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# Analyse cellulaire et moléculaire de la transmission précoce de la borréliose de Lyme: rôle de l'interface cutanée

Quentin Bernard

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**Quentin BERNARD**

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**Analyse cellulaire et moléculaire de la transmission précoce de la borréliose de Lyme : rôle de l'interface cutanée**

THÈSE dirigée par :

Mme. BOULANGER Nathalie  
M. JAULHAC Benoit

Docteur, université de Strasbourg  
Professeur, université de Strasbourg

RAPPORTEURS :

Mme. BONNET Sarah I.  
M. MENARD Robert

Docteur, Ecole nationale vétérinaire d'Alfort, Paris  
Docteur, Institut Pasteur, Paris

AUTRES MEMBRES DU JURY :

Mme. FROSSARD Nelly  
M. FUSAI Thierry

Docteur, Faculté de pharmacie, Strasbourg  
Docteur, Institut de Recherche Biomédicale des Armées, Lyon

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## Liste des abréviations

ACA : Acrodermatite Chronique Atrophiante  
AD : Transactivator Domain  
ADN : Acide Désoxyribonucléique  
AIM2 : Absent In Melanoma 2  
Arg/Gln : Arginine /Glutamine  
ARN : Acide Ribonucléique  
B. burgdorferi sl : Borrelia burgdorferi sensu lato  
B. burgdorferi ss : Borrelia burgdorferi sensu stricto  
BIP : B Cell Inhibitor Protein  
BIR : Baculoviral Inhibitor of Apoptosis Repeat  
BSK : Barbour Stoenner Kelly  
CARD : Caspase Recruitment Domain  
CCL : Chemokine ligand  
CD : Cluster de Différenciation  
CDC : Centers Diseases Control  
CLR : C-type Lectin Receptor  
CRASP : Complement regulator-acquiring surface protein  
CRD : Carbohydrate-Recognition Domain  
DAI : DNA-dependent activator of IFN-regulatory factors  
DAMP : Damage-Associated Molecular Patterns  
db : Double Brin  
DbpA/B : Decorin Binding Protein A/B  
DC : Cellule Dendritique  
DC-SIGN : dendritic cell-specific ICAM3-grabbing nonintegrin  
DNGR-1 : DC NK lectin group receptor-1  
ELISA : Enzyme-Linked Immunosorbent Assay  
EM : Erythème Migrans  
EMM : Erythème Migrans Multiple  
FB : Fibroblaste  
hBD : human Beta Defensin  
HMGB1 : High-Mobility Group Box 1  
HSP : Heat Shock Protein  
I. scapularis : Ixodes scapularis  
IFN : Interféron  
Ig : Immunoglobuline  
IL : Interleukine  
Kb : kilobases  
KC : keratinocyte  
kDa : kilo Dalton  
KO : Knock-Out  
LB : Lymphocytome borrélien  
LBD1 : Ligand Binding Domain 1  
LCR : Liquide Céphalorachidien  
Lmp1 : surface-located membrane protein 1  
LPS : Lipopolysaccharide  
LTA : Acide lipotéchoïque



MAP : Mitogen Associated Protein  
MC : Mastocyte  
MCP-1/CCL2 : Monocyte Chemoattractant Protein-1  
MMP : Matrix Metalloproteinase  
MMR : Macrophage Mannose Receptor  
MyD88 : myeloid differentiation primary response 88  
NET : Nuclear Extra-cellular Trap  
NK : Natural Killer  
NLR : NOD Like Receptor  
OMP : Outer Membrane Protein  
OMS : organisation mondiale de la santé  
Osp : Outer Surface Protein  
PAMP : Pathogen-Associated Molecular Patterns  
PBMC : Cellule Mononucléée Périphérique  
PCR : Polymerase Chain Reaction  
PDGF : Platelet-Derived Growth Factor  
PGE2 : Prostaglandine E2  
PRR : Pathogen Recognition Receptor  
PYD : Pyrin Domain  
RANTES : Regulated on Activation, Normal T Cell Expressed and Secreted  
RLR : RIG-I-Like Receptor  
ROS : Reactive Oxygen Species  
RT-PCR : Reverse Transcription Polymerase Chain Reaction  
S. aureus : Staphylococcus aureus  
SCF : Stem Cell Factor  
SCID : Severe combined immunodeficiency  
Th : T helper/T auxiliaire  
t-HRF : tick histamine release factor  
TLR : Toll Like Receptor  
TNF : Tumor Necrosis Factor  
TRIF : TIR-domain-containing adapter-inducing interferon- $\beta$   
TSLPI : Tick Salivary Lectin Pathway Inhibitor  
VlsE : Variable major protein-like sequence

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# Introduction

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Partie I

LA BORRELIOSSE DE LYME

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## I. Historique de la maladie

C'est en 1883 que la description de manifestations cliniques associées à la Borréliose de Lyme, première maladie vectorielle de l'hémisphère nord, apparaît pour la première fois. Le docteur allemand Alfred Buchwald décrit une anomalie de peau semblable à l'acrodermatite chronique atrophiante (ACA), l'une des manifestations tardives de la maladie de Lyme (Buchwald 1883). Toutefois, le lien avec une piqûre de tique n'est pas directement établi. En 1909, le dermatologue Arvid Afzelius remarque l'apparition d'une lésion en forme de cible apparaissant à la suite de piqûres de tiques (Afzelius 1910). Cette lésion, manifestation précoce caractéristique de la borréliose de Lyme, sera qualifiée d'érythème migrans (EM) quelques années plus tard par Lipschütz (Lipschütz 1913). En 1922, les médecins français Garin et Bujadoux décrivent pour la première fois une manifestation neurologique associée à la piqûre d'une tique et à l'apparition d'un EM (Garin and Bujadoux 1993). C'est alors que les hypothèses d'un virus, de bactérie du genre *Rickettsia* ou de toxines de tiques comme agents responsables de ces symptômes sont émises. En 1948, Lennhoff propose la présence d'entités comparables à des spirochètes comme responsables de l'EM, mais l'hypothèse sera par la suite réfutée (Lennhoff 1948). En 1951, l'efficacité du traitement à la pénicilline confirme toutefois l'origine infectieuse de la borréliose de Lyme (Hollstrom 1951).

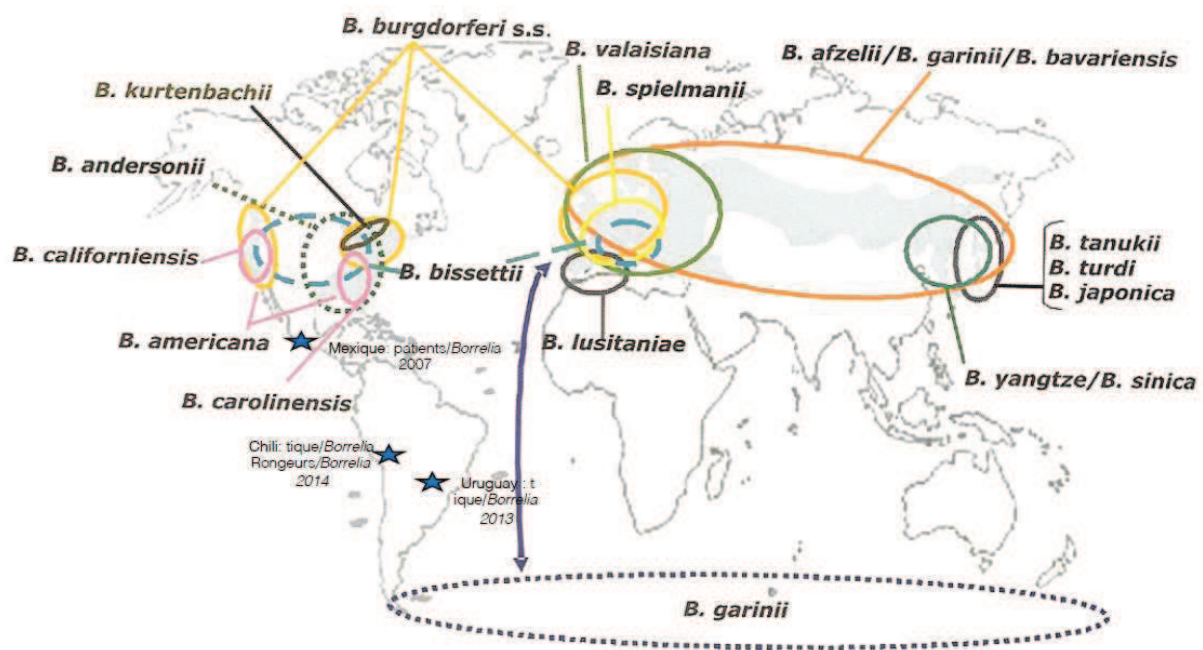
L'histoire se poursuit aux États-Unis lorsqu'en 1969, le premier cas d'EM est décrit par Scrimenti dans l'état du Wisconsin (Scrimenti 1970). Dans les années 70, Allen Steere observe une prévalence d'oligoarthrite, une autre manifestation tardive de la maladie, cent fois plus élevée dans la ville de Lyme que dans le reste des États-Unis (Steere et al. 1977). Les patients rapportent presque systématiquement avoir été piqué par une tique les quelques mois précédant le début des douleurs articulaires. De plus, ils décrivent l'apparition peu de temps après la piqûre de la tique d'un EM. À la suite de ces différentes observations et de la caractérisation des différents symptômes possibles, le nom de « maladie de Lyme » commence à être évoqué. Parallèlement, en Australie, des cas d'arthrite de Lyme commencent à être décrits (Stewart et al. 1982). En 1981, Alan Barbour parvient à cultiver les spirochètes suspectées d'induire la maladie de Lyme à l'aide d'un milieu de Kelly modifié. Il faudra cependant attendre 1983 pour que Willy Burgdorfer incrimine définitivement les spirochètes isolés de tique qui après inoculation chez des lapins induisent après plusieurs semaines un EM (Barbour et al. 1983a).

Dans les années qui suivent, la pathologie infectieuse est officiellement appelée « maladie/Borréliose de Lyme ». L'isolement de la bactérie se fait alors aussi en Europe sur tiques, sur prélèvement sanguin, biopsie cutanée et liquide céphalorachidien (LCR). C'est Johnson en 1984, après

avoir publié ses travaux sur la taxonomie de la bactérie, qui propose de nommer cette dernière *Borrelia burgdorferi* en hommage au professeur Willy Burgdorfer (Johnson et al. 1984).

## II. Epidémiologie de la borréliose de Lyme

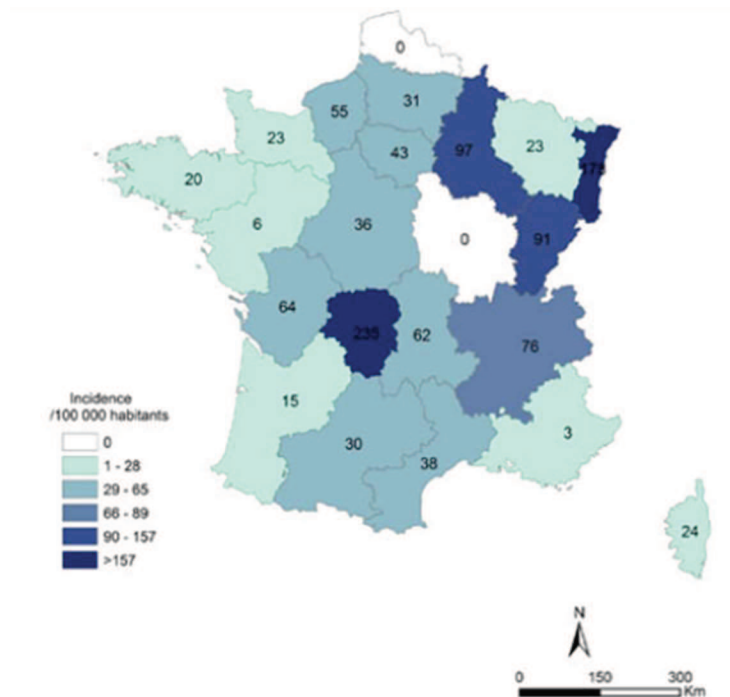
Différentes espèces de bactéries au sein du complexe *Borrelia burgdorferi* sensu lato (sl) sont capables d'induire la borréliose de Lyme (**Figure 1**). Les trois espèces majoritairement responsables de la maladie chez l'Homme sont *B. burgdorferi* sensu stricto (ss), *B. afzelii* et *B. garinii*. Les trois espèces sont présentes sur le continent européen alors que seule *B. burgdorferi* ss est retrouvée sur le continent américain (Sonenshine and Roe 2014c).



**Figure 1.** Distribution géographique de l'agent infectieux de la borréliose de Lyme (Sonenshine and Roe 2014c). ★ Mise en évidence de *Borrelia* dans l'hémisphère sud

En tant que maladie vectorielle la plus répandue de l'hémisphère nord, la borréliose de Lyme touche plus de 100 000 personnes par ans. En Europe, 85 000 cas par an sont estimés (Rapport de l'OMS, 2006) contre 30 000 cas aux États-Unis (Rapport du CDC, 2013). Cependant, ce nombre serait largement sous-estimé. Les zones les plus touchées correspondent aux aires boisées, exposant les forestiers, les chasseurs, les agriculteurs ou encore les randonneurs à un risque plus élevé (Lindgren and Jaenson 2006). Puisque la transmission de la bactérie à l'hôte vertébré se fait par l'intermédiaire d'un

vecteur, la tique du genre *Ixodes*, le risque de contracter la maladie est directement lié à l'abondance de ce dernier. Un pic d'activité de la maladie est ainsi généralement observé pendant la période printemps/été, correspondant au pic d'activité des tiques, mais aussi à l'abondance d'activités humaines en environnement extérieur (Kurtenbach et al. 2006). De plus, les régions touchées par la borréliose de Lyme correspondent aux territoires qui présentent un biotope permettant la survie et le développement des tiques du genre *Ixodes*. En France entre 2009 et 2011 (**Figure 2**), l'incidence moyenne de la borréliose de Lyme était d'environ 43 pour 100 000 habitants, avec des régions comme l'Alsace où celle-ci dépasse les 150 pour 100 000 habitants (inVS 2013).



**Figure 2.** Estimation de l'incidence de la borréliose de Lyme en France en 2009-2011

(D'après inVS/Cires 2013)

### III. Aspect clinique

L'évolution de la borréliose de Lyme suit théoriquement un schéma en trois étapes. Une phase localisée précoce, une phase disséminée précoce et une phase disséminée tardive. Cependant, le type et la cinétique des symptômes associés à ces trois étapes peuvent varier selon le continent et l'espèce bactérienne considérés. De plus, l'observation clinique n'est pas toujours en adéquation totale avec



l'évolution théorique en plusieurs étapes (Wormser et al. 2006). En effet, certaines études décrivent des patients ayant développé des manifestations tardives sans apparition préalable de manifestations précoces (Strle and Stanek 2009).

## 1. La phase localisée précoce

La transmission à l'hôte vertébré de la bactérie, par la tique, se fait au niveau de l'interface cutanée. C'est donc logiquement à ce niveau que va apparaître, dans 89% des cas, le premier symptôme caractéristique : l'EM (**Figure 3**) (Stanek et al. 2012). Cette lésion inflammatoire circulaire apparaît généralement une à deux semaines après la transmission de la bactérie. Elle peut perdurer plusieurs mois et atteindre un diamètre de 80 cm si le patient n'est pas traité (Boyé 2007). Elle reflète la réponse inflammatoire de l'hôte vertébré face à la diffusion de la bactérie dans les tissus cutanés. L'inspection visuelle de l'EM, manifestation la plus caractéristique de la borréliose de Lyme, suffit dans la plupart des cas au diagnostic précoce de la maladie. Des méthodes de cultures ou de PCR sur des biopsies d'EM peuvent être utilisées à des fins de recherche, mais elles ne sont pas nécessaires pour le diagnostic clinique de routine.



**Figure 3.** La manifestation caractéristique précoce de la borréliose de Lyme : l'érythème migrans (Stanek et al. 2012).

## 2. La phase disséminée précoce

Les symptômes de la phase disséminée précoce interviennent plusieurs semaines à plusieurs mois après l'infection par la bactérie. Cette dernière a alors disséminé principalement par voie sanguine pour atteindre les différents organes cibles : la peau à distance, les articulations, le cerveau et/ou le cœur.

## A. Les manifestations cutanées

Il existe principalement deux types de manifestations cutanées associées à la dissémination hémotogène de la bactérie : l'érythème migrans multiple (EMM) et le lymphocytome borrélien (LB) (**Figure 4**). L'EMM, qui intervient dans 5 à 20% des cas, dépasse rarement le nombre de 7 lésions même si certains cas dépassant les 30 lésions ont été observés (Constantin et al. 2000). Le LB, observé en Europe, mais pas aux États Unis, est un nodule non douloureux qui intervient dans 2% des cas au niveau de l'oreille, l'aréole mammaire ou le scrotum. Le rapprochement entre le LB et la borréliose de Lyme est effectué à l'aide d'un test sérologique (Stanek et al. 2012).



**Figure 4.** Les manifestations précoces disséminées de la borréliose de Lyme : L'érythème migrans multiple et le Lymphocytome borrélien (Stanek et al. 2012)

## B. Les manifestations articulaires

Dans le contexte de la borréliose de Lyme, l'arthrite touche environ 5% des personnes infectées par la bactérie. Elle se caractérise par un gonflement des grosses articulations telles que les genoux. Le diagnostic se confirme généralement à l'aide d'une sérologie ciblant les IgG et peut être complété par une PCR ciblant la bactérie à partir du liquide synovial.

## C. Les manifestations neurologiques

Les manifestations neurologiques interviennent dans environ 3% des cas. Les symptômes chez l'adulte sont de l'ordre de la méningoradiculite, de la méningite ou encore de la paralysie faciale périphérique. Chez les enfants, les symptômes sont relativement identiques à l'exception de la méningoradiculite (Stanek et al. 2011, 2012). Le diagnostic se fait généralement à l'aide d'un prélèvement intrathécale du LCR sur lequel une détection d'anticorps dirigés contre la bactérie est effectuée.

## D. Les manifestations cardiaques

Les manifestations cardiaques représentent les symptômes les plus rares de la maladie de Lyme. Elles englobent des troubles soudains de la conduction auriculo-ventriculaire, des troubles du rythme cardiaque et parfois des myocardites ou des péricardites (van der Linde 1991).

### 3. La phase disséminée tardive

La phase disséminée tardive correspond à des manifestations chroniques des symptômes précédemment énumérés. Bien que les différentes espèces de *Borrelia* responsables de la maladie de Lyme soient capables d'induire l'ensemble des symptômes décrits, l'organotropisme selon l'espèce de bactérie considérée semble varier. Ainsi en Europe et en Asie, *B. afzelii* peut persister dans la peau pendant des années avant de provoquer une ACA (**Figure 5**) (Steere et al. 2004). Toujours sur les mêmes continents, *B. garinii* est davantage retrouvée au niveau du système nerveux central. Elle peut alors induire plusieurs symptômes neurologiques tels que des encéphalomyélites ou des encéphalopathies (Oschmann et al. 1998). Enfin, *B. burgdorferi*, la seule espèce présente sur le continent américain, est plus spécifiquement associée aux symptômes d'arthrites chroniques. Environ 60% des patients non traités subissent des poussées d'arthrites chroniques plusieurs mois après l'infection, généralement au niveau du genou (Steere et al. 1987). Tous symptômes confondus, 80% des borrélioses de Lyme impliquent des manifestations cutanées (Mullegger 2004). Cette incidence élevée pointe du doigt l'importance de la peau dans l'établissement de la maladie.



**Figure 5.** L'évolution chronique de la borréliose de Lyme affectant la peau : l'acrodermatite chronique atrophiante. (copyright 1996 – 2015 DermIS)

## 4. Diagnostic et traitement de la Borréliose de Lyme

### A. Le diagnostic de la maladie

La présence d'un EM, l'inflammation caractéristique précoce de la borréliose de Lyme, est le seul symptôme permettant d'établir un diagnostic uniquement à partir de l'observation clinique. Le LB, la méningoradiculite et l'ACA sont fortement corrélés à l'infection par *Borrelia* mais nécessitent d'être validés en laboratoire. Les autres symptômes possibles ne sont pas spécifiques de la borréliose de Lyme. Afin de confirmer une infection par *Borrelia*, différents tests biologiques peuvent être effectués. Classiquement, une sérologie par ELISA et/ou western-blot (IgM/IgG) est effectuée afin de confirmer ou d'infirmer la présence d'une infection par *Borrelia*. Cependant le test sérologique est limité. Les anticorps persistant pendant des années, il ne permet pas de distinguer entre une infection active et une infection passée. Idéalement, la mise en évidence par culture dans un milieu spécifique (BSK) de bactéries vivantes ou d'ADN par PCR ciblant le gène de la flagelline à partir de biopsie d'un tissu infecté par *Borrelia*, du fluide cérébrospinal ou synovial ou du sang permet de confirmer le diagnostic.

### B. Le traitement de la maladie

Le traitement par antibiotiques est effectué à tous les stades de la maladie. Cependant, plus le traitement est effectué de manière précoce, plus il est efficace (Stanek et al. 2012). Les antibiotiques classiquement utilisés sont la doxycycline et l'amoxicilline. Selon l'état d'avancement de la maladie, d'autres antibiotiques tels que la ceftriaxone, l'azithromycine ou la pénicilline peuvent être prescrits. Globalement, l'efficacité du traitement dépend de l'évolution de la maladie, mais aussi du choix de l'antibiotique, de la dose, de la durée du traitement et des potentiels effets secondaires de ce dernier. L'administration des antibiotiques se fait par voie orale, sauf dans le cas d'atteintes neurologiques où elle peut être effectuée par intraveineuse (Stanek et al. 2012). Certains symptômes notamment articulaires semblent réapparaître malgré le traitement chez certains patients. L'efficacité du traitement ayant été prouvée (Wormser 2003), les douleurs persistantes peuvent être dues à des séquelles liées aux dommages tissulaires induits par la bactérie lorsqu'elle était présente, ou dans certains cas, à une nouvelle infection.

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Partie II

L'AGENT PATHOGENE ET  
SON VECTEUR

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# I. L'agent pathogène : *Borrelia burgdorferi* sensu lato

## 1. Taxonomie

*B. burgdorferi* sl est un complexe de bactéries appartenant à la classe des spirochètes (Figure 6). Trois familles de bactéries composent cette classe : les Brachyspiraceae, les Leptospiraceae et les Spirochaetaceae. *Borrelia* appartient à la famille des Spirochaetaceae. Cette famille comprend d'autres agents pathogènes importants en santé humaine tels que *Treponema pallidum*, l'agent responsable de la syphilis et le genre *Leptospira*. Il existe deux groupes bien distincts au sein des *Borrelia*. L'un regroupe les espèces responsables de fièvres récurrentes, l'autre les espèces à l'origine de la borréliose de Lyme. Parmi les espèces capables d'induire la maladie de Lyme, trois sont majoritairement représentées : *B. burgdorferi* ss, *B. afzelii* et *B. garinii*. D'autres espèces du même complexe mais moins fréquentes sont aussi capables de provoquer la borréliose de Lyme : *B. bavariensis*, *B. bissettii*, *B. kurtenbachii*, *B. lusitaniae*, *B. spielmanii* et *B. valaisiana* (Rudenko et al. 2011).

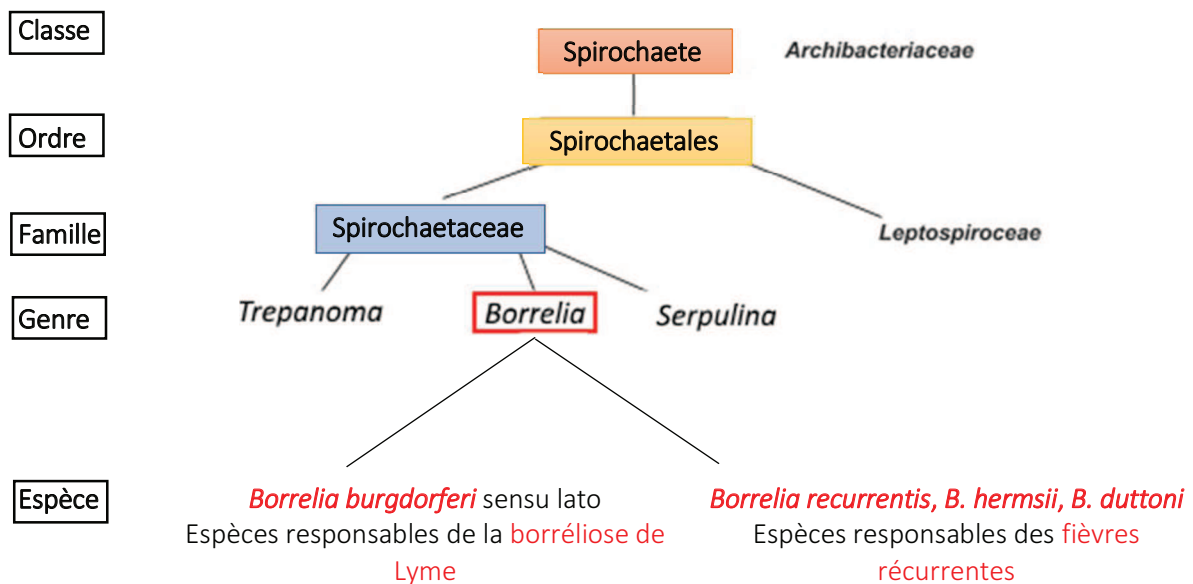


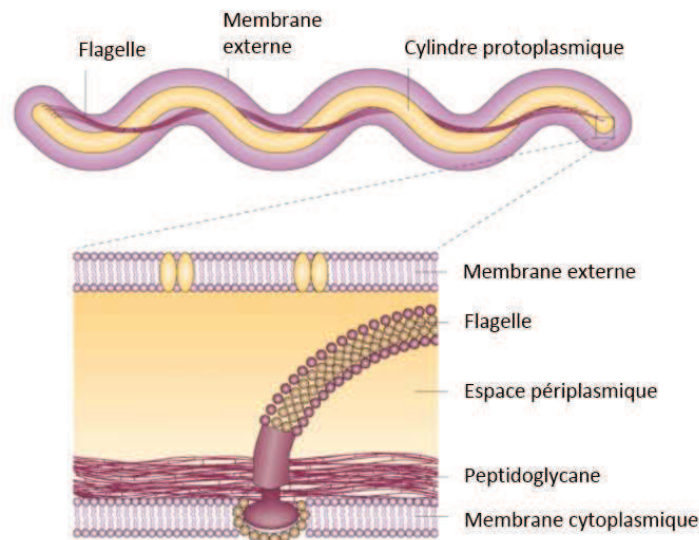
Figure 6. Classifications des *Borrelia* au sein de la classe des spirochètes

(D'après Schröder et al. 2008)

## 2. Caractéristiques morphologiques

Les *Borrelia*, comme l'ensemble des bactéries de la classe des spirochètes, ont une apparence spiralée. *B. burgdorferi* est caractérisée par une longueur de 20 à 30 µm et une largeur de 0,2 à 0,3 µm. De l'extérieur vers l'intérieur, la structure des *Borrelia* est constituée d'une membrane externe, d'un

espace périplasmique contenant notamment des flagelles et une couche de peptidoglycanes puis d'une membrane interne (**Figure 7**). Les flagelles sont ancrés de part et d'autre du corps protoplasmique tout en étant enroulés autour de ce dernier, donnant la forme spiralee caractéristique aux *Borrelia* en plus d'assurer leur mobilité (Barbour and Hayes 1986). Contrairement aux bactéries à Gram négatif classiques, les *Borrelia* ne possèdent pas de lipopolysaccharides (LPS). La structure des *Borrelia* leur permet une mobilité plus efficace en milieu visqueux. L'expression des protéines impliquées dans la mobilité de la bactérie est régulée par environ 6% de son génome (Karami 2012).



**Figure 7.** Caractéristiques morphologiques de *Borrelia*, d'après (Rosa et al. 2005)

### 3. Génétique

Le génome de *Borrelia* est composé d'un chromosome linéaire d'environ 900 kb ainsi que de plusieurs plasmides linéaires et circulaires d'une taille d'environ 400 kb en moyenne. Les génomes des trois espèces *B. burgdorferi* ss, *B. afzelii* et *B. garinii* ont été séquencés. Le chromosome principal contient 90% de séquences codantes essentiellement attribuées à des gènes de ménage de la bactérie. Il est constant en contenu et en structure au sein du genre *Borrelia*. Par opposition, les plasmides codent essentiellement les gènes de virulence de la bactérie, notamment les protéines de surface, et leurs séquences diffèrent d'une espèce de *Borrelia* à une autre. Ils se caractérisent aussi par la présence de nombreuses séquences paralogues, de pseudogènes et même de gènes essentiels.

Catégorie	% de gènes chez <i>B. burgdorferi</i>
Métabolisme intermédiaire	4.9
Biosynthèse de petites molécules	3.1
Métabolisme des macromolécules	22.2
Structure de la cellule	37
Processus cellulaires	7.4
Autres fonctions	5.6
Fonctions inconnues	19.8

**Tableau 1.** Composition du génome de *B. burgdorferi* B31 réparti selon les fonctions cellulaires associées (Karami 2012)

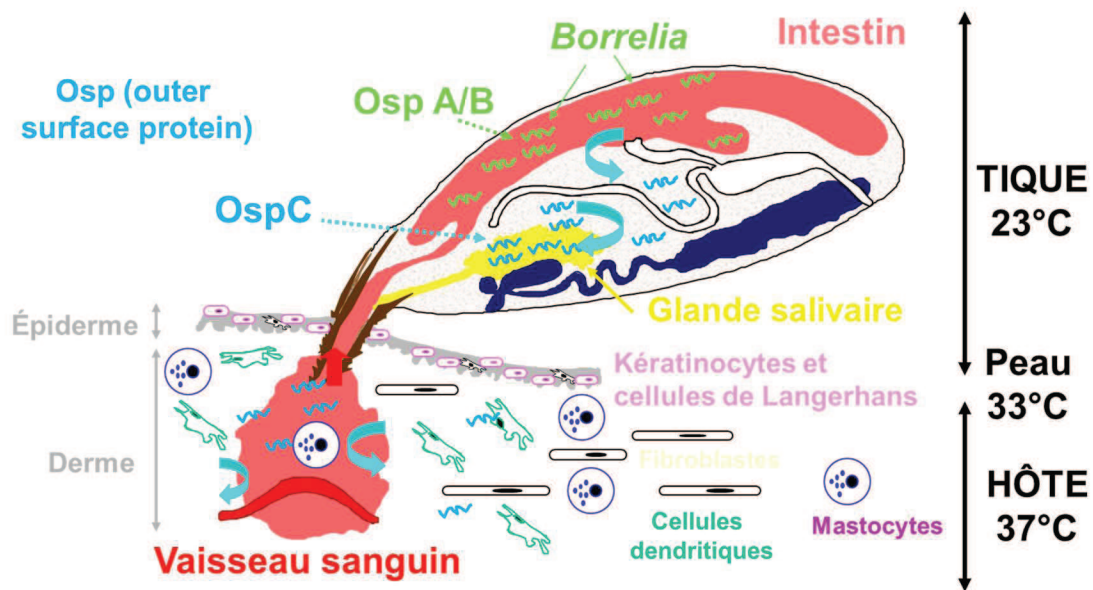
Le génome le mieux connu appartient à la souche B31 (**Tableau 1**) qui appartient à l'espèce *B. burgdorferi* ss. Ce génome est composé d'un chromosome linéaire et de 12 plasmides linéaires (lp5, lp17, lp21, lp25, lp28 (1-4), lp36, lp38, lp54 et lp56) et 9 plasmides circulaires (cp9, cp26, cp32 (1-9)). Deux des plasmides linéaires sont essentiels à l'infection de l'hôte vertébré. Ces deux plasmides, lp25 et lp28-1, sont particulièrement instables lors de la culture *in vitro* de la bactérie et ont tendance à disparaître après plusieurs générations. Parmi les plasmides circulaires, les plasmides de 32 kb appelés cp32s semblent jouer un rôle dans le transfert génétique horizontal (Rosa et al. 2005). Il peut en exister jusqu'à 9 variants différents au sein d'une même bactérie.

#### 4. Les protéines de surface de *Borrelia* : facteurs d'interaction avec l'hôte vertébré et le vecteur

Le cycle de vie de *Borrelia* nécessite l'alternance entre un hôte vertébré et un vecteur. À l'hôte vertébré et au vecteur correspondent deux environnements différents auxquels la bactérie doit pouvoir s'adapter. La bactérie modifie alors l'expression de ses gènes en fonction de l'environnement dans lequel elle se situe (**Figure 8**). Dans le but d'identifier de nouvelles protéines de surface de *Borrelia* associées à ces deux environnements, de nombreuses expériences de microarrays ont été réalisées dans les années 2000. Ce type d'expériences a permis de mesurer la réponse globale de l'expression des



gènes de *Borrelia* à la suite de la mise en culture de la bactérie dans des conditions mimant l'environnement de l'hôte vertébré, ou après une modification de la température de culture (Revel et al. 2002; Brooks et al. 2003; Ojaimi et al. 2003). Lors de ces expériences, l'élévation de la température permettait l'analyse des gènes normalement exprimés lors du passage des *Borrelia* de la tique (23°C) à l'hôte vertébré (33°C puis 37°C). Parmi ces gènes surexprimés, cinquante d'entre eux codaient des protéines associées à des peptides signaux, indiquant un rôle potentiel de ces protéines au niveau de la membrane externe. Les études qui suivirent ont permis de caractériser de nombreuses protéines de surface, qui peuvent être divisées en deux catégories : les lipoprotéines ancrées dans la surface via la



partie lipidique (Outer surface protein = Osp) et les protéines possédant un ou plusieurs domaines transmembranaires (Outer Membrane Protein = OMP).

**Figure 8.** Cycle de transmission de *Borrelia* de la tique du genre *Ixodes* à l'hôte vertébré (d'après Nathalie Boulanger)

## A. Les lipoprotéines majeures de *Borrelia*

### a. De OspA à OspF

Les propriétés antigéniques de **OspA** et **OspB** ont permis d'identifier ces deux lipoprotéines (Barbour et al. 1983b; Howe et al. 1985). Ce sont deux molécules d'une trentaine de kilodaltons chacune, transcrites à partir du même promoteur sur le plasmide lp54 (Fraser et al. 1997). Ces deux protéines sont exprimées lors de la présence de *Borrelia* dans l'intestin de la tique, puis sont réprimées

lors du début du repas sanguin (Schwan et al. 1995; Schwan and Piesman 2000; Hefty et al. 2002b). Ces deux protéines sont essentielles à l'acquisition de la bactérie par la tique, et à la persistance de *Borrelia* dans le vecteur. Cette persistance se fait notamment grâce à la liaison d'OspA avec le récepteur TROSPA présent dans l'intestin de la tique (Pal et al. 2004a). **OspD** est une protéine de 28 kDa aussi capable de se lier aux extraits d'intestin de tiques (Li et al. 2007). Cependant, le fait qu'il existe des isolats cliniques dépourvus d'OspD indique que cette lipoprotéine n'est pas essentielle à la survie de la bactérie (Marconi et al. 1994; Li et al. 2007).

**OspC** est la plus étudiée des lipoprotéines de *Borrelia*. Cependant, le rôle de cette protéine de 22 kDa n'a toujours pas été complètement élucidé. OspC est surexprimée lors du passage de *Borrelia* de la tique à l'hôte vertébré, tandis qu'OspA et OspB sont réprimées (Radolf and Caimano 2008). OspC semble être une lipoprotéine essentielle à la transmission de la bactérie puisque la bactérie mutante pour OspC est incapable d'infecter la souris (Grimm et al. 2004; Tilly et al. 2006). Il a été démontré que cette protéine est essentielle dans l'infection précoce et qu'elle n'intervient pas dans la persistance chez l'hôte vertébré. La capacité d'OspC à lier une protéine de la salive de tique, **Salp15**, permettant de camoufler la bactérie du système immunitaire indique un rôle dans la survie de la bactérie (Ramamoorthi et al. 2005). La protéine OspC n'est pas seulement impliquée dans l'échappement immunitaire puisque le mutant OspC est toujours incapable d'infecter des souris immunodéficientes (mutantes pour le facteur de différenciation myéloïde 88 (MyD88) ou SCID) (Stewart et al. 2006). Plus récemment, il a été proposé qu'OspC est impliquée dans la liaison à une hypothétique molécule de l'hôte vertébré par l'intermédiaire d'un domaine LBD1 (Ligand Binding Domain 1) permettant sa dissémination (Earnhart et al. 2010). Son importance et son immunogénicité ont fait d'OspC un candidat vaccinal idéal. Cependant, il existe une variation importante des séquences d'OspC au sein des différentes espèces et souches de *Borrelia* qui rend le développement d'un vaccin difficile (Wilske et al. 1996; Wang et al. 1999b; Brown et al. 2005; Earnhart et al. 2005, 2007).

**OspE** et **OspF** ont été découvertes chez la souche de *B. burgdorferi* ss N40 co-transcrits sur le même plasmide (Lam et al. 1994). Cependant, chez les autres souches, ces deux gènes sont présents sur des plasmides différents. OspE peut se lier au plasminogène, ce qui pourrait jouer un rôle dans la dissémination de *Borrelia*. Le rôle d'OspF est moins connu, mais semble être lié au tropisme tissulaire lors de la transmission de *Borrelia* (Hefty et al. 2002a, 2002b; Miller et al. 2003; Antonara et al. 2007)

## b. Les protéines d'adhésion au sein de l'hôte vertébré

Parmi les protéines de surfaces de *Borrelia*, certaines permettent à la bactérie de se fixer aux cellules afin d'assurer sa dissémination. Les plus étudiées sont **DbpA**, **DbpB** et **BBK32**. Les deux premières sont capables de lier la décorine, un protéoglycane qui peut interagir avec la fibronectine (Guo et al. 1995), un constituant majeur de la matrice extracellulaire. Leur expression est augmentée lors de l'infection (Carroll et al. 2000; Revel et al. 2002; Ojaimi et al. 2003). Cependant, elles ne sont pas essentielles à la transmission de *Borrelia*, bien que leur présence augmente la virulence de la bactérie (Blevins et al. 2008). BBK32 est une glycoprotéine capable de lier la fibronectine, l'héparine et le dermatan sulfate permettant à *Borrelia* de se mouvoir dans la matrice extracellulaire (Szczepanski et al. 1990; Probert and Johnson 1998). Tout comme DpbA et DpbB, la protéine BBK32 participe à la virulence de *Borrelia*, mais n'est pas essentielle à la transmission (Li et al. 2006).

## c. Les protéines de l'échappement immunitaire

La plupart des lipoprotéines de surface de *Borrelia* jouent un rôle dans l'adhésion aux cellules de mammifères ou à la dissémination de la bactérie. Un autre rôle important impliquant les lipoprotéines est l'échappement immunitaire. OspC est capable de protéger la bactérie en liant la protéine Salp15 de la salive de tique, mais d'autres lipoprotéines sont capables d'inhiber directement des constituants impliqués dans l'immunité de l'hôte vertébré. Parmi celles-ci, on trouve les **CRASPs** (« Complement Regulator-Acquiring Surface Proteins ») et **OspE** (Hellwage et al. 2001). OspE, en plus de lier le plasminogène (Brisette et al. 2009), est capable de lier le facteur H du système du complément, permettant d'inhiber son action délétère vis-à-vis de la bactérie. Parmi les CRASPs, CspA et CspZ ont aussi été caractérisées. Ces deux protéines sont aussi capables de lier le facteur H (Brooks et al. 2005; Hartmann et al. 2006; Kenedy et al. 2009). Cependant, CspA n'est pas exprimé dans l'hôte vertébré. Il est ainsi suggéré que CspA joue un rôle protecteur dans la tique lors de l'initiation du repas sanguin (McDowell et al. 2006; Bykowski et al. 2007). CspZ est exprimé dans l'hôte vertébré, mais ne semble pas jouer un rôle essentiel dans l'infection de celui-ci (Coleman et al. 2008).

Une autre protéine spécialisée dans l'échappement immunitaire de *Borrelia*, mais n'appartenant pas à la catégorie des CRASPs est la protéine **VlsE**, pour « Variable major protein-like sequence » (Zhang et al. 1997). Cette protéine est codée à partir de 15 cassettes présentes sur le plasmide lp28-1. Chacune des cassettes possède six régions variables et six régions constantes. La partie variable est la plus exposée aux anticorps de l'hôte vertébré (Kumaran et al. 2001). La conversion

génique de *VlsE* commence dès 4 jours après l'infection, et continue tout au long de celle-ci (Zhang and Norris 1998). La variation de la séquence de *VlsE* est moins fréquente chez les souris immunodéficientes, soulignant l'importance de facteurs immunitaires de l'hôte vertébré dans la conversion génique de *VlsE*.

## B. Les protéines de surface transmembranaires

Les protéines transmembranaires de *Borrelia* (OMPs), contrairement aux Osps, ne possèdent pas de partie lipidique en N-terminal. La plupart des OMPs sont codées à partir de *loci* chromosomiques stables, indiquant un rôle physiologique essentiel de ces dernières. La faible quantité et immunogénicité des OMPs rend l'étude de celles-ci compliquée (Radolf et al. 1994; Fraser et al. 1997).

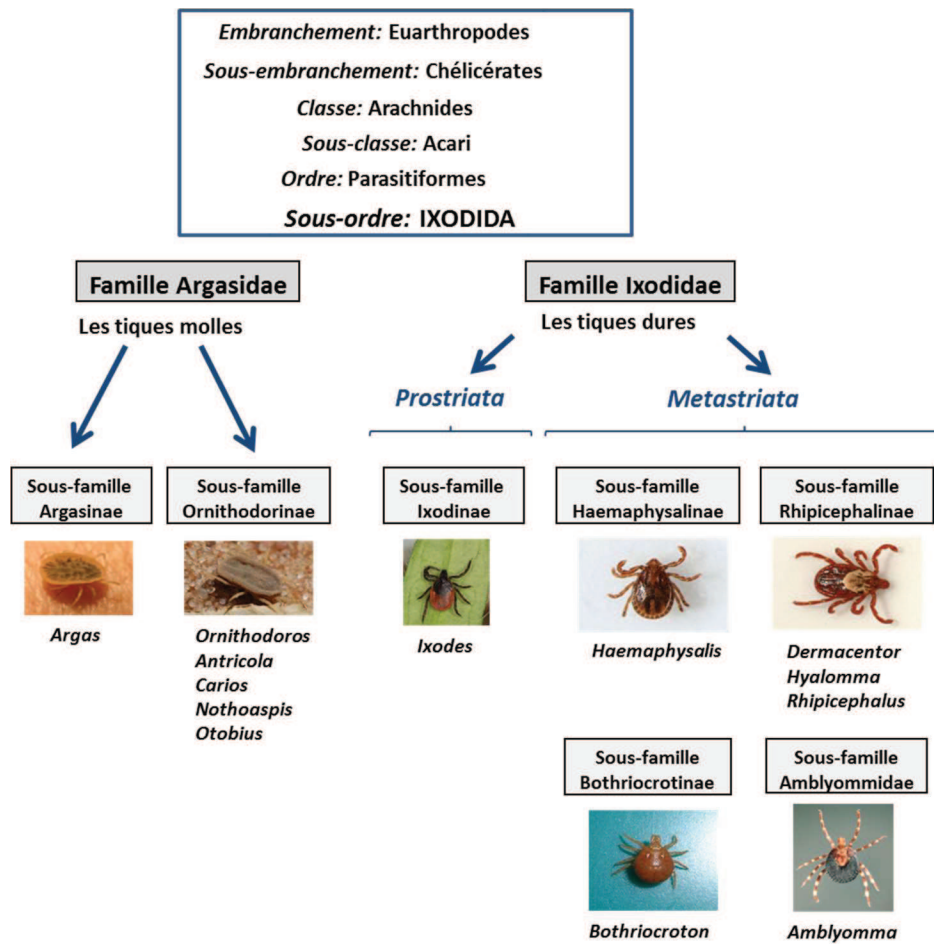
Parmi ces OMPs, **P66** est une porine non spécifique, voltage-dépendante. C'est une protéine relativement conservée au sein du genre *Borrelia* (Skare et al. 1997). Cette protéine de 66 kDa, surexprimée lors du repas de la tique, est aussi capable de se lier aux cellules de mammifères par l'intermédiaire des chaînes  $\beta 1$  et  $\beta 3$  des intégrines (Coburn and Cugini 2003). Ainsi, elle joue sûrement un rôle dans la dissémination de la bactérie.

**Lmp1** (surface-located membrane protein 1) est une protéine codée au niveau du chromosome de la bactérie, qui semble impliquée dans la protection vis à vis du système immunitaire adaptatif (Yang et al. 2009). Les *Borrelia* mutantes pour Lmp1 ne persistent pas lors de l'infection chez la souris. Cependant, la persistance est à nouveau rétablie lors de l'infection de souris SCID. Ces travaux ont permis de déterminer le rôle joué par Lmp1 dans l'échappement de l'immunité adaptative.

## II. Le vecteur : la tique du genre *Ixodes*

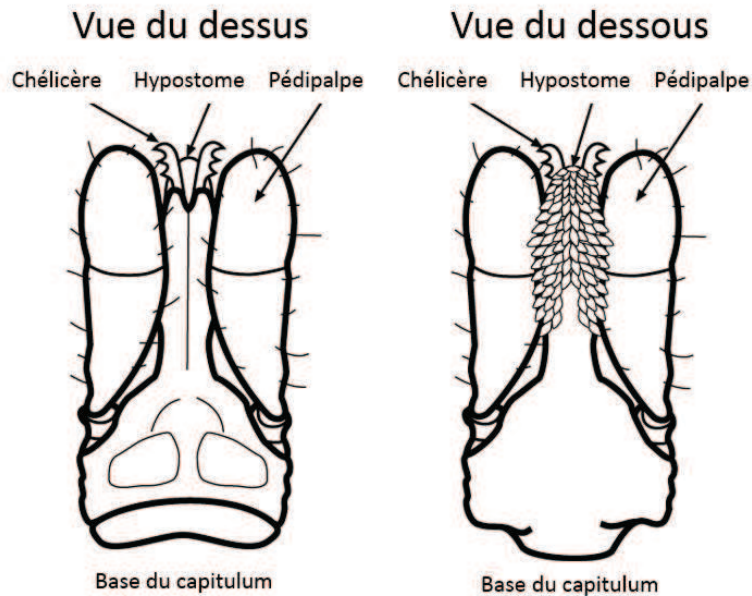
### 1. Taxonomie

Les tiques, de l'ordre des Ixodida, sont des arthropodes qui appartiennent au sous-embanchement des Chelicerata, à la classe des Arachnida, à la sous-classe des Acari et au superordre des Parasitoformes. Selon une étude de 2011 (Mans et al. 2011), leur origine remonterait au début de l'ère du Permien, soit il y a 300 millions d'années environ. L'ordre des Ixodida regroupe actuellement environ 900 espèces (Guglielmone et al. 2010) réparties en 3 familles : Ixodidae (tiques dures), Argasidae (tiques molles) et Nuttalliellidae (**Figure 9**).



**Figure 9.** Classification des tiques moles et tiques dures, d'après Nathalie Boulanger

L'anatomie des *Ixodida* est caractéristique de celle des acariens : un corps en deux parties avec quatre paires de pattes chez les stades matures. Les *Ixodida* sont ainsi composée d'une partie antérieure et postérieure : respectivement le capitulum et l'idiosome. Le capitulum porte les pièces piqueuses de la tique. Ces dernières sont définies par la présence d'un hypostome (permet l'ancrage dans la peau de l'hôte vertébré), de chélicères (dilacèrent les tissus) et de pédipalpes (organes sensoriels) (**Figure 10**). Les tiques molles diffèrent des tiques dures par la présence du capitulum au niveau de la face ventrale. De plus, alors que les tiques *Ixodes* effectuent trois repas sanguins longs (3 à 10 jours) au cours de leur vie, les tiques Argasidae peuvent en effectuer plusieurs sur de courtes durées (quelques minutes). Enfin, contrairement aux tiques dures, l'idiosome des tiques molles ne possède pas de scutum, une plaque sclérifiée présente au niveau de la face dorsale (Sonenshine and Roe 2014a).



**Figure 10.** Représentation schématique du capitulum des tiques dure (*Ixodes*)

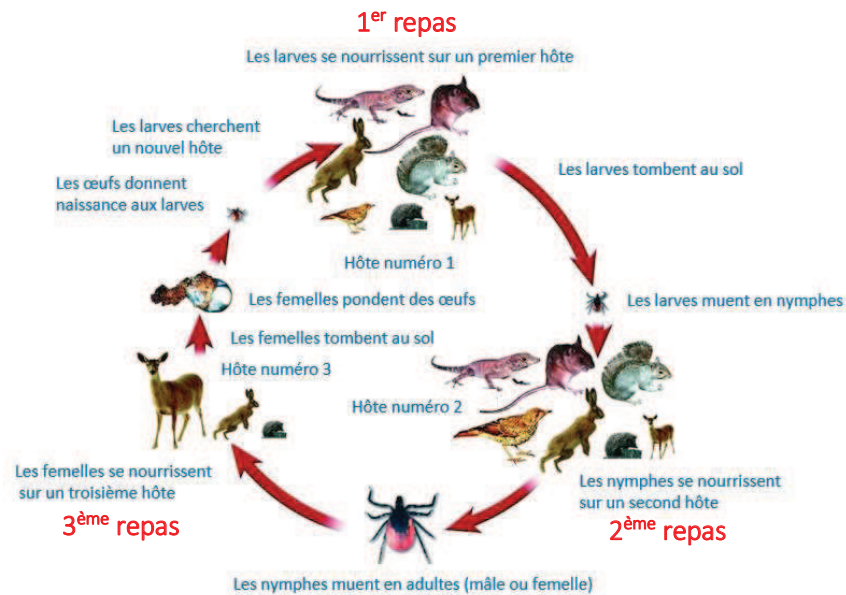
(D'après Scott Charlesworth, Purdue University)

## 2. Caractéristiques de la tique du genre *Ixodes*

### A. Cycle de vie

Les tiques du genre *Ixodes* sont des ectoparasites hématophages obligatoires. Elles détectent leurs hôtes vertébrés à l'aide de l'organe de Haller situé sur la première paire de pattes, notamment sensible aux phéromones et au dioxyde de carbone (Sonenshine 2006). À la suite de son ancrage dans la peau de l'hôte vertébré à l'aide de ses pièces piqueuses, la tique crée une poche de lyse au niveau du derme lui permettant de se nourrir. Ces repas sanguins sont nécessaires à la survie de la tique ainsi qu'à son évolution d'une stase à l'autre. Au nombre de trois au cours de la vie de la tique, ces repas sanguins assurent la mue de la larve en nymphe, puis de la nymphe en adulte et enfin la ponte de centaines d'œufs par la femelle (**Figure 11**). La larve (1 à 2 mm) se nourrit classiquement sur de petits mammifères tels que les rongeurs (ex : *Apodemus silvaticus/flavicollis*), sur les oiseaux ou sur les reptiles. Son repas sanguin dure 2 à 3 jours. La larve mue alors en nymphe, qui se nourrit à son tour sur des hôtes vertébrés similaires pendant 5 jours environ. Enfin, la nymphe mue en adulte mâle ou femelle. Seule la femelle effectuera systématiquement un dernier repas d'une dizaine de jours afin de lui permettre de pondre les œufs qui donneront naissance à de nouvelles larves (Mannelli et al. 2012). Le cycle de vie de la tique

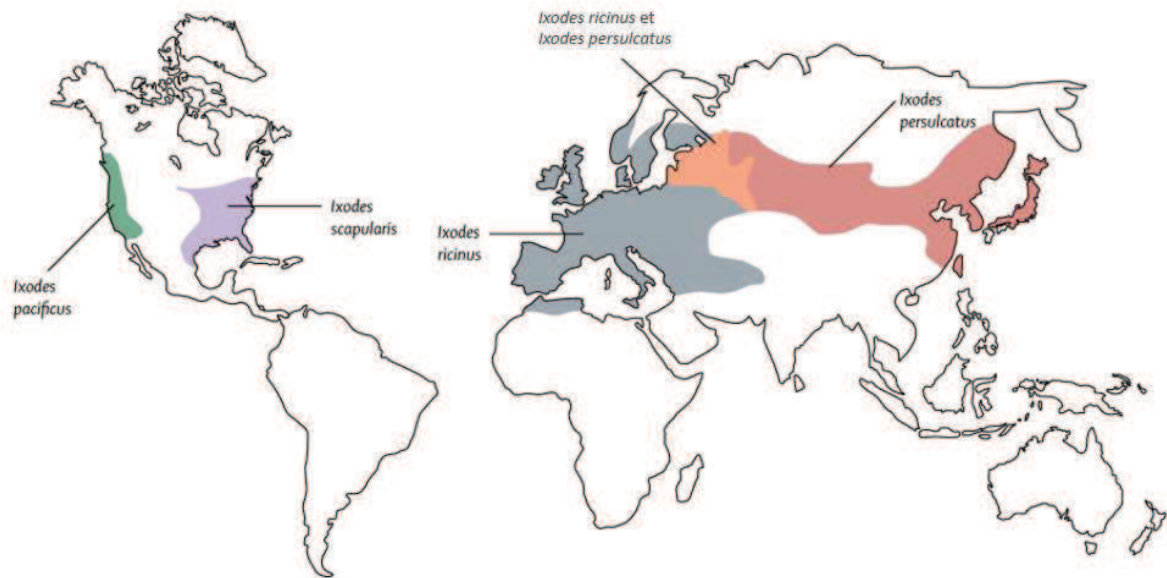
est d'environ deux ans, ce qui lui confère un rôle non négligeable en tant que réservoirs d'agents pathogènes.



**Figure 11.** Cycle de vie de la tique du genre *Ixodes* (Mannelli et al. 2012)

## B. Répartition géographique

L'épidémiologie de la borréliose de Lyme est directement influencée par la répartition géographique des tiques du genre *Ixodes*, vectrice de *Borrelia*. Les tiques vectrices de la borréliose de Lyme vivent dans des zones tempérées, humides et boisées principalement dans l'hémisphère nord. Elles peuvent cependant aussi être retrouvées dans des zones urbaines ou semi-urbaines (Dister et al. 1997; Junttila et al. 1999). Les espèces majeures impliquées dans la transmission de la bactérie sont *Ixodes pacificus* et *I. scapularis* aux Etats-Unis, *I. ricinus* en Europe et *I. persulcatus* en Asie (**Figure 12**). D'autres espèces de tiques telles qu'*I. uriae* et *I. hexagonus* participent au maintien de *Borrelia* dans la nature même si leur implication dans la transmission de la bactérie à l'homme n'a pas été démontrée.



**Figure 12.** Répartition géographiques des espèces de tiques du genre *Ixodes* principalement impliquées dans la transmission de *Borrelia* sp. (Stanek et al. 2012)

### 3. La tique du genre *Ixodes*, un problème de santé publique

#### A. La tique : un vecteur de nombreux agents pathogènes

Les tiques du genre *Ixodes* peuvent être responsables de différents problèmes sanitaires. Dans un premier temps, la piqûre de tique elle-même, de par l'injection de salive à l'hôte vertébré, peut causer des allergies menant à des chocs anaphylactiques. Elle peut induire chez certaines personnes des paralysies ascendantes, potentiellement mortelles si la tique n'est pas retirée (Edlow and McGillicuddy 2008). Plus récemment, la piqûre de la tique *I. ricinus* a été décrite comme pouvant induire une allergie à la viande rouge par l'intermédiaire d'IgE spécifique de l'alpha-galactose (Hamsten et al. 2013). Outre la pathogénicité directe des tiques du genre *Ixodes*, celles-ci est aussi capable de transmettre différents agents pathogènes tels que des bactéries, des virus et des parasites (**Tableau 2**).



Maladie	Agents pathogènes
<i>Babésiose</i>	<i>Babesia microti/divergens/EU1</i>
Encéphalite à tique	Tick-borne encephalitis virus ( <i>Flavivirus</i> )
Rickettsioses	<i>Rickettsia sibirica/australis/japonica/honei/monacensis</i>
Anaplasmose	<i>Anaplasma phagocytophilum</i>
Borréliose de Lyme	<i>Borrelia burgdorferi sensu lato</i>
Fièvre récurrente	<i>Borrelia miyamotoi</i>
Tularémie	<i>Francisella tularensis</i>

**Tableau 2.** Différents exemples d'agents pathogènes de type parasitaire, viral et bactérien transmissibles par la tique du genre *Ixodes* (d'après Sonenshine and Roe 2014b) ; parasite, virus, bactérie.

## B. La transmission de la borréliose de Lyme

La larve acquiert *Borrelia* lorsqu'elle effectue son repas sanguin sur un hôte vertébré déjà infecté. Celle-ci mue alors en nymphe, et peut infecter un nouvel hôte vertébré sain lors de son deuxième repas sanguin (**Figure 11**). Si la nymphe mue en tique adulte femelle, celle-ci peut alors infecter l'hôte vertébré lors du troisième repas du cycle de vie de la tique. L'homme est un hôte vertébré accidentel au cours de ce cycle. Ce sont les nymphes qui sont majoritairement responsables de son infection (Matuschka et al. 1992). Lorsque la tique initie son repas sanguin, la bactérie initialement présente au niveau de l'intestin de la tique entame une migration vers les glandes salivaires de l'arthropode. Cette migration est possible notamment grâce à l'induction de l'expression de l'une de ses protéines de surface, OspC, au détriment de l'expression d'OspA (Schwan et al. 1995). La bactérie est ensuite co-inoculée à l'hôte vertébré avec la salive de tique. Il est généralement admis que la transmission de la bactérie à l'hôte a lieu après une durée de 24 heures. Cependant, selon les espèces de tiques ou de *Borrelia* considérées, ce délai peut varier (Crippa et al. 2002; Wilhelmsson et al. 2013). Notamment, la transmission à certains modèles animaux peut se faire en moins de 16 heures (Cook

2015). Globalement, plus la tique reste longtemps attachée à l'hôte vertébré, plus le risque de transmission de la bactérie est élevé. Dans le cas de tiques infectées de manière systémique, les *Borrelia* peuvent être présentes dans les glandes salivaires avant le début du repas sanguin, augmentant les chances d'être transmises à l'hôte vertébré de façon précoce (Leuba-Garcia et al. 1994). Le nombre de spirochètes nécessaires à une infection efficace lors de la transmission par la tique n'est pas clairement identifié. Cependant, il semblerait qu'un nombre d'environ 20 bactéries soit suffisant pour infecter 50% des hôtes vertébrés lorsque les spirochètes sont injectées à la seringue en présence d'extrait de glandes salivaires de tiques (Lima et al. 2005).

## C. La salive de tique

### a. Un composant essentiel au repas sanguin

À la suite de la piqûre de la tique, l'hôte vertébré devrait normalement réagir par l'activation de la coagulation, la vasoconstriction des vaisseaux sanguins et l'initiation d'une réponse immunitaire permettant la réparation des tissus et le rejet de la tique. Cependant, la tique parvient à court-circuiter l'ensemble de ces mécanismes par l'intermédiaire de molécules actives présentes dans sa salive, injectées dans la peau lors du repas sanguin. L'ensemble de ces molécules anti-hémostatiques et anti-inflammatoires est essentiel au bon déroulement du repas de la tique, et *a fortiori*, à son développement (Fontaine et al. 2011; Mans 2011). La composition de la salive de tiques est complexe, preuve de la complexité et de la redondance même des mécanismes de défense de l'hôte vertébré. La salive contient des molécules protéiques ou non exprimées de manière spécifique au cours du repas sanguin, associées à des activités d'inhibition de la coagulation, vasodilatatrices, immunomodulatrices ou encore inhibitrices de la douleur et de la démangeaison (Kazimírová and Štibrániová 2013; Liu and Bonnet 2014).

### b. Son activité anti-hémostatique

L'hémostase est un mécanisme complexe permettant de contrôler l'hémorragie à la suite d'une lésion des vaisseaux sanguins. Elle fait intervenir la vasoconstriction des vaisseaux puis l'agrégation des plaquettes pour former un bouchon consolidé par un caillot à base de fibrine (Hoffman et al. 2009). Afin de mener à bien son repas sanguin, la tique doit éviter la coagulation sanguine. Elle y parvient grâce à de nombreuses molécules présentes dans sa salive capables de cibler plusieurs composants nécessaires

à la coagulation sanguine (**Tableau 3**). Ces molécules font de la salive de tiques une source intéressante pour le développement de nouveaux médicaments (Kazimírová and Štibrániová 2013).

Parmi l'ensemble de ces molécules, certaines sont associées à des activités vasodilatatrices (ex : **prostacycline, tick histamine release factor (t-HRF), serpine**), inhibitrices de l'agrégation plaquettaire (Ex : **apyrase, IRS-2**) et inhibitrices de la cascade de la coagulation (ex : antithrombine, anti-facteur Xa). La redondance de l'effet de ces différentes molécules sur l'hémostase souligne la nécessité d'inhiber ce mécanisme de l'hôte vertébré pour la survie de la tique.

Espèce de tique	Molécule	Cible/fonction
<b>Vasodilatation</b>		
<i>Ixodes scapularis</i>	Prostacycline	Vasodilatation
<i>I. scapularis</i>	tHRF	Vasodilatation
<i>Ixodes ricinus</i>	IRS-2 (Serpine)	Cathepsine G/ Chymase
<b>Agrégation plaquettaire</b>		
<i>I. scapularis</i>	Apyrase	ATP/ADP
<i>I. scapularis/Ixodes pacificus</i>	Ixodegrine	Antagoniste de l'intégrine
<i>I. ricinus</i>	IRS-2 (Serpine)	Thrombine
<b>Anticoagulation</b>		
<i>I. scapularis</i>	Ixolaris	Inhibiteur de TF (tissue factor)
<i>I. scapularis</i>	Salp14	Voie intrinsèque
<i>I. scapularis</i>	TIX-5	Inhibiteur du facteur V
<i>I. ricinus</i>	Ir-CPI	Voie intrinsèque

**Tableau 3.** Molécules capables de moduler l'hémostase caractérisées à partir de la salive de tiques du genre *Ixodes* (Kazimírová and Štibrániová 2013)

### c. Son activité anti-inflammatoire

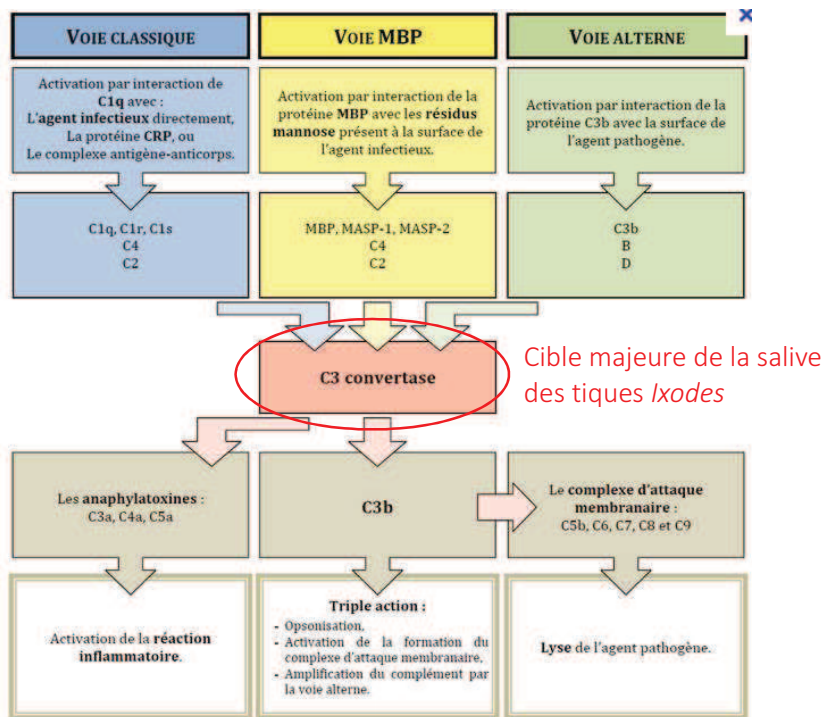
Les tiques ont évolué afin d'être capables de moduler l'immunité innée et adaptative de l'hôte vertébré grâce à leur salive. De cette manière, elles évitent l'interruption de leur repas sanguin. L'effet d'extraits de glandes salivaires de tiques sur le système du complément, mais aussi sur de nombreuses cellules de l'immunité (granulocytes, macrophages, cellules NK, lymphocytes T et B, kératinocytes) est largement décrit (Ramachandra and Wikel 1992; Schoeler et al. 2000; Mejri et al. 2002; Hannier et al. 2003; Kern et al. 2011a; Marchal et al. 2011). Classiquement, l'intrusion au niveau cutané de la tique devrait induire une réponse inflammatoire locale capable de rejeter le parasite. Cependant, la salive de tique possède des molécules aux activités immunosuppressives ciblant les acteurs de l'immunité innée,

du complément et de l'immunité adaptative (**Tableau 4**). Un des premiers événements lors de l'initiation de l'inflammation est l'attraction de neutrophiles au point de piqûre de la tique. La **lipocaline** présente dans la salive de tique est capable d'inhiber le chimiotactisme de ces cellules *in vitro* et leur activation *in vivo* (Beaufays et al. 2008a). De plus, la salive permet d'inhiber la sécrétion d'IL-8, une chimiokine importante pour le recrutement des neutrophiles (Hajnická et al. 2001). La salive inhibe aussi la sécrétion d'autres cytokines telles que l'IL-2, MCP-1, CCL3, RANTES et CCL11 (Hajnická et al. 2005). Il existe au sein de la salive de certaines espèces de tiques des molécules, les **évasines**, capables de lier différentes chimiokines afin d'empêcher leur fonctionnement (Frauensschuh et al. 2007; Déruaz et al. 2008). **ISL 929** et **ISL 1373** sont deux protéines isolées de la salive d'*I. scapularis* qui réduisent l'expression de la  $\beta 2$  intégrine, inhibant ainsi l'adhérence des polynucléaires (Guo et al. 2009). La salive peut aussi contenir des molécules non protéiques telles que la **prostaglandine PGE<sub>2</sub>** pouvant inhiber l'expression de cytokines pro-inflammatoires comme le TNF- $\alpha$  ou l'IL12p40 (Oliveira et al. 2011).

Espèce de tique	Molécule	Cible/fonction
<b>Inhibiteurs du complément</b>		
<i>Ixodes scapularis</i>	Isac	C3 convertase
<i>I. scapularis</i>	Salp 20	C3 convertase
<i>I. ricinus</i>	IRAC I, II, pralogues de Isac	C3 convertase
<b>Immunosuppresseurs</b>		
<i>I. scapularis</i>	Salp 15	Inhibe production IL-2, prolifération lymphocytes T, protège <i>Borrelia</i> des anticorps
<i>I. scapularis</i>	IL-2 binding protein	lymphocytes T
<i>I. scapularis</i>	ISL 929 et ISL 1373	Inhibe adhérence des polynucléaires
<i>I. scapularis</i>	Sialostatine L et L2	Cathepsine L
<i>I. ricinus</i>	Iris	Inhibe réponse lymphocytes T et macrophages, favorise réponse TH2
<i>I. ricinus</i>	BIP	Inhibe prolifération lymphocytes B
<i>I. ricinus</i>	Ir-LBP (Lipocaline)	Neutrophiles
<i>I. scapularis</i>	Prostaglandine PGE <sub>2</sub>	Inhibe la production de TNF- $\alpha$ et d'IL-12

**Tableau 4.** Molécules capables de moduler l'inflammation caractérisées à partir de la salive de tiques du genre *Ixodes* (D'après Kazimírová and Stibrániová 2013)

Le complément est un mécanisme de défense à l'interface entre l'immunité innée et l'immunité adaptative. Il est composé de trois voies principales : la voie alterne, la voie classique et la voie des lectines (**Figure 13**). La voie alterne, impliquée essentiellement dans la réponse contre les agents pathogènes, joue aussi un rôle dans le rejet des tiques. Il semble exister une spécificité de l'activité anti-complément de la salive de tique entre les parasites et leurs hôtes vertébrés respectifs (Lawrie et al. 1999). Plusieurs molécules ayant une activité anti-complément ont été identifiées, notamment chez *I. scapularis* : **Salp 20**, **Isac** et **Isac-1** (Valenzuela et al. 2000a; Tyson et al. 2007), et *I. ricinus* : **IRAC I et II** (Daix et al. 2007a; Couvreur et al. 2008a). Ces molécules empêchent la formation de la C3 convertase, une enzyme essentielle à la voie alterne du complément.



**Figure 13.** Les trois voies majeures du système du complément (d'après Janeway et al. 2005)

La salive de tique possède aussi une activité inhibitrice ciblant l'immunité adaptative malgré la présentation d'immunogènes issues de celle-ci par des cellules de Langerhans aux lymphocytes (Andrade et al. 2005). De manière générale, il a été montré que la salive de tique polarise la réponse immunitaire adaptative vers une réponse du type Th2 (IL-4, IL-5, IL-6, IL-10, IL-12), au détriment de l'expression des cytokines liées à la réponse T auxiliaire (Th) Th1 (IL-2, IFN- $\gamma$ ) (Gillespie et al. 2000; Schoeler and Wikel 2001; Wikel and Alarcon-Chaidez 2001; Brossard and Wikel 2008). **P36**, une protéine de 36 kDa capable d'inhiber la multiplication des cellules T a été identifiée dans la salive de *Dermacentor*

*andersoni* (Bergman et al. 2000). Une autre protéine, **Iris**, a été caractérisée à partir de la salive d'*I. ricinus*. Cette protéine est exprimée lors du repas sanguin de la tique et permet d'inhiber la prolifération des cellules T et la sécrétion d'IL-6 et de TNF- $\alpha$  (Leboulle et al. 2002). Une autre protéine très étudiée est **Salp15**, retrouvée dans la salive d'*I. scapularis*. Les homologues chez *I. ricinus* sont appelés **Iric-1**, **Iric-2** et **Iric-3** (Hovius et al. 2007). Cette protéine est capable d'altérer la signalisation intracellulaire des cellules T en se liant à la molécule CD4, mais aussi l'activation des cellules dendritiques (DC) en se liant au récepteur dendritique cell-specific ICAM3-grabbing nonintegrin (DC-SIGN). De plus, la **sialostatine L** est présente dans la salive de tique et régule négativement la prolifération des cellules T cytotoxiques et la maturation des DCs (Kotsyfakis et al. 2006). La salive de tique altère aussi l'activation des cellules B par l'intermédiaire des **BIP** (B cell inhibitory protein), inhibant la production d'anticorps spécifiques pouvant induire le rejet de la tique (Hannier et al. 2004a).

#### d. Importance de la salive dans la transmission de *Borrelia*

La salive de tique a évolué afin de permettre à l'arthropode d'effectuer correctement son repas sanguin, mais un autre organisme a appris à tirer profit de ses capacités immunomodulatrices : *Borrelia* (Liu and Bonnet 2014). L'interaction entre *Borrelia* et la salive de tique est un bon témoin de la relation unique qui existe entre la bactérie et son vecteur. En effet, la présence de la bactérie chez la tique stimule l'expression d'une protéine anti-inflammatoire de la salive de tique : **Salp15** (Ramamoorthi et al. 2005). En plus de ses capacités à cibler directement l'inflammation vues précédemment, cette protéine est capable de lier la protéine de surface de *Borrelia*, OspC afin d'altérer la reconnaissance de la bactérie par le système immunitaire de l'hôte vertébré, notamment par les anticorps (Hovius et al. 2008a). Une autre protéine de la salive de tique, **Salp20**, permet de protéger la bactérie contre la lyse induite par le complément (Tyson et al. 2007). Les inhibiteurs de lectine (Tick Salivary Lectin Pathway Inhibitor : **TSLPI**) de la salive de tique permettent aussi de protéger *Borrelia* en altérant la voie des lectines du complément ainsi que la phagocytose de la bactérie par les neutrophiles (Schuijt et al. 2011a). Lors de l'infection de la souris par *Borrelia*, l'ajout d'extrait de glandes salivaires de tiques augmente la bactériémie, la dissémination dans les organes, la transmission de la bactérie de l'hôte vertébré à la tique et inhibe l'expression de cytokines pro-inflammatoires (Pechová et al. 2002; Zeidner et al. 2002) et de peptides antimicrobiens (Marchal et al. 2011). Globalement, elle inhibe aussi l'afflux de lymphocytes et de neutrophiles au point de piqûre ainsi que dans les ganglions lymphatiques, facilitant la transmission de *Borrelia* (Severinová et al. 2005). Spécifiquement, la salive altère l'activation

et la prolifération des DCs en ciblant notamment les voies d'activation liées à l'IFN- $\gamma$  et au récepteur Toll 2 (TLR2) (Skallová et al. 2008; Lieskovska and Kopecky 2012). De plus, la salive de tique augmente la résistance des *Borrelia* face aux macrophages notamment en inhibant la production d'ions superoxyde et d'oxyde nitrique (Kuthejlová et al. 2001). La salive de tique réduit aussi l'efficacité des neutrophiles en inhibant leur adhésion et leur capacité à phagocyter *Borrelia* (Montgomery et al. 2004). La salive permet d'orienter davantage vers une réponse Th2 lors de l'infection par *Borrelia*, conduisant à l'expression d'IL-4 inhibant la production d'anticorps IgG2a anti-*Borrelia* (Christe et al. 2000). De plus, les BIP notamment présentes dans la salive d'*I. ricinus* inhibent la prolifération des lymphocytes B induit par la protéine de surface de *Borrelia* OspC (Hannier et al. 2003).

La salive permet également à la bactérie de disséminer plus facilement en augmentant l'efficacité de son passage vers la tique par co-feeding. Le co-feeding permet à la bactérie de passer d'une tique à une autre lorsque celles-ci piquent un même hôte vertébré en étant proche l'une de l'autre (Voordouw 2015). Cependant, sur le long terme, la salive peut être un frein à la transmission de *Borrelia*. En effet, les piqûres répétées peuvent entraîner le développement d'une réponse immunitaire spécifique contre les molécules de la salive de tique entraînant le rejet de cette dernière, et par la même occasion la protection contre *Borrelia* (Wikel et al. 1997; Nazario et al. 1998; Burke et al. 2005; Narasimhan et al. 2007).

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Partie III

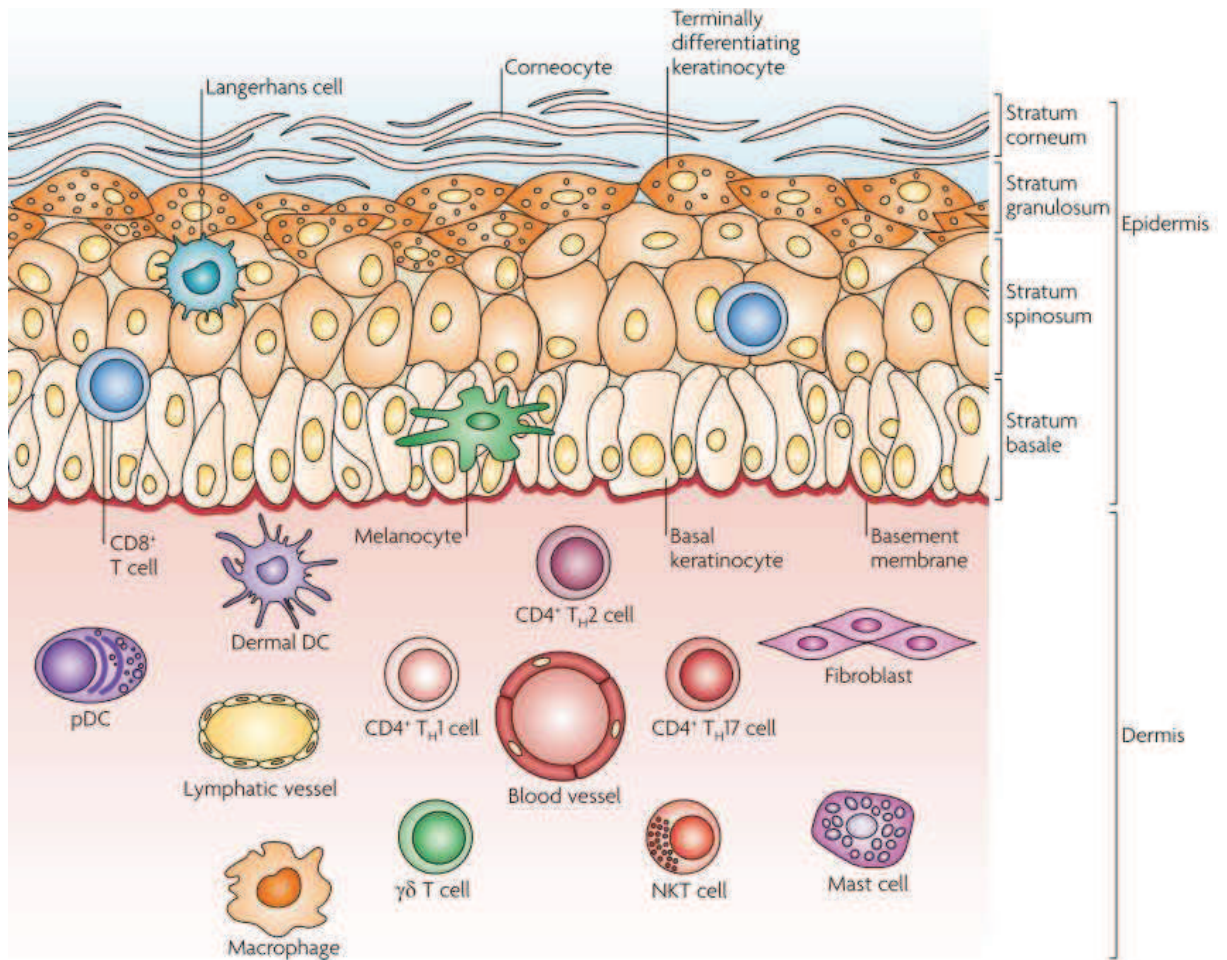
L'INTERFACE CUTANEE

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## I. La structure de la peau

La peau humaine est composée de deux compartiments majeurs : l'épiderme et le derme. Le compartiment le plus externe, l'épiderme, composé majoritairement de kératinocytes (KCs) est associé à quatre couches distinctes. Le stratum germinativum, la couche la plus interne, est composé de KCs basaux responsables du renouvellement des couches supérieures. Les nouveaux KCs migrent alors vers la couche suivante appelée stratum spinosum où ils commencent à se différencier. Ce mécanisme de maturation est associé à un changement de forme des cellules qui deviennent polygonales et à l'expression d'une kératine distincte de celle exprimée par les KCs basaux. La couche suivante, nommée stratum granulosum, est caractérisée par la présence de grains de kératohyaline dans le cytoplasme, par l'expression de kératine et de lipide précurseurs de la graisse épidermique, ainsi que la présence de nombreuses jonctions serrées (Kubo et al. 2009). Enfin, le stratum corneum, la couche la plus externe, est composé de KCs morts, appelés cornéocytes, qui constituent une barrière physique efficace contre les agents toxiques et pathogènes extérieurs et contre la déshydratation. L'épiderme est aussi composé dans une moindre mesure de mélanocytes, produisant un pigment, la mélanine, mais aussi de DCs (cellules de Langerhans) et de cellules T, principalement des CD8 (Nestle et al. 2009). Le compartiment le plus interne, le derme, est composé essentiellement de fibroblastes (FBs). Ces cellules fusiformes sécrètent les molécules nécessaires à la formation de la matrice extracellulaire : glycoprotéine (collagène) et protéoglycanes. Cette matrice sert ainsi de soutien à l'épiderme, mais aussi de structure aux différents vaisseaux (sanguins, lymphatiques, nerveux) qui permettent aux cellules immunitaires (DCs, T, NK, NKT,  $T_{\gamma}^{\delta}$ , macrophages et mastocytes) de migrer à travers la peau (Nickoloff 1993) (**Figure 14**). Le derme est aussi le support de structures complexes comme les follicules pileux, les glandes sébacées et les glandes sudoripares. La peau, notamment le stratum corneum, en plus de jouer un rôle défensif direct contre les infections, est aussi le support d'une flore commensale complexe (bactéries, champignons, virus, parasites) dont le rôle protecteur est de plus en plus décrit (Belkaid and Segre 2014). Dans le cas des maladies vectorielles telles que la borréliose de Lyme, les agents pathogènes ont appris à contourner cette barrière qu'est la peau en utilisant la morsure/piqûre de vecteurs tels que la tique ou le moustique. Cependant, une fois les agents pathogènes injectés dans la peau, ces derniers doivent franchir la barrière immunitaire.



**Figure 14.** Structure anatomique de la peau (Nestle et al. 2009)

## II. La peau : le siège d'une niche immunitaire complexe

### 1. Kératinocytes et fibroblastes

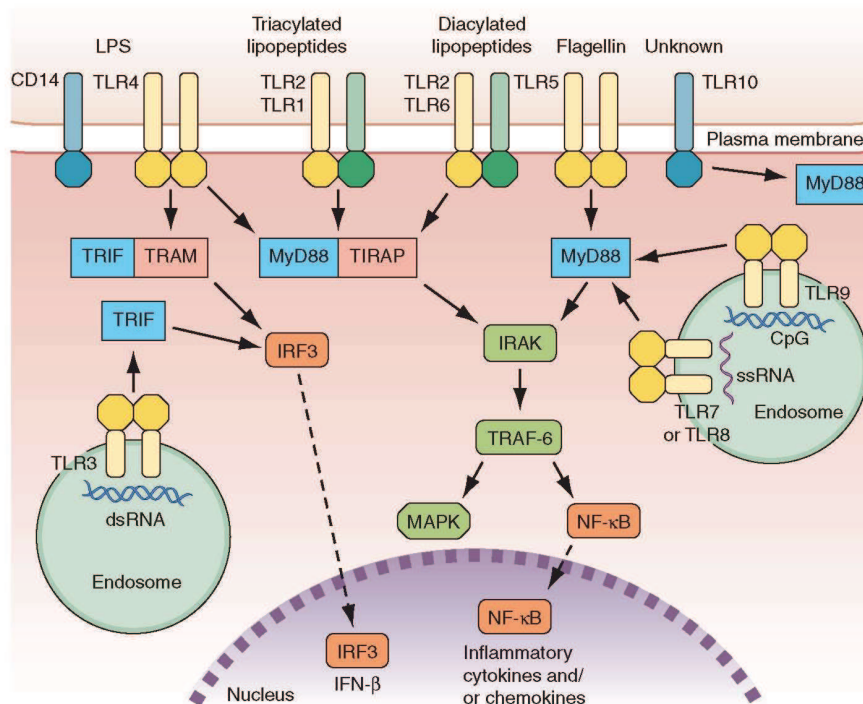
Dans un premier temps, les KCs et les FBs forment une barrière physique efficace contre le monde extérieur. Dans un second temps, ils sont capables de participer à la mise en place d'une réponse inflammatoire coordonnée avec l'ensemble des cellules immunitaires résidentes ou de passage au sein de la peau. Les KCs et les FBs possèdent des récepteurs immunitaires leur permettant de reconnaître les signaux de dangers liés à aux agents pathogènes (Pathogen associated molecular pattern : PAMP) ou aux dommages tissulaires (Damage associated molecular pattern : DAMP) : **les Pattern Recognition Receptors (PRR)**. De plus, KCs et FBs sont capables de présenter directement les antigènes aux cellules T (Umetsu et al. 1986; Nickoloff and Turka 1994). Les KCs sont ainsi capables de répondre à la présence des agents pathogènes en sécrétant des cytokines/chimiokines telles que l'interleukine IL-22, le TNF- $\alpha$ ,

l'IL-8, l'IL-1 $\beta$ , l'IL-6 ou encore l'IL-10 et des peptides antimicrobiens tels que les  $\beta$ -défensines et les cathélicidines (LL-37 chez l'homme et CRAMP chez la souris). L'ensemble des molécules inflammatoires ainsi sécrétées par les KCs et des FBs permettent une communication avec les cellules immunitaires présentes (les cellules de Langerhans, les DCs dermiques, les mastocytes, les macrophages et les cellules NK) et l'attraction de nouvelles cellules (Neutrophiles, Lymphocytes).

## A. Les Récepteurs Toll

On retrouve parmi les PRRs les TLRs, les Nod Like Receptors (NLR), les RIG-I Like Receptors (RLR) et les C-type lectin Receptors (CLR). Les TLRs ont une origine ancienne au sein de l'évolution puisqu'ils sont retrouvés chez des organismes tels que *Drosophila melanogaster*, *Caenorhabditis elegans* ou encore chez les plantes. Chez l'homme, ils sont présents au niveau de la membrane cellulaire plasmique (TLR1, TLR2, TLR4, TLR5, TLR6) et au niveau de la membrane des endosomes/lysosomes (TLR3, TLR7, TLR8, TLR9). Les TLRs ne sont pas présents systématiquement dans tous les types cellulaires. Par exemple, les TLR7 et TLR8 ne sont pas exprimés de manière constitutive par les KCs (Lebre et al. 2007). Les TLRs sont capables de reconnaître différents motifs conservés au sein des agents pathogènes (ARN, ADN, lipoprotéines, acide lipotéichoïque (LTA), LPS, flagelline), faisant de ces récepteurs des piliers de l'immunité innée (**Figure 15**). La nature universelle des molécules reconnues telles que l'ADN ou l'ARN permet aux TLRs de reconnaître aussi bien les molécules issues d'un agent pathogène que celles issues du soi, sans pouvoir systématiquement les distinguer. La compartimentalisation de ces différentes molécules est alors une notion essentielle au bon fonctionnement de ces récepteurs. Lorsque celle-ci est rompue, lors de la nécrose par exemple, les molécules du soi peuvent être reconnues par les TLRs, conduisant à l'activation de l'inflammation (Yang et al. 2010; Bernard et al. 2012). Cette activation peut être bénéfique pour l'hôte vertébré en initiant les processus de réparation tissulaire ou en préparant le système immunitaire contre une potentielle future infection. Elle peut aussi cependant être néfaste et conduire à des évènements d'auto-immunité (Marshak-Rothstein 2006; Pradhan et al. 2012). Le fonctionnement des TLRs repose sur une structure sous forme de dimère de protéines transmembranaires. Les TLRs sont composés de 700 à 1100 acides aminés permettant la mise en place d'un domaine extracellulaire, transmembranaire et cytoplasmique. La partie extracellulaire contient la poche de liaison à l'antigène. Les associations dimériques sont généralement du type homodimère, mais certains TLRs forment des hétérodimères (TLR1-2 / TLR1-6). De plus, les TLRs peuvent former des complexes avec des molécules extérieures à la famille des TLRs telles que les protéines MD2 ou CD14 afin d'augmenter la diversité des motifs antigéniques reconnus. La liaison des TLRs avec l'antigène est

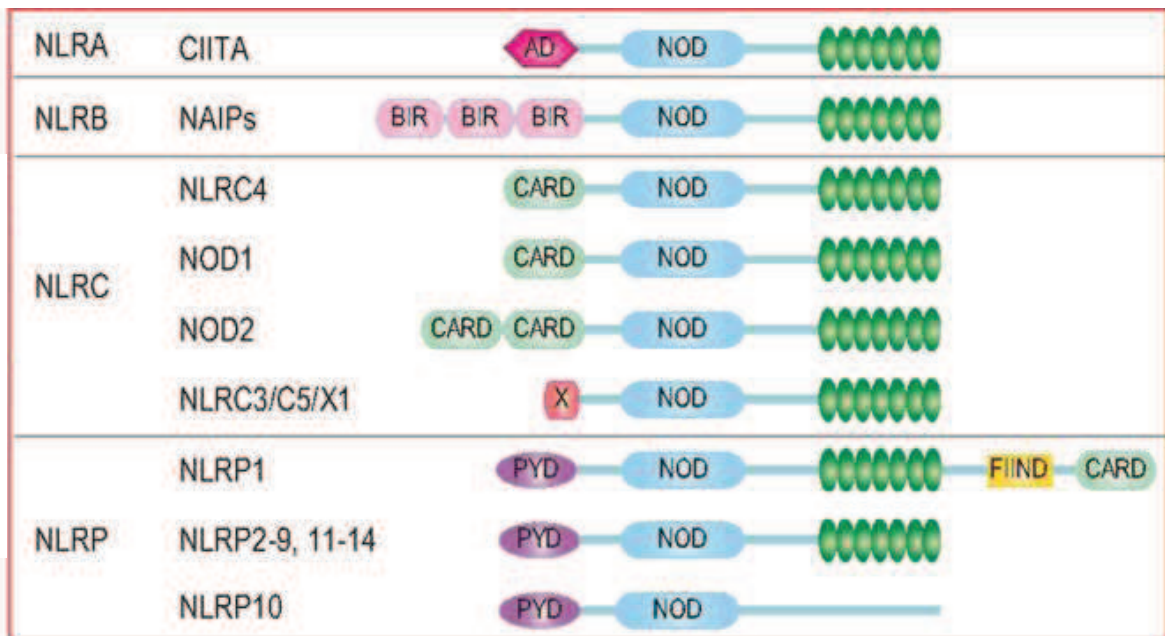
suivie par la mise en place de l'une des deux voies d'activations majeures : la voie TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) ou la voie MyD88. Chacune des deux voies est suivie d'une cascade d'activation de différentes protéines kinases permettant finalement l'activation de facteurs de transcription tels que NF- $\kappa$ B, IRF-3, IRF-7 ou AP-1 conduisant à l'expression de molécules inflammatoires (interférons, cytokines, chimiokines et peptides antimicrobiens) (**Figure 15**).



**Figure 15.** Les récepteurs Toll et les voies d'activation associées (van Duin et al. 2006)

## B. Les Récepteurs NOD

Les NLRs sont des récepteurs cytoplasmiques, ou mitochondriaux pour l'un d'entre eux (NLRX1) (Hong et al. 2012), capables de reconnaître des motifs moléculaires conservés. Vingt-deux NLRs ont été découverts jusqu'à présent chez l'homme (Motta et al. 2015). Ces récepteurs peuvent être divisés en quatre familles selon le domaine présent au niveau de la partie N-terminal (**Figure 16**). Ces domaines sont les suivants : Caspase Recruitment Domain (**CARD**), Pyrin Domain (**PYD**), Baculoviral Inhibitor of Apoptosis Repeat (**BIR**) et Transactivator Domain (**AD**). Les quatre familles de NLRs portent ainsi un nom en rapport avec le domaine présent : **NLRC**, **NLRP**, **NLRB** et **NLRA**.



**Figure 16.** Organisation des quatre familles de récepteurs NOD (Zhong et al. 2013)

Outre les récepteurs de la famille NLRC, ceux de la famille NLRP ont aussi été l'objet de nombreuses études. La famille NLRP regroupe quatorze membres. Ces récepteurs sont notamment connus pour leur implication dans la formation d'inflammasomes. Deux types d'inflammasomes sont bien décrits : l'inflammasome NLRP1 et l'inflammasome NLRP3. NLRP1 est activé par le peptidoglycane et l'anthrax, une toxine produite par *Bacillus anthracis*. NLRP3 peut être activé par différents ligands tels que des bactéries entières (ex : *Staphylococcus aureus*), de l'ARN bactérien ou encore des cristaux d'acide urique (Martinon and Tschopp 2004; Boyden and Dietrich 2006; Kanneganti et al. 2006; Mariathasan et al. 2006)

### C. Les Récepteurs Rig-I Like

Les RLRs sont des hélicases cytoplasmiques essentielles pour la défense antivirale. Les deux RLRs les mieux connus sont RIG-I et MDA5, qui sont capable de détecter la présence d'ARN double brin (ARN db) comme TLR3. L'ARN db est une structure moléculaire généralement adoptée par les virus à ARN lors de leur réplication. La détection de ce composé par les RLRs mène en principe à la production d'interféron par les cellules infectées (Yoneyama and Fujita 2007). L'importance des RLRs vis-à-vis de TLR3 semble être cellule spécifique. Par exemple, TLR3 semble jouer un rôle majeur dans les DCs

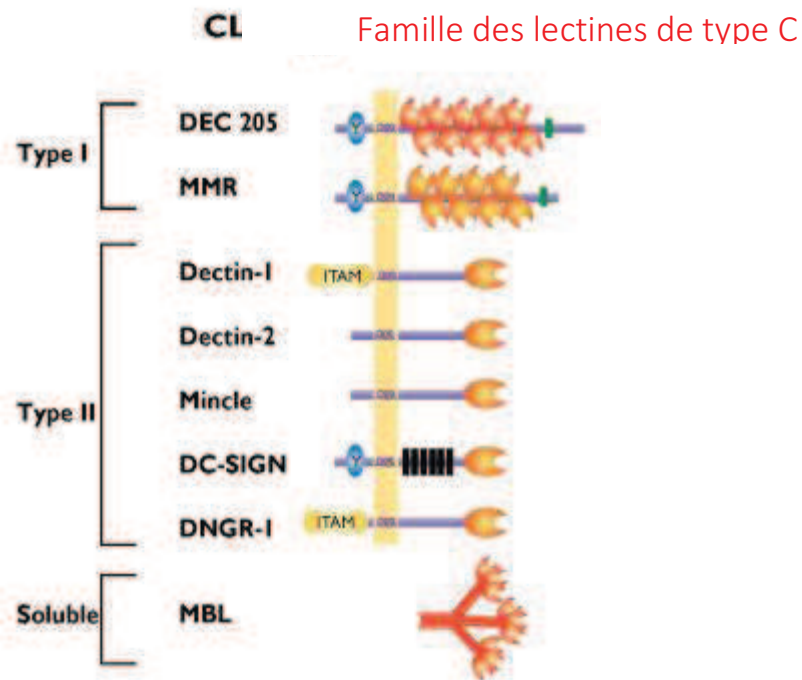
plasmacytoïdes alors que les RLRs RIG-I et MDA5 sont nécessaires pour les DCs conventionnelles, les macrophages et les FBs (Kato et al. 2005).

Depuis peu, une nouvelle classe de RLRs est de plus en plus étudiée : Les RLRs spécifiques de l'ADN (simple ou double brin). Le premier à avoir été identifié, DAI (DNA-dependent activator of IFN-regulatory factors), est capable de détecter l'ADN double brin cytoplasmique et d'induire l'expression d'interférons (Takaoka et al. 2007). AIM2 (absent in melanoma 2), p202 et LRRFIP1 sont aussi des détecteurs d'ADN cytoplasmique connus à ce jour. AIM2 peut former un inflammasome avec la caspase 1 (Jones et al. 2010). Au contraire, p202 inhibe l'activation des caspases (Roberts et al. 2009). Globalement, la reconnaissance de l'ADN cytoplasmique repose sur différents mécanismes complexes, dont certains dépendants de récepteurs extérieurs à la famille des TLRs et de RLRs sont toujours inconnus à ce jour.

## D. Les récepteurs lectines de type C

Les CLRs correspondent à des récepteurs capables de lier des carbohydrates de manière calcium-dépendante. Cette activité est véhiculée par les Carbohydrate-recognition domains (CRD). Il existe deux types de CLRs attachés à la membrane et un type soluble (**Figure 17**). Les CLRs transmembranaires de type I, DEC-205 et le Macrophage Mannose Receptor (MMR), possèdent plusieurs CRDs. Au contraire, les CLR membranaires de type II, Dectin-1, Dectin-2, macrophages-inductible C-type lectin (Mincle), DC-SIGN et DC NK lectin group receptor-1 (DNGR-1), possèdent un seul CRD.

On retrouve les CLR sur la plupart des cellules capables de phagocyter les glycoprotéines issues des agents pathogènes telles que les macrophages ou les DCs. Dectin-1 et Dectin-2 sont impliquées dans la reconnaissance de carbohydrates respectivement  $\beta$ -glucans et  $\alpha$ -mannans issus de champignons tels que *Saccharomyces cerevisiae* (Brown et al. 2003; Drummond et al. 2011). L'activation des Dectins 1 et 2 mène à la mise en place de l'inflammasome NLRP3 (Sancho and Reis e Sousa 2012). DC-SIGN lui est un récepteur présent sur les DCs dérivées de monocytes, impliqué dans la reconnaissance de nombreux virus, bactéries, levures et parasites. Il peut moduler l'activation des TLRs au niveau du facteur de transcription NF- $\kappa$ B (den Dunnen et al. 2009). De manière générale, les autres CLR sont impliqués dans la défense antifongique.



**Figure 17.** Les différents récepteurs lectines de type C (D'après (Figdor et al. 2002))

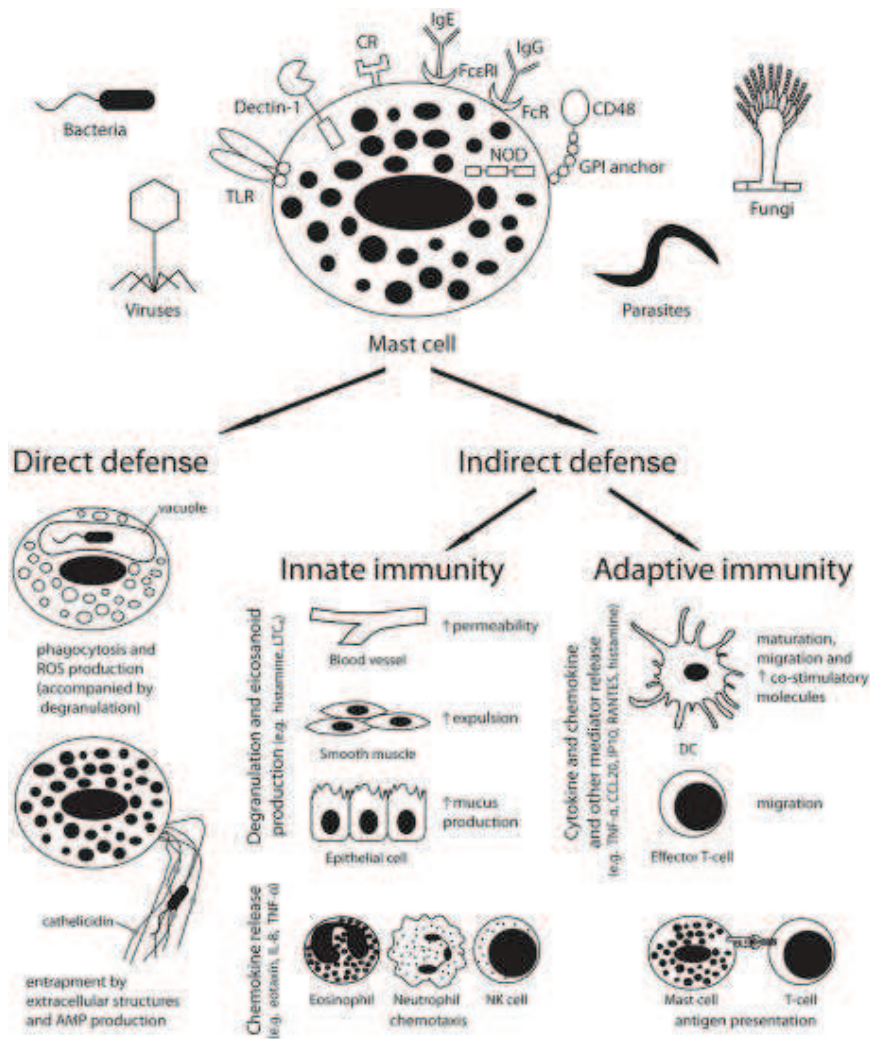
## 2. Mastocytes

Les mastocytes (MCs) sont des cellules généralement associées aux phénomènes allergiques, de par leur position stratégique au niveau des tissus en contact avec les allergènes extérieurs (peau, muqueuse respiratoire et intestinale) (Harvima et al. 2008). Cependant, ces cellules ont aussi été associées à un rôle protecteur au niveau de la peau contre certaines infections bactériennes et parasitaires (Maurer et al. 2006; Siebenhaar et al. 2007; Metz et al. 2009). De plus, tout comme les macrophages, ces cellules peuvent intervenir dans le processus de guérison des tissus après une lésion (Huttunen et al. 2000; Weller et al. 2006).

Les MCs peuvent être divisés en trois catégories selon le type de protéinases qu'ils contiennent. Les MC<sub>TC</sub> qui contiennent de la tryptase, de la chymase, de la carboxypeptidase et de la cathepsine G-like protéinase, les MC<sub>T</sub> contenant seulement de la tryptase et les MC<sub>C</sub> contenant de la chymase et de la carboxypeptidase (Irani et al. 1989; Schechter et al. 1990; Weidner and Austen 1993). La grande majorité des MCs de la peau appartiennent au groupe des MC<sub>TC</sub>, alors que les MC<sub>T</sub> sont majoritaires au niveau des muqueuses respiratoires et intestinales. Cette répartition suggère un rôle spécifique de la chymase, la carboxypeptidase et la cathepsine G-like protéinase au niveau de la peau. Les MCs

possèdent un rôle de pivot au sein de l'immunité (**Figure 18**). Les MCs peuvent directement cibler les agents pathogènes en les phagocytant, et en les détruisant à l'aide de ROS (Malaviya et al. 1994). Indirectement, les MCs peuvent répondre à la stimulation par des anticorps IgE et IgG par l'intermédiaire de ses récepteurs FcεRI, FCγRI et FCγRIIa (Tkaczyk et al. 2002; Zhao et al. 2006). Les MCs peuvent aussi détecter la présence des agents pathogènes à l'aide des PRRs, et notamment les TLRs (Dawicki and Marshall 2007). Les MCs peuvent aussi répondre à des stimuli endogènes tels que les facteurs du complément (C3a et C5a), le TNF ou encore le stem cell factor (SCF) (van Overveld et al. 1991; el-Lati et al. 1994; Dvorak et al. 1998). Une fois activés, les MCs peuvent dégranuler complètement ou partiellement, ce qui consiste au relargage de médiateurs (protéinases, histamine) dans les tissus (Dvorak and Kissell 1991). Le relargage de médiateurs, notamment d'histamine, permet d'augmenter la perméabilité vasculaire, et ainsi d'augmenter le flux de cellules immunitaires au site d'infection. Les MCs peuvent aussi sécréter des peptides antimicrobiens comme la cathélicidine, des cytokines et des chimiokines permettant notamment d'attirer les neutrophiles, les cellules NK, les DCs et les cellules T. Elles sont une source importante de TNF-α préformé au niveau de la peau (Walsh et al. 1991). Enfin, les MCs sont aussi capables de migrer jusqu'aux ganglions lymphatiques afin de présenter directement les antigènes aux cellules T CD8, et d'activer les cellules T à l'aide du TNF-α ou bien par contact direct par l'intermédiaire du ligand OX40 (Kashiwakura et al. 2004; Nakae et al. 2005, 2006; Hershko and Rivera 2010). Les MCs peuvent aussi avoir un rôle suppresseur de l'inflammation. Aussi, ces cellules peuvent aussi être responsables d'un dysfonctionnement de l'immunité lorsque la libération sanguine d'histamine mène à une dégranulation systémique des MCs.





**Figure 18.** Le rôle central des mastocytes au sein de l'immunité (Urb and Sheppard 2012)

### 3. Cellules dendritiques, macrophages et cellules T

Les DCs peuvent être classées selon leur localisation au niveau de la peau. On retrouve ainsi les cellules de Langerhans au niveau de l'épiderme et les DCs dermiques au niveau du derme. En plus de caractériser les DCs selon leur localisation, on peut les classer selon leurs propriétés inflammatoires. Ainsi, les DCs inflammatoires sont spécialisées dans la sécrétion de cytokines pro-inflammatoires, les DCs plasmacytoïdes sécrètent des interférons de type I et les CD103+ sont spécialisées dans la présentation croisée. Globalement, les DCs expriment plusieurs PRRs leur permettant de détecter la présence des agents pathogènes. Les cellules de Langerhans se distinguent par l'expression de CD1a au niveau de leur surface chez l'homme. Elles possèdent des organelles cytoplasmiques appelés le granule de Birbeck. Ces cellules sont capables d'ingérer les antigènes lipidiques et microbiens afin de les

présenter aux cellules T, notamment après migration dans les ganglions lymphatiques. Il semble que ces cellules soient préférentiellement associées au développement d'une réponse de type Th2, et donc à l'induction d'une immunotolérance. Une autre population de DC possédant le cluster de différenciation CD206 est présente dans l'épiderme en cas d'inflammation. Ces DCs semblent pouvoir véhiculer les réponses inflammatoires de type allergéniques. Les DCs dermiques sont quant à elles capables de migrer rapidement au niveau des ganglions lymphatiques afin d'activer les cellules T à la suite d'une infection. Il en existe plusieurs types que l'on différencie par rapport aux molécules exprimées au niveau de leur surface membranaire (langerin, CD1C...).

Les macrophages de la peau sont des cellules sédentaires, qui peuvent aussi sous certaines conditions migrer jusqu'aux ganglions lymphatiques. Lors d'une infection, ces cellules sont capables de phagocyter, de présenter les antigènes aux cellules T et B et d'exprimer des molécules pro-inflammatoires. De plus, en cas de lésion, ces cellules participent au phénomène de cicatrisation.

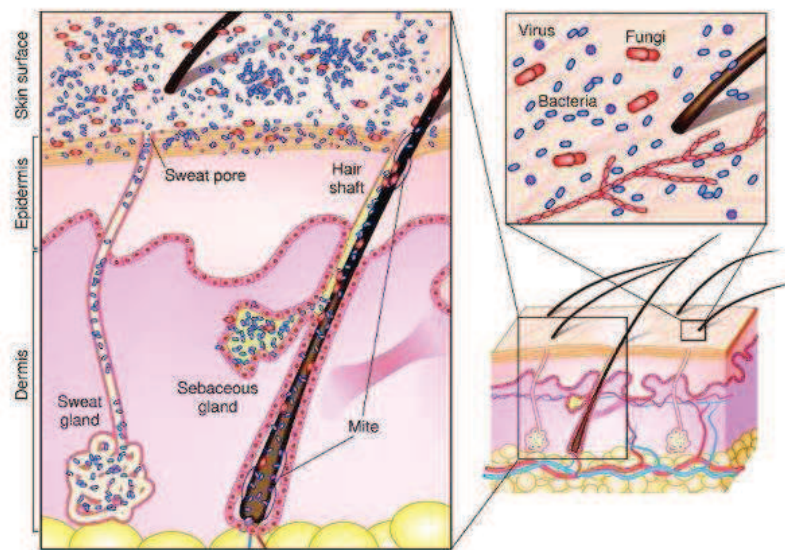
Les KCs, MCs, DCs ou encore les macrophages permettent notamment grâce à leur fonction de cellules présentatrices d'antigènes d'activer les cellules T, effecteurs de l'immunité adaptative. La peau contient plus de  $2 \times 10^{10}$  cellules T résidentes. Les cellules T de l'épiderme, essentiellement des T CD8  $\alpha\beta$ , sont présentes majoritairement au niveau basal, proche des cellules de Langerhans. Les cellules T du derme sont situées essentiellement au niveau des veinules post-capillaires. Globalement, les cellules T présentes dans la peau sont des cellules T CD4 et CD8 mémoires. L'implication des cellules T dans le développement de maladies auto-immunes de la peau a été démontrée. L'importance de ces cellules lors d'infections cutanées a aussi été explorée. De plus, les cellules Th17 sont capables, par l'intermédiaire de l'IL-17 et l'IL-22, d'augmenter la production de peptide antimicrobiens par les KCs afin d'accroître la défense immunitaire envers les agents pathogènes. Les cellules T non-conventionnelles, les lymphocytes T  $\gamma\delta$  et les NKT, sont aussi présentes dans la peau. Les cellules T  $\gamma\delta$  peuvent contrôler l'intégrité des cellules épithéliales notamment par la sécrétion de facteurs de croissance. Ces cellules sont aussi capables de sécréter directement des peptides antimicrobiens.

### III. La peau et son microbiome

#### 1. Un microbiome d'une diversité complexe

Tout comme l'intestin, la peau est un organe qui dispose d'une flore commensale diversifiée : bactéries, champignons, virus et parasites (Kong and Segre 2012; Duerkop and Hooper 2013; Schommer and Gallo 2013). Ces organismes, présents par milliards sur la peau ont été identifiés d'abord par culture

et plus récemment par séquençage. Globalement, deux types de microorganismes sont retrouvés : les résidents et les transitoires. Les facteurs intrinsèques tels que l'âge, le génome et le système immunitaire de l'hôte vertébré influencent la composition du microbiome. Les facteurs environnementaux tels que le climat ou extrinsèques tels que l'hygiène peuvent aussi avoir une influence. La complexité de la structure de la peau apporte autant de niches permettant l'établissement d'une flore diversifiée (**Figure 19**). Par exemple, *Propionibacterium acnes* sera essentiellement retrouvé au niveau des glandes sébacées, ou le sébum sécrété sera hydrolysé par la bactérie permettant ainsi de générer des acides gras augmentant son adhérence (Marples et al. 1971).



**Figure 19.** La colonisation des différents compartiments de la peau par le microbiome (Kong and Segre 2012)

## 2. La relation entre le microbiome et la peau

La présence de cette flore, notamment la composante bactérienne (**Figure 20**), a été décrite comme jouant un rôle important dans la physiologie de la peau (cicatrisation) (Lai et al. 2009) et dans le développement et le fonctionnement de son immunité (Naik et al. 2012). Il a ainsi été démontré que *Staphylococcus epidermidis* était capable d'amplifier par l'intermédiaire de TLR2 l'inflammation induite lors d'une lésion cutanée impliquant l'activation de TLR3 (Lai et al. 2009, 2010). Les bactéries commensales ne sont pas pathogènes en conditions normales (bonne hygiène, structure et immunité de la peau intacte). Cependant, elles peuvent le devenir à la suite d'une perturbation. Par exemple, *S. epidermidis*, une bactérie commensale résidente de la peau, peut devenir pathogène chez les patients

immunodéprimés (Otto 2009). De plus, *S. aureus* peut être présent en tant que bactérie commensale résidente non pathogène, et provoquer des infections chez des patients atteints de dermatite atopique (Hanifin 2009). Bien que la peau soit un passage obligatoire pour les agents pathogènes transmis par un vecteur, l'influence du microbiome cutané sur la transmission de ces derniers reste pour l'instant inexplorée.

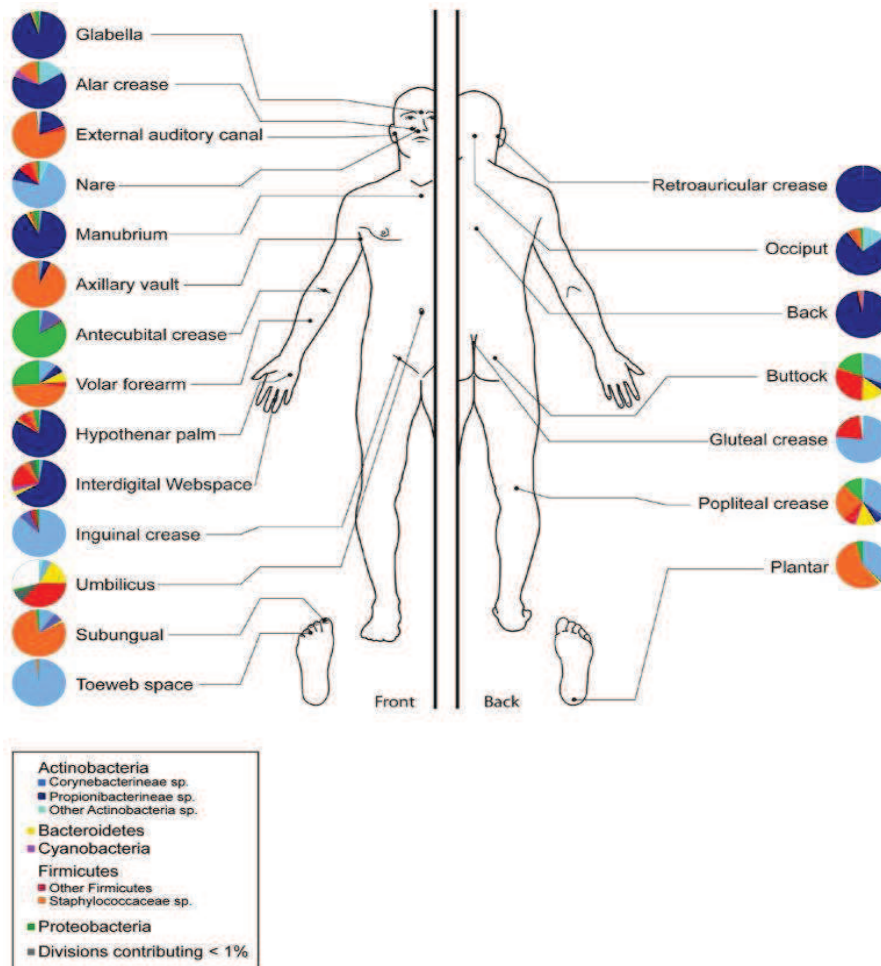


Figure 20. Répartition de la composante bactérienne du microbiome cutané (Kong and Segre 2012)

#### IV. La réponse inflammatoire cutanée face à *Borrelia*

Selon les espèces de *Borrelia* et l'hôte vertébré considéré, il est admis que la bactérie est injectée au niveau de l'interface cutanée 24 à 48 heures après la piqûre de la tique. Il est cependant possible dans certains cas que la transmission se fasse en un temps inférieur à 16 heures (Cook 2015). Une fois ce délai passé, la bactérie se retrouve directement en contact avec les cellules de la peau : KCs,

FBs, DCs/cellules de Langerhans, cellules T, cellules endothéliales et MCs. La peau constitue ainsi un organe primordial dans la défense contre les agents pathogènes transmis par un vecteur (Bernard et al. 2014) (cf. revues ci-joint), et donc *a fortiori* contre *Borrelia*, puisqu'elle est le lieu où est initiée la réponse immunitaire contre l'agent pathogène.

## 1. La réponse immunitaire innée

### A. Le système du complément

La réponse immunitaire innée est essentielle dans la mise en place d'une immunité précoce lors d'infections bactériennes. Elle est non spécifique et permet de contrôler l'infection sans la mise en place d'une mémoire immunitaire. Elle implique de nombreux types cellulaires : les cellules épithéliales (KCs, FBs), endothéliales et immunitaires (macrophages, DCs, neutrophiles, cellules NK), ainsi que le système du complément.

Le système du complément permet d'induire la lyse directe de la bactérie ou le recrutement de cellules immunitaires au niveau du site d'infection. La voie principale du complément est la voie dite classique (**Figure 13**). Elle consiste en un clivage de la molécule C3 en C3a et C3b par la C3 convertase. C3b peut recouvrir la surface des bactéries afin d'induire l'opsonisation de la bactérie notamment par l'intermédiaire des anticorps (Gros et al. 2008). Les cellules hôtes possèdent des protéines, dont le facteur H fait partie, qui permettent de les protéger du système du complément en inactivant la molécule C3b. De nombreuses bactéries utilisent des protéines similaires au facteur H afin de se protéger elles aussi du complément. La pathogénicité des espèces de *Borrelia* est notamment liée à leur capacité à interférer avec le système du complément, leur permettant d'acquérir une certaine résistance vis-à-vis du sérum de l'hôte vertébré (Ek Dahl et al. 2007). Parmi les différentes espèces de *Borrelia*, *Borrelia afzelii* est résistante au complément des rongeurs, alors que *B. garini* y est majoritairement sensible et *B. burgdorferi* ss montre un profil intermédiaire. En revanche, *B. garini* est réfractaire au complément des oiseaux (Kurtenbach et al. 1998, 2006)

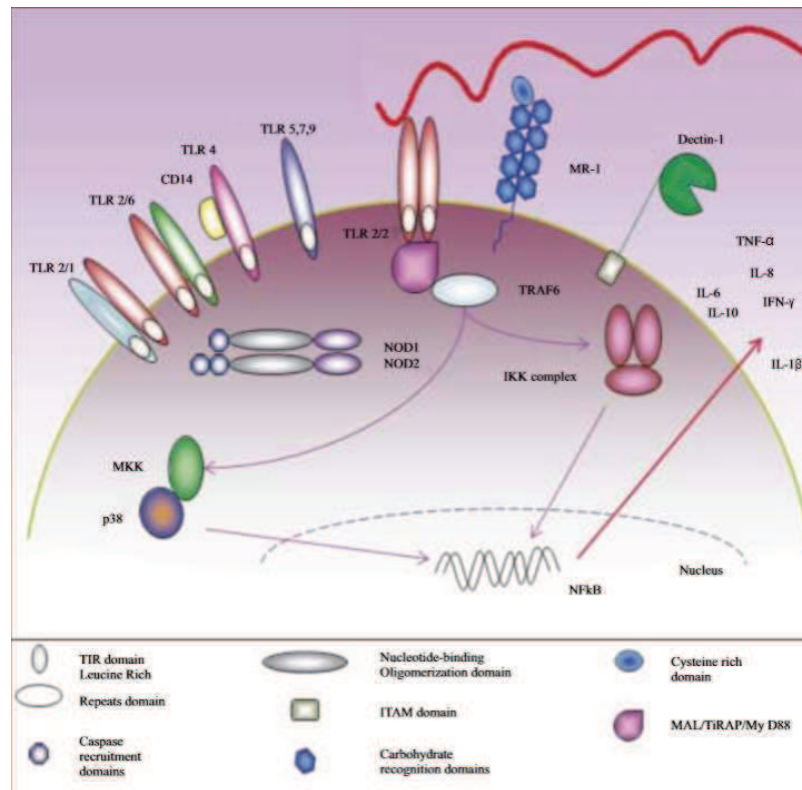
### B. Les récepteurs de l'immunité innée

L'immunité innée repose globalement sur la reconnaissance de PAMPs à l'aide des PRRs. Dans le cadre de la reconnaissance de *Borrelia*, l'implication des TLRs, des NLRs et des « C-type » lectines a

été clairement identifiée (**Figure 21**). Le rôle de **TLR2** a notamment été bien étudié. Celui-ci est surexprimé par les neutrophiles de patients infectés par *Borrelia* (Jablonska and Marcinczyk 2006). De plus, des patients possédants la mutation Arg753Gln au sein du gène de TLR2 possèdent des monocytes sanguins périphériques incapables de répondre à *Borrelia* (Schröder et al. 2005). Cependant, TLR2 n'est pas le seul TLR à intervenir puisque les macrophages TLR2 knock out (KO) sont capables de répondre à la présence de la bactérie lysée (Wooten et al. 2002). L'implication de TLR2 est surtout associée à l'immunité précoce contre *Borrelia*. La charge bactérienne au niveau des organes cibles chez des souris TLR2-KO a été déterminée 100 fois supérieure à celle chez les souris sauvages en début d'infection. Cependant, après 4 semaines, elle était comparable entre les deux types de souris (Wang et al. 2004). TLR2 peut aussi avoir un rôle délétère lors de l'infection par *Borrelia*. En effet, il a été montré que les patients présentant la mutation Arg753Gln sont réfractaires aux manifestations articulaires tardives (Schröder et al. 2005). Les autres TLRs dont l'implication dans la reconnaissance de *Borrelia* a été mise en évidence sont **TLR5**, **TLR7**, **TLR8**, **TLR9** et le co-récepteur **CD14**. L'implication de TLR5 semble cependant se faire au détriment de celle de TLR2, puisque son expression est réduite lorsque TLR2 est activé par la bactérie (Cabral et al. 2006). Le blocage du co-récepteur CD14, surexprimé chez les patients atteints de la borréliose de Lyme, réduit la capacité des neutrophiles à répondre à la présence de *Borrelia*, mais la réponse n'est pas abolie (Zhao et al. 2007a). Ce co-récepteur n'est donc pas le récepteur principal, mais permet plutôt de faciliter la réponse inflammatoire. Concernant TLR9, son rôle semble être mineur. L'ADN CpG de *Borrelia* est capable d'activer des cellules murines. Cependant, il n'a pas été possible de déceler des différences d'induction de cytokines entre des cellules sauvages et des cellules issues de souris TLR9-KO après incubation avec *Borrelia* (Shin et al. 2008a). De plus, après stimulation d'astrocytes avec la bactérie, aucune augmentation de l'expression de l'ARNm de TLR9 n'a été observée (Bernardino et al. 2008). TLR7 et TLR8 sont deux autres récepteurs Toll endosomiques capables d'induire la production d'interféron  $\alpha$  et  $\beta$  après phagocytose des *Borrelia* par des monocytes et des DCs plasmacytoïdes (Cervantes et al. 2014).

L'implication de **NOD2** dans la réponse inflammatoire envers *Borrelia* a été suggérée à la suite d'un travail sur la microglie murine. L'activation de celle-ci par *Borrelia* augmente l'expression de NOD2, mais pas celle de NOD1 (Sterka and Marriott 2006). De plus, il a été démontré que les personnes possédant une version non fonctionnelle de NOD2 expriment moins de cytokines inflammatoires que les autres. Ainsi, le rôle des NLRs dans la borréliose de Lyme nécessite d'être exploré davantage. Les récepteurs « c-type lectines », notamment le récepteur au mannose, semblent aussi jouer un rôle dans la réponse immunitaire innée contre *Borrelia*. L'expression du récepteur au mannose est augmentée

chez les DCs après incubation avec la bactérie (Cinco et al. 2001). De plus, ces récepteurs peuvent lier *Borrelia*. Cependant, la répercussion sur l'inflammation de l'activation de ce récepteur au mannose par *Borrelia* reste à être élucidée.



**Figure 21.** La réponse immunitaire innée contre *Borrelia* (Berende et al. 2010a)

### C. Les molécules effectrices de la réponse immunitaire innée

L'activation des différents récepteurs par *Borrelia* se poursuit par la mise en place de différentes voies de signalisation. La voie d'activation dépendante du facteur MyD88 semble nécessaire à la destruction de la bactérie (Liu et al. 2004). Mais elle n'est pas la seule puisque la voie dépendante de TRIF permet aussi la transduction de signaux de reconnaissance de *Borrelia* (Petnicki-Ocwieja et al. 2013a). De plus, les MAP (mitogen-activated protein) kinases jouent aussi un rôle important dans la réponse inflammatoire contre la bactérie (Olson et al. 2006). L'activation de ces différentes voies a pour but d'activer la synthèse de molécules inflammatoires. *Borrelia* induit typiquement *in vitro* la sécrétion d'IL-6, d'IL-1 $\beta$ , d'IL-12, de TNF- $\alpha$  ou encore d'IFN- $\gamma$  par les cellules mononucléées périphériques (PBMCs) et les MCs. Des chimiokines (IL-8), adhésines (E-selectine, VCAM-1, ICAM-1) et des cytokines anti-

inflammatoires (IL-10) sont aussi induites par la bactérie (Berende et al. 2010a). Finalement, les processus de cette immunité innée permettent le recrutement de cellules immunitaires, l'expression de molécules de co-stimulation par les cellules présentatrices d'antigènes et *a fortiori* la mise en place de l'immunité adaptative.

## 2. La réponse immunitaire adaptative

### A. Les lymphocytes T

Les lymphocytes T sont des cellules immunitaires capables de cibler directement les agents pathogènes (cellules T CD8 cytotoxiques) ou d'activer d'autres cellules qui agiront plus directement sur les agents pathogènes (T CD4 auxiliaires). Dans le contexte de la borréliose de Lyme, une orientation de la réponse des cellules T majoritairement Th1 a été mise en évidence au niveau des organes cibles (Kang et al. 1997). Le développement de la réponse Th1 souligne l'importance des phénomènes de phagocytose et de cytotoxicité dans la clairance de la bactérie, et explique l'infiltration de macrophages et de cellules T observé au niveau de l'érythème migrans. Plus exactement, la réponse immédiate nécessite l'intervention d'une réponse de type Th1 avant d'être progressivement remplacée par une réponse de type Th2 afin de contrôler l'infection tout en évitant les symptômes inflammatoires tels que l'arthrite. Chez les patients, lorsque l'IFN- $\gamma$  (cytokine liée à la réponse Th1) persiste et n'est pas remplacé par la sécrétion d'IL-4 (cytokine liée à la réponse Th2), ils développent des symptômes chroniques (Widhe et al. 2004).

### B. Les Lymphocytes B

La réponse immunitaire humorale, faisant intervenir les anticorps, est depuis longtemps considérée comme essentielle dans la lutte contre l'infection par *Borrelia*. La production continue d'anticorps contre la bactérie lors de la dissémination de celle-ci démontre l'implication des cellules B (Ekdahl et al. 2007). La présence d'anticorps anti-OspA et anti-OspB a été corrélée à une sévérité accrue des manifestations articulaires (Akin et al. 1999). De nombreuses *Borrelia* sont sensibles à la voie classique du complément médiée par les anticorps, et il semblerait que les neutrophiles soient un type cellulaire important dans ce processus (Singh and Girschick 2006). La prédominance des isotypes IgG1 et IgG3, deux isotypes associés au système du complément, dans le sérum de patients infectés par *Borrelia* démontre aussi l'importance de ce mécanisme dans le contrôle de l'infection (Widhe et al. 1998).



### 3. L'échappement immunitaire cutanée de *Borrelia*

Malgré la combinaison des différents mécanismes immunitaires pour contenir l'infection par *Borrelia*, la bactérie a évolué afin d'échapper à ces derniers. Comme il a été mentionné auparavant, la bactérie est capable de bénéficier de l'assistance de la salive de tique afin d'échapper à l'immunité de l'hôte vertébré. Il a notamment été montré que la bactérie peut fixer la protéine Salp15, produite par la tique *I. scapularis*, afin d'échapper au système immunitaire. De plus, la salive de tiques contient des inhibiteurs du complément. La bactérie elle-même peut inhiber le complément en fixant le facteur H à l'aide de ses CRASPs et de ses Erps. Un des mécanismes phares de l'échappement de *Borrelia* est la variation antigénique, à laquelle les protéines telles que VlsE, OspA, OspB et OspC sont soumises. Un autre mécanisme d'échappement consiste en l'échange latéral d'information génétique entre les bactéries. Les bactériophages interviennent dans ce processus, permettant l'échange de plasmides entre différentes espèces de *Borrelia* (Belmeli and Piffaretti 1996; Eggers and Samuels 1999; Wang et al. 1999a). Aussi, l'absence de nécessité de fer pour le développement de *Borrelia* permet à cette dernière de résister au mécanisme de privation de cet élément (Posey 2000). Enfin, il semblerait que la bactérie puisse adopter des formes rondes afin d'accroître sa résistance, même si de nombreuses controverses existent sur ce sujet (Meriläinen et al. 2015).

Globalement, la capacité de *Borrelia* à échapper au système immunitaire au niveau cutané fait de la peau un organe de persistance idéal pour la bactérie. La capacité de *Borrelia* à être ignoré du système immunitaire lors qu'elle est présente dans la peau pourrait expliquer sa persistance même après traitement antibiotique chez les modèles animaux (Embers et al. 2012; Hodzic et al. 2014). La peau, dans laquelle la bactérie se multiplie avant de disséminer notamment au niveau de la peau à distance, présente des structures idéales au sein desquelles la bactérie pourrait rester quiescente. Parmi ces structures pourraient figurer les follicules pileux dont la capacité à fournir un environnement immunoprivilégié pour plasmodium, le parasite du paludisme, a été démontrée (Gueirard et al. 2010).

# Smuggling across the Border: How Arthropod-Borne Pathogens Evade and Exploit the Host Defense System of the Skin

Quentin Bernard<sup>1</sup>, Benoit Jaulhac<sup>1,2</sup> and Nathalie Boulanger<sup>1,2</sup>

The skin is a critical barrier between hosts and pathogens in arthropod-borne diseases. It harbors many resident cells and specific immune cells to arrest or limit infections by secreting inflammatory molecules or by directly killing pathogens. However, some pathogens are able to use specific skin cells and arthropod saliva for their initial development, to hide from the host immune system, and to establish persistent infection in the vertebrate host. A better understanding of the initial mechanisms taking place in the skin should allow the development of new strategies to fight these vector-borne pathogens that are spread worldwide and are of major medical importance.

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## INTRODUCTION

The skin constitutes a protective barrier, controlling temperature homeostasis, sensing danger signals from the environment, and organizing the early innate immune response, all of which are accomplished because of the epidermis and the dermis. In case of injury, pharmacological (coagulation, platelet aggregation, and vasoconstriction) as well as immune processes occur in the skin (Schröder *et al.*, 2006; Miller and Modlin, 2007; Nestle *et al.*, 2009; Kennedy-Crispin and Billick, 2011). These cells sense the presence of damage-associated molecule patterns or pathogen-associated molecule patterns through their Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) leading to the secretion of inflammatory cytokines, type I IFN, chemokines, and antimicrobial peptides (Kawai and Akira, 2010). Dermal dendritic cells (DCs) activate and

sustain the expansion of skin-resident CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Moreover, they expand the immune response by activating and recruiting naive T cells in the nearest draining lymph node. Activated T cells stimulate other skin cells, including keratinocytes (KCs), to amplify the immune response by a positive feedback loop (Nestle *et al.*, 2009). The skin represents an essential immunologic organ that coordinates host defense by its functions in both innate and adaptive immunity (Schröder *et al.*, 2006; Gallo and Hooper, 2012).

Some pathogens have developed strategies to efficiently infect vertebrate hosts by taking advantage of the skin barrier disruption initiated by the bite of arthropods such as insects and ticks. The impact of arthropod-borne diseases such as malaria, leishmaniasis, dengue, and Lyme disease among others (Table 1) (Dana, 2009; Colpitts *et al.*, 2012) constitutes a major public health concern (Del Giudice *et al.*, 2005; Eisen and Eisen, 2011). The triad, pathogen, arthropod, and vertebrate host, interact tightly with each other and alter the immune defense outcome. Pathogens use arthropods to modify their antigenic surfaces and to amplify their virulence (Ramamoorthi *et al.*, 2005; Boulanger *et al.*, 2006; de Moura *et al.*, 2007; Neelakanta *et al.*, 2010; Kajla *et al.*, 2011; Chouikha and Hinnebusch, 2012) and seem to transform the skin barrier into an immune-tolerant organ for their own development (Guilbride *et al.*, 2012). The skin of the vertebrate host is a key interface for arthropod-borne diseases as it is there that pathogens and arthropod saliva are co-inoculated (Frischknecht, 2007), and the outcome of infections usually differs if pathogens are injected intravenously, intradermally, or via the arthropod (Inoue and Culleton, 2011; Kern *et al.*, 2011). The importance of the skin in certain arthropod-borne diseases is clearly visualized by the presence of different clinical inflammatory manifestations (e.g., erythema migrans for Lyme disease, severe lesions in leishmaniasis, trypanome in trypanosomiasis, and eschar for certain *Rickettsiae*) (Table 1).

In this review, we will first illustrate how some pathogens use specific skin cells for their development in arthropod-borne diseases, and how they subvert this environment to hide from the immune system or use it to control the immune response to facilitate their multiplication (Table 2). Next, we will describe how the vector contributes to pathogen virulence, notably by co-injecting its saliva with the pathogens, allowing a minimum of inoculated pathogens to survive and multiply in a hostile environment.

<sup>1</sup>EA7290, Virulence Bactérienne Précoce, Groupe Borréliose de Lyme, Institut de Bactériologie, Université de Strasbourg, Strasbourg, France and <sup>2</sup>Centre National de Référence Borrelia, Centre Hospitalier Universitaire, Strasbourg, France

Correspondence: Nathalie Boulanger, EA7290, Virulence Bactérienne Précoce, Groupe Borréliose de Lyme, Institut de Bactériologie, Université de Strasbourg, 3 Rue Kœberle, 67000 Strasbourg, France. E-mail: nboulanger@unistra.fr

Abbreviations: DC, dendritic cell; DENV, dengue virus; FB, fibroblast; IL, interleukin; KC, keratinocyte; KO, knockout; MO, macrophage; MC, mast cell; NO, nitric oxide; Treg, T regulator; TLR, Toll-like receptor

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**Table 1. Most important arthropod-borne diseases and their cutaneous manifestations in the vertebrate host**

Disease (pathogens)	Vector	Cutaneous manifestations	References
<b>Viruses</b>			
Dengue fever (dengue virus, DENV-1–4)	<i>Aedes</i> spp. (mosquito)	Macular, maculopapular, scarlatiniform, or petechial eruption	Gubler, 1998
Tick-borne encephalitis (tick-borne encephalitis virus, Western, Siberian, and Far-Eastern)	<i>Ixodes</i> and <i>Haemaphysalis</i> (ticks)	No	Dumpis <i>et al.</i> , 1999
West Nile virus	<i>Culex</i> (mosquitoes)	Maculopapular exanthema	Ferguson <i>et al.</i> , 2005
<b>Bacteria</b>			
Lyme disease ( <i>Borrelia burgdorferi sensu lato</i> )	<i>Ixodes</i> (ticks)	Skin lesion (erythema migrans)	Stanek <i>et al.</i> , 2012
Anaplasmosis ( <i>Anaplasma phagocytophilum</i> )	<i>Ixodes</i> or <i>Dermacentor</i> (ticks)	Rare	Bakken and Dumler, 2006
Rocky Mountain spotted fever ( <i>Rickettsia rickettsii</i> )	<i>Dermacentor</i> (ticks)	Maculopapular rash	Lin and Decker 2012
African tick bite fever ( <i>Rickettsia africae</i> )	<i>Amblyomma</i> , <i>Dermacentor</i> , or <i>Rhipicephalus</i> (ticks)	Maculopapular rash	Jensenius <i>et al.</i> , 2003
Boutonneuse fever ( <i>Rickettsia conorii</i> )	<i>Rhipicephalus</i> (ticks)	Maculopapular rash	Moraga <i>et al.</i> , 1982
Murine typhus ( <i>Rickettsia typhi</i> )	<i>Xenopsylla cheopis</i> (flea)	Erythematous papules	Gorchynski <i>et al.</i> , 2009
<b>Parasites</b>			
Malaria ( <i>Plasmodium falciparum</i> )	<i>Anopheles</i> (mosquitoes)	<i>Purpura fulminans</i> (very rare)	Kato <i>et al.</i> , 2007
Leishmaniasis ( <i>Leishmania</i> )	Sandfly	Skin lesions, nodules (eventually ulcers)	Reithinger <i>et al.</i> , 2007
Chagas disease ( <i>Trypanosoma cruzi</i> )	Triatominae bug	Chagoma	Rassi <i>et al.</i> , 2010
Sleeping sickness ( <i>Trypanosoma brucei rhodesiense</i> and <i>T. b. gambiense</i> )	Tsetse fly	Chancre, pruritus, and edema	Malvy and Chappuis, 2011

**PARASITES TRANSMITTED BY ARTHROPODS**

**Leishmaniasis**

This parasitic infection is due to a trypanosomidae flagellate parasite, *Leishmania*, transmitted by a sandfly, *Phlebotomus* (Old World) or *Lutzomia* (New world) (Titus and Ribeiro, 1990). It can stay localized in the skin or disseminate to the rest of the body according to the parasite species. The first clinical manifestation is a skin lesion that can persist for several weeks (Murray *et al.*, 2005). For decades it was thought that the parasite infects macrophages (MOs) directly and solely through complement-based opsonic ingestion and lectin-lipophosphoglycan phagocytosis (Wilson and Pearson, 1988; McNeely and Turco, 1990; Guy and Belosevic, 1993). In MOs, the parasite multiplies within the phagolysosome in which it differentiates from flagellated (promastigotes) to unflagellated parasite (amastigote forms) (Chang and Dwyer, 1976). During infection, the Th-1 response confers protection, whereas the Th-2 response makes the mouse model more susceptible to infection. More recently, it was shown that the first target cell for the parasite is the neutrophil (Laskay *et al.*, 2003; van Zandbergen *et al.*, 2004). Neutrophils, short-lived phagocytic cells, are among the first cell types to appear during an infection (Nauseef, 2007). One to two days after activation, they become apoptotic cells and eventually are internalized by DCs or MOs to be degraded or processed. They migrate to the site of a sand fly bite within 30 min in the presence or absence of parasite.

A study using fluorescence microscopy has shown that *Leishmania* colocalizes first within neutrophils and only afterwards within MOs (Peters *et al.*, 2008). This mechanism could

help the protozoan to escape the immune system and/or improve the efficiency of MO infection (Jochim and Teixeira, 2009). Infected neutrophils are preferentially internalized by these cells (Ribeiro-Gomes *et al.*, 2012). This could be linked to an increase in the expression of apoptotic markers induced by *Leishmania* accelerating its uptake by MOs, or by DCs (Langerhans and migratory DCs) inhibiting adaptive immune activation (Ribeiro-Gomes and Sacks, 2012). Mast cells also seem to have an important role in host protection, as mast-cell knockout mice develop larger lesions with more parasites and greater dissemination compared with wild-type mice (Maurer *et al.*, 2006). The exacerbation is likely owing to the decrease of local DC recruitment and to the shift of the Th-1 toward a Th-2 response. Similarly, KCs seem to be involved in the early immune response. Epidermal cytokines such as IL-12, IL-1 $\beta$ , IL-4, and IL-6 promote Th-1 differentiation in mice that are resistant to infection (Ehrchen *et al.*, 2010). Other cells known to control the Th-1/Th-2 balance that are present in the skin are T-regulatory cells (Treg). In mice, Langerhans cells have an immunosuppressive role during *L. major* infection of driving the expansion of Treg and inhibiting the Th-1 response, which is favorable for the survival of the pathogen (Peters and Sacks, 2006; Kautz-Neu *et al.*, 2011).

Recently, another study using CCR6 knock-out mice, a receptor shared by Th-17 and T-reg, has corroborated the fact that Treg have an important role in the development of *Leishmania* infection, notably decreasing the IFN- $\gamma$  response, which is crucial to control the infection (Barth *et al.*, 2012). In addition to resident skin cells, antimicrobial peptides

**Table 2. Major publications on the interaction between pathogens and host skin cells with potential effects of arthropod saliva**

Disease (pathogens)	Skin cells	Major effects on host cells with or without arthropod saliva	Major references	
<b>Viruses</b>				
Dengue ( <i>Flavivirus</i> )	Dendritic cells (DCs)	Invasion of DCs by binding to DC-SIGN receptors	Tassaneetrithep <i>et al.</i> , 2003	
		Protection of DC functions by saliva	Ader <i>et al.</i> , 2004	
	Macrophages	Sequestration of the virus into the endosomes of dermal macrophages expressing CD209	Kwan <i>et al.</i> , 2008	
	Keratinocytes (KCs), fibroblasts (FBs)	Invasion of KCs and FBs by the virus and secretion of antimicrobial peptides and IFN	Surasombattana <i>et al.</i> , 2011 Bustos-Arriaga <i>et al.</i> , 2011	
	Mast cells (MCs)	Limitation of the infection by MCs, recruiting NK T cells	John <i>et al.</i> , 2011 Yoshida <i>et al.</i> , 2012	
Tick-borne encephalitis (TBE virus)	DCs	Increase of the infection efficiency owing to the tick saliva	Fialová <i>et al.</i> , 2010	
<b>Bacteria</b>				
Lyme disease ( <i>Borrelia burgdorferi sensu lato</i> )	Neutrophils	Recruitment of neutrophils and production of O <sub>2</sub> <sup>-</sup>	Ribeiro <i>et al.</i> , 1990	
		Inhibition of integrin expression by tick saliva (ISL 929 and ISL 1373)	Guo <i>et al.</i> , 2009	
		Inhibition of phagocytosis and oxidative burst at the infection site	Hartiala <i>et al.</i> , 2008	
			Survival of some bacteria in NETs	Menten-Dedoyart <i>et al.</i> , 2012
	MCs	Degranulation upon infection	Talkington <i>et al.</i> , 1999	
	DCs	Inhibition of DC maturation: decrease of the expression of major histocompatibility complex (MHC) II by the bacteria and the saliva	Silberer <i>et al.</i> , 2000 Hovius <i>et al.</i> , 2008	
	Fibroblasts/macrophages	Increase of metalloprotease (MMP9) expression to escape the skin	Zhao <i>et al.</i> , 2003, 2007a, 2007b	
	FBs	Internalization for latency	Wu <i>et al.</i> , 2011	
KCs	Inhibition of secretion of antimicrobial peptides and chemokines by tick saliva	Marchal <i>et al.</i> , 2011a, 2011b		
<b>Parasites</b>				
Malaria ( <i>Plasmodium</i> ), leishmaniasis ( <i>Leishmania</i> )	MCs	Facilitating action of mosquito saliva: secretion of IL-4 and IL-10	Depinay <i>et al.</i> , 2006 Schneider <i>et al.</i> , 2011	
	DCs/macrophages	Decrease of MHC-II expression	da Silva <i>et al.</i> , 2012	
	Tregs	Increase of the suppressive activity of Tregs	da Silva <i>et al.</i> , 2012	
	Macrophages Neutrophils	Apoptosis of <i>Leishmania</i> -infected neutrophils internalized by macrophages, the target cells	Laskay <i>et al.</i> , 2003 Peters <i>et al.</i> , 2008	
		Inhibition of NO synthesis by the vector saliva	Gomes <i>et al.</i> , 2012	
		Increase of TGF-β secretion	Van Zandbergen <i>et al.</i> , 2004	
	DCs	Inhibition of maturation by maxadilan, saliva protein of the vector	Wheat <i>et al.</i> , 2008	
		Uptake of <i>Leishmania</i> -infected neutrophils and decrease of MHC class II expression	Ribeiro-Gomes <i>et al.</i> , 2012	
	MCs	Stimulation of Th1 response to improve the host response	Maurer <i>et al.</i> , 2006	
	KCs	Early inflammation driven by KCs by promoting Th1 response	Ehrchen <i>et al.</i> , 2010	
	Tregs/DCs	Stimulate the expansion of Tregs, which favor the inhibition of IFN-γ, and so improve the infection	Peters and Sacks, 2006 Kautz-Neu <i>et al.</i> , 2011 Barth <i>et al.</i> , 2012	

such as defensins and cathelicidins are also important effectors of innate immunity in the skin (Bernard and Gallo, 2011). In a mouse model, cathelicidin (*Camp*) has been shown to directly target the parasite. In mouse knock out for *Camp*, the level of the anti-inflammatory cytokines (IL-10) is high, whereas the level of pro-inflammatory cytokines (IL-12 and IFN- $\gamma$ ) is reduced (Kulkarni et al., 2011). Cathelicidin is secreted by epithelial cells and mast cells (Nardo et al., 2008), and is chemotactic for neutrophils and other immune cells.

Although a significant number of *Leishmania* antigens have been identified as potential candidates (Launois et al., 2008), no vaccine is available against leishmaniasis. Further investigations are necessary to better understand the initial pathogenesis of the parasite in the skin, especially the precise role of neutrophils in the initiation of the disease (Ritter et al., 2009) as well as the role of Treg (Bourreau et al., 2009; Barth et al., 2012). The use of *in vivo* imaging with fluorescent parasites varying in virulence should help clarify these two aspects.

#### Malaria

*Plasmodium*, a protozoan Apicomplexa responsible for malaria, causes >600,000 deaths per year in tropical countries (World Malaria Report, 2013). The invasive forms, the sporozoites, are inoculated into the dermis by *Anopheles* mosquitoes without inducing a skin lesion. They initially invade hepatocytes, where an intense multiplication occurs, and then the red blood cells (Sinnis and Coppi, 2007). For decades, malarialogists believed that sporozoites injected by mosquitoes rapidly left the skin within minutes to reach the liver without specific cell interactions in the skin (Sinnis and Sim, 1997; Mota et al., 2004). However, recent studies have now shown that some sporozoites remain in the skin and initiate an immune response (Vanderberg and Frevert, 2004; Guilbride et al., 2012). In fact, sporozoites take several hours to leave the inoculation site (Amino et al., 2006; Yamauchi et al., 2007); 40% of them remain in the skin after 6h, 11% after 24h, and 20% go to the draining lymph nodes (LNs) (Gueirard et al., 2010). In mice, between 0.5 and 1.8% of the inoculated sporozoites develop into skin exoerythrocytic forms and survive for weeks in the immune-privileged site constituted by hair follicles (Voza et al., 2012). A possible effect of exoerythrocytic forms in the skin may be the development of tolerance. In addition, the mosquito bite induces the recruitment of migratory immune cells to the inoculation site (Amino et al., 2006) and sporozoites increase the mobility of DCs and Treg in the skin. The major histocompatibility complex II expression of DCs is lower after an infected-mosquito bite than after a noninfected-mosquito bite, transforming these cells into tolerogenic ones. The Treg recruitment also has a key role in this strategy; however, their specific role remains to be investigated (da Silva et al., 2012).

For a while, *Plasmodium* has been thought to reach hepatocytes directly after being injected by mosquitoes. However, recent intravital imaging studies have helped to improve our understanding of the initial phase of malaria in

the skin (Ménard et al., 2013). An immune response is primed into the skin against these *Plasmodium* skin forms. Thus, analyzing precisely how cutaneous immune cells and resident cells (KCs and fibroblasts (FBs)) respond to these special exoerythrocytic form antigens may be helpful in developing efficient vaccines.

#### VIRUS TRANSMITTED BY ARTHROPODS

##### Dengue

Among arthropod-borne pathogens, viruses are well represented. One of the most studied, the dengue virus (DENV), is transmitted by *Aedes* mosquitoes. The disease threatens 2.5 billion people, mainly in tropical countries, and can be responsible for lethal hemorrhagic fever. The DENV is a *Flavivirus* with a single-strand positive RNA, with four human DENV serotypes (DENV-1–4). It attaches to the host cell receptor through its E glycoprotein, enters the cell via endosomes, and after an acidification process reaches the cytoplasm where the nucleocapsid replicates (Smit et al., 2011). Alternatively, DENVs can enter cells directly through the plasma membrane (Vancini et al., 2012), confirming early observations (Hase et al., 1989). In the skin, the virus first infects resident DCs by binding to the DC-SIGN molecules (L-SIGN, CD209) (Navarro-Sanchez et al., 2003; Tassaneeritthep et al., 2003). KCs are also susceptible to DENV infection (Surasombatpattana et al., 2011). Given the high proportion of KCs in the skin, this ability to infect KCs gives the virus a chance to expand rapidly before the activation of a strong immune response by specific immune cells. However, KCs are able to respond to DENV infection in a MyD88-dependent pathway by inducing a high expression of IFN- $\beta$ , IFN- $\gamma$ , and antimicrobial peptides. The response is mediated by double-strand RNA sensors such as TLR3/IRF7, RIG-I, and MDA5 (Nasirudeen et al., 2011). On the other hand, molecules secreted by KCs attract immune cells, MOs, and DCs in which DENV can replicate. Similarly, FBs also contribute to the expansion of the DENV (Bustos-Arriaga et al., 2011). They are permissive to the virus and can constitute a cell reservoir.

DENV binds FB and KC membrane through its NS1 protein and the heparan sulfate and chondroitin sulfate E molecules (Avirutnan et al., 2007). However, FBs can fight the infection by secreting IFN, TNF- $\alpha$ , and antimicrobial peptides. This response occurs through a TLR3/IRF3-dependent pathway, but not through IRF7 as in the case of DCs and KCs. Whether FBs act mainly as reservoirs for the virus or whether they mainly contribute to elimination of the virus by participating in the pro-inflammatory response activation remains to be investigated.

Mast cells (MCs) are also important for the immune surveillance of DENV infection (John et al., 2011). MCs are essential for resistance to vaccinia infection, another virus of the skin (Wang et al., 2012a, b). Even if MCs are very resistant to the DENV infection, intracellular virus RNA is detected and internalized via MDA5 and RIG-I receptors. The virus induces the degranulation of MCs present at the infection site, inducing the recruitment of NK and NKT cells and the release of cytokines (TNF- $\alpha$  and IFN- $\alpha$ ) and chemokines (CCL5, CXCL12, and CX3CL1). The TNF- $\alpha$  secretion

could facilitate the recruitment of immune cells by increasing vascular permeability. MCs seem to participate in the virus clearance by limiting the viral invasion of the LNs even if their involvement is not clear (Yoshida *et al.*, 2012). Alternatively, they could also help the virus to spread out more efficiently by making access to the bloodstream easier (Shresta *et al.*, 2006). Dermal-type MOs also show an inherent resistance to DENV infection. They could limit the infection by sequestering the virus into poorly acidified phagosomes (Kwan *et al.*, 2008).

#### BACTERIA TRANSMITTED BY ARTHROPODS

##### Lyme borreliosis

As the first arthropod-borne disease of the Northern Hemisphere, Lyme borreliosis has been particularly well studied. It is a bacterial spirochete, *Borrelia burgdorferi sensu lato*, transmitted by a hard tick, *Ixodes* sp. (Radolf *et al.*, 2012). It is first characterized by a cutaneous inflammation, erythema migrans, then by a multisystemic disorder affecting the nervous system, the joints, and the skin. Currently, it is considered to be an extracellular bacterium, even if some recent studies have shown that *B. burgdorferi* can be internalized in certain cells such as the FBs (Chmielewski and Tylewska-wierzbnowska, 2010; Wu *et al.*, 2011). Early studies describe the role of DCs (Hulínská *et al.*, 1994; Filgueira *et al.*, 1996; Beermann *et al.*, 2000). *B. burgdorferi* inhibits the expression of class II molecules on epidermal Langerhans cells and thus interferes with the immune response against the bacteria (Silberer *et al.*, 2000). In addition, the tick bite induces the migration of neutrophils to the site of bacterial inoculation (Hartiala *et al.*, 2008).

Owing to its OspB protein, *B. burgdorferi* inhibits neutrophil phagocytosis and the generation of oxidative bursts (Hartiala *et al.*, 2008). Recently, these cells have been shown to trap bacteria into their neutrophil extracellular traps (NETs) (Menten-Dedoyart *et al.*, 2012). Some of the spirochetes are killed, but others survive into NETs, forming nonmotile round bodies. In addition to direct interaction with skin cells, *B. burgdorferi* upregulates some host proteins secreted by resident skin cells for its own benefit. Lacking metalloproteases (MMPs) to migrate through skin extracellular matrix, it induces MMP-9 secreted by FBs and/or MOs (Zhao *et al.*, 2003, 2007a, b). In this disease, the skin constitutes an amplification site at which the bacteria multiply intensively (Kern *et al.*, 2011) and it seems to constitute a 'filter' in which, among the heterogeneous population of *Borrelia*, specific bacterial clones are selected to disseminate in the vertebrate host (Brisson *et al.*, 2011). The mechanism of this selection remains to be identified and deserves further investigations.

#### VECTOR SALIVA, A CRITICAL PARAMETER FOR EFFECTIVE PATHOGEN TRANSMISSION

Arthropod saliva contains molecules with anti-hemostatic, anti-inflammatory, and immune-modulatory properties to facilitate the uptake of the blood meal that is essential for their survival (Titus *et al.*, 2006; Francischetti *et al.*, 2010;

Kazimírová and Stibrániová, 2013). Interestingly, pathogens use saliva from such arthropods to facilitate their transmission. Thus vectors are not just syringes that inoculate pathogens, but are an essential part of the triangular interaction characterizing arthropod-borne diseases.

Studies on Lyme borreliosis and leishmaniasis have clearly revealed a major contribution of arthropod saliva in the transmission of pathogens (Gillespie *et al.*, 2000; Brossard and Wikel, 2004; Nuttall and Labuda, 2004; Titus *et al.*, 2006; Fontaine *et al.*, 2011). Transcriptomic and proteomic analyses of arthropod salivary glands have shown that pathogens upregulate some proteins to facilitate the vertebrate host infection (Ribeiro, 1987; Titus and Ribeiro, 1990; Dixit *et al.*, 2009; Girard *et al.*, 2010; Ramabu *et al.*, 2010; Sim *et al.*, 2012). The presence of these arthropod proteins increases pathogen virulence, so that fewer pathogens are necessary to induce host infection as shown for *Leishmania* by sandfly saliva (Titus and Ribeiro, 1988). For example, a peptide called maxadilan has been identified as a facilitating molecule (Lerner *et al.*, 1991). It increases the infection by driving the Th-1 immune response to a Th-2 response. It also downregulates nitric oxide expression by MOs and the expression of co-stimulatory molecules by DC and T-cell activation (Wheat *et al.*, 2008). The saliva also induces neutrophil apoptosis associated with increased parasite burden (Prates *et al.*, 2011). Other molecules, LJM11, LJM19, PpSP15, and Linb-11 in sand fly saliva, have been correlated with host protection after vaccination trials in mice (Gomes and Oliveira, 2012; Gomes *et al.*, 2012; de Moura *et al.*, 2013). However, their roles during the transmission of the parasite require further investigations.

Similar observations have been made with ticks. Owing to the long-lasting blood meal of ticks, the arthropod has to develop different strategies to stay attached to the host skin for several days, thus explaining the high number of molecules with different properties identified in tick saliva (Kazimírová and Stibrániová, 2013). In Lyme borreliosis, *Borrelia* specifically upregulates a protein in *Ixodes* saliva, Salp15, which has been shown to affect different host immune cells such as DCs, CD4 T cells, or KCs (Anguita *et al.*, 2002; Ramamoorthi *et al.*, 2005; Hovius *et al.*, 2008; Marchal *et al.*, 2011b), thus improving the transmission of the bacteria.

Tick saliva contains additional proteins inhibiting T- and B cell proliferation, DC maturation, neutrophil functions, and complement activation (Ribeiro *et al.*, 1990; Leboulle *et al.*, 2002; Sá-nunes *et al.*, 2007; Guo *et al.*, 2009; Francischetti *et al.*, 2010). The tick saliva of *Ixodes* can also facilitate the transmission of the bacteria, *Anaplasma phagocytophilum*, by downregulating cytokine expression of MOs (Chen *et al.*, 2012). A similar phenomenon has been observed for tick-borne encephalitis, in which the virus modifies the composition of tick saliva to improve the infection by increasing the DC infection efficiency. This leads to a decrease in virus-induced TNF- $\alpha$  and IL-6 production (Robertson *et al.*, 2009; Fialová *et al.*, 2010).

Mosquito saliva also seems to increase *Plasmodium* infectivity. It was earlier observed that sporozoites inoculated via

infected mosquito were more infectious than those inoculated intravenously (Vaughan *et al.*, 1999; Leitner *et al.*, 2010). When washed sporozoites were co-inoculated with or without mosquito saliva intradermally, the presence of saliva induced IL-4 and IL-10. Parasitemia was higher and cerebral malaria developed more frequently (Depinay *et al.*, 2006; Schneider *et al.*, 2011; Mauduit *et al.*, 2012). The saliva of *Aedes aegypti* also exhibits immunomodulatory properties by inducing lymphocyte but not DC apoptosis in a caspase 3- and 8-dependent pathway (Bizzarro *et al.*, 2013).

The role of vector saliva is usually protective for pathogens. However, the immune response developed against saliva may sometimes be detrimental. In the case of repeated sandfly bites, a delayed hypersensitivity reaction makes the bite site refractory to the parasite, notably by increasing the amount of Th-1 cytokine production (Kamhawi, 2000; Gomes and Oliveira, 2012). In *Anopheles stephensi*, the saliva seems to indirectly protect the host from pathogens by increasing neutrophil attraction (Owhashi *et al.*, 2001). In *Aedes aegypti*, the saliva decreases the DCs' vulnerability to DENV and increases their production of cytokines (Ader *et al.*, 2004).

Vector saliva is an amazing source of pharmacologic and immunosuppressive molecules. The role of saliva has been particularly well studied in the long-lasting blood meal of ticks, but its role in, for example, malaria should be precisely investigated. Moreover, its essential role in the initial pathogenesis of vector-borne diseases makes it a preferential target for vaccine development. However, the exact mechanism of protection induced by arthropod saliva needs to be elucidated to determine precisely how long it persists in the skin and acts on the different skin cells, for its eventual safe use in human vaccines.

## CONCLUSION

The skin of vertebrates as an essential organ for immune defense is exploited during the transmission of arthropod-borne pathogens. Its key role is clearly demonstrated by the cutaneous manifestations that develop at the site of inoculation in diseases such as leishmaniasis, Lyme disease, and/or rickettsial diseases. Through the action of its resident and recruited cells, the skin is a primary participant in the physiopathology of these diseases. Recently, the role of the skin has been reevaluated by the discovery of new target cells such as neutrophils in leishmaniasis and in certain developmental stages in malaria. The interaction of *Plasmodium* skin stages and resident skin cells remains to be investigated. As resident cells such as KCs and FBs are probably also important for the development of immunity as in Lyme Borreliosis (Marchal *et al.*, 2011a; Wu *et al.*, 2011) and dengue (Surasombattana *et al.*, 2011), *in vitro* approaches such as skin organotypic cultures may help to explore this aspect. For most of these diseases, the skin seems to be a site of immune tolerance and pathogen latency, in which the role of Treg is essential (Barth *et al.*, 2012; Guilbride *et al.*, 2012). Such research may help to establish future effective vaccines combining pathogens and vector saliva compounds (Nuttall *et al.*, 2006; Schuijt *et al.*, 2011; Parizi *et al.*, 2012).

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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# Présentation du sujet de thèse

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Dans le contexte de la Borréliose de Lyme, la peau joue un rôle majeur puisqu'elle est la première interface rencontrée par *Borrelia burgdorferi* sensu lato lors de la transmission à l'hôte vertébré. La tique, du genre *Ixodes*, induit une lésion cutanée par sa piqûre telmophage en insérant ses pièces piqueuses ainsi qu'en injectant sa salive, créant un microhématome et une cavité lui permettant de se nourrir. Ce processus facilite le repas sanguin, mais aussi la transmission de *Borrelia* (Sonenshine and Roe 2014a). Une fois injectées, les bactéries et la salive interagissent avec les cellules de la peau telles que les KCs de l'épiderme, et les FBs et cellules immunitaires du derme. La peau est donc un organe crucial lors de la transmission de *Borrelia* puisqu'elle est le lieu où est initiée la réponse immunitaire contre la bactérie. Ce travail de thèse repose sur l'étude de l'initiation de l'inflammation cutanée lors de la transmission précoce de *Borrelia* par la tique du genre *Ixodes*. Pour cela, le sujet a été divisé en trois axes majeurs.

Dans un premier temps, nous avons étudié l'impact de la piqure de la tique *Ixodes ricinus* sur l'inflammation précoce contre *B. burgdorferi* ss. Pour cela, nous avons étudié le rôle des KCs, cellules résidentes de l'épiderme, dans l'inflammation induite par la lésion tissulaire, associée à la piqûre de tique, et par la bactérie. Lors de la transmission de *Borrelia*, les pièces piqueuses de la tique dilacèrent les tissus de la peau lésant les cellules qui s'y trouvent. De plus, la salive de tique participe à la lyse cellulaire en ciblant les FBs (Schramm et al. 2012). La lésion tissulaire pourrait induire la libération d'ARN par les cellules lésées renforçant ainsi l'inflammation via l'activation de TLR3 (Karikó et al. 2004a; Cavassani et al. 2008; Lai et al. 2009; Lim and Wang 2011). D'autre part, il est établi que la bactérie, via une lipoprotéine majeure de surface OspC, active le récepteur TLR2 conduisant à une inflammation précoce (Wooten et al. 2002; Marchal et al. 2011). Puisque le rôle de la blessure et du récepteur TLR3 lors de la transmission de la borréliose de Lyme n'a pas été étudié, nous avons décidé d'analyser le rôle de la co-activation TLR2/TLR3 sur un modèle *in vitro* de KCs humains primaires. L'influence de la salive de tique sur cette co-activation a aussi été investiguée. Un modèle d'inflammation *in vivo* chez la souris fut utilisé par la suite pour compléter l'étude de ce phénomène. Enfin, nous avons déterminé, en collaboration avec une équipe de chimistes (L. Sabatier, IPHC, Strasbourg), la protéine de salive de tique responsable de la lyse des FBs.

Dans un deuxième temps, nous avons analysé l'importance de la bactérie lors de l'inflammation cutanée. *Borrelia* voit l'expression de ses gènes varier au cours de son passage du vecteur à l'hôte vertébré. De nombreux gènes associés à la virulence de la bactérie sont exprimés lors du séjour de ces dernières au sein de la peau. Nous avons cherché à caractériser les gènes responsables de la virulence d'un clone de la souche 297 de l'espèce *B. burgdorferi* ss aux capacités disséminantes particulièrement

efficaces. Dans un premier temps, les protéines exprimées par le clone ont été analysées par spectrométrie de masse par une équipe de chimiste, puis comparées aux protéines exprimées par la souche native. Dans deuxième temps, nous avons validé l'expression des gènes associés à ces protéines par la bactérie lors de l'infection *in vivo* chez la souris.

Finalement, le rôle de niche immunitaire de la peau a été exploré. Pour cela, nous nous sommes concentrés sur l'étude d'un type de cellules immunitaires résidentes de la peau, les MCs, dans l'inflammation contre *Borrelia*. Les MCs ont très peu été étudiées dans le contexte de la borréliose de Lyme (Talkington and Nickell 1999). Nous avons donc décidé d'analyser le comportement de ces cellules issues de chez la souris face à ces bactéries. Leurs capacités à dégranuler et à exprimer des molécules inflammatoires ont été analysées en présence de la bactérie entière, de sa lipoprotéine de surface OspC et de la salive de tique. L'importance de la peau a ainsi été explorée à la fois du point de vue du vecteur, de l'agent pathogène et de l'hôte vertébré, les trois acteurs essentiels au développement de la borréliose de Lyme.

# Résultats

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Les résultats sont présentés sous forme d'articles

# L'acteur 1 : la tique



Nymphes *Ixodes ricinus* gorgées et non gorgées (Photographie d'après N. Boulanger)

## ARTICLE 1:

*Ixodes* tick saliva suppresses the keratinocyte cytokine response to TLR2/TLR3 ligands during early exposure to Lyme borreliosis

Quentin Bernard, Richard L. Gallo, Benoît Jaulhac, Teruaki Nakatsuji, Benjamin Luft, Xiahou Yang,

Nathalie Boulanger

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Au cours des maladies à transmission vectorielle, la peau de l'hôte vertébré constitue la première interface rencontrée par les agents infectieux. Ils y sont inoculés avec la salive des arthropodes et interagissent avec les cellules résidentes de la peau (KCs, FBs et cellules immunitaires). Lors du repas sanguin des tiques dures s'effectuant sur plusieurs jours, la tique du genre *Ixodes* dilacère la peau et inocule sa salive, induisant une poche d'inoculation autour de ses pièces piqueuses (« Feeding pool ») (Sonenshine and Roe 2014a). Cette blessure pourrait être potentiellement utilisée par les agents infectieux transmis par les tiques : elle libère de l'espace et des nutriments en lysant les cellules qui peuvent être utilisés par les agents pathogènes pour se développer dans la peau; elle peut induire une immunosuppression des cellules immunitaires attirées au point de pique par le biais de la salive de tique.

Nous avons pris la borréliose de Lyme comme modèle pour étudier ce phénomène. Nous avons montré précédemment que la salive de tique du genre *Ixodes* lyse *in vitro* les FBs humains, ce qui pourrait expliquer la poche d'inoculation et la nécrose observée après piqûre de tique (Schramm et al. 2012). Les FBs lysés génèrent des ARNs double brin, qui pourraient participer à l'inflammation en activant le récepteur Toll TLR3 sur les cellules de la peau (KCs et FBs) (Karikó et al. 2004a; Lim and Wang 2011; Wanke et al. 2011). Pour tester cette hypothèse, nous avons utilisé le poly (I:C) pour mimer l'ARN double brin généré par les cellules nécrotiques et testé son effet potentiel sur l'interaction KCs-*Borrelia in vitro*. L'interaction poly (I:C)-TLR3 augmente l'inflammation produite par l'interaction TLR1/2-*Borrelia* ou TLR1/2-OspC, une lipoprotéine de surface de la bactérie essentielle lors de la transmission à l'hôte vertébré. La salive de tique inhibe cette inflammation. L'interaction TLR2-TLR3 n'est cependant que partiellement observée chez la souris lorsque le poly (I:C) et la protéine L-OspC sont co-injectés au niveau de l'oreille, soulignant la différence entre le modèle *in vitro* sur cellules humaines primaires et le modèle murin. D'autre part, il existe une différence majeure entre la souris et l'homme : seul ce dernier présente un érythème migrant (Müllegger and Glatz 2008).

Nous proposons le modèle suivant pour la transmission de *Borrelia* sp. De la tique à l'hôte vertébré : la salive de tique (1) lyse les FBs et participe à la formation de la poche d'inoculation, puis (2) génère un tissu nécrotique qui active TLR3 et le neutralise aussitôt. Cependant, quand la tique s'est détachée et que la bactérie migre vers les sites à distance, l'activation de TLR3 pourrait expliquer l'inflammation observée lors de la borréliose de Lyme : l'EM, puis les manifestations systémiques dans les organes à distance (articulation, système nerveux central et peau à distance).



## *Ixodes* tick saliva suppresses the keratinocyte cytokine response to TLR2/TLR3 ligands during early exposure to Lyme borreliosis

Quentin Bernard<sup>1</sup>, Richard L. Gallo<sup>2</sup>, Benoît Jaulhac<sup>1,4</sup>, Teruaki Nakatsuji<sup>2</sup>, Benjamin Luft<sup>3</sup>, Xiahou Yang<sup>3</sup> and Nathalie Boulanger<sup>1,4</sup>

<sup>1</sup>EA7290 Virulence bactérienne précoce: groupe borréliose de Lyme, Fédération de médecine Translationnelle et Faculté de Pharmacie de Strasbourg, Université de Strasbourg, Strasbourg, France; <sup>2</sup>Department of Dermatology, University of California, San Diego, CA, USA; <sup>3</sup>Department of Medicine, State University of New York, Stony Brook, NY, USA; <sup>4</sup>Centre National de Référence Borrelia, Centre Hospitalier Universitaire, Strasbourg, France

Correspondence: Nathalie Boulanger, EA7290: Virulence bactérienne précoce: Groupe Borréliose de Lyme, Institut de bactériologie, 3 rue Koeberlé, 67 000 Strasbourg, France. Tel.: 33 3 69 55 14 49 / 33 3 68 85 41 25, Fax: 33 3 69 55 16 98, e-mail: nboulanger@unistra.fr

**Abstract:** *Ixodes* hard tick induces skin injury by its sophisticated biting process. Its saliva plays a key role to enable an efficient blood meal that lasts for several days. We hypothesized that this feeding process may also be exploited by pathogens to facilitate their transmission, especially in the context of arthropod-borne diseases. To test this, we used Lyme borreliosis as a model. This bacterial infection is caused by *Borrelia burgdorferi* sensu lato transmitted by *Ixodes*. We co-incubated *Borrelia* with human keratinocytes in the presence of poly (I: C), a dsRNA TLR3 agonist generated by skin injury. This induced a strong cytokine response from human primary keratinocytes that was much greater than that induced by *Borrelia* alone. OspC, a TLR2/1 agonist and a major surface

lipoprotein of *Borrelia* also amplified the process. Interestingly, tick saliva inhibited cytokine responses by keratinocytes to these TLR agonists. We propose that *Borrelia* uses the immunoprivileged site produced by tick saliva to facilitate its transmission.

**Abbreviations:** AMPs, antimicrobial peptides; EM, erythema migrans; FB, fibroblast; IFN, interferon; IL, interleukin; KC, keratinocyte; L-OspC, lipidated OspC; Osps, outer Surface proteins; TLR, Toll-like receptor; UL-OspC, unlipidated OspC.

**Key words:** borrelia – lyme – ticks – TLR2 – TLR3

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### Introduction

In arthropod-borne diseases, the skin of the vertebrate host plays a key role as the first interface encountered by pathogens. There, arthropod saliva and pathogens are co-inoculated and interact with resident skin cells, keratinocytes (KCs), fibroblasts (FBs) and immune cells (1,2). Ticks stay anchored to the skin for several days by their sophisticated mouthparts, the hypostome and the two chelicerae (3,4). During this long-lasting blood meal, *Ixodes* ticks injure the skin and inoculate bioactive compounds released by the tick saliva. This process contributes to the formation of a feeding pool around the tick mouthparts (4), facilitating blood uptake, transmission and local multiplication of pathogens (5). *Ixodes* saliva targets different immune cell functions: dendritic cell (DC) maturation, T and B cell proliferation, neutrophil and KC activation (6–14). It also affects complement activation (15). Different tick salivary proteins have been characterized. Prostaglandin E2 inhibits DC maturation while evasin-E3 inhibits interleukin-8 (IL-8) secretion, a chemokine involved in neutrophil attraction (16). Salp15 of *I. scapularis* (7,17) targets different receptors of immune cells (CD4 T cells, DCs) to inhibit inflammation during *Borrelia burgdorferi* transmission. During *Anaplasma phagocytophilum* infection, an intra-cellular bacterium transmitted also by *Ixodes* ticks, the tick salivary protein, sialostatin L2, inhibits caspase 1-mediated inflammation, reducing IL-1 $\beta$  and IL-18 secretion by murine macrophages as well as other cytokines (18,19). Overall, the *Ixodid* tick saliva strongly decreased inflammatory response against vector-borne pathogens (20).

The skin injury induces a cascade of pharmacological events: vasoconstriction, platelet aggregation and coagulation. These are immediately counteracted by tick saliva (20,21). Necrosis and ulceration have been observed in mouse skin experimentally bitten by ticks (3,22). Skin injury induces tissue necrosis: high-mobility group box 1 protein (HMGB1), uric acid, heat-shock proteins (HSPs) and nucleotides are released by necrotic cells and can activate inflammation (23). Among nucleotides molecules, double-stranded RNA activates inflammation through Toll-like receptor 3 (TLR3) (24–26).

In Lyme borreliosis, upon the infectious *Ixodes* tick bite, the first interaction between *B. burgdorferi* sensu lato (sl) and the vertebrate host occurs at the skin interface (21). For most people, an erythema migrans (EM) develops before the onset of disseminated manifestations affecting the joints, the nervous system or the distant skin (27). *Borrelia* is largely covered by surface lipoproteins, the Osps (outer surface proteins) (28). Bacterial transmission correlates with a major antigenic modification of these lipoproteins. Within the tick, the spirochaetes migrate from the gut to the salivary glands and before being injected into the host skin, OspC expression is upregulated to the detriment of OspA expression (29). OspC is an essential protein for the early transmission of the spirochaete from the tick to the host (30–32). Its role is not totally understood. It might help *Borrelia* dissemination through the manipulation of the host vasculature (33). The triacylated lipoprotein OspC is also known to trigger an inflammatory response by activating a TLR2/1-dependent pathway with its lipid part

(Pam3Cys) (34–36). This inflammatory response is facilitated by CD14 receptor and characterized by cytokine secretion, including tumor necrosis factor (TNF- $\alpha$ ) and antimicrobial peptides (AMPs) notably by KCs (8,37–41). AMPs (defensins and cathelicidins) and TLRs are effectors of skin innate immunity and have been shown to be essential in skin disorders such as atopic dermatitis and psoriasis (42,43). AMPs are secreted *in vitro* by human primary KCs upon the interaction of *Borrelia* or OspC with TLR2 (39). During *Borrelia* infection, innate immunity is also triggered by other TLRs, including TLR5, TLR7, TLR8, and TLR9 (35,44–46).

The potential role of TLR3 during *Borrelia* infection has never been investigated so far. Recently, TRIF, the adaptor molecule of TLR3 has been described to be used in TLR2 signalling (47). In addition, a crosstalk between TLR3 and TLR2 has been shown in the context of skin injury and a commensal bacteria, *Staphylococcus epidermidis*. Through its lipoteichoic acid moiety, this commensalism controls excessive skin inflammation (25,48). As TLR2 interacts with *B. burgdorferi*, we analysed the potential effect of TLR3 activation generated by the tick injury on *Borrelia*-induced inflammation. Our data suggest that the TLR3–TLR2 crosstalk could play an important role during the early transmission of *B. burgdorferi* sl.

## Materials and methods

### Bacteria

*Borrelia burgdorferi* sensu stricto (ss) N40, passage 8 (8 bacterial subcultures as its direct isolation from the infected tick), cultured in BSK-H medium (Sigma-Aldrich) at 33°C were used at the late-log-phase and washed twice by centrifugation (30 min, 5000 g). *B. burgdorferi* ss 297, OspC-deficient and complemented mutants, have been described previously (49).

### Tick salivary glands

*I. ricinus* salivary gland extract (SGE) was used at 20  $\mu$ g/ml as previously described (8). This concentration corresponds to the SGE concentration obtained from one tick after dissection. SGE was tested by the Limulus assay to check for the presence of endotoxins and was found to contain <0.3 endotoxin units.

### TLR agonists and other reagents

The surface proteins, lipidated OspC (L-OspC) in 20 mM Tris-Cl (pH 7.5), 0.3% Triton X-100 and unlipidated OspC (UL-OspC) were cloned and purified from *B. burgdorferi* ss B31 as previously described (50). They were used in the presence of polymyxin B, at 50  $\mu$ g/ml (Euromedex, Strasbourg, France). The triacylated lipopeptide Pam3CysSerLys4 (Pam3CSK4) and polyinosinic-polycytidylic acid [poly (I:C)] high molecular weight (HMW) were from InvivoGen.

### Primary cell culture of keratinocytes and stimulation

Human foreskins were obtained from patients at the CHU Strasbourg (France) after written consent. This procedure was approved by the hospital's institutional review board.

After trypsin-EDTA digestion (0.25%; Gibco-ThermoScientific, Illkirch, France) for 45 min at 37°C of foreskin tissues, keratinocytes (KCs) were grown in KGM-Gold™ media supplemented with BulletKit™ supplements (Promocell, Heidelberg, Germany) until passage 3–4. KC purity was checked by microscopic observation. At 80–90% confluence, KCs were first stimulated for 4 h with poly (I:C) at 1  $\mu$ g/ml, then either with Pam3CSK4 at 1  $\mu$ g/ml ( $6.6 \times 10^{-7}$  M), L-OspC at 50 ng/ml ( $2.3 \times 10^{-9}$  M), UL-OspC at 50 ng/ml ( $2.4 \times 10^{-9}$  M) or

*B. burgdorferi* ss (MOI 50:1) for an additional 20 h. To test the effect of tick SGE on KCs inflammation, L-OspC was first incubated for 30 min at room temperature with 20  $\mu$ g/ml of SGE then added to KCs. For all *in vitro* inflammatory assays, supernatants and RNAs were collected separately.

### ELISA

To measure IL-8 or TNF- $\alpha$  secreted by KCs, enzyme-linked immunosorbent assays (ELISAs) were performed on cell supernatants. Protocols were based on sandwich techniques as described by the manufacturer (R&D, France).

### RNA extraction and quantitative RT-PCR

After *in vitro* stimulation, supernatants were collected and cells were resuspended in TRIzol to extract RNAs according to the manufacturer's protocol (Invitrogen-ThermoScientific, Illkirch, France). Concerning the *in vivo* model, mouse ears were processed with a 3-mm biopsy punch and then homogenized with 2.8-mm ceramic bead media (Mobio, Carlsbad, USA). 1  $\mu$ g of total RNA was treated with DNase (Ambion-ThermoScientific, Illkirch, France) and then reverse transcribed with the Superscript II first-strand synthesis system (Invitrogen). The quantitative PCR (qPCR) was performed on the Applied Biosystems 7500 Real-Time PCR system (Life Technologies) with Power SYBR Green PCR Master Mix or TaqMan Universal PCR Master Mix (Life Technologies, USA). The primers used are listed in Table S1. IL-8, human beta-defensin-2 (hBD-2), TNF- $\alpha$  and macrophage inflammatory protein 2-alpha (MIP-2) genes expression were normalized to the GAPDH (human) or to the actin (mouse) genes expression and compared to untreated conditions.

### RNA interference (RNAi)

The transfection of KCs was performed according to the Amaxa protocol T-18. Briefly, 1 nmol of SMARTpool On-Target (Thermo Scientific) plus TLR2, TLR3, TRIF, Myd88 or control siRNAs was added to 3 million KCs, resuspended in 100  $\mu$ l of keratinocyte nucleofactor solution. A SMARTpool On-Target consists of the combination of four different siRNA targeting the same mRNA. Immediately following the transfection, cells were plated at a density of  $6 \times 10^4$  cells/well in 24-well plates and incubated overnight. The medium was changed the following day, and the cells were used for activation assays 48 h after the transfection.

### In vivo model of cutaneous inflammation

The Institutional Animal Care, CREMEAS (Comité Régional d'Ethique en Matière d'Experimentation Animale) has reviewed and approved officially this research. Protocols for all animal experiments were prepared according to their guidelines. The cutaneous inflammation of the ears of C57/BL6 mice was induced by subcutaneous injection of poly (I:C) (100  $\mu$ l/10  $\mu$ g) then 2 h later L-OspC (100  $\mu$ l/250 ng) into the ear lobes. After an additional 6 or 24 h, ear thickness was measured with a micrometre (Mitutoyo, USA), or a 3-mm skin biopsy punch was collected for further gene analyses (qPCR) as described previously (25).

### Statistical analyses

Each experiment was carried out at least three times in independent trials. The most representative experiment is shown. Results represent the mean ( $\pm$ SEM) of at least triplicates of one experiment and were analysed by two-tailed Student's *t*-test or ANOVA with Tukey post-test using GraphPad software. Differences in values were considered significant if  $P < 0.05$  (\* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ).

## Results

### Poly (I: C) upregulated *Borrelia*- and OspC-induced KC inflammation

Human KCs secrete several pro-inflammatory molecules including hBD-2 and IL-8 following exposure to bacteria (8,51,52) or to dsRNA released during skin injury (25,26). As the tick bite induces skin injury, we studied how it might influence cytokine and AMP response triggered by *Borrelia*. A strong increase in IL-8 or TNF- $\alpha$  release, or hBD-2 mRNA, was observed when poly (I: C), a synthetic analogue of dsRNAs used to mimic those released by necrotic cells, and *Borrelia* were co-incubated with human primary KCs (Fig. 1). As the surface lipoprotein OspC is expressed during *Borrelia* transmission to the vertebrate host, we also tested the effect of poly (I: C) on KCs activated by lipitated OspC (L-OspC), unlipitated OspC (UL-OspC) or the lipid moiety of the protein, Pam<sub>3</sub>CSK<sub>4</sub>. We observed a stronger IL-8 and TNF- $\alpha$  protein increase and hBD-2 mRNA induction when poly (I: C) was co-incubated with the different inducers (Fig. 1), except for HD2 induction in the presence of L-OspC and poly (I: C) (Fig. 1c).

However, lipoproteins other than L-OspC upregulated the cytokine response in the presence of poly (I: C) – as OspC-KO mutants still increased IL-8 secretion (Fig. 2a). Interestingly, this was not the case for TNF- $\alpha$  secretion and hBD-2 induction where the increase in the response was abolished with *Borrelia* OspC-KO (Fig. 2b and c). Type I IFN was poorly or not induced in our experiments (data not shown).

### The response to *Borrelia* or L-OspC/TLR2 and poly (I: C)/TLR3 is mediated by both the TRIF and the MyD88 pathways

Different TLR molecules detect *Borrelia* during infection. Triacylated bacterial lipoproteins including OspC are known to interact specifically with TLR2/1 heterodimer (34). Poly (I: C) interacts with TLR3 receptor mimicking host inflammatory signals generated by skin injury. Using RNA silencing, we analysed the involve-

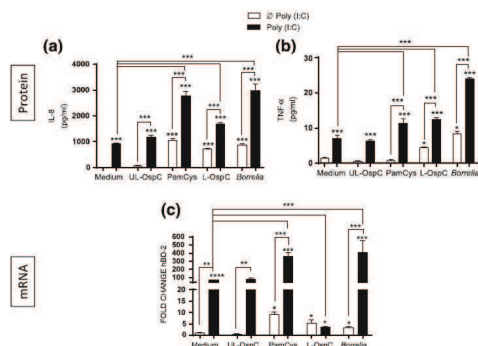
ment of both pathways in the positive crosstalk between *Borrelia* or L-OspC and the injury signal.

TLR2 silencing significantly inhibited IL-8 secretion induced by *Borrelia* or L-OspC (Fig. S1a and b). However, it did not modify the TNF- $\alpha$  secretion at 24 h (Fig. S1c and d). hBD-2 expression was reduced after TLR2 silencing only when cells were activated by L-OspC but not *Borrelia* (Fig. S1e and f). TLR3 silencing almost entirely inhibited poly (I: C)-induced cytokine response, and it strongly affected the crosstalk between *Borrelia* or L-OspC and poly (I: C) for all the inflammatory genes tested (Fig. S1). The effect of TLR2 silencing on the crosstalk was less efficient.

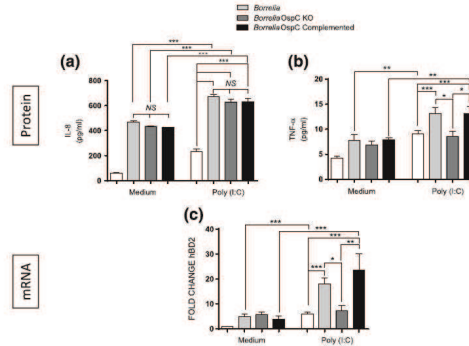
TLR2 signals through MyD88 adaptor molecule, while TLR3 signals through TRIF adaptor molecule. We confirmed the involvement of both pathways in the response. As expected, MyD88 silencing affected IL-8, TNF- $\alpha$  and hBD-2 expression induced by *Borrelia* or L-OspC (Fig. S2). It also decreased the expression of the same inflammatory genes when KCs were stimulated with poly (I: C). TRIF silencing altered the response induced by poly (I: C) for all tested genes. It also affected TNF- $\alpha$  secretion induced by *Borrelia* or L-OspC. Both MyD88 and TRIF silencing strongly downregulated the crosstalk-associated inflammation between poly (I: C) and *Borrelia*/L-OspC for all genes measured.

### Tick saliva strongly inhibited the TRIF/MyD88-mediated response to L-OspC and poly (I: C)

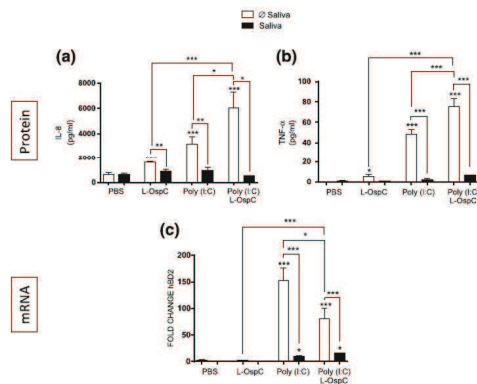
We have previously shown (8) that tick saliva inhibits L-OspC or *Borrelia*-induced KC inflammation via TLR2. We then analysed the anti-inflammatory effect of *I. ricinus* salivary gland extracts on TLR3–TLR2/1 crosstalk. Tick saliva inhibited not only the inflammatory cytokine response via TLR2 but also the ones triggered via TLR3 for IL-8, TNF- $\alpha$  and hBD-2 (Fig. 3). It also affected the TLR3–TLR2 response to L-OspC and poly (I: C) by strongly decreasing all tested inflammatory molecules.



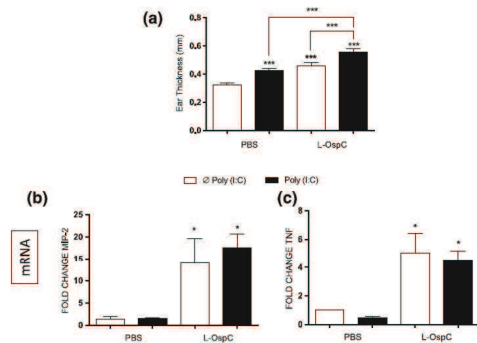
**Figure 1.** Positive crosstalk between poly (I: C) and different TLR2/1-agonists. Undifferentiated KCs were activated for 4 h with 1  $\mu$ g/ml of poly (I: C). Then, Pam<sub>3</sub>CSK<sub>4</sub> [1  $\mu$ g/ml ( $6.6 \times 10^{-7}$  M)], L-OspC [50 ng/ml ( $2.3 \times 10^{-9}$  M)], UL-OspC [50 ng/ml ( $2.3 \times 10^{-9}$  M)] or *Borrelia* (50:1) were added for an additional 20 h. IL-8 (a) and TNF- $\alpha$  (b) concentrations were measured in supernatant by ELISA. RNAs were collected in TRIzol, reverse transcribed and then analysed by RT-qPCR. The fold change for hBD-2 (c) was measured relative to the non-treated conditions and normalized to the actin gene expression. Data represent means (SEM) of at least triplicates. Each experiment was carried out at least 3 times, and one representative experiment is shown.



**Figure 2.** The positive cooperation between *Borrelia* and poly (I: C) does not depend only on L-OspC. Undifferentiated KCs were activated for 4 h with 1  $\mu$ g/ml of poly (I: C) before adding *Borrelia burgdorferi* sensu stricto (ss) strain 297, OspC-KO *B. burgdorferi* ss 297 or OspC-complemented *B. burgdorferi* ss 297 (50:1) for 20 h. IL-8 (a) and TNF- $\alpha$  (b) concentrations in supernatant were measured by ELISA. Total KC RNAs were collected in TRIzol, reverse transcribed and then analysed by RT-qPCR. The fold change for hBD-2 (c) was measured relative to the non-treated conditions and normalized to the actin gene expression. Data represent means (SEM) of at least triplicates. Each experiment was carried out at least 3 times, and one representative experiment is shown.



**Figure 3.** Tick saliva totally inhibits the inflammation induced by poly (I: C), L-OspC or both. KCs were activated for 4 h with 1  $\mu$ g/ml of poly (I: C). Then, L-OspC, first incubated for 30 min with tick saliva, was added for 20 h. IL-8 (a) and TNF- $\alpha$  (b) concentrations were measured in supernatant by ELISA. RNAs were collected in Trizol, reverse transcribed and then analysed by RT-qPCR. The fold change for hBD-2 (c) was measured relative to the non-treated conditions and normalized to the actin gene expression. Data represent means (SEM) of at least triplicates. Each experiment was carried out at least 3 times, and one representative experiment is shown.



**Figure 4.** In the mouse model of ear inflammation, the crosstalk between poly (I: C) and L-OspC differs from *in vitro*. PBS or poly (I: C) (10  $\mu$ g) was injected intradermally in the ear of C57BL/6 mice. Then, 2 h later, L-OspC (250 ng) or PBS was injected. The ear thickness was measured 24 h later (a). The induction of inflammatory genes such as MIP-2 (b) and TNF- $\alpha$  (c) was measured at 6 h. Data represent means (SEM) ( $n > 6$  per group). Each experiment was carried out at least 3 times, and one representative experiment is shown.

#### L-OspC-induced inflammation is also upregulated by poly (I: C) *in vivo* in mouse model

To validate our observations *in vivo*, we used a model of ear inflammation in C57BL/6 mice (25). When mice were co-inoculated with L-OspC and poly (I: C), we observed a higher cell infiltrate than when they were only inoculated with OspC or poly (I: C) alone, as shown by the ear thickness measurement at 24 h (Fig. 4a). However, when we looked at the gene expression, the positive crosstalk was observed neither for MIP-2 [a murine counterpart of IL-8 (53,54)] nor for TNF- $\alpha$  at 6 h (Fig. 4b). mBD-3 (a

mouse analogue of human defensin hBD-2) was not significantly induced in our experiments. We observed in this model that L-OspC concentration was very critical and induced tissue necrosis if used at too high concentrations (data not shown).

#### Discussion

During Lyme borreliosis, the contribution of the skin injury induced by the tick bite on inflammation and transmission is largely unknown. The host cells damaged by tick mouthparts might generate dsRNAs that activate TLR3. It could potentially impact the TLR2-*Borrelia* inflammatory process. KCs, as major cells of the epidermis, play a pivotal role as sentinels to prevent excessive inflammation (43,55,56). They are directly involved during skin injury (25,56–60) and are directly affected by tick bite. On their surface, they harbour TLR molecules that are key receptors in the regulation of inflammation during injury by secreting cytokines, chemokines and AMPs (56). TLR molecules, triggered either by PAMPs or by danger signals, are essential at epithelial interfaces to protect from infection and to maintain homeostasis (61).

Although TLR pathways can be activated independently, crosstalks are now described, especially for the TLR2 pathway. Crosstalk between TLR2 and TLR3 has already been observed in murine dendritic cells (62). TLR3-dependent inflammation and TLR2/6 activated by commensal bacteria have been shown to act together to inhibit excessive inflammation (25). Human integrin  $\alpha 3 \beta 1$  regulates TLR2 recognition of lipopeptides from endosomal compartments (63). TLR2 inhibits type I interferon induced by TLR7/9 through IRAK1 depletion (64). TLR2 activation pathways require the adaptor molecules MyD88 for surface membrane TLRs and for endosomal TLRs such as TLR7, TLR8 and TLR9. TRIF is another adaptor molecule specific for TLR3, while TLR4 utilizes MyD88 to signal from its plasma membrane location and TRIF to signal from endosomal compartments (65). Recently, TLR2 has been shown to use both MyD88 and TRIF during *Borrelia* phagocytosis by bone marrow-derived macrophages (47).

In Lyme disease, TLR2/TLR1 is involved in Lyme inflammation (34,46,66–68). The injury induced by the tick bite generates tissue damage and a feeding pool around the mouthparts (3,69), which could potentially activate the TLR3 pathway by released dsRNAs (24,70,71). Crosstalk between TLR3 and TLR1/2 has never been investigated in this context. We report for the first time TLR3 involvement in the inflammatory process observed during the early transmission of Lyme disease. *In vitro*, TLR3 activation increases *B. burgdorferi*- and OspC-induced inflammation of KCs, as shown by the induction of IL-8 and TNF- $\alpha$ . For hBD-2, similar phenomenon was observed except when L-OspC and poly (I: C) were co-incubated. This could be due to the structure of the ligand itself, L-OspC. Moreover, for this defensin, the state of KC differentiation is essential to detect significant amount of hBD-2 (52). The lipid moiety is clearly a critical parameter for the crosstalk as UL-OspC does not increase TLR3-induced inflammation. On the other hand, the need to use a 300-fold higher concentration of Pam3CSK4 than L-OspC to observe the positive crosstalk points out the major role of the protein. However, it has been previously described that synthetic lipopeptides are less soluble than intact proteins and then they are not as efficient to induce an immune activation (72). The difference in concentration between OspC and the lipopeptide is therefore not as high, as calculated theoretically. This TLR crosstalk might also involve micro-

RNAs as they have been shown to be induced in KC inflammatory response induced by different TLR agonists (73) and they are present in salivary glands of unfed and fed ticks (74,75). This aspect deserves further investigation as these miRNAs regulate gene expression and are involved in numerous processes such as cell proliferation, apoptosis and stress response.

The silencing of the TLR molecules mainly affected the TLR3–poly (I:C) pathway and the crosstalk TLR2–TLR3. Compared to MyD88 silencing, TLR2 silencing was less apparent especially for TNF- $\alpha$  and hBD-2 when *Borrelia* was used. Other MyD88-dependent pathways than TLR2 likely play a role in the KC inflammatory response. The silencing of the adaptor molecule MyD88 and TRIF produced very strong effect on the expression of inflammatory molecules whatever the initial TLR agonist, *Borrelia* or OspC. MyD88 has been involved in the control of *Borrelia* phagocytosis, but not in the production of immunoglobulins (76). Even if KC phagocytosis of *Borrelia* has never been observed, our results are consistent with previous studies which showed the involvement of MyD88 in innate but not acquired immunity (76,77). Unexpectedly, poly (I:C)-induced inflammation was shown to depend not only on TRIF but also on MyD88. A link between MyD88 and TLR3 has been already established where MyD88 acts as a negative regulator of the TLR3/TRIF pathway in corneal inflammation (78). A recent study has shown that the inflammation induced by *Borrelia* was mediated in part by the TRIF pathway in macrophages (47). Finally, during the process of *Borrelia* transmission, the tick saliva plays a key role locally by modulating the host immune response. Immunosuppression of TLR-dependent inflammation (TLR2, TLR4) by *Ixodes* saliva has already been shown in macrophages in the context of *A. phagocytophilum* infection (19). We show for the first time that tick saliva reduces the TLR3 activation and the crosstalk between TLR3 and TLR2. The mechanism underlying this saliva effect remains to be further investigated. The strong IL-8 inhibition by *Ixodes* saliva might be due to salivary proteins such as evasins, already involved in chemokine inhibition by *Ixodid* saliva (16). IL-10 inhibits inflammatory cytokine production by macrophages and dendritic cells after activation by *Borrelia* (79). It would be of interest to investigate how this cytokine is regulated by tick saliva.

We then tried to validate *in vivo*, some of our *in vitro* observations. In a mouse model, we analysed the potential effect of TLR3 activation on OspC-induced inflammation. The cell infiltrate was more pronounced when both TLR agonists were inoculated as shown by the ear thickness measurement at 24 h. In contrast to our *in vitro* data, the crosstalk was not observed when we measured MIP-2 and TNF- $\alpha$  expression. In humans, EM develops 3–32 days after the infectious tick bite (80). The TLR2–TLR3 crosstalk we described in human KCs might participate to this early skin inflammation, explaining, in part, the presence of elevated

IL-8 concentration in patient serum with EM (81). The absence of inflammatory crosstalk might be explained by the absence of EM in this mouse model of Lyme disease. Moreover, in the whole skin, different resident and immune cells interact with each other, likely explaining that the crosstalk was different as the one observed *in vitro*. This study also underlines the complex process of *Borrelia* transmission with different TLR pathway activations and crosstalks, which likely involves dendritic cells and fibroblasts as well as Treg *in vivo*.

In conclusion, we propose that *Borrelia* transmission during the cutaneous phase is a two-step process. During the initial phase of transmission when tick saliva is present, TLR3 and TLR2 activations are inhibited by tick saliva, allowing the local multiplication of *Borrelia* in the feeding pool. Tick saliva induces an immune tolerance against the bacteria. This effect could last several days as long as the saliva is present in the skin. Then, during the extracellular migration of *Borrelia* towards target host tissues (skin, nervous system and joints) and their multiplication, it is likely that tissue damage induced by the bacteria triggers local inflammation. This crosstalk between pathogenic bacteria–TLR2 activation and host necrotic cells–TLR3 activation would explain the deleterious effect of *Borrelia* for the vertebrate host and perhaps the initial process of EM.

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#### Author contributions

Q. Bernard, T. Nakatsuji and N. Boulanger performed the research. Q. Bernard, R.L. Gallo, B. Jaulhac and N. Boulanger designed the research study. R.L. Gallo, B. Luft, X. Yang and B. Jaulhac contributed essential reagents or tools. Q. Bernard, N. Boulanger, R.L. Gallo, B. Luft and X. Yang analysed the data. Q. Bernard and N. Boulanger wrote the manuscript.

#### Conflict of interest

The authors state no conflict of interest.

#### Supporting Information

Additional supporting data may be found in the supplementary information of this article.

**Figure S1.** The crosstalk between poly (I:C) and *Borrelia*/L-OspC is mediated by TLR3 and TLR2.

**Figure S2.** The crosstalk between poly (I:C) and *Borrelia*/L-OspC is mediated by both TRIF and MyD88 pathways.

**Table S1.** Primer sets used for qPCR.

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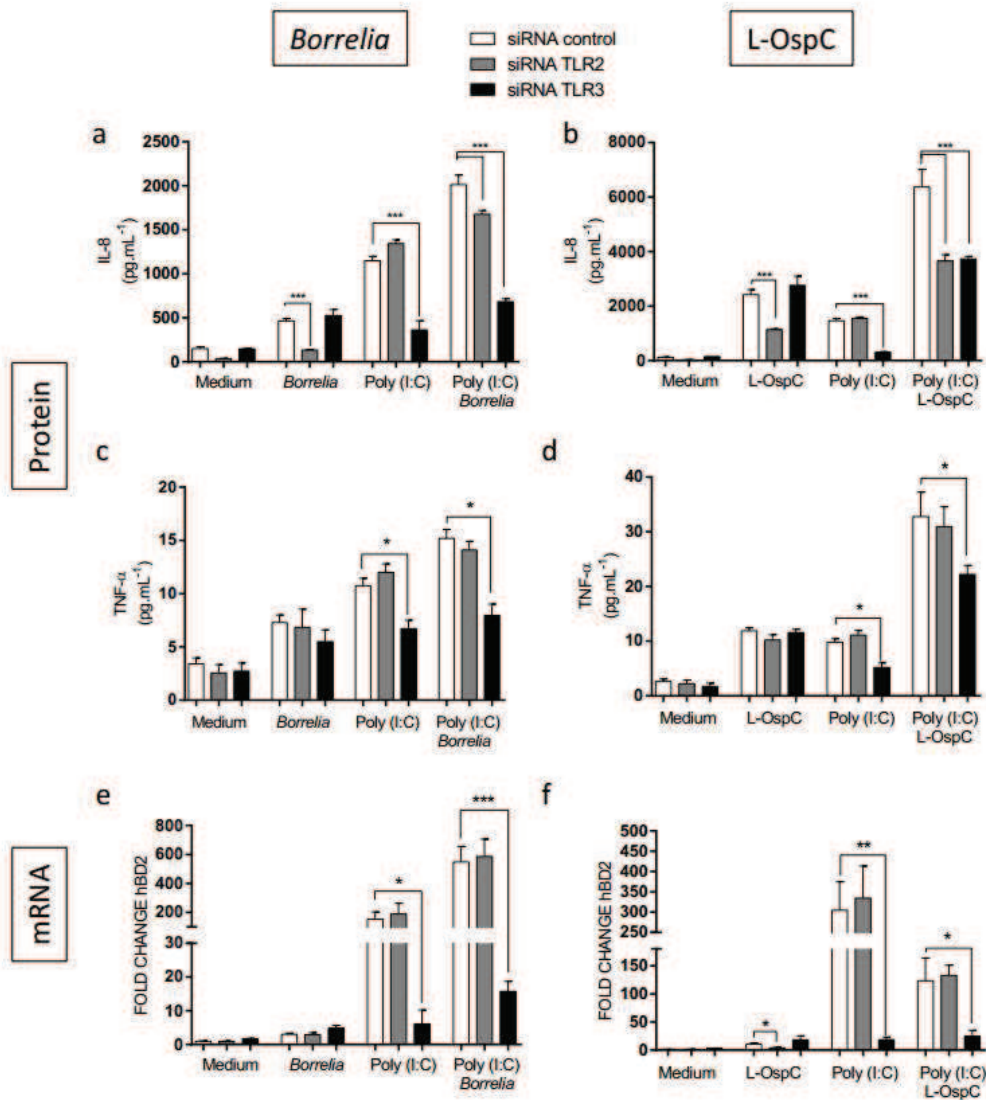


Figure S1

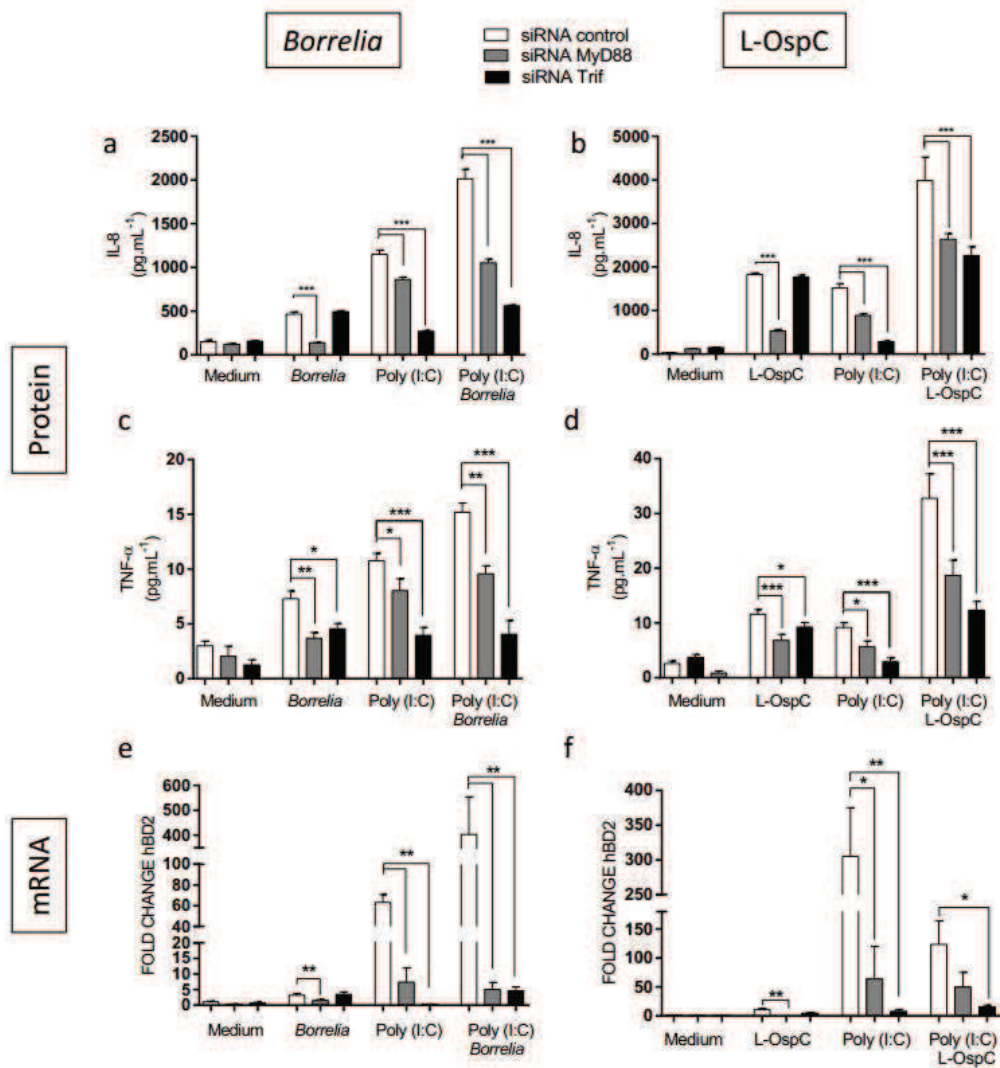


Figure S2



## ARTICLE 2:

# Identification and characterization of a histone h4 with lytic effect on human dermal fibroblasts in ixodes ricinus salivary gland extract: potential roles in borrelia transmission

Amandine Boeuf, Nathalie Boulanger, Gilles Schnell, Quentin Bernard, Aurelie Kern, Benoît

Westermann, Frederic Schramm, Benoît Jaulhac, Laurence Ehret-Sabatier

**EN PREPARATION**

La salive de tique contient de nombreux composés capables notamment de contrer les mécanismes associés à l'hémostase (vasoconstriction, agrégation plaquettaire) et à la réponse immunitaire. Lors d'une précédente étude menée au laboratoire, la capacité de la salive de tique à lyser les FBs a été observée. Cette lyse cellulaire pourrait participer à la formation du « feeding pool », une cavité qui se forme autour de l'hypostome de la tique au niveau de la peau afin de faciliter le repas sanguin de cette dernière. Nous avons ainsi identifié l'une des protéines responsables de la lyse cellulaire afin caractériser le rôle potentiel de celle-ci dans la transmission de *Borrelia*, l'agent infectieux de la borréliose de Lyme transmis par la tique du genre *Ixodes*.

Dans un premier temps, des extraits de glandes salivaires de tiques totaux ont été fractionnés par micro RP-HPLC. La capacité à lyser les FBs des différentes fractions obtenues a par la suite été évaluée. Parmi les différentes fractions capables de lyser les cellules, une seule protéine en commun fut déterminée par digestion enzymatique et spectrométrie nano LC-MS/MS : **l'histone H4**.

Dans un deuxième temps, l'activité lytique de l'extrait de glandes salivaires de tiques a été comparée à celle d'histone H4 commerciale. L'histone H4 commerciale a lysé les FBs, cependant de façon moins importante que l'extrait de glandes salivaires de tiques.

Dans un troisième temps, nous avons analysé les propriétés antimicrobiennes de l'histone H4 sur *Borrelia*, puisque qu'une telle activité avait déjà été associée à l'histone H4 lors d'études

précédentes sur *S. aureus* (Lee et al. 2009a). Nous avons observé une activité antimicrobienne de l'histone H4 sur *S. epidermidis* et *Micrococcus luteus*, deux bactéries commensales de la peau humaine, mais pas contre *B. burgdorferi* ss.

La lyse des FBs associée à la présence de l'Histone H4 peut être essentielle au bon déroulement du repas sanguin de la tique. L'activité lytique associée à l'activité antimicrobienne pourrait aussi participer à l'augmentation de l'efficacité de la transmission de *Borrelia*. Si le relargage de l'histone H4 au niveau du point de piqûre s'avère être essentiel au bon déroulement du repas sanguin, alors l'histone H4 pourrait être un bon candidat vaccinal pour bloquer la transmission de *Borrelia*, voire d'autres agents infectieux transmis par la tique du genre *Ixodes*. Des expériences de vaccination sur souris sont actuellement en cours d'évaluation au laboratoire. De plus, le niveau d'expression de l'histone H4 dans les glandes salivaires de tiques au cours du repas sanguin va être évalué par microscopie à fluorescence afin de confirmer l'origine salivaire de cette protéine.

# Identification and characterization of a histone h4 with lytic effect on human dermal fibroblasts in *Ixodes ricinus* salivary gland extract: potential roles in *Borrelia* transmission

Amandine Boeuf<sup>1</sup>, Nathalie Boulanger<sup>2,3</sup>, Gilles Schnell<sup>1</sup>, Quentin Bernard<sup>2</sup>, Aurelie Kern<sup>2</sup>, Benoît Westermann<sup>1</sup>, Frederic Schramm<sup>2</sup>, Benoît Jaulhac<sup>2,3</sup>, Laurence Ehret-Sabatier<sup>1</sup>

<sup>1</sup>Laboratoire de Spectrométrie de Masse BioOrganique, Institut Pluridisciplinaire Hubert Curien, UMR 7178 CNRS-Université de Strasbourg, France

<sup>2</sup>EA7290, Virulence bactérienne précoce, groupe Borréliose de Lyme, Facultés de médecine et de pharmacie, Université de Strasbourg, France.

<sup>3</sup>Centre National de Reference *Borrelia*, Strasbourg, France.

#Correspondence to:

[laurence.sabatier@unistra.fr](mailto:laurence.sabatier@unistra.fr)

ECPM - 25 rue Becquerel

67087 Strasbourg Cedex 2, France

Tel: +33 3 68 85 27 26

Fax: +33 3 68 85 27 25

[nboulanger@unistra.fr](mailto:nboulanger@unistra.fr)

EA7290 : Virulence bacterienne precoce : groupe Borrelia

Institut de Bacteriologie

3 rue Koeberle

67 000 Strasbourg, France

Tel: +XX

Fax: +XX

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Abbreviations: SGE, salivary gland extract; FB, fibroblast; PTM, Post Translational Modification; AMP, antimicrobial peptides

## Abstract

Understanding the mechanism of pathogen transmission is essential for the development of strategies to reduce arthropod borne diseases. Saliva of insects and acarids inoculated with pathogens plays a key role in the efficiency of the transmission by its pharmaco- and immunomodulatory properties. As the skin is a key interface in arthropod borne diseases, we have developed a strategy to identify tick molecules involved in the transmission of pathogens, based on micro-HPLC and mass spectrometry, and *in vitro* assays on human skin cells. From salivary gland extract of *Ixodes ricinus*, we have identified histone H4 with a lytic effect on human primary fibroblasts and that can be essential in the early phase of the arthropod bite by its antimicrobial activity. This histone might be involved in the formation of the feeding pool, responsible of the tissue necrosis of the vertebrate host. This histone would facilitate pathogen transmission, among those *Borrelia burgdorferi sensu lato*.

## INTRODUCTION

Hard ