn's disease: a pivotal site of Th1-shifted immune response? *Lab. Invest.* 85, 137–145. doi: 10.1038/labinvest.3700189

Park, K., and Scott, A. L. (2010). Cholesterol 25-hydroxylase production by dendritic cells and macrophages is regulated by type I interferons. *J. Leukoc. Biol.* 88, 1081–1087. doi: 10.1189/jlb.0610318

Puissegur, M.-P., Botanch, C., Duteyrat, J.-L., Delsol, G., Caratero, C., and Altare, F. (2004). An *in vitro* dual model of mycobacterial granulomas to investigate the molecular interactions between mycobacteria and human host cells. *Cell. Microbiol.* 6, 423–433. doi: 10.1111/j.1462-5822.2004.00371.x

Ramakrishnan, L. (2012). Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* 12, 352–366. doi: 10.1038/nri3211

Raoult, D. (2012). Chronic Q fever: expert opinion versus literature analysis and consensus. *J. Infect.* 65, 102–108. doi: 10.1016/j.jinf.2012.04.006

Raoult, D., Marrie, T., and Mege, J. L. (2005). Natural history and pathophysiology of Q fever. *Lancet Infect. Dis.* 5, 219–226. doi: 10.1016/S1473-3099(05)70052-9

Redente, E. F., Higgins, D. M., Dwyer-Nield, L. D., Orme, I. M., Gonzalez-Juarrero, M., and Malkinson, A. M. (2010). Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation. *J. Leukoc. Biol.* 88, 159–168. doi: 10.1189/jlb.0609378

Russell, D. G., Cardona, P.-J., Kim, M.-J., Allain, S., and Altare, F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat. Immunol.* 10, 943–948. doi: 10.1038/ni.1781

Schoggins, J. W., Wilson, S. J., Panis, M., Murphy, M. Y., Jones, C. T., Bieniasz, P., et al. (2011). A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472, 481–485. doi: 10.1038/nature09907

Shaler, C. R., Horvath, C. N., Jeyanathan, M., and Xing, Z. (2013). Within the Enemy's Camp: contribution of the granuloma to the dissemination, persistence and transmission of *Mycobacterium tuberculosis*. *Front. Immunol.* 4:30. doi: 10.3389/fimmu.2013.00030

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P.-L., and Ideker, T. (2011). Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* 27, 431–432. doi: 10.1093/bioinformatics/btq675

Srigley, J. R., Vellend, H., Palmer, N., Phillips, M. J., Geddie, W. R., Van Nostrand, A. W., et al. (1985). Q-fever. The liver and bone marrow pathology. *Am. J. Surg. Pathol.* 9, 752–758. doi: 10.1097/00000478-198510000-00007

Textoris, J., Capo, C., and Mège, J.-L. (2012). "Type I interferons and bacterial infectious diseases: new features," in *Recent Research Developments in Immunology*, ed S. G. Pandalai (Trivandrum: Research Signpost), 49–74.

Tezuka, H., Abe, Y., Asano, J., Sato, T., Liu, J., Iwata, M., et al. (2011). Prominent role for plasmacytoid dendritic cells in mucosal T cell-independent IgA induction. *Immunity* 34, 247–257. doi: 10.1016/j.immuni.2011.02.002

Travis, L. B., Travis, W. D., Li, C. Y., and Pierre, R. V. (1986). Q fever. A clinicopathologic study of five cases. *Arch. Pathol. Lab. Med.* 110, 1017–1020.

Ulrichs, T., and Kaufmann, S. H. E. (2006). New insights into the function of granulomas in human tuberculosis. *J. Pathol.* 208, 261–269. doi: 10.1002/path.1906

Vesosky, B., Rottinghaus, E. K., Stromberg, P., Turner, J., and Beamer, G. (2010). CCL5 participates in early protection against *Mycobacterium tuberculosis*. *J. Leukoc. Biol.* 87, 1153–1165. doi: 10.1189/jlb.1109742

Wahl, J. R., Goetsch, N. J., Young, H. J., Van Maanen, R. J., Johnson, J. D., Pea, A. S., et al. (2005). Murine macrophages produce endothelin-1 after microbial stimulation. *Exp. Biol. Med. (Maywood)* 230, 652–658.

Xiao, Y., Motomura, S., and Podack, E. R. (2008). APRIL (TNFSF13) regulates collagen-induced arthritis, IL-17 production and Th2 response. *Eur. J. Immunol.* 38, 3450–3458. doi: 10.1002/eji.200838640

Zumla, A., and James, D. G. (1996). Granulomatous infections: etiology and classification. *Clin. Infect. Dis.* 23, 146–158. doi: 10.1093/clinids/23.1.146

Zvilich, M., Williams, J. C., Waag, D., Rill, W. R., Malli, R. J., Bell, P., et al. (1995). Characterization of the non-specific humoral and cellular antiviral immunity stimulated by the chloroform-methanol residue (CMR) fraction of *Coxiella burnetii*. *Antiviral Res.* 27, 389–404. doi: 10.1016/0166-3542(95)00022-E

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Myeloid decidual dendritic cells and immunoregulation of pregnancy: defective responsiveness to *Coxiella burnetii* and *Brucella abortus*

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Dendritic cells (DCs) are a component of the placental immune system, but their role in pregnancy is still poorly understood. Decidual DCs (dDCs) were selected from at-term pregnancy on the basis of CD14 and CD11c expression. A phenotypic analysis revealed that dDCs are characterized by the expression of monocyte-derived DC (moDCs) markers and specific markers such as HLA-G and its ligand ILT4. As demonstrated by whole-genome microarray, dDCs expressed a specific gene program markedly distinct from that of moDCs; it included estrogen- and progesterone-regulated genes and genes encoding immunoregulatory cytokines, which is consistent with the context of foeto-maternal tolerance. A functional analysis of dDCs showed that they were unable to mature in response to bacterial ligands such as lipopolysaccharide or peptidoglycan, as assessed by the expression of HLA-DR, CD80, CD83, and CD86. When dDCs were incubated with bacteria known for their placenta tropism, *Coxiella burnetii* and *Brucella abortus*, they were also unable to mature and to produce inflammatory cytokines. It is likely that the defective maturation of dDCs and their inability to produce inflammatory cytokines is related to the spontaneous release of IL-10 by these cells. Taken together, these results suggest that dDCs exhibit an immunoregulatory program, which may favor the pathogenicity of *C. burnetii* or *B. abortus*.

Keywords: placenta, dendritic cell, phenotype, microarray, immunoregulation

INTRODUCTION

Dendritic cells (DCs) are sentinels that instruct the adaptive immune system at the interface of the host with environment. Following an encounter with microorganisms, they develop a maturation program associated with their migration to draining lymph nodes. The maturation program of DCs includes loss of endocytosis ability, dramatic changes in surface markers such as CD80, CD83, CD86, and membrane translocation of MHC class II molecules. Once mature, DCs are able to present the antigen to resting T cells (Banchereau and Steinman, 1998). The maturation program of DCs can be induced by microbial components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) and modulated by the cytokine context. Hence, interferon (IFN)-γ or Tumor Necrosis Factor (TNF) drive inflammatory activation while interleukin (IL)-4, IL-10, or Transforming Growth Factor (TGF)-β induce an immunoregulatory response of DCs. This leads to Th1 or Th2 response, respectively (Akdis et al., 2012; Dzopalic et al., 2012). The functional properties of human DCs are dependent on DC location. Skin DCs are composed of epidermal Langerhans cells and different subtypes of dermal DCs that favor cell-mediated and antibody-mediated responses (Von Bubnoff et al., 2004; Kaplan et al., 2005; He et al., 2006). Intestinal DCs are essential to instruct the immune system about the presence of penetrating microorganisms but also to maintain tolerance toward commensals (Fleeton et al., 2004).

