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Etude des mécanismes de survie des bactéries intracellulaires dans les macrophages

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Sommaire

Résumé

A travers l'évolution, les agents pathogènes ont développé des stratégies leur permettant de survivre au sein de leur hôte en interférant avec la biogénèse des phagolysosomes. Comme *C. burnetii* vit dans un phagosome acide incapable de fusionner avec les lysosomes et que sa virulence est associée à l'expression de son LPS, nous avons étudié le rôle du LPS de *C. burnetii* dans le détournement de la conversion phagosomale. En effet, nous avons montré que *C. burnetii* virulent ainsi que son LPS se localisent dans des compartiments Lamp-1+ qui n'acquièrent pas la CathepsineD et Rab7 n'est pas recruté à leur surface. Contrairement au LPS du variant avirulent de *C. burnetii,* qui est localisé dans les lysosomes, le LPS de *C. burnetii* virulent (vLPS) n'induit pas l'activation de la MAPKinase p38, empêche le recrutement de Rab7 à la surface des compartiments en déstabilisant le complexe HOPS. Finalement, nous avons démontré que la bactérie virulente exprimant le vLPS détourne la conversion phagosomale pour survivre et pour se multiplier dans les macrophages en évitant l'activation de l'axe MAPK-p38/Vps41-HOPS.

Nous avons également étudié les mécanismes permettant à *Tropheryma whipplei*, l'agent de la maladie de Whipple, de se répliquer dans les macrophages puisque les macrophages sont la cible in vivo de cette bactérie. Nous avons montré que *T. whipplei* bloque la conversion de son phagosome. En effet, après purification des phagosomes contenant *T. whipplei*, nous avons observé par western blot et microscopie confocale que *T. whipplei* survit dans un phagosome immature possédant des caractéristiques à la fois des phagosomes précoces et tardifs (présence conjointe de Rab5 et Rab7) rendant impossible sa fusion avec les lysosomes.

L'IL-16 étant connue pour induire la réplication de *T. whipplei*, nous avons étudié l'effet de cette cytokine sur la biogénèse du phagosome de *T. whipplei*. L'utilisation d'anticorps bloquants l'IL-16 induit la mort bactérienne et rétablit la maturation du phagosome en phagolysosome. L'effet de la bactérie sur des souris KO pour le gène de l'IL-16 a également été étudié, mettant en évidence une élimination de *T. whipplei*. Cette cytokine joue donc un rôle dans le défaut de conversion du phagosome en empêchant les phagosomes tardifs contenant *T. whipplei* de fusionner avec les lysosomes.

Une meilleure compréhension des mécanismes de survie des bactéries intracellulaires pourrait être utile au développement de nouvelles stratégies rationnelles d'éradication de ces agents pathogènes.

Summary

Through evolution, pathogens have developed strategies to survive within their host by interfering with the biogenesis of phagolysosomes. As it is known that *C. burnetii* lives in acidic phagosome which is unable to fuse with the lysosomes and that its virulence is associated with the expression of LPS, we studied the role of *C. burnetii* LPS in hijacking of phagosomal conversion. Indeed, we showed that the virulent *C. burnetii* and its LPS are located in Lamp-1+ compartments which do not acquire CathepsineD and Rab7 is not recruited to their surface. Contrary to LPS of avirulent *C. burnetii*, which is located in the lysosomes, the LPS of virulent *C. burnetii* (vLPS) does not induce activation of the p38 MAPKinase and it prevents the recruitment of Rab7 to the surface of compartments by destabilizing the HOPS complex. Finally, we demonstrated that virulent bacteria expressing virulent LPS hijack phagosomal conversion to survive and multiply in macrophages by preventing activation of p38-MAPK/Vps41-HOPS axis.

We also studied the mechanisms by which *Tropheryma whipplei*, the agent of Whipple's disease, replicates in macrophages, which are its target *in vivo*. We have shown that *T. whipplei* blocks the conversion of its phagosome. Indeed, after purification of phagosomes containing-*T. whipplei*, we observed by Western blot and confocal microscopy that *T. whipplei* survives in an immature phagosome with characteristics of both early and late phagosomes (presence of Rab5 and Rab7) making it unable to fuse with lysosomes.

As the IL-16 is known to induce replication of *T. whipplei*, we studied the effect of this cytokine on the phagosome biogenesis of *T. whipplei*. The use of antibodies blocking IL-16 induces bacterial death and restores phagosomal maturation into the

phagolysosome. The effect of bacteria on IL16-/- mice has also been studied, which shows the elimination of *T. whipplei*. This cytokine plays an important role in preventing the conversion of phagosome by inhibiting late phagosomes containing *T. whipplei* to fuse with lysosomes.

A better understanding of the survival mechanisms of intracellular bacteria may be useful in developing new rational strategies to eradicate these pathogens.

AVANT-PROPOS

De nombreuses bactéries pathogènes possèdent un mode de vie intracellulaire qui les met à l'abri de la réponse immune. On distingue les bactéries intracellulaires facultatives, capables de se multiplier hors des cellules, des bactéries intracellulaires strictes, qui ne peuvent survivre que dans leurs cellules hôtes. Différents types cellulaires peuvent être infectés par les bactéries intracellulaires mais les cellules myéloïdes sont des hôtes privilégiés de ces bactéries. Les macrophages jouent un rôle essentiel dans la mise en place de la réponse immune, étant à la fois initiateurs et effecteurs de la lutte antibactérienne. Initiateurs puisque, dès la pénétration de la bactérie dans l'organisme, certaines fonctions des macrophages telles que le chimiotactisme, la phagocytose et la production de cytokines sont activées. Ces fonctions constituent l'immunité naturelle. Les macrophages sont aussi initiateurs de la réponse immune spécifique puisqu'ils présentent l'antigène aux lymphocytes T. Grâce à des molécules de costimulation, ils activent les lymphocytes T qui acquièrent alors la capacité de produire des cytokines essentielles dans la lutte contre les microorganismes intracellulaires. Ces cytokines d'origine lymphocytaire, et en particulier l'interféron gamma (IFN-γ), activent à leur tour le potentiel lytique des macrophages qui deviennent de puissants effecteurs de la lutte antibactérienne. Tandis que nombre de germes extracellulaires sont détruits par les macrophages avant même l'activation de l'immunité adaptative,

l'élimination des microorganismes intracellulaires nécessite la coopération entre les macrophages et les lymphocytes T. Les puissantes capacités lytiques des macrophages activés nécessitent d'être finement régulées. Ainsi, le macrophage est en permanence soumis à l'action de cytokines activatrices et inhibitrices, de diverses origines cellulaires, agissant de façon autocrine et/ou paracrine. Ces cytokines constituent un réseau complexe qui contrôle l'état d'activation des macrophages et donc leur potentiel lytique.

La capacité phagocytaire des macrophages et leur longue durée de vie en font des hôtes privilégiés pour les microorganismes à développement intra-cellulaire. Ainsi, de façon paradoxale, les bactéries intracellulaires résident principalement dans des cellules normalement destinées à les détruire. Pour survivre et se multiplier dans des cellules hôtes dotées d'un tel pouvoir lytique, les agents pathogènes ont donc été contraints d'élaborer divers mécanismes d'échappement à la réponse immunitaire. De nombreuses étapes de l'activation des macrophages sont ainsi susceptibles d'être affectées. Les bactéries intracellulaires peuvent moduler l'activation des monocytes/macrophages en induisant la synthèse de cytokines inhibant leurs propriétés microbicides. Elles peuvent également altérer le trafic intracellulaire et se retrouver alors dans une vacuole permettant leur survie et leur développement.

L'objectif de ma thèse est d'étudier les mécanismes mis en place par des agents pathogènes tels que *Coxiella burnetii* (articles I et II) et *Tropheryma whipplei* (articles III et IV) pour bloquer la maturation du phagosome dans lequel elles survivent.

Par souci de clarté, j'ai divisé ce manuscrit en deux parties :

I- le trafic intracellulaire de *Coxiella burnetii*,

II- le trafic intracellulaire de *Tropheryma whipplei*.

INTRODUCTION

Les monocytes sont des cellules sanguines d'un diamètre allant de 10 à 18 micromètres. Leur noyau volumineux est irrégulier, d'aspect parfois réniforme, rarement nucléolé avec une chromatine fine. Leur cytoplasme très pâle contient de fines granulations azurophiles et est aisément identifié par des colorations mettant en jeu une activité estérase ou phosphatase acide. Les monocytes circulent dans le flux sanguin et peuvent migrer à travers les vaisseaux sanguins et s'établir dans les tissus : ils s'y différencient en macrophages. Les monocytes et les macrophages sont des phagocytes professionnels capables d'ingérer toutes sortes de particules inertes ou vivantes destinées à être éliminées par l'organisme. La phagocytose se déroule en trois étapes : une étape de reconnaissance de la particule (fixation), prélude à la phase d'ingestion de la particule qui se traduit par une formation d'un phagosome englobant la particule. L'étape de dégradation des particules ingérées est rendue possible par la conversion du phagosome en phagolysosome et l'activation des mécanismes microbicides dépendants de l'oxygène. Le potentiel lytique des macrophages est tel qu'il impose d'être strictement contrôlé. Celui des macrophages quiescents est limité. Suite à leur activation, les macrophages acquièrent leur pleine capacité lytique. Ils répondent également à différents signaux inhibant leur activité lytique, ce qui permet l'arrêt de la réaction et d'éviter l'altération des tissus environnants.

Il est maintenant clairement établi que l'activité microbicide des macrophages est extrêmement sensible aux cytokines auxquelles ils sont exposés. Les macrophages sont tout à la fois cibles et producteurs de cytokines. Leur activation s'effectue en deux étapes et nécessite au moins deux signaux. Le premier leur permet de passer de l'état résident (quiescent) à l'état « primé » (sensibilisé) et le second de l'état sensibilisé à l'état activé (macrophage cytotoxique et microbicide). Le contact des macrophages avec les microorganismes, en particulier avec le lipopolysaccharide (LPS) des bactéries à Gram négatif, fournit le premier signal, le second étant fourni par la présence de cytokines activatrices telle que l'IFN-γ ou le « Tumoral Necrosis Factor » (TNF). L'activité microbicide des macrophages est inhibée par des cytokines qu'elles soient purement lymphocytaires telles que l'interleukine (IL)-4 ou d'origines diverses (lymphocytaires et macrophagiques) telles que l'IL-10. En effet, les macrophages sont non seulement un acteur essentiel de la réponse immunitaire innée mais également de la réponse immunitaire acquise puisqu'ils apprêtent et présentent l'antigène aux lymphocytes et leur fournissent molécules de costimulation et cytokines régulatrices (IL-12 par exemple). En retour, leur activité microbicide est contrôlée par des cytokines sécrétées par différents acteurs de la réponse immune telles que l'IFN-γ qui stimule cette activité microbicide ou l'IL-4 qui l'inhibe. Enfin, la phagocytose des agents infectieux par les macrophages est favorisée par leur opsonisation

via les anticorps sécrétés par les lymphocytes B et les plasmocytes, cette opsonisation conduisant le plus souvent à leur élimination grâce au déclenchement de la machinerie microbicide des macrophages via les récepteurs des immunoglobulines.

Le potentiel lytique des monocytes/macrophages est tel qu'il impose d'être strictement contrôlé et il est maintenant bien connu que leur activité microbicide est extraordinairement sensible aux cytokines auxquelles ils sont exposés. En effet, l'introduction d'un agent infectieux dans l'organisme active le système immunitaire avec libération d'un ensemble de cytokines dont le mode d`action peut être autocrine, paracrine ou endocrine. Les monocytes / macrophages se situent au cœur de ce réseau de cytokines, étant à la fois cibles et producteurs de cytokines proinflammatoires et immunorégulatrices. Ils sont en effet la cible des cytokines de la réponse immunitaire cellulaire Th1 telles que l'IFN-γ et 1'IL-12 et de la réponse Th2 (IL-10 et IL-4) **[1, 2]**. Ils produisent en outre des cytokines proinflammatoires telles que le TNF, l'IL-1 et l'IL-6 en réponse à de nombreux produits bactériens, en particulier le lipopolysaccharide (LPS) bactérien.

Un concept général d'activation des macrophages est apparu ces dernières années. S'appuyant sur la dichotomie classique Th1/Th2, il porte le nom de macrophages M1/M2 **[2, 3]**. Des études convergentes montrent que les macrophages sont polarisés en macrophages M1 ou M2 en fonction des médiateurs qu'ils

rencontrent. C'est ainsi que des molécules telles que l'IFN-γ et le TNF induisent une polarisation M1. Les macrophages M1 produisent un certain nombre de molécules qui rendent compte de leur activité tumoricide et bactéricide. Les macrophages M2 représentent, eux, une population hétérogène quant à leurs voies d'induction, leurs caractéristiques phénotypiques et leurs propriétés. En effet, les macrophages M2 correspondent à un continuum d'états différents et on distingue actuellement trois types de macrophages M2, les macrophages M2a, les macrophages M2b et les macrophages M2c. Ces macrophages M2 sont considérés comme faiblement microbicides **[3]**.

I. Contrôle de l'infection bactérienne par les cytokines

Les mécanismes d'action des cytokines sur les fonctions macrophagiques commencent à être élucidés. Ainsi, I'IFN-γ augmente l'activité microbicide des macrophages dirigés contre des agents pathogènes intracellulaires via l'induction des dérivés actifs de l'oxygène (ROI) et de l'azote (RNI) et de cytokines telles que le TNF **[4]**. Le TNF induit, lui, l'amorçage de l'explosion oxydative tout en activant les propriétés microbicides des macrophages **[5]**. A l'opposé des cytokines précédentes, 1'IL-10, l'IL-4 et le TGF-β « désactivent » les monocytes/macrophages en contrecarrant les effets de l'IFN-γ et du TNF. Ces cytokines immunorégulatrices inhibent la production des cytokines inflammatoires induites, en

particulier, par le LPS ou l'IFN-γ ainsi que la production des ROI et des RNI **[6]**. Il a été montré en outre que l'IL-10 favorise la survie de *Legionella pneumophila* **[7]**, que l'IL-4 inhibe l'activité antileishmanienne des macrophages **[8]** et que le TGF-β favorise la réplication de *Mycobacterium tuberculosis* **[9]**. Cependant, dans certaines conditions, les cytokines peuvent conduire à la survie et la réplication des agents pathogènes avec une certaine sélectivité puisque, si le TGF-β favorise la réplication de *M. avium*, l'IL-10 ne l'affecte pas **[10]**. Par ailleurs, les agents pathogènes sont susceptibles de détourner le réseau de cytokines à leur avantage [**Revue I**]. Ainsi le virus d'Epstein-Barr synthétise-t-il une protéine proche de l'IL-10 humaine et les leishmanies contrôlent-elles la synthèse de diverses cytokines (IL-2, IL-3, Granulocyte Macrophage-Colony Stimulating Factor). Les souches virulentes de *Brucella abortus* induisent la synthèse d'un inhibiteur du TNF, ce qui favorise leur survie dans les macrophages humains **[11]**.

II. La reconnaissance des agents infectieux par les macrophages

Le système immunitaire inné constitue la première ligne de défense de l'hôte durant l'infection et joue un rôle important dans la reconnaissance et l'activation d'une réponse inflammatoire **[12].** La réponse immune innée est initiée par la reconnaissance par les PRRs (*Pattern Recognition Receptors*) macrophagiques de structures microbiennes conservées au cours de l'évolution, appelées PAMPs (*Pathogen-Associated Molecular Patterns*). Après reconnaissance des

PAMPs, les PRRs présents à la surface ou au sein des cellules signalent la présence d'une infection et initient des réponses proinflammatoires et antimicrobiennes en activant toute une série de voies de signalisation incluant des molécules adaptatrices, des kinases et des facteurs de transcription **[13]**.

Un nombre relativement limité de PRRs permet la reconnaissance d'un grand nombre d'agents infectieux. C'est ainsi que des microbes de composition biochimique différente et avec des cycles de vie entièrement différents peuvent être reconnus par un seul type de PRRs **[14]**. Les mécanismes de reconnaissance des agents infectieux sont également redondants puisque plusieurs PRRs peuvent participer à la reconnaissance d'un seul type d'agent infectieux **[15]**.

La paroi des bactéries à gram négatif est constituée de peptidoglycanes, de phospholipides, de LPS et de différentes protéines. Le LPS est le composant majeur de cette paroi. Il est composé de l'antigène O, qui est la partie la plus externe, d'un core oligosaccharide et d'un lipide A par lequel la molécule est ancrée dans la membrane externe de la bactérie. Le LPS, en particulier son lipide A, est l'un des PAMPs le plus connu et il est en grande partie responsable de la réponse inflammatoire engendrée en réponse à l'infection. Libéré de la paroi des bactéries à gram négatif, le LPS s'associe avec la protéine de la phase aiguë extracellulaire (LBP) et se lie ensuite au corécepteur CD14 exprimé à la surface des momocytes. Cet événement permet le

transfert du LPS à la molécule accessoire MD2 qui est associée avec le domaine extracellulaire d'un PRR appelé *Toll-Like Receptor* (TLR)-4, ce qui provoque l'oligomérisation de TLR4 et la cascasde transductionnelle qui en découle **[12].** D'autres PRRs tels que le récepteur du complément CR3, TLR2 ou les *Nucleotide-binding Oligomeriza- tion Domain-containing proteins* (NOD)-1/2 sont d'autres candidats comme récepteurs du LPS.

Quant aux bactéries à gram positif, les TLR2 jouent un rôle majeur dans la reconnaissance de l'acide lipoteichoïque, des lipoprotéines et des peptidoglycanes **[16, 17]**. En outre, le CpG de l'ADN bactérien est reconnu par les TLR9 **[18]**. Les récepteurs cytoplasmiques comme NOD et l'inflammasome NALP1 sont activés par le MDP (*muramyl dipeptide*) **[19, 20]**, une molécule dérivée des peptidoglycanes.

De façon plus générale, les agents infectieux sont reconnus par des récepteurs très variés présents à la surface des phagocytes, dont certains sont impliqués dans la phagocytose : c'est notamment le cas des intégrines (α_vß3 et ß3), des récepteurs Fc des immunoglobulines (FcR), des récepteurs scavengers (SR), des récepteurs du complément (CR) et des récepteurs du mannose (MR).

III. La conversion phagosomale

Une fois l'agent infectieux internalisé par les macrophages, il se retrouve dans une structure, le phagosome. Le phagosome naissant suit une série d'étapes de maturation. Au cours de l'étape finale de sa maturation, le phagosome fusionne avec les lysosomes pour donner le phagolysosome, lieu de destruction des particules phagocytées **(Figure 1) [21-23]**. Les endosomes et les phagosomes sont des structures dynamiques interagissant entre elles de façon homotypique ou hétérotypique **[22, 24]**. Cette dynamique concourt à la biogénèse d'un endosome précoce en endosome tardif ou d'un phagosome en phagolysosome via l'acquisition ou la perte de divers composants membranaires ou cytosoliques. C'est ainsi que le phagosome subit plusieurs étapes de fusions/fissions avec d'autres organelles. Il passe d'un état précoce caractérisé par la présence de protéines telles que *early endosome antigen* (EEA)-1 ou la GTPase Rab5 vers un état tardif caractérisé, lui, par la présence de *Lysosomal-Associated Membrane Protein* (LAMP)-1, d'une pompe V-H^+-ATPase qui permet l'acidification du phagosome et de la GTPase Rab7. A ce phagosome tardif succède une étape de fusion avec les lysosomes caractérisée par la présence de LAMP-1 et de cathepsine D **[21]**. Trois modèles ont été proposés pour expliquer la biogénèse des endosomes et des phagosomes : le modèle de « vesicle shuttle » dans lequel des vésicules de transport se connectent aux organelles endocytiques préexistantes; le modèle de « maturation » dans lequel les vésicules

endocytiques sont graduellement transformées en vésicules endocytiques matures; le modèle « kiss and run » qui suggère que la maturation du phagosome ne serait pas due à des fusions mais à de brefs échanges de matériel (membranes et contenu) entre les phagosomes et les endosomes **[25, 26]**.

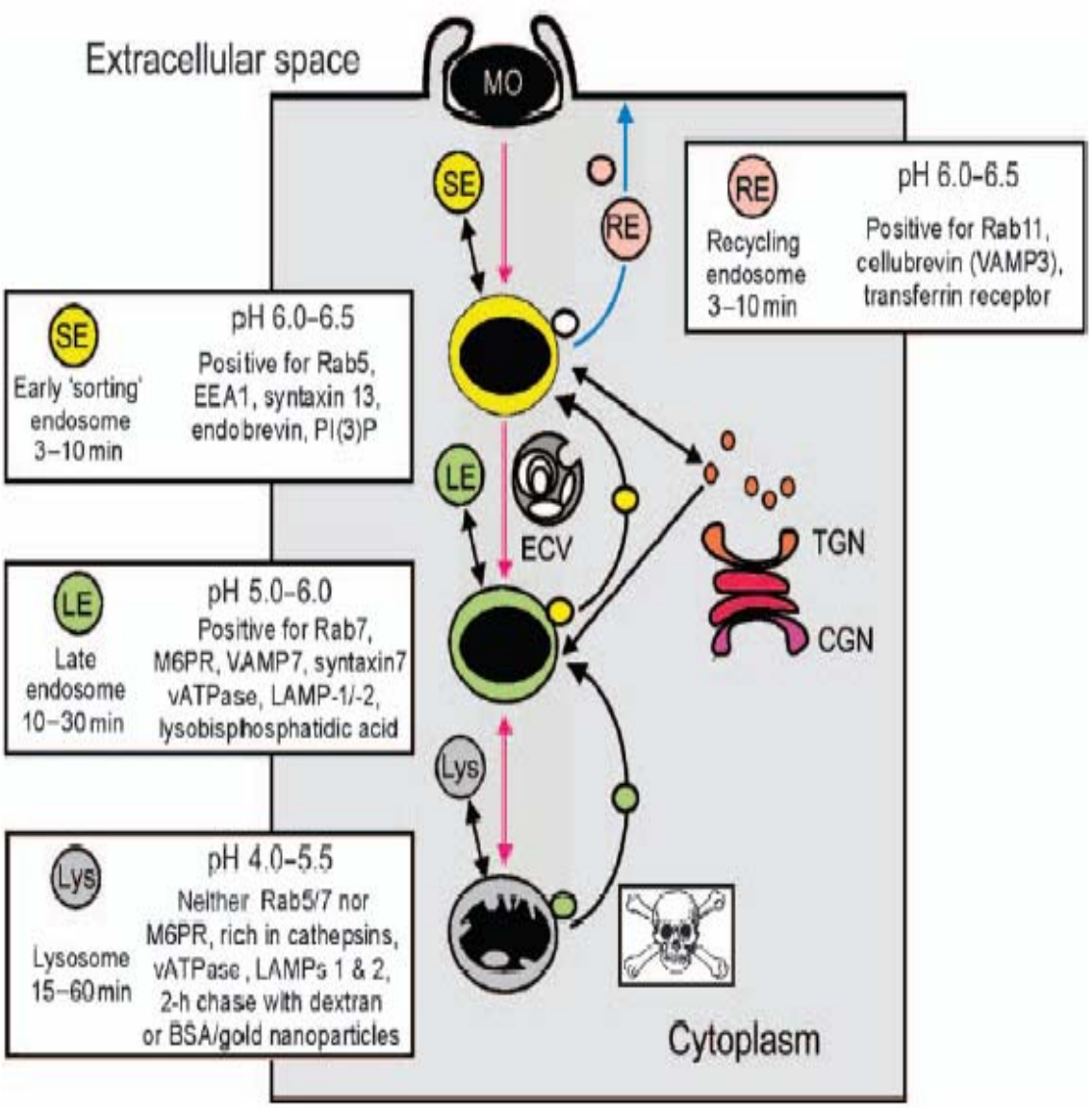

Figure 1. Schéma simplifié des voies d'endocytose (phagocytose) (Tiré de Haas, Traffic 8: 311-330 (2007))

Dans ce dernier modèle, phagosomes et endosomes n'acquièrent pas simultanément les membranes endocytiques et leur contenu, à l'exception de la fusion complète décrite dans les modèles de « maturation » ou de « vesicle shuttle ». Bien que l'on puisse concevoir la formation du phagolysosome comme un simple processus de maturation, la cinétique de cette maturation montre un tableau complexe de réactions. Les raisons de cette complexité sont nombreuses: elles relèvent du large spectre de particules phagocytées et des différents systèmes cellulaires utilisés dans chaque étude. Elles relèvent également de la multiplicité des points d'interaction entre le phagosome et la voie d'endocytose. La complexité de la maturation phagosomale rend difficile la dissection moléculaire des événements impliqués dans ce processus.

Les molécules impliquées dans la biogénèse des endosomes et des phagosomes sont nombreuses. Plus de deux cents molécules ont été trouvées dans la membrane des phagosomes mais seules quelques-unes sont bien connues **[22, 27, 28]**. Chaque étape de la biogénèse des endosomes et des phagosomes est caractérisée par l'acquisition et la perte de molécules spécifiques. Le contrôle et le maintien de l'intégrité de la voie endocytique et phagosomale s'effectue grâce à deux grands groupes de protéines: les petites protéines G (*GTP-binding protein*) de la famille Ras **[29-31]** et les protéines SNARE (*soluble NSF-attachment protein receptor*) **[32]**.

Les protéines Rab représentent la plus grande famille des GTPases, avec plus de quarante molécules décrites dans la littérature **[30, 31]**. Les différentes protéines Rab sont spécifiquement associées à des compartiments intracellulaires distincts et forment des domaines permettant les interactions entre compartiments. Ainsi Rab5 et Rab7 **[33]** contrôlent-ils la séquence des interactions avec les endosomes précoces et tardifs. Les petites protéines G Rab4, Rab11 et ARF6 ont pour fonction de contrôler le recyclage des protéines et des membranes des compartiments endosomiaux vers la membrane plasmique **[34]**. Les interactions entre le réseau trans-golgien (TGN) et les endosomes tardifs sont, elles, contrôlées par Rab9 **[35]**. Cependant, les protéines Rab ne sont pas des molécules strictement membranaires et peuvent être retrouvées dans les cellules sous deux conformations différentes: une forme cytosolique inactive associée au GDP et une forme membranaire active, couplée au GTP **[36]**. De nombreuses molécules sont capables d'interagir avec les protéines Rab et de réguler leur état d'activation et leur localisation intracellulaire en inhibant, par exemple, la dissociation du complexe Rab-GDP ou en activant l'hydrolyse du GTP **[36]**. Les molécules Rab sont proposées comme étant des interrupteurs ou des montres moléculaires qui contrôlent non seulement la spécificité de l'interaction entre deux compartiments intracellulaires mais aussi son déroulement grâce au contrôle du cycle GTPasique par différents effecteurs **[36, 37]**. Le mécanisme d'action précis des

protéines Rab demeure néanmoins obscur. L'activation du complexe SNARE grâce à l'interaction avec les t-SNARE est la fonction la plus couramment associée à ces petites protéines G **[38]**. La composition des domaines Rab et les mécanismes d'interaction entre les compartiments intracellulaires commencent à être cependant élucidés.

Il faut noter que, lors de la conversion endo-lysosomale ou phago-lysosomale dependant de Rab7, le complexe HOPS (*homotypic fusion and protein sorting*) joue un rôle important dans le recrutement et l'activation de Rab7 à la membrane des endosomes ou des phagosomes. En effet, deux sous-unités accessoires, Vps39 et Vps41, s'associent avec le core du Vps-C pour former le complexe HOPS. HOPS contrôle la fusion des membranes vacuolaires aux lysosomes. Le Vps39 fonctionne dans les levures comme un GEF (*guanosine exchange factor*) qui active le Rab Ypt7 orthologue de Rab7 **[39]**, alors que le Vps 41 se lie à Ypt7 en stabilisant l'homocomplexe HOPS **[40]**. HOPS s'associe aussi à des phospholipides acides et des SNAREs **[41, 42]**.

Les petites protéines GTPases Rab sont impliquées dans la biogénèse des phagosomes : leur contrôle par certaines bactéries pathogènes constitue pour elles un moyen de moduler la maturation phagosomale et d'assurer leur survie intracellulaire [33, 43]. Le rôle exact des molécules Rab et du complexe HOPS dans la biogénèse des phagosomes, des endosomes et le processus de

contrôle de leur activité dans un contexte infectieux reste à être précisé.

IV. Transduction du signal et maturation du phagosome

Le signal des récepteurs inflammatoires dans la maturation phagosomale peut :

Cibler le protéome du phagosome

Il pourrait cibler la famille des protéines Rab qui sont considérées comme les principaux régulateurs du trafic endocytique. Rab5 module la fusion des endosomes précoces, et les cycles entre la forme active (liée au GTP) et la forme inactive (liée au GDP) de Rab5 sont régulés par les *guanine nucleotide exchange factors* (GEFs) et les *GTPase activating proteins* (GAPs) **[44]**, qui pourraient potentiellement être ciblées par les TLRs. La phosphorylation des *Guanine Nucleotide Dissociation Inhibitors* (GDI) par p38, une MAP kinase (MAPK), induit la formation du complexe cytosolique GDI-Rab5 et se traduit par une augmentation du trafic endocytique (**Figure 2**) **[45, 46].**

De nombreux exemples montrent que signal et trafic endocytique sont liés (Cavalli *et al.* (2001a), Di Fiore et De Camilli (2001), McPherson *et al.* (2001) et Gonzalez-Gaitan (2003)). C'est ainsi que le signal induit par des récepteurs tels que *l'Epidermal Growth Factor Receptor* (EGFR) ou le *Nerve Growth Factor Receptor* (NGFR)

accroît l'endocytose en augmentant le recrutement de la clathrine et la formation des puits recouverts de clathrine (CCPs).

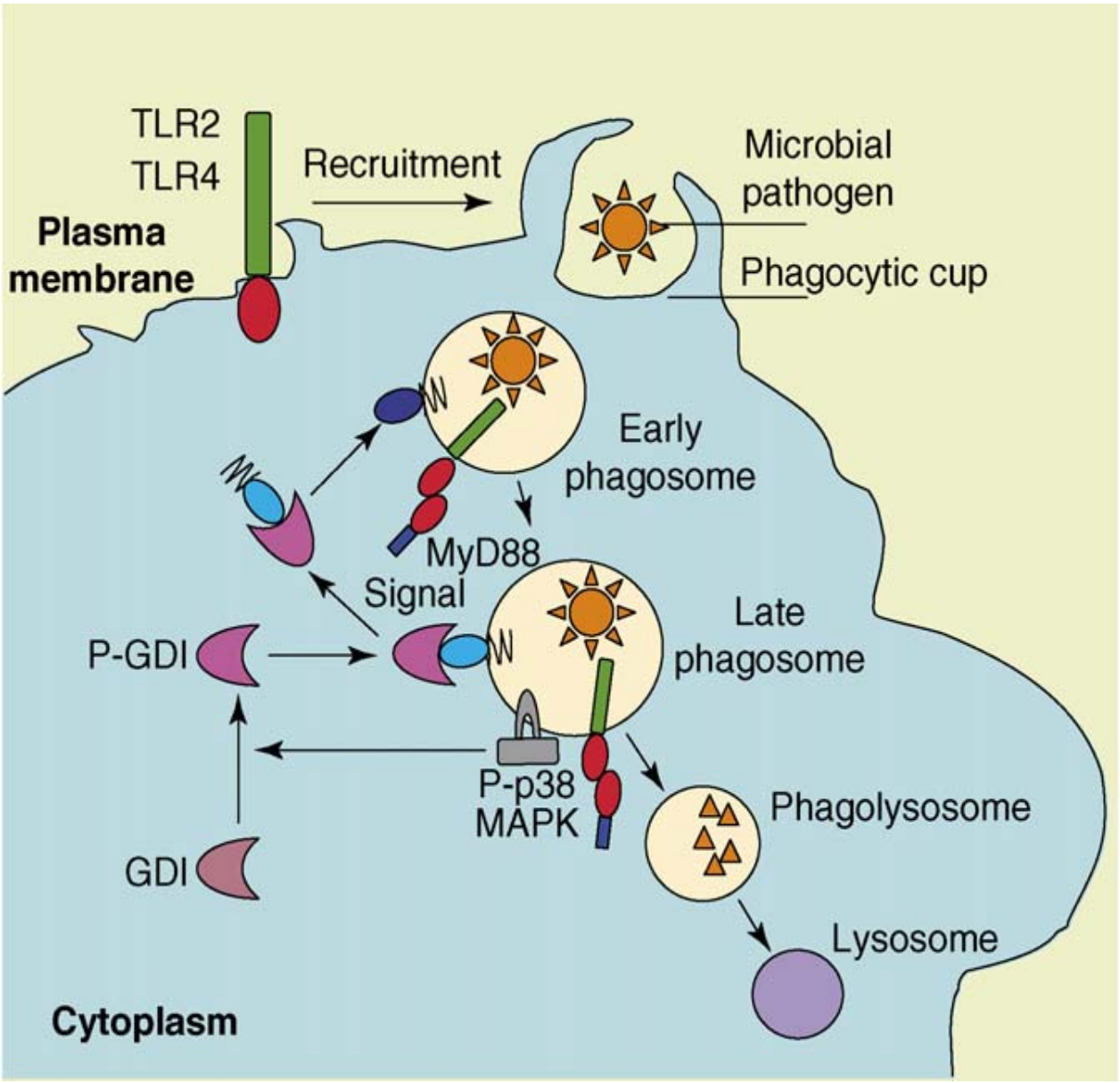

Figure 2. Contrôle de la maturation du phagosome par le signal du TLR est médié par MyD88 et MAPK p38

(Tiré de Blander, Trends Immunol, 28:19-25(2006))

Le signal à travers l'EGFR induit l'activation de Rab5 et la phosphorylation sur résidus de tyrosine de Eps15, une protéine régulatrice de l'endocytose (Barbieri *et al*, 2000 ; Confalonieri *et al*, 2000).

Lors de la phagocytose des agents infectieux, les TLRs présents sur la membrane plasmique tels que TLR2 et TLR4 sont engagés par des structures microbiennes, ce qui engendre différents signaux via la protéine adaptatrice MyD88 et la MAPK p38 (Blander and Medzhitov, 2004) puis la maturation du phagosome selon des cinétiques et des conséquences fonctionnelles qui varient selon l'agent infectieux considéré (*Escherichia coli*, *Staphylococcus aureus* ou *Salmonella typhimurium*).

Cibler la mobilité du phagosome

Une autre voie potentielle dans laquelle le signal peut cibler la maturation phagosomale est d'augmenter le taux auquel les phagosomes se déplacent le long des microtubules. Cette mobilité des phagosomes est guidée par des complexes de protéines motrices tels que kinesine/kinectine et dynéine/dynactine (Blocker *et al .,* 1998 ; Karcher *et al*., 2002). La présence de la dynactine sur les membranes des phagosomes recrute la dynéine cytosolique, et cela semble exiger un événement de phosphorylation par une kinase associée à la dynactine (Hamm-Alvarez *et al.*, 1993 ; Blocker *et al.*, 1997).

V. Détournement de la conversion phagosomale

Les bactéries pathogènes ont su développer de multiples stratégies pour éviter l'activité microbicide des macrophages **[47] (Figure 3)**. Une première stratégie mise en place par certaines bactéries extracellulaires consiste en l'évitement de la phagocytose et, par conséquent, des événements lytiques qui l'accompagnent. La stratégie mise en place par les bactéries intracellulaires est radicalement différente. Elles s'accommodent de leur phagocytose mais se doivent alors de résister aux mécanismes lytiques des macrophages. Cette résistance peut être classée en plusieurs catégories. Une première consiste à rendre inopérants les effecteurs microbicides. On peut classer dans cette même catégorie les bactéries qui inhibent les effecteurs microbicides tels que les ROI, par exemple en les détoxifiant. Autre stratégie que développent certaines bactéries, leur compétition avec leurs cellules hôtes pour un nutriment tel que le fer, compétition qui leur permet de se répliquer au sein de ces cellules hôtes. Une troisième consiste à empêcher la mise en place des effecteurs microbicides. C'est ainsi que nombre de bactéries pathogènes détournent ou inhibent la conversion phagosomale qui conduit normalement à la lyse bactérienne.

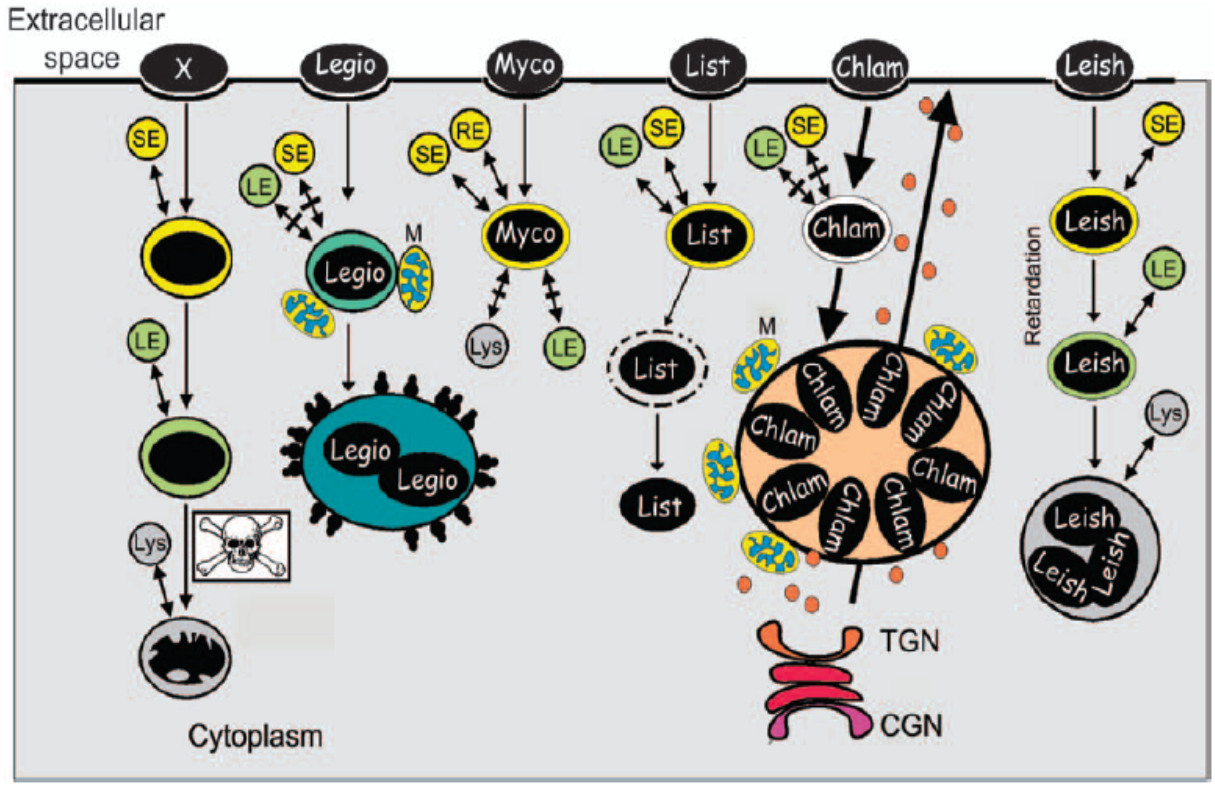

Figure 3. Où comment les bactéries pathogènes échappent à leur élimination dans les macrophages

(Tiré de Haas A, Traffic 8: 311-330 (2007))

A – Bactéries s'échappant dans le cytosol

Une des stratégies déployées par certains agents infectieux pour éviter l'environnement délétère du phagolysosome est la lyse du phagosome. *Shigella flexneri*, *Listeria monocytogenes*, *Rickettsia conorii* **[48, 49]** lysent leur phagosome et s'échappent vers le cytoplasme, lieu de leur réplication.

B – Bactéries quittant la voie d'endocytose

Chlamydia, *Legionella pneumophila* et *Brucella abortus*, dès leur ingestion, évitent la voie d'endocytose qui conduirait à leur lyse dans les phagolysosomes. Les compartiments intracellulaires dans lesquels elles résident sont pauvres en hydrolases ou contiennent des hydrolases sous forme de précurseurs inactifs. Ainsi, *Chlamydia* est retrouvée 40 heures après infection dans un compartiment non acide capable de fusionner avec des vésicules issues de l'appareil de Golgi. Ces vésicules, riches en glycosphingolipides et autres substrats, constituent une source de nutriments nécessaires à la réplication de *Chlamydia*. La phosphoprotéine IncA semble être l'un des facteurs membranaires responsable de la localisation intracellulaire de *Chlamydia* **[50]**. Le phagosome de *L. pneumophila* est totalement isolé de la voie d'endocytose ; la bactérie réside dans une vacuole recouverte par les ribosomes et qui présente des similarités morphologiques avec les autophagosomes **[51]**. Les autophagosomes sont des compartiments qui se forment lors du

recyclage ou de la dégradation des molécules cytoplasmiques et participent également à la restructuration cellulaire **[52]**. *B. abortus* évite, elle aussi, la voie d'endocytose. Si, tôt après la phagocytose, son phagosome est caractérisé par la présence de marqueurs des compartiments précoces de la voie d'endocytose (EEA1), il est rapidement localisé dans un compartiment multi-membranaire de type autophagosome puis, plus tardivement, présent dans le réticulum endoplasmique dans lequel *B. abortus* se réplique **[53]**.

C – Bactéries bloquant la voie d'endocytose

Certains agents pathogènes, tels *Mycobacterium tuberculosis* ou *M. avium*, se développent au sein d'un phagosome qui possède les caractéristiques des endosomes précoces mais qui n'acquiert pas celles des endosomes tardifs et des lysosomes **[54, 55]**. Rab5 est séquestré à la surface du phagosome et on observe une inhibition partielle de la production de phosphatidylinositol (3,4,5)-triphosphate (PI3P) nécessaire à la conversion phagosomale **[56]**. Le phagosome mycobactérien est également caractérisé par une absence d'acidification due à un défaut d'acquisition des V-H^+-ATPases **[57]**, par une acquisition limitée de LAMP-1, par la présence d'une forme immature de la cathepsine D et l'absence des récepteurs du mannose-6-phophate **[55]**. Il garde cependant sa capacité fusogène puisqu'il peut interagir avec la voie d'exocytose

et la voie d'endocytose **[58]**, ce qui permet un remodelage constant du phagosome.

D'autres bactéries exploitent le pH acide des phagosomes tardifs pour y survivre. Cet environnement, à priori hostile, peut même constituer une source de nutriments pour ces bactéries et le pH acide peut être nécessaire à leur métabolisme. Ainsi, la vacuole de phagocytose de *Salmonella enterica* sérotype typhimurium est caractérisée par une acquisition transitoire d'EEA-1 et de récepteurs de la transferrine (TfR) ; elle est suivie par un enrichissement progressif en V-H^+-ATPase et en glycoprotéines lysosomiales, mais pas en hydrolases acides. Des vésicules enrichies en LAMP-1 et Rab7, mais ne contenant pas de cathepsine D, sont accumulées de façon transitoire au voisinage des vacuoles bactériennes avec lesquelles elles interagissent. Ces vésicules pourraient correspondre à des compartiments intermédiaires formés au cours de la voie d'endocytose, ce qui expliquerait pourquoi la vacuole bactérienne acquiert certaines glycoprotéines lysosomiales et exclut les hydrolases acides normalement concentrées dans les lysosomes. Lorsque les bactéries commencent à se répliquer, elles induisent la formation de structures membranaires tubulaires connectées aux vacuoles. La fonction de ces tubules reste à déterminer. Des effecteurs bactériens tels que le système de sécrétion Spi/Sca inhibent la fusion entre le phagosome bactérien et les lysosomes **[59]**.

D – Bactéries survivant dans une vacuole acide

Bien que la plupart des bactéries aient développé une stratégie d'évitement de l'environnement acide et hydrolytique du phagolysosome, certaines d'entre elles se répliquent dans un environnement acide. C'est ainsi que *C. burnetii* se réplique dans une vacuole acide **[60, 61]** nécessaire à son métabolisme **[62]** mais qui est incapable de fusionner avec les lysosomes puisqu'elle est dépourvue d'un marqueur lysosomial tel que la cathepsine D. **[60]**. *Francisella tularensis* a longtemps été décrit comme une bactérie survivant dans un phagolysosome **[63]**. Toutefois, des travaux récents montrent qu'en réalité *F. tularensis* lyse la membrane de son phagosome afin de rejoindre le compartiment cytoplasmique dans lequel elle se divise **[64]**. Aucune bactérie, à ce jour, n'a été décrite comme survivant dans les phagolysosomes.

VI- Facteurs de virulence et détournement de la conversion phagosomale

Des facteurs dits de virulence permettent aux bactéries pathogènes de survivre et d'édifier une niche réplicative au sein de l'organisme hôte. Certains de ces facteurs sont des systèmes de sécrétion de type III : ce sont les protéines SpiC, Yops et ExoS sécrétées respectivement par *S. enterica* serotype typhimurium, *Yersinia* et *Pseudomonas aeruginosa* (**Tableau 1**). *Salmonella* injecte ainsi la protéine SpiC dans le cytosol des macrophages où elle interagit avec la molécule Hook3 pour inhiber la fusion phagosome-lysosome **[66].** Une autre protéine sécrétée par *Salmonella*, SopB/SigD, une phosphoinositide phosphatase, semble être impliquée dans la survie des bactéries. SopB catalyse la formation du PtdIns(3)P (phosphatidylinositol (3)-phosphate) apartir du PtdIns(3,5)P_2 (phosphatidylinositol (3,5)-diphosphate) sur les endosomes et empêche la fusion des vacuoles contenant *Salmonella* (SCVs) avec les lysosomes**[67]**. Les protéines ExoS et YopE assurent des fonctions similaires. L'extrémité N-terminale de ExoS présente une homologie de structure avec la cytotoxine YopE de *Yersinia*. Injectées dans le cytoplasme des macrophages, ces deux protéines exercent une activité antiphagocytaire **[68, 69]**en détruisant le cytosquelette d'actine **[70]**.

Protein	Biochemical function	Effect on host cells
Salmonella (SPI-1)		
SipA	binds plastin and actin/promotes actin bundling	promotes localized cytoskeletal rearrangements
SipB	activation of caspase-1	induces apoptosis
SipC	actin nucleation	localizes cytoskeletal rearrangements
SopB	inositol phosphate phosphatase	promotes cytoskeletal rearrangements/promotes Cl^- secretion
SopE	GTPase exchange factor	promotes cytoskeletal rearrangements/activates MAP kinase pathway
SptP	tyrosine phosphatase/GTPase activating protein	rebuilds cytoskeleton
Salmonella (SPI-2)		
SpiC	unknown	inhibits phagosome-lysosome fusion
Shigella		
IpaA	binds vinculin/promotes actin depolymerization	stimulates bacterial entry
IpaB	activation of caspase-1	induces apoptosis
IpaC	unknown	stimulates bacterial entry
IpgD	putative inositol phosphate phosphatase	unknown
Yersinia		
YopE	putative GTPase-activating protein	disrupts cytoskeleton/antiphagocytosis
YopH	tyrosine phosphatase	disrupts cytoskeleton/antiphagocytosis
YopJ/P	inhibition of MAP kinase kinase activity	induces apoptosis
YpkA/YopO	serine/threonine kinase	unknown
P. aeruginosa		
ExoS	ADP-ribosyl transferase/putative GTPase-activating protein	disrupts cytoskeleton/antiphagocytosis
ExoT	ADP-ribosyl transferase	cytotoxicity
ExoU	unknown	cytotoxicity
ExoY	adenylate cyclase	cytotoxicity
PpkA	serine/threonine kinase	unknown
EPEC		
Tir	intimin receptor	formation of actin pedestals

Tableau 1. Exemples d'effecteurs du système de sécrétion de type III de certains bactéries et leur mode d'action.
(**tiré de** A. Sukhan, CMLS 57 : 1033-1049 (2000))

D'autres agents pathogènes utilisent des molécules de surface pour survivre dans les cellules hôtes. La forme promastigote de *Leishmania donovani*, un parasite, survit et se multiplie dans les macrophages en détournant la conversion phagosomale. Une fois dans le phagosome, grâce au lipophosphoglycane exprimé à sa surface, *L. donovani* déstructure les microdomaines de son phagosome et empêche sa fusion avec les endosomes tardifs, bloquant ainsi la conversion phagosomale **[71]**. Le lipoarabinomannane des mycobactéries inhibe l'axe Ca^{2+}/Calmodulin-PI3kinase (Vps34) empêchant ainsi la formation du PI(3)P à partir du PI. Comme le PI3P est indispensable au recrutement de EEA1, un effecteur de Rab5, l'absence de recrutement de EEA1 arrête la maturation du phagosome au stade précoce **[72]**. Le LPS constitue probablement un des facteurs bactériens majeurs impliqués dans les mécanismes d'échappement à l'activité microbicide des macrophages. Il a été montré que la survie de *Salmonella* nécessite la présence de l'antigène O de son LPS puisque 80% des mutants de *Salmonella* n'exprimant pas l'antigène O sont localisés dans les lysosomes contre 20% des bactéries sauvages **[73]**. Il a été également observé que *Brucella suis*, via l'antigène O de son LPS, entre dans les macrophages murins dans des régions membranaires riches en rafts lipidiques, ce qui empêche la fusion des phagosomes contenant la bactérie avec les lysosomes **[74]**.

RESULTATS

REVUE I

Hijacked phagosomes and leukocyte activation: an intimate relationship

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Nous avons rassemblé au sein de cette revue les bases de la conversion phagosomale et les mécanismes les mieux connus du détournement de la conversion phagosomale par les agents infectieux. Le rôle joué par les Rab-GTPases et le complexe VpsC-HOPS dans la régulation de la conversion phagosomale qui requièrent les molécules SNARES, impliquées dans les événements de fusion de la membrane est ainsi évoqué. Les différents marqueurs permettant d'identifier chaque stade de la maturation phagosomale sont cités (**Tableau 1**). Nous décrivons également la nature des phagosomes contenant *Mycobacterium tuberculosis* et tout particulièrement la nature du phagosome de *Coxiella burnetii* en relation avec le devenir intracellulaire de ces bactéries (**Tableau 3**). Nous avons enfin tenté de lier conversion phagosomale et état d'activation des macrophages puisque les cytokines semblent moduler un certain nombre de molécules impliquées dans la conversion phagosomale. Cette dernière partie sera détaillée dans la revue II intitulée « *Role of the leukocyte activation in phagosome maturation process* » (cf. Annexe).

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JLB

Review

Hijacked phagosomes and leukocyte activation: an intimate relationship

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ABSTRACT

Intracellular pathogens have developed different strategies to survive within host cells. For example, these pathogens might interfere with the biogenesis of phagolysosomes, thereby forming replicative vacuoles. Although the complex mechanisms used by pathogens to hijack the biogenesis of phagolysosomes have been elucidated in naive leukocytes, the role of leukocyte activation in this process has not yet been investigated. Leukocytes are known to be activated by cytokines, and several reports have demonstrated that several cytokines modulate the endocytic pathway and thereby, affect phagosome biogenesis. These observations provide molecular evidence that endocytosis can be regulated by the immune environment. In this review, we highlight the effect of leukocyte activation by cytokines on the endocytic pathway and on phagosome biogenesis. We briefly describe the mechanism of phagolysosome formation before focusing on the strategies used by two bacterial pathogens, *Coxiella burnetii* and *Mycobacterium tuberculosis*, to hijack phagolysosome biogenesis. Finally, we emphasize the effect of leukocyte activation on the endocytic pathway and on phagolysosome formation, which has not been highlighted to date. *J. Leukoc. Biol.* 89: 000–000; 2011.

INTRODUCTION

Infectious diseases are major threats to human health worldwide, and intense effort has been directed toward understanding the mechanisms by which infectious intracellular pathogens compromise humans. Bacterial pathogens have evolved multiple strategies to survive within host cells. Several pathogens interfere with the biogenesis of phagolysosomes, thus resulting in the formation of phagosomes that are incapable of fusing with lysosomes (as reviewed in ref. [1]). Indeed, *Mycobacterium*-containing phagosomes fuse with early endosomes but are unable to fuse with late endosomes and consequently, with lysosomes [1, 2]. *Coxiella burnetii* survives within acidic late phagosomes that do not fuse with lysosomes [3, 4]. However, most of these studies used naive cells before stimulation with immune mediators. When a microorganism interacts with its host, it stimulates innate and adaptive immune responses. The innate immune response leads to the activation of host cells such as monocytes, macrophages, and DCs and drives the production of cytokines, which act in autocrine and paracrine manners. The adaptive immune system engages effectors such as antibodies and polarized T cells with their cytokines. Little is known about the effect of macrophage activation on the maturation of phagosomes (also called phagosome conversion) into phagolysosomes. We believe that cytokines modulate the composition and maturation of phagosomes, and this hypothesis is supported by recently published data. Proinflammatory cytokines, such as IFN-γ, IL-12, and IL-6, and anti-inflammatory cytokines, such as IL-10, have been shown to affect the endocytic pathway and thereby, modify phagosome biogenesis. This finding provides molecular evidence that endocytic processes can be regulated by the immune environment. In this review, we will summarize the general mechanisms of phagosome maturation and the specific mechanisms used by bacterial pathogens such as *Mycobacterium* spp. and *C. burnetii* to subvert phagosome maturation. We will detail the current mechanisms by which cytokines control the endocytic pathway and phagosome maturation. The exhaustive listing of the interplay of cytokines with endocytic processes established in this review provides fertile ground to better define the physiological roles of cytokines in modulating endocytic pathways and the intracellular fate of bacterial pathogens.

Abbreviations: EEA1=early endosome autoantigen-1, GDI=guanyl-nucleotide dissociation inhibitor, HOPS=homotypic protein-sorting, LAM=lipoarabinomannan, Lamp-1=lysosomal-associated membrane protein-1, M6PR=mannose 6-phosphate receptor, MptpB=*Mycobacterium* protien-tyrosine-phosphatase B, PI=phosphatidylinositol, PI(3)P=phosphatidylinositol-3-phosphate, RILP=Rab-interacting lysosomal protein, SapM=putative acid phosphatase *Mycobacterium*, T4SS=type IV secretion system, VAMP=vesicle-associated membrane protein, Vps=vacuolar protein sorting

PHAGOLYSOSOME BIOGENESIS

Once internalized, bacteria are localized within a nascent phagosome that undergoes a series of fusion and fusion-

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fission ("kiss and run") events with different populations of endocytic organelles. The final product of these events is the formation of a phagolysosome, which is a complex structure formed by more than 200 proteins that destroy bacteria [5]. The transformation of nascent phagosomes to phagolysosomes begins immediately after phagosome sealing and occurs in four successive stages: early, intermediate, and late phagosomes and the phagolysosome. The mechanisms that govern the progression of phagosome maturation are exceedingly complex and involve regulated interactions with various endocytic organelles. The discovery of the kiss and run mechanism, which consists of a succession of multiple transient fusion-fission events [6], the identification of the role of Rab proteins as membrane organizers (as reviewed in ref. [7]), the characterization of endosome conversion processes [8], and the analysis of phagolysosome composition [5] have delineated some general principles that are applicable to the biogenesis of phagosomes. It is widely accepted that the mechanism of progression from early phagosomes to phagolysosomes is similar to the process that governs endosome progression (endosome conversion). We substitute the term *phagosome maturation*, which suggests a progressive evolution of a well-known entity, by the term of *phagosome conversion*, which seems more appropriate to describe discontinuous events, such as successive fusion-fission events. Endosome and phagosome conversion is temporally coordinated through the sequential activities of molecules such as Rab GTPases, which regulate the endocytic pathway and require SNARE molecules, which are involved in membrane fusion events.

Immediately after sealing, nascent phagosomes interact with early and recycling endosomes by fusion and fusion-fission events. They gain several proteins necessary for the conversion of nascent phagosomes into early phagosomes (**Fig. 1A**). These proteins include the small GTPase Rab5, EEA1, syntaxin-13, Vps34 (a type III PI3K), and PI(3)P generated by Vps34. Subsequently, by successive fusion-fission events, early phagosomes transform into intermediate compartments prior to the formation of late phagosomes. These intermediate phagosomes have the properties of early and late phagosomes (Fig. 1A), as their surfaces contain molecules characteristic of early endosomes [Rab5, PI(3)P, EEA1] and late endosomes (Rab7, RILP, Lamp-1) [1, 9]. Although the small GTPases Rab5 and Rab7 are critical mediators of phagosome conversion [10, 11], it appears that the transition from intermediate phagosomes to late phagosomes might be coordinated by the VpsC-HOPS complex [1, 8], which mediates the transition of Rab5-positive endosomes to Rab7-positive endosomes [8]. Late phagosomes are characterized by the presence of proteins such as Lamps (Lamp-1, -2, and -3), RILP, and the small GTPase Rab7, which progressively replace early and intermediate phagosomal markers [1, 12] (Fig. 1A). The small GTPase Rab7 mediates exchanges between phagosomes and late endosomes or lysosomes [13, 14]. Finally, late phagosomes interact with lysosomes through M6PRs. This terminal step of phagosome conversion leads to the formation of phagolysosomes that are rich in lysosomal hydrolytic enzymes such as cathepsin D [1, 12] (Fig. 1A). In addition to changes in composition, phagosome conversion involves physical modifications. Whereas the pH of early phagosomes is ~6.0, acquisition of the vacuolar proton pump ATPase (V-H$^+$-ATPase) by late phagosomes induces a drop in the intraphagosomal pH to 5.5 in late phagosomes and 4.5 in phagolysosomes [1, 12]. It is possible to discriminate between the different stages of phagosome conversion using characteristic markers or specific probes (**Table 1**). The complex machinery that drives phagosome conversion is a preferential target for numerous pathogens capable of hijacking the process of phagolysosome formation (**Table 2**).

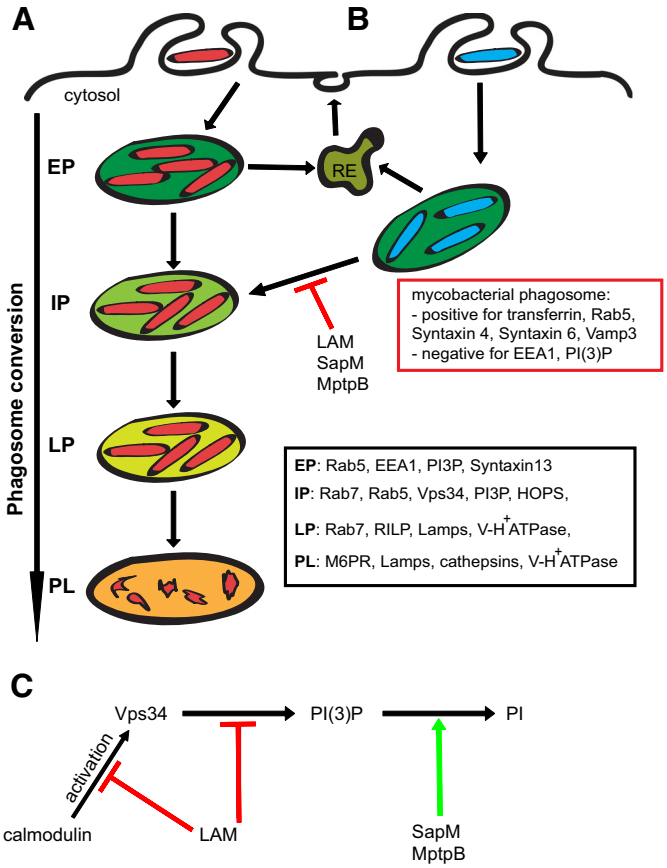

Figure 1. Overview of *Mycobacterium* phagosome hijacking mechanisms. (A) Immediately after sealing, nascent phagosomes interact with endosomes by fusion and fusion-fission events. They gain several protein necessary for the conversion of nascent phagosomes into phagolysosome. (B) The phagosome harboring *Mycobacterium tuberculosis* is blocked at the early stage and is accessible to early endosomes and transferrin-bound iron. This phagosome harbors Rab5, VAMP3, syntaxin 4, and syntaxin 6 but is devoid of EEA1, PI(3)P, and V-H$^+$-ATPase. Three mycobacterial products act in concert (LAM, SapM, and MptpB) to inhibit the conversion of the *Mycobacterium*-containing phagosome into a phagolysosome, within which the bacteria would be classically destroyed. (C) LAM inhibits the production of PI(3)P by Vps34 and the activation of Vps34 by calmodulin. SapM and MptpB, two phosphatases, hydrolyze PI(3)P to form PI, EP, Early phagosomes; IP, intermediate phagosomes; LP, late phagosomes; PL, phagolysosomes; RE, recycling endosomes.

TABLE 1. Simplified View of Endocytic Compartments Identity

Compartments	Composition
Early endosomes, early phagosomes	pH 6.0 – 6.5; Rab5; EEA1; Vps34; PI(3)P; rabenosyn-5
Late endosomes; late phagosomes	pH 5.0 – 6.0; Rab7; Lamps; V-H^+-ATPase; M6PR; RILP
Lysosomes; phagolysosomes	pH 4.0 – 5.5; V-H^+-ATPase; Lamps; mature lysosomal enzymes (cathepsins)
Recyling endosomes	Rab4; transferrin receptor; EEA1
Autophagosomes	LC3; p62; Lamps, beclin, Vps34

THE MYCOBACTERIAL PHAGOSOME: A DYNAMIC EARLY STRUCTURE

The human pathogens *M. tuberculosis* and *Mycobacterium avium* and a variety of nontuberculosis mycobacterial species, such as *Mycobacterium bovis* and *Mycobacterium marinum*, persist and replicate within macrophages. These organisms survive within specialized immature phagosomes by interfering with phagolysosome biogenesis (Fig. 1B) [2]. The principal characteristics of mycobacterial phagosomes are the incomplete acidification of the lumen, which results from exclusion of the V-H^+-ATPase [22], and the absence of lysosomal hydrolases such as cathepsin D [23]. A biochemical analysis of purified phagosomes has revealed that Rab5 is acquired and accumulates in these phagosomes, whereas neither Rab7 nor Lamp-1 is present even after several days [24, 25]. This blockade at the Rab5-positive stage suggests that *M. tuberculosis* is able to manipulate the functions of the Rab GTPases to replicate within host cells [10]. To understand how a phagosome harboring *M. tuberculosis* is blocked at the early phagosome stage, Rab5-interacting partners and regulatory proteins of the early endosomal trafficking and phagosome conversion process have been closely examined [24, 26, 27]. It appears that a phagosome harboring *M. tuberculosis* is characterized by the absence of EEA1 on its surface [25, 26]. This absence could be explained by decreased amounts of PI(3)P [28–30], as PI(3)P is a critical regulator of the recruitment of Rab5 effectors such as EEA1 [31]. It is important to note that the inhibition of phagosome conversion by mycobacteria cannot be reduced to a straightforward defect in EEA1 acquisition and an alteration in the PI(3)P level, as a number of additional modifications have been described for mycobacterial phagosomes. For example, SNAREs, such as VAMP3, syntaxin 4, and syntaxin 6, are retained on the surface of mycobacterial phagosomes [26, 27, 32]. It is known that SNARE proteins are required for membrane fusion events and for interactions of endosomes and/or lysosomes with phagosomes. The retention of SNAREs at the phagosomal membrane could explain the observation that the mycobacterial phagosome is not a static organelle; it has been clearly demonstrated that the mycobacterial phagosome is accessible to early endosomes. Indeed, access to transferrin-bound iron [22, 33] and the trafficking of transferrin receptors through mycobacterial phagosomes have been found to occur efficiently [33]. However, explanations for why VAMP3, syntaxin 4, and syntaxin 6 are retained in the phagosome membrane have not been elucidated.

Finally, three *M. tuberculosis* products inhibit PI(3)P production (Fig. 1C): LAM, which is the major component of the mycobacterial membrane [10], and SapM and MptpB, which are PI phosphatases [34, 35]. Because of its similarity with mammalian PIs, LAM inhibits the production of PI(3)P by Vps34 [36, 37]. LAM also inhibits the calmodulin kinase II-dependent activation of Vps34 by blocking the accumulation of cytosolic calcium [38]. SapM and MptpB dephosphorylate monophosphorylated PIs, leading to the inhibition of phagosome fusion with late endosomes and lysosomes [28, 35]. Therefore, LAM, SapM, and MptpB act in concert to deplete the mycobacterial phagosome of PI(3)P.

TABLE 2. Intracellular Lifestyle of Few Bacterial Pathogens

Bacteria	Intracellular localization	References
Brucella abortus	early phagosome	[15]
Chlamydia spp	nonacidified inclusion	[10]
C. burnetii	acidic late phagosome	[16]
Francisella tularensis	cytosol	[17]
Legionella pneumophila	ER-derived phagosome	[1]
Listeria monocytogenes	cytosol	[18]
M. tuberculosis	early phagosome	[2]
Rickettsia spp	cytosol	[19]
Salmonella typhimurium	late phagosome	[20]
Shigella flexnerii	cytosol	[19]
Yersinia pestis	autophagosome	[21]

THE *C. BURNETII* PHAGOSOME

C. burnetii, an obligate intracellular bacterium, is the causative agent of Q fever. This zoonosis is prevalent in most places throughout the world except New Zealand [39] and has a seroprevalence of 4.03 per inhabitant in France [40]. *C. burnetii* is a potential bioweapon that has been classified as a group B agent by the Centers for Disease Control and Prevention in the United States [39]. The infective form of *C. burnetii* found in patients with Q fever is called phase I, whereas avirulent variants, which are also known as phase II bacteria, are obtained following a long-term laboratory culture of phase I *C. burnetii* [39, 40]. Phase I and phase II *C. burnetii* behave differently depending on the host cell type. In myeloid cells, which are the host cells of *C. burnetii*, phase I *C. burnetii* survive and replicate, whereas phase II organisms are eliminated (as reviewed in ref. [16]). In contrast, virulent and avirulent organisms replicate in non-microbicidal cells such as CHO, L929, Vero, and HeLa cells [41–45] or trophoblastic cell lines (unpublished results).

TABLE 3. Studies on the Nature of Vacuoles Harboring Virulent *C. burnetii* or Avirulent Variants

C. burnetii	Localization	Markers used in the studies	Cell types	Microbicidal activity	References
avirulent	phagolysosome	acid phosphatase	Vero	no	[46]
avirulent	phagolysosome	Lamp-1, Lamp-2, M6PR, acridine orange, V-H$^+$-ATPase	Vero	no	[43]
avirulent	phagolysosome	Lamp-1, EEA1	Vero	no	[47]
avirulent	lysosomal-like compartment	acridine orange, Lamp-1, EEA1	Vero	no	[42]
avirulent	lysosomal compartment	flotillin, cholesterol	Vero	no	[48]
avirulent	autophagosome	LC3, Rab24	CHO	no	[44]
avirulent	autophagosome	Rab5, Rab7, EEA1, LC3, delayed fusion with lysosomes	CHO	no	[45]
avirulent	autophagosome	Rab7, LC3	HeLa	no	[41]
avirulent	phagolysosome	pH determination	P388D1; L929	no	[49]
avirulent	lysosomal compartment	Lamp-1, acridine orange	Vero	no	[50]
avirulent	lysosomal compartment	Lamp-2	mBMDM	yes	
avirulent	lysosomal compartment	Lamp-1	hMDM	yes	
avirulent	lysosomal compartment	Lamp-1	DCs	no	
avirulent	lysosomal compartment	CD63	DCs	no	[51]
avirulent	phagolysosome	Lamp-1, cathepsin D, M6PR, Rab7, EEA1, pH determination, V-H$^+$-ATPase, NIRF	THP-1-PMA	yes	[3]
avirulent	phagolysosome	Lamp-1, cathepsin D	human	yes	[4]
avirulent	phagolysosome	NADPH oxidase	monocytes neutrophil	yes	[52]
virulent	not phagolysosome	acid phosphatase	L cell	no	[46]
virulent	phagolysosome	acid phosphatase, 5′-nucleolidase	CEF	no	[53]
virulent	phagolysosome	thorium dioxide, pH determination, acid phosphatase activity	J774A.1	weakly	[54]
virulent	delayed formation of phagolysosome	thorium dioxide	J774A.1	weakly	[55]
virulent	acidified late phagosome	pH determination, Lamp-1, Rab7, M6PR, EEA1, V-H$^+$-ATPase, NIRF, cathepsin D	THP-1-PMA	yes	[3]
virulent	late phagosome	Lamp-1, cathepsin D	human monocytes	yes	[4]
virulent	late phagosome	Lamp-2, acridine orange	mBMDM	yes	[50]
virulent	not phagolysosome	cathepsin D	hMDM	yes	[56]
virulent	lysosomal compartment	CD63	DCs	no	[51]
virulent	not autophagosome	p62	hMDM	yes	[a]
virulent	phagolysosome	Lamp-1, cathepsin D	trophobiast	no	[a]

[a]Unpublished results. Vero, monkey epithelial cell; CEF, chicken embroy fibroblast; CHO, chinese hamster ovary cells, HeLa: epithelial cell line; hMDM, human MDMS; J774A.1, macrophage-like cell line; L929, mouse fibroblast cell line, L cell, cell line derived from mouse fibroblast cell line; mBMDM, mouse bone marrow-derived macrophages; P388D1, Lymphoblast cell line; THP-1, human myelomonocytic cell line; THP-1-PMA, THP-1 cells treated with PMA; NIRF, near-infrared fluorescence.

Intracellular localization of *C. burnetii*

The nature of the compartment in which *C. burnetii* resides is debated, in great part, for historical reasons. During the 1980s, *C. burnetii* was shown to reside in an acidic compartment in several cell types. Consequently, it has been assumed that *C. burnetii* resides in phagolysosomes, as the acidity of the compartment was determined with reference markers for lysosomes. This assumption was based on an insufficient characterization of *C. burnetii*-containing phagosomes, as it is currently known that several markers are required to characterize a

TABLE 4. *C. burnetii:* A Multifaceted Compartment

Bacteria	Compartments	Composition	Cells
virulent	acidified phagosome	Lamp-1, Lamp-2, V-H$^+$-ATPase, Low Rab7, Low M6PR	microbicidal
avirulent variants	phagolysosome	Lamp-1, Lamp-2, M6PR, V-H$^+$-ATPase, Rab7, lysosomal enzymes	microbicidal
virulent and avirulent variants	phagolysosome-autophagosome	Lamp-1, Lamp-2, M6PR, V-H$^+$-ATPase, Rab7, lysosomal enzymes, LC3, cholesterol, Rab24	non-microbicidal

given compartment (Table 1). In addition, most of the results were obtained using avirulent variants of *C. burnetii* (**Table 3**) and were extrapolated to virulent *C. burnetii*, which led to a misinterpretation of the intracellular localization of *C. burnetii* (as reviewed in refs. [57–59]). At present, *C. burnetii* appears to survive within an acidic late phagosome in monocytes and macrophages by preventing phagolysosome biogenesis (**Table 4**) [16].

The absence of phagosome-lysosome fusion in macrophages infected with *C. burnetii* has been discussed. Howe and Mallavia [55] claim that lysosomal markers, such as acid phosphatases, decorate *C. burnetii* phagosomes in the mouse macrophage cell line J774A.1, which are weakly microbicidal. However, after 6 h of infection, only 50–60% of the bacteria colocalize with acid phosphatases; the fate of *C. burnetii* was not investigated beyond 6 h. The increased colocalization of *C. burnetii* with acid phosphatases might be a result of the transient elimination of *C. burnetii* in phagolysosomes, as a transient elimination of *C. burnetii* has been observed in macrophages [60]. The discrepancies in the literature and the generalization of data obtained with avirulent variants to virulent *C. burnetii* are likely associated with the heterogeneous host cells used in diverse reports and the nature of the engaged receptors. Thus, in non-microbicidal cells and in cell lines such as CHO, L929, Vero, and HeLa cells particularly, *C. burnetii* resides in phagolysosomes [41–45]. Similar results have been obtained with trophoblastic cell lines (unpublished results).

In contrast, in monocytes and macrophages, *C. burnetii* survives within acidic late phagosomes [3]. The route of *C. burnetii* internalization may also influence the fate of the bacteria. Indeed, *C. burnetii* is internalized by monocytes and macrophages through the engagement of surface proteins such as $\alpha v\beta 3$ integrin, whereas phase II bacteria also engage complement receptor 3 (CD11b/CD18), a receptor specific to myeloid cells [61]. The importance of the nature of the infected cells and the route of internalization of bacterial pathogens have been clearly demonstrated for mycobacteria. In mouse J774A.1 macrophages, *M. bovis* is transiently eliminated during the first days of infection; bacteria that are not eliminated survive but are unable to replicate. In contrast, in human MDMs, *M. bovis* begins to replicate immediately without a transient elimination phase [62].

Similar differences have been reported for other mycobacterial species [12, 63]. The opsonization of mycobacteria modifies bacterial trafficking. Indeed, opsonized mycobacteria replicate within phagolysosomes, whereas unopsonized mycobacteria replicate within early phagosomes. This last observation suggests that the nature of the receptor engaged during bacterial entry may govern the molecular mechanisms involved in phagosome conversion.

Intracellular localization of avirulent variants of *C. burnetii*

In contrast to virulent *C. burnetii*, **avirulent variants of** *C. burnetii* (phase II organisms) are eliminated in phagolysosomes of human monocytes and macrophages [16], and their replication is controlled in resident mouse peritoneal macrophages [64]. In non-microbicidal cells, phase II bacteria replicate within a compartment that displays all of the characteristics of phagolysosomes, as is decorated with Lamp-1, Lamp-2, M6PR, V-H^+-ATPase, Rab7, cathepsin D, and acid phosphatases (Table 4) [41–43, 58]. However, several markers of autophagosomes, such as LC3, Rab24, and cholesterol, are detected at the surface of the compartment that contains phase II bacteria [41, 44, 45]. These data suggest that avirulent *C. burnetii* do not replicate in a phagolysosome but rather, in a chimeric compartment that shares properties of phagolysosomes and autophagosomes. It is likely that avirulent bacteria re-route phagolysosomes through the autophagosome pathway. We can also suppose that this environment is more suitable for bacterial metabolism than the harsh environment of phagolysosomes, at least in part, as these chimeric organelles are rich in the cholesterol required for bacterial replication [48, 65].

Virulence factors of *C. burnetii*

The mechanisms used by *C. burnetii* to hijack phagosome conversion remain unknown. However, several bacterial virulence factors might be involved. The genome of *C. burnetii* contains several genes that encode components of a T4SS (IcmT, IcmS, and IcmK) [66]. These genes are mechanistically related to the *Legionella* Dot/Icm apparatus [67], which contributes to the hijacking of phagosome maturation by *Legionella*. It is tempting to speculate that this T4SS plays a major role in the biogenesis of *C. burnetii*-containing phagosomes [59, 68, 69]. Finally, the virulence of *C. burnetii* has been correlated with the expression of LPS. *C. burnetii* displays antigenic variations that are similar to the smooth-rough variations of enterobacteria. It has been reported that LPS might govern the intracellular localization of several pathogens such as *B. abortus* [70, 71], *Y. pestis* [72], and *S. flexneri* [73]. Published [74] and unpublished results suggest that the LPS of phase I *C. burnetii* is involved in the hijacked conversion of bacterial phagosomes. We can speculate that *C. burnetii* LPS affects phagosome conversion in a manner similar to that of *M. tuberculosis* LAM (see above) or the lipophosphoglycan from *Leishmania donovani*, which disrupts phagosome microdomains and consequently inhibits phagosome-lysosome fusion [75].

LEUKOCYTE ACTIVATION AND PHAGOSOME CONVERSION

Various cytokines regulate the ability of monocytes and macrophages to clear pathogens and elicit a sustained immune response. Recent studies have shown that phagosome conversion depends on the balance between proinflammatory cytokines, such as IFN-γ, IL-12 and IL-6, and anti-inflammatory cytokines, such as IL-10, which indicates that cytokines modulate phagosome conversion during bacterial infections. However, the mechanisms underlying the cytokine-mediated modulation of intracellular pathogen trafficking remain to be elucidated.

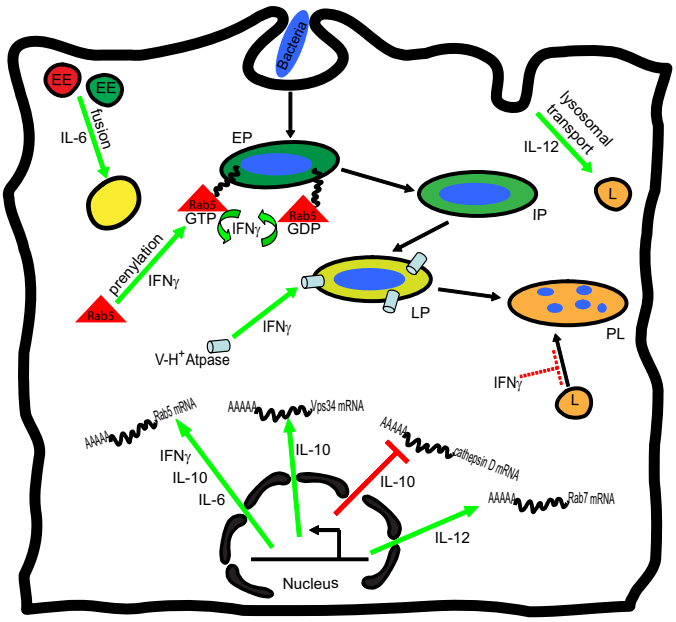

Figure 2. Cytokines that affect endocytic components. Several key endocytic regulators that play a critical role in membrane trafficking, endosome conversion, and phagosome conversion may be modulated by cytokines. EE, Early endosomes, L, lysosomes; green arrows, positive effect; red stroke, negative effect; red dotted stroke, delayed effect.

Molecular effects of cytokines on endocytosis and endocytosis effectors

Cytokines modulate key endocytic regulators, such as Rab5 and Rab7, which play a critical role in membrane trafficking, endosome conversion, and phagosome conversion (**Fig. 2**). Several years ago, it was demonstrated that IFN-γ selectively up-regulates the expression of Rab5a mRNA and protein and induces Rab5a guanine nucleotide exchange activity in mononuclear cells [76]. In addition, IFN-γ increases Rab5 prenylation, which leads to increased levels of membrane-associated Rab5a:GTP. The effect of IFN-γ on Rab5a synthesis and processing is selective; the levels of other Rab GTPases, including Rab5b, Rab5c, Rab7, and Rab11, remain unaffected. It is likely that the prenylation of Rab5a is responsible for the selective retention of Rab5a at the membranes of endosomes and phagosomes. We can suppose that the enhanced activity of Rab5a proteins modifies the composition of the phagosome membrane and accelerates the rate of phagosome conversion into phagolysosomes [76]. We have observed recently that IL-10 strongly decreases the expression of Rab5 and Vps34 transcripts, whereas IFN-γ does not affect the levels of Vps34 mRNA (unpublished results). We can speculate that immunoregulatory cytokines such as IL-10 decelerate endosome and phagosome conversion by controlling the protein levels of critical endocytic effectors such as Rab5 and Vps34 kinase. Treatment of macrophages with IL-6 specifically induces the expression of Rab5 through the activation of ERK, whereas IL-12 exclusively up-regulates the expression of Rab7 through the activation of p38 MAPK. In addition, IL-12 is associated with the up-regulation of lysosomal transport, whereas IL-6 stimulates fusion events between early compartments in macrophages [77]. Taken together, these observations confirm that cytokines affect endocytic processes and show that ERKs are tightly linked to endocytosis. It is important to note that p38 MAPK phosphorylates endocytic effectors such as EEA1 and Rab5 GDI [78, 79], thus enhancing endosome conversion.

Other membrane networks are targeted by cytokines. Indeed, IFN-β induces alkalinization of the trans-Golgi network by inhibiting V-H$^+$-ATPase activity [80], which limits endocytic exchanges. IL-10 decreases the expression of cathepsin D in monocytes from patients with inflammatory bowel disease [81] and affects fluid-phase and mannose receptor-mediated endocytosis in human primary macrophages [82]. Consequently, it is likely that lysosomal biogenesis is limited, and the delivery of cathepsin D to lysosomes is decreased. The data obtained during the last few years clearly show that several cytokines act on the molecules involved in the endocytic process, endosome conversion, and phagosome conversion. This leads to changes in the membrane composition of endocytic organelles and a decrease or an increase in endocytic exchanges.

TABLE 5. Cytokines that Affect Phagosome Conversion

Cytokine	Effects	References
IFN-γ	induces *Mycobacterium* killing: rescues PL fusion; increases V-H$^+$-ATPase phagosomal recruitment and phagosomal acidification; inhibits tranferrin accessibility to phagosomes; induces PL fusion; induces Lamp-1 and cathepsin D recruitment	[83]
	induces *L. pneumophila* killing: inhibits phagosome remodeling into ER-derived compartment; induces Lamp-2 and cathepsin D recruitment	[83]
	induces *C. burnetii* killing: rescues PL fusion; induces vacuole alkalinization	[83]
	induces *L. monocytogenes* killing: induces Rab5a expression	[83]
IL-22	induces *Mycobacterium* killing: rescues PL fusion	[83]
IL-12	induces *Salmonella* killing: increases Rab7 expression; induces lysosomal transport	[80]
IL-6	increases *Salmonella* survival: induces Rab5 expression; inhibits PL fusion	[80]
IL-10	IL-10 knockout mice: increased colocalization of mycobacteria with lysosomal markers	[80]
	modulates *C. burnetii* killing through phagosome conversion; IL-10-neutralizing antibodies rescue phagosome conversion and bacteria in monocytes from patients with chronic Q fever	[4]

Several key endocytic regulators that play a critical role in phagosome conversion may be modulated by cytokines. PL, Phagolysosome.

Effects of cytokines on phagosome composition and conversion

Effects of IFN-γ on phagosome composition and conversion. Several studies have reported that IFN-γ acts directly on the conversion of pathogen-containing phagosomes (**Table 5**). IFN-γ allows *Mycobacterium* killing by macrophages by rescuing phagosome-lysosome fusion [84, 85]. Several phagosomal modifications are observed during IFN-γ treatment. Phagosomes containing mycobacteria are not accessible to transferrin, which inhibits the exchange of mycobacterial phagosomes with early endosomes. In addition, the pH of mycobacterial phagosomes is 5.2 in IFN-γ-activated macrophages, whereas it is 6.3 in resting macrophages. Biochemical analysis of mycobacterial phagosomes confirmed that the lower intraphagosomal pH is correlated with the increased accumulation of V-H^+-ATPase [84], which suggests that IFN-γ rescues phagosome fusion with late endosomes and the recruitment of V-H^+-ATPase at the phagosomal membrane. In addition, individual bacterial vacuoles that contain a single mycobacterium have been observed to coalesce into communal vacuoles containing multiple mycobacteria, which suggests that phagosome–phagosome homotypic fusion events increase during IFN-γ treatment [84]. Finally, IFN-γ induces the colocalization of mycobacteria with lysosomal markers such as Lamp-1 and cathepsin D, which indicates that phagosomes are converted to phagolysosomes, in which the bacteria are destroyed [86]. IFN-γ also inhibits the remodeling of *L. pneumophila*-containing phagosomes into ER-derived vesicles via their conversion into Lamp-2 and cathepsin D-expressing phagolysosomes [87]. Consequently, these data are consistent with the hypothesis that IFN-γ directs the endocytic pathway toward the degradative pathway. *C. burnetii* phagosomes fuse with lysosomes in the presence of IFN-γ, as demonstrated by the acquisition of cathepsin D and the killing of phase I *C. burnetii* [3]. IFN-γ mediates these events through two different mechanisms. First, the addition of IFN-γ to *C. burnetii*-infected macrophages stimulates phagosome-lysosome fusion but does not affect the vacuolar pH. Second, treatment of macrophages with IFN-γ prior to *C. burnetii* infection induces alkalinization of *C. burnetii* vacuoles independently of V-H^+-ATPase exclusion [3]. These findings are in contrast with reports showing that IFN-γ decreases the pH of *Mycobacterium*-containing phagosomes [84]. The mechanism of IFN-γ-mediated vesicle alkalinization in the *C. burnetii* infection model remains to be elucidated. In a model of *L. monocytogenes* infection, IFN-γ induced Rab5a expression and phagosome conversion, which play a central role in *Listeria* destruction [88]. These findings reveal the importance of Rab5a as a mediator of the listericidal activity of IFN-γ, and IFN-γ-induced Rab5 causes remodeling of the phagosomal environment, facilitates the translocation of Rac2 to phagosomes harboring *Listeria*, and regulates the activity of this GTPase. Rac2 governs phagocyte NADPH oxidase activity and the subsequent production of reactive oxygen intermediates [88]. These events likely facilitate the transition of early phagosomes to late phagosomes, thus inhibiting the membrane lysis of *L. monocytogenes* phagosomes. Recently, Trost et al. [83] used quantitative proteomic and bioinformatic approaches to profile the changes in composition, abundance, and phosphorylation of proteins in latex bead-containing phagosomes in resting and IFN-γ-activated macrophages. They found that 2415 phagosomal proteins and 2975 unique phosphorylation sites are modulated by IFN-γ. Furthermore, IFN-γ modulates 167 proteins found on phagosomes, and 90% of these proteins are up-regulated. These proteins include enzymes that are expected to enhance microbial degradation, trigger the macrophage immune response, promote antigen loading on MHC class I molecules, and modulate phagosome conversion. Several phagosomal proteins involved in fusion with endocytic compartments, such as syntaxin 7, syntaxin 13, Rab7, lysosomal integral membrane protein 2, and VAMP8, are up-regulated by IFN-γ. In addition, IFN-γ delays the phagosomal acquisition of lysosomal hydrolases and peptidases [83]. These data clearly demonstrate that IFN-γ dramatically changes the composition of phagosome membranes and likely modulates phagosome conversion.

Effects of other cytokines on phagosome conversion. In addition to IFN-γ, other proinflammatory cytokines, such as IL-22, IL-12, and IL-6, have been shown to modulate the conversion of phagosomes into phagolysosomes (Table 5). IL-22 allows mycobacterial killing by macrophages by rescuing phagosome-lysosome fusion [84, 85]. In cells infected with *Salmonella*, the *Salmonella*-containing vacuole is targeted to lysosomes when cells are treated with IL-12, whereas the transport of *Salmonella* to lysosomes is inhibited in the presence of IL-6. In these instances, IL-12 induces lysosomal transport, whereas IL-6 stimulates fusion between early compartments in macrophages, thereby modulating *Salmonella* trafficking and survival in macrophages [77]. The survival of *Salmonella* in macrophages is inhibited significantly in IL-12-treated cells in comparison with untreated control cells. IL-12 increases the expression of Rab7 in macrophages, which enhances *Salmonella* killing by targeting the bacteria to lysosomes. In contrast, IL-6 up-regulates Rab5 expression, which promotes the fusion of *Salmonella*-harboring phagosomes with early endosomes and thereby, inhibits their transport to lysosomes [77]. The modulation of phagosome conversion in infectious models is not limited to proinflammatory cytokines, as anti-inflammatory cytokines such as IL-10 have also been shown to affect phagosome conversion (Table 5). Indeed, in macrophages derived from the bone marrow of IL-10 knockout mice, the colocalization of mycobacteria with lysosomal markers is enhanced relative to macrophages from control mice, which suggests an increase in the acidification of mycobacterial phagosomes [86]. IL-10 is overproduced during chronic Q fever [89, 90], and monocytes from patients with chronic Q fever are unable to kill *C. burnetii* and exhibit defective phagosome conversion; phagosomes harboring *C. burnetii* are not converted in phagolysosomes. In contrast, monocytes from patients who are recovering from acute Q fever kill *C. burnetii* efficiently, and this killing is associated with phagosome conversion [4]. These observations are related to the activity of the disease, as phagosome-lysosome fusion and *C. burnetii* killing are restored in patients who have recovered from Q fever endocarditis. Interest-

ingly, *C. burnetii* killing and phagosome conversion into phagolysosomes are related. The use of specific antibodies to neutralize IL-10 in monocytes from patients with chronic Q fever rescues phagosome conversion and bacterial killing to the levels detected in cured patients. In contrast, the addition of rIL-10 to monocytes from patients who have recovered from Q fever endocarditis blocks phagosome conversion, prevents the phagosome from colocalizing with cathepsin D, and inhibits *C. burnetii* killing [4]. The molecules of the endocytic pathway that are modulated by IL-10 remain unknown. As IL-10 strongly decreases the expression of Rab5 and Vps34 transcripts (unpublished data), we can speculate that IL-10 acts via a down-modulation or repression of key modulators of endocytosis. It is also possible that IL-10 inhibits Rab prenylation or GDI activity, as the function of IL-10 is in opposition to that of IFN-γ.

Finally, despite the observed control of the endocytic pathway through changes in the phagosome luminal environment, cytokines may have a direct effect on bacterial survival. Several cytokines, such as IL-1β [91], IL-6 [92], and TNF [93], are able to bind to bacteria. Importantly, this binding may affect bacterial growth. Indeed, IL-1β binds to virulent strains of *Escherichia coli* and stimulates their growth [91]. IL-6 induces the replication of *M. avium* [92]. Human epidermal growth factor favors the growth of *M. tuberculosis* and *M. avium* when these bacteria are cultured extracellularly [94]. *S. typhimurium* and *S. flexneri* bind TNF-α, and this binding leads to greater bacterial invasion of HeLa cells [93]. The direct effect of cytokines on bacterial replication is unknown, and the synthesis of a hypothesis to explain this phenomenon is difficult with the available evidence.

CONCLUDING REMARKS

This study highlights the relationship between phagosome-dependent microbicidal pathways and the counter-strategies used by bacteria. By manipulating the dynamics and integrity of the host cell membrane, pathogens can create niches that are suitable for their survival and replication. The formal identification of phagosomes that contain pathogens is difficult, as these pathogens manipulate the composition of the compartments they inhabit, thus leading to a mixed identity. The use of a large number of markers is clearly necessary to characterize compartments in which bacteria reside. A partial characterization may lead to oversimplifications and misinterpretations. Another difficulty is the increased number of molecules that is necessary to define the identity of a phagosome. It is likely that only a global approach, such as proteomics, will be suitable to define bacterial phagosomes precisely, as immunofluorescence provides only partial information. As the number of emerging pathogens is increasing, it will be important to develop large-scale proteomics methods to purify the diverse types of bacterial phagosomes and to define the "phagobiome." Another element of complexity in defining the identity of bacterial phagosomes is the recent discovery that phagosome conversion is directed by the microenvironment of host cells, especially immune cells. Indeed, the treatment of macrophages with inflammatory cytokines induces the conversion of bacterial phagosomes into phagolysosomes and bacterial killing; in contrast, immunoregulatory cytokines inhibit phagosome conversion and favor bacterial growth. In addition, the contact between leukocytes and bacterial pathogens may lead to cytokine production, thus creating amplification or inhibitory loops. These processes may explain the discrepancies observed in the intracellular localization of some pathogens that infect leukocytes or other cell types that are less sensitive to cytokines, such as endothelial cells. The cross-talk among endocytosis, bacterial phagosome conversion, and leukocyte activation requires further investigation, such as full-genome RNA interference screening and knock-in/knock-out approaches. A better knowledge of this cross-talk will likely be important for the development of new, rational strategies for the eradication of bacterial pathogens.

AUTHORSHIP

E.G. designed and wrote the review. A.O.B. and J-L.M. were in charge of one chapter of this review.

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KEY WORDS:
cytokines · phagolysosomes · endosomes · bacteria

Trafic intracellulaire de *Coxiella burnetii*

Article I et Article II

Coxiella burnetii, une bactérie à gram négatif, est responsable de la fièvre Q. *C. burnetii*, classifiée dans la subdivision gamma des protéobactéries, est une bactérie strictement intracellulaire qui réside dans les cellules myéloïdes en inhibant la conversion phagosomale en phagolysosomes **[75]**.

Les facteurs de virulence bactériens impliqués dans le blocage / détournement de la conversion phagosomale sont divers, le plus souvent exprimés à la surface des bactéries. Ce sont en particulier le lipoarabinomannane (LAM) **[76]**, le lipophosphoglycane (LPG) **[71]** et le LPS **[77, 78]**. Le LPS, le composant le plus abondant de la membrane externe des bactéries à gram négatif, est une molécule complexe qui présente de multiples activités biologiques **[79]**. Le LPS libéré dans les fluides corporels par les bactéries mortes ou vivantes est rapidement ingéré par les phagocytes professionnels par pinocytose, macropinocytose, phagocytose ou endocytose dépendante de récepteurs, la voie la plus efficace **[77]**. Le LPS est constitué de trois régions séparées : le lipide A, le core oligosaccharidique et la chaîne O **[80]** (**Figure 4**). La chaîne O ou antigène O, la partie la plus externe de la molécule, est l'antigène reconnu par les anticorps : elle est typiquement composée d'hexoses.

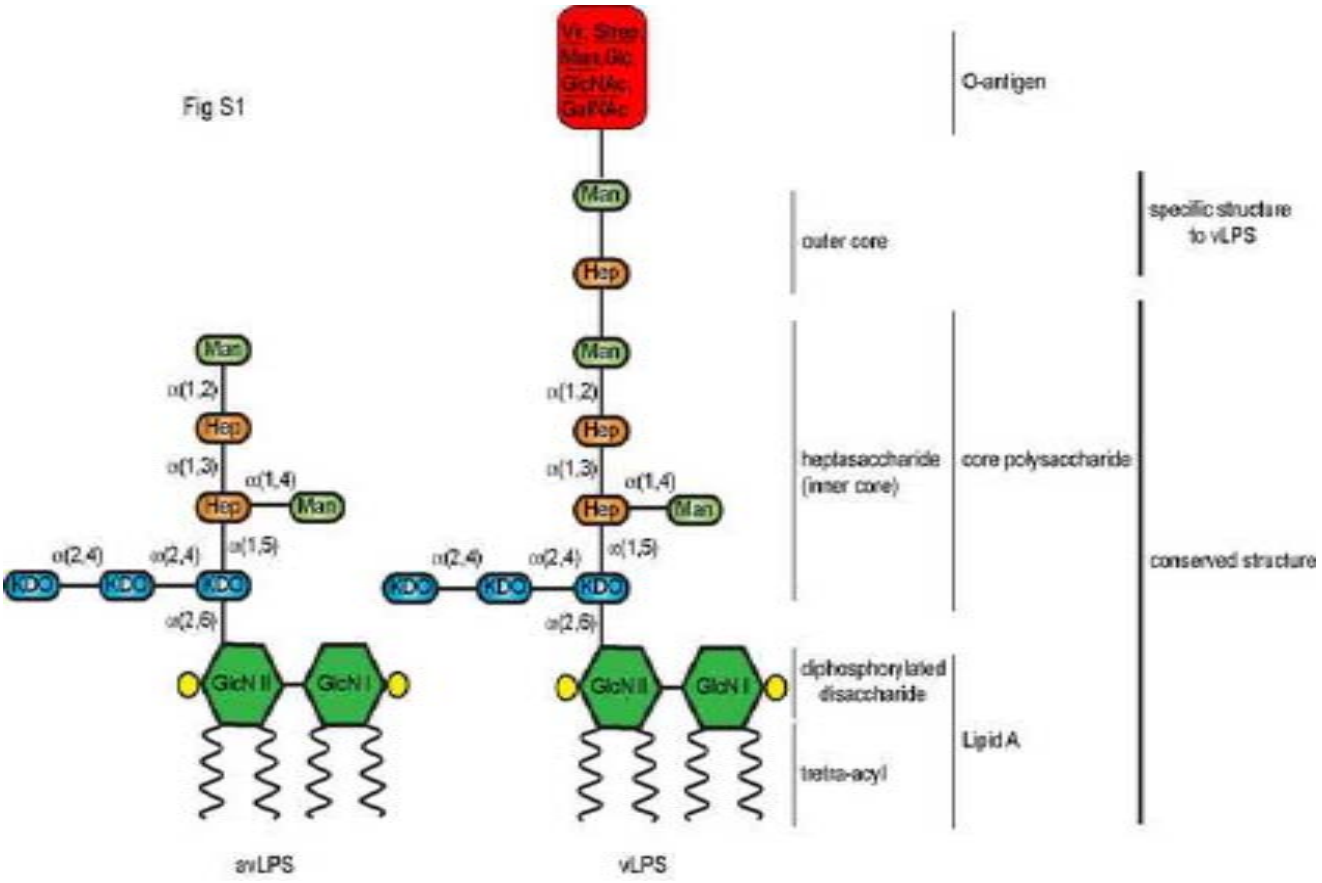

Figure 4 : Structure du LPS

Les unités répétitives de la région O sont constituées de 1 à 8 résidus glycosyl et leur contenu en sucres, leur séquence, leurs types de liaisons chimiques ou leur degré de substitution diffèrent selon les espèces bactériennes **[79, 80]**. Le core oligosaccharidique est subdivisé en core interne et en core externe. Le core interne est typiquement constitué d'hexoses communs tels que le glucose, le galactose, la N-acétylgalactosamine et la N-acétyl-glucosamine : sa structure est généralement plus variable que celle du core interne qui est, lui, caractérisé par des sucres rares, particulièrement le 2-keto-3-deoxyoctanoate (Kdo) et l'heptose. La multiplication bactérienne exige a minima la liaison d'un résidu Kdo au lipide A **[79, 80]**. Le lipide A est typiquement composé d'un disaccharide D-GlcN-(1-6)-D-GlcN(D-glucosamine-(1-6)-D-glucosamine)
portant deux groupements phosphates en position 1 et 4' auxquels peuvent se substituer de l'éthanolamine ou de l'éthanolamine-phosphate. Sur ce disaccharide sont attachés par des liaisons ester ou amide des groupements acyls de longueur variable. C'est à travers son lipide A que le LPS interagit avec les cellules eucaryotes, en particulier avec les macrophages/monocytes **[80]**. Le lipide A est le centre endotoxique de la molécule du LPS. Les facteurs majeurs contribuants à son endotoxicité sont le nombre et la longueur des chaînes acylées et l'état de phosphorylation du disaccharide *backbone*.

La nature, le nombre, la longueur, l'ordre et la saturation des chaînes acylées du lipide A varient selon les espèces bactériennes (**Figure 5**).

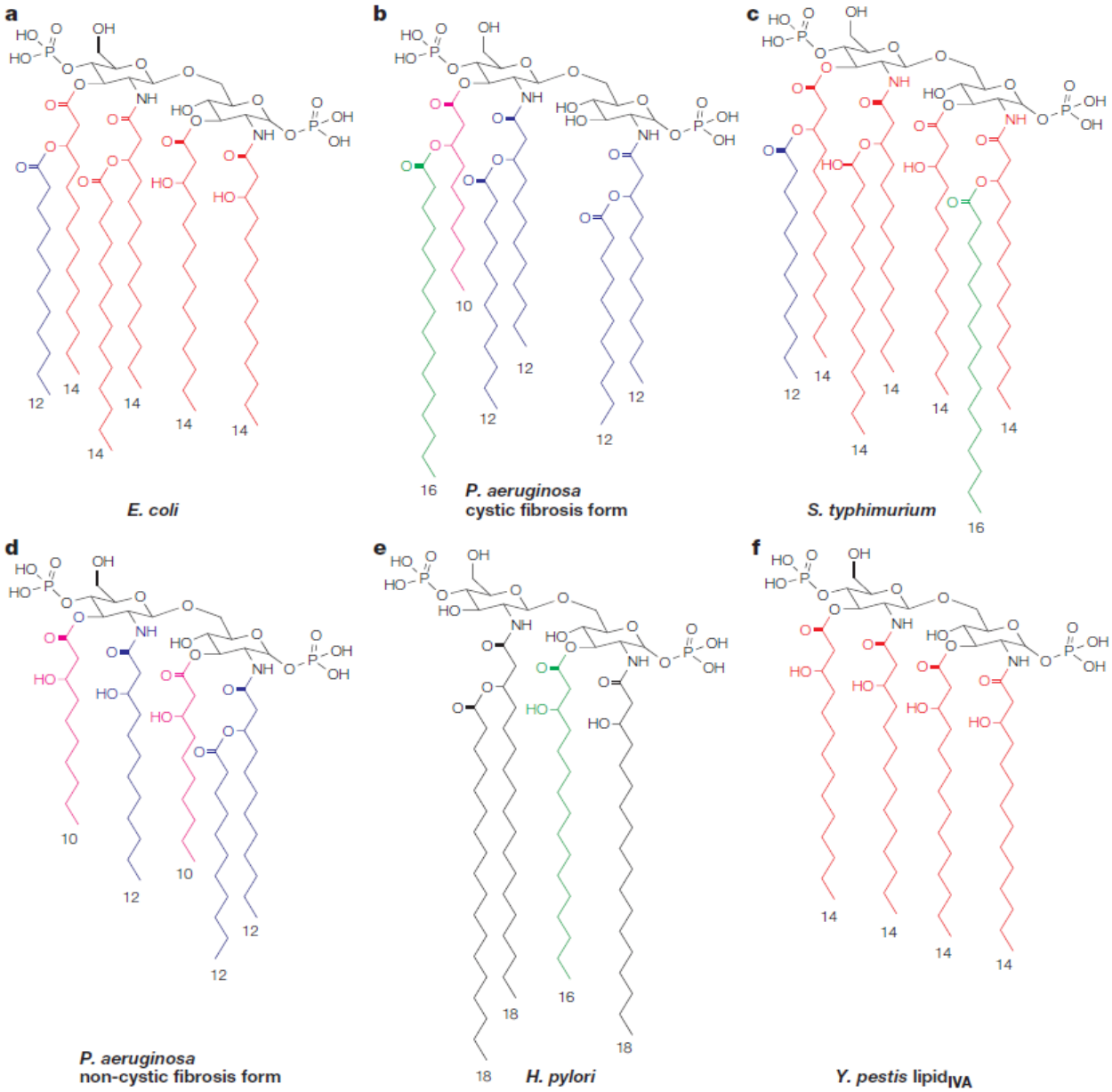

Figure 5: La diversité structurale du lipide A des organismes à gram-négatif

(Tiré de Samuel I. Miller *et al*. (*Nature reviews microbiology*, 3: 36-46 (2005))

Le LPS de *Escherichia coli* : *E. coli* provoque des infections de la voie urinaire et des maladies gastrointestinales. La haute toxicité de son LPS est due à sa structure. Son lipide A hexa-acylé et diphosphorylé avec des chaînes acylées longues de 12 à 14 atomes de carbone est considéré comme proche de la structure optimale induisant une activation maximale de TLR4.

Le LPS de *Salmonella minnesota* : les salmonelles sont responsables de nombreuses gastroentérites. Leur LPS est presque autant endotoxinique que le LPS de *E. coli* mais celui de *Salmonella minnesota*, avec un lipide A hepta-acylé, présente une activité fortement réduite.

Le LPS d'*Hélicobacter pylori :* Les chaînes acylées de son LPS sont plus longues que celles du LPS d'*E. coli* avec en moyenne 16 à 18 atomes de carbone. Son activité endotoxique est 1000 fois plus réduite que celle de *Salmonella typhimurium* que ce LPS soit tétra-acylé (R-LPS) ou hexa-acylé (S-LPS).

Le LPS de *Bactéroides fragilis* : *B. fragilis* est une bactérie commençale de l'intestin humain. Traditionnellement, l'activité endotoxinique de son LPS est 100-1000 fois plus réduite que celles des LPS de *Salmonella* et d'*E. coli.* Cette faible endotoxicité est due à un lipide A monophosphorylé avec 5 acides gras relativement longs de 15 à 16 atomes de carbone.

En résumé, le LPS est l'un des activateurs les plus puissants de la réponse immune des mammifères et il est essentiel à la viabilité des bactéries à gram négatif. De très nombreux LPS sont exprimés dans la nature mais tous partagent des motifs communs, un diglucosamine *backbone* phosphorylé lié à plusieurs chaînes acylées et au moins à un résidu de Kdo.

Les différences entre l'ensemble des types de LPS affectent toutes ses parties mais sont particulièrement importantes dans le O-polysaccharide, moindres dans la région du core et peu dans le lipide A, le centre endotoxinique de la molécule (**Figure 5**).

Outre son rôle endotoxinique, le LPS altère la conversion phagosomale permettant ainsi la survie des bactéries pathogènes au sein des macrophages. C'est ainsi que le LPS d' *E. coli* inhibe partiellement la pinocytose et la phagocytose, retarde les mécanismes de conversion des organelles telles que les endosomes, les macropinosomes et les phagosomes **[77]**. Le LPS de *B. abortus* inhibe la fusion phagosome-lysosome via sa chaîne O **[81]**. Il interfère également avec la présentation de l'antigène aux cellules T $CD4^+$ en induisant une exocytose lipidique massive **[82]**. Le LPS de *S. flexneri* utilise une voie d'endocytose non classique faisant appel en grande partie à la voie de recyclage, ce qui suggère que la localisation intracellulaire du LPS libre pourrait jouer un rôle dans la localisation intracellulaire des agents pathogènes **[83]**.

Le LPS est considéré comme un élément majeur de la virulence de *C. burnetii*. Après plusieurs passages en culture, *C. burnetii* subit une transition de phase de son LPS similaire à la transition LPS lisse - LPS rugueux des entérobactéries. Cette transition phase I - phase II s'accompagne de la perte de virulence des bactéries **[75]**. La composition en sucres des LPS de phase I et de phase II est différente. Le LPS de phase I contient des sucres tels que le L-virenose, le dihydrohydroxystreptose et la galactosamine uronyl-α-(1,6)-glucosamine, qui sont absents du LPS de phase II **[84]**. De larges délétions chromosomiques sont présentes chez les bactéries de phase II comparées aux bactéries de phase I **[85]**.

Nous avons posé l'hypothèse que le LPS est impliqué dans le détournement de la conversion du phagosome par *C. burnetii*. Nous avons ainsi étudié le trafic du LPS de *C. burnetii* dans les macrophages et son rôle dans la biogénèse des lysosomes.

Nous avons montré que le LPS des bactéries virulentes (vLPS), le seul facteur de virulence décrit à ce jour pour cette bactérie, affecte la biogénèse des lysosomes dans les macrophages en n'induisant pas l'activité de la MAPK p38, contrairement au LPS de variants bactériens avirulents (avLPS) (article I). A l'mage de leur LPS, les bactéries virulentes n'activent pas p38 alors que les variants avirulents l'activent (article II). Nous avons aussi montré que p38 régule le niveau de phosphorylation de Vps 41, une unité

accessoire du complexe HOPS. Nous en concluons que *C. burnetii* évite l'engagement de l'axe MAPK p38/Vps 41-HOPS pour échapper à sa destruction dans les phagolysosomes.

ARTICLE I

Impaired stimulation of p38α-MAPK/Vps41-HOPS by virulent LPS from pathogenic *Coxiella burnetii* prevents bacteria trafficking to microbicidal phagolysosomes

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(*Manuscript in revision*)

Dans cette étude, nous montrons que le trafic intracellulaire du LPS virulent (vLPS) de *Coxiella burnetii* et du LPS de ses variants avirulents (avLPS) semble être respectivement similaire aux trafics des bactéries virulentes et de leurs variants avirulents. Le avLPS est localisé dans des lysosomes et Rab7 est transitoirement présent à leur surface. Le vLPS est, lui, localisé dans un compartiment tardif exprimant Lamp-1 mais qui n'exprime Rab7 à sa surface et n'acquiert pas la Cathepsine D. Nous avons aussi montré que la non localisation du vLPS dans les lysosomes n'est pas due à sa séquestration dans un autophagosome ni à son transit par le trans-golgi ou le réticulum endoplasmique. Le mécanisme mis en jeu par le vLPS pour inhiber la fusion avec les lysosomes implique un défaut d'activation de la MAPK p38 alors que le avLPS active p38 et se retrouve dans un compartiment lysosomial. En outre, le p38 activé par le avLPS phosphoryle Vps41, un composant clé du complexe HOPS impliqué dans la fusion dépendante de Rab7 des phagosomes ou des endosomes avec les lysosomes. Nous avons ainsi montré que les bactéries qui expriment le vLPS détournent la conversion phagosomale pour se répliquer dans les macrophages en évitant l'activation de l'axe MAPK p38/Vps 41-HOPS/Rab7.

Impaired stimulation of p38α-MAPK/Vps41-HOPS by virulent LPS from pathogenic *Coxiella burnetii* prevents bacteria trafficking to microbicidal phagolysosomes

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Running Title: p38α-MAPK/Vps41-HOPS axis in *C. burnetii* virulence

Keywords: LPS, lysosomes; *Coxiella burnetii*; RAB7; p38α-MAPK; phagosomes; HOPS

SUMMARY

Internalisation and subsequent destruction of bacteria in phagolysosomes by macrophages protects the host against pathogens. We demonstrate that lipopolysaccharide (vLPS) from pathogenic *Coxiella burnetii* (v*Cb*) is a major virulence determinant that does not stimulate bacteria trafficking to phagolysosomes. Indeed, stimulation of p38α-MAPK signalling by avirulent LPS (avLPS) is required for proper trafficking of avirulent *C. burnetii* to phagolysosomes, which does not occur with vLPS. The defect in the targeting of v*Cb*/vLPS to degradative compartments is due to the engagement of TLR4 by vLPS and to a lack of p38α-MAPK-driven phosphorylation and recruitment of Vps41-HOPS to vLPS-containing vesicles. The expression of an upstream activator of p38α-MAPK or of the phosphomimetic mutant Vps41-S796E allows the targeting of vLPS and v*Cb* to phagolysosomes. Thus, v*Cb* can evade the immune response via variations in LPS composition and p38α-MAPK and its cross-talk with Vps41 play a central role in the trafficking of bacteria to phagolysosomes.

INTRODUCTION

Coxiella burnetii, the causative agent of the zoonosis Q fever, is a potential biowarfare and bioterrorism agent (Madariaga et al., 2003; Regis, 1999) and is also responsible for lethal endocarditis (Raoult et al., 2005). Molecular variations in lipopolysaccharide (LPS), a major outer membrane component, primarily determine the pathogenic properties of *C. burnetii* (Lukacova et al., 2008; Toman et al., 2009). Therefore, these variations represent unique biomarkers of pathogenic *C. burnetii* strains (Toman et al., 2009). The composition and structure of lipid A and the core polysaccharide of LPS are conserved between virulent and avirulent LPS (vLPS and avLPS, respectively) **(Fig. S1)** (Toman and Vadovic, 2011). By contrast, the vLPS O-antigen contains virenose and dihydrohydroxystreptose residues (Toman and Vadovic, 2011). Much remains to be learned regarding the molecular basis underlying the crosstalk between the LPS variants and host cells, which determine the outcome of the infection.

In response to LPS stimulation, Toll-like receptor (TLR) signalling controls distinct innate immune defence programmes, particularly the maturation of macrophage phagosomes (Blander and Medzhitov, 2004, 2006). This process involves crosstalk between mitogen-activated protein kinase (MAPK) signalling and components of the vesicular trafficking machinery that have yet to be defined (Blander and Medzhitov, 2006; Fontana and Vance, 2011; Symons et al., 2006). Pathogenic *C. burnetii* is an obligate intracellular bacterial pathogen that must evade phagolysosomal degradation to survive and replicate (Ghigo et al., 2002; Ghigo et al., 2009). In macrophages, virulent *C. burnetii* bacteria reside and replicate in compartments known as “phagolysosome-like vacuoles”, which have properties of both late endosomes and lysosomes. These compartments do not harbour lysosomal enzymes or the small GTPase RAB7, but have acidic properties and are positive for lysosomal-associated membrane protein-1 (LAMP-1) (Barry et

al., 2011). These findings suggest that disruption of phagolysosome maturation by *C. burnetii* is a major trait of pathogenic strains. The fusion of a phagosome with a lysosome (the phagolysosomal conversion process) involves the vacuolar protein-sorting (VPS)-C complex HOPS (homotypic fusion and protein sorting), which cooperates with Ypt7/RAB7 and its GEF Mon1/Sand1 in endosomal maturation (Kinchen et al., 2008; Nordmann et al., 2010; Poteryaev et al., 2010; Rink et al., 2005; Wang et al., 2011). Studies conducted in yeast have indicated that the Vps41 protein, a key component of the HOPS complex, is required for the stabilisation of the HOPS complex on the endosomal membrane prior to fusion with the vacuole and is controlled by the vacuolar casein kinase Yck3 (Cabrera et al., 2009; LaGrassa and Ungermann, 2005; Nickerson et al., 2009).

In this study, we establish how the crosstalk between p38α-MAPK and the HOPS-component Vps41 dictates the proper trafficking of vesicles containing avirulent *C. burnetii* to phagolysosomes and how this determines the differential intracellular survival properties of virulent/avirulent bacteria.

RESULTS

Virulent *C. burnetii* and vLPS do not reach lysosomal compartments

Early in this study, we observed a direct correlation between variation in vesicular trafficking of virulent (v*Cb*) or avirulent (av*Cb*) strains of *C. burnetii* and their differential capacity to survive in bone marrow–derived macrophages (BMDMs). This observation was made by studying the pathogenic *C. burnetii* RSA493 strain (vCb), which is a phase I, biosafety level 3 strain known to cause Q fever in humans and productive infection in mice (Russell-Lodrigue et al., 2009). Indeed, av*Cb* bacteria localised in phagolysosomal compartments, which are defined by the presence of LAMP-1, the soluble lysosomal hydrolase cathepsin D (catD) **(Fig. 1A),** and RAB7 **(Fig. 1B)**. In parallel, we determined that 90% of av*Cb* bacteria were killed in macrophages, in contrast to v*Cb* bacteria, which were not degraded in lysosomes and were able to replicate **(Figs. 1A** and **1C)**. An analysis of the intravesicular localisation of v*Cb* revealed the preferential localisation of these bacteria to compartments that were devoid of catD **(Fig. 1A)** and RAB7 **(Fig. 1B)**. By monitoring the intravesicular trafficking of *C. burnetii* vLPS and avLPS, we were able to attribute this difference in routing to variations in LPS composition between the strains (Lukacova et al., 2008; Pretat et al., 2009) **(Figs. 2** and **S2)**. Macrophage compartments containing either type of LPS showed a progressive acquisition of LAMP-1 (>85% LPS/LAMP-1 colocalisation at 120 minutes; **Figs. 2A, S2A, 2B, S2B** and **2E)**. Compartments containing avLPS also progressively acquired catD (> 90% LPS/catD colocalisation at 120 minutes; **Figs. 2B, S2B** and **2F)**. By contrast, we observed a lack of colocalisation between catD and vLPS (< 10% vLPS/catD colocalisation at 120 minutes; **Figs. 2A, S2A and 2F)**. Experiments conducted with α-N-acetylglucosaminidase, a soluble lysosomal hydrolase (Ficko-Blean et al., 2008), confirmed that vLPS did not traffic to

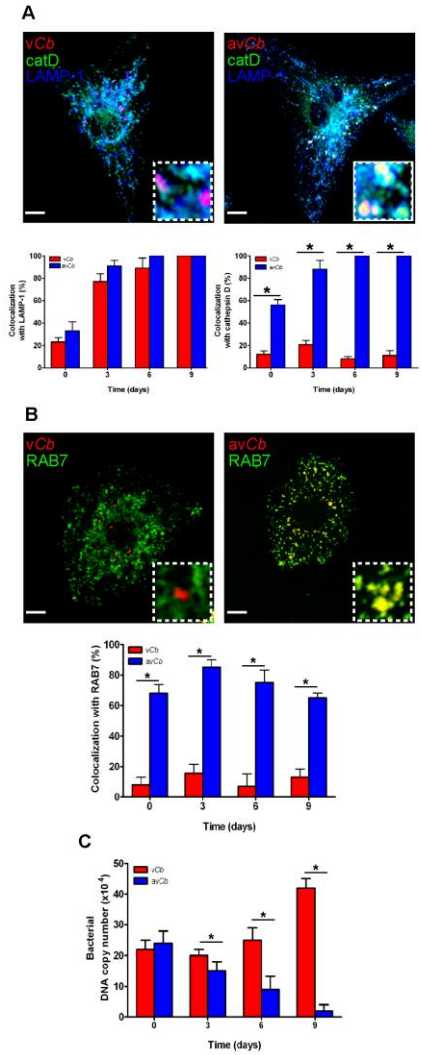

Figure 1. Differential intracellular localisation and growth of virulent (v*Cb*) and avirulent (av*Cb*) *C. burnetii* strains in infected bone marrow-derived macrophages (BMDMs).

The intracellular localisation of the virulent (v*Cb*) and avirulent (av*Cb*) *C. burnetii* strains within macrophages **(A** and **B)** was assessed via confocal microscopy. **(A)** The colocalisation of *C. burnetii* (red) with LAMP-1 (blue) or cathepsin D (green) was quantified, and the results are expressed as the mean ± SD (*n*=3). The scale bar indicates 5 µm. av*Cb*, but not v*Cb*, localised within phagolysosomes. **(B)** The colocalisation of *C. burnetii* (red) and RAB7 (green) was quantified, and the results are expressed as the mean ± SD (*n*=3). The scale bar indicates 5 µm. av*Cb*, but not v*Cb*, localised within RAB7-containing compartments. **(C)** BMDMs were infected with strains of *C. burnetii* expressing either v*Cb* or av*Cb*, and bacterial replication was determined using qPCR. The results are expressed as the DNA copy number (mean ± SD; *n*=4). The v*Cb* bacteria replicated within macrophages, whereas the av*Cb* bacteria were eliminated.

lysosomes **(Fig. S3A)**. We also confirmed that the absence of vLPS within the lysosomes was not a result of a general delay in vesicular trafficking **(Fig. S3B)** or due to the sequestration of vLPS within autophagosomes **(Fig. S4A)**. In addition, we excluded any potential delay in the trafficking of vLPS via routing through the Golgi apparatus and/or the endoplasmic reticulum **(Figs. S4B** and **S4C)** (Giles and Wyrick, 2008; Latz et al., 2002). Therefore, our analysis identified a defect in the trafficking of virulent *C. burnetii* into phagolysosomes that we attributed to vLPS.

Defective RAB7 recruitment to vLPS-containing late endosomes

RAB proteins are small GTPases that control specific steps of vesicular trafficking (Stenmark, 2009). RAB5 localises to early endosomes and is involved in the transition from early to late endosomes (Zerial and McBride, 2001). RAB7 localises to late endosomes, where it governs the biogenesis of lysosomes and phagolysosomes (Bucci et al., 2000; Wang et al., 2011). Based on this information, we analysed the distribution of these RAB proteins on the membranes of vLPS- or avLPS-containing compartments. We hypothesised that alterations in the recruitment of RAB5 and/or RAB7 might explain the defective targeting of vLPS to lysosomes. Immediately after internalisation, we observed a transient colocalisation of avLPS and RAB5 in BMDMs **(Figs. S5B** and **S5C)**. This colocalisation was followed by a strong colocalisation of avLPS and RAB7 at later time periods (> 80% avLPS/RAB7 colocalisation at 90 minutes; **Figs. 2D, S2D** and **2G)**. vLPS, regardless of an initial time delay, also transiently colocalised with RAB5 **(Figs. S5A** and **S5C)**. However, in contrast to avLPS, it did not reach RAB7-positive compartments (<10% vLPS/RAB7colocalisation at 90 minutes; **Figs. 2C, S2C** and **2G).** Importantly, the absence of colocalisation between vLPS and RAB7 was not a result of a delay in routing to lysosomes **(Fig. S3B)**. These

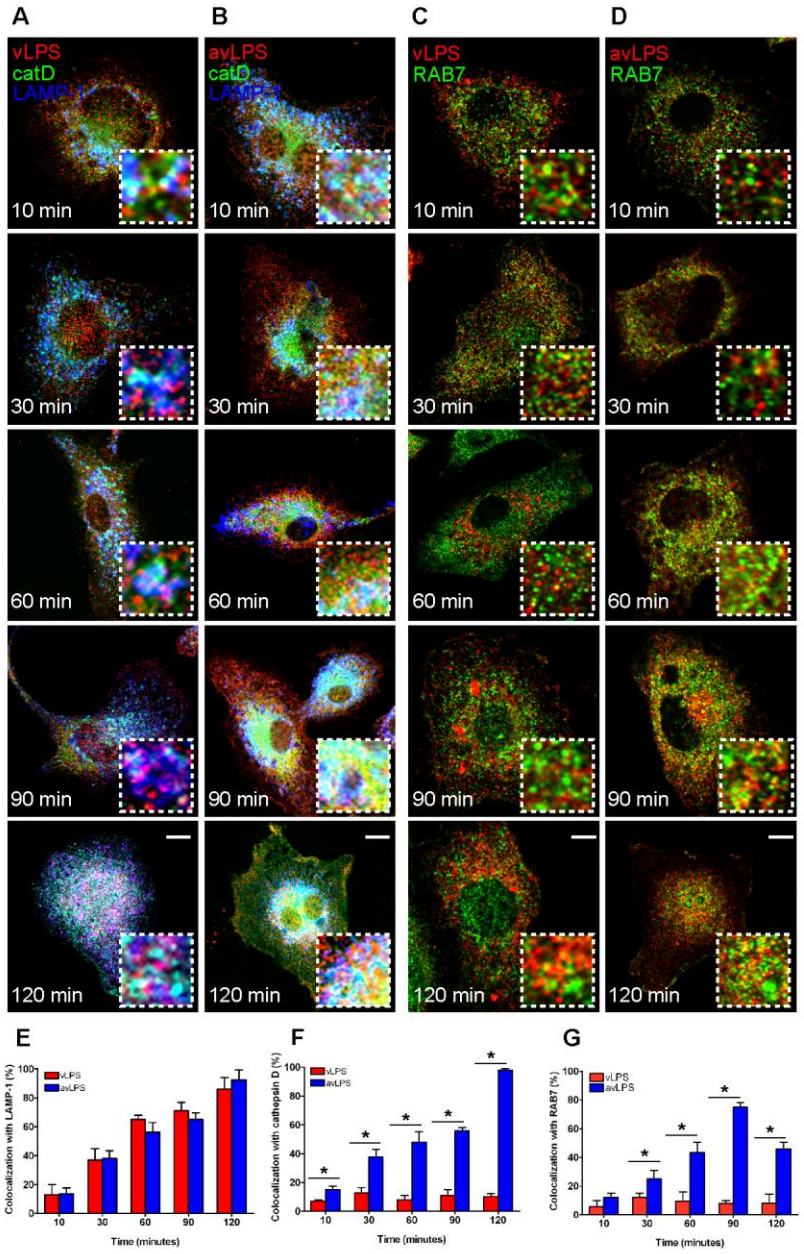

Figure 2. In contrast to avLPS, vLPS prevents lysosomal targeting. BMDMs were treated with vLPS **(A** and **C)** or avLPS **(B** and **D)** from *C. burnetii*. The maturation of the intracellular compartments containing LPS was monitored via confocal microscopy. Panels **A** and **B** show the levels of colocalisation between LPS (red), LAMP-1 (blue) and cathepsin D (green). Panels **C** and **D** show the levels of colocalisation between LPS (red) and RAB7 (green). The colocalisation levels were quantified **(E, F, G)**, and the results are expressed as the mean ± SD (**E** and **F,** n=4**; G,** n=5**)**. The scale bar indicates 5 μm. In contrast to avLPS, vLPS did not colocalise with cathepsin D– or RAB7-positive compartments.

data collectively reveal a defect in the recruitment of RAB7 to the membrane of vLPS-containing endosomes, as observed for v*Cb*.

p38α-MAPK activation is required for the trafficking of v*Cb* and vLPS to lysosomes

We next aimed to determine the mechanism underlying the defects in phagolysosomal conversion and its involvement in bacterial virulence. Mitogen-activated protein kinase (MAPK) signalling pathways have crucial functions in the transduction of signals downstream of TLR stimulation by LPS (Blander and Medzhitov, 2004, 2006). Interestingly, these kinases also play a role during the early stages of membrane trafficking to favour RAB5 activation (Scita and Di Fiore, 2010; Sorkin and von Zastrow, 2009). We performed immunoblotting experiments to monitor MAPK activation by *C. burnetii* avLPS and vLPS. Within 30 minutes of treatment with either form of LPS, we detected stimulation of the ERK1/2 and JNK signalling pathways in BMDMs **(Fig. 3A)**. At this time point, we observed an absence of phosphorylated p38α-MAPK in BMDMs challenged with vLPS, in contrast to avLPS-challenged BMDMs, in which we detected phosphorylated p38α-MAPK **(Fig. 3A)**. In support of this finding, we also observed an absence of p38α-MAPK phosphorylation in BMDMs infected with v*Cb* compared with those infected with avCb **(Fig. S6** and (Boucherit et al., 2012)). These results define p38α-MAPK as a key host factor differentially engaged during infection with avirulent and virulent strains of *C. burnetii*.

Further analysing these signalling defects, we found that the p38α-MAPK upstream activator MKK3/6 was not activated in cells challenged with vLPS unlike the activation observed in cells challenged with avLPS **(Fig. 3B)**. Consistent with a difference in bacterial strain recognition by the host cells, we measured a 10-fold lower production of the pro-inflammatory cytokines interleukin (IL)-6 and tumour

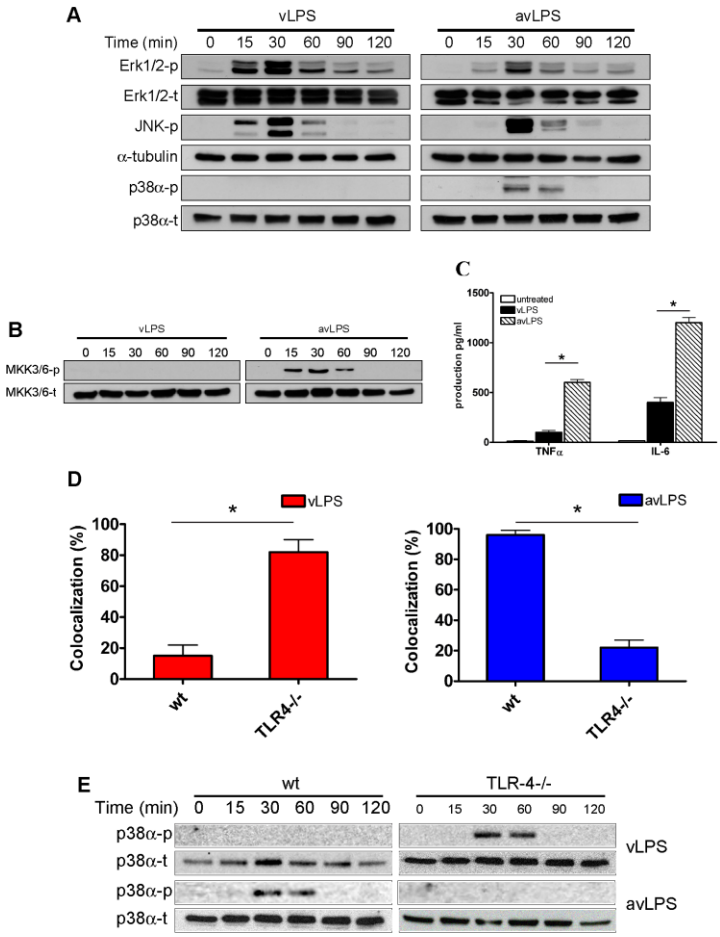

Figure 3. avLPS, but not vLPS, induces p38α-MAPK signalling.
(A) BMDMs were stimulated with vLPS or avLPS. Representative immunoblot showing the levels of total (t) and phosphorylated (p) MAPK ERK1/2, JNK and p38α (*n*=3). This result shows that vLPS did not induce p38α MAPK activation. **(B)** BMDMs were stimulated with *C. burnetii* vLPS or avLPS. Representative immunoblot showing the levels of total (t) and phosphorylated (p) MAPK MKK3/6 (*n*=3). This result shows that vLPS did not induce MKK3/6 activation. **(C)** The production (pg/ml) of TNF-α and IL-6 by BMDM that were stimulated for 16 hours with LPSs (1 µg/ml) was determined via ELISA. The results are expressed as the mean ± SD from three experiments. This result shows that the TLR4-Myd88 signalling pathway is not induced by vLPS. **(D)** The colocalisation between cathepsin D and the LPSs within TLR4-deficient BMDMs was visualised via confocal microscopy and quantified using ImageJ software. The results are expressed as the mean ± SD from two experiments. **(E)** BMDMs from wild-type or TLR4-deficient mouse macrophages were treated with vLPS or avLPS, and the levels of the total (t) and phosphorylated (p) p38α-MAPK were analysed via immunoblotting. The presented blot is representative of three experiments. TLR4 is required for vLPS to inhibit p38α-MAPK phosphorylation.

necrosis factor-α (TNF-α) in cells stimulated with vLPS compared with cells stimulated with avLPS **(Fig. 3C)**. The p38α-MAPK signalling pathway operates downstream of TLR4 (Kopp and Medzhitov, 2003), and the colocalisation of LPSs in TLR4-deficient macrophages confirmed the requirement for TLR4 in the targeting of LPSs to lysosomes **(Fig. 3D)**. Thus, we evaluated p38α-MAPK phosphorylation in wild-type and TLR4-deficient macrophages challenged with *C. burnetii* LPS **(Fig. 3E).** TLR4 appeared to be required for the differential stimulation of p38α-MAPK (Fig. 3E). Taken together, these data demonstrate that the p38α-MAPK pathway–dependent trafficking of LPSs to lysosomes requires TLR4.

We next attempted to analyse the relevance of p38α-MAPK activity in the intracellular trafficking of vLPS. We first evaluated whether the forced activation of p38α-MAPK could lead to the proper localisation of v*Cb* and vLPS to degradative phagolysosomes. Therefore, we expressed a constitutively active form of MKK6 (GFP-MKK6DD), an upstream activator of p38α-MAPK. Expression of GFP-MKK6DD in macrophages restored the colocalisation of vLPS or v*Cb* with phagolysosomal markers **(Figs. 4A** and **4B)**. Under these conditions, vLPS also colocalised with RAB7 **(Fig. 5A)**. Importantly, the expression of GFP-MKK6DD also resulted in the killing of v*Cb* in macrophages **(Fig. S7)**. Complementary to these findings, avLPS did not reach lysosomes in p38$\alpha^{-/-}$ MEFs; this finding is in contrast to that observed for wild-type MEFs, where avLPS reached the lysosomes **(Fig. 4C)**. More specifically, in p38$\alpha^{-/-}$ MEFs, we detected less than 10% avLPS/lysosomal marker colocalisation compared with 90% colocalisation in wild-type MEFs. Additionally, avLPS and av*Cb* failed to reach the lysosomes of macrophages treated with a p38α-MAPK inhibitor **(Figs. S8A** and **S8B)**. Overall, these functional analyses establish a critical role for p38α-MAPK signalling in *C. burnetii* fate and trafficking to lysosomes.

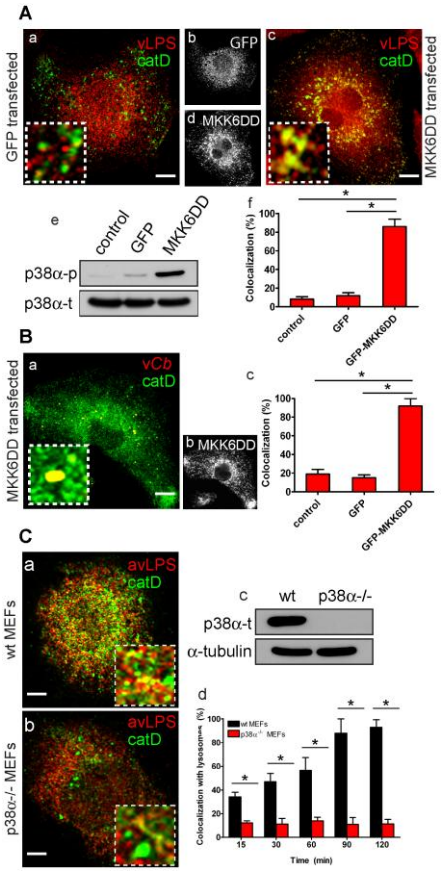

Figure 4. avLPS, but not vLPS, induces p38α-MAPK signalling, which drives endolysosomal conversion.

(A) BMDMs were transfected with lentiviruses expressing GFP (a, b) or GFP-MKK6DD (c, d). After 120 minutes of vLPS treatment, the colocalisation of vLPS (red) and cathepsin D (green) was determined (a and c) and quantified (f). The results are expressed as the mean ± SD (*n*=3). Panels b and d show the expression patterns of GFP and GFP-MKK6DD, respectively. Immunoblot analysis was used to confirm the increased levels of p38α-MAPK phosphorylation after the expression of MKK6DD compared with the untransduced (control) and GFP-expressing cells (e). (**B**) The colocalisation of v*Cb* (red) and cathepsin D (green) (panel a) and the quantification (panel c) of MKK6DD-expressing cells are shown. Panel b shows the expression pattern of GFP-MKK6DD. The results are expressed as the mean ± SD (*n*=2). The expression of MKK6DD in macrophages induced the colocalisation of vLPS and v*Cb* with lysosomes. **(C)** Wild-type MEFs and p38α$^{-/-}$ MEFs were treated with avLPS for 120 minutes, and the colocalisation of avLPS (red) and cathepsin D (green) was determined (a and b) and quantified (d). Immunoblot analysis confirmed that in contrast to the wild-type MEFs, the p38α$^{-/-}$ MEFs did not express p38α (c). The results are expressed as the mean ± SD (*n*=4). The scale bar indicates 5 µm. The absence of p38α-MAPK affected the targeting of avLPS to the lysosomes.

vLPS of *C. burnetii* does not induce Vps41 phosphorylation, preventing delivery into phagolysosomes

The role of p38α-MAPK in the trafficking of *C. burnetii* avLPS to lysosomes prompted us to examine the molecular mechanisms involved. The expression of wild-type RAB7 or the constitutively active mutant RAB7:Q67L did not result in the colocalisation of vLPS with RAB7 (less than 10% colocalisation; **Fig. 5A)**. This expression also failed to induce the trafficking of vLPS into lysosomes (less than 15%; **Fig. 5A)**. Therefore, we concluded that RAB7 expression and activation were not sufficient to target vLPS to lysosomes. Interestingly, colocalisation of RAB7 with compartments containing vLPS was observed following expression of MKK6DD **(Fig. 5A)**. Therefore, we hypothesise that MKK6DD/p38α-MAPK acts on a protein that might regulate or cooperate with RAB7.

Because p38α-MAPK is a serine/threonine kinase, we searched for proteins involved in endolysosomal fusion that have been described as a substrate of serine/threonine kinases. Studies in yeast have indicated that the Vps41 protein is required for the stabilisation of the HOPS complex at the endosomal membrane prior to fusion with vacuoles. Moreover, serine (Ser) phosphorylation of Vps41 by the vacuolar casein kinase Yck3 is critical for HOPS function and for transport to the vacuoles (Cabrera et al., 2009; LaGrassa and Ungermann, 2005; Nickerson et al., 2009). Therefore, we aimed to determine whether LPS-induced p38α-MAPK activation has an effect on the phosphorylation of Vps41. BMDMs were challenged with *C. burnetii* LPSs, and the phosphorylation of Vps41 was monitored. This set of experiments revealed that avLPS triggered the phosphorylation of Vps41 on serine residues **(Fig. 5B**). This result also demonstrated that the phosphorylation of Vps41 is specifically triggered by avLPS because no effect was observed when vLPS was used **(Fig. 5B)**. Experiments performed in non-starved conditions

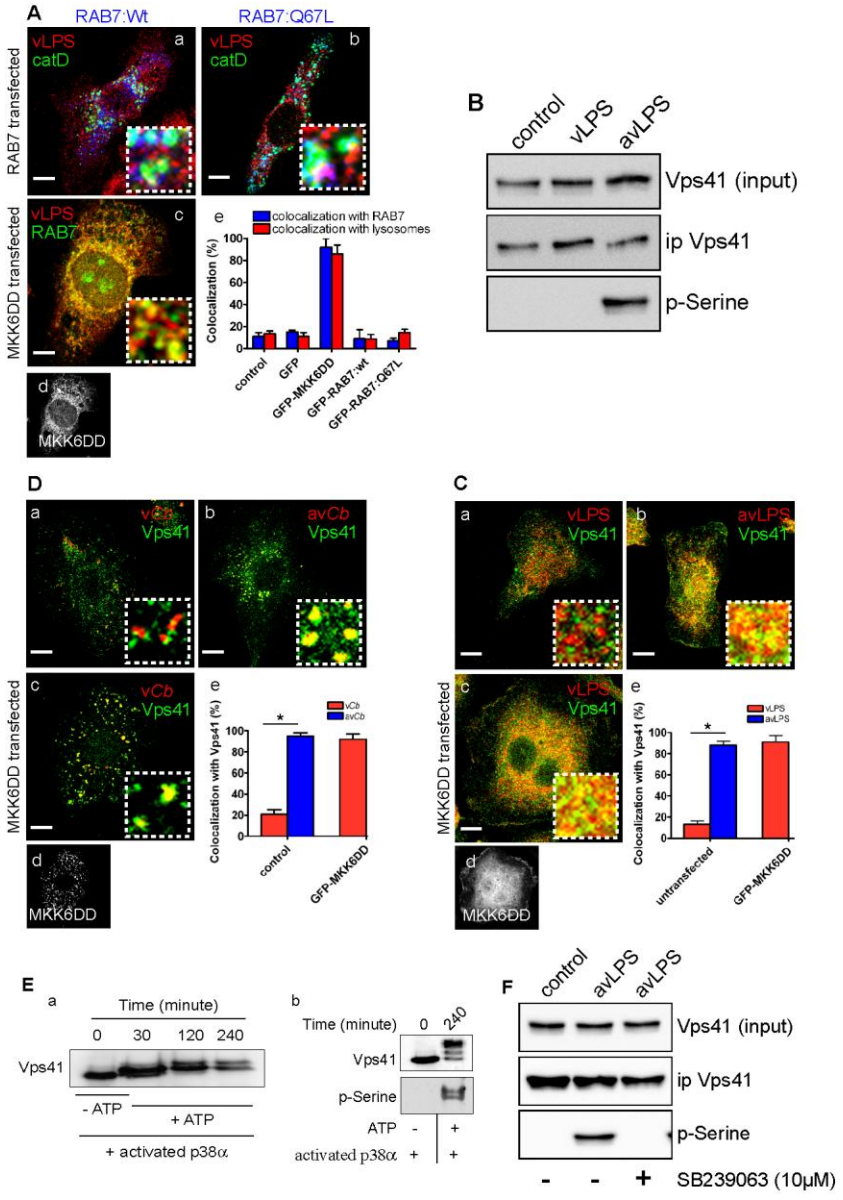
A
RAB7:Wt
RAB7:Q67L
RAB7 transfected
MKK6DD transfected
vLPS
catD
RAB7
MKK6DD
colocalization with RAB7
colocalization with lysosomes
Colocalization (%)
control
GFP
GFP-MKK6DD
GFP-RAB7:wt
GFP-RAB7:Q67L
B
control
vLPS
avLPS
Vps41 (input)
ip Vps41
p-Serine
D
vCb
avCb
Vps41
Colocalization with Vps41 (%)
C
vLPS
avLPS
untransfected
E
Time (minute)
0
30
120
240
Vps41
- ATP
+ ATP
+ activated p38α
p-Serine
ATP
activated p38α
F
SB239063 (10μM)

Figure 5. The association between p38α-MAPK, RAB7 and Vps41 activation. **(A)** BMDMs were transfected with plasmids expressing GFP-RAB7:wt (a), GFP-RAB7:Q67L (b) or GFP-MKK6DD (c and d). Panels a and b show the colocalisation of vLPS (red) and lysosomes labelled with cathepsin D (green) and GFP-RAB7:wt or GFP-RAB7:Q79L (blue). Panel c shows the colocalisation of vLPS (red) with endogenous RAB7 (green). In panel d, the expression of GFP-MKK6DD is visualised. The colocalisation of LPS with either cathepsin D or RAB7 was quantified (e). The expression of MKK6DD induced the colocalisation of vLPS and RAB7. By contrast, the expression of neither RAB7:wt nor GFP-RAB7:Q79L induced the colocalisation of vLPS and RAB7. **(B)** BMDMs in starved conditions were untreated or treated with vLPS or avLPS. Vps41 was immunoprecipitated from the cell lysates, and the serine-phosphorylated forms (p-Serine) were visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of Vps41. The presented blot is representative of 3 experiments. Serine phosphorylation was not detected in the presence of vLPS. **(C)** *C. burnetii* vLPS (a) and avLPS (b) were internalised by BMDMs, and vLPS (c) was internalised by BMDMs expressing MKK6DD. The colocalisation of LPS (red) and Vps41 (green) was analysed. In panel d, the expression of GFP-MKK6DD is presented. The colocalisation of LPS and Vps41 was quantified using ImageJ software (e). The results are expressed as the mean ± SD (n=3). **(D)** v*Cb* (a) and av*Cb* (b) were internalised by BMDMs, and v*Cb* (c) was internalised by BMDMs expressing MKK6DD. The bacterial (red) colocalisation with Vps41 (green) was analysed using confocal microscopy. In panel d, the expression of GFP-MKK6DD is shown. The colocalisation of the bacteria and Vps41 was quantified using ImageJ software (e). The results are expressed as the mean ± SD, n=3 experiments. The scale bar indicates 5 μm. Neither vLPS nor v*Cb* colocalised with Vps41. MKK6DD overexpression induced the colocalisation of vLPS and v*Cb* with Vps41. **(E)** (a) Vps41 and activated p38α-MAPK were incubated with or without ATP, and the shift in mobility due to the phosphorylation of Vps41 by activated p38α-MAPK was analysed via immunoblotting. Vps41 was phosphorylated by p38α-MAPK. (b) Vps41 and activated p38α were incubated with or without ATP, and the serine phosphorylation of Vps41 was assessed via immunoblotting. **(F)** BMDMs in starved conditions were mock treated or pretreated with 10 μM SB239063 (Sigma-Aldrich), an inhibitor of p38α-MAPK, for 1 hour and then were incubated with or without avLPS. Vps41 was immunoprecipitated from the cell lysates, and the serine-phosphorylated (p-Serine) forms were visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of Vps41. The presented blot is representative of three experiments. Serine phosphorylation was not detected in cells treated with the p38-MAPK inhibitor.

showed that there was a pool of activated p38α-MAPK that could phosphorylate Vps41, although at low efficiency **(Fig. S9)**.

Functional analyses revealed that avLPS and av*Cb* colocalised with Vps41 (88 ± 4% and > 90% colocalisation, respectively) in contrast to vLPS and v*Cb*, which did not colocalise with Vps41 (13 ± 3.5% and 20% colocalisation, respectively; **Figs. 5C and 5D)**. Furthermore, we observed that v*Cb* and vLPS colocalised with Vps41 after the expression of MKK6DD in macrophages **(Figs. 5C** and **5D)**. Using a different activator of p38α-MAPK, such as IFN-γ (Matsuzawa et al.), we were also able to force the colocalisation of vLPS and Vps41 **(Fig. S10)**. In addition, we blocked the colocalisation of av*Cb* and Vps41 by treating macrophages with a p38α-MAPK inhibitor **(Fig. S8C)**. We confirmed that inhibition of p38α-MAPK blocked the phosphorylation of Vps41 in BMDMs challenged with avLPS **(Fig. 5F)**. Complementary to these findings, we observed that p38α-MAPK co-immunoprecipitated with Vps41, and vice-versa, demonstrating their interaction in avLPS-treated BMDMs **(Fig. S11)**. Taken together, these findings prompted us to investigate whether p38α-MAPK directly targets Vps41. Using an *in vitro* phosphorylation assay, we observed a shift in the gel mobility of Vps41 in the presence of activated p38α-MAPK and ATP **(Fig. 5E)**. The phosphorylation of Vps41 was confirmed by performing anti-phosphorylated serine immunoblotting **(Fig. 5E)**. Using bioinformatics analyses, we screened available databases for p38α-MAPK sites within the Vps41-sequence (NetphosK, http://www.cbs.dtu.dk/services/NetPhosK/) and identified two potential phosphorylation sites at positions S83 and S796 **(Fig. 6A)** with scores of 0.53 and 0.59, respectively. To confirm the importance of Vps41 phosphorylation, the putative serine residues (S83 and S796) identified by the bioinformatic analyses **(Fig. 6A)** were mutated into glutamic acid or alanine residues. The resulting Vps41 phosphomimetic mutants (Vps41:S83E, Vps41:S796E) and Vps41:S796A were

expressed in p38α$^{-/-}$ MEFs. Remarkably, we found that in contrast to Vps41:wt and Vps41:S83E, the Vps41:S796E mutant promoted the colocalisation of vLPS with cathepsin D- **(Figs. 6B** and **6C)** and RAB7- **(Figs. 6B** and **6C)** containing compartments. Similar data were obtained for avLPS using p38α$^{-/-}$ MEFs expressing Vps41:S796E **(Figs. 6D** and **6E)**.

In summary, our data support the hypothesis that Vps41 is a target of p38α-MAP kinase and demonstrate the importance of this signalling axis in the targeting of avLPS to lysosomes.

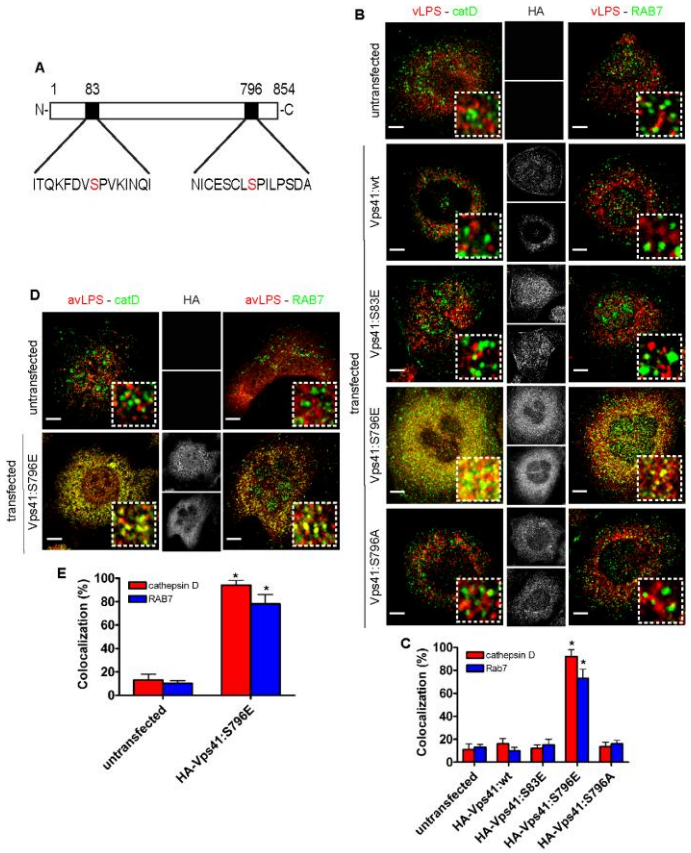

Figure 6. The Vps41 phosphorylation status affects LPSs localisation.
(A) Identification of serine phosphorylation sites on Vps41. The potential p38α-MAPK phosphorylation sites in Vps41 were identified using the online NetphosK software (http://www.cbs.dtu.dk/services/NetPhosK/). The p38α-MAPK target sequence is highlighted. **(B)** $p38\alpha^{-/-}$ MEFs were not transfected or transfected with HA-VPS41:wt, HA-VPS41:S83E, HA-VPS41:S796E, or HA-VPS41:S796A. Panels show the colocalisation of vLPS (red) and lysosomes labelled with cathepsin D or RAB7 (green). In middle panel, the expression of HA-VPS41:wt, HA-VPS41:S83E, HA-VPS41:S796E, or HA-VPS41:S796A is shown. **(C)** The colocalisation of vLPS with either cathepsin D or RAB7 was quantified after 120 minutes. The expression of HA-VPS41:S796E induced vLPS to colocalise with cathepsin D and RAB7 in contrast to the expression of HA-VPS41:wt, HA-VPS41:S83E, or HA-VPS41:S796A, which did not induce this colocalisation. **(D)** $p38\alpha^{-/-}$ MEFs were not transfected or transfected with HA-VPS41:S796E-expression plasmid. Panels show the colocalisation of avLPS (red) and lysosomes labelled with cathepsin D or RAB7 (green). In middle panel, the expression of HA-VPS41:S796E is shown. **(E)** The colocalisation of avLPS with either cathepsin D or RAB7 was quantified. The expression of HA-VPS41:S796E induced vLPS to colocalise with cathepsin D and RAB7. The results are expressed as the mean ± SD (n=3).

DISCUSSION

In this study, we demonstrate that the pathogenicity of *C. burnetii* primarily resides in the ability of its LPS variants to block the activation of p38α-MAPK signalling, which is required for the proper targeting of the bacteria to microbicidal lysosome compartments. We provide evidence that p38α-MAPK interacts with and phosphorylates the Vps41 subunit of the HOPS complex. Complementary to these findings, our functional analyses also suggest that phosphorylation of Vps41 by p38α-MAPK at serine 796 promotes the trafficking of avirulent *C. burnetii* bacteria and LPS to lysosomes.

MAP kinases relay signals in response to environmental cues, such as the presence of bacterial agents, to drive the expression of genes involved in innate immunity (Davis, 2000). Growing evidence also points to a direct role for MAPK kinases in the biogenesis and dynamics of endocytic compartments (Sorkin and von Zastrow, 2009). In particular, p38α-MAPK resides at the centre of stress-induced signalling cascades (Davis, 2000) and regulates early endocytic events in the endocytic pathway (Cavalli et al., 2001; Mace et al., 2005). Previous studies have established that p38α-MAPK controls early endocytic steps by phosphorylating two RAB5 effectors, EEA1 and rabenosyn-5 (Mace et al., 2005). Furthermore, studies using inhibitors of the p38α-MAPK pathway have suggested its involvement in the phagocytic process (Blander and Medzhitov, 2004; Souza et al., 2006, 2007). Consistent with a defect in p38α-MAPK activity, we also observed a time delay in the colocalisation of RAB5 with vLPS (Figs. S5A and S5C), and an internalisation delay has been previously described for v*Cb* (Capo et al., 2003). Most importantly, in this study, we show that p38α-MAPK also plays a key role at a late stage of vesicular trafficking via the engagement of Vps41-HOPS. The HOPS heteromeric complex is comprised of six subunits, among which Vps39 and Vps41 bind RAB7, and it is required for both the transition of late endosomes into lysosomes and the

conversion of late phagosomes into phagolysosomes (Huotari and Helenius, 2011; Wang et al., 2011). In yeast, serine phosphorylation of Vps41 by Yck3 has been shown to promote targeting of the HOPS complex to endosome-vacuole fusion sites and to confer a requirement for GTP-bound Ypt7 for membrane tethering and fusion *in vitro* (Cabrera et al., 2009; Zick and Wickner, 2012). In this study, we demonstrate the crosstalk between p38α-MAPK and Vps41, and that this crosstalk plays a key role in the innate immune response to *C. burnetii*. Consistent with the function of Vps41 as stabilizing factor of the HOPS/Ypt7 complex at the membrane in yeast, our functional analysis identified a defect in the recruitment of RAB7 to vLPS-containing phagosomes. This defect is abrogated upon p38α-MAPK activation or overexpression of phosphomimetic activated Vps41. RAB7 activation was not sufficient to rescue the endolysosomal conversion of compartments containing vLPS. However, the relationship between the HOPS complex and Ypt7/RAB7 at the endosomes and lysosomes is complex (Nickerson et al., 2009). Indeed, HOPS may play dual roles as it can act both upstream (by interacting with the RAB7 GEF Mon1/Sand1) and downstream (by functioning as a tethering effector of RAB7) to facilitate endosomal membrane fusion. (Nickerson et al., 2009; Peralta et al., 2010). Further investigation in mammalian cells and under pathogenic conditions will be required to understand the cooperation between HOPS and Rab7. Remarkably, recent studies have shown a conserved neuroprotective function of human Vps41 against Parkinson's disease toxins in mammalian cells and *Caenorhabditis elegans* neurons, supporting Vps41 as potential novel therapeutic target for the treatment of synucleinopathies (Peralta et al., 2010; Ruan et al., 2010). Our data define the critical role of p38α-MAPK at a late stage of the degradative pathway via the engagement of the Vps41-HOPS host factor.

Remarkably, macrophages from Q fever patients present a defect in phagosome maturation (Ghigo et al., 2004). Major advances have been made in characterising how pathogen virulence factors disrupt macrophage signalling via the posttranslational modification of critical host factors (Ribet and Cossart, 2010; Rosenberger and Finlay, 2003). In this study, we demonstrate a critical role of the virulent LPS variant from pathogenic *C. burnetii,* which is responsible for the lack of targeting of the bacteria to phagolysosomes. We demonstrate that the differential activation of p38α-MAPK signalling by avLPS and vLPS determines the outcome of macrophage infection at the level of phagolysosome conversion. v*Cb* and av*Cb* have been shown to modulate p38α-MAPK in cells other than macrophages (Hussain et al., 2010; Voth and Heinzen, 2009). Thus, the survival of intracellular *C. burnetii* involves the lack of p38-MAPK phosphorylation due to a variation in its LPS composition. Variation in LPS composition is one strategy used by pathogens to interfere with cell activation following TLR engagement (Miller et al., 2005). In this study, we demonstrate that the vLPS of virulent phase I *C. burnetii* has a unique property to blunt host induction of the p38α-MAPK signalling that is required for the targeting of bacteria to lysosomes, which also requires the expression of TLR4 (Jimenez de Bagues et al., 2005). The specific determinants that distinguish avLPS from vLPS in enterobacteria reside in the O-antigen of vLPS, which bears two unusual sugars, virenose and dihydrohydroxystreptose (Toman and Vadovic, 2011; Vadovic et al., 2011; Vadovic et al., 2005). Bacterial outer-membrane components, such as the lipophosphoglycan of leishmania and the lipoarabinomannan of mycobacteria, are able to impair the conversion of phagosomes into phagolysosomes. This virulence strategy involves the LPG-induced chelation of cholesterol, which subsequently disrupts membrane rafts (Lodge and Descoteaux, 2008) or the inhibition of the production of phosphatidylinositol 3-phosphate by Vps34 (Philips, 2008). LPS from *Brucella*

354 *abortus* also acts as a trafficking diversion agent. Indeed, *Brucella* mutants with an
355 altered LPS O-side chain are trafficked to phagolysosomes (Porte et al., 2003).
356 However, how this mechanism is controlled has not been elucidated. Here, we
357 demonstrate a complex crosstalk between the LPS variants and TLR4 host factors
358 that results in the differential phosphorylation of p38α-MAPK. In addition, avLPS
359 fails to induce Vps41 phosphorylation in TLR4$^{-/-}$ BMDMs **(Fig. S12)**. Our results
360 suggest that TLR4 is necessary for the inhibition of p38α-MAPK phosphorylation.
361 Collectively, our data reveal the importance of the p38α-MAPK/Vps41-
362 HOPS axis for avirulent *C. burnetii* trafficking to lysosomes and the role of LPS
363 variants in corrupting this innate immune mechanism.
364

EXPERIMENTAL PROCEDURES

Ethics statement. All of the animal experiments were conducted according to the Guiding Principles of Animal Care and Use defined by the Conseil Scientifique du Centre de Formation et de Recherche Expérimentale Médico-Chirurgicale (CFREMC) and according to the rules of Décret N° 87-848 as of 10/19/1987. All of the animal experiments were also approved by the ethics board of the university where the experiments were performed (Faculté de Médecine, Marseille, experimentation permit number 13.385).

Antibodies and fluorescent compounds. See Supplemental Experimental Procedures.

Cell culture. BMDMs were generated from 6- to 8-week-old C57BL/6 mice and TLR4$^{-/-}$ mice, as previously described (Cook et al., 2007; Ren et al., 2005). Differentiated BMDMs and p38α$^{-/-}$ MEFs (Adams et al., 2000) were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2.

Cell transfection. BMDMs were transfected using a modified lentivirus encoding GFP-MKK6DD, GFP-RAB7:wt, or GFP-RAB7:Q67L **(see Supplemental Experimental Procedures)**. After 72 hours, approximately 70–80% of the BMDMs had been transfected.

p38α$^{-/-}$ MEFs were transfected with HA-Vps41:wt, HA-Vps41:S83E, HA-Vps41:S796E or HA-Vps41:S796A **(see Supplemental Experimental Procedures)** using Lipofectamine 2000 (Invitrogen). After 24 hours, approximately 89–90% of the p38α$^{-/-}$ MEFs had been transfected.

Bacteria and LPS preparations. See Supplemental Experimental Procedures**.**

LPS endocytosis. Cells were incubated with 1 µg/ml *C. burnetii* LPS for 1 hour at 4°C and then extensively washed with culture medium to remove unbound LPS. The cells were then incubated in culture medium at 37°C for different time periods (10 to 120 minutes) and processed for immunofluorescence staining.

***C. burnetii* infection.** Cells were incubated with *C. burnetii* (200 virulent bacteria or 50 avirulent bacteria per cell) for 4 hours at 37°C. After washing to remove free bacteria (day 0), the cells were cultured, and the infection was quantified using quantitative real time PCR (qPCR), as previously described (Meghari et al., 2007). For confocal microscopy experiments, the cells were fixed with PFA and processed for immunofluorescence staining as described below. Colocalisation was analysed as previously described (Ghigo et al., 2010).

Confocal microscopy. The cells were prepared for immunofluorescence labelling as previously described (Chu and Ng, 2004; Forestier et al., 1999; Ghigo et al., 2010) and imaged using an inverted Leica TCS SP5 and SP6 confocal laser-scanning microscope (Leica, Heidelberg, Germany) **(see Supplemental Experimental Procedures)**.

Immunoblot analysis. BMDMs were stimulated with 1 µg/ml vLPS or avLPS. At the designated times, the BMDMs were washed with ice-cold PBS, and immunoblotting was performed as previously described (Al Moussawi et al., 2010). The detection of total ERK1/2, total p38α, MKK3/6 and α-tubulin was performed on stripped membranes (Restore Western Blot, Pierce). The immunoblots were

visualised using an Amersham Biosciences revelator or an LAS 4000 camera system (GE Healthcare).

Immunoprecipitation

BMDMs were treated with or without LPS and lysed. Vps41 was immunoprecipitated, and its phosphorylation was analysed via immunoblotting **(see Supplemental Experimental Procedures)**.

Vps41 phosphorylation assay. Vps41 was phosphorylated *in vitro*, and the phosphorylation of Vps41 was analysed using mobility shift assays, as previously described (Cabrera et al., 2009) **(see Supplemental Experimental Procedures)**.

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

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Supplementary figures

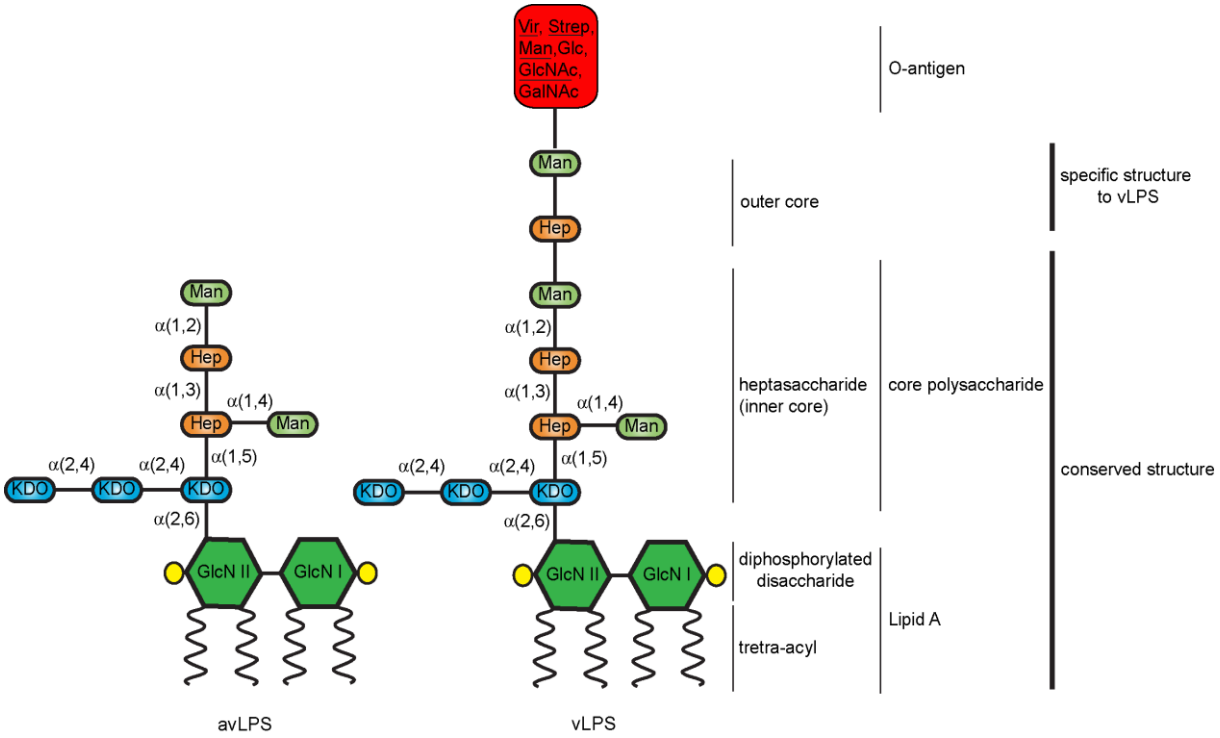

Figure S1. Structure of the *C. burnetii* LPS.
C. burnetii LPS is composed of lipid A, core polysaccharide and O-antigen moieties. The composition and structure of lipid A and the core polysaccharide are conserved between vLPS and avLPS. **i)** Analysis of *C. burnetii* lipid A revealed two major tetra-acylated molecular species that share the classic backbone of a diphosphorylated GlcN disaccharide in which both GlcN I and GlcN II are composed of amide-linked iso or normal (n) C16:0(3-OH). The lipid A structure differs considerably from the published classic form of enterobacterial lipid A. **ii)** The core polysaccharide contains a heptasaccharide in its lipid A proximal region that is composed of two terminal D-mannoses (Man), 2- and 3,4-linked D-*glycero*-D-*manno*-heptoses, and terminal 4- and 4,5-linked 3-deoxy-D-*manno*-oct-2-ulosonic acid residues. **iii)** The molecular basis of the variation between the two forms of LPS resides in the O-antigen. In contrast to vLPS, avLPS is truncated and does not possess the O-antigen and thus does not contain the two unusual sugars (virenose and dihydrohydroxystreptose) (Alexander and Rietschel, 2001; Schramek et al., 1985; Skultety et al., 1996; Toman et al., 2004; Toman et al., 2003; Toman and Skultety, 1996; Toman et al., 2009; Toman and Vadovic, 2011; Vadovic et al., 2011; Vadovic et al., 2005).

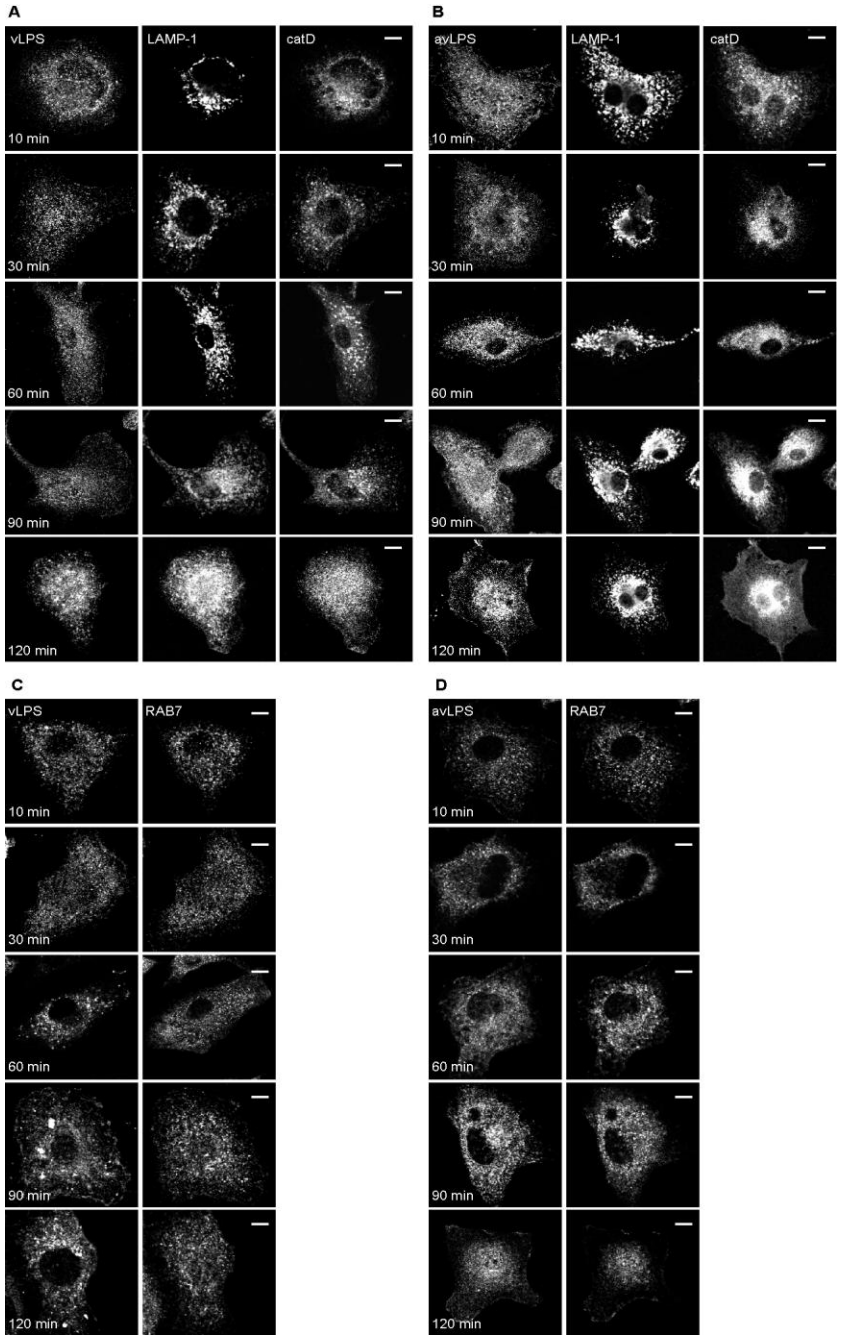

Figure S2. In contrast to avLPS, vLPS prevents lysosomal targeting. BMDMs were treated with vLPS **(A** and **C)** or avLPS **(B** and **D)** from *C burnetii*. The maturation of the intracellular compartments containing LPS was monitored via confocal microscopy. Panels **A** and **B** show the levels of colocalisation between LPS, LAMP-1 and cathepsin D. Panels **C** and **D** show the levels of colocalisation between LPS and RAB7. The scale bar indicates 5 µm. In contrast to avLPS, vLPS did not colocalise with cathepsin D- or RAB7-positive compartments.

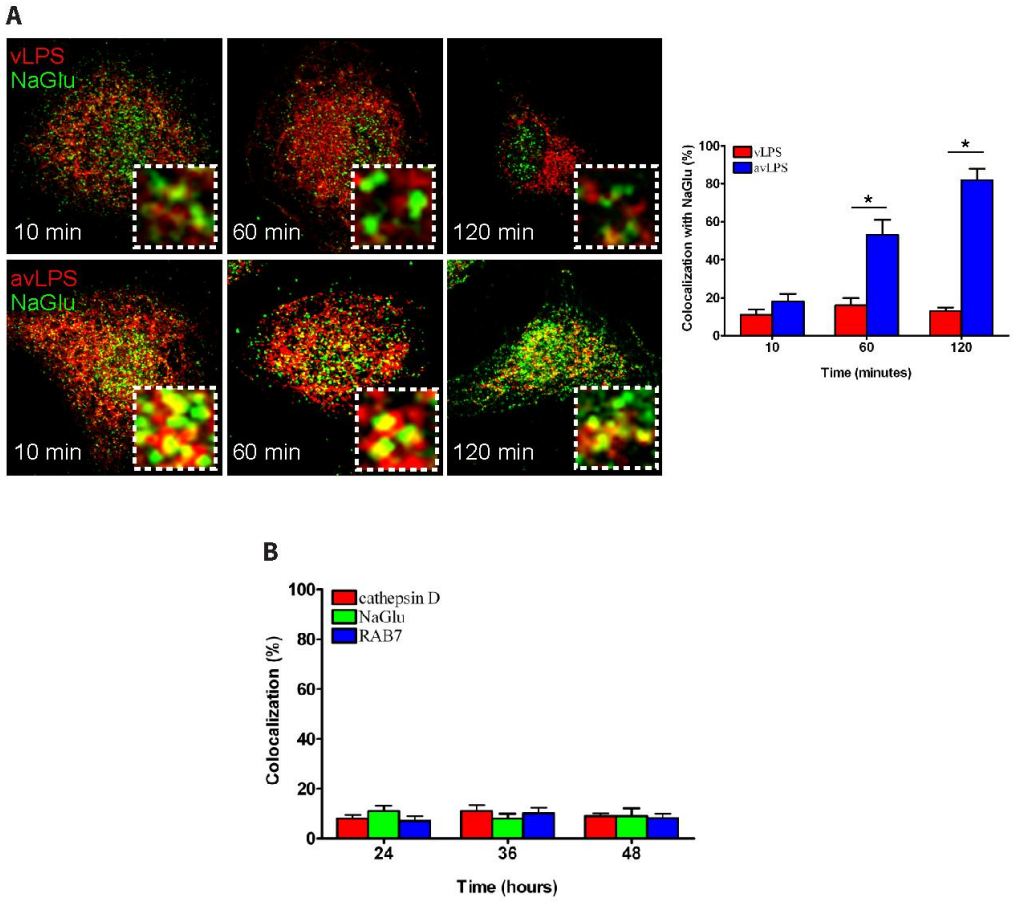

Figure S3. Colocalisation of LPS with lysosome and autophagosome markers.

BMDMs were treated with *C. burnetii* vLPS or avLPS. **(A)** The colocalisation of vLPS or avLPS (red) with α-N-acetyl Glucosaminidase (NaGlu, green) was analysed using confocal microscopy and quantified using ImageJ software. In contrast to avLPS, vLPS did not colocalise with α-N-acetylglucosaminidase. The results are expressed as the mean ± SD (*n*=3). **(B)** vLPS did not colocalise with cathepsin D, α-N-acetylglucosaminidase or Rab7, demonstrating that the defective localisation of vLPS within lysosomes did not result from the delayed acquisition of Rab7 or the lack of lysosomal delivery. The results are expressed as the mean ± SD (*n*=2).

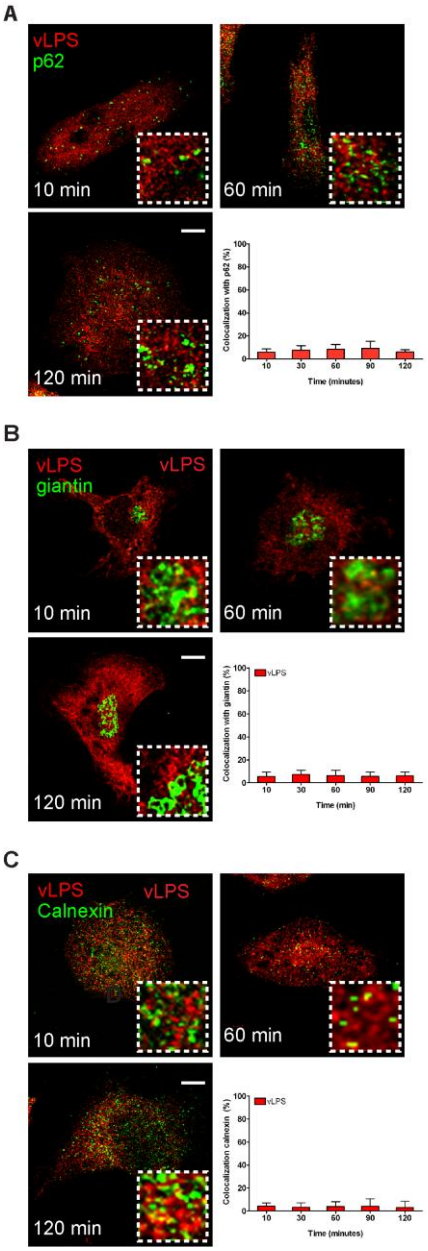

Figure S4. vLPS does not colocalise with markers of autophagosomes, the Golgi apparatus or the endoplasmic reticulum.
BMDMs were treated with *C. burnetii* vLPS, and the colocalisation of vLPS (red) with **(A)** p62 (green), a marker of autophagosomes; **(B)** giantin (green), a marker of the Golgi apparatus; or **(C)** calnexin (green), a marker of the endoplasmic reticulum, was determined using confocal microscopy. The colocalisation of LPS with p62, giantin or calnexin was quantified using ImageJ software. The results are expressed as the mean ± SD (n=2). The scale bar indicates 5 µm.

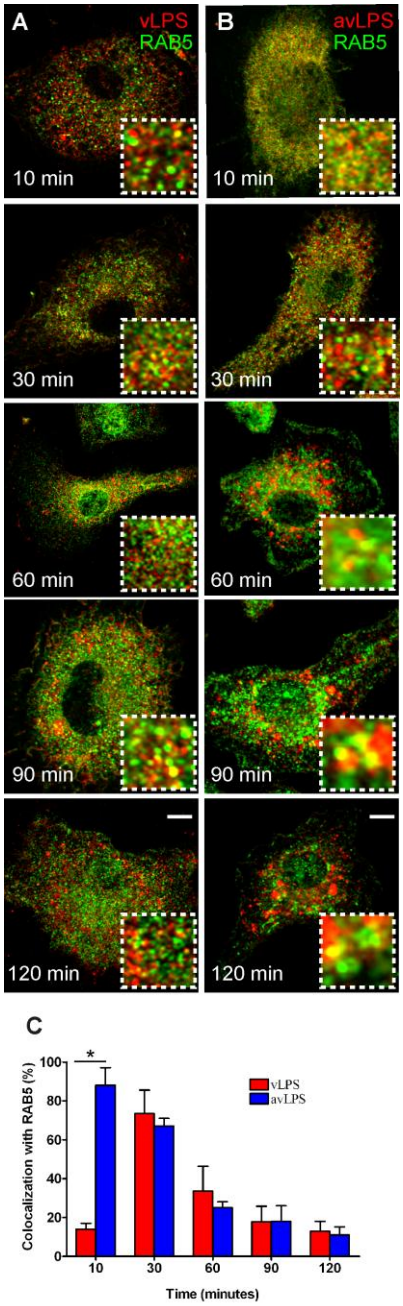

Figure S5. vLPS and avLPS colocalise with RAB5.
BMDMs were stimulated with either *C. burnetii* vLPS **(A)** or avLPS **(B)**. The colocalisation of RAB5 (green) and LPS (red) was determined via confocal microscopy and quantified using ImageJ software **(C)**. vLPS and avLPS transiently colocalised with RAB5, but the colocalisation of vLPS with RAB5 was delayed compared with the colocalisation observed with avLPS. The results are expressed as the mean ± SD (*n*=3). The scale bar indicates 5 µm.

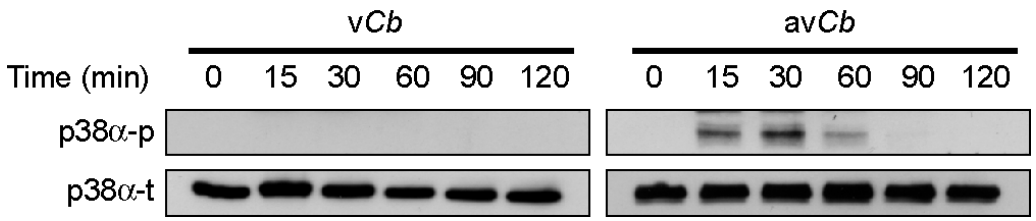

Figure S6. The absence of p38α-MAPK phosphorylation in BMDMs infected with a virulent strain of *C. burnetii*.
BMDMs were stimulated with virulent (v*Cb*) or avirulent (av*Cb*) *C. burnetii*. The levels of total (t) and phosphorylated (p) p38α-MAPK were analysed via immunoblotting. The presented blot is representative of three experiments.

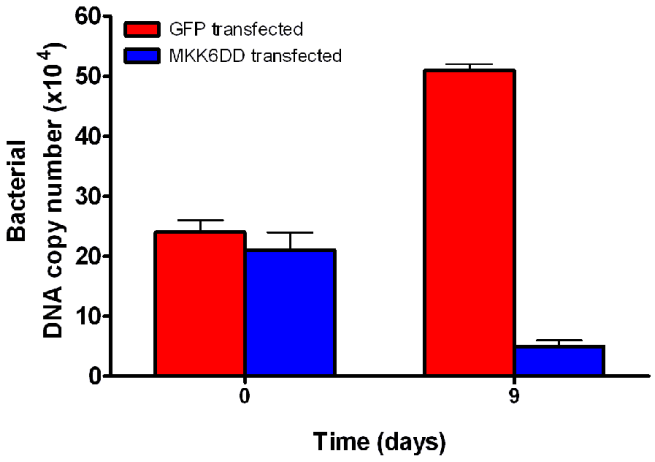

Figure S7. Rescue of bacterial killing by p38α-MAPK activation. BMDMs were transfected with lentiviruses expressing either GFP or GFP-MKK6DD. The BMDMs were then infected with v*Cb* or av*Cb* for 4 hours (T0), washed to remove unphagocytosed bacteria and incubated for the indicated time period. Bacterial replication was assessed by determining the bacterial DNA copy number using qPCR. The results are expressed as the mean ± SD (*n*=3).

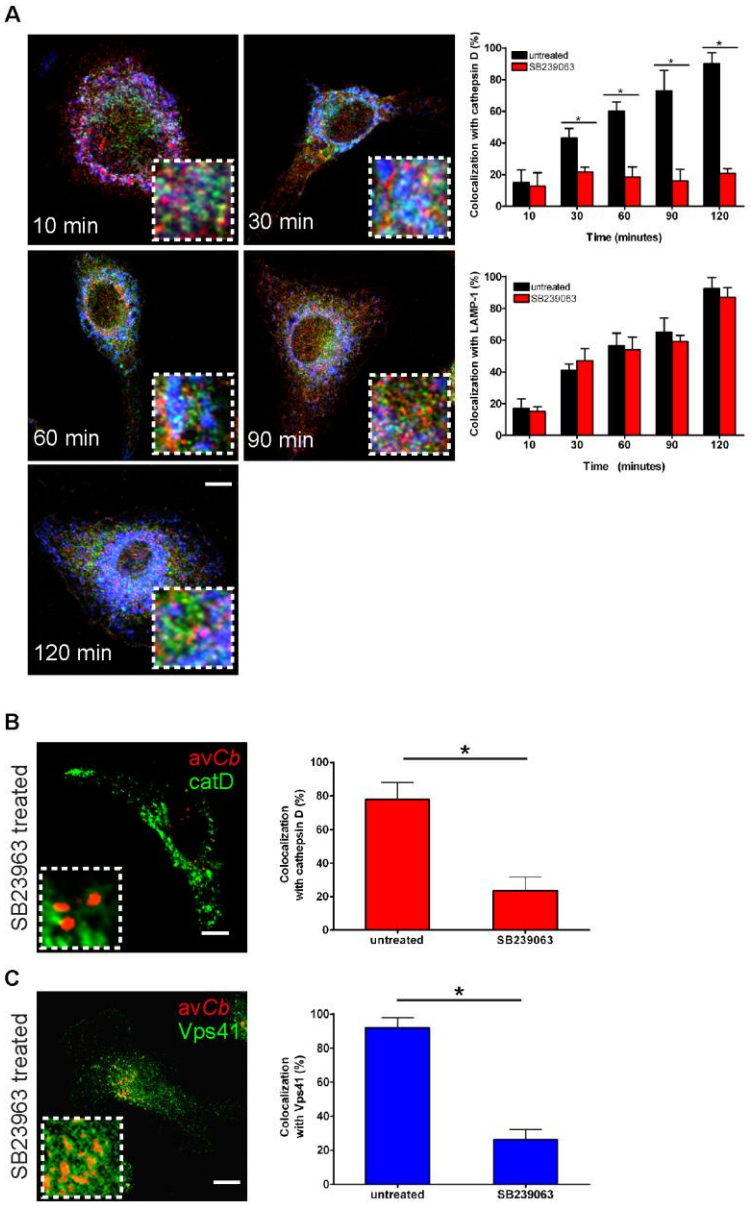

Figure S8. avLPS and v*Cb* fail to reach the lysosomes of macrophages treated with a p38α-MAPK inhibitor.

BMDMs were mock treated or pretreated with 10 µM SB239063 (Sigma-Aldrich), an inhibitor of p38-MAPK activity, for 1 hour. **(A)** avLPS was then internalised for the indicated time period. The colocalisation of avLPS (red) with cathepsin D (green) or LAMP-1 (blue) was monitored via confocal microscopy and quantified using ImageJ software. The results are expressed as the mean ± SD (*n*=3). The scale bar indicates 5 µm. **(B** and **C)** SB239063-treated BMDMs were infected with av*Cb*. The colocalisation of av*Cb* (red) with **(B)** cathepsin D (green) or **(C)** Vps41 (green) was monitored via confocal microscopy and quantified using ImageJ software. The results are expressed as the mean ± SD (*n*=3). The scale bar indicates 5 µm.

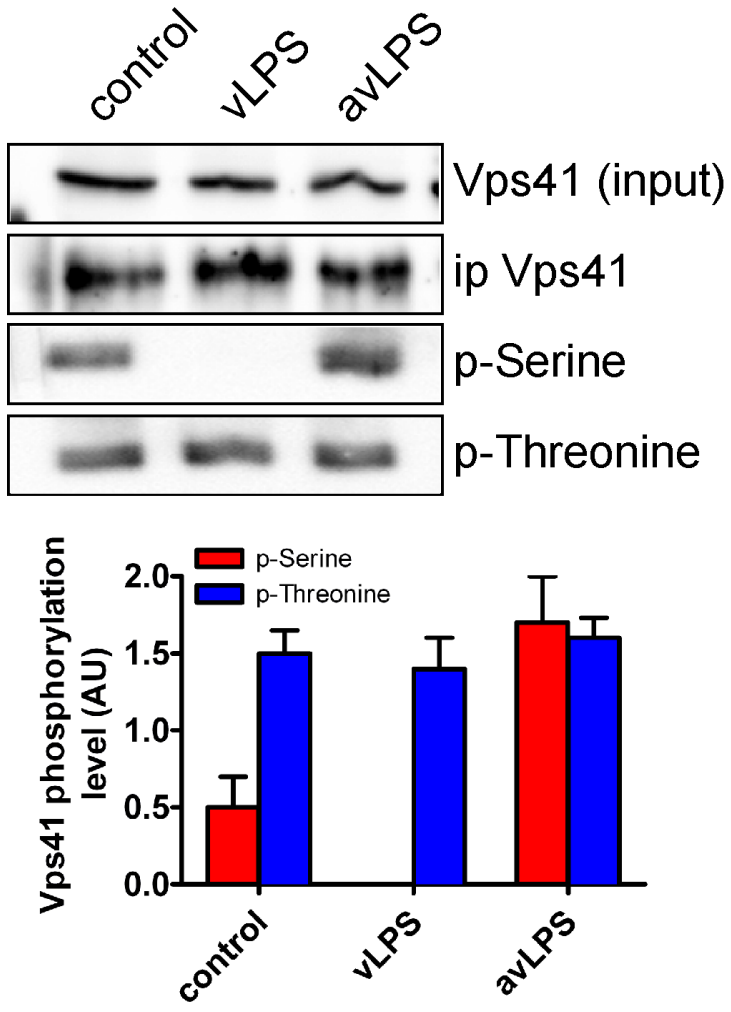

Figure S9. The effects of vLPS on Vps41 phosphorylation in non-starved conditions.

BMDMs in non-starved conditions were either left untreated or treated with vLPS or avLPS. Vps41 was immunoprecipitated from the cell lysates, and the serine- and threonine-phosphorylated forms (p-Serine and p-Threonine, respectively) were visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of Vps41. The presented blot is representative of three experiments. The level of phosphorylation was calculated based on the total amount of immunoprecipitated Vps41 using ImageJ software. The level of threonine phosphorylation is similar in all experimental conditions; however, serine phosphorylation was not detected in the presence of vLPS.

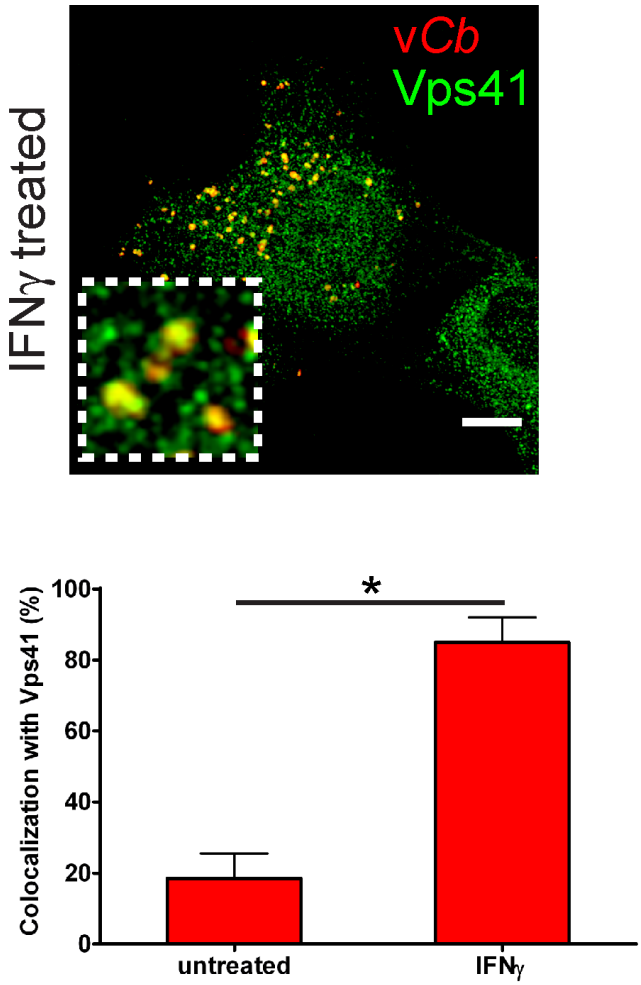

Figure S10. IFN-γ, an activator of p38α-MAPK, induces the colocalisation of Vps41 and v*Cb*.
BMDMs were mock treated or pretreated with recombinant mIFN-γ (200 IU/ml, R&D systems), an activator of p38-MAPK, for 4 hours. v*Cb* was then internalised for the indicated time period. The colocalisation of v*Cb* (red) and Vps41 (green) was monitored via confocal microscopy and quantified using ImageJ software. The results are expressed as the mean ± SD (*n*=2). The scale bar indicates 5 µm.

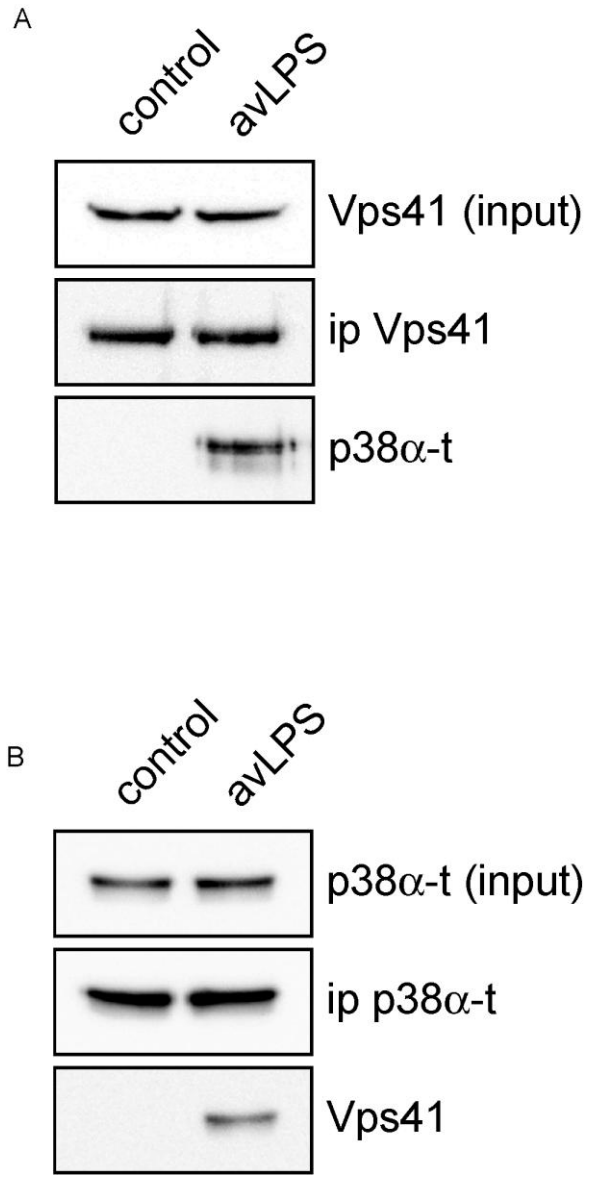

Figure S11. p38α-MAPK and Vps41 interact reciprocally.
Starved BMDMs were not treated or treated with avLPS. (A) Vps41 was immunoprecipitated from the cell lysates, and the co-immunoprecipitation with total (t) p38α-MAPK was visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of Vps41. The presented blot is representative of two experiments. (B) Total (t) p38α-MAPK was immunoprecipitated from the cell lysates, and the co-immunoprecipitation with Vps41 was visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of total (t) p38α-MAPK. The presented blot is representative of two experiments. These results show that p38α-MAPK and Vps41 interact reciprocally.

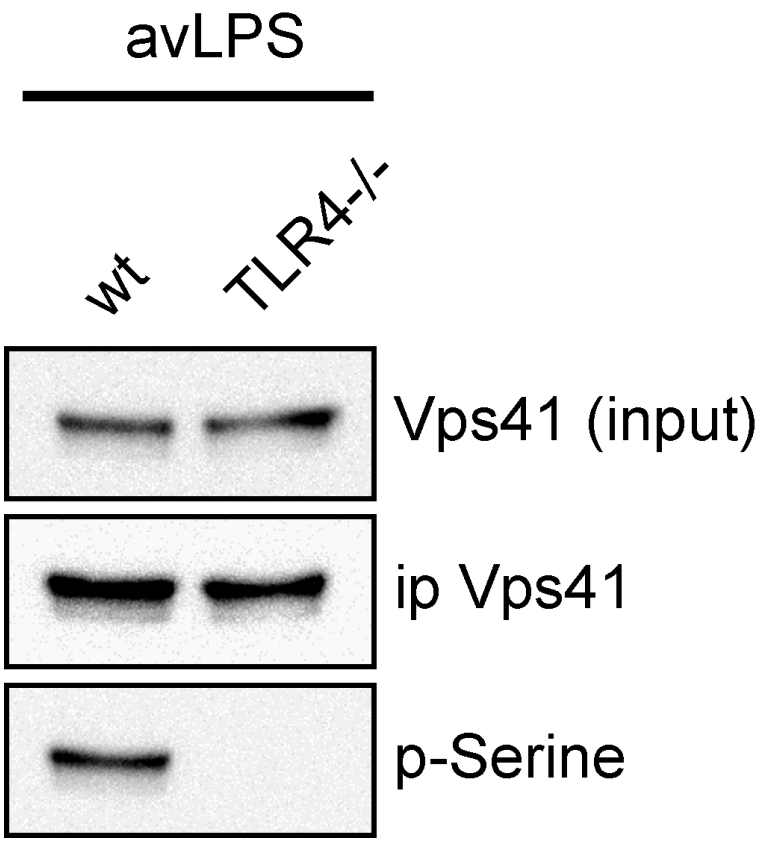

Figure S12. TLR4 engagement is required for the phosphorylation of Vps41.
Starved wild-type and TLR4-deficient BMDMs were either left untreated or treated with avLPS. Vps41 was immunoprecipitated from the cell lysates, and the serine-phosphorylated form (p-Serine) was visualised via immunoblotting. An aliquot of the lysate (input) was also loaded to demonstrate the total amount of Vps41. The presented blot is representative of two experiments. These data demonstrate that TLR4 engagement is required for Vps41 serine phosphorylation.

Supplemental Experimental Procedures

Antibodies and fluorescent compounds. *C. burnetii* bacteria and LPS were detected using human antibodies specific for *C. burnetii* (Ghigo et al., 2002). Rabbit antibodies specific for p62 and active cathepsin D were a gift from S. Méresse (Centre d'Immunologie de Marseille-Luminy, Marseille, France) and Dr S. Kornfeld (Washington University School of Medicine, St. Louis, MO), respectively. Rat antibodies specific for LAMP-1 and rabbit antibodies specific for RAB7 were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) and Sigma-Aldrich, respectively. Rabbit antibodies specific for RAB5, giantin and α-N-acetylglucosaminidase and mouse antibodies specific for calnexin and goat anti-Vps41 were purchased from Abcam. Antibodies specific for phospho-p38α (p38α-p), total p38α (p38α-t), phospho-ERK1/2 (Erk1/2-p), total ERK1/2 (Erk1/2-t), phospho-JNK (JNK-p), phospho-MKK3/6 (MKK3/6-p), total MKK3/6-t (MKK3/6-t) and α-tubulin were purchased from Cell Signaling Technology. The mouse anti-phosphothreonine and anti-phosphoserine antibodies were purchased from Millipore. Secondary antibodies were purchased from Invitrogen. Mouse recombinant IFN-γ was purchased from R&D Systems. SB28963 was purchased from Sigma-Aldrich. Mouse antibodies specific for HA were purchased from Santa Cruz.

Bacteria and LPS preparations. The RSA 493 and RSA 439 Nile Mile strain of *C. burnetii* were phenotyped and cultured as previously described (Capo et al., 1999; Ghigo et al., 2002; Glazunova et al., 2005; Howe et al., 2010). LPS from virulent (vLPS) and avirulent (avLPS) *C. burnetii* was 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 transfection. BMDMs were transiently transfected using lentiviral technology (Invitrogen). Briefly, MKK6DD was cloned from pEFmlink-MKK6DD into the pEGFP vector. Then, GFP, GFP-MKK6DD (the constitutively active form of the p38α MAPK activator MKK6), GFP-RAB7:wt and GFP-RAB7:Q67L (the constitutively active form of RAB7) were subcloned into the entry clone pENTR TOPO vector using the TOPO cloning system, as recommended by the manufacturer (Invitrogen). The inserted DNA was transferred into the destination vector to create a lentivirus encoding GFP, GFP-MKK6DD, GFP-RAB7:wt or GFP-RAB7:Q67L using the ViraPower HiPerform Gateway Expression System (Invitrogen), as recommended by the manufacturer. Viruses were produced using 293T cells, and viral supernatants were harvested 48 and 72 hours after transfection. The supernatants were centrifuged at 1,600 × *g* for 15 minutes at 4°C, filtered through 0.45 μm filters, collected and concentrated using PEG-*it* virus precipitation solution (System Biosciences) and then stored at -80°C.

Confocal microscopy. Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS pH 7.4) and prepared for immunofluorescence labelling, as previously described (Chu and Ng, 2004; Forestier et al., 1999; Ghigo et al., 2010). Coverslips were mounted in Mowiol, and the cells were imaged using an inverted Leica TCS SP5 confocal laser-scanning microscope (Leica, Heidelberg, Germany). Image acquisition was performed using the Leica Confocal software. The collected images were processed using Adobe Photoshop v7.0.1 software. The cells were evaluated as follows: 25 fields with at least three cells per field were examined for each experimental condition; in total, approximately 100 cells were examined per

experimental condition. The colocalisation analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij) and the JaCoP plug-in (http://rsbweb.nih.gov/ij/Plugins/track/jacop.html), as previously described (Barr et al., 2008; Bolte and Cordelieres, 2006). The calculation of overlap is based on the calculation of the Manders coefficients M1 and M2, reflecting channel1/channel2 overlap and channel2/channel1 overlap.

ELISA

BMDM were stimulated with 1 µg/mL vLPS or avLPS for 16 hours. The supernatants were collected, stored at -80°C and then evaluated for the presence of IL-6 and TNFα using commercially available ELSA kits, as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA).

Immunoprecipitation

Wild-type or TLR4-deficient BMDMs were treated with or without LPSs (1 µg/ml) for 30 minutes 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. Vps41 or p38α-MAPK was immunoprecipitated via overnight incubation of total protein with anti-Vps41 (Abcam) or anti-p38α-MAPK antibodies followed by incubation with protein A-Sepharose beads (Roche). The immunoprecipitated pellets were washed and analysed via immunoblotting on 6% polyacrylamide gels using a mouse anti-phosphothreonine, anti-phosphoserine, anti-Vps41 or anti-p38α-MAPK antibody. The detection of Vps41 or p38α-MAPK from the input sample was performed using 50 µg of protein. The immunoblots were visualised using an LAS 4000 camera system (GE Healthcare) or an Amersham Biosciences revelator. In some experiments the phosphorylation levels were assessed using ImageJ software.

Vps41 mutagenesis

Point mutations in Vps41 were generated using the Quickchange Site-Directed Mutagenesis Kit from Agilent Technologies. The following primers were used to generate the point mutations: S83E (5 CATCACTCAGAAGTTTGATGTAG AGCCTGTGAAGATAAATCAGATTAG and 3 CTAATCTGATTTATCT TCACAGGCTCTACATCAAACTTCTGAGTGATG), S796E (5-CATCTGTG AGTCGTGCCTTGAGCCTATTCTTCCATCAGATGC and 3-GCATCTGATG GAAGAATAGGCTCAAGGCACGACTCACAGATG), S796A (5-CATCTGT GAGTCGTGCCTTGCGCCTATTCTTCCATCAGATGC and GCATCTGATG GAAGAATAGGCGCAAGGCACGACTCACAGATG).

Vps41 phosphorylation assay

In the presence or absence of ATP (50 µM), 5 µg of recombinant Vps41 (Abnova) was incubated with 1 µg of active recombinant p38α (Biaffin GmbH & Co KG) at 30°C for different periods of time in a specific buffer (20 mM HEPES pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT). The Vps41 mobility shift was analysed via immunoblotting, as previously described (Cabrera et al., 2009), using an anti-Vps41 antibody, and Vps41 serine phosphorylation was analysed using an anti-phosphoserine antibody.

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ARTICLE II

Effects of *Coxiella burnetii* on MAP kinase phosphorylation

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FEMS immunology and medical microbiology, 2011

La fièvre Q est une zoonose causée par une bactérie intracellulaire obligatoire, *Coxiella burnetii*. Les symptômes de la fièvre Q aiguë sont une fièvre, une pneumonie, une hépatite et des désordres neurologiques. Chez les valvulopathes ou les immunodéprimés, la fièvre Q peut devenir chronique avec comme conséquence principale une endocardite. La fièvre Q chronique est caractérisée par un défaut de réponse immune spécifique, une activité microbicide altérée des monocytes, une production importante d'anticorps anti- *C. burnetii* et une surproduction d'IL-10.

La reconnaissance des microorganismes par les phagocytes entraîne une cascade transductionnelle impliquant entre autres voies l'activation des MAPKs. Ces MAPKs sont connues pour jouer un rôle critique dans la réponse immune. La réponse immune étant déréglée dans la fièvre Q, nous avons étudié l'activation des MAPKs (p38, JNK et ERK) par *C. burnetii.*

Nous avons montré que *C. burnetii* et ses variants avirulents induisent l'activation de JNK et ERK. En revanche, seuls les variants avirulents activent p38. Nous avons également montré que p38 est activé dans les macrophages dérivés des monocytes de donneurs sains, de patients atteints d'une fièvre Q aiguë et de patients ayant guéri dune fièvre Q chronique alors que p38 n'est pas activé dans les macrophages des patients présentant une fièvre Q chronique active. Ces résultats suggèrent que la détermination de l'activation de p38 peut servir d'outil pronostique de la fièvre Q chronique.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

SHORT COMMUNICATION

Effects of *Coxiella burnetii* on MAPKinases phosphorylation

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MAPKs; Q fever; macrophages; p38; intracellular bacteria; signaling.

Abstract

Q fever is a disease caused by *Coxiella burnetii*, an obligate intracellular bacterium. Acute Q fever is characterized by efficient immune response, whereas chronic Q fever is characterized by dysregulated immune response as demonstrated by the lack of granulomas, the failure of *C. burnetii* to induce lymphoproliferation, and interferon-γ production. The mitogen-activated protein kinase (MAPK) signaling pathway plays crucial roles in innate immune responses and control of bacterial infections. However, its role in Q fever has not been addressed. First, we investigated the activation of MAPKs p38, c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2 in murine macrophages stimulated with *C. burnetii*. *Coxiella burnetii* NM phase I (virulent) and NM phase II (avirulent) induced the activation of JNK and ERK1/2. Avirulent *C. burnetii* activate p38, whereas *C. burnetii* did not induce the phosphorylation of p38. Second, the level of p38 activation was studied in Q fever patients. We found that p38 was activated in monocyte-derived macrophages from healthy donors and patients with acute Q fever in response to a potent agonist such as lipopolysaccharide. Interestingly, p38 was not activated in patients with active chronic Q fever and was activated in patients with cured chronic Q fever. These results suggest that the determination of p38 activation may serve as a tool for measuring Q fever activity.

Q fever is a zoonosis with worldwide distribution caused by *Coxiella burnetii*, an obligate intracellular bacterium. Acute Q fever symptoms consist of self-limited febrile illness, pneumonia, granulomatous hepatitis, neurologic disorders, and miscellaneous manifestations (Raoult *et al.*, 2005). The major symptom of chronic Q fever is endocarditis, which usually occurs in patients with previous valvular disease or in a context of immunosuppression. Although acute Q fever is spontaneously resolutive, the chronic disease is characterized by impaired immune response, defective microbicidal activity of monocytes, production of anti-*C. burnetii* antibodies (Raoult *et al.*, 2005), and overproduction of IL-10 (Honstettre *et al.*, 2003).

Recognition of microorganisms by macrophages is mediated by pattern recognition receptors (PRR) that bind conserved pathogen-associated molecular patterns (PAMPs). PRR engagement by PAMPs activates a major signaling cascade that ultimately leads to the activation of mitogen-activated protein kinases (MAPKs). The MAPK signaling pathway plays critical roles in innate immune responses. Gram negative bacteria are potent activators of three MAPKs pathways (Guha & Mackman, 2001): the extracellular signal-regulated kinase (ERK) pathway, the c-jun N-terminal kinase (JNK) pathway, and the p38 pathway. As the immune response is dysregulated in Q fever, we investigated the activation of MAPK p38, JNK, and ERK1/2 induced by *C. burnetii* in macrophages. For this purpose, mouse bone marrow-derived macrophages (BMDMs) were stimulated with 200 virulent bacteria (Nine Mile in phase I, NM I, strain RSA493) per cell or 50 avirulent bacteria (Nine Mile in phase II, NM II) per cell as previously described (Ghigo *et al.*, 2002) for different periods. MAPK activation was revealed by Western blot analysis using specific antibodies against phospho-p38 (p38-p), total p38 (p38-t), phospho-Erk1/2 (Erk1/2-p), total Erk1/2 (Erk1/2-t), phospho-JNK (JNK-p), and α-tubulin (Cell Signaling). NM I

induced a transient phosphorylation of Erk 1/2 that was maximum after 30 min, whereas NM II stimulated activation of Erk 1/2 in a delayed manner with a maximum at 60 min (Fig. 1a). JNK was activated by both NM I and NM II within 15–30 min. However, the level of activation of JNK by NM II variants was 15-fold higher (quantified by densitometry) than NMI (Fig. 1a). Surprisingly, the MAPK p38 was not phosphorylated in response to NM I; in contrast the NM II induced the activation of p38 with a maximum of phosphorylation at 30 min (Fig. 1a). Similar results have been observed in dendritic cells (Shannon *et al.*, 2005). The mechanisms used by NM I to interfere with p38 activation remain unclear. Only a few reports, *in vitro*, describe altered p38 activation by bacterial and/or parasite ligands. *Salmonella* SpvC removes phosphate groups from phosphothreonine residues, thus inactivating MAPKs in host cells (Zhu *et al.*, 2007). It has also been demonstrated that *Leishmania major* inactivates p38 by cleaving p38 upstream adaptor TAB 1 (Halle *et al.*, 2009). As p38 is regulated through specific phosphatases (Cuadrado & Nebreda, 2010), we can hypothesize that NM I activates specific p38 phosphatases.

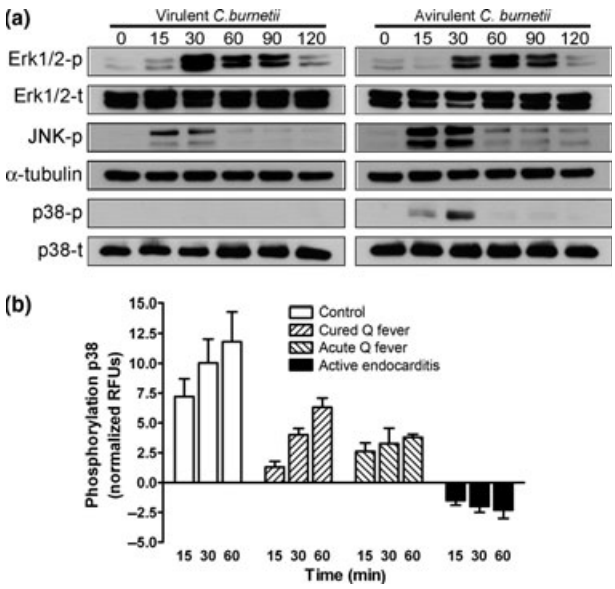

Fig. 1. Activation of MAPKs. (a) BMDMs were stimulated with virulent *Coxiella burnetii* and avirulent variants for different periods (min). MAPK activation was monitored by Western blot using antibodies directed against phospho-p38 (p38-p), total p38 (p38-t), phospho-Erk1/2 (Erk1/2-p), total Erk1/2 (Erk1/2-t), phospho-JNK (JNK-p), and α-tubulin. One representative experiment is shown ($n = 3$). (b) MDMs from four healthy donors or 12 patients with Q fever (four patients for each type of Q fever; cured chronic, acute and active endocarditis) were stimulated with lipopolysaccharide, and the phosphorylation of p38 was determined using phospho-p38 MAPK cell-based ELISA. The results expressed as normalized RFU represent the mean ± SEM of experiments performed in triplicate.

We then wondered if the lack of p38 activation is associated with *C. burnetii* infection. Peripheral blood mononuclear cells from healthy donors and patients with Q fever were purified using a Ficoll gradient, and monocytes were isolated using positive selection CD14. Monocytes were differentiated into macrophages for 7 days, and monocyte-derived macrophages (MDMs) were stimulated using *Escherichia coli* lipopolysaccharide (1 μg mL^{-1}) for different periods. The phosphorylation of p38 was assessed using phospho-p38 MAPK cell-based ELISA (R&D Systems). Results are expressed in normalized Relative Fluorescent Unit (RFU). The statistical comparison between groups was performed using Tukey's multiple comparison test. In MDMs from four healthy controls, lipopolysaccharide induced p38 activation in a time-dependent manner (Fig. 1b). In MDMs from four patients with acute Q fever and four patients with cured endocarditis, p38 was phosphorylated, but its activation level was significantly ($P < 0.05$) decreased compared with time-matched controls. Interestingly, in MDMs from four patients with active Q fever endocarditis, p38 was not phosphorylated, whatever the time of stimulation ($P < 0.001$) (Fig. 1b). These results may be related to the defective immune response observed in active Q fever endocarditis (Ghigo *et al.*, 2004; Raoult *et al.*, 2005). To our knowledge, this is the first demonstration that p38 activity is defective in an infectious disease.

In conclusion, we show that *C. burnetii* activated ERK1/2, JNK MAPKs, but not p38 in murine macrophages. This effect was specific to *C. burnetii* NM phase I (virulent), as *C. burnetii* NM phase II (avirulent) activated p38. The absence of p38 phosphorylation might reflect either a lack of p38 activation or a specific inactivation of p38 by virulent *C. burnetii*. As the absence of p38 phosphorylation also characterized macrophages from patients with active chronic Q fever and was partially observed in patients with acute or cured chronic Q fever, it might be used as marker of the disease activity. Monitoring p38 phosphorylation using a rapid and accurate method should improve the prognosis of patients with Q fever.

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Trafic intracellulaire de *Tropheryma whipplei*

Articles III et IV

George Hoyt Whipple a décrit en 1907 une nouvelle maladie qu'il définit comme lipodystrophie intestinale. Suite à la présence de graisses dans les selles, l'intestin et les ganglions mésentériques, il envisage une maladie du métabolisme des acides gras. Ce n'est qu'en 1961, avec l'avènement de la microscopie électronique, que l'étiologie bactérienne de la maladie de Whipple est confirmée **[65]**. Sur des critères de PCR, l'agent de la maladie de Whipple a été considéré comme une actinomycète **[86]**. Plus récemment encore, une première souche a été isolée à partir de la valve mitrale d'un patient **[87]**.

Le nom de *Tropheryma whipplei*, l'agent de la maladie de Whipple, a alors été proposé en hommage au pathologiste américain **[88]**. Près de 100 ans après sa description, *T. whipplei* a pu être retrouvée rétrospectivement dans le cas rapporté en 1907 par Whipple **[89]**. La culture de *T. whipplei* a aussi permis le séquençage complet de son génome **[90, 91]**, ce qui a ouvert la voie à l'établissement d'un milieu de culture axénique **[92]**.

Les biopsies duodénales montrent un infiltrat important des tissus lésés par des macrophages dits spumeux dans lesquels il est probable que les bactéries se répliquent. C'est la raison pour laquelle a été entreprise une étude de l'interaction des cellules myéloïdes avec *T. whipplei* **[93]**. Alors que *T. whipplei* est éliminée par les monocytes circulants, elle se réplique dans les macrophages dérivés de ces mêmes monocytes. La réplication bactérienne est

associée à l'expression de l'IL-16 par les macrophages. En effet, l'ajout d'anticorps bloquants anti-IL-16 aux macrophages conduit à l'élimination de *T. whipplei*. L'ajout d'IL-16 aux monocytes conduit, lui, à la réplication des bactéries.

Notre travail a consisté à identifier le compartiment réplicatif de *T. whipplei* dans les macrophages humains et murins. Nous avons montré que *T. whipplei* se réplique dans un phagosome atypique incapable de fusionner avec les lysosomes (**Article III**). L'existence de ce compartiment est associée à l'expression de l'IL-16 puisque son inhibition par des anticorps spécifiques ou l'inactivation de son gène entraînent la maturation du phagosome de *T. whipplei* en phagolysosome (**Article IV**).

Article III

The small GTPases Rab5 and Rab7 coexist on the phagosome harbouring *Tropheryma whipplei*, the agent of Whipple's disease

(Manuscrit en préparation)

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Nous avons montré en utilisant des phagosomes purifiés sur gradient de sucrose que *T. whipplei* réside dans un phagosome tardif incapable de fusionner avec les lysosomes. Nous avons alors étudié le recrutement de Rab5 et de Rab7, des régulateurs majeurs de la conversion phagosomale, par ces phagosomes. De façon inattendue, Rab5 et Rab7 sont simultanément présents à la surface du phagosome de *T. whipplei*. Ce phagosome est ainsi dans un état transitoire de conversion puisqu'il est à la fois phagosome précoce et phagosome tardif. Une dérégulation de l'expression transcriptionnelle de Ra5 et Rab7 ne peut être mise en cause. Le recrutement à la surface du phagosome du PI(3)P requis pour la conversion du phagosome est lui aussi normal. Le mécanisme permettant à *T. whipplei* de bloquer la conversion de son phagosome reste à élucider. Cependant, l'analyse bioinformatique du génome et du protéome de *T. whipplei* révèle la présence d'une protéine présentant une forte homologie avec la GAPDH nom de *L. monocytogenes* connue pour moduler l'activité de Rab5. Cette GAPDH pourrait expliquer le blocage de la conversion phagosomale par *T. whipplei*.

The small GTPases Rab5 and Rab7 coexist on the phagosome harbouring *Tropheryma whipplei*, the agent of Whipple's disease

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Running title: A chimeric phagosome for the Whipple's disease agent

Key Words: Rab, phagosome, bacteria, *Tropheryma whipplei*

Abstract

The survival strategies of bacterial pathogens into host cells imply the control of phagosome conversion. Here, we showed for the first time that a microbial pathogen, namely *Tropheryma whipplei*, the agent of Whipple disease, survived and replicated within murine bone marrow-derived macrophages by blocking the phagosome conversion at the Rab5-Rab7 stage. Indeed, the analysis of purified phagosomes and confocal microscopy revealed that *T. whipplei*-containing phagosomes shared the properties of early phagosomes, known to express Rab5 and, transiently, PI(3)P, and late phagosomes, known to express, Rab7 and Lamp-1. These *T. whipplei*-containing phagosomes were unable to fuse with lysosomes since they did not express a lysosomal hydrolase such as cathepsin D. We also demonstrated that the blockage of phagosome conversion was not associated with a transcriptional modulation of Rab5, Rab7 or Vps34 transcripts, or altered PI(3)P production. Bioinformatic analysis revealed that *T. whipplei* encodes a protein homologue to *Listeria monocytogene*s GAPDH, described to interfere with Rab5 activity, suggesting that this protein might be a key for the defective conversion of *T. whipplei*-containing phagosome.

Introduction

Once internalized by host cells, particles including bacteria are localized within phagosomes that undergo a series of fusion/fission events with different populations of endocytic organelles, such as endosomes and lysosomes, leading to the maturation or conversion of phagosomes in phagolysosomes where bacteria are destroyed. The mechanisms underlying phagosome conversion progression are exceedingly complex. However, some general principles can be discerned. Immediately after phagosome sealing, the first step of phagosome conversion is intermingled fusion/fission events with early endosomes. Phagosomes gain several markers of early endosomes, such as Early Endosome Autoantigen-1 (EEA1) and the small GTPase Rab5, a Rab protein from the small G protein superfamily. The early phagosomes (pH 6.0) gradually transform into late phagosomes that present features of late endosomes such as the small GTPase Rab7 and Lysosomal membrane-associated protein (Lamp)-1. Late endosomal markers progressively replace early endosomal markers (Flannagan et al., 2009; Kinchen and Ravichandran, 2008). In addition, late phagosomes acquire the vacuolar proton pump ATPase ($V\text{-}H^+$-ATPase) that allows the intraphagosomal pH to reach an acidic pH (around 4.0) (Flannagan et al., 2009; Kinchen and Ravichandran, 2008). Finally, acidic late phagosomes interact with lysosomes leading to phagolysosomes that contain lysosomal hydrolytic enzymes such as cathepsin D (Flannagan et al., 2009; Kinchen and Ravichandran, 2008). It is

now believed that Rab proteins coordinate signaling 'hubs" through which membrane traffic is regulated (Gurkan et al., 2005). Indeed, Rab5 and Rab7 are critical for endosome and phagosome conversion (Henry et al., 2004; Rink et al., 2005). Rab proteins Rab5 and Rab7 are involved in the coordination of the membrane traffic continuum (Brumell and Scidmore, 2007; Zerial and McBride, 2001) and the endosome and phagosome conversion (Henry et al., 2004; Rink et al., 2005). A transition between Rab5 and Rab7 stage is required for the normal endosome and phagosome conversion (Henry et al., 2004; Rink et al., 2005). During this stage, Rab5 and Rab7 co-exist for a short period of time, on same endomes or phagosomes (Henry et al., 2004; Rink et al., 2005). This step is require for the endosome and phagosome conversion process.

Bacterial pathogens evolve multiple strategies to survive within their host cells. One of the major mechanisms permitting bacterial pathogens to survive within their host cells is their ability to alter the maturation of their phagosomes (Flannagan et al., 2009). *Mycobacterium* phagosomes fuse with early endosomes but are unable to fuse with late endosomes (Flannagan et al., 2009; Philips, 2008). *Coxiella burnetii* survives in an acidified late phagosome that does not fuse with lysosomes (Ghigo et al., 2009). *Salmonella thyphimurium* resides in an atypical phagosome, which is neither a late nor an early phagosome (Steele-Mortimer, 2008). As Rab GTPases are critical in endosome conversion, it is not surprising

that bacterial pathogens that target Rab functions creat a compartment suitable for their replication in host cells (Brumell and Scidmore, 2007). Indeed, *M. tuberculosis* retains Rab5 at the surface of its phagosome and inhibits the PI(3)P production required for the recruitment of Rab5 effectors (Brumell and Scidmore, 2007). To replicate, *Helicobacter pilori* induces the formation of Rab7 positive vacuole (Brumell and Scidmore, 2007). *Listeria monocytogenes* interfers with Rab5 activity and consequently reside in early phagosome before to escape in cytosol (Alvarez-Dominguez et al., 2008).

The Whipple's disease is an infectious disease caused by the actinomycete *Tropheryma whipplei* (Marth and Raoult, 2003). Whipple's disease, a rare systemic disease, is associated with an initial phase of migratory polyarthritis, fatigue, weight loss, and anemia followed by a progressive syndrome of abdominal pain, steatorrhea, and cachexia with lymphadenopathy and hyperpigmentation (Moos et al., 2006). The course of Whipple's disease is fatal, unless antibiotics treatment is initiated (Schneider et al., 2008). *T. whipplei* replicates in monocyte-derived macrophages, but not in monocytes, suggesting that its cell targets are exquisitely specific (Desnues et al., 2005). Several data unpublished and published (Ghigo et al., 2002) have suggested that T. whipplei inhibit the phagosome conversion.. In this paper, we hypothesized that *T. whipplei* replicates in macrophages by hijacking the normal conversion of its phagosome. Our study revealed that the

compartment containing *T. whipplei* was a chimeric phagosome expressing simultaneously Rab5, Rab7 and Lamp-1, but was unable to fuse with lysosomes. The absence of Rab5/ Rab7 transition cannot be explained by altered PI(3)P recruitment at the phagosome surface. Bioinformatic analysis reveal that *T. whipplei* code an annotated GAPDH protein presenting homology with the *L. monocytogenes* GAPDH, which was descrided to be involved in the blocage of the Rab5 GTPase activity (Alvarez-Dominguez et al., 2008).

Results

T. whipplei survived and replicated in BMDM

Mouse bone marrow-derived macrophages (BMDM) were infected with *T. whipplei* (bacterium-to-cell ratio of 50:1) for 4 hours (day 0), washed to eliminate free organisms and incubated for additional periods. The intracellular fate of *T. whipplei* was assessed by quantitative real time PCR (**Figure 1A**). At day 0, 3.8 x 10^3 bacterial DNA copies were detected, showing that BMDM ingested organisms (see also Fig. 3). The number of bacterial DNA copies transiently decreased at day 3, suggesting a killing phase as previously described in human macrophages (Desnues JI 2005). Thereafter, the number of DNA copies steadily increased from day 6 to day 12 (3.1 x 10^4 bacterial DNA copies at day 12), demonstrating that *T. whipplei* intensively replicated within BMDM.

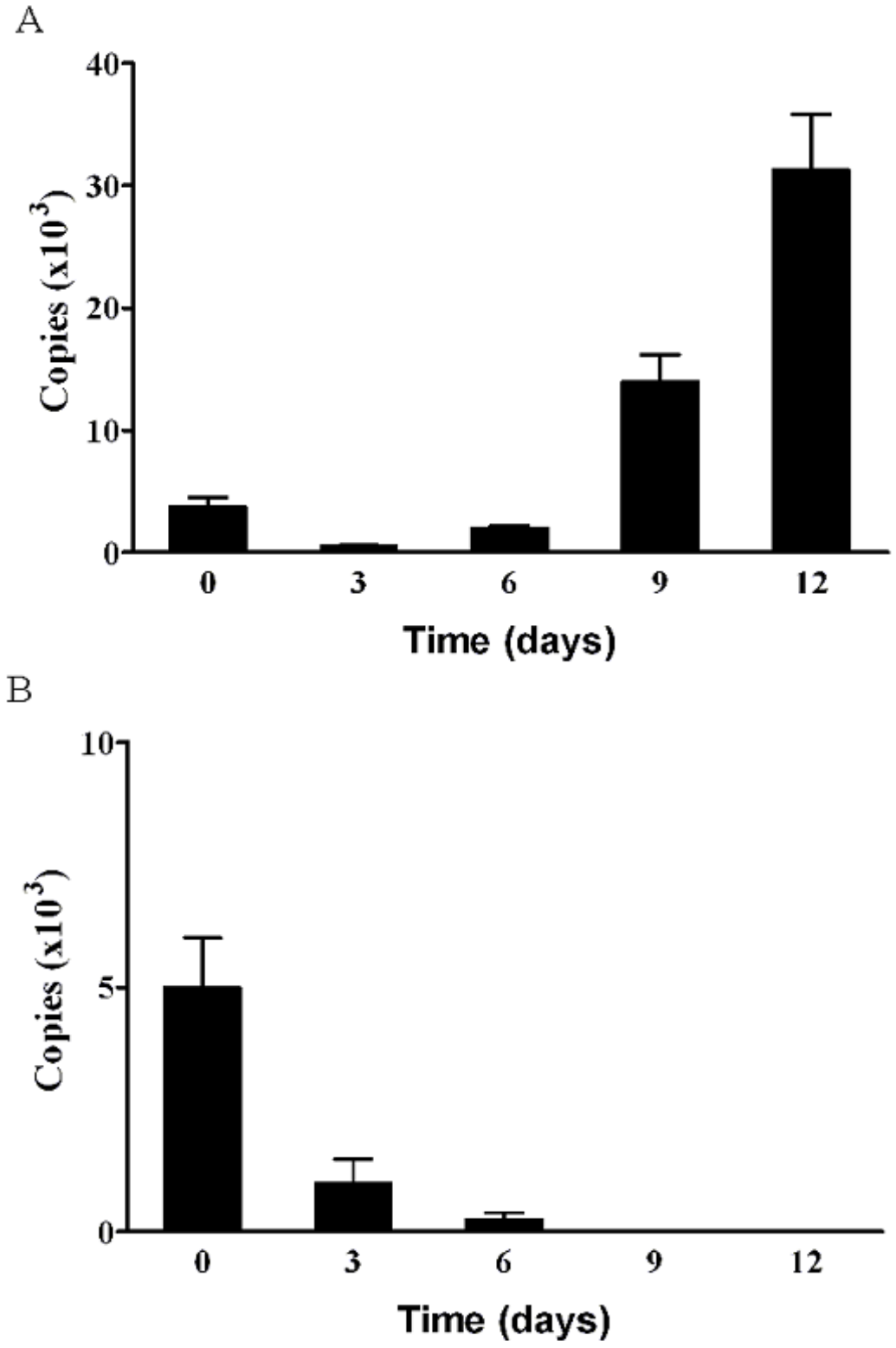

Figure 1. *T. whipplei* replicated within BMDM
BMDM were incubated with *T. whipplei* (bacterium-to-cell ratio of 50:1) for 4 hours **(A)** or *M. smegmatis* (bacterium-to-cell ratio of 10:1) for 1 hour **(B)**. They were then washed to remove free bacteria and incubated for additional periods. The number of bacterial DNA copies was determined by qPCR. Results are the mean ± SEM of 4 experiments.

As *T. whipplei* replication may be due to defective microbicidal activity of BMDM we infected BMDM with *Mycobacterium smegmatis* (bacterium-to-cell ratio of 10:1) for 1 hour, and the intracellular fate of *M. smegmatis* was evaluated by qPCR (**Figure 1B**). BMDM internalized *M. smegmatis* (5.0×10^3 bacterial DNA copies at day 0) and progressively eliminated organisms (0.25×10^3 bacterial DNA copies at day 6, and no copies found at days 9 and 12).These results suggested that BMDM microbicidal activities were not altered.

T. whipplei was localized in late phagosomes unable to fuse with lysosomes

We investigated the nature of the compartment in which *T. whipplei* replicated. For that purpose, we purified 12 day-compartment that contained *T. whipplei* (TwC) or latex beads (LBC), as control since latex beads are known to trafic through early endosomes and late endosomes to reach a phagolysosomal compartment (Desjardins et al., 1994; Via et al., 1997). Phagosomes were isolated using sucrose gradient, according previously described methods (Desjardins et al., 1994) (**Figure 2A**). Latex bead compartments were recovered relire and collected at the 10 to 25 % sucrose interface, as previouly described (**Figure 2A**) (Desjardins et al., 1994).

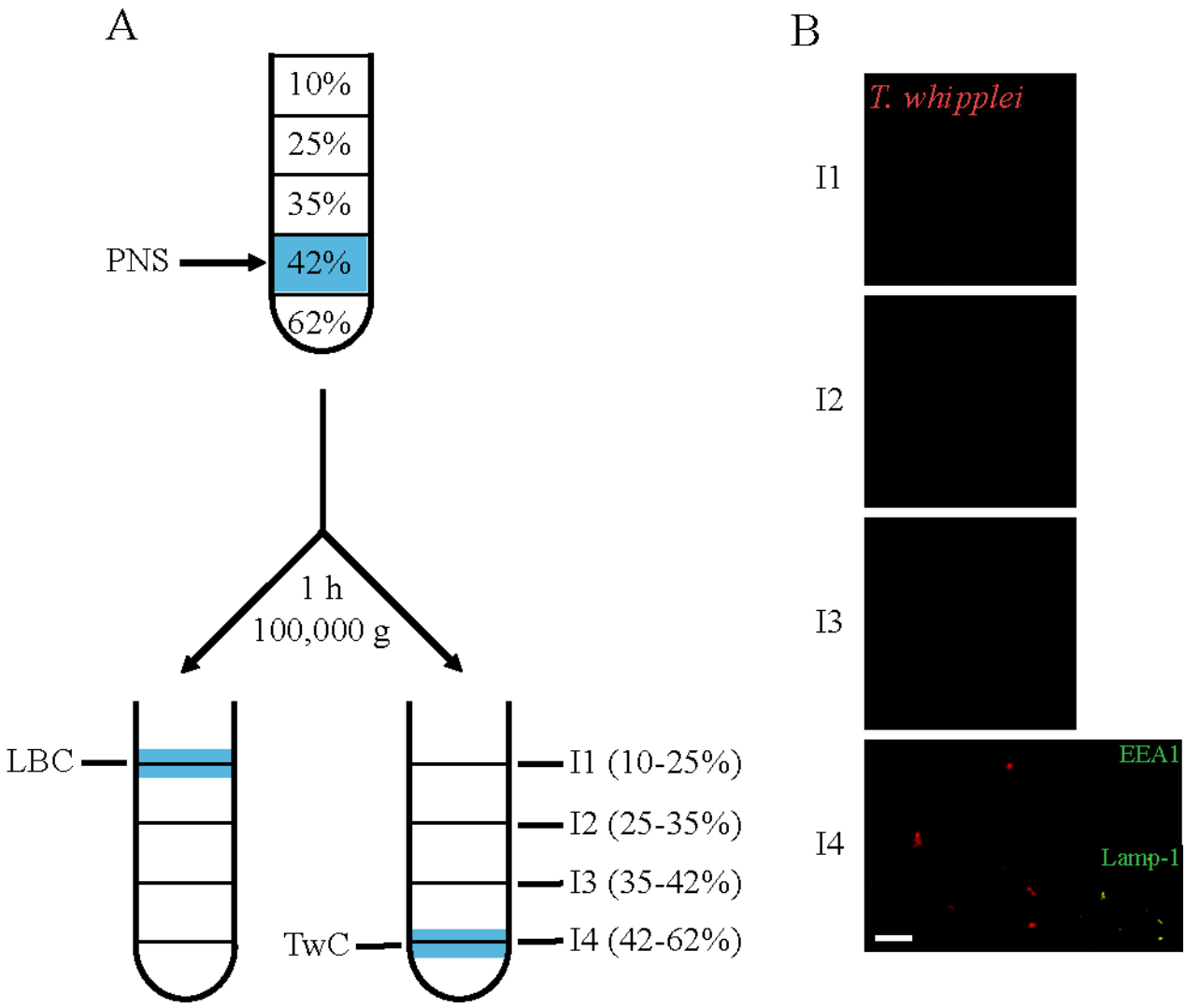

Figure 2. Purification of the *T. whipplei*-containing compartment

BMDM were incubated with latex beads or *T. whipplei* for 1 h and 4 h, respectively, and then incubated for 12 days. The compartments containing latex beads (LBC) or *T. whipplei* (TwC) were deposited on sucrose gradients, and centrifuged for 1 h at 100,000 x *g*. **(A)** Schematic view of the purification procedure. **(B)** Sucrose fractions (I1 to I4 fractions) were analyzed by immunofluorescence for the presence of *T. whipplei*, EEA1 or Lamp-1.

To purified phagosomes containing *T. whipplei*, we have applied the same protocol (**Figure 2A**). The sucrose fraction containing bacteria was identified by detection of *T.whipplei* using immunofluorescence. *T. whipplei* organisms were recovered at the 42 to 62% sucrose interface (**Figure 2B**). The presence of contaminant endosomes in the 42 to 62% sucrose interface was investigated using anti-EEA1 and anti-Lamp-1 antibodies (to detect early endosomes and late endosomes, respectively). Neither early endosomes nor late endosomes were detected in the fraction containing bacteria (**Figure 2B**). Lamp-1 was detected at the surface of the compartment containing *T. whipplei* (**Figure 2B**). These results demonstrated that the integrity of *T. whipplei*- containing phagosomes was conserved and that the sucrose fraction containing bacteria was devoided of late or early endosome endosome.

We compared the protein profiles of isolated phagosomes using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS). Numerous peaks were detected in TwC whereas the profile of *T. whipplei* organisms was poor in proteins and completely different. In addition, the protein fingerprints of TwC and LBC were clearly different (supplementary data S1A). The hierarchical clustering of m/z peaks showed that purified TwC and LBC shared 12 proteins. Eighteen specific proteins were detected on purified LBC whereas 9 specific proteins were associated with TwC **(supplementary data**

S1B). Purified TwC and LBC were analyzed by western blot for the presence of late phagosome (Lamp-1) and lysosome markers (cathepsin D). Both LBC and TwC were positive for Lamp-1. A densitometric analysis reveal that the recrutment of Lamp-1 by the TwC is 1.46 time less than observed for LBC **(Figure 3A)**. In contrast to LBC in which cathepsin D was detected, TwC did not recruit cathepsin D **(Figure 3A)**. A densitometric analysis confirmed the different band intensities of LBC and TwC for cathepsin D (**Figure 3A)**. Taken together, these data clearly suggested that the compartment in which *T. whipplei* replicated was not a classical phagolysosome in which latex beads resided.

The lack of cathepsin D recruitment by *T. whipplei*-containing phagosomes was further investigated by immunofluorescence and confocal microscopy analysis. Lamp-1 was present on cytoplasmic vesicles typical of late endosomes and lysosomes, and also colocalized with *T. whipplei* since bacteria were surrounded by Lamp-1 (**Figure 3B**). At 30 minutes post-infection, 22 ± 11 % of phagosomes containing *T. whipplei* colocalized with Lamp-1; this percentage increased progressively and reached 61 ± 8 % at 4 hours post-infection. After 9 ot 12 days of infection, all detected bacteria were colocalized with Lamp-1 (**Figure 3B**). Cathepsin D was present within cytoplasmic vesicles typical of lysosomes (**Figure 3C**). A time course study of the colocalization of *T. whipplei* with cathepsin D showed two successive phases **(Figure 3C, right)**.

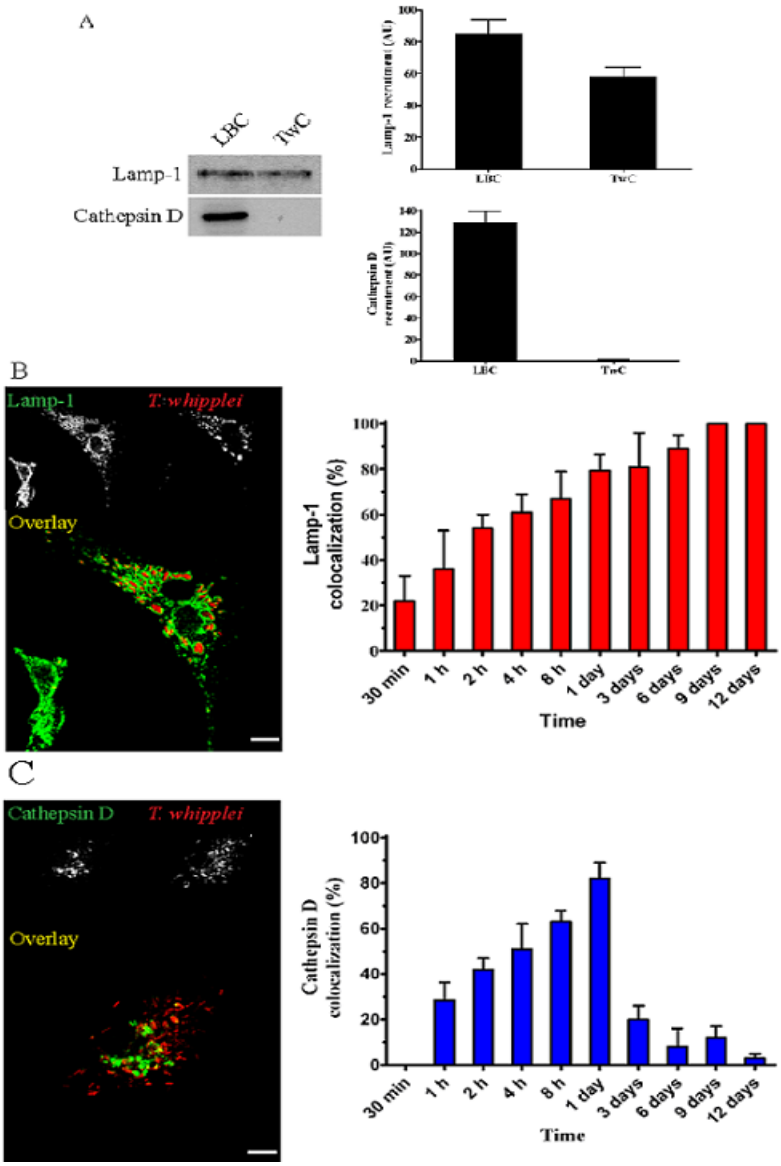

Figure 3. *T. whipplei* phagosomes did not fuse with lysosomes

(A) Purified TwC and LBC were analyzed for the presence of Lamp-1 and cathepsin D by western blot. The western blot picture was representative of 3 experiments. The bands were quantified, and the results are expressed in arbitrary units (AU). The results were the mean of 3 experiments **(B)** In BMDM, The colocalization of *T whipplie* with Lamp-1 was analysed by immunofluorescence and confocal microscopy. Pictures are representative of day 12. Colocalization with Lamp-1 was quantified and results were expressed in percentage of colocalization (n=4). Results are the mean ± SEM of 3 experiments. **(C)** The colocalization of *T whipplie* with cathepsin D in BMDM was analysed by immunofluorescence and confocal microscopie. Pictures are representative of day 12. Colocalization of *T. whipplei* with cathepsin D was quantified and results were expressed in percentage of colocalization. Results are the mean ± SEM of 3 experiments.

After 1 h of incubation with *T. whipplei*, 28.4 ± 8 % of phagosomes acquired cathepsin D; this percentage increased to reach 63 ± 5 % after 8 h and was maximum after one day (82 ± 7 %). This phase of cathepsin D acquisition by *T. whipplei* phagosomes is correlated to the elimination of the majority of organisms by BMDM (see Fig. 1A). It is likely that the minority of bacteria that did not colocalize with cathepsin D after one day were able to replicate in a later stage. Indeed, when *T. whipplei* organisms replicated within BMDM (between days 3 and 12, see Fig. 1A), the percentage of *T. whipplei* phagosomes that contained cathepsin D decrease slowly to reach 8 ± 8 and 3 ± 2 after 6 days and 12 days (**Figure 3C**). Taken together, these data suggested that replicating bacteria were located within a late phagosome unable to fuse with lysosomes since it did not acquire lysosomal enzymes.

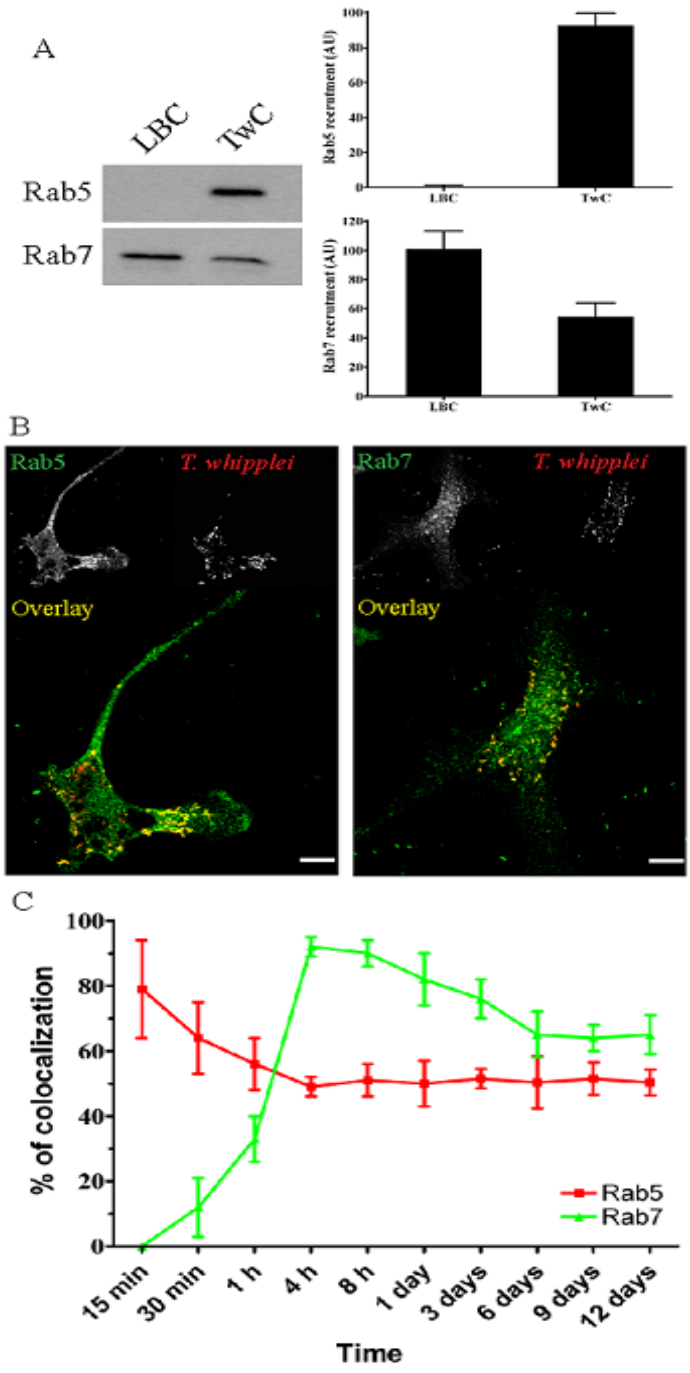

Figure 4. *T. whipplei* phagosomes continuously harbored both Rab5 and Rab7

(A) Purified TwC and LBC were analyzed for the presence of Rab7 and Rab5 by western blot.. The micrograph was representative of 3 experiments (left). The bands were quantified, and the results are expressed in arbitrary units (AU). The results were the mean of 3 experiments (right). **(B)** In BMDM, the colocalization of *T whipplie* with Rab5 and Rab7 was analysed by immunofluorescence and confocal microscopy. Pictures are representative of day 12. **(C)** Colocalization of *T. whipplei* with Rab5 and Rab7 was quantified and results were expressed in percentage of colocalization. Results are the mean ± SEM of 3 experiments.

T. whipplei-containing phagosomes expressed both Rab5 and Rab7

As the maturation of endosomes and phagosomes needs Rab5 and Rab7 exchange, we hypothesized that this exchange is altered in *T. whipplei* phagosomes. Purified TwC were analyzed by western blot for the presence of Rab5 and Rab7 **(Figure 4A)**. At day 12 post-infection, Rab7 was detected on both TwC and LBC. As expected, Rab5 was undetectable in LBC. In contrast, high levels of Rab5 were present in TwC. The time course of the persistence of Rab5 on *T. whipplei* phagosomes was further investigated by immunofluorescence and confocal microscopy analysis. First, *T. whipplei* phagosomes acquired Rab7 in a progressive manner between 30 min and 4 h, and more than 60% of phagosomes expressed Rab7 between 1 and 12 days (Fig. 4, B and C). Second, *T. whipplei* phagosomes early acquired Rab5 since about 80% of them expressed Rab5 within 15 min. However, they were unable to eliminate Rab5 from their surface since about 50% of these phagosomes expressed Rab5 when incubated for prolonged periods varying from 4 h to 12 days (Fig. 4, B and C). These results showed that *T. whipplei* was localized in a chimeric phagosome expressing both Rab5 and Rab7, and suggested that *T. whipplei* blocks the conversion of its phagosome in a transition stage between early and late phagosomes.

PI(3)P was normally recruited on *T. whipplei* phagosome

As PI(3)P was reported to be an essential factor for the endosome and phagosome conversion, indeed it is involved in the coordination of the recrutment of Rab5 at the phagosome membrane. Since Rab5 and Rab7 are both present to the phagosome, we have hypothesized that the altered production of PI(3)P affected this exchange in *T. whipplei* phagosomes. We investigated the presence of PI(3)P at the surface of *T. whipplei* phagosomes using the transfection of BMDM with a GFP-2xFyve construct since Fyve domains recognize PI(3)P with great selectivity and considerable affinity (Gaullier et al., 1998). After 15 min of infection with *T. whipplei*, 68 ± 11% of *T. whipplei* phagosomes were surrounded by GFP-2xFyve **(Figure 5A top, and Figure 5B)**. This percentage decreased ‘39 ± 6 %) after 8 hours of infection and was undetectable thereafter **(Figure 4A bottom, and Figure 5B)**. As the recruitment of PI(3)P on *T. whipplei* phagosome is similar to that found for LBC (Henry et al., 2004), it is likely that the defective Rab5/Rab7 exchange was not due to a defective recruitment of PI(3)P at the surface of *T. whipplei* phagosome.

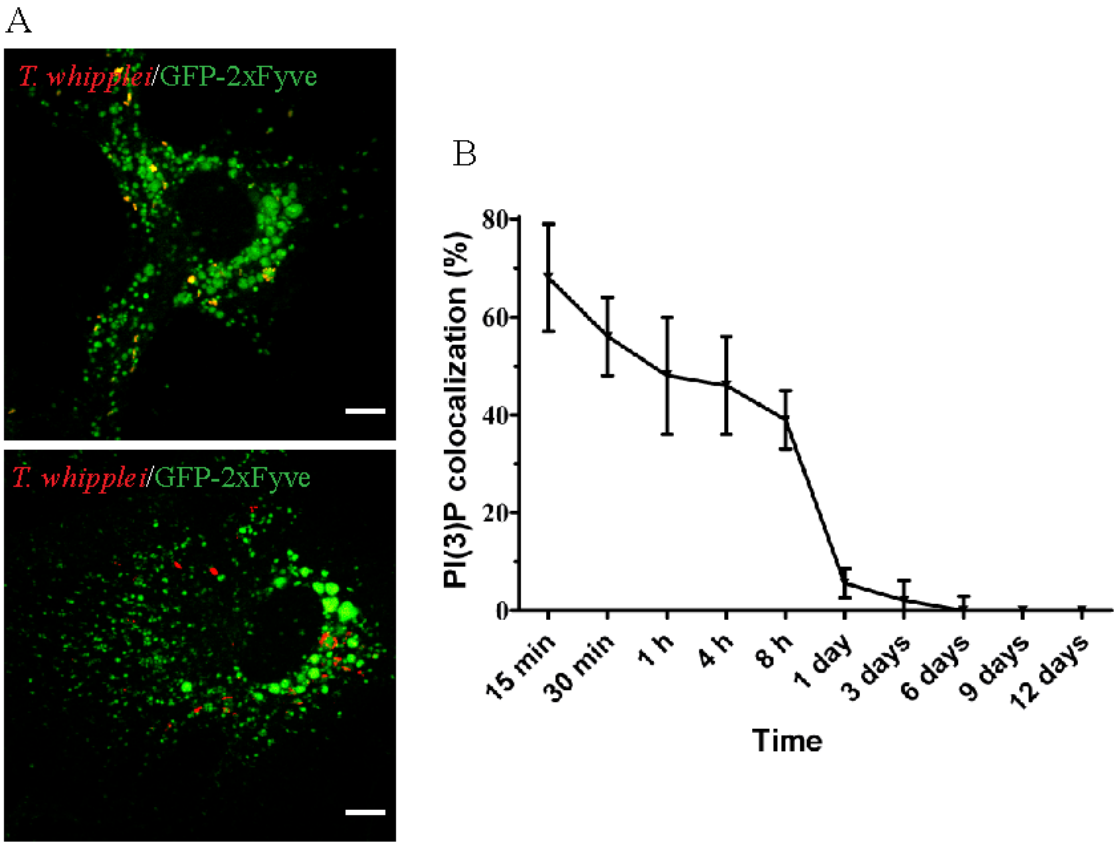

Figure 5. *T. whipplei* phagosomes expressed PI(3)P

BMDM were transfected with 2xFyve-GFP using lentiviral vector and were infected with *T. whipplei* at a bacterium-to-cell ratio of 50:1 Cf fig 3 et 4 for different periods. The colocalization of *T. whipplei* with 2xFyve was analyzed by immunofluorescence and confocal microscopy. Top: 15 minutes; bottom: 1 day. day 12 post-infection. Right: time course of the colocalization of *T. whipplei* with PI(3)P. More than 400 phagosomes were examined per experimental condition, and the results are the mean ± SEM of 3 experiments.

Discussion

Clinical and experimental (Desnues et al., 2005; Schneider et al., 2008) data have showed that *T whipplei* replicates within human macrophages, but the nature of the compartment in which *T. whipplei* resides is not known. We showed here that, in an early phase, a large amount of bacteria was eliminated but that, in a late phase *T. whipplei* intensively replicated within murine BMDM, as previously found in human macrophages (Desnues et al., 2005). The defective clearance of *T. whipplei* by BMDM was not due to defective microbicidal properties since they were able to eliminate other intracellular bacteria such as *M. smegmatis*. The nature of the compartment containing *T. whipplei* has been studied using different experimental approaches. First, phagosomes containing *T. whipplei* or latex beads, as control, were purified by sucrose gradient, and their protein content was compared by MALDI TOF MS. Clearly, the compartment in which *T. whipplei* replicated was distinct from classical phagolysosomes (in which latex beads resides). Second, western blot analysis of this compartment also showed that it expressed a marker of late endosomes/lysosomes such as Lamp-1, but was devoided of a lysosomal enzyme such as cathepsin D. Third, immunofluorescence and confocal microscopy analysis highlighted the relationship between the intracellular fate of *T. whipplei* and the conversion of its phagosome.

Indeed, when the majority of *T. whipplei* organisms was eliminated by BMDM, *T. whipplei* are localised within a phagolysosome that expressed both Lamp-1 and cathepsin D, and only a minority of organisms survived in a phagosome that did not express cathepsin D..During the phase of bacterial replication, almost all organisms resided in a late phagosome that expressed Lamp-1 but was unable to fuse with lysosomes since it did not express cathepsin D.

We found that the inhibition of phagoso-lysosome formation is associated with a defect of Rab5/Rab7 conversion. The transition from early to late endosomes is highly related to Rab5/Rab7 conversion (Roberts et al., 2006). In addition, Rab5 and Rab7 co-exist for few minutes at the membrane of the compartment (Henry et al., 2004; Rink et al., 2005)Thus, western blot analysis and confocal microscopy revealed that *T. whipplei* phagosome was a chimeric phagosome since it harbored for long periods of time both Rab5 and Rab7. The chimeric nature of *T. whipplei* phagosomes cannot be explained by an increased level of expression of Rab5 and Rab7 proteins, since The expression of the genes encoding Rab5 and Rab7 was not up-regulated in response to *T. whipplei* **(Supplementary data S2)**.. Second, since PI(3)P are involved in endosome conversion and that bacteria such *Mycobacterium tuberculosis* (Philips, 2008) block the phagosome conversion *by* inhibition of PI(3)P production, we have investigated the presence of PI(3)P on the phagosome containing *T. whipple*i. Using lentiviral transfection of 2xFyve-GFP, we found

that PI(3)P was recruited by *T. whipplei* phagosomes, as described for phagosomes containing latex beads (Henry et al., 2004). In addition, *T. whipplei* have no effect on the transcriptional level of Vps34, kinase involved in PI(3)P production **(Supplementary data S2)**.

To our knowledge, it is the first time that the phagosome containing a bacterial pathogen was described as chimeric. Even several microorganisms are known to block the phagosome conversion by acting on Rab proteins or Rab effectors (Schneider et al., 2008), none was described to induce a phagosome which stably express both Rab5 and Rab7. *M. tuberculosis* is described to block the conversion of the phagosome at the Rab5 stage enable to recruit Rab7 (Philips, 2008). At the surface of the mycobacterial phagosome, Rab5 is continously detected, however, markers of the later stage were not detected; *L. monocytogenes* also blocks the phagosome conversion at the Rab5 stage before escaping in cytosol (Prada-Delgado et al., 2005). The phagosome containing *Helicobacter pylori* is positive for Lamp-1, Rab7, but not for the lysosomal enzymes (Brumell and Scidmore, 2007). Several hypotheses may explain the chimeric nature of *T. whipplei* phagosome. During the normal endosome conversion. The inhibition of the GTPase activity of Rab5 might lead to its accumulation in active form (Rab5:GTP) at the surface of phagosome, and to the absence of transition Rab5:GTP to Rab5:GDP, might inhibit the transition Rab5 to Rab7, in despite

of recruitment of the Rab7 at the phagosome membrane. The HOPS complex is a regulator of Rab5/Rab7 transition (Henry et al., 2004; Rink et al., 2005). We can suppose that the HOPS complex is unoperative. Preliminary results obtained by bioinformatic analysis suggest that the genome of *T. whipplei* encodes a G3PDH protein presenting some homology with *L. monocytogenes* G3PDH. *L. monocytogenes* G3PDH was described to interfer with Rab5 activity(Alvarez-Dominguez et al., 2008). We can hypothesize that *T. whipplei* G3PDH acts in a similar way, which can explain the blockage of the phagosome containing *T. whipplei* in Rab5-Rab7 stage.

In conclusion, our results described a new strategy used by a bacterial pathogen to escape to the microbicidal activity of macrophages. The phagosome containing *T. whipplei* expresses continuously Rab5 and Rab7, whereas their co-expression is solely transient in normal endosome conversion. Consequently, the *T. whipplei* phagosome is unable to fuse with lysosomes, thus leading to bacterial replication within macrophages.

Acknowledgments

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Disclosures

The authors have no competing interests and no financial conflict of interest.

Materials and Methods

Bacteria. The *Twist*-Marseille strain of *T. whipplei* (CNCM I-2202) was cultured within HEL cells (CCL-37; American Type Culture Collection) and purified as described previously (Raoult et al., 2000). Bacteria were counted by Gimenez staining and indirect immunofluorescence, and their viability was assessed using the Live and Dead BacLight bacterial viability kit (Invitrogen) (Ghigo et al., 2002). *M. smegmatis* was kindly provided by Pr. M. Drancourt (Marseille).

Cell culture. BMDM were generated from 6- to 8-week-old C57BL/6 mice killed by cervical dislocation, as previously described (Cook et al., 2007). Briefly, the bone marrow was flushed out from mice femurs and tibias in RPMI supplemented with 10% of fetal calf serum (FCS), 2 mM L-glutamine, 100 UI/ml penicillin and 100 µg/ml streptomycin (RPMI medium). Macrophage differentiation was obtained by incubating bone marrow cells in RPMI medium supplemented with 15% of L929 cell supernatant, rich in macrophage colony-stimulating factor M-CSF.. For immunofluorescence studies, BMDM were seeded (1 x 10^5 cells per well containing a glass coverslip) in 24-well tissue culture plates in supplemented RPMI medium. Then, BMDM were infected with *T. whipplei* (bacterium-to-cell ratio of 50:1) for 4 h or *M. smegmatis* (bacterium-to-cell ratio of 10:1) for 1 h (Wagner et al., 2005), washed to remove free bacteria, and incubated for designated periods (Desnues et al., 2005). In some experiments, BMDM were infected by *T. whipplei* for shorter periods.

qPCR. Macrophages were lyzed, and DNA was extracted using the QIAamp DNA MiniKit (Qiagen). PCR was performed using the LightCycler-FastStart DNA Master SYBR Green system (Roche), and conducted with primers specific for *T. whipplei* 16S-23S ribosomal intergenic spacer region (tws3f and tws4r), as described previously (Fenollar et al., 2002) **(Table 1)**. In each PCR

run, a standard curve was generated using serial dilutions ranging from 10 to 10^8 copies of the intergenic spacer region, and established by the LightCycler 5.32 software (LC-Run version 5.32; Roche).

Genes	Left primers	Right primers
T. whipplei	ccggtgacttaacctttttggaga	Tcccgaggcttatcgcagattg
β-actin	tggaatcctgtggcatccatgaaac	Taaaacgcagctcagtaacagtccg
Rab5	cgggccaaatactggaaata	Aggacttgcttgcctttgaa
Rab7	gagcggactttctgaccaag	Ccggtcattcttgtccagtt
Vps34	gtgacctggacatcaacgtg	Gcttgttctgccaggagttc

Table 1. List of primers

The primer sequences of the targeted genes are designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi.).

RT-PCR. Infected BMDM were lyzed, and RNA extracted using the QIAamp RNA MiniKit (Qiagen). cDNA was synthesized from 1 μg of total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen). Specific primers for each gene were designed using Primer3Plus (http://frodo.wi.mit.edu/primer3/), and their sequences are listed in **Table 1**. PCR was performed using Hotstar *Taq* polymerase (Qiagen) following manufacturer's recommendations, and PCR products were electrophoresed through 1% agarose gel containing ethidium bromide. Data were acquired with Gel Doc 2000 (BioRad) and gene expression was normalized to the β-actin gene.

Phagosome purification. BMDM (10^8 cells) were incubated for 1 h with latex beads (1 μm, fluorescent red, Sigma-Aldrich) at the 1/5000 dilution or for 4 h with *T. whipplei* (bacterium-to-cell ratio: 50:1). Then, BMDM were washed to remove free particles or bacteria, and incubated for 12 days in RPMI 1640 containing 10% FCS. The compartments containing latex beads (LBC) or *T. whipplei* (TwC) were purified according the procedure described by Desjardins et al. (Desjardins et al., 1994). Briefly, BMDM were washed in cold PBS containing protease inhibitors (Complete, Roche) and scraped with a rubber policeman at 4°C. The cells were pelleted and homogenized and washed in homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4) containing

protease inhibitor (Complete, Roche) at 4°C. Then resuspended in 1 ml of homogenization buffer containing protease inhibitor, and homogenized on ice in a cells homogenizer. The homogenate was carried out until about 90% of cells were broken. Unbroken cells were pelletedcentrifuged at 1200 rpm for 5 min and the supernatant (PNS) was recovered and deposited on a sucrose gradient as decribed in Figure 1. The LBC and TwC were then isolated on a sucrose step gradient (all sucrose solutions are wt/wt in 3 mM imidazole, pH 7.4) as follows. The supernatant (PNS) containing LBC and TwC was brought to 40% sucrose by adding the same volume of a 62% sucrose solution. This 40% sucrose supernatant was loaded on top of a 1 ml cushion of 62 % sucrose. We then added 2 ml of 35 % sucrose, 2 ml of 25 % sucrose, and 2 ml of 10% sucrose solutions. Centrifugation was performed in swinging bucket rotor (SW40, Beckman Instruments) at 100,000 *g* for 1 h. The LBC and TwC were collected, suspended in 12 ml of cold PBS containing protease inhibitors and pelleted by a 15 min centrifugation at 40,000 g in an SW 40 rotor at 4°C. LBC and TwC were storage at -80°C after nitrogen snap frozen, in PBS containing protease inhibitor.

Western blotting. The protein content of purified phagosomes was examined by the Bradford method using γ–globulin as standard. Phagosomes (25 μg) were loaded onto 15% SDS polyacrylamide gels, electrophoresed and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked in PBS with 0.05% Tween 20 (PBST) supplemented with 3% powdered milk and then incubated with primary Abs against Rab5 (Ab13253, Abcam), Rab7 (R4779, Sigma), Lamp-1 (1D4B, DSHB) or cathepsin D (a gift from Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, MO). The blots were washed with PBST and incubated with a secondary Ab, either HRP-conjugated anti-rabbit or anti-mouse immunoglobulins (Pierce) in PBST containing 3% powdered milk. The blots were then revealed using Immobilon Western Chemiluminescent HRP substrate (Millipore).

MALDI-TOF MS. The protein profiles of TwC, LBC and *T. whipplei* was analyzed by MALDI-TOF MS using a previously described method (Bittar et al., 2009; Fournier et al., 2009; Seng et al., 2009). One μl of TwC, LBC or *T. whipplei* was deposited on a target plate (Bruker Daltonics, Bremen, Germany). Two μl of matrix solution (saturated cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid) were then added to the plate and allowed to co-crystallise with the sample. Analysis was

performed in a MALDI-TOF-MS spectrometer (337 nm) (Autoflex; Bruker Daltonics) with the FLEX control software (Bruker Daltonics). Positives ions were extracted with an accelerating voltage of 20 kV in linear mode. The spectra were analyzed in a mass-to-charge ratio (m/z) range of 2000–20,000. The profiles of multiple replicates were compared and analyzed using BioTyper 2.0 software and, finally, MS data were collected and analyzed using MeV v4.4.1 software (http://www.tm4.org/), generating a hierarchical clustering.

Colocalization experiments. BMDM (1 x 10^5 cells/assay) were infected with *T. whipplei* (50 bacteria/cell), extensively washed to discard unbound bacteria and then incubated for different periods of time before fixation in 3% paraformaldehyde. Fixed macrophages were permeabilized with 0.1% Triton X-100 or saponin 0.1% and immunofluorescence labeling was performed according to standard procedures (Chu and Ng, 2004). Polyclonal mouse anti *T. whipplei* antibodies were generated in the laboratory. Rat antibodies specific for Lamp-1 (1D4B) were purchased from DSHB (Iowa, USA), and rabbit antibodies specific for cathepsin D (a gift from Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, MO), Rab5 (Ab13253, Abcam), Rab7 (R4779, Sigma). Secondary Alexa antibodies, goat anti-mouse alexa 488, goat anti-rat IgG and anti-rabbit IgG coupled with alexa 555, were

purchased from Invitrogen. BMDM were examined by laser scanning microscopy using a confocal microscope (Leica TCS SP5, Heidelberg, Germany) with a 63X/1.32-0.6 oil objective and an electronic Zoom 1.5X. Optical sections of fluorescent images were collected at 0.15-µm intervals using Leica Confocal Software and processed using Adobe Photoshop© V7.0.1. At least 150 cells were examined for each experimental condition and results are expressed as the percentage of bacteria that colocalized with fluorescent markers. Cells were selected as following: 50 microscope fields, with at least 3 cells per field containing at least 3 to 5 phagosomes were randomly selected. In total, more than 400 phagosomes were examined per experimental condition.

Vector construction and lentiviral vector production. pCCL.WPS.PKG-eGFPmax constructs were generated by insertion of GFPmax sequence PCR-amplified from pGFPmax (Amaxa) within pCCL.WPS.PKG (gift from P. Soubeyran, INSERM U-624). Then, 2xFyve sequence PCR-amplified from 2xFyve-PGFP (a gift from M. Zerial, MPI-CBG, Germany) was inserted in pCCL.WPS.PKG-eGFPmax, generating the pCCL.WPS.PKG-eGFP-2xFyve construct. To produce lentiviral vector, 293T cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing D-glucose, 10% FCS, 2 mM L-glutamine, 100 UI/ml penicillin and 100 µg/ml streptomycin.

Cells were then co-transfected with the pCCL.WPS.PKG-eGFP-2xFyve and VSV, DH plasmid (gift from P. Soubeyran, INSERM U-624) using Lipofectamine (Invitrogen). The supernatant was harvested two days post-transfection and was added to BMDM

Statistical analysis. Results are expressed as means ± SEM and compared with the non-parametric Mann-Whitney *U* test. Differences were considered significant when $p<0.05$.

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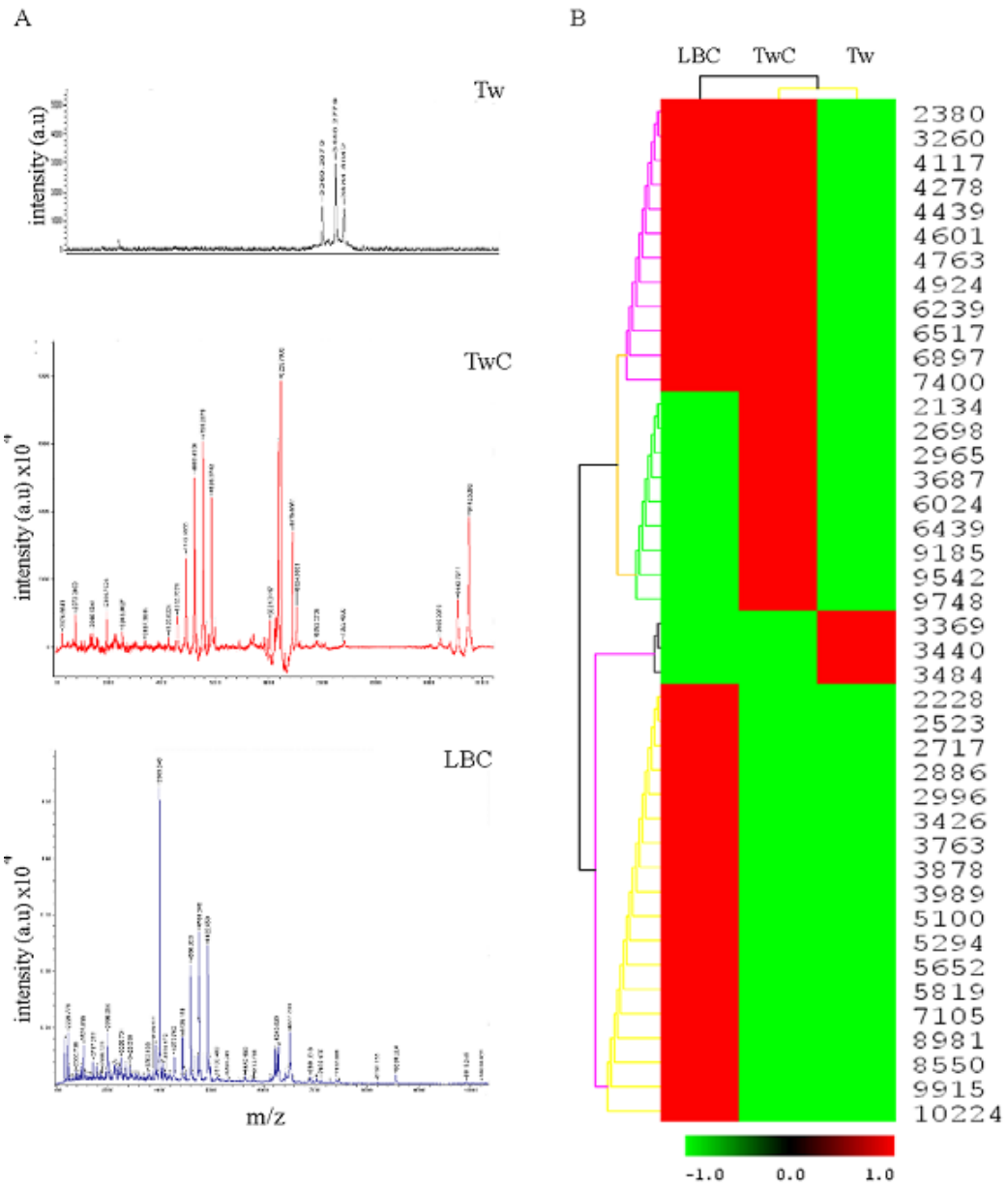
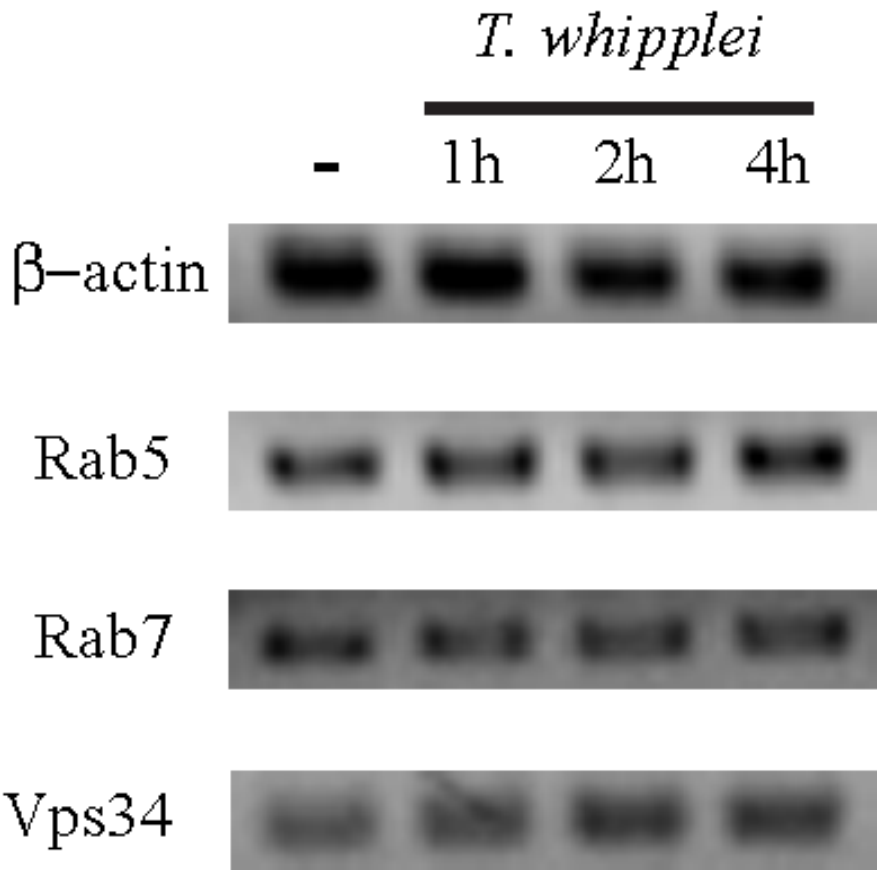


Supplementary data S1. Phagosome profiles in MALDI-TOF mass spectrometry

(A) MALDI-TOF MS was performed using *T. whipplei* (Tw) organisms, and purified compartment containing *T. whipplei* organisms (TwC) or latex beads (LBC). **(B)** Data were collected and analyzed using **the** MeV v4.4.1 software (http://www.tm4.org/) that generates a hierarchical clustering.



Supplementary data S2. *T. whippei* did not modulate the transcription of molecules involved in phagosome conversion

BMDM were infected with *T. whipplei* (bacterium-to-cell ratio of 50:1) for different periods. Total RNA was extracted and transcribed in cDNA. After amplification, PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The figure is representative of three experiments.

ARTICLE IV

IL-16 Promotes *T. whipplei* Replication by Inhibiting Phagosome Conversion and Modulating Macrophage Activation

Eric Ghigo, Abdoulaye Oury Barry, Lionel Pretat, Khatoun Al Moussawi, Benoît Desnues, Christian Capo, Hardy Kornfeld, Jean-Louis Mege

PLoS ONE, 2010

Au cours de ce travail, nous avons montré que l'IL-16 affecte la voie d'endocytose puisque, dans les macrophages non traités par l'IL-16, *T. whipplei* est localisée dans un phagosome tardif incapable de fusionner avec les lysosomes. La neutralisation de l'IL-16 endogène par des anticorps ou l'utilisation de souris invalidées pour le gène codant l'IL-16 entraîne la maturation du phagosome de *T. whipplei* en phagolysosomes et l'élimination de la bactérie. Cet effet est spécifique puisque le traitement par une autre cytokine désactivatrice comme l'IL-10 n'affecte pas la conversion du phagosome. L'effet de l'IL-16 n'est pas dû à un défaut de modulation transcriptionnelle de Rab5 et de Rab7, ou de modulation traductionnelle de Lamp-1 ou de la cathepsine D. Comme l'IFN-γ est essentiel à la compétence des macrophages et que sa production est inhibée dans la maladie de Whipple, nous avons voulu savoir si l'effet de l'IL-16 est relié à la modulation de l'IFN-γ. L'IFN-γ induit la mort de *T. whipplei* ainsi que la conversion du phagosome en phagolysosome. C'est la première fois que l'IL-16 est décrite comme affectant le trafic intracellulaire d'une bactérie intracellulaire.

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IL-16 Promotes *T. whipplei* Replication by Inhibiting Phagosome Conversion and Modulating Macrophage Activation

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Abstract

The replication of *Tropheryma whipplei* (the agent of Whipple's disease) within human macrophages is associated with the expression of IL-16, a cytokine known for its chemotactic and inflammatory properties. In this study, we asked whether IL-16 acts on *T. whipplei* replication by interfering with the endocytic pathway. We observed that in macrophages, *T. whipplei* was located within late phagosomes that were unable to fuse with lysosomes; in monocytes, *T. whipplei* was eliminated in phagolysosomes. Moreover, adding IL-16 to monocytes induced bacterial replication and inhibited phagolysosome formation. On the other hand, blocking IL-16 activity, either with anti-IL-16 antibodies in human macrophages or by using murine IL-16$^{-/-}$ bone marrow-derived macrophages, inhibited *T. whipplei* replication and rescued phagolysosome biogenesis. Furthermore, we propose that IL-16-mediated interference with the endocytic pathway is likely related to macrophage activation. First, IFNγ induced *T. whipplei* elimination and phagolysosome formation and inhibited IL-16 production by macrophages. Second, the full transcriptional response of murine macrophages to *T. whipplei* showed that *T. whipplei* specifically modulated the expression of 231 probes in IL-16$^{-/-}$ macrophages. Gene Ontology analysis revealed that 10 of 13 over-represented terms were linked to immune responses, including proinflammatory transcriptional factors of the NF-κB family. Our results demonstrated a previously unreported function for IL-16 in promoting bacterial replication through inhibited phagolysosome biogenesis and modulated macrophage activation program.

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Introduction

Whipple's disease (WD) is a chronic multisystemic infection caused by *Tropheryma whipplei* [1]. The classical manifestations of WD are weight loss, diarrhea, polyarthralgia, fever, and lymphadenopathy. In addition, cardiac and central nervous system symptoms may also be associated with WD [2]. The course of WD is fatal unless antibiotic treatment is initiated [3]. Specific immune deficiencies and genetic traits have been suggested to be involved in WD development. Indeed, impaired interferon (IFN) γ production associated with interleukin (IL)-12 deficiency and Th2 repolarization of the immune response has been considered to be critical for WD physiopathology [2,4]. Consistent with these observations, we previously found that macrophages from intestinal lesions are polarized into alternatively activated macrophages, also known as M2 macrophages [5]. Furthermore, we have shown that *T. whipplei* replication in human macrophages is related to IL-16 production [6]. IL-16, constitutively produced as cytosolic pro-IL-16, is secreted after caspase-3-mediated processing [7,8,9] by numerous cell types [10,11,12]. IL-16 is a chemoattractant for CD4-expressing immune cells, such as T cells [10], monocytes [13], dendritic cells [14] and eosinophils [15]. Aside from its role as a chemoattractant, IL-16 may also be involved in the innate immune response because it favors the production of inflammatory cytokines by monocytes [16] and may act on antigen-presenting cells as well [17]. IL-16 is also known to modulate the adaptive immune response by favoring Th1 responses. Indeed, IL-16 primes T cells to IL-15 production and CD25 expression, and thus renders them more susceptible to the presence of IL-2 [18].

One of the microbicidal mechanisms of macrophages is based on the formation of phagolysosomes. Specifically, macrophages internalize microorganisms into phagosomes, which undergo extensive remodeling involving an active exchange of material with plasma membrane, endosomes, lysosomes, Golgi- and endoplasmic-derived vesicles. Phagosomes fuse with early endosomes and then with late endosomes, as demonstrated by the acquisition of specific markers such as early endosome antigen-1, the small GTPases Rab5 and Rab7, and lysosome-associated membrane protein (Lamp)-1, respectively. Finally, late phagosomes fuse with lysosomes and acquire hydrolase enzymes such as cathepsin D [19]. Microorganisms are destroyed in these

phagolysosomes, which are associated with both Lamp-1 and cathepsin D [20]. However, several types of pathogenic microorganisms have been shown to manipulate host cell organelles and membrane trafficking processes to survive and replicate within host cells [21,22]. For example, *Salmonella* resides in an atypical phagosome that is neither an early nor a late phagosome [23]. *Mycobacterium*-containing phagosomes fuse with early endosomes but are unable to fuse with late endosomes [24,25]. *Coxiella burnetii*, the agent of Q fever, survives in macrophages within an acidic late phagosome that does not fuse with lysosomes [26].

It is well-established that the microbicidal activity of monocytes/macrophages is regulated by cytokines that polarize macrophages [27]. IFNγ, a cytokine known to confer microbicidal competence to macrophages, controls *Listeria monocytogenes* infection and its clearance [28]. However, only a few reports have attempted to address the relationship between cytokines and phagosome conversion. It has been shown that treatment of macrophages with IFNγ allows the elimination of mycobacteria through the conversion of bacterial phagosomes to phagolysosomes [29,30]. IFNγ triggers the listericidal competence of macrophages by up-regulating the small GTPase activity required for phagosome conversion [31]. IFNγ stimulates *C. burnetii* elimination and induces the maturation of *C. burnetii* phagosomes in phagolysosomes [26]. Conversely, IL-10, an immunosuppressive cytokine, promotes the intracellular localization of mycobacteria within phagosomes that are unable to fuse with lysosomes [29] and stimulates *C. burnetii* replication by increasing the ability of *C. burnetii* to traffic into an acidified late phagosome [32,33].

We have previously shown that *T. whipplei* is localized within an acidic compartment associated with Lamp-1 unable to fuse with lysosomes [34] and that *T. whipplei* replication in human macrophages is related to IL-16 production [6]. However, the mechanisms triggered by IL-16 that affect the intracellular fate of *T. whipplei* remain largely understudied. In this study, we found that IL-16 was required for *T. whipplei* replication within late phagosomes in both human and murine macrophages. The lack of IL-16 led to *T. whipplei* elimination within phagolysosomes. The effect of IL-16 on *T. whipplei* replication in late phagosomes was repressed by inhibiting IL-16 production by IFNγ. Microarray analysis of IL-16$^{-/-}$ macrophages indicated roles for IL-16 in the innate immune response and the NF-κB pathway. Taken together, our study demonstrates novel functions of IL-16 in intracellular trafficking and macrophage regulation.

Results

Intracellular localization of *T. whipplei*

In the first series of experiments, human monocytes and macrophages were infected with *T. whipplei* (50 bacteria/cell) for four hours (designated as day 0) and washed to discard unphagocytosed bacteria. The intracellular fate of *T. whipplei* was assessed for 12 days. As published before [6], monocytes eliminated *T. whipplei*, whereas *T. whipplei* replicated within macrophages after a transient phase of *T. whipplei* elimination (**Figure 1**).

We next studied the nature of the *T. whipplei*-containing compartments in both monocytes and macrophages. In monocytes, *T. whipplei* was eliminated within phagolysosomes that acquired both Lamp-1 and cathepsin D. Specifically, 62±3% of *T. whipplei* phagosomes colocalized with Lamp-1, and 47±8% colocalized with cathepsin D at day 0. These percentages increased to 80% at day 1, and intact bacteria were no longer detected thereafter (**Figure 2A**). In macrophages, bacteria were located in late phagosomes. Specifically, 28±8% of *T. whipplei*

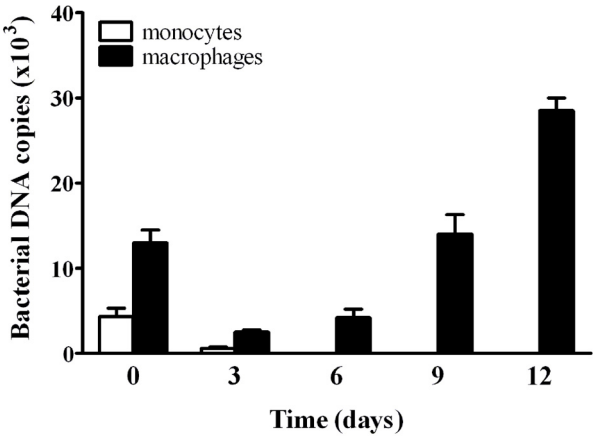

Figure 1. *T. whipplei* replicates in macrophages but not in monocytes. Monocytes and macrophages were incubated with *T. whipplei* for four hours (50 bacteria/cell), washed to discard unphagocytosed bacteria and incubated for different time periods. Bacterial uptake and replication were assessed by determining the bacterial DNA copy number by qPCR. The results are expressed as the mean ± SEM of four experiments performed in triplicate.
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phagosomes were positive for Lamp-1 at day 0; this percentage increased to 71±9% and 94±6% after 3 and 12 days, respectively. In a first phase, *T. whipplei* colocalized with cathepsin D (**Figure 2B**): 33±5% of bacteria colocalized with cathepsin D at day 0, and 37±8% colocalized with it at day 1, suggesting that bacteria were transiently eliminated within phagolysosomes. This phenomenon might be related to the dramatic decrease in the *T. whipplei* DNA copy number observed between day 0 and 3 (**Figure 1**). In a second phase (day 3–12), the percentage of *T. whipplei* colocalizing with cathepsin D steadily decreased: at day 12, it was only 11±4% (**Figure 2B**). This phenomenon might be related to the increase in the *T. whipplei* DNA copy number observed between days 3 and 12 (**Figure 1**). Taken together, these results suggest that *T. whipplei* replication in macrophages is associated with the presence of bacteria within late phagosomes that are unable to fuse with lysosomes.

Effect of IL-16 on *T. whipplei* localization

Given that IL-16 release is related to *T. whipplei* replication [6], we asked if exogenous IL-16 inhibits the conversion of *T. whipplei* phagosomes to phagolysosomes. To address this question, monocytes were treated for 16 hours with 10 ng/ml of recombinant human (rh) IL-16 prior to *T. whipplei* infection [6]. The pretreatment of monocytes with IL-16 did not significantly alter bacterial uptake (**Figure 3A, inset**). *T. whipplei* replication occurred to an extent similar to that observed in macrophages **(comparison between Figure 3A and Figure 1)** as published before [6]. However, *T. whipplei* colocalized with Lamp-1 but not with cathepsin D in IL-16-treated monocytes (**Figure 3B**). The percentage of phagosomes containing *T. whipplei* colocalizing with cathepsin D fell significantly ($p<0.05$) (two-fold) between days 0 and 1. At day 12, *T. whipplei* was only present in IL-16-treated cells, in which it resided in phagosomes associated with Lamp-1, but not with cathepsin D (**Figure 3C**). We also studied the effect of exogenous IL-16 on the intracellular fate of *T. whipplei* in macrophages. IL-16 did not affect *T. whipplei* uptake but it significantly ($p<0.05$) increased bacterial replication (**Figure 4A**) as published before [6]. It also inhibited the acquisition of

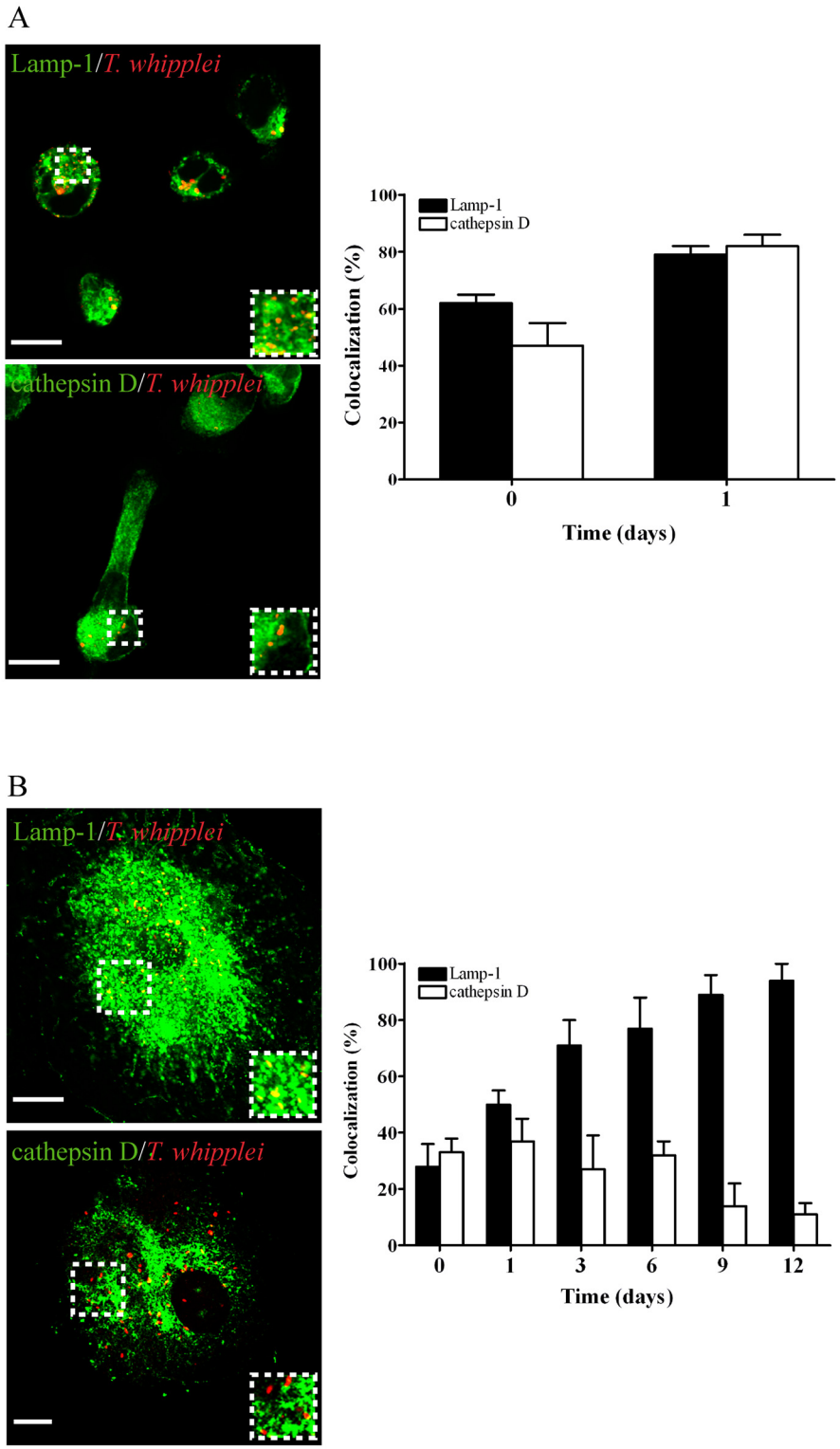

Figure 2. *T. whipplei* localization in monocytes and macrophages. The intracellular localization of *T. whipplei* within monocytes at day 1 (**A**) and macrophages at day 12 (**B**) was assessed by indirect immunofluorescence and laser scanning microscopy. Scale bars represent 5 µm. The percentages of *T. whipplei* that colocalized with cathepsin D or Lamp-1 in monocytes (**A**) and macrophages (**B**) were determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of three independent experiments.
doi:10.1371/journal.pone.0013561.g002

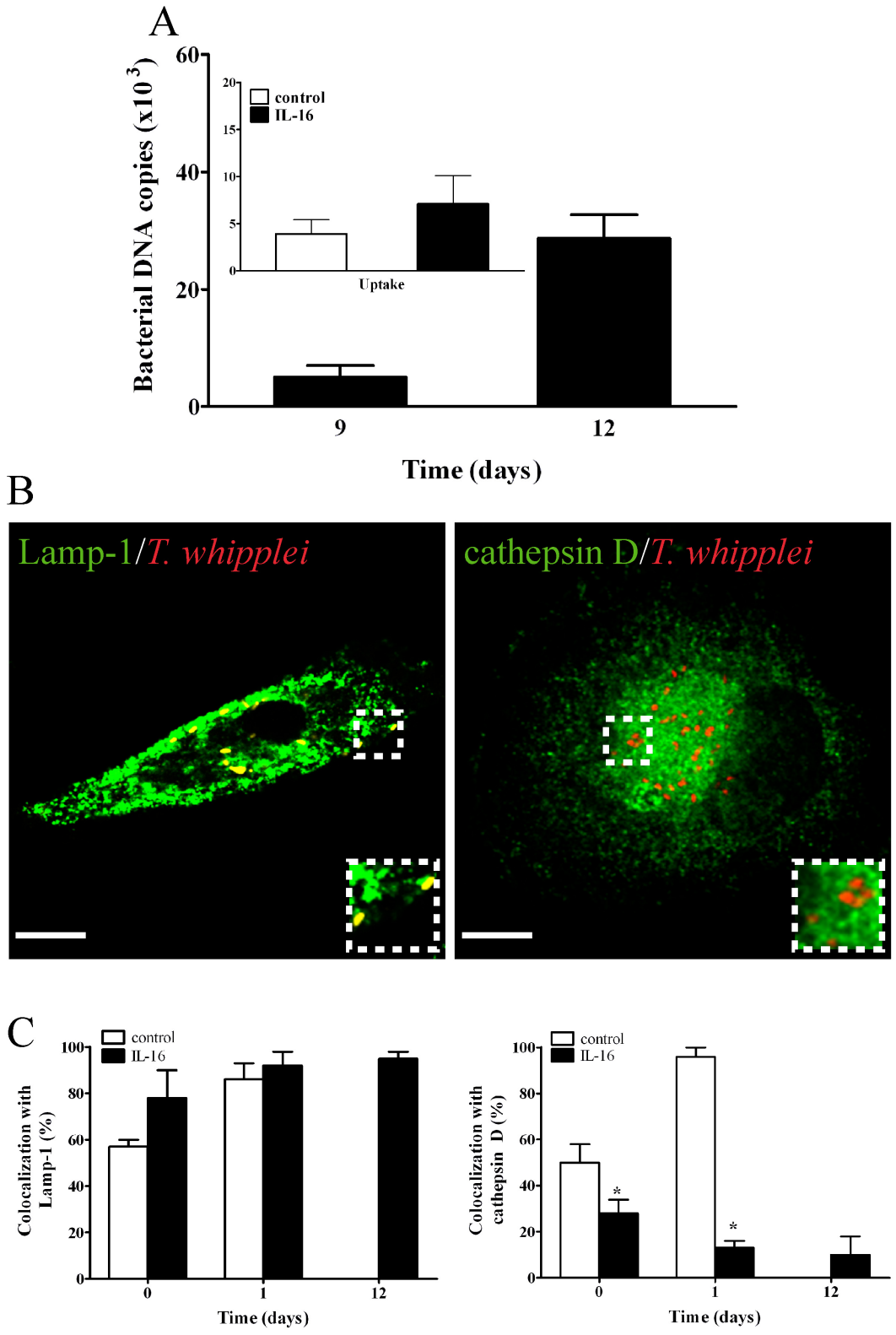

Figure 3. Effect of exogenous IL-16 on *T. whipplei* fate in monocytes. Monocytes were treated with or without IL-16 for 18 hours, incubated with *T. whipplei* for four hours (50 bacteria/cell), washed to remove unphagocytosed bacteria and incubated for additional time periods. (**A**) *T. whipplei* uptake (**inset**) and replication were assessed by determining the bacterial DNA copy number by qPCR after 9 and 12 days of infection. The results are expressed as the mean ± SEM of four experiments performed in triplicate. (**B**) The intracellular localization of *T. whipplei* within IL-16-treated monocytes was assessed by indirect immunofluorescence and laser scanning microscopy at day 12. Scale bars represent 5 µm. (**C**) The percentages of *T. whipplei* that colocalized with cathepsin D or Lamp-1, respectively, were determined ($n=5$). More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of five independent experiments. $p<0.05$.
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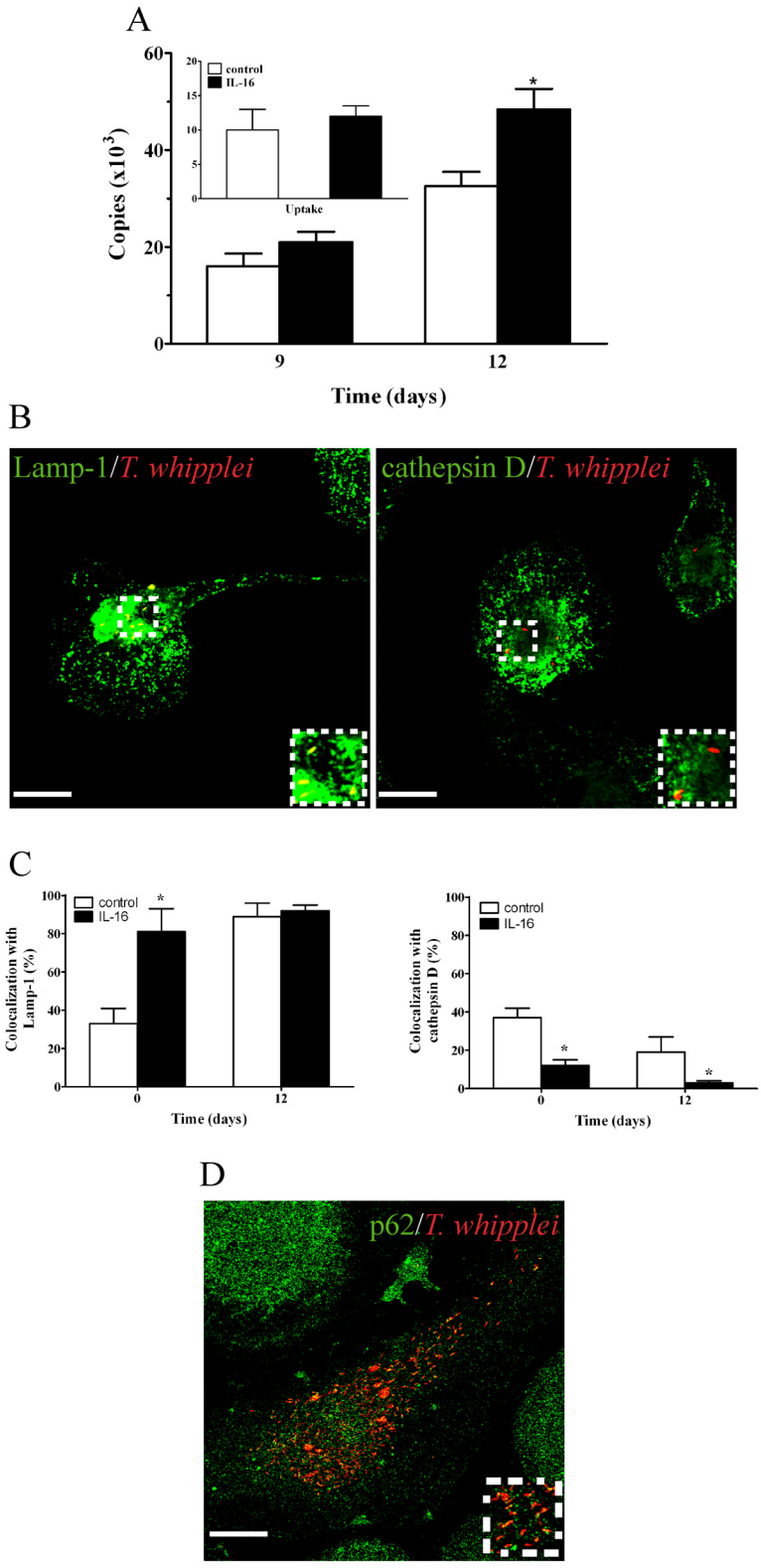

Figure 4. Effect of exogenous IL-16 on *T. whipplei* fate in macrophages. Macrophages were treated with rhIL-16 as described in Figure 3. (**A**) *T. whipplei* uptake (**inset**) and replication were determined by qPCR. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate. (**B**) The intracellular localization of *T. whipplei* was analyzed by indirect immunofluorescence and laser scanning microscopy at day 12. Scale bars represent 5 µm. (**C**) The percentage of organisms that colocalized with Lamp-1 or cathepsin D, respectively, was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of four independent experiments. $p<0.05$. (**D**) The localization of organisms with p62, a specific marker for autophagosomes, was assessed by indirect immunofluorescence and laser scanning microscopy. More than 200 phagosomes were examined in two independent experiments.
doi:10.1371/journal.pone.0013561.g004

cathepsin D by *T. whipplei* phagosomes (**Figure 4, B and C**). Next, we wondered whether IL-16 re-routed *T. whipplei* phagosomes towards the autophagosome pathway. We found that Ab directed against p62, a marker for autophagosomes [35], did not colocalize with *T. whipplei* (**Figure 4D**). Finally, we investigated if the effect of IL-16 on *T. whipplei* trafficking was specific using latex beads. Latex beads were located within phagolysosomes at days 1 and 12 post-ingestion. IL-16 did not modify the latex beads localization with either of the two markers (**Figure S1**), demonstrating that IL-16 specifically acted on *T. whipplei* trafficking. Taken together, these data show that incubating monocytes or macrophages with exogenous IL-16 can increase *T. whipplei* replication within phagosomes that are unable to be converted to phagolysosomes.

Inhibition or absence of IL-16 rescued the maturation of *T. whipplei* phagosomes

We then investigated the effect of IL-16 inhibition on the replication and localization of *T. whipplei* in macrophages. Treatment of macrophages with anti-IL-16 antibodies (Abs) did not alter *T. whipplei* uptake but it dramatically inhibited bacterial replication (**Figure 5A**) as published before [6]. It also induced the conversion of *T. whipplei* phagosomes to phagolysosomes (**Figure 5B**). Specifically, about 80% of *T. whipplei* phagosomes colocalized with Lamp-1 and cathepsin D at day 1, and bacteria were no longer detected thereafter (**Figure 5C**). Hence, IL-16 was required to maintain *T. whipplei* localization within late phagosomes. Second, the intracellular fate and localization of *T. whipplei* were studied in bone marrow-derived macrophages (BMDM) from IL16$^{-/-}$ mice. *T. whipplei* uptake by IL-16$^{-/-}$ and wild type (wt) BMDMs was similar (**Figure 6A, inset**). In wt BMDMs, bacterial replication was intense at days 9 and 12 post-infection (**Figure 6A**) as published before [36] and similar to that observed in human macrophages (**see Figure 1**). In IL-16$^{-/-}$ BMDMs, the bacterial DNA copy number was 3.5 times lower ($p<0.05$) than that in wt BMDMs at days 9 and 12 (**Figure 6A**), demonstrating that *T. whipplei* replication was inhibited in the absence of IL-16. This phenomenon was related to *T. whipplei* colocalization with phagolysosomes. Specifically, in wt and IL-16$^{-/-}$ BMDMs, the percentage of *T. whipplei* phagosomes that colocalized with cathepsin D was higher in IL-16$^{-/-}$ BMDMs than in wt BMDMs ($74\pm4\%$ vs. $56\pm6\%$ at day 0, $p<0.05$; $55\pm6\%$ vs. $21\pm2\%$ at day 12, $p<0.04$) (**Figure 6B and 6C**). Taken together, these results demonstrate that the microbicidal competence of macrophages towards *T. whipplei* can be restored in the absence of IL-16.

Effect of IFNγ on *T. whipplei* intracellular localization and IL-16 production

Given that the absence of IL-16 is associated with *T. whipplei* elimination by macrophages, and that impaired IFNγ production may be a cause of the delayed *T. whipplei* elimination in WD patients [4], we looked for a connection between IL-16 and IFNγ in *T. whipplei* infection. To this end, macrophages were treated for 16 hours with recombinant IFNγ prior to *T. whipplei* infection. IFNγ-treated macrophages internalized *T. whipplei* more efficiently than untreated macrophages ($p<0.05$), but *T. whipplei* replication was abolished at day 12 ($p<0.05$) (**Figure 7A**). The colocalization of *T. whipplei* with Lamp-1 in untreated and IFNγ-treated macrophages was close to 80% at day 0 and did not change thereafter (**Figure 7B**). In contrast, while the percentage of *T. whipplei* colocalizing with cathepsin D decreased from $66\pm6\%$ to $17\pm2\%$ after 12 days in untreated cells, more than 85% of *T. whipplei* phagosomes colocalized with cathepsin D in IFNγ-treated macrophages (**Figure 7C**). We then asked if IFNγ stimulates *T. whipplei* elimination by macrophages by interfering with IL-16 production. IL-16 production induced by *T. whipplei* was completely inhibited when macrophages were pretreated with IFNγ (**Table 1**). Furthermore, IFNγ inhibited the induction of *T. whipplei* replication by IL-16 (**Table 2**). These data clearly show that IFNγ regulates IL-16 production, and IL-16 in turn mediates *T. whipplei* replication.

Microarray analysis of IL-16$^{-/-}$ macrophages stimulated with *T. whipplei*

As wt BMDMs infected with *T. whipplei* have been shown to display an atypical activation program combining M2 polarization, a type I IFN response and apoptosis [36], host responses towards *T. whipplei* in IL-16$^{-/-}$ BMDM were monitored using a full genome microarray, and data were compared to those previously reported [36] (GEO database at NCBI, accession number GSE16180). After a six-hour stimulation with *T. whipplei*, 356 and 273 probes were significantly modulated in wt and IL-16$^{-/-}$ BMDMs, respectively. Among them, only 42 probes were similarly modulated in both wt and IL-16$^{-/-}$ BMDMs (**Figure 8A and Table S1**). Next, the genes were annotated according to functional classes. Gene Ontology (GO) biological processes at level 3 did not detect major differences between wt and IL-16$^{-/-}$ BMDM responses to *T. whipplei* (**Figure 8B**). Indeed, the GO terms of immune response (GO: 0006955), defense response (GO: 0006952), response to external stimulus (GO: 0009605), regulation of biological process (GO: 0050789) and cytokine production (GO: 0001816) were significantly over-represented in both wt and IL-16$^{-/-}$ BMDMs. In contrast, when GO biological processes were analyzed at lower levels, functional differences were identified. For example, analysis of GO biological processes at level 5 revealed 2 over-represented GO terms in wt BMDMs, different from the 13 over-represented GO terms in IL-16$^{-/-}$ BMDMs. Importantly, among these 13 GO terms, 10 were linked to immune response (**Figure 8B**). A closer analysis of GO biological processes at level 8 revealed that the regulation of the I-κB kinase/NF-κB cascade (GO: 0043122) was over-represented in IL-16$^{-/-}$ BMDMs but not in their wt counterparts (**Figure 8B**). These observations were further confirmed by an analysis of over-represented genes according to their transcription factors. Specifically, c-Rel and STAT were over-represented in wt BMDMs (**Figure 9A**), in accordance with recent data demonstrating that *T. whipplei* induces type I IFN-dependent responses in these cells [36]. In contrast, in IL-16$^{-/-}$ BMDMs, the main transcription factors regulated by *T. whipplei* were NF-κB, CP2/LBP-1C/LSF and c-Rel (**Figure 9B**), all involved in inflammatory responses. Taken together, these results show that the activation programs induced by *T. whipplei* in wt and IL-16$^{-/-}$ BMDMs are different, and that IL-16 might be involved in macrophage activation. This effect was specific because no significant terms were found between wt and IL-16$^{-/-}$ BMDMs stimulated with lipolysaccharide (LPS). Using the FatiGO Compare tool, 11,235 and 12,107 features were significantly modulated in wt and IL-16$^{-/-}$ BMDMs, respectively. The large majority (9121) of them were common to both wt and IL-16$^{-/-}$ BMDMs (**Figure S2**).

Discussion

We describe here a previously unreported ability of IL-16 to modulate anti-bacterial defenses. IL-16 inhibited *T. whipplei* killing by inhibiting the maturation of bacterial phagosomes into phagolysosomes. The intracellular fate of *T. whipplei* differed dramatically between monocytes and macrophages. In monocytes,

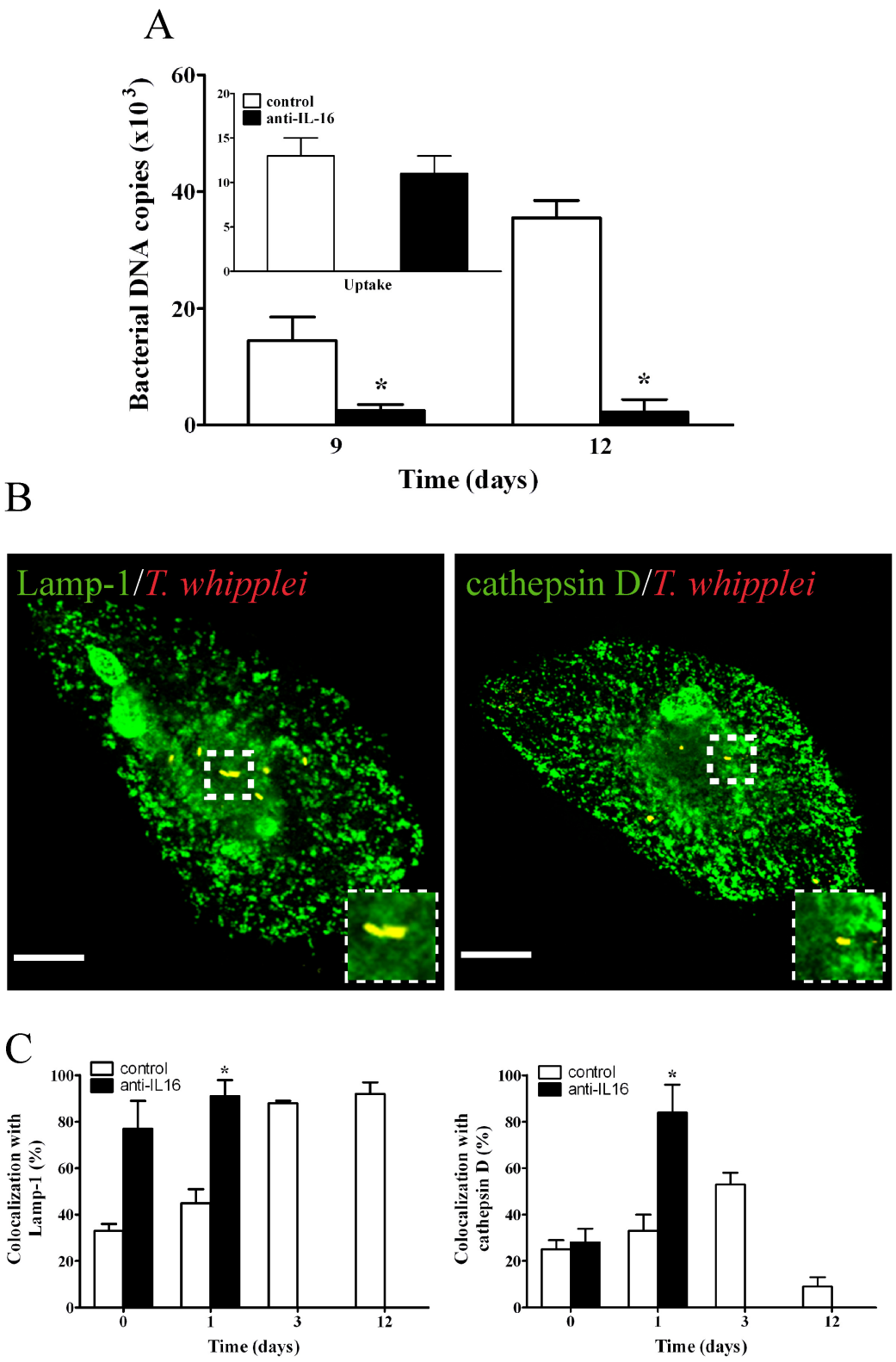

Figure 5. Effect of anti-IL-16 Abs on *T. whipplei* fate in macrophages. Macrophages were treated with or without anti-IL-16 blocking Abs for 18 hours, incubated with *T. whipplei* for 4 hours, washed to remove unphagocytosed bacteria and incubated for additional time periods in the presence of blocking Abs. (**A**) *T. whipplei* uptake (**inset**) and replication were assessed by determining the bacterial DNA copy number by qPCR. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate. (**B**) The intracellular localization of *T. whipplei* within macrophages treated with anti-IL-16 Abs was assessed by indirect immunofluorescence and laser scanning microscopy at day 1. Scale bars represent 5 µm. (**C**) The percentage of *T. whipplei* that colocalized with cathepsin D or Lamp-1, respectively, was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of three independent experiments. $p<0.05$.
doi:10.1371/journal.pone.0013561.g005

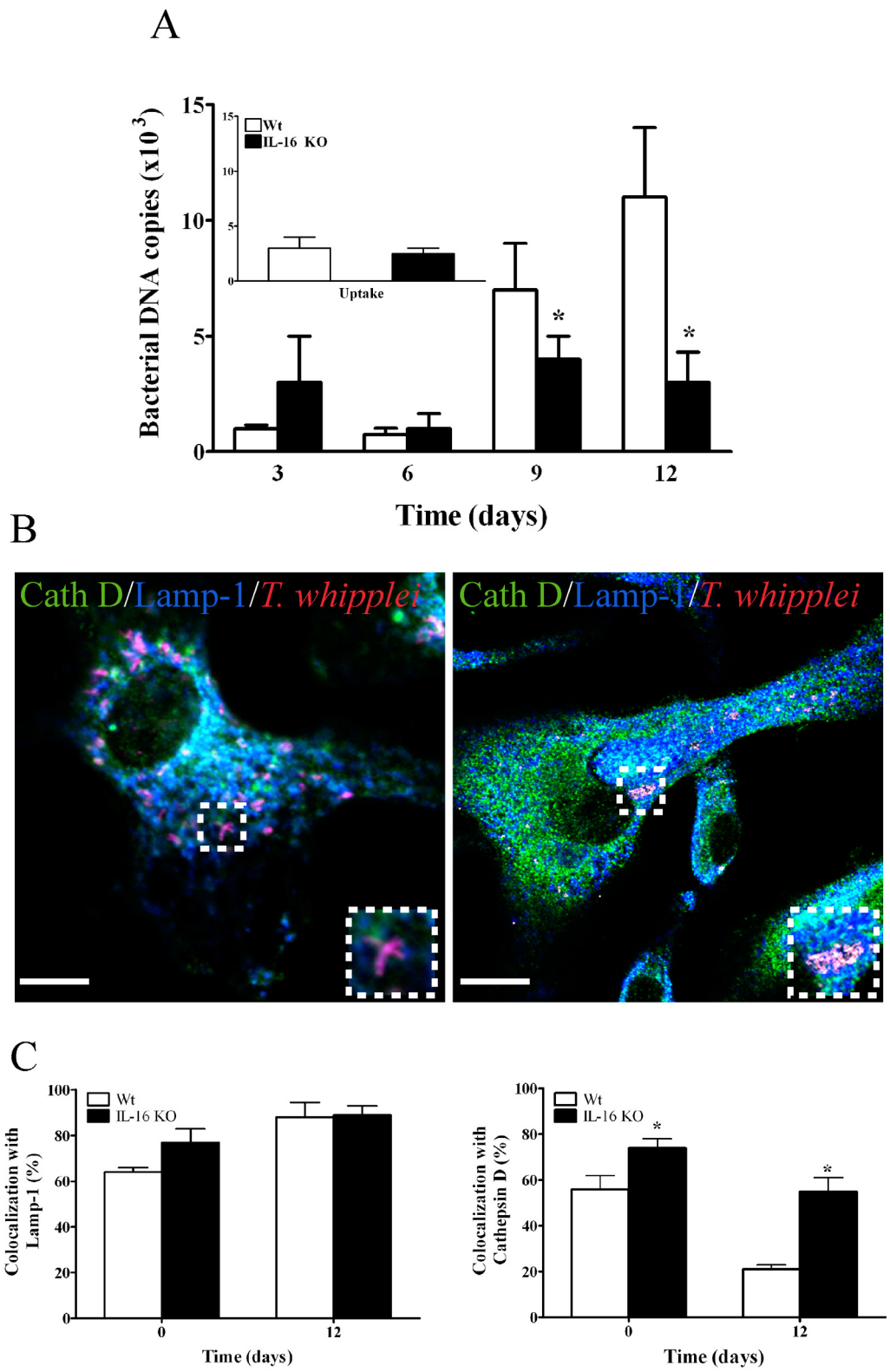

Figure 6. ***T. whipplei*** **fate in IL-16$^{-/-}$ BMDM.** BMDMs from wt and IL-16$^{-/-}$ mice were infected with *T. whipplei* similarly to the infection of human macrophages. (**A**) *T. whipplei* uptake (inset) and replication were determined by qPCR. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate. (B) The intracellular localization of *T. whipplei* within wt (left panel) and IL-16$^{-/-}$ (right panel) BMDMs was assessed by indirect immunofluorescence and laser scanning microscopy at day 12. Scale bars represent 5 μm. (**C**) The percentage of *T. whipplei* that colocalized with Lamp-1 or cathepsin D, respectively, was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of three independent experiments. The colocalization of *T. whipplei* (red) with cathepsin D (green) appears as yellow; the colocalization of *T. whipplei* with Lamp-1 (blue) appears as purple; and the colocalization of *T. whipplei* with Lamp-1/cathepsin D appears as white. $p<0.05$.
doi:10.1371/journal.pone.0013561.g006

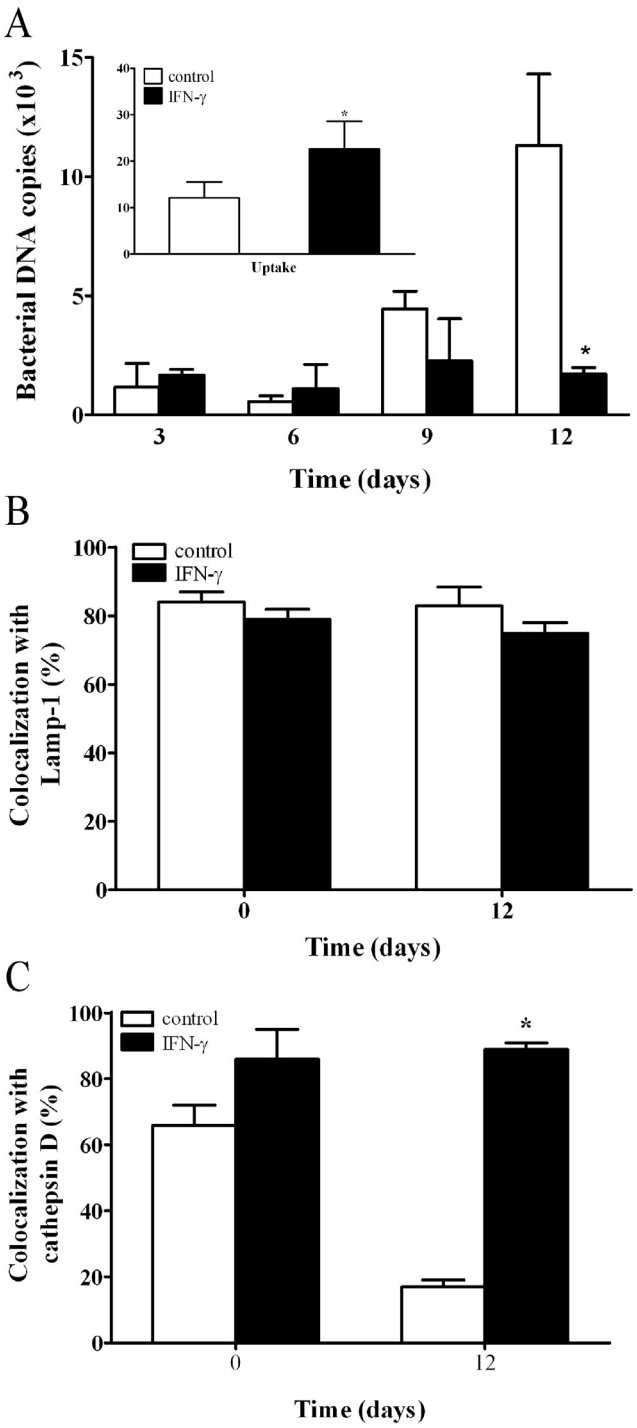

Figure 7. Effect of exogenous IFNγ on *T. whipplei* fate in macrophages. Macrophages were treated with rhIFNγ (500 UI/ml) and then infected with *T. whipplei* (50 bacteria/cell). (**A**) *T. whipplei* uptake (**inset**) and replication were determined by qPCR. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate. (**B, C**) The intracellular localization of *T. whipplei* within IFNγ-treated macrophages was assessed by indirect immunofluorescence and laser scanning microscopy. The percentage of *T. whipplei* that colocalized with Lamp-1 (**B**) or cathepsin D (**C**) was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of five independent experiments. $p<0.05$.
doi:10.1371/journal.pone.0013561.g007

Table 1. Effect of IFNγ on IL-16 secretion[a].

IFNγ	−		+	
T. whipplei	−	+	−	+
IL-16 secretion	8.2±5	700±101	<6.2	<6.2

[a]Macrophages were pre-treated with or without rhIFNγ (500 UI/ml) for 16 hours and then infected with *T. whipplei* (50 bacteria/cell). Supernatants were collected after 48 hours and assayed for the presence of IL-16 by immunoassay. The results are expressed as the mean ± SEM (pg/ml) of three independent experiments performed in triplicate.
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T. whipplei was rapidly eliminated in phagolysosomes characterized by the presence of Lamp-1 and cathepsin D. In macrophages, a large number of bacteria were eliminated in an early phase, but the remaining bacteria survived and intensively replicated in a late phase. Similar results were obtained in a *Brucella* infection model, in which the majority of bacteria were eliminated, while only few bacteria survived and then replicated within a suitable compartment [37]. When *T. whipplei* organisms replicated, they were localized in late phagosomes harboring Lamp-1 but devoid of cathepsin D, suggesting a blockage of phagosome-lysosome fusion.

We have also found that IL-16 inhibits the ability of both human monocytes and macrophages to eliminate *T. whipplei*. First, adding exogenous IL-16 to monocytes induced *T. whipplei* replication and increased bacterial replication in macrophages as published before [6]. Second, blocking IL-16 Abs induced *T. whipplei* elimination as published before [6]. Third, *T. whipplei* replicated in murine BMDMs, whereas bacterial replication was inhibited in BMDMs from IL-16$^{-/-}$ mice. The effect of IL-16 on the microbicidal responses of monocytes and macrophages cannot be attributed to a deactivation effect similar to that induced by IL-10 known to stimulate *C. burnetii* replication within macrophages [38]. Indeed, IL-10 was unable to induce *T. whipplei* replication in monocytes or to increase *T. whipplei* replication in macrophages (**Figure S3**). On the other hand, the effect of IL-16 on *T. whipplei* replication was specific since IL-16 had no effect on *C. burnetii* replication (**Figure S4**). These results indicate that IL-16 specifically favors *T. whipplei* replication.

The inhibition of *T. whipplei* elimination by IL-16 was likely related to the inhibited phagosome-lysosome fusion. In IL-16-treated monocytes and macrophages, *T. whipplei* resided in late phagosomes unable to fuse with lysosomes. Blocking IL-16 by specific Abs led to *T. whipplei* elimination within phagolysosomes. In mouse BMDMs that did not express IL-16, *T. whipplei* were also eliminated in phagolysosomes. The effect of IL-16 on the maturation of *T. whipplei* phagosomes was specific because IL-10 did not affect *T. whipplei* localization within phagolysosomes in monocytes or that within late phagosomes in macrophages

Table 2. Effect of IFNγ on IL-16-stimulated replication of *T. whipplei*[a].

	control	IL-16	IL-16+IFNγ
T. whipplei DNA copies (×10^3)	28.6±2.1	63.4±5.5	0

[a]Macrophages were pretreated with or without rhIL-16 (10 ng/ml) for 16 hours, infected with *T. whipplei* (50 bacteria/cell) in the absence or presence of rhIFNγ, washed to discard unphagocytosed bacteria and incubated for nine days in the absence or presence of IFNγ. Bacterial DNA copies were quantified by qPCR at day 9. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate.
doi:10.1371/journal.pone.0013561.t002

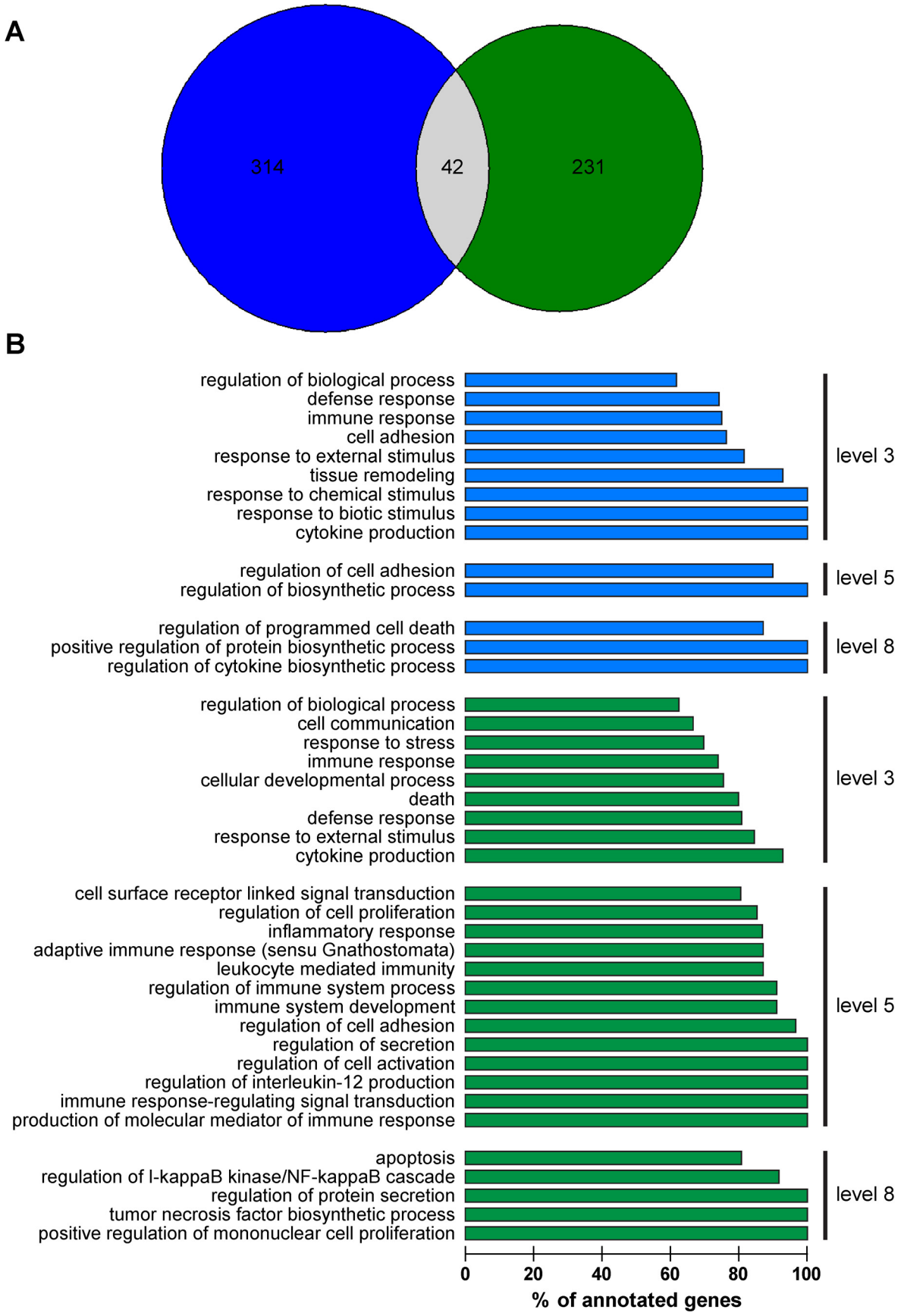
A
314
42
231
B
regulation of biological process
defense response
immune response
cell adhesion
response to external stimulus
tissue remodeling
response to chemical stimulus
response to biotic stimulus
cytokine production
level 3
regulation of cell adhesion
regulation of biosynthetic process
level 5
regulation of programmed cell death
positive regulation of protein biosynthetic process
regulation of cytokine biosynthetic process
level 8
regulation of biological process
cell communication
response to stress
immune response
cellular developmental process
death
defense response
response to external stimulus
cytokine production
level 3
cell surface receptor linked signal transduction
regulation of cell proliferation
inflammatory response
adaptive immune response (sensu Gnathostomata)
leukocyte mediated immunity
regulation of immune system process
immune system development
regulation of cell adhesion
regulation of secretion
regulation of cell activation
regulation of interleukin-12 production
immune response-regulating signal transduction
production of molecular mediator of immune response
level 5
apoptosis
regulation of I-kappaB kinase/NF-kappaB cascade
regulation of protein secretion
tumor necrosis factor biosynthetic process
positive regulation of mononuclear cell proliferation
level 8
0
20
40
60
80
100
% of annotated genes

Figure 8. Analysis of transcriptional responses of BMDMs to *T. whipplei*. BMDMs were stimulated with *T. whipplei* (50 bacteria/cell) for six hours and host responses were analyzed using whole genome microarrays. (**A**) Significant features were compared between wt (blue) and IL-16$^{-/-}$ (green) BMDMs and represented by a Venn diagram. Common significant features are displayed in grey. (**B**) Significantly over-represented GO biological processes in *T. whipplei*-stimulated wt (blue) and IL-16$^{-/-}$ (green) BMDM were determined by applying the two-tailed Fisher's exact test ($p<0.05$).
doi:10.1371/journal.pone.0013561.g008

(**Figure S5**). IL-10 has been previously demonstrated to favor the localization of mycobacteria within early phagosomes [29] and to increase the capacity of *C. burnetii* to traffic into a non-mature late phagosome [32,33]. In addition, IL-16 specifically controlled *T. whipplei* phagosome conversion because IL-16 had no effect on latex bead and *C. burnetii* (**Figure S4**) trafficking. Cytokines such as IFNγ, IL-4 and IL-13 have been described to induce or inhibit autophagy. Specifically, IFNγ has been shown to re-route the phagosome conversion pathway towards the autophagy pathway [39]. We demonstrated that the effect of IL-16 on the intracellular localization of *T. whipplei* was not associated with a re-routing of the phagosome conversion pathway towards the autophagosome pathway because p62, a marker for autophagosomes [35], did not colocalize with *T. whipplei*.

The mechanisms elicited by IL-16 that enable *T. whipplei* replication by blocking the late endosome stage were not related to defective recruitment of several molecules involved in phagosome conversion and phagolysosome biogenesis. It has been demonstrated that cytokines such as IL-6 and IL-12 specifically affect the expression of the GTPases Rab5 and Rab7, which are essential for membrane trafficking events leading to phagosome-lysosome fusion [40]. IFNγ also selectively induces Rab5a synthesis [41]. We showed here that IL-16 did not affect the transcription of either Rab5 or Rab7 (**Figure S6**). IL-16 might also inhibit the fusion of *T. whipplei* phagosomes with lysosomes by modulating the protein level of cathepsin D present on macrophages. Our results clearly showed that IL-16 did not affect the protein expression of cathepsin D or Lamp-1 (**Figure S6**). IFNγ has been demonstrated to affect the abundance, recruitment and phosphorylation of several proteins involved in phagosome conversion [28] and to affect Rab prenylation [41]. We therefore hypothesize that IL-16 might also affect post-transduction events in molecules engaged in phagolysosome biogenesis. Note that cathepsin D and Lamp-1 arrive at lysosomes via different trafficking routes. Lamp-1 is delivered to late endosomes and concentrates in lysosomes whereas cathepsin D is delivered to late endosomes via the mannose-6-phosphate receptor. We cannot exclude that IL-16 affects specifically one of these routes. An another hypothesis is that IL-16 acts on *T. whipplei* replication and phago-lysosome fusion by favoring a faster maturation of monocytes into macrophages since IL-16 has been described to accelerate differentiation of monocytes into macrophages [6].

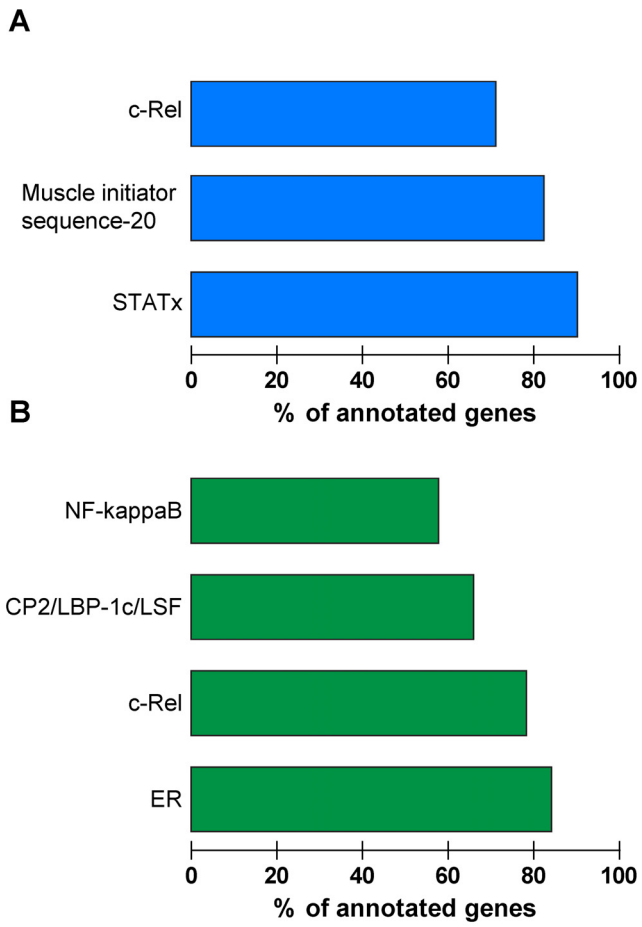

Figure 9. Transcription factors activated by *T. whipplei* in BMDMs. BMDMs were stimulated with *T. whipplei* for six hours and host responses were analyzed using whole genome microarrays. Significantly over-represented transcription factors involved in the transcriptional responses of *T. whipplei*-stimulated wt (**A**) and IL-16$^{-/-}$ (**B**) BMDMs were determined using the Fatiscan tool.
doi:10.1371/journal.pone.0013561.g009

Although IL-16 is considered a Th1-related cytokine [18], our data suggest that IL-16 may also behave as a Th2-related cytokine. In accordance with this hypothesis, IL-16 has been shown to activate Stat-6, which is known to play a role in the polarization of CD4$^+$ T cells to the Th2 phenotype [42]. Consequently, we assumed that an imbalance between IL-16 and IFNγ was likely to occur in our case. First, IFNγ induced both the elimination of *T. whipplei* by macrophages in a dose-dependent manner (**Table S2**) and phagosome conversion to phagolysosomes. Second, IFNγ inhibited both IL-16 production by macrophages and IL-16-stimulated *T. whipplei* replication. It is likely that IFNγ stimulates the microbicidal response of macrophages towards *T. whipplei* by impairing the IL-16 pathway.

To further investigate the downstream effectors of IL-16, we analyzed the transcriptomic response to *T. whipplei* in IL-16$^{-/-}$ macrophages using a whole genome array and previouly reported data [36]. In the absence of IL-16, the expression of 273 probes was modulated by *T. whipplei*. The great majority of these probes were not affected by *T. whipplei* in macrophages expressing IL-16. This response was different from that to LPS treatment, in which most modulated genes were common to macrophages with and without IL-16 expression. The analysis of GO biological processes revealed the enrichment of 13 GO terms linked to immune response. Specifically, there was over-representation of the I-κB kinase/NF-κB cascade. This finding is in agreement with our recent publication in which the activation of transduction pathways by *T. whipplei* involves a detrimental type I IFN signaling pathway and a poor NF-κB response [36]. In the absence of IL-16, we speculate that the activation of macrophages by *T. whipplei* might favor the NF-κB pathway instead of the type 1 IFN pathway. As a consequence, we can assume that IL-16 does not

polarize macrophages toward M2 cells. First, only genes from the NF-κB pathway were modulated in IL-16$^{-/-}$ BMDMs stimulated with *T. whipplei* suggesting a partial effect of IL-6 on macrophages polarization. Second, the effect of IL-16 on *T. whipplei*-stimulated response was specific because the transcriptional response induced by LPS were similar in wt and IL-16$^{-/-}$ BMDMs.

In conclusion, this study demonstrates for the first time that IL-16 induces *T. whipplei* replication by blocking the normal conversion of *T. whipplei* phagosomes into phagolysosomes. Our data also suggest that IL-16 inhibits the fusion of *T. whipplei* phagosomes with lysosomes by inducing a macrophage activation program that causes the IFNγ and NF-κB pathways to become essential to the elimination of *T. whipplei*. These results might explain why IFNγ was efficient for the treatment of refractory WD. They also suggest that IL-16 blockade by IFNγ, specific Abs or RNAi might contribute to the therapeutic elimination of *T. whipplei* in WD patients.

Methods

T. whipplei culture

The *Twist*-Marseille strain of *T. whipplei* (CNCM I-2202) was cocultured with HEL cells (CCL-37; American Type Culture Collection) and purified as previously described [34]. Bacteria were counted by Gimenez staining and indirect immunofluorescence, and their viability was assessed using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from leukopacks (Etablissement Français du Sang) by Ficoll gradient (MSL, Eurobio). Monocytes were then isolated using CD14$^+$ columns as recommended by the manufacturer (Miltenyi Biotec). More than 95% of the cells were CD14$^+$ monocytes as determined by flow cytometry. Macrophages were derived from monocytes by a seven-day culture in RPMI 1640 containing human AB serum: more than 95% of cells were considered as macrophages since they expressed CD68 as determined by flow cytometry. Monocytes and macrophages (10^5 cells/assay) were incubated in flat-bottom 24-well plates containing glass coverslips for immunofluorescence experiments. Then, they were infected with *T. whipplei* for four hours, washed to remove unphagocytosed bacteria and incubated for designated periods in RPMI 1640 containing 10% fetal calf serum (FCS) [6]. In some experiments, rhIL-16 (10 ng/ml) [6], rhIL-10 (10 ng/ml) or rhIFNγ (500 UI/ml) (R&D Systems) was added to monocytes and macrophages 18 hours prior to infection. Endogenous IL-16 was neutralized using 1 µg/ml anti-IL-16 mAbs (R&D Systems) as previously described [6].

BMDMs were generated from 6- to 8-week-old IL-16$^{-/-}$ C57BL/6 and littermate controls, as previously described [43]. Briefly, mice were euthanized by cervical dislocation, and bone marrow was flushed out from femurs and tibias. Bone marrow progenitors were seeded in Petri dishes in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 100 UI/ml penicillin and 100 µg/ml streptomycin supplemented with 15% L929 cell supernatant and allowed to differentiate for seven days [44]. Differentiated BMDMs were seeded (10^5 cells/well containing a glass coverslip for immunofluorescence analysis) in 24-well tissue culture plates in RPMI medium.

Ethics Statement

All animal experiments followed the guiding principles of animal care and use defined by the Conseil Scientifique du Centre de Formation et de Recherche Experimental Médico-Chirurgical (CFREMC) with the rules of Décret N° 87-848 of 10/19/1987 and were approved by the ethics board of the university at which the experiments were performed (Faculté de Médecine de la Timone, Experimentation permit number 13.385).

Quantitative real-time PCR (qPCR)

Cells were lyzed, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). PCR was performed using the LightCycler-FastStart DNA Master SYBR Green system (Roche) with primers specific for the *T. whipplei* 16S-23S ribosomal intergenic spacer region (tws3f and tws4r) as previously described [45]. The sequences of primers for *T. whipplei* were as follows: ccggtgacttaacctttttggaga (left primer) and tcccgaggcttatcgcagattg (right primer). In each PCR run, a standard curve was generated using serial dilutions ranging from 10 to 10^8 copies of the intergenic spacer region, and the results were calculated using the LightCycler 5.32 software (LC-Run version 5.32, Roche).

Colocalization experiments

Cells (10^5 cells/assay) were infected with *T. whipplei* (50 bacteria/cell) for four hours, extensively washed to discard unphagocytosed bacteria and then incubated for different time periods before fixation in 3% paraformaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100 or 0.1% saponin, and immunofluorescence labeling was performed according to standard procedures [46]. Polyclonal rabbit and monoclonal mouse anti-*T. whipplei* Abs were generated in our laboratory. Rat and mouse Abs specific for Lamp-1 (clone 1D4B and H4A3) were purchased from DSHB (Iowa, USA). Rabbit Ab specific for cathepsin D was a gift from Dr. S. Kornfeld (Washington University School of Medicine, St. Louis, MO) and Rabbit Ab specific for p62 was a gift from S. Meresse (Centre d'Immunologie de Marseille Luminy, Marseille, France). Secondary Alexa Abs, goat anti-mouse IgG conjugated with Alexa 488, goat anti-rat IgG and anti-rabbit IgG coupled with Alexa 555 were purchased from Invitrogen. In human monocytes and macrophages, the colocalization of *T. whipplei* with Lamp-1 or cathepsin D was studied using the couples mouse Lamp-1 Abs with rabbit anti-*T. whipplei* Abs and rabbit anti-cathepsin D Abs with mouse anti-*T. whipplei* Abs, respectively. In BMDMs, the combination of mouse anti-*T. whipplei* Abs, rat anti-Lamp-1 Abs and rabbit anti-cathepsin D Abs was used to directly assess the colocalization of *T. whipplei* with both Lamp-1 and cathepsin D. Cells were then examined by laser scanning microscopy using a confocal microscope (Leica TCS SP5, Heidelberg, Germany) with a 63X/1.32-0.6 oil objective and an electronic Zoom 1.5X. Optical sections of fluorescent images were collected at 0.15-µm intervals using Leica Confocal Software and processed using Adobe Photoshop© V7.0.1 software. At least 100 cells were examined for each experimental condition. The results are expressed as the percentage of bacteria that colocalized with fluorescent markers. Cells were selected as follows: 25 microscope fields, with at least four cells per field containing at least three phagosomes were randomly selected. More than 300 phagosomes were examined per experimental condition.

Microarray analysis

BMDMs were infected with *T. whipplei* (50 bacteria/cell) for six hours, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). The quality and the quantity of prepared RNA were assessed using the 2100 Bioanalyzer (Agilent Technologies). Sample labeling and hybridization were performed according to the manufacturer's recommendations (One-Color Microarray-Based Gene Expression Analysis). Briefly, labeled cDNA was synthesized using the Low RNA Input Fluorescent Amplification

Kit (Agilent Technologies) with 300 ng of total RNA and cyanine 3-labeled CTP. Hybridizations of 4X44k Mouse Whole Genome microarrays (Agilent Technologies) were performed in triplicate for 17 hours at 65°C using the *In situ* Hybridization Kit Plus (Agilent Technologies). Slides were scanned at a 5-µm resolution by a G2505B DNA microarray scanner (Agilent Technologies), and images were analyzed with the Agilent Feature Extractor Software 9.5.1.1. Global normalization by trimmed means was applied to raw datasets using the Excel software (Microsoft). A threshold-free functional profiling of significant features of the microarray was applied to avoid any loss of information as previously described [47]. Significant features were selected by applying the Student's *t* test to the input data with a p value<0.01. GO annotation and enrichment of GO biological process were performed with the freely available online tools FatiGO Search and Fatiscan (http://babelomics.bioinfo.cipf.es/). The two-tailed Fisher's exact test was used to determine functional classes of genes significantly over-represented or under-represented ($p<0.05$).

GEO Database

All data are MIAME compliant and the raw data have been deposited in a MIAME compliant database (GEO). All transcriptional profile files are available in the GEO database at NCBI (accession number GSE20210).

Immunoassays

Macrophages were treated with or without rhIFNγ for 16 hours and then infected with *T. whipplei* (50 bacteria/cell) for 48 hours in the presence or absence of IFNγ. Cell supernatants were assessed for the presence of IL-16 by immunoassay (R&D Systems). The sensitivity of kits is about 6.2 pg/ml.

Statistical analysis

Results are expressed as means ± SEM and were analyzed by the non-parametric Mann-Whitney *U* test. Differences were considered significant when $p<0.05$.

Supporting Information

Figure S1 Effect of IL-16 on the intracellular localization of latex beads. Macrophages were pretreated with rhIL-16 for 18 hours, incubated with latex beads (dilution 1/5000, Sigma Aldrich) for 4 hours, washed to remove unphagocytosed beads and incubated for additional time periods. The intracellular localization of the latex beads was analyzed by indirect immunofluorescence and laser scanning microscopy. The percentage of beads that colocalized with (A) Lamp-1 or (B) cathepsin D was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of two independent experiments.
Found at: doi:10.1371/journal.pone.0013561.s001 (0.56 MB TIF)

Figure S2 Analysis of transcriptional responses of BMDMs to LPS. BMDMs were stimulated with LPS (100 ng/ml) for six hours, and host responses were analyzed by microarrays. Gene expression values were normalized by trimmed means. Significant features were then compared between wt (blue) and IL-16−/− (green) BMDMs and represented by a Venn diagram. Common significant features are displayed in grey.
Found at: doi:10.1371/journal.pone.0013561.s002 (0.58 MB TIF)

Figure S3 Effect of IL-10 on *T. whipplei* replication. Monocytes and macrophages were pretreated with or without rhIL-10 (10 ng/ml) for 18 hours, incubated with *T. whipplei* (50 bacteria/cell) for 4 hours, washed to remove unphagocytosed bacteria and incubated for additional time periods. *T. whipplei* uptake (inset) and replication in monocytes (A) and macrophages (B) were assessed by determining the bacterial DNA copy number by qPCR. The results are expressed as the mean ± SEM of four independent experiments.
Found at: doi:10.1371/journal.pone.0013561.s003 (0.53 MB TIF)

Figure S4 Effect of IL-16 on *C. burnetii* replication and intracellular localization. Human macrophages were pretreated with or without rhIL-16 (10 ng/ml) for 18 hours, incubated with *C. burnetii* (200 bacteria/cell) for 4 hours, washed to remove unphagocytosed bacteria and incubated for additional time periods. (A) *C. burnetii* replication was assessed by determining the bacterial DNA copy number by qPCR. The results are expressed as the mean ± SEM of three independent experiments. (B) The intracellular localization of *C. burnetii* within IL-16 treated cells was assessed by indirect immunofluorescence and laser scanning microscopy. The percentage of organisms that colocalized with lysosomes (Lamp-1 and cathepsin D) was determined. More than 150 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of three independent experiments.
Found at: doi:10.1371/journal.pone.0013561.s004 (0.52 MB TIF)

Figure S5 Effect of IL-10 on T. whipplei intracellular localization. Monocytes (A, B) and macrophages (C, D) were pretreated with or without IL-10 (10 ng/ml) for 18 hours, incubated with T. whipplei for 4 hours (50 bacteria/cell), washed to remove unphagocytosed bacteria and incubated for additional time periods. The intracellular localization of *T. whipplei* within IL-10-treated cells was assessed by indirect immunofluorescence and laser scanning microscopy. The percentage of organisms that colocalized with Lamp-1 (A and C) or cathepsin D (B and D) was determined. More than 300 phagosomes were examined per experimental condition, and the results are expressed as the mean ± SEM of five independent experiments.
Found at: doi:10.1371/journal.pone.0013561.s005 (0.76 MB TIF)

Figure S6 IL-16 does not modulate molecules involved in phagosome conversion. Macrophages treated with rhIL-16 (10 ng/ml) for different time periods were lysed and RNA was extracted using the QIAamp RNA Mini Kit (Qiagen). cDNA was synthesized from 1 µg of total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen). Specific primers for each gene were designed using the Primer3Plus software (http://frodo.wi.mit.edu/primer3/). The primer sequences were as follows: for Rab5, cgggccaaatactggaaata (left primer) and aggacttgcttgcctctgaa (right primer); for Rab7, ggccttctacagaggtgcag (left primer) and ccggtcattcttgtccagtt (right primer); for β-actin used as an internal control, ggaaatcgtgcgtgacatta (left primer) and aggaaggaaggctggaagag (right primer). PCR was performed using Hotstart Taq polymerase (Qiagen) following the manufacturer's recommendations. PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide. Data were acquired with a Gel Doc 2000 (BioRad), and gene expression was normalized to β-actin. The figure is representative of three experiments. (B) Macrophages were stimulated with or without rhIL-16 (10 ng/ml) for 16 hours and washed with ice-cold PBS. Western blotting was performed as previously described (Al Moussawi et al. 2010). In brief, cells were lysed in ice-cold RIPA buffer containing protease inhibitor (Complete, Roche) and phosphatase inhibitor (Phosphostop, Roche) cocktails. After clearing, cell lysates were loaded onto 12% SDS polyacrylamide gels, electrophoresed and transferred onto nitrocellulose membranes (Millipore). The membranes were incubated with primary Abs directed against α-tubulin (Cell Signaling), Lamp-1 (H4A3, Abcam) or cathepsin D and then

incubated with peroxidase-conjugated Abs directed against anti-rabbit or anti-mouse IgG (Pierce). The blots were then revealed using the Immobilon Western Chemiluminescent HRP substrate (Millipore). Each blot is a representative of three independent experiments.
Found at: doi:10.1371/journal.pone.0013561.s006 (0.68 MB TIF)

Table S1 Transcripts significantly induced by *T. whipplei* in both wt and IL-16−/− BMDMs.
Found at: doi:10.1371/journal.pone.0013561.s007 (0.08 MB DOC)

Table S2 IFNγ induces *T. whipplei* elimination in a dose-dependent manner. Macrophages were treated with different concentrations of rhIFNγ and infected with *T. whipplei* (50 bacteria/cell). Bacterial replication was assessed by determining the bacterial DNA copy number by qPCR and cell viability was determined using Trypan blue exclusion. The results are expressed as the mean ± SEM of four independent experiments performed in triplicate.
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Author Contributions

Conceived and designed the experiments: EG JLM. Performed the experiments: EG AOB LP KAM. Analyzed the data: EG BD CC. Contributed reagents/materials/analysis tools: EG AoB LP KAM BD HK. Wrote the paper: EG CC HK JLM.

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DISCUSSION ET CONCLUSIONS

Les macrophages sont des cellules phagocytaires qui jouent un rôle important dans la réponse immune. Lors d'une infection, les macrophages sont capables de phagocyter les agents infectieux pour ensuite les éliminer dans un phagolysosome. Mais nombre de maladies infectieuses sont provoquées par des bactéries ou des parasites qui se développent dans des niches intracellulaires. Certains de ces agents pathogènes interfèrent avec la biogénèse des phagolysosomes pour survivre et se répliquer. Par exemple, *B. abortus* interagit avec le réticulum endoplasmique, mais pas avec le réseau endocytique classique. Les phagosomes contenant *Mycobacterium* fusionnent avec les endosomes précoces mais sont incapables de fusionner avec les endosomes tardifs. *C. burnetii*, l'agent étiologique de la fièvre Q, est décrite comme une bactérie capable de survivre dans une vacuole acide dans les monocytes et les macrophages en altérant la conversion phagosomale. Mais les mécanismes par lesquels cette bactérie détourne la conversion phagosomale restent à élucider. Après plusieurs passages en culture, *C. burnetii* exprime une variation antigénique qui est associée avec une perte de sa virulence **[75]**. Ce variant avirulent, contrairement à la bactérie virulente, est dégradé dans un phagolysosome. Le LPS est considéré comme un facteur majeur de la virulence de *C. burnetii* et diffère selon que les bactéries sont virulentes (v*Cb*) ou avirulentes (av*Cb*) **[84]**. C'est la raison pour laquelle on s'est demandé si le LPS de *C. burnetii* est impliqué dans le détournement de la conversion phagosomale.

Le LPS constitue probablement un des facteurs bactériens majeurs impliqués dans les mécanismes d'échappement à l'activité microbicide des macrophages. C'est ainsi que le LPS de *E. coli* inhibe partiellement la pinocytose et la phagocytose, retarde les mécanismes de conversion d'organelles telles que les endosomes, les macropinosomes et les phagosomes **[77].** Le LPS de *B. abortus* inhibe la fusion phagosome-lysosome via la chaîne O qui le constitue **[74]**. Le LPS de *S. flexneri* utilise une voie d'endocytose non classique qui fait appel en grande partie à la voie de recyclage **[83]**.

Dans ce projet, nous avons d'abord observé une corrélation directe entre la localisation des bactéries et leur survie. Les variants avirulents de *C. burnetii* sont localisés dans un phagolysosome qui se caractérise par la présence de LAMP-1 et de la cathepsine D : 90% d'entre eux sont dégradés. Les bactéries virulentes échappent, elles, à la dégradation lysosomale et se répliquent. Ces différences de localisation sont associées à la différence de structure de leurs LPS. En effet, les compartiments contenant les deux types de LPS acquièrent progressivement LAMP-1. Les compartiments contenant avLPS acquièrent transitoirement Rab7 puis la cathepsine D, contrairement à ceux contenant le vLPS qui n'acquièrent ni Rab7 ni la cathepsine D. L'étude de la colocalisation du vLPS avec le alpha-N-acetylglycosaminase, une hydrolase lysosomale, a confirmé la non localisation de ce LPS dans les lysosomes. La cinétique de transit du avLPS est similaire à

celle de l'albumine **[94]** ou à celle du LPS de *S. flexneri* **[83, 94]**. L'absence de localisation du vLPS dans les lysosomes n'est pas due à un simple retard d'acquisition, à sa séquestration dans des autophagosomes ou à un transit à travers le trans-golgi ou le réticulum endoplasmique.

L'activation de la voie de signalisation des TLRs par des bactéries peut réguler la phagocytose à différents stades tels que l'étape d'internalisation ou celle de la maturation du phagosome **[95]**. En outre, la cascade de signalisation des TLRs engendre l'activation des MAPKs qui sont elles aussi impliquées dans la conversion phagosomale ; en particulier, la MAPK p38 activée par les TLRs peut accélérer la maturation des phagosomes**[95]**. Nous avons montré que le avLPS et les variants avirulents de *C. burnetii* induisent l'activation des MAPKs ERK, JNK et p38 alors que le vLPS et les bactéries virulentes activent ERK et JNK, mais pas p38. Le défaut d'engagement de p38 entraîne un défaut de la phosphorylation de Vps41 et le recrutement de Rab7 à la surface du phagosome. L'absence de Rab7 n'empêche pas le recrutement de LAMP-1 et l'acidification du compartiment dans lequel réside *C. burnetii.* Le compartiment contenant *C. burnetii* présente quelques similarités avec les phagosomes contenant *Leishmania donovani.* En effet, les phagosomes contenant la forme promastigote de *L. donovani* n'acquièrent pas Rab7 bien qu'ils acquièrent LAMP-1 et soient acides (Scianimanico, 1999, 70).

Nos résultats établissent également l'importance de la phosphorylation par p38 de Vps41, une sous-unité du complexe HOPS, ce qui entraîne le recrutement de Rab7 à la membrane phagosomale et la conversion du phagosome en phagolysosome. Les MAPKs sont des éléments clés impliqués dans la réponse immune innée et dans le contrôle de la biogénèse et de la dynamique des compartiments endocytiques (Sorkin, 2009, 119). La MAPK-p38 régule les événements précoces durant l'endocytose des récepteurs opioïdes (Mace, 2005, 85 ; Cavalli, 2001, 86) via la phosphorylation de EEA1 et de Rabenosyn-5, deux effecteurs de Rab5. En outre, l'activation de p38 bloque la maturation du phagosome contenant *Mycobacterium* en réduisant le recrutement de la protéine effectrice de Rab5 (EEA1) **[96]**. Le complexe HOPS est requis pour permettre la conversion des phagosomes tardifs en phagolysosomes (Huotari, 2011, 144 ; Wang, 2011, 125). HOPS est un complexe comprenant six protéines, parmi lesquelles on peut citer les sous-unités Vps39 et Vps41 qui contrôlent l'activité de Rab7. Vps39 avec son activité GEF active Rab7 et Vps41 stabilise le complexe HOPS/Rab7. Chez la levure, la phosphorylation de Vps41 par YCK3 favorise le transport endo-lysosomal (Cabrera, 2009, 123). Nous étendons ces résultats aux cellules de mammifères puisque p38 phosphoryle Vps41, ce qui entraîne son recrutement à la membrane du phagosome et le recrutement de Rab7. Nos résultats montrent ainsi que p38, en contrôlant la stabilité de HOPS/Rab7, joue un rôle important dans le contrôle

de la biogénèse des phagolysosomes et que des agents pathogènes ont mis en place une stratégie élaborée ciblant p38 et la cascade moléculaire et fonctionnelle qui en découle.

Outre le contrôle de la voie endocytique à travers des changements dans l'environnement intraphagosomal, les cytokines peuvent avoir un effet direct sur la survie bactérienne. L'IL-10 est une cytokine impliquée dans la persistance bactérienne et l'évolution des maladies infectieuses. L'infection primaire par *C. burnetii* est généralement asymptomatique ; toutefois, elle devient chronique chez les sujets valvulopathes ou chez les immunodéprimés. L'évolution de la maladie est en partie contrôlée par l'IL-10. Une surproduction d'IL-10 est observée durant la fièvre Q chronique, et les monocytes des patients atteints d'une fièvre Q chronique sont incapables de dégrader *C. burnetii*. Dans ces monocytes, un défaut de la conversion phagosomale est observé puisque le phagosome contenant *C. burnetii* est incapable de fusionner avec les lysosomes. En revanche, les monocytes de patients atteints d'une fièvre Q aiguë détruisent efficacement *C. burnetii*. Ces résultats montrent ainsi qu'élimination de *C. burnetii* et conversion du phagosome en phagolysosome sont associées.

Nous avons également abordé dans cette thèse l'étude des mécanismes mis en jeu par *Tropheryma whipplei* pour survivre dans les macrophages. *T. whipplei*, l'agent de la maladie de Whipple, a

pour cellule hôte le macrophage tout comme de nombreux agents pathogènes qui évitent la dégradation dans les phagolysosomes en altérant la conversion phagosomale. Nous avons montré que *T. whipplei* ne déroge pas à cette règle. Nous avons en effet observé que *T. whipplei* est localisé dans un phagosome incapable de fusionner avec les lysosomes. Des résultats similaires avaient été observés dans des cellules non microbicides telles que les cellules HeLa **[60]**. Ce blocage par *T. whipplei* de la conversion phagosomale est complexe puisque le phagosome de *T. whipplei* présente à la fois des marqueurs d'endosomes précoces comme Rab5 et des marqueurs d'endosomes tardifs tels que Rab7 et LAMP-1. Il s'agit donc d'un phagosome bloqué dans une étape de transition de la conversion phagosomale. C'est, à notre connaissance, la première fois que ce type de phagosome est décrit. En effet, les phagosomes bactériens décrits dans la littérature sont bloqués soit dans une étape précoce comme celui de *M. tuberculosis* qui arbore des marqueurs tels que Rab5 soit dans une étape tardive comme celui de *C. burnetii* qui arbore LAMP-1. Des agents pathogènes tels que, par exemple, *C. trachomatis* peuvent également transformer le phagosome en une structure n'ayant plus les propriétés classiques de la voie d'endocytose. Le mécanisme utilisé par *T. whipplei* pour inhiber la conversion du phagosome implique clairement la voie endosomale. Le mécanisme par lequel coexistent de façon prolongée Rab5 et Rab7 n'est pas élucidé. Nous pouvons d'ores et déjà exclure une modulation

transcriptionnelle aberrante de Rab5 et de Rab7. *M. tuberculosis* est connu pour bloquer la conversion du phagosome en inhibant la production de PI(3)P **[55]**. Nous excluons également une altération du recrutement de PI(3)P par le phagosome de *T. whipplei* puisque le PI(3)P requis pour la conversion phagosomale est recruté normalement à la membrane. La production de PI(3)P ne semble pas être non plus modulée puisque Vps34 (une phosphatidylinositol 3-kinase produisant ce PI(3)P) n'est pas altérée sur le plan transcriptionnel. Enfin, la création par *T. whipplei* d'un phagosome chimère pourrait être due à une altération de l'activité de Rab5 ou de Rab7. La comparaison bioinformatique du génome de *T. whipplei* et de certaines bactéries pathogènes connues pour affecter le phagosome indique que *T. whipplei* est dotée d'un gène similaire au gène codant la GAPDH chez *L. monocytogenes* et qui inhibe l'activité GTPase de Rab5. Si l'on admet que ce gène code une protéine chez *T. whipplei*, ce facteur bactérien pourrait être la clé du blocage de la conversion phagosomale par *T. whipplei*.

L'IL-16 est connue pour induire la réplication de *T. whipplei* dans les monocytes et exacerber la réplication de *T. whipplei* dans les macrophages. En étudiant la nature du phagosome de *T. whipplei* en présence ou en absence d'IL-16, nous avons montré que l'IL-16 bloque la formation du phagolysosome. En effet, la déplétion d'IL-16 par utilisation d'anticorps neutralisants ou l'utilisation de macrophages provenant de souris invalidées pour

l'IL-16 entraînent la formation de phagolysosomes qui conduisent à l'élimination de *T. whipplei*. L'effet de l'IL-16 n'est pas une simple inhibition des propriétés microbicides des macrophages puisqu'une cytokine désactivatrice telle que l'IL-10 n'affecte ni la réplication de *T. whipplei* ni la conversion de son phagosome. L'effet des cytokines sur la voie d'endocytose n'est pas, à ce jour, élucidé. Plusieurs études montrent que des cytokines telles que l'IL-10, l'IFN-γ, l'IL-6 ou l'IL-12 **[97]** affectent l'expression de molécules-clés de l'endocytose ou modifient la composition des endosomes et que ces modifications peuvent rendre compte de la survie ou de la mort des agents pathogènes. Nous avons montré que l'IFN-γ inhibe la production d'IL-16 entraînant ainsi l'élimination de *T. whipplei*, ce qui pourrait expliquer l'absence d'élimination de *T. whipplei* chez les patients atteints de la maladie de Whipple qui présentent un défaut de production d'IFN-γ. L'absence d'IFN-γ comme régulateur de la production d'IL-16 pourrait expliquer la production importante d'IL-16 chez les patients infectés à *T. whipplei* **[93]** et donc la réplication de cet agent infectieux dans les tissus atteints.

En conclusion, au cours de cette étude, nous avons mis en évidence les mécanismes par lesquels *C. burnetii* interfère avec la biogénèse des phagolysosomes. Nous avons pu montrer que, via son LPS, *C. burnetii* évite la stimulation de l'axe p38-MAPK/HOPS pour échapper à sa dégradation dans les

phagolysosomes. Ces résultats obtenus in vitro éclairent d'un jour nouveau des données physiopathologiques. En effet, les patients atteints d'une fièvre Q chronique active présentent un défaut de maturation du phagosome contenant *C. burnetii* et une absence d'activation de p38, contrairement à des contrôles sains, des patients atteints d'une fièvre Q aiguë ou des patients guéris d'une fièvre Q chronique. En d'autres termes, la détermination d'un défaut éventuel d'activation de p38 pourrait être utile au suivi des patients atteints d'une fièvre Q chronique. Nous avons également montré que *T. whipplei* réside dans un phagosome chimère ayant à la fois des propriétés des phagosomes précoces et des phagosomes tardifs et que l'IL-16 contrôle la maturation de ce phagosome. Cependant, il serait intéressant d'élucider avec précision le mécanisme par lequel coexistent de façon prolongée Rab5 et Rab7 sur le phagosome contenant *T. whipplei* en identifiant le facteur de virulence de la bactérie ainsi que son mode d'action. Il serait aussi pertinent d'établir un lien direct entre l'IL-10 et le défaut de recrutement de Rab7 à la surface du phagosome abritant *C. burnetii*. Enfin, une meilleure compréhension des mécanismes de survie des bactéries devrait permettre d'identifier les effecteurs bactériens et leurs cibles dans la cellule hôte. Comme la plupart de ces agents pathogènes sont localisés dans des compartiments peu accessibles aux antibiotiques conventionnels, ces recherches sont essentielles pour la mise en place de thérapies préventives ou curatives innovantes.

ANNEXES

Nous présentons en annexes un chapitre de livre intitulé

« *Role of the leukocyte activation in phagosome maturation process* »

(Revue II)

et un article intitulé

« *Antibiotic susceptibility and intracellular localization of Diplorickettsia massiliensis* »

REVUE II

Role of the leukocyte activation in phagosome maturation process

Abdoulaye Oury Barry, Nicolas Boucherit and Eric Ghigo

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Les leucocytes sont des cellules de la réponse immune connues pour être activées par des cytokines. L'endocytose peut également être régulée par un environnement immun. Dans cette revue, nous avons évoqué l'effet des cytokines sur la voie endocytique et sur la biogénèse des phagosomes. En effet, le traitement des macrophages avec des cytokines inflammatoires induit la conversion des phagosomes bactériens en phagolysosomes et la dégradation bactérienne. En revanche, les cytokines immunorégulatrices inhibent la conversion phagosomale et favorisent la croissance bactérienne. Comme l'interaction entre les leukocytes et les bactéries peut entraîner la production de cytokines, il est possible de voir apparaître des boucles d'amplification ou inhibitrices. Cet aspect de la réponse à l'infection est cependant largement méconnu. Une meilleure compréhension de ce dialogue entre activation leukocytaire et activité endocytique pourrait être utile au développement de nouvelles stratégies rationnelles d'éradication des agents pathogènes.

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3. Role of the leukocyte activation in phagosome maturation process

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Abstract. To survive within host cells, intracellular pathogens interfere with the biogenesis of phagolysosomes, thereby forming replicative vacuoles. Whereas the complex mechanisms used by pathogens to hijack the biogenesis of phagolysosomes have been elucidated in naive leukocytes, the role of leukocyte activation in this process has not yet been investigated. Leukocytes are known to be activated by cytokines and several reports provide molecular evidence that endocytosis can be regulated by the immune environment. In this chapter, we will discuss the effect of leukocyte activation by cytokines on the endocytic pathway and on phagosome biogenesis.

Introduction

Pathogens have evolved multiple strategies to survive within host cells. Several pathogens interfere with the biogenesis of phagolysosomes, thus resulting in the formation of phagosomes that are enable of interact with lysosomes (as reviewed in [1]). Most of these studies used naive cells before stimulation with immune mediators. However, when a microorganism interacts with its host, it stimulates both innate and adaptive immune responses. The innate immune

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response leads to the activation of host cells, such as monocytes, macrophages, and dendritic cells, and drives the production of cytokines. The adaptive immune system engages effectors such as antibodies and polarized T cells with their cytokines. Little is known about the effect of macrophage activation on the maturation of phagosomes (phagosome conversion) into phagolysosomes. Various cytokines regulate the ability of monocytes and macrophages to clear pathogens and elicit a sustained immune response. Recent studies have shown that phagosome conversion depends on the balance between pro-inflammatory cytokines, such as IFNγ, IL-12 and IL-6, and anti-inflammatory cytokines, such as IL-10, which indicates that cytokines modulate phagosome conversion during bacterial infections. This finding provides molecular evidence that endocytic processes can be regulated by the immune environment.

Hereby, we briefly describe the mechanism of phagolysosome formation before focusing on the the effect of leukocyte activation on the endocytic pathway and on phagolysosome formation.

1. Phagolysosome genesis

Once internalized, bacteria are localized within a nascent phagosome that undergoes a series of fusion and fusion-fission ("kiss and run") events with different populations of endocytic organelles. The final product of these events is the formation of a phagolysosome, which is a complex structure composed by more than 200 proteins that destroy bacteria [2]. . The mechanisms that govern the progression of phagosome maturation are exceedingly complex and involve regulated interactions with various endocytic organelles. It is widely considered similar to the process that governs endosome progression (endosome conversion). We substitute the term "phagosome maturation", which suggests a progressive evolution of a well-known entity, by the term of "phagosome conversion", which is more appropriate to describe discontinuous events such as successive fusion-fission events. Endosome and phagosome conversion are temporally coordinated through the sequential activities of molecules such as Rab GTPases, which regulate the endocytic pathway and require SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) molecules, which are involved in membrane fusion events.

The transformation of nascent phagosomes to phagolysosomes occurs in four successive stages **(Figure 1)**. After sealing, nascent phagosomes interact with early and recycling endosomes and gain several proteins necessary for the conversion of nascent phagosomes into early phagosomes. These proteins include the small GTPase Rab5, early endosome autoantigen-1 (EEA1),

syntaxin-13, vacuolar protein sorting 34 (Vps34), a type III phosphatidylinositol-3-kinase (PI3K), and phosphatidylinositol-3-phosphate (PI(3)P) generated by Vps34. Subsequently, by successive fusion-fission events, early phagosomes transform into intermediate compartments prior to the formation of late phagosomes. These intermediate phagosomes have the properties of both early and late phagosomes **(Figure 1)**, because their surfaces contain molecules characteristic of both early endosomes (Rab5, PI(3)P, EEA1) and late endosomes (Rab7, RILP, lysosomal-associated membrane protein-1 (Lamp-1)) [1; 3]. The small GTPases Rab5 and Rab7 are critical mediators of phagosome conversion [4; 5] and the transition from intermediate phagosomes to late phagosomes is coordinated by the VpsC-homotypic protein-sorting (HOPS) complex [1; 6]. The HOPS complex mediates the transition of Rab5-positive endosomes to Rab7-positive endosomes [6]. Late phagosomes are characterized by the presence of proteins such as Lamps (Lamp-1, -2, -3), RILP and the small GTPase Rab7, which progressively replace early and intermediate phagosomal markers [1; 7] **(Figure 1)**. The small GTPase Rab7 mediates exchanges between phagosomes

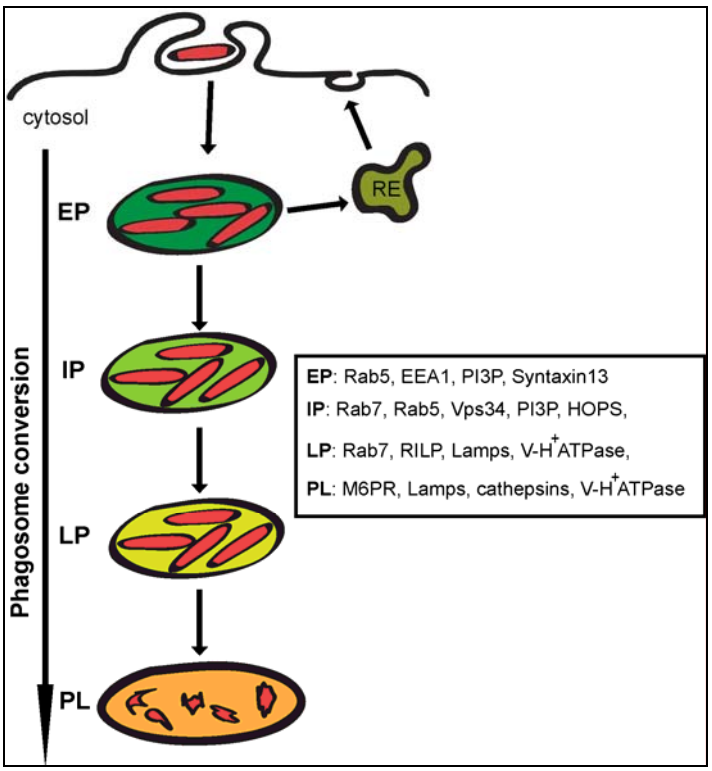

Figure 1. Overview of phagosome maturation mechanisms. Immediately after sealing, nascent phagosomes interact with endosomes by fusion and fusion-fission events. They gain several protein necessary for the conversion of nascent phagosomes into phagolysosome. EP, Early phagosomes; IP, intermediate phagosomes; LP, late phagosomes; PL, phagolysosomes; RE, recycling endosomes.

and late endosomes or lysosomes [8; 9]. Finally, late phagosomes interact with lysosomes through mannose 6-phosphate receptors (M6PRs). This terminal step of phagosome conversion leads to the formation of phagolysosomes that are rich in lysosomal hydrolytic enzymes such as cathepsin D [1; 7] **(Figure 1)**.

In addition to changes in composition, phagosome conversion also involves physical modifications. Whereas the pH of early phagosomes is approximately 6.0, acquisition of the vacuolar proton pump ATPase ($V\text{-}H^+$-ATPase) by late phagosomes induces a drop in the intraphagosomal pH to 5.5 in late phagosomes and 4.5 in phagolysosomes [1; 7]. It is possible to discriminate between the different stages of phagosome conversion using characteristic markers or specific probes **(Table 1)**.

Table 1. Endocytic compartments identity.

Compartments	Composition
early phagosomes	pH 6.0-6.5; Rab5; EEA1; Vps34; PI(3)P; rabenosyn-5
late phagosomes	pH 5-6; Rab7; Lamps; $V\text{-}H^+$ATPase; M6PR; RILP
phagolysosomes	pH 4.0-5.5; $V\text{-}H^+$ATPase; Lamps; mature lysosomal enzymes

The complex machinery that drives phagosome conversion is a preferential target for numerous pathogens capable of hijacking the process of phagolysosome formation **(Table 2)**.

Table 2. Intracellular localization of some bacterial pathogens.

Bacteria	Intracellular localization	References
Francisella tularensis	cytosol	[10]
Listeria monocytogenes	cytosol	[11]
Rickettsia spp	cytosol	[12]
Shigella flexnerii	cytosol	[12]
Brucella abortus	early phagosome	[13]
Mycobacterium tuberculosis	early phagosome	[14]
Coxiella burnetii	late phagosome	[15]
Salmonella typhimurium	late phagosome	[16]
Chlamydia spp	non acidified inclusion	[4]
Legionella pneumophila	ER-derived phagosome	[1]
Yersinia pestis	autophagosome	[17]

2. Molecular effects of cytokines on endocytosis effectors

Cytokines modulate key endocytic regulators that play a critical role in membrane trafficking, endosome conversion and phagosome conversion **(Table 3)**. It has been demonstrated that IFNγ selectively up-regulates the expression of Rab5a mRNA and protein and induces Rab5a guanine nucleotide exchange activity in mononuclear cells [18]. IFNγ increases Rab5 prenylation, which leads to increased levels of membrane-associated Rab5a:GTP. The effect of IFNγ on Rab5a synthesis and processing is selective because the levels of other Rab GTPases, including Rab5b, Rab5c, Rab7 and Rab11, remain unaffected. It is likely that the prenylation of Rab5a is responsible for the selective retention of Rab5a at the membranes of endosomes and phagosomes. The enhanced activity of Rab5a protein modifies the composition of the phagosome membrane and accelerates the rate of phagosome conversion into phagolysosomes [18]. We have observed that IL-10 strongly decreases the expression of Rab5 and Vps34 transcripts,

Table 3. Effects of cytokines on key endocytic regulators.

Cytokine	Effects
IFNγ	increase Rab5 prenylation
	increase the turn over Rab5:GDP to Rab5:GTP
	induce Rab5 mRNA transcription
	induces Rab5a expression
	induces V-H^+-ATPase phagosomal recruitment
	induces Lamp-1,-2 and cathepsin D recruitment
	reduce the lysosome fusion
	stimulate the phagolysosome biogenesis
IL-22	rescues phagolysosome biogenesis fusion
IL-12	induce Rab7 mRNA expression
	induces lysosomal transport
IL-6	induces Rab5 mRNA expression
	induce early endosome homotypic fusion
	inhibits phagolysosome biogenesis fusion
IL-10	induces Rab5 mRNA expression
	induces Vps34 mRNA expression
	inhibit cathepsin D mRNA expression
	limit lysosomal fusion

whereas IFNγ does not affect the levels of Vps34 mRNA (unpublished results). We can speculate that immunoregulatory cytokines such as IL-10 interfere with the endosome and phagosome conversion by controlling the protein levels of critical endocytic effectors such as Rab5 and Vps34 kinase. Treatment of macrophages with IL-6 specifically induces the expression of Rab5 through the activation of extracellular signal-regulated kinase (ERK), whereas IL-12 exclusively up-regulates the expression of Rab7 through the activation of p38 mitogen-activated protein kinase (MAPK). In addition, IL-12 is associated with the up-regulation of lysosomal transport, whereas IL-6 stimulates fusion events between early compartments in macrophages [19]. These observations confirm that cytokines affect endocytic processes. The involvement of MAPKs in the control of endocytic pathway is not surprising since it is now accepted that signalling cascades can regulate endocytosis [20-22]. It is important to note that p38 MAPK phosphorylates endocytic effectors such as EEA1 and Rab5 guanyl-nucleotide dissociation inhibitor (GDI) [23; 24], thus enhancing endosome conversion.

Several other membrane networks are targeted by cytokines. Indeed, IFNγ induces alkalinization of the trans-Golgi network by inhibiting V-H^+-ATPase activity [25], which limits endocytic exchanges. IL-10 decreases the expression of cathepsin D in monocytes from patients with inflammatory bowel disease [26] and affects fluid-phase and mannose receptor-mediated endocytosis in human primary macrophages [27]. Consequently, it is likely the generation of hydrolase-defective lysosomes.

3. Cytokines effects on phagosome composition and conversion

3.1. IFNγ

IFNγ might act directly on the conversion of pathogen-containing phagosomes **(Table 3)**. IFNγ allows *Mycobacterium* killing by macrophages by rescuing phagosome-lysosome fusion [28; 29]. Several phagosomal modifications are observed during IFNγ treatment. Phagosomes containing mycobacteria are not accessible to transferrin, which inhibits the exchange of mycobacterial phagosomes with early endosomes. In addition, the pH of mycobacterial phagosomes is 5.2 in IFNγ-activated macrophages, whereas it is 6.3 in resting macrophages. Biochemical analysis of phagosomes containing mycobacteria confirmed that the lower intraphagosomal pH is correlated with the increased accumulation of V-H^+-ATPase [28], suggesting that IFNγ rescues phagosome fusion with late endosomes ??and the recruitment of V-H^+-ATPase at the phagosomal membrane. In addition, individual bacterial vacuoles that contain a single mycobacterium have been

observed to coalesce into communal vacuoles containing multiple mycobacteria, thus suggesting that phagosome-phagosome homotypic fusion events increase during IFNγ treatment [28]. Finally, IFNγ induces the co-localization of mycobacteria with lysosomal markers such as Lamp-1 and cathepsin D, indicating that phagosomes are converted to phagolysosomes in which the bacteria are eliminated [30]. IFNγ also inhibits the remodeling of *Legionella pneumophila*-containing phagosomes into endoplasmic reticulum-derived vesicles via their conversion into Lamp-2 and cathepsin D-expressing phagolysosomes [31]. *C. burnetii* phagosomes fuse with lysosomes in the presence of IFNγ, as demonstrated by the acquisition of cathepsin D and the killing of phase I *C. burnetii* [32]. IFNγ mediates these events through two different mechanisms. First, the addition of IFNγ to *C. burnetii*-infected macrophages stimulates phagosome-lysosome fusion but does not affect the vacuolar pH. Second, treatment of macrophages with IFNγ prior to *C. burnetii* infection induces alkalinization of *C. burnetii* vacuoles independently of V-H^+-ATPase exclusion [32]. These findings are in contrast with reports showing that IFNγ decreases the pH of *Mycobacterium*-containing phagosomes [28]. The mechanism of IFNγ-mediated vesicle alkalinization in the *C. burnetii* infection model is unknown.

In a model of *L. monocytogenes* infection, IFNγ induced Rab5a expression and phagosome conversion, which play a central role in *Listeria* destruction [33]. These findings reveal the importance of Rab5a as a mediator of the listericidal activity of IFNγ. IFNγ-induced Rab5 causes remodeling of the phagosomal environment, facilitates the translocation of Rac2 to phagosomes harboring *Listeria* and regulates the activity of this GTPase. Rac2 governs phagocyte NADPH oxidase activity and the subsequent production of reactive oxygen intermediates [33]. These events likely facilitate the transition of early phagosomes to late phagosomes, thus inhibiting the membrane lysis of *L. monocytogenes* phagosomes.

Trost *et al.* used quantitative proteomic and bioinformatic approaches to profile the changes in composition, abundance, and phosphorylation of proteins in latex bead-containing phagosomes in both resting and IFNγ-activated macrophages, [34]. They found that more than 2,000 phagosomal proteins are modulated by IFNγ. These proteins include enzymes that are expected to enhance microbial degradation, trigger the macrophage immune response, promote antigen loading on major histocompatibility complex class I molecules and modulate phagosome conversion. Several phagosomal proteins involved in fusion with endocytic compartments, such as syntaxin 7, syntaxin 13, Rab7, LIMP2, and VAMP8, are up-regulated by IFNγ. In conclusion, IFNγ dramatically changes the composition of phagosome membranes and likely modulates phagosome conversion.

3.2. Other cytokines

IL-22, IL-12 and IL-6, have been shown to modulate the conversion of phagosomes into phagolysosomes **(Table 3)**. IL-22 allows mycobacterial killing by macrophages by rescuing phagosome-lysosome fusion [28; 29]. In cells infected with *Salmonella*, the *Salmonella*-containing vacuole is targeted to lysosomes when cells are treated with IL-12, whereas the transport of *Salmonella* to lysosomes is inhibited in the presence of IL-6. In these instances, IL-12 induces lysosomal transport, whereas IL-6 stimulates fusion between early compartments in macrophages, thereby modulating *Salmonella* trafficking and survival in macrophages [19]. The survival of *Salmonella* in macrophages is significantly inhibited in IL-12-treated cells in comparison to untreated control cells. IL-12 increases the expression of Rab7 in macrophages, which enhances *Salmonella* killing by targeting the bacteria to lysosomes. In contrast, IL-6 up-regulates Rab5 expression, which promotes the fusion of *Salmonella*-harboring phagosomes with early endosomes and thereby inhibits their transport to lysosomes [19].

3.3. Effects of anti-inflammatory cytokines on phagosome conversion

The modulation of phagosome conversion is not limited to pro-inflammatory cytokines **(Table 3)**. In macrophages derived from the bone marrow of IL-10 knockout mice, the co-localization of mycobacteria with lysosomal markers is enhanced compared to macrophages from control mice, which suggests an increase in the acidification of mycobacterial phagosomes [30]. IL-10 is overproduced during chronic Q fever [35; 36], and monocytes from patients with chronic Q fever are unable to kill *C. burnetii* and exhibit defective phagosome conversion: phagosomes harboring *C. burnetii* are not converted in phagolysosomes. In contrast, monocytes from patients that are recovering from acute Q fever kill *C. burnetii* efficiently, and this killing is associated with phagosome conversion [37]. These observations are related to the activity of the disease, because phagosome-lysosome fusion and *C. burnetii* killing are restored in patients that have recovered from Q fever endocarditis. Interestingly, *C. burnetii* killing and phagosome conversion into phagolysosomes are related. The use of specific antibodies to neutralize IL-10 in monocytes from patients with chronic Q fever rescues phagosome conversion and bacterial killing to the levels detected in cured patients. In contrast, the addition of recombinant IL-10 to monocytes from patients that have recovered from Q fever endocarditis blocks phagosome conversion, prevents the phagosome from co-localizing with lysosomal enzymes and inhibits *C. burnetii* killing [37]. The molecules of the endocytic pathway that

are modulated by IL-10 remain unknown. Since IL-10 strongly decreases the expression of Rab5 and Vps34 transcripts (unpublished data), it can be speculated that IL-10 acts via a down-modulation or repression of key modulators of endocytosis. It is also possible that IL-10 inhibits Rab prenylation or GDI activity, because the function of IL-10 is in opposition to that of IFNγ.

Concluding remarks

By manipulating the dynamics and integrity of the host cell membrane, pathogens can create niches that are suitable for their survival and replication. The formal identification of phagosomes that contain pathogens is difficult, because these pathogens manipulate the composition of the compartments they inhabit, thus leading to a mixed identity. Another element of complexity in defining the identity of bacterial phagosomes is the recent discovery that phagosome conversion is directed by the microenvironment of host cells, especially immune cells. Indeed, the treatment of macrophages with inflammatory cytokines induces the conversion of bacterial phagosomes into phagolysosomes and bacterial killing. In contrast, immunoregulatory cytokines inhibit phagosome conversion and favor bacterial growth. In addition, the contact between leukocytes and bacterial pathogens may lead to cytokine production, thus creating amplification or inhibitory loops. These processes may explain the discrepancies observed in the intracellular localization of some pathogens that infect leukocytes or other cell types that are less sensitive to cytokines (i.e. endothelial cells). The cross-talk between endocytosis, bacterial phagosome conversion and leukocyte activation requires further investigation. A better knowledge of this cross-talk will likely be important for the development of new rational strategies for the eradication of bacterial pathogens.

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ARTICLE V

Antibiotic susceptibility and intracellular localization of *Diplorickettsia massiliensis*

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FEMS immunology and medical microbiology, 2011

Mon expérience en microscopie m'a permis d'être associé à cet article qui porte sur la susceptibilité de *Diplorickettsia massiliensis* à certains antibiotiques tels que la doxycycline, la ciprofloxacine, la levofloxacine et la rifampicine.

D. massiliensis a été récemment isoléeà partir de *Ixodes ricinus*, une espèce commune de tiques connue pour piquer l'homme. C'est une bactérie intracellulaire obligatoire de la famille des *Coxiellaceae* qui se réplique dans les cellules humaines (Mediannikov et al. 2010). De nombreuses maladies sont transmises par *I. ricinus* à l'homme en Europe (Socolovschi et al., 2009) mais il existe nombre de cas sans étiologie prouvée (Hofmann, 1996 ; Sharma *et al.* , 2010). *D. massiliensis* pourrait être impliquée dans certaines de ces maladies.

Nous avons décrit pour la première fois la susceptibilité de *D. massiliensis* à une variété d'antibiotiques. La doxycycline, la ciprofloxacine, la levofloxacine et la rifampicine inhibent la croissance de *D. massiliensis* mais pas d'autres molécules telles que la pénicilline G, le chlorure d'ammonium, la gentamycine, l'omeprazole, la bafilomycine A1 ou la chloroquine. Nous avons également observé que *D. massiliensis* est localisé dans un compartiment acide dans lequel il se multiplie. Ce compartiment n'est ni un phagosome précoce ni un phagosome tardif ni un phagolysosome. L'acidification de ce compartiment ne semble pas contribuer à la résistance des bactéries aux antibiotiques puisqu'elles peuvent croître dans un environnement à pH neutre.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Antibiotic susceptibility and intracellular localization of *Diplorickettsia massiliensis*

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D. massilienisis; antibiotic assay; intracellular localization.

Abstract

Diplorickettsia massiliensis is an obligate intracellular bacterium from the *Coxiellaceae* family recently isolated from *Ixodes ricinus* ticks. The inhibitory effects of antimicrobial agents were assessed by two different methods, immunofluorescence and Gimenez staining assay. Different markers (EEA1, Lamp-1, Cathepsin D, and LysoTracker Red DND99) were used to reveal the nature of the vacuole containing the bacterium. Ciprofloxacin, levofloxacin, and rifampin had MIC values of 2 μg mL^{-1}. We found that 4 μg mL^{-1} of Doxycycline inhibited the growth of *D. massiliensis* strain. Surprisingly, *D. massiliensis* was resistant to chloramphenicol up to the concentration of 64 μg mL^{-1}. We found that penicillin G, ammonium chloride, gentamycin, omeprazole, bafilomycin A1, and chloroquine were not active against *D. massiliensis*. Studies performed with markers EEA1, Lamp-1, Cathepsin D, and LysoTracker Red DND99 showed that *D. massiliensis* is localized within an acidic compartment that is not an early phagosome, but a late phagosome or a phagolysosome. Gimenez staining stays a good method that will work with a very low number of bacteria and can be used to determine the MICs of new therapeutic antibiotics precisely. The resistance profile of *D. massiliensis* was found to be quite unusual for intracellular Gram-negative bacterium with marked resistance to chloramphenicol. Despite of localization in acidic compartment, pH-neutralizing agents do not significantly inhibit intracellular growth of bacterium. The results of these studies prove that antibiotic resistance does not depend on pH of vacuole. This pH-related mechanism seems not to play a contributing role in the overall resistance of *D. massiliensis*.

Introduction

The recently described bacterium *Diplorickettsia massiliensis* is an obligate intracellular Gram-negative bacterium (Mediannikov *et al.*, 2010). The only available strain of *D. massiliensis* was isolated from *Ixodes ricinus* tick collected from southeastern part of the Slovak republic forest Rovinka in 2006. Comparative sequence analysis of the 16S rRNA gene showed that it is phylogenetically related to the genus *Rickettsiella*; further, it can be grouped into the family *Coxiellaceae* and the order *Legionellales* of γ-proteobacteria. The order *Legionellales* is comprised of two families, *Legionellaceae* and *Coxiellaceae*. Many species of *Legionella* in this monotypic family cause legionellosis, most notably *Legionella pneumophila*. The *Coxiellaceae* family comprises two genera, *Coxiella* and *Rickettsiella* (La Scola *et al.*, 2001); *Coxiella burnetii*, a *Coxiella* family member and intracellular bacterium, is the causative agent of Q fever (Maurin & Raoult, 1999). The genus *Rickettsiella* currently includes four officially recognized species: *Rickettsiella popilliae*, *Rickettsiella grylli*, *Rickettsiella chironomi*, and *Rickettsiella stethorae*. These intracellular pathogens infect a wide range of arthropods including insects, crustaceans, and arachnids (Fournier & Raoult, 2005). The tick *Ixodes ricinius* from which *D. massiliensis* was isolated harbors a wide spectrum of microorganisms that are pathogenic to both humans and animals including: *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Babesia* spp., *Ehrlichia* spp., *Anaplasma phagocytophilum*, *Bartonella henselae*, and *Bartonella quintana* (Reye *et al.*, 2010). Recently, we observed three cases from Marseille, France (one has just returned from Slovenia). Patients symptoms were fever, skin eschar, arthralgia, and myalgia (Subramanian *et al.*, 2011).

As *D. massiliensis* is an obligate intracellular bacterium (Mediannikov *et al.*, 2010) localized within a compartment, *in vitro* studies to assess antibiotic susceptibility necessitate the use of cell culture systems. The intracellular environment is likely to influence both the susceptibility of the intracellular pathogen and drug activity. It has long been assumed that the intracellular accumulation of an antibiotic is indicative of efficient activity against intracellular bacteria. Antimicrobial therapy targeting an intracellular pathogen is more complex than an extracellular target; intracellular antimicrobial activity additionally depends on drug penetration into and accumulation within the cell, cellular metabolism, sub-cellular disposition, and the bioavailability of the drug (Van *et al.*, 2006). Further, the efficiency of an antibiotic is related to the intracellular localization of the targeted bacteria. Pathogenic bacteria, including *Mycobacterium* spp., *Ehrlichia* spp., *Salmonella* spp., *Francisella tularensis*, *Legionella* spp., *Brucella* spp., and *Yersinia enterocolitica* (Maurin & Raoult, 1997), reside in atypical phagosomes; in general, these atypical phagosomes are more or less permissive for antibiotics or contain (i.e. pH) affecting the chemical properties of the antibiotic. Thus, the relative inefficiency of doxycycline treatment in Q fever (Maurin & Raoult, 1999) is closely related to the fact that *C. burnetii* is localized in an acidic compartment of the host cell (Beron *et al.*, 2002). In this study, we have determined the kinetics of activity on growth and the minimum inhibitory concentration (MIC) of 12 antibiotics against *D. massiliensis*; results were obtained through the observation of bacterial growth in shell vials using Gimenez staining for visualization. We also studied the intracellular localization of *D. massiliensis* to examine different patterns of antibiotic resistance.

Materials and methods

Bacterial strain and MIC determination

Diplorickettsia massiliensis was cultured as described previously (Mediannikov *et al.*, 2010). Briefly, *D. massiliensis* was cultured in XTC-2 cells at 28 °C with Leibovitz-15 media (Invitrogen) supplemented with 2% fetal bovine serum (FBS) and 2% tryptose-phosphate broth solution (Sigma-Aldrich, Ayrshire, UK). *Diplorickettsia massiliensis* was also grown in the human MRC-5 and human erythroleukemia (HEL) cell lines (Mediannikov *et al.*, 2010) at 32 °C using minimum essential media containing 5% fetal calf serum and supplemented with 2 mM L-glutamine (Gibco).

Cells became heavily infected usually after 5 days of incubation for XTC-2 cells and 7 days of incubation for MRC-5 cells. Cell supernatants were then discarded from the flasks, and the infected cells were detached using sterile glass beads. The supernatants were diluted 1 : 200 in the culture media. This dilution was used to infect XTC-2 and MRC-5 monolayers in shell vials. The XTC-2 vials were incubated at 28 °C, and the MRC-5 vials were incubated at 37 °C in 5% CO_2.

Antibiotics and other tested substances used in this study were tested in serial twofold dilutions as follows: doxycycline, 0.25–8 μg mL^{-1} (Pfizer, Neuilly, France); chloramphenicol, 0.25–64 μg mL^{-1} (Roussel, Paris, France); erythromycin, 0.25–132 μg mL^{-1} (Roussel); rifampin, 0.25–8 μg mL^{-1} (Merrel Dow, Neuilly/Seine, France); ciprofloxacin, 0.25–8 μg mL^{-1} (Bayer Pharma, Sebs, France); levofloxacin, 0.25–8 μg mL^{-1} (Hoechst Marion, Roussel); penicillin G, 0.25–132 μg mL^{-1} (Diamant); gentamicin, 0.25–132 μg mL^{-1} (Dakota Pharm, Creteil, France); ammonium chloride, 1 to 100 mM (Coger, Paris, France); bafilomycin A1, 2.5–80 nM, (Sigma, Germany); omeprazole (Sigma, France); and chloroquine, 0.25–8 μg mL^{-1} (France). Stock solutions were prepared according to the manufacturer's instructions and stored at −80 °C until used. Working solutions were prepared just prior to antibiotic treatment by diluting stock solutions in the respective media required. Dilutions of each antibiotic were added to rows of infected cells; positive controls consisted of infected cells in the absence of antibiotics, and negative controls consisted of uninfected cells in the presence of antibiotics. We harvested cells in 15-day period to be sure that the effect of antibiotic or other substances developed fully. After incubation, shell vials were stained with the Gimenez stain. Smears were prepared on glass slides with a centrifugal slide maker (Cytospin II; Shandon, Cheshire, UK), and the number of intracellular *D. massiliensis* was counted at 100× magnification. The MIC was defined as the lowest antibiotic concentration that inhibited growth of the *D. massiliensis*. Experiments were performed three times with an interval time to confirm the results.

Determination of degree of infection

The percentage of infected cells was determined by the direct microscopic examination of cells stained utilizing the Gimenez technique (Gimenez, 1964). A minimum of 200 cells were examined in each prepared slide to determine the proportion of infected cells. The percentage of infected cells was assessed at a magnification of 100×.

A minimum of 20–40 cells were observed for *D. massiliensis* counting of per host cell under the microscope. So we checked and calculated the number of bacteria per cells.

Indirect immunofluorescence assay (IFA)

IFA was used to confirm the bacteriostatic activity of antibiotics by the inhibition of growth in shell vials (Raoult

et al., 1991). After 15 days of growth, infected cells were fixed in the shell vials for 10 min with absolute ethanol, rehydrated for 5 min in phosphate-buffered saline (PBS), and incubated for 30 min at 37 °C with 0.3 mL of anti-*D. massiliensis* mouse serum. Polyclonal antibodies directed against *D. massiliensis* were generated in our laboratory and suspended in PBS (titer, 1 : 400). Next, cover slips were rinsed, washed three times with PBS (10 min each), and then incubated for 30 min at 37 °C with 0.3 mL of fluorescein-conjugated goat anti-mouse immunoglobulin (1 : 100) (Bio-Merieux, Charbonnieres-les-Bains, France). After incubation, the cover slips were washed three times for 5 min each in PBS, mounted on slides, and examined by fluorescence microscope at a magnification of 40×. The MIC was defined as the lowest antibiotic concentration that caused growth inhibition of bacteria compared with the control sample of day 0. Several visible intracellular or extracellular bacteria in the field of light microscope were not considered as bacterial growth.

Intracellular localization of *D. massiliensis*

Infected cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100, and then immunofluorescence labeling was performed. Rat antibodies specific for Lamp-1 (clone 1D4B) (DSHB, IA, USA) and rabbit antibodies specific for EEA-1 (M. Zerial, MPI-CBG, Dresden, Germany) and Cathepsin D (M. Zerial, MPI-CBG) were purchased. Secondary Alexa antibodies were purchased from Invitrogen. To study the acidity of the compartments, infected cells were incubated with LysoTracker Red DND99 (Molecular Probes) at 100 nM for 2 h and then washed and fixed with 3% paraformaldehyde. After fluorescent labeling, these cells were mounted with Mowiol and examined by laser scanning microscopy using a confocal microscope (Leica TCSSP5) with a 63×/1.32-0.6 oil CS lens and an electronic Zoom 4×. Optical sections of fluorescent images were collected at 0.25-μm intervals using Leica confocal software and processed using ADOBE PHOTOSHOP V5.5 software. At least 60 cells were examined for each experimental condition; results are expressed as the percentage of *D. massiliensis* that colocalized with each of the fluorescent markers.

Results

Antibiotic susceptibility using immunofluorescence staining and Gimenez staining

The degree of infection, defined by the proportion of cells infected by *D. massiliensis* over the total number of morphologically identifiable cells, corresponded to the

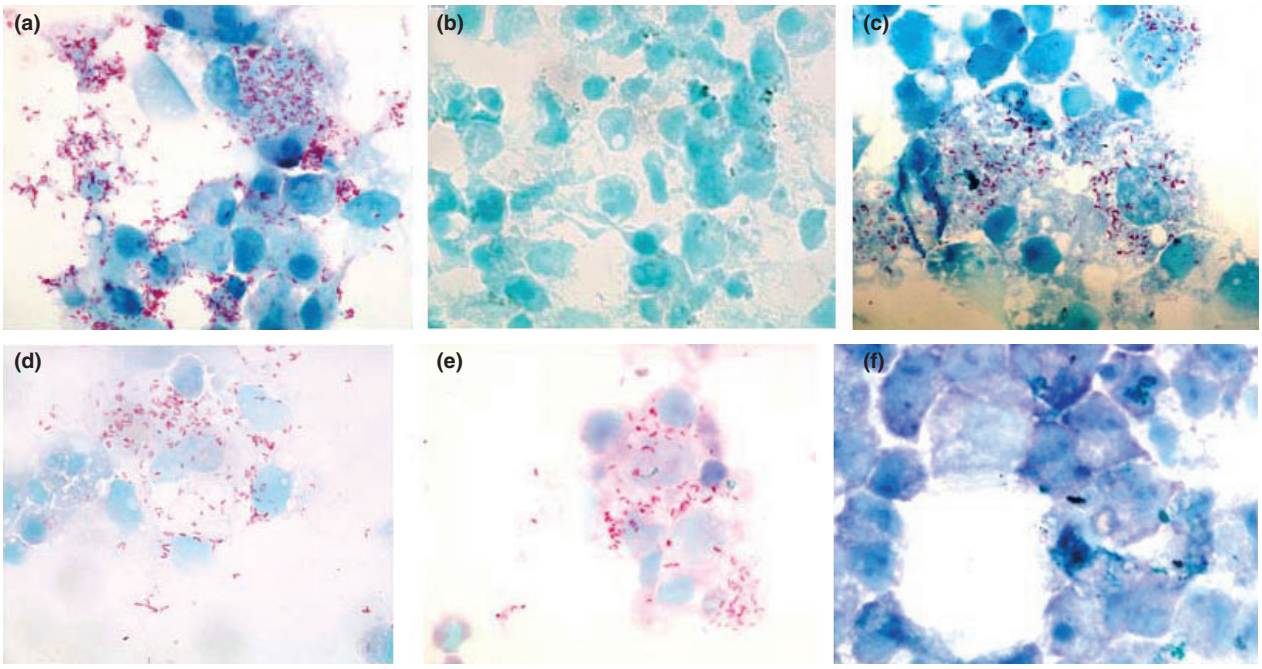

Fig. 1. Photomicrographs illustrating the effect of antibiotic treatment on *Diplorickettsia massiliensis*-infected XTC cells by Gimenez staining (100× magnification). Control, 15 days postinfection (a); negative control (b); rifampin (0.25 μg mL^{-1}) (c); rifampin (0.5 μg mL^{-1}) (d); rifampin (1 μg mL^{-1}) showed little infection compare with control (e); rifampin (2 μg mL^{-1}) showed no sign of infection after 15 days of treatment (f).

Table 1. Comparison of antibiotic MICs for *Diplorickettsia massiliensis* using Gimenez and immunofluorescence staining

	MIC of antibiotics (XTC-2 and MRC-5)	
Antibiotics	Gimenez staining	IFA
Penicillin G	>64 μg mL^{-1}	>64 μg mL^{-1}
Chloramphenicol	>64 μg mL^{-1}	>64 μg mL^{-1}
Erythromycin	>64 μg mL^{-1}	>64 μg mL^{-1}
Bafilomycin	>80 nM mL^{-1}	>80 nM mL^{-1}
Ammonium chloride	>100 mM mL^{-1}	>100 mM mL^{-1}
Omeprazole	>8 μg mL^{-1}	>8 μg mL^{-1}
Chloroquine	>8 μg mL^{-1}	>8 μg mL^{-1}
Gentamicin	16 μg mL^{-1}	32 μg mL^{-1}
Doxycycline	4 μg mL^{-1}	8 μg mL^{-1}
Ciprofloxacin	2 μg mL^{-1}	8 μg mL^{-1}
Levofloxacin	2 μg mL^{-1}	4 μg mL^{-1}
Rifampin	2 μg mL^{-1}	4 μg mL^{-1}

mean number of bacteria per cell. These numbers were used in the evaluation of the bacteriostatic effects of the antibiotics and other chemical substances tested (Fig. 3). As expected, IFA was found to be more sensitive than Gimenez staining in the visualization of infected cells (Fig. 1).

Although the Gimenez staining protocol was easy to perform, the counting of the organisms was laborious and time-consuming, as was the subsequent calculation of the percent reduction in infection. The MIC results for the 12 antibiotics tested against *D. massiliensis* obtained using Gimenez staining showed no significant differences to the MIC values obtained utilizing IFA (Table 1, and Figs 1 and 2).

All experiments were run concurrently and in triplicate. While doxycycline was able to inhibit *D. massiliensis*

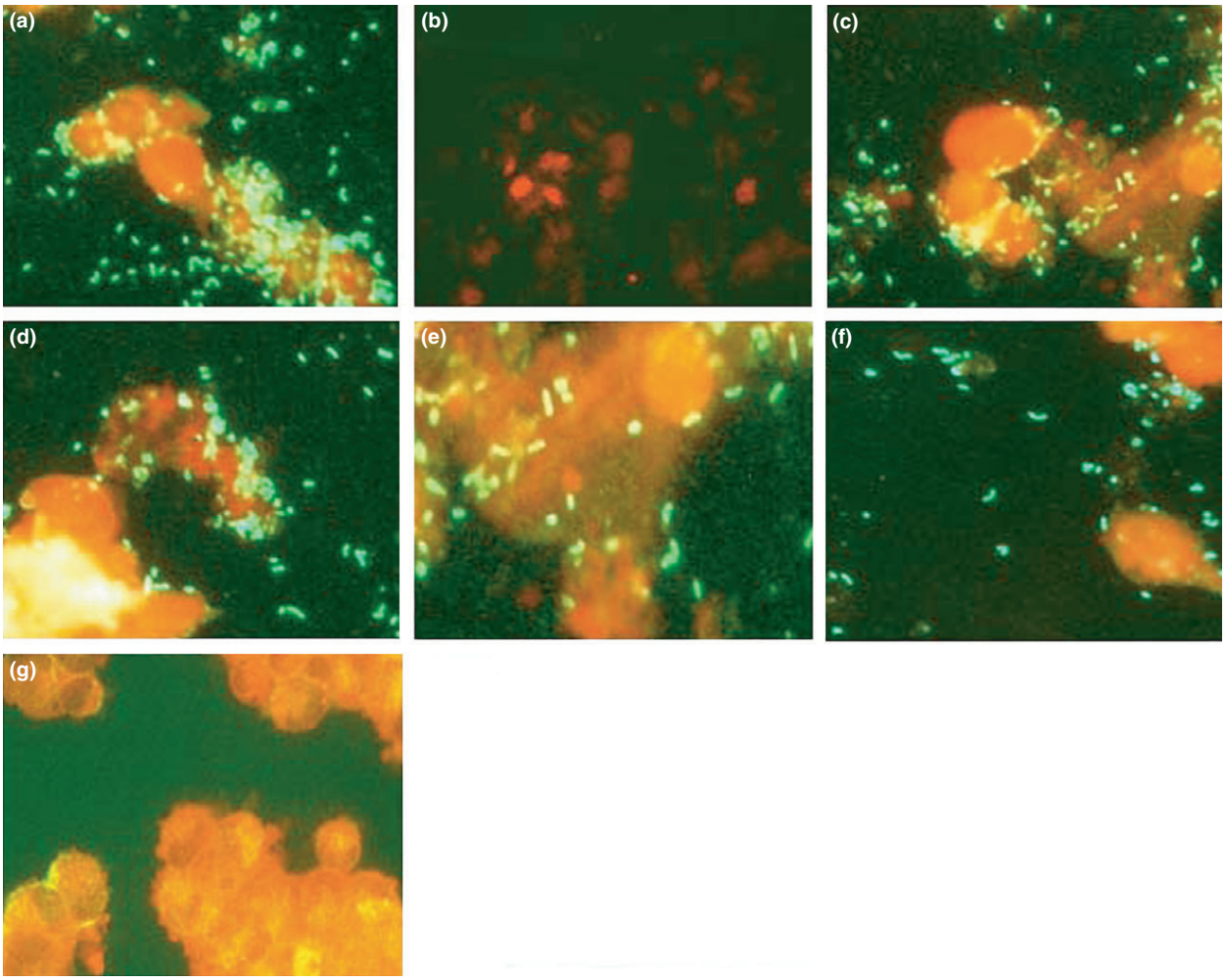

Fig. 2. Photomicrographs illustrating the effect of antibiotic treatment on *Diplorickettsia massiliensis*-infected XTC cells by IFA, 100×. Control, 15 days postinfection (a); negative control (b); rifampin (0.25 μg mL^{-1}) (c); rifampin (0.5 μg mL^{-1}) (d); rifampin (1 μg mL^{-1}) (e); rifampin (2 μg mL^{-1}) showed little infection compare with control (f); rifampin (4 μg mL^{-1}) showed no sign of infection after 15 days of treatment (g).

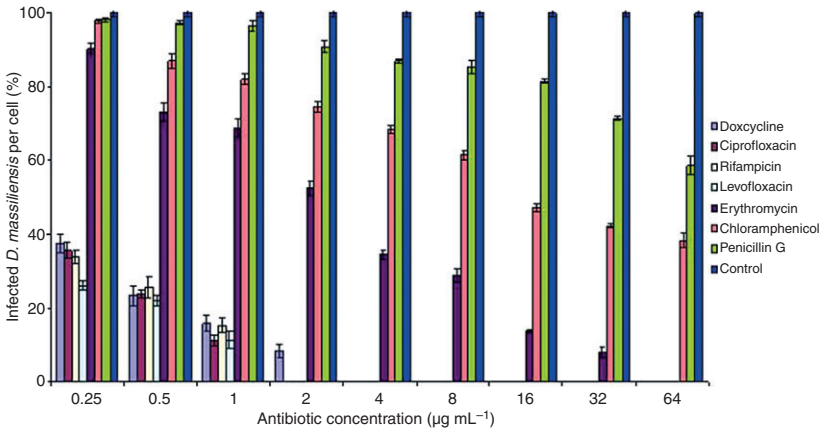

Fig. 3. Effect of different antibiotic treatments on *Diplorickettsia massiliensis*-infected XTC cells counting bacteria per cell. Effects of chloramphenicol, penicillin G, erythromycin, gentamycin, rifampin, ciprofloxacin, levofloxacin, and doxycycline on *D. massiliensis* in XTC-2 cell lines. A minimum of 20–40 cells were examined for each concentration. Results were compared with a positive control as the standard (minimum 100 bacteria per cell). The results are reported as the means and standard errors for the three replicates. The ANOVA, four drugs (doxycycline, ciprofloxacin, rifampin, and levofloxacin) states that the variation among the treatments (drugs) and the effect of dosage (4 μg mL^{-1}) are highly significant ($P < 0.01$).

growth at a concentration of 4 μg mL^{-1}, the MIC of gentamicin was 16 μg mL^{-1}. Surprisingly, *D. massiliensis* was resistant to chloramphenicol up to 64 μg mL^{-1}. Bacteria were also resistant to erythromycin, with a MIC value of 64 μg mL^{-1}. In contrast, ciprofloxacin, levofloxacin, and rifampin had MIC values of 2 μg mL^{-1} (Table 1).

Figures 3 and 4 illustrate the percentage of infection for each concentration. Doxycycline, ciprofloxacin, levofloxacin, and rifampin each showed a reduction in the percentage of infected cells, with no infection detected at concentrations as low as 4 and 2 μg mL^{-1}. However, in the cases of omeprazole (proton pompe inhibitor), penicillin G, and chloramphenicol, *D. massiliensis* bacteria were observed at all concentrations. Additionally, no bacteriostatic activity was detected for ammonium chloride (nonspecific inhibitor of phagocytosis), bafilomycin (inhibitor of vacuolar-type H+-ATPase), and chloroquine (lysosomotropic at low pH), substances that are all considered to be vacuole directed.

Combinations of chemicals

The experiments were also repeated with various combinations of drugs and antibacterial substances, including those that influence on phagocytosis and intravacuolar environment. The effect of chloramphenicol (from 0.25 to 64 μg mL^{-1}) was assessed in the presence of 20 nM of bafilomycin. The results of this experiment are shown in Table 1. The combination of bafilomycin and chloramphenicol was observed to be more effective than chloramphenicol only; no growth was observed by Gimenez staining at concentrations of 4 μg mL^{-1} of chloramphenicol and

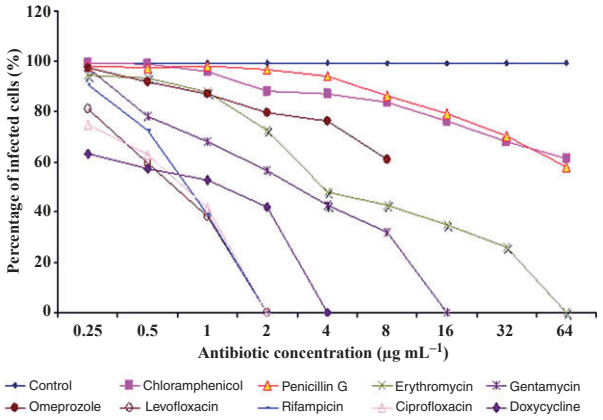

Fig. 4. Determination of degree of infection. A minimum 200 cells were counted for each concentration. We found that 90–99% of cells were infected in the control samples. Ciprofloxacin, levofloxacin, rifampin, and doxycycline showed that at higher concentrations, there are no bacteria. However, chloramphenicol and penicillin G showed an even higher concentration infection that was more than 50% when compared with the control. Also the interaction effects between the drugs and its dosage is also highly significant ($P < 0.01$). Among lowest drug, dosage of 0.5 ug could show significant difference ($P < 0.01$) from the control. The significant highest static effect was shown by at 0.5 ug compared with other drugs. Also the *Diplorickettsia* effect of rifampin at 0.5 (μg mL^{-1}) and 4 μg mL^{-1}) was not significantly different even at *P* value 5%. So rifampin can be considered at best and only Diploricketistatic candidate at 0.5 ug. A significant ($P < 0.01$) effect was observed in three drugs at a dosage of 2 (μg mL^{-1}) in ciprofloxacin, rifampin, and levofloxacin.

20 nM mL^{-1} of bafilomycin. Interestingly, when used individually, bafilomycin and chloramphenicol failed to exhibit an inhibitory effect on *D. massiliensis* growth.

Intracellular localization of *D. massiliensis*

Electron microscopy has shown that *D. massiliensis* is localized within vacuoles in the cells, indicating that the bacteria does not replicate in the cytosol. (Mediannikov *et al.*, 2010) However, the nature of the vacuole containing the bacterium is unknown. Hypothesizing that the cellular localization of *D. massiliensis* might play a role in antibiotic resistance and susceptibility, we investigated the vacuoles containing *D. massiliensis*. To examine the nature of the vacuoles, we utilized markers of the early phagosome (EEA1, early endosome-1), late phagosome and phagolysosome (Lamp-1, lysosomal-associated membrane protein-1), and phagolysosomes (active Cathepsin D).

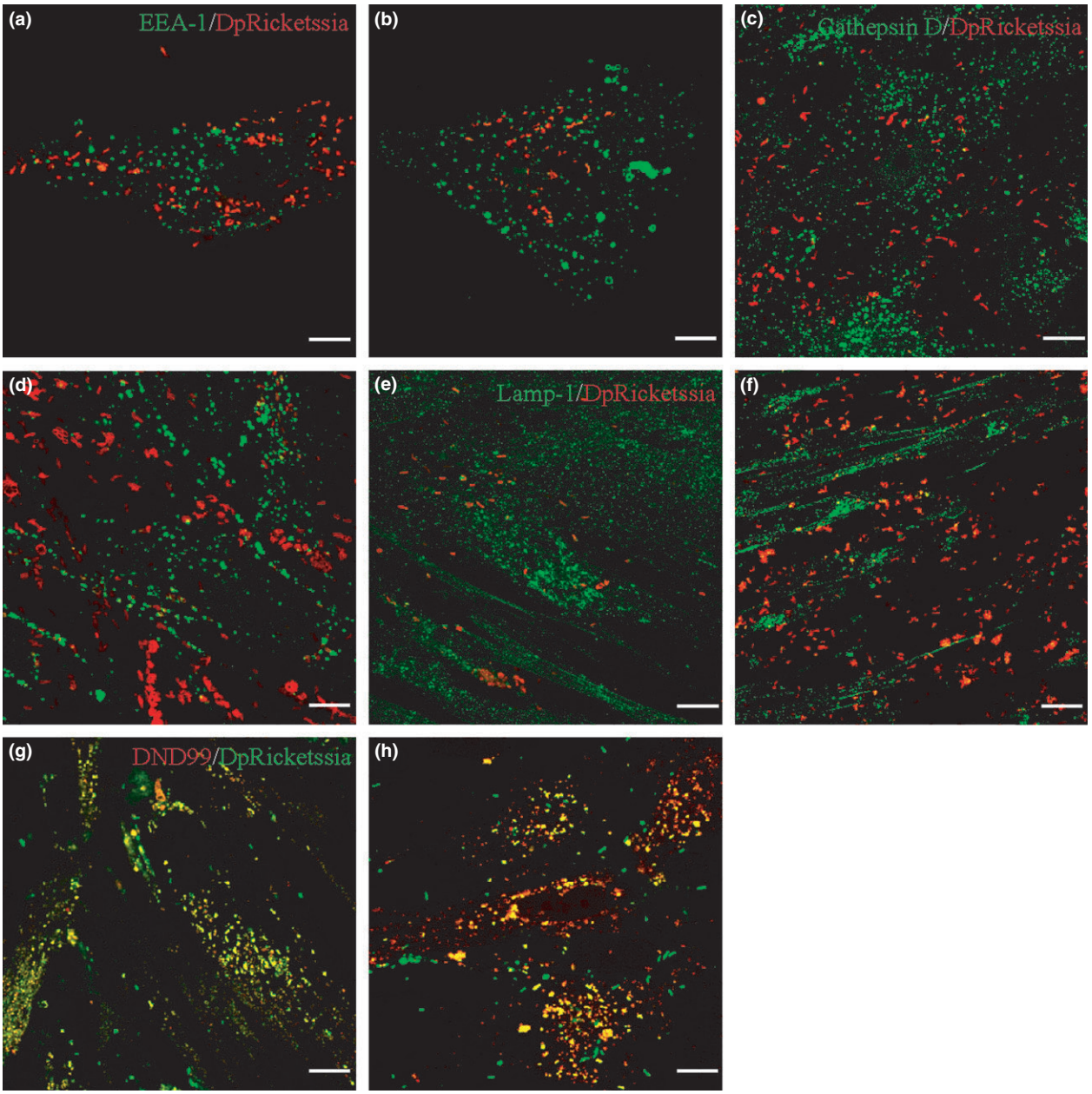

Fig. 5. Intracellular localization of *Diplorickettsia massiliensis* with HEL and MRC-5 cells. Intracellular localization *D. massiliensis* was studies in HEL cells (a, c, e, g) and in MRC-5 cells (b, c, f, h) by immunofluorescence and confocal microscopy. (a, b) Colocalization of *D. massiliensis* (red) with EEA-1 (green). (c, d) Colocalization of *D. massiliensis* with Cathepsin D (green). (e, f) colocalization of *D. massiliensis* (red) with Lamp-1 (green). (g, f) colocalization of *D. massiliensis* (green) with Lysotracker DND99 (red).

Table 2. Colocalization of *Diplorickettsia massiliensis* with compartment markers

	Percentage of colocalization with			
Cell lines	EEA-1	Cathepsin D	Lamp-1	DND99
HEL	0 ± 0	0	2 ± 1	97 ± 2
MRC-5	1 ± 0.5	0	3 ± 2	91 ± 7

Cells were incubated with *D. massiliensis*, after 7 days the number of bacteria that colocalized with EEA-1, Cathepsin D, Lamp-1, and Lysotracker red DND99 was scored. The results are expressed as the percentage of *D. massiliensis* that colocalized with EEA-1, Cathepsin D, Lamp-1, and Lysotracker red DND99 are the mean ± SD of three experiments.

First, we investigated EEA1, a marker of the early phagosome; *D. massiliensis* does not colocalize either with EEA1 in either HEL cells (Fig. 5a and Table 2) or in MRC-5 cells (Fig. 5b and Table 2). We also investigated markers for late phagosomes and phagolysosomes (Lamp-1) and phagolysosomes (Cathepsin D). *Diplorickettsia massiliensis* was not observed to colocalize with Cathepsin (Fig. 5c and Table 2) or Lamp-1 (Fig. 5d and Table 2) in HEL cells. Similar results were obtained with MRC-5 cells (Fig. 5e and f, and Table 2). These data suggest that *D. massiliensis* is not localized in early phagosomes, late phagosomes, or phagolysosomes. Next, the acidic nature of the compartments containing *D. massiliensis* was investigated using LysoTracker Red DND99, a weakly basic amine that selectively accumulates in compartments with low pH. Confocal microscopy revealed that more than 90% (Table 2) of *D. massiliensis* bacteria colocalized with DND99 in HEL (Fig. 5g) and MRC-5 cells (Fig. 5h). These data suggest that *D. massiliensis* is localized inside an acidic compartment within cells.

Discussion

The recently described bacterium *D. massiliensis* is phylogenetically related to *Rickettsiella* spp., *Legionella* spp., and *C. burnetii*, the etiologic agent of Q fever. This bacterium has been shown to grow successfully in humans cells (Mediannikov *et al.*, 2010). Epidemiologically, the bacterium is associated with the tick *I. ricinus*, a species known to bite humans. Despite of a number of infectious diseases transmitted by *Ixodes* ticks in Europe (Socolovschi *et al.*, 2009), there is still many cases without a proven etiological diagnosis (Hofmann, 1996; Sharma *et al.*, 2010). Some of these cases may be due to *D. massiliensis* infection.

In our study, the inhibitory effects of antimicrobial agents were assessed by two different methods, immunofluorescence and Gimenez staining. Intracellular bacteria in shell vial cultures were clearly visualized when infected cells were stained by immunofluorescence using mouse anti-*D. massiliensis* polyclonal antibody. We did not use real-time PCR for the evaluation because of its disadvantages: genomic DNA evaluation does dot correspond to live bacteria and does not take into account the degradation of bacterial DNA; cDNA reflect the number of metabolically active bacteria but is too sensitive and dependent of a gene selection.

In summary, our report describes for the first time the susceptibilities of *D. massiliensis* to a wide range of antibiotics as determined by Gimenez staining and immunofluorescence staining. The efficacies of all antibiotics tested in the control of *D. massiliensis* are summarized in Table 1. Our results show that doxycycline, ciprofloxacin, levofloxacin, and rifampin are effective in inhibiting *D. massiliensis* growth; however, we found that penicillin G, chloramphenicol, erythromycin, ammonium chloride, gentamycin, omeprazole, bafilomycin A1, and chloroquine were not.

The efficacies of antibiotics tested in the control of *D. massiliensis* were compared with *Coxiella*, *F. tularensis*, and *L. pneumophila* (Table 3). Consistent with our findings, the two quinolone antibiotics, ciprofloxacin, and levofloxacin as well as the drugs doxycycline and rifampin were more effective against *Coxeilla* (Raoult *et al.*, 1991), *F. tularensis* (Maurin & Raoult, 2001), and *L. pneumophila* (Edelstein & Meyer, 1980; Roch & Maurin, *2005*).

The resistance documented to chloramphenicol is quite unusual for most gamma-proteobacteria (Vester & Douthwaite, 2001; Maurin *et al.*, 2003). However, resistance has been reported in strict intracellular bacteria, such as *Ehrlichia chaffeensis* and *A. phagocytophilum* (Maurin *et al.*, 2003). The mechanisms whereby *D. massiliensis* gains resistance to chloramphenicol are yet unknown, but it could be explained by the activity of chloramphenicol acetyltransferase, which is encoded by the *cat* gene in *Escherichia coli* (Potrykus & Wegrzyn, 2001).

Doxycycline, rifampin, and macrolides have been shown to be highly active against strict intracellular bacteria such as *Rickettsia* spp., *C. burnetii*, and *Ehrlichia* spp. (Spicer *et al.*, 1981; Maurin & Raoult, 1996, 2001) In contrast, resistance to macrolides was identified in the case of *D. massiliensis*. In contrast, resistance to macrolides was identified in the case of *D. massiliensis*. One possible explanation is the alteration of specific nucleotides in the 23S rRNA gene, contained within the large ribosomal subunit (Vester & Douthwaite, 2001). We analyzed the 23S gene of *D. massiliensis* derived from the genome-sequencing project (data not shown). The sequence of the gene coding for 23S rRNA of *D. massiliensis* was compared with sequences from bacteria resistant to erythromycin (Branger *et al.*, 2004). However, no mutation corresponding for erythromycin resistance was

Table 3. Antibiotic MICs of *Diplorickettsia massiliensis*, *Coxiella burnetii*, *Legionella pneumophila*, and *Francisella tularensis* using Gimenez staining

		MIC (μg mL^{-1})				
		Coxiella burnetii				
S. no	Antibiotic	Nine mile	Q212	*Legionella pneumophila*	*Francisella tularensis*	*Diplorickettsia massiliensis*
1	Penicillin G	NA	NA	4–16	256	>64
2	Gentamycin	>10	>10	0.25–2	4	>16
3	Erythromycin	4	2	1	4	64
4	Doxycycline	4	2	1	8	4
5	Rifampin	4	4	≤ 0.001	0.5	2
6	Ciprofloxacin	4	2	0.06	0.25	2
7	Levofloxacin	2	2	0.03	0.12	2
8	Chloramphenicol	>8	>8	<1	4	>64

NA, data not available.

found. Other ribosomal genes may also be involved (protein L4, L22) as well as erm genes and/or efflux pumps; however, these remain to be investigated.

We investigated the effect of pH-neutralizing agents on *D. massiliensis* survival by Gimenez staining. The agents were used at the lowest concentrations that neutralized intracellular pH without affecting the viability of XTC-2 and MRC-5 cells. We found that *D. massiliensis* growth is not inhibited at a neutral pH with high concentrations of bacteria being detected.

Bafilomycin A1 is toxic macrolide antibiotic derived from *Streptomyces griseus* (Werner *et al.*, 1984). It is a strong inhibitor of the vacuolar-type H+-ATPase *in vitro* at nanomolar concentrations (Bowman *et al.*, 1988; Hanada *et al.*, 1990). In this study, we showed that when used alone, bafilomycin A1 is not effective against *D. massiliensis* infection. Nevertheless, in combination with chloramphenicol, it was active at a lower concentration. Decreased susceptibility of *D. massiliensis* to the antibiotics may be due to an alteration in the target of the drugs. Alkalinization of vacuoles has been shown to result in bactericidal activity of doxycycline against *C. burnetii* (Maurin *et al.*, 1992) omeprazole and bafilomycin and chloroquine when used individually have been found to be ineffective. A combination of doxycycline and chloroquine also displays bactericidal activity against *C. burnetii* (Raoult *et al.*, 1990).

Our study shows that *D. massiliensis* does not require an acidic pH for growth and that it can grow even under neutral pH conditions. The results of these studies indicate that antibiotic resistance does not depend on vacuolar pH; thus, a pH-related mechanism does not appear to play a contributing role in the overall resistance profile of *D. massiliensis*. *Diplorickettsia massiliensis* is localized within an acidic compartment that is not an early phagosome, late phagosome, or a phagolysosome. Therefore, although *D. massiliensis* survival does not require an acidic pH, it localizes within an acidic compartment inside infected cells.

In conclusion, our results suggest that the Gimenez staining method will work with a very low number of bacteria and can be used to determine the MICs of new therapeutic antibiotics more precisely. Additionally, this tool can be used in the future to help better define mechanisms of antibiotic resistance and to aid in the screening of new therapeutic drugs. According to possible acute infection because of *D. massiliensis* after tick bite, Doxycycline should be used as a reference treatment.

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