The placenta is a tissue dedicated to the exchange between mother and fetus and to feto-maternal tolerance. This latter relies on the presence of immune cells, mainly consisting of NK cells but also T lymphocytes, macrophages, and DCs (Erlebacher, 2013). While the role of NK cells and macrophages in feto-maternal tolerance is now being understood (Ben Amara et al., 2013; Erlebacher, 2013), the role of DCs remains unclear. DCs are present at the feto-maternal interface (Tagliani and Erlebacher, 2011), cycling endometrium and decidua. Their number is relatively low as compared to placenta macrophages (Erlebacher, 2013). The placenta-associated DCs are heterogeneous. Further, it has been described that decidua may contain mature DCs expressing CD83, which are found in clusters with CD3 T cells (Kämmerer et al., 2000), and immature DCs expressing CD14 and DC-SIGN (dendritic cell specific ICAM-grabbing non integrin, CD209) (Kämmerer et al., 2003). Decidual DCs (dDCs) play both antigen-presenting role and immunoregulatory role (Miyazaki et al., 2003; Blois et al., 2007; Gregori et al., 2010; Amodio et al., 2013).

In this report, we isolated and characterized dDCs from at-term placentas. They expressed classical phenotypic DC markers and also HLA-G and ILT4. The dDCs were characterized by a gene program in which estrogen and progesterone-regulated genes and genes encoding immunoregulatory cytokines were enriched. These DCs were unable to mature in response to bacteria-derived ligands such as LPS or PGN, and to bacteria known for their placenta tropism such as *Coxiella burnetii* and *Brucella abortus*. The spontaneous secretion of IL-10 combined with the defective production of inflammatory cytokines likely accounts for the immunoregulatory profile of dDCs. These results suggest that dDCs play an immunoregulatory role in feto-maternal tolerance, which is not broken down by *C. burnetii* and *B. abortus* and may contribute to their pathogenicity.

MATERIALS AND METHODS

PREPARATION OF PLACENTAL CELLS

Fifteen at-term placentas obtained by vaginal delivery were collected in the Gynecology-Obstetrics Department of the Hôpital de la Conception (Marseille, France) after written informed consent of healthy pregnant women. The study was approved by the Ethics Committee from Aix-Marseille University (N° 08-012). The placenta samples (approximately 150 g) were incubated in a solution consisting of Hank's Balanced Salt Solution (HBSS, Invitrogen, Cergy Pontoise, France), $MgSO_4$, DNase I (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 2.5% trypsin (Invitrogen) buffered with HEPES for 45 min and were then incubated for 30 min under gentle agitation at 37°C, as described previously (Ben Amara et al., 2013). The digestion products were then filtered through 100-μm pores, incubated in 50-ml tubes containing 2 ml fetal calf serum (FCS) and centrifuged at 1000× *g* for 15 min. The cells were counted, deposited on a Ficoll cushion and centrifuged at 700× *g* for 20 min. Mononuclear cells were recovered, and macrophages were discarded using magnetic beads coated with anti-CD14 Abs (Miltenyi Biotech, Paris, France). $CD14^-$ cells were recovered and $CD11c^+$ cells were sorted using magnetic beads (Miltenyi Biotec) coupled with anti-CD11c antibodies (Abs, Beckman Coulter, Villepinte, France). The purity of $CD11c^+$ cells was higher than 95%.

Trophoblasts were isolated as previously described (Salcedo et al., 2013) with slight modifications. Briefly, isolated cells from placental samples were deposited on 25 and 60% Percoll (Sigma-Aldrich) phases and centrifuged at 1200× *g* for 20 min. Trophoblasts were isolated using anti-epidermal growth factor R (EGFR) Abs (Santa Cruz, Heidelberg, Germany) coupled to magnetic beads (Miltenyi Biotech). The purity of isolated trophoblasts was checked by flow cytometry using EGFR Abs and was higher than 96%. Trophoblasts were cultured in DMEM-F12 containing 10% FCS and antibiotics. Cell supernatants were collected 2 days after confluence and stored at −20°C.

PREPARATION OF moDCs

Blood from healthy donors was provided by the Etablissement Français du Sang (Marseille, France). Peripheral blood mononuclear cells (PBMCs) from buffy coats were recovered from the Ficoll-Hypaque interface after a 700× *g* centrifugation for 20 min. Monocytes were isolated from PBMCs using magnetic beads coupled with Abs specific for CD14, as previously described (Gorvel et al., 2014). Monocyte purity was higher than 98%. To obtain moDCs, monocytes were incubated in RPMI 1640 containing 20 mM HEPES, 2 mM glutamine, 10% FCS, 1 ng/ml IL-4, and 1 ng/ml granulocyte macrophage colony-stimulating factor (R&D Systems, Lille, France) for 7 days. The purity of moDCs was assessed by the absence of CD14 and the presence of CD11c, and purity was higher than 98%.

STIMULATION OF moDCs AND dDCs

moDCs and dDCs (2×10^5 cells per assay) were stimulated with *Escherichia coli* LPS (Sigma-Aldrich, 100 ng/ml) and PGN (Sigma-Aldrich, 1 μg/ml) for 18 h. They were also incubated with *C. burnetii* (MOI 20:1) and *B. abortus* (MOI 20:1) for 18 h. *C. burnetii* organisms (RSA493 Nile Mile strain) were obtained by culture in L929 cells, as previously described (Barry et al., 2012). *B. abortus* strain 2308 was grown on tryptic soy agar (Sigma-Aldrich) at 37°C for 4–5 days, as previously described (Pizarro-Cerdá et al., 1998).

FLUORESCENCE MICROSCOPY

The moDCs and dDCs (10^5 cells per assay) were cultured on glass slides for 18 h. After fixation in 3% paraformaldehyde for 15 min, they were permeabilized by 0.1% TritonX-100 for 2 min and then incubated for 30 min with bodipy phallacidin (Invitrogen) to label filamentous actin (F-actin). Cell nuclei were labeled with DAPI (Invitrogen) for 10 min and slides were mounted on Mowiol (Invitrogen). Pictures were taken using a confocal microscope DMI16000 (Leica, Nanterre, France) and analyzed using Image J software (National Institute of Health, USA).

In some experiments, moDCs and dDCs were incubated with *C. burnetii* and *B. abortus* for 18 h. *C. burnetii* and *B. abortus* organisms were revealed using human and bovine specific Abs, respectively. Secondary Abs consisted of anti-human and -bovine Abs coupled with 555 Alexa fluor. Pictures were taken using a confocal microscope DMI16000 (Leica, Nanterre, France) and merged using Image J software (National Institute of Health, USA). Superposition of red and green labeling induced yellow color on the picture.

FLOW CYTOMETRY

The moDCs and dDCs (10^5 cells per assay) were incubated with HLA-DR and CD11c Abs (Beckman Coulter) in 400 μl of PBS containing 2% BSA for 30 min at 4°C. moDCs and plaDCs were then incubated with DC-SIGN, ASGPR, MARCO, Dectin-1, HLA-ABC, CD80, CD83, CD86, HLA-G, ILT4, BDCA-1 mAbs or isotypic controls (Beckman Coulter, Villepinte, France) for 30 min. They were then labeled with aquadead Amcyan (CellTrace) to exclude dead cells, as recommended by the manufacturer. After centrifugation, moDCs and dDCs were fixed in 3% paraformaldehyde for 15 min, washed in phosphate-buffered saline and analyzed using a Canto II flow cytometer associated with the software FACS Diva (Becton Dickinson, Pont de Claix, France). The dDCs were gated according CD11c expression and approximately 10,000 events were numerated. The results are given in percentage of positive cells.

MICROARRAYS

Total RNA of moDCs and dDCs (2×10^6 cells per well) was extracted using the RNeasy minikit (Qiagen, Courtaboeuf, France) and DNAse treatment (Gorvel et al., 2014). The expression of modulated genes was analyzed using 4X44k Human Whole Genome microarrays (Agilent Technologies, Les Ulis, France) and three biological replicates per experimental condition, as recently described (Ben Amara et al., 2010). Sample labeling and hybridization were performed using One-Color Microarray-Based Gene Expression Analysis. Slides were scanned at a 5 μm resolution with a G2505C DNA microarray scanner (Agilent Technologies, Les Ulis, France). Image analysis and intra-array signal corrections were performed using Agilent Feature Extractor Software A.9.1.3.

Microarray data analysis was performed using the R (v.2.15) and Bioconductor libraries, as recently described (Moal et al., 2013). In brief, raw data were filtered and normalized using the Agi4x44PreProcess library. Unsupervised and supervised analyses were carried out using hierarchical clustering, principal component analysis (PCA) (made4 library, Culhane et al., 2005), and Significance Analysis of Microarray (SAM) algorithm (siggenes library). Genes were considered to be differentially expressed when absolute fold change (FC) was above 2.0. Functional enrichment analysis was performed on selected genes with DAVID tools (Dennis et al., 2003), using the Gene Ontology (GO) (Ashburner et al., 2000), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Okuda et al., 2008) pathways. Functional pathways were designed using the Cytoscape and Inkscape softwares.

WESTERN BLOTTING

The moDCs and dDCs (10^6 cells per assay) were scrapped in ice-cold RIPA buffer, as previously described (Barry et al., 2012). Proteins were separated by electrophoresis (40 μg of loaded proteins) and transferred onto nitrocellulose membranes (Amersham, Courtaboeuf, France). The membranes were probed with mouse Abs directed against chorionic somatomammotropin hormone 1 (CSH-1), also known as placental lactogen hormone, or α-tubulin (R&D Systems) for 18 h. The blots were incubated with horseradish peroxidase-conjugated Abs directed against mouse IgG (Pierce, Rockford, IL, USA). Bound Abs were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore). The expression of CSH-1 was quantified by densitometric scanning and was normalized against α-tubulin. The results are expressed as relative intensities.

IMMUNOASSAYS

Supernatants from stimulated moDCs and dDCs were collected and freezed at −80°C. The concentrations of released cytokines were assessed using commercial ELISA kits. The sensitivity of IL-10 and IL-12p70 kits (R&D Systems) is 3.9 pg/ml and 2.5 pg/ml, respectively, and that of IL-6 kit (Becton Dickinson) is 4 pg/ml. The intra- and inter-variability of kits was less than 10%.

STATISTICAL ANALYSIS

The results are expressed as the means ± SD and were compared using the non-parametric Mann–Whitney *U*-test. When the comparisons involved more than two conditions, the analysis was made with ANOVA test. *P*-values less than 0.05 were considered significant.

RESULTS

PHENOTYPIC CHARACTERIZATION OF dDCs

We isolated myeloid DCs from decidual mononuclear cells by combining negative selection with anti-CD14 Abs and positive selection with anti-CD11c Abs. The morphology of the resulting $CD14^-CD11c^+$ DC subset, called dDCs, was similar to that of moDCs, another type of myeloid DCs, using bodipy phallacidin and confocal microscopy (**Figure 1A**). We then analyzed dDC expression of classical myeloid DC markers, including MARCO (macrophage R with collagenous structure), ASGPR (ascialoglycoprotein receptor), Dectin-1, DC-SIGN and BDCA-1 using flow cytometry. Both dDCs and moDCs were positive for CD11c. Less than 15% of dDCs and moDCs expressed membrane MARCO;

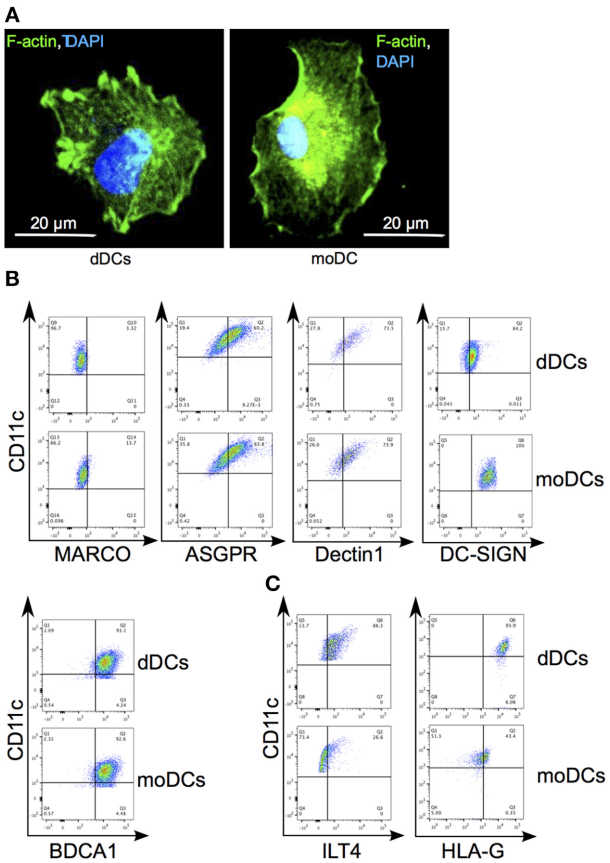

FIGURE 1 | Phenotypic characterization of dDCs. (A) dDCs and moDCs were labeled with bodipy phallacidin (in green) and DAPI (in blue). One representative DC is shown. **(B)** The expression by dDCs of canonical molecules of DCs was assessed by flow cytometry. Dot-plots represent the expression of MARCO, ASGPR, DC-SIGN, and Dectin1 by $CD11c^+$ cells. Quadrant gate represent the limit set by isotype control analysis. **(C)** The expression of HLA-G and ILT4 by plaDCs and moDCs by $CD11c^+$ cells was assessed by flow cytometry. Quadrant gates represent the limit set by isotype control analysis. Dot-plots are representative of five different placentas.

more than 50% of dDCs and moDCs expressed BDCA-1, ASGPR and Dectin-1; DC-SIGN was largely expressed by dDCs and moDCs (**Figure 1B**). In contrast, dDCs strongly expressed HLA-G and its interacting molecule ILT4, which were poorly and not expressed by moDCs, respectively (**Figure 1C**). Taken together, these results show that dDCs differed from moDCs on the basis of HLA-G and ILT4 expression.

TRANSCRIPTIONAL ANALYSIS OF dDCs

As dDCs were phenotypically close to moDCs with the exception of the specific expression of HLA-G and ILT-4, we wondered if the analysis of gene expression would reveal specific transcriptional features. We found that 1525 genes were significantly modulated in dDCs compared with moDCs (using a $FC > 2.0$ and a False Discovery Rate (FDR) < 0.01) (**Figure 2A**). They consisted of

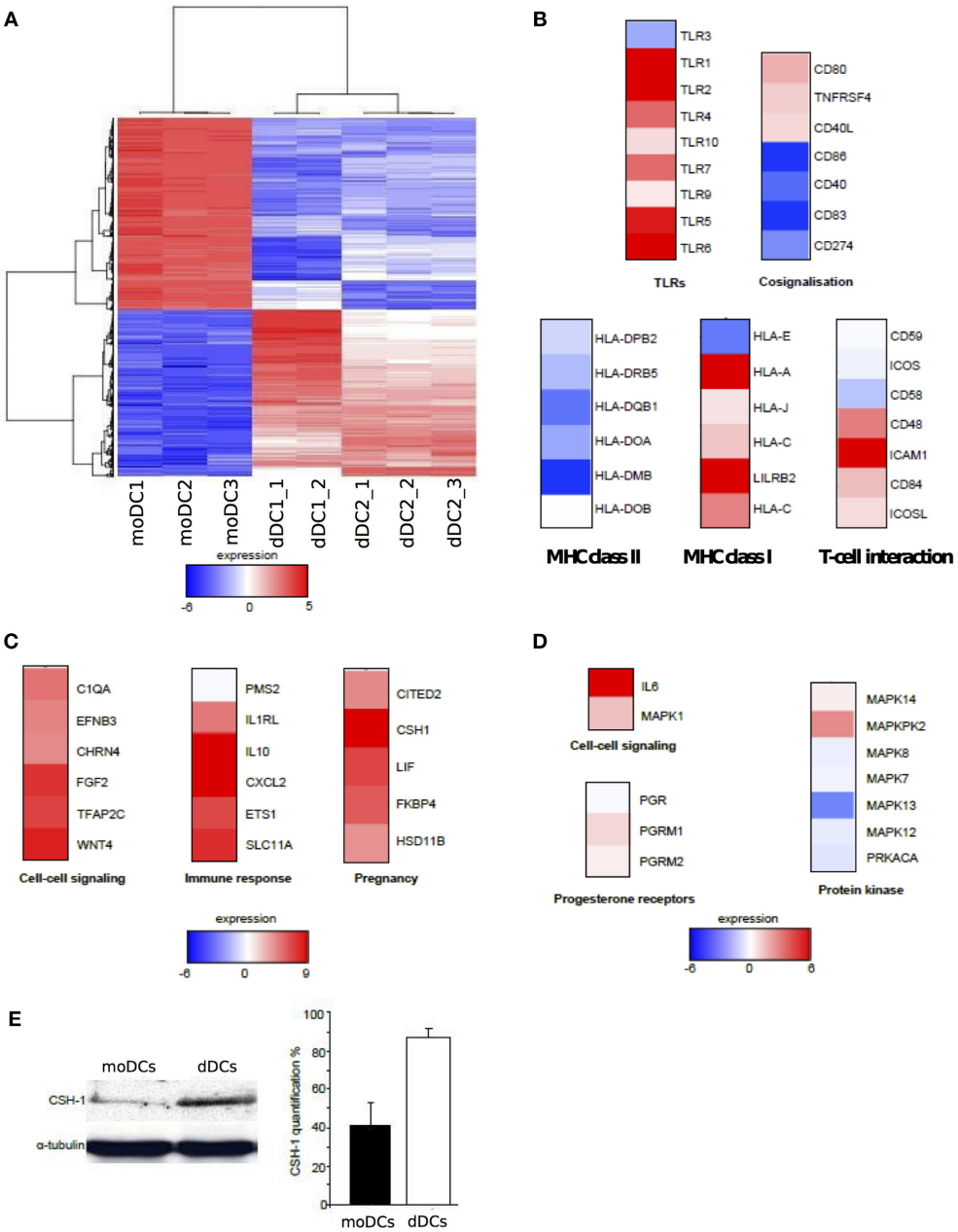

FIGURE 2 | Transcriptional analysis of dDCs. (A–D) dDCs and moDCs were recovered, and microarray analysis was performed after RNA extraction. Only genes modulated in dDCs compared with moDCs were retained and the heatmap represents up-modulated (in red) and down-modulated (in blue) expression of genes **(A)** The functional annotation of the genes related to DC markers and those modulated in dDCs compared to moDCs is presented **(B)** Estrogen- and progesterone-modulated genes (**C** and **D**, respectively) were analyzed in dDCs compared with moDCs and functional annotations are shown. **(E)** The production of CSH-1 by dDCs and moDCs was assessed by western blot. The quantification was performed by densitometric scanning after normalization with α-tubulin. The results are representative of three different placentas.

672 up-modulated genes and 853 down-modulated genes. We then selected clusters of genes involved in DC phenotype and functions, namely pathogen recognition (TLRs), co-signalisation and antigen presentation (MHC class I and II molecules, T-cell interaction), and investigated their transcriptional expression (**Figure 2B**). The expression of the genes encoding TLR1, TLR2, TLR4, TLR5, TLR6, and TLR7 was up-modulated in dDCs, whereas the expression of TLR9 and TLR10 was similar in dDCs and moDCs. TLR3 was found to be slightly depressed in dDCs. Most of the genes involved in DC co-signalisation function, *CD86*, *CD40*, *CD83*, and *CD274* (also known as *PDL1*), and those encoding MHC class II molecules were strongly down-modulated. In contrast, the majority of the genes encoding MHC class I molecules, and specifically *HLA-G* and its ligand *ILT4* (*LILRB2*) were up-modulated in dDCs. Finally, among the genes involved in the interaction with T-cells, *ICAM1* was highly up-modulated (**Figure 2B**). Taken together, these results showed that most of the genes involved in DC functions were down-modulated in dDCs compared with moDCs with the exception of *TLR* genes and genes encoding MHC class I molecules.

The second feature of transcriptional signature of dDCs is the impact of the placenta microenvironment. As estrogens and progesterone are major components of placenta microenvironment, we investigated estrogen and progesterone-regulated genes in dDCs compared with moDCs. We found that 17 genes known to be regulated by estrogens and 12 progesterone-regulated genes were modulated in dDCs. The 17 estrogen-regulated genes belong to the GO terms "cell–cell signaling," "immune response" and "pregnancy". These genes were up-modulated with the exception of PMS2 (**Figure 2C**). The 12 progesterone-modulated genes consisted of "cell–cell signaling," "progesterone receptors and sub-units," and "protein kinase" GO terms. These genes were poorly modulated at the exception of the *IL6* gene that was highly up-modulated (**Figure 2D**). The *CSH1* gene was highly expressed in dDCs and CSH-1 is strongly produced during pregnancy (Huddleston and Schust, 2004). We determined the presence of CSH-1 in dDCs and moDCs by immunoblotting. We found that dDCs, but not moDCs, constitutively produced CSH-1 (**Figure 2E**). These results suggested that the differences between dDCs and moDCs rely largely on their hormonal microenvironment, especially estrogens.

MODULATION OF CYTOKINE PATHWAYS IN dDCs

Since dDCs exhibited a transcriptional program in which genes associated with DC maturation were essentially down-modulated, we wondered if signaling pathways were altered. We selected two pathways, IL-10 and TGF-β known for their role in fetal tolerance. We found that the expression of the genes encoding IL-10 and TGF-β was higher in dDCs than in moDCs (**Figure 3**). In the *IL10* pathway, the genes encoding IL-10R (*IL10RA* and *IL10RB*) were also up-modulated, but effector molecules such as *STAT5* and *CREBBP* were down-modulated. It is noteworthy that *STAT5* inhibitors (*PIAS3* and *FKBP4* genes) were up-modulated in dDCs. The *TGF*β pathway was mainly up-modulated in dDCs, especially *SMAD* molecules. Only *SMAD6*, which is an inhibitor of the SMAD signaling cascade, was down-modulated. Finally, genes encoding nuclear transcription factors such as CITED2 (MSG related protein 1), MYC and SP1 were up-modulated whereas *CREBBP* and *CITED1* genes were down-modulated (**Figure 3**). Taken together, these results suggested that the *TGF*β pathway was fully active in dDCs whereas the *IL10* pathway was partially activated.

MATURATION OF dDCs AND RESPONSE TO MICROBIAL LIGANDS

As the transcriptional signature of dDCs reflects an immunoregulatory profile, we investigated their ability to mature in the presence of LPS or PGN, known to induce the maturation of moDCs, as determined by the membrane expression of HLA-DR, CD80, CD83, and CD86. In the absence of stimulation, HLA-DR was strongly expressed by moDCs. In contrast, 90% of dDCs weakly expressed HLA-DR whereas only 8% of dDCs expressed HLA-DR at a high level. LPS and PGN increased the expression of HLA-DR in moDCs, but were unable to increase the membrane expression of HLA-DR in the majority of dDCs (**Figure 4A**). The expression of CD80, CD83, and CD86 was similar in unstimulated dDCs and moDCs. LPS and PGN markedly increased the expression of CD80, CD83, and CD86 in moDCs but were unable to substantially increase their expression in dDCs (**Figure 4A**). Hence, dDCs were unable to fully mature in response to TLR ligands. We then investigated the ability of dDCs to release inflammatory and immunoregulatory cytokines. The unstimulated production of IL-12p70 and IL-6 was similar in dDCs and moDCs. While LPS and PGN stimulated IL-12p70 and IL-6 release by moDCs, no effect was observed in dDCs (**Figure 4B**). The profile of IL-10 production was completely different. Indeed, unstimulated dDCs, but not moDCs, spontaneously released high levels of IL-10. The stimulation of dDCs by TLR ligands such as LPS and PGN did not affect the release of IL-10 by dDCs but markedly increased IL-10 release by moDCs (**Figure 4B**). Taken together, these results show that dDCs were unable to mature in response to ligands that induce the maturation of moDCs and to produce inflammatory cytokines in response to TLR ligands.

MATURATION OF dDCS AND RESPONSE TO INTRACELLULAR BACTERIA

The inability of dDCs to mature in response to TLR ligands may create a favorable context for intracellular bacteria and specifically for the bacteria with a tropism for placenta such as *C. burnetii* and *B. abortus*. We tested the ability of *C. burnetii* and *B. abortus* to induce the membrane expression of DC maturation markers. The expression of HLA-DR, CD80, CD83, and CD86 was increased in *C. burnetii*-stimulated moDCs and that of CD80 and CD86 in *B. abortus*-stimulated moDCs. In contrast, *C. burnetii* did not affect the expression of HLA-DR, CD80, CD83, and CD86 by dDCs. The stimulation of dDCs by *B. abortus* remained silent at the exception of a weak increased expression of CD86 (**Figure 5A**). We verified that the lack of response to bacteria was not due to a lack of interaction. *C. burnetii* and *B. abortus* entered DC as shown in **Figure 5B**. These results showed that dDCs did not mature in response to *C. burnetii* and *B. abortus*, suggesting an intrinsic deficiency of dDC maturation.

DISCUSSION

In this paper, we characterized myeloid dDCs from third-trimester placentas. This DC population was isolated by negative selection through a CD14 column, followed by a positive

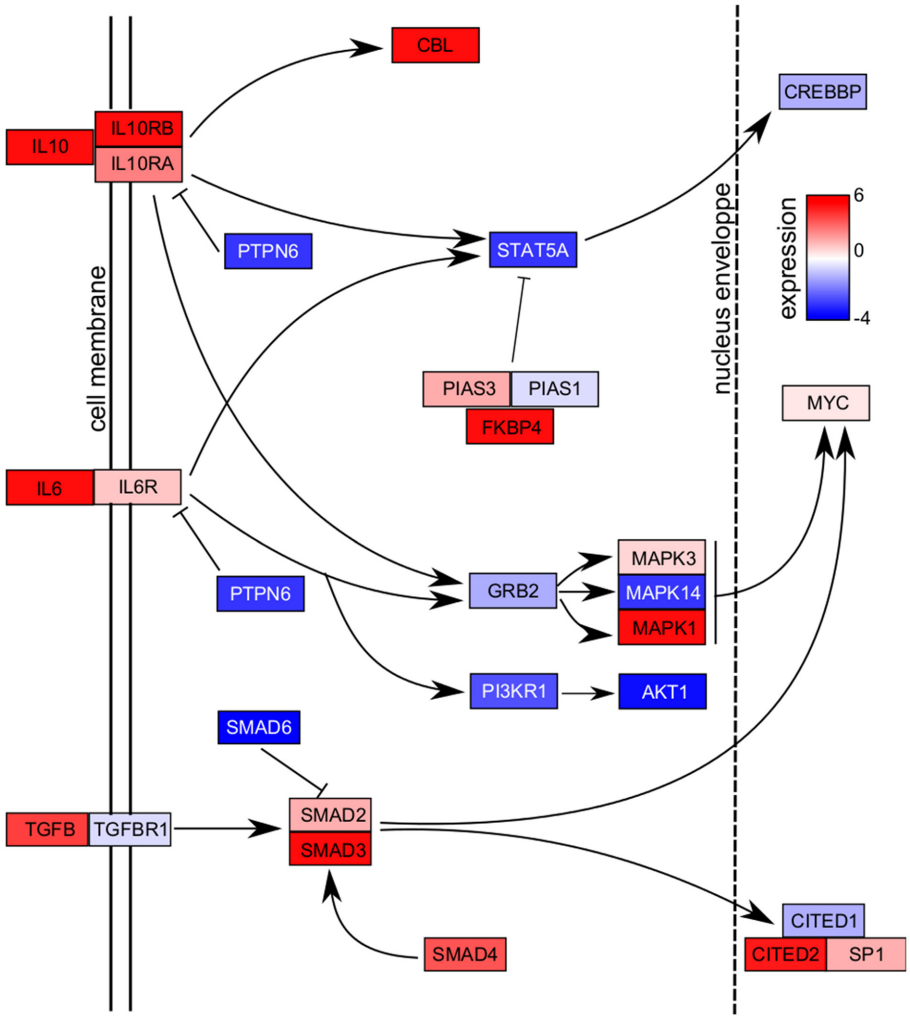

FIGURE 3 | Cytokine pathways. The dDCs and moDCs were recovered, and microarray analysis was performed after RNA extraction. The genes modulated in dDCs compared with moDCs were retained and the *IL10*, *IL6*, and *TGFB* pathways are presented. Up-modulated molecules are shown in red and down-modulated molecules in blue. Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog; CBL, E3 ubiquitin protein ligase; CITED, Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain; CREBBP, CREB-binding protein; FKBP4, F506-binding protein 4; GRB, growth factor R-bound protein; MYC, v-myc avian myelocytomatosis viral oncogene homolog; PIAS, protein inhibitor of activated STAT; PTPN6 protein tyrosine phosphatase non receptor type 6; SP, specificity protein; STAT5A, signal transducer and activator of transcription 5A.

selection through CD11c column. This procedure excluded placenta macrophages that highly express CD14 (Ben Amara et al., 2013) and also myeloid DCs that express CD14 and DC-SIGN (Erlebacher, 2013). The $CD14^{-}CD11c^{+}$ dDCs expressed BDCA-1, ASGPR, Dectin-1, and DC-SIGN. The expression of lectins such as ASGPR and Dectin-1 is characteristic of immature DCs. Indeed, IL-10-producing $CD4^{+}$ T cells maintain DC in an immature status in which ASGPR is expressed (Kaisho and Akira, 2003; Li et al., 2012). DC-SIGN is expressed mainly by immature DCs and defective signaling through DC-SIGN may be involved in immune tolerance (Valladeau et al., 2001; Geijtenbeek et al., 2004). The $CD11c^{+}BDCA1^{+}$ dDCs are also present in the first trimester (Erlebacher, 2013), suggesting a stability of DC subsets during pregnancy.

We also provided evidence that dDCs were characterized by a specific transcriptional repertoire when compared with moDCs. They expressed TLR2 and TLR4, which are involved in the recognition of PGN and LPS, respectively (Kaisho and Akira, 2003), suggesting that dDCs recognize gram-negative and gram-positive bacteria. Interestingly, the genes encoding MHC class II molecules and associated pathways were down-modulated in dDCs compared with moDCs whereas the expression of genes encoding MHC class I molecules and associated pathways were mostly up-modulated. We can suppose that dDCs had a lower ability to process and present vacuolar antigens. The transcriptional signature of cytokines also evokes an immunoregulatory role for dDCs. Indeed, *IL6* and *IL10* genes were up-modulated in dDCs as compared with moDCs even if their downstream

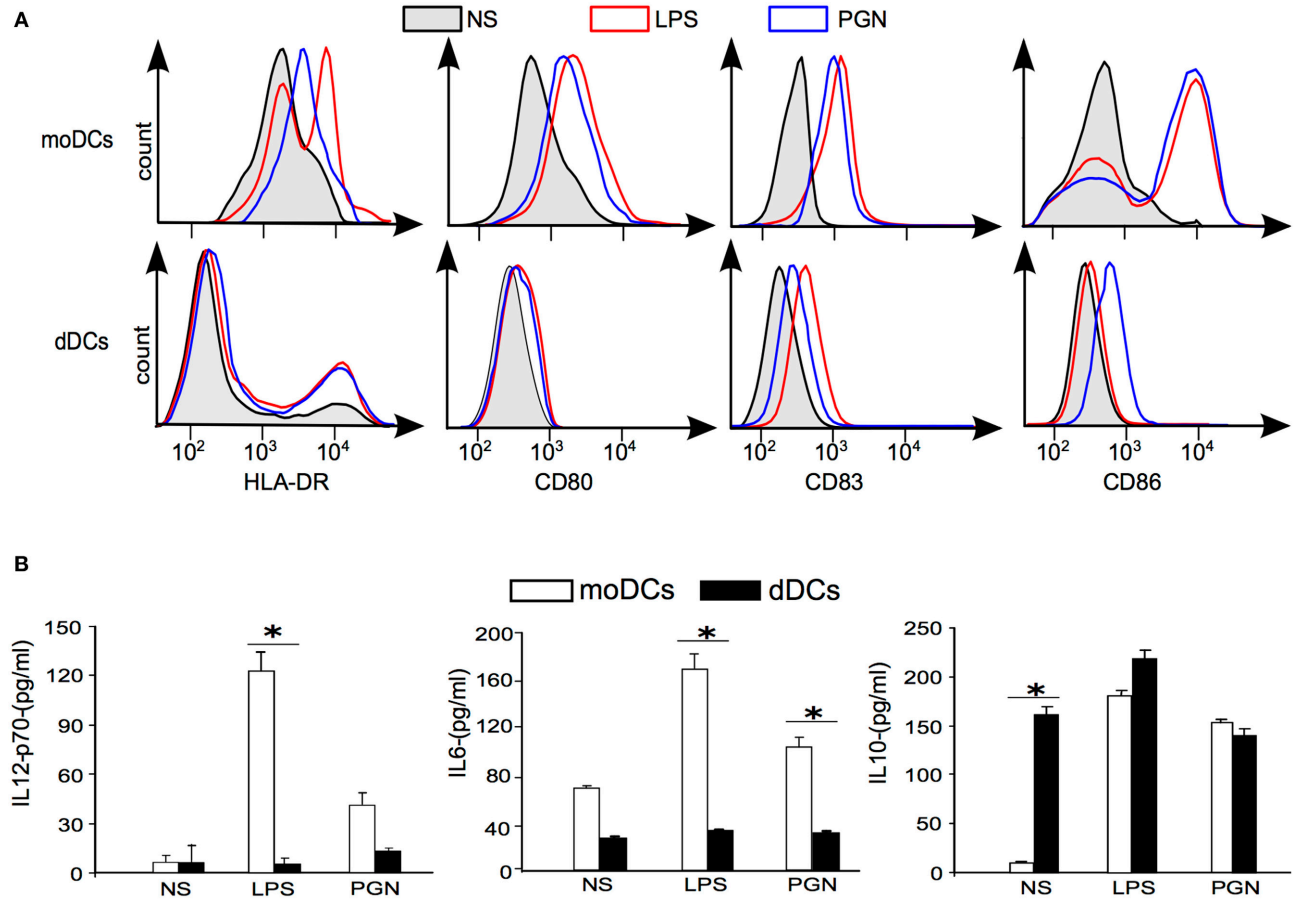

FIGURE 4 | plaDCs and TLR ligands. (A) The expression of maturation markers was determined by flow cytometry in moDCs and dDCs stimulated with LPS or PGN for 18 h. Black lines and gray areas represent naive moDCs and dDCs; in red, LPS stimulation; in blue, PGN stimulation. **(B)** moDCs and dDCs were either stimulated or not by LPS or PGN for 18 h. Cytokine release was assessed by ELISA, and the results representative of five different experiments are expressed in pg/ml. "∗" represent significative variations between conditions (+/− SD).

signaling effectors were down-modulated. The *TGF*β pathway was clearly up-modulated in dDCs, suggesting that this pathway may play a role in fetal tolerance (Svajger et al., 2010; Erlebacher, 2013). The comparison of the transcriptional program of dDCs with that of decidual $CD14^+$ macrophages and multinucleated giant cells (MGCs) revealed that dDCs were close to $CD14^+$macrophages with which they exhibited a common placental signature. They were markedly distinct from moDCs and macrophages derived from monocytes or MGC (Supplementary Material).

As phenotypic and transcriptional features of dDCs suggested a tolerogenic signature, we tested the ability of dDCs to mature in response to TLR agonists known to induce the maturation of moDCs. A few proportions of dDCs expressed HLA-DR in contrast to moDCs upon stimulation with microbial ligands. In addition, these latter increased only marginally the membrane expression of CD83 and CD86 in dDCs. This was markedly different from first trimester $CD83^+$ dDCs that express a mature DC phenotype (Kämmerer et al., 2000). We also found that dDCs were poorly inflammatory. Indeed, they did not release significant levels of IL-6 and IL-12p70 in response to LPS and PGN in contrast to moDCs. The dDCs spontaneously released high levels of IL-10, as previously found for trophoblasts, decidual macrophages, and uterine NK cells (Huddleston and Schust, 2004). As a consequence, LPS and PGN were unable to increase the release of IL-10 by dDCs while these agonists dramatically increased the release of IL-10 by moDCs. This demonstrates that dDCs were hyporeactive to inflammatory agonists. The tolerogenic signature of dDCs may prevent the development of immune response to intracellular bacteria with placenta tropism. Hence, *C. burnetii* and *B. abortus* were unable to induce their maturation This response was specific because *C. burnetii* or *B. abortus* were able to induce the maturation of moDCs. It has been also reported that dDCs locally present the antigen to decidual T cells in ways that minimize Th1 responses and reinforce the immunodepression associated with pregnancy, thus favoring the replication of pathogens within the placenta, such as *Listeria monocytogenes* (Abram et al., 2003), *C. burnetii* (Ben Amara et al., 2010) or *B. abortus* (Salcedo et al., 2013).

The mechanisms underlying dDC properties that favor the persistence of intracellular bacteria may involve other mechanisms than the lack of inflammatory cytokines. Indeed, dDCs

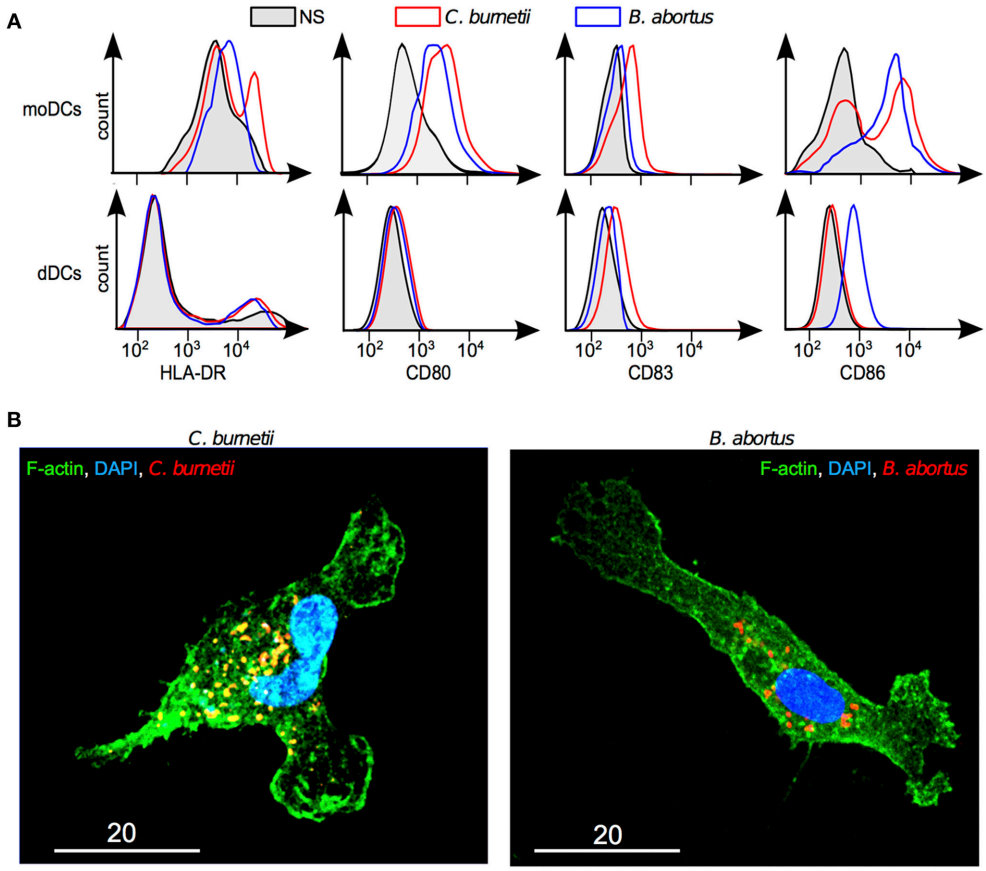

FIGURE 5 | Response of dDCs to *C. burnetii* and *B. abortus*. DDCs were incubated with *C. burnetii* or *B. abortus* for 18 h. **(A)** The expression of maturation markers was determined by flow cytometry in moDCs and dDCs stimulated or not by *C. burnetii* or *B. abortus*. In black and gray areas, naive moDCs and dDCs; in red, *C. burnetii* stimulation; in blue, *B. abortus* stimulation. The results are representative for five experiments. **(B)** After washing, bacteria were labeled with specific Abs, F-actin with bodipy phallacidin and nuclei with DAPI. dDCs were observed by confocal microscopy. The micrographs reveal bacteria (in red or yellow), F-actin (in green) and nuclei (in blue).

expressed specific molecules such as HLA-G and its ligand ILT4. HLA-G is known for its immunosuppressive properties in normal and pathological conditions (Ristich et al., 2005). In the presence of soluble HLA-G tetramers, moDCs are not able to completely mature (McIntire and Hunt, 2005). The interaction of HLA-G with ILT4 limits trophoblast lysis by NK cells and increases the production of TGF-β and immunosuppressive cytokines in decidual macrophages and $CD83^+$ DCs from first trimester (McIntire and Hunt, 2005). Sex hormones likely play a major role in the activity of placenta cells. It has been shown that progesterone increases the expression of HLA-G and induces a tolerogenic profile in uterine DCs (Szekeres-Bartho et al., 2009). Even if estrogens have been described as enhancers of DC maturation (Hughes and Clark, 2007; Nofer, 2012; Seillet et al., 2013), they have also been described as inhibiting DC maturation during RNA-virus infections (Escribese et al., 2008) and limiting the production of defensins by moDCs and myeloid DCs (Escribese et al., 2011). It is likely that estrogens are more potent than progesterone to affect the functional activity of dDCs, as suggested by microarray analysis. Among the estrogen-regulated genes, we found that *CSH1* gene and CSH-1 protein that is involved in prolactin secretion were highly up-modulated in dDCs. CSH-1 and prolactin participate to normal development of pregnancy because a decreased production of prolactin or CSH-1, as found in preeclampsia, an inflammatory disease of the placental tissue, results in retarded growth (Männik et al., 2012). We hypothesized that the hormonal context affected the ability of dDCs to mature and to produce inflammatory cytokines.

In this report, we found that dDCs exhibit specific features in addition to the markers of myeloid DCs. They were unable to mature and to produce inflammatory cytokines in response to agonists known to induce DC maturation and were strongly influenced by their tolerogenic hormonal microenvironment. These properties may contribute to the feto-maternal tolerance and to the pathogenicity of intracellular bacteria with placenta tropism, as dDCs were also unable to respond properly to such pathogens.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/journal/10.3389/fcimb.2014.00179/abstract

REFERENCES

Abram, M., Schlüter, D., Vuckovic, D., Wraber, B., Doric, M., and Deckert, M. (2003). Murine model of pregnancy-associated *Listeria monocytogenes* infection. *FEMS Immunol. Med. Microbiol.* 35, 177–182. doi: 10.1016/S0928-8244(02)00449-2

Akdis, M., Palomares, O., van de Veen, W., van Splunter, M., and Akdis, C. A. (2012). TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection. *J. Allergy Clin. Immunol.* 129, 1438–1449. doi: 10.1016/j.jaci.2012.05.003

Amodio, G., Mugione, A., Sanchez, A. M., Viganò, P., Candiani, M., Somigliana, E., et al. (2013). HLA-G expressing DC-10 and CD4(+) T cells accumulate in human decidua during pregnancy. *Hum. Immunol.* 74, 406–411. doi: 10.1016/j.humimm.2012.11.031

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245–252. doi: 10.1038/32588

Barry, A. O., Boucherit, N., Mottola, G., Vadovic, P., Trouplin, V., Soubeyran, P., et al. (2012). Impaired stimulation of p38α-MAPK/Vps41-HOPS by LPS from pathogenic *Coxiella burnetii* prevents trafficking to microbicidal phagolysosomes. *Cell Host Microbe.* 12, 751–763. doi: 10.1016/j.chom.2012.10.015

Ben Amara, A., Ghigo, E., Le Priol, Y., Lépolard, C., Salcedo, S. P., Lemichez, E., et al. (2010). *Coxiella burnetii*, the agent of Q fever, replicates within trophoblasts and induces a unique transcriptional response. *PLoS ONE* 5:e15315. doi: 10.1371/journal.pone.0015315

Ben Amara, A., Gorvel, L., Baulan, K., Derain-Court, J., Buffat, C., Vérollet, C., et al. (2013). Placental macrophages are impaired in chorioamnionitis, an infectious pathology of the placenta. *J. Immunol.* 191, 5501–5514. doi: 10.4049/jimmunol.1300988

Blois, S. M., Kammerer, U., Soto, C. A., Tometten, M. C., Shaikly, V., Barrientos, G., et al. (2007). Dendritic cells: key to fetal tolerance? *Biol. Reprod.* 77, 590–598. doi: 10.1095/biolreprod.107.060632

Culhane, A. C., Thioulouse, J., Perrière, G., and Higgins, D. G. (2005). MADE4: an R package for multivariate analysis of gene expression data. *Bioinformatics* 21, 2789–2790. doi: 10.1093/bioinformatics/bti394

Dennis, G. Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., et al. (2003). DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* 4, P3. doi: 10.1186/gb-2003-4-5-p3

Dzopalic, T., Rajkovic, I., Dragicevic, A., and Colic, M. (2012). The response of human dendritic cells to co-ligation of pattern-recognition receptors. *Immunol. Res.* 52, 20–33. doi: 10.1007/s12026-012-8279-5

Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annu. Rev. Immunol.* 31, 387–411. doi: 10.1146/annurev-immunol-032712-100003

Escribese, M. M., Kraus, T., Rhee, E., Fernandez-Sesma, A., López, C. B., and Moran, T. M. (2008). Estrogen inhibits dendritic cell maturation to RNA viruses. *Blood* 112, 4574–4584. doi: 10.1182/blood-2008-04-148692

Escribese, M. M., Rodríguez-García, M., Sperling, R., Engel, S. M., Gallart, T., and Moran, T. M. (2011). Alpha-defensins 1-3 release by dendritic cells is reduced by estrogen. *Reprod. Biol. Endocrinol.* 9:118. doi: 10.1186/1477-7827-9-118

Fleeton, M. N., Contractor, N., Leon, F., Wetzel, J. D., Dermody, T. S., and Kelsall, B. L. (2004). Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. *J. Exp. Med.* 200, 235–245. doi: 10.1084/jem.20041132

Geijtenbeek, T. B. H., van Vliet, S. J., Engering, A., Hart, B. A., and van Kooyk, Y. (2004). Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu. Rev. Immunol.* 22, 33–54. doi: 10.1146/annurev.immunol.22.012703.104558

Gorvel, L., Textoris, J., Banchereau, R., Ben Amara, A., Tantibhedhyangkul, W., von Bargen, K., et al. (2014). Intracellular bacteria interfere with dendritic cell functions: role of the type I interferon pathway. *PLoS ONE* 9:e99420. doi: 10.1371/journal.pone.0099420

Gregori, S., Tomasoni, D., Pacciani, V., Scirpoli, M., Battaglia, M., Magnani, C. F., et al. (2010). Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* 116, 935–944. doi: 10.1182/blood-2009-07-234872

He, Y., Zhang, J., Donahue, C., and Falo, L. D. Jr. (2006). Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity.* 24, 643–656. doi: 10.1016/j.immuni.2006.03.014

Huddleston, H., and Schust, D. J. (2004). Immune interactions at the maternal–fetal interface: a focus on antigen presentation. *Am. J. Reprod. Immun.* 51, 283–289. doi: 10.1111/j.1600-0897.2004.00157.x

Hughes, G. C., and Clark, E. A. (2007). Regulation of dendritic cells by female sex steroids: relevance to immunity and autoimmunity. *Autoimmunity* 40, 470–481. doi: 10.1080/08916930701464764

Kaisho, T., and Akira, S. (2003). Regulation of dendritic cell function through Toll-like receptors. *Curr. Mol. Med.* 3, 373–385. doi: 10.2174/1566524033479726

Kämmerer, U., Eggert, A. O., Kapp, M., McLellan, A. D., Geijtenbeek, T. B. S., Dietl, J., et al. (2003). Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. *Am. J. Pathol.* 162, 887–896. doi: 10.1016/S0002-9440(10)63884-9

Kämmerer, U., Schoppet, M., McLellan, A. D., Kapp, M., Huppertz, H. I., Kämpgen, E., et al. (2000). Human decidua contains potent immunostimulatory CD83+ dendritic cells. *Am. J. Pathol.* 157, 159–169. doi: 10.1016/S0002-9440(10)64527-0

Kaplan, D. H., Jenison, M. C., Saeland, S., Shlomchik, W. D., and Shlomchik, M. J. (2005). Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 23, 611–620. doi: 10.1016/j.immuni.2005.10.008

Li, D., Romain, G., Flamar, A.-L., Duluc, D., Dullaers, M., Li, X. H. et al. (2012). Targeting self- and foreign antigens to dendritic cells via DC-ASGPR generates IL-10-producing suppressive CD4+ T cells. *J. Exp. Med.* 209, 109–121. doi: 10.1084/jem.20110399

Männik, J., Vaas, P., Rull, K., Teesalu, P., and Laan, M. (2012). Differential placental expression profile of human Growth Hormone/Chorionic Somatomammotropin genes in pregnancies with pre-eclampsia and gestational diabetes mellitus. *Mol. Cell. Endocrinol.* 355, 180–187. doi: 10.1016/j.mce.2012.02.009

McIntire, R., and Hunt, J. (2005). Antigen presenting cells and HLA-G-a review. *Placenta* 26, S104–S109. doi: 10.1016/j.placenta.2005.01.006

Miyazaki, S., Tsuda, H., Sakai, M., Hori, S., Sasaki, Y., Futatani, T., et al. (2003). Predominance of Th2-promoting dendritic cells in early human pregnancy decidua. *J. Leukoc. Biol.* 74, 514–522. doi: 10.1189/jlb.1102566

Moal, V., Textoris, J., Ben Amara, A., Mehraj, V., Berland, Y., Colson, P., et al. (2013). Chronic hepatitis E virus infection is specifically associated with an interferon-related transcriptional program. *J. Infect. Dis.* 207, 125–132. doi: 10.1093/infdis/jis632

Nofer, J.-R. (2012). Estrogens and atherosclerosis: insights from animal models and cell systems. *J. Mol. Endocrinol.* 48, R13–R29. doi: 10.1530/JME-11-0145

Okuda, S., Yamada, T., Hamajima, M., Itoh, M., Katayama, T., Bork, P., et al. (2008). KEGG Atlas mapping for global analysis of metabolic pathways. *Nucleic Acids Res.* 36, W423–W426. doi: 10.1093/nar/gkn282

Pizarro-Cerdá, J., Méresse, S., Parton, R. G., van der Goot, G., Sola-Landa, A., Lopez-Goñi, I., et al. (1998). *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect. Immun.* 66, 5711–5724.

Ristich, V., Liang, S., Zhang, W., Wu, J., and Horuzsko, A. (2005). Tolerization of dendritic cells by HLA-G. *Eur. J. Immunol.* 35, 1133–1142. doi: 10.1002/eji.200425741

Salcedo, S. P., Chevrier, N., Lacerda, T. L. S., Ben Amara, A., Gerart, S., Gorvel, V. A., et al. (2013). Pathogenic brucellae replicate in human trophoblasts. *J. Infect. Dis.* 207, 1075–1083. doi: 10.1093/infdis/jit007

Seillet, C., Rouquié, N., Foulon, E., Douin-Echinard, V., Krust, A., Chambon, P., et al. (2013). Estradiol promotes functional responses in inflammatory and steady-state dendritic cells through differential requirement for

activation function-1 of estrogen receptor α. *J. Immunol.* 190, 5459–5470. doi: 10.4049/jimmunol.1203312

Svajger, U., Anderluh, M., Jeras, M., and Obermajer, N. (2010). C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. *Cell. Signal.* 22, 1397–1405. doi: 10.1016/j.cellsig.2010.03.018

Szekeres-Bartho, J., Halasz, M., and Palkovics, T. (2009). Progesterone in pregnancy; receptor-ligand interaction and signaling pathways. *J. Reprod. Immunol.* 83, 60–64. doi: 10.1016/j.jri.2009.06.262

Tagliani, E., and Erlebacher, A. (2011). Dendritic cell function at the maternal-fetal interface. *Expert Rev. Clin. Immunol.* 7, 593–602. doi: 10.1586/eci.11.52

Valladeau, J., Duvert-Frances, V., Pin, J. J., Kleijmeer, M. J., Ait-Yahia, S., Ravel, O., et al. (2001). Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. *J. Immunol.* 167, 5767–5774. doi: 10.4049/jimmunol.167.10.5767

Von Bubnoff, D., Bausinger, H., Matz, H., Koch, S., Häcker, G., Takikawa, O., et al. (2004). Human epidermal langerhans cells express the immunoregulatory enzyme indoleamine 2,3-dioxygenase. *J. Invest. Dermatol.* 123, 298–304. doi: 10.1111/j.0022-202X.2004.23217.x

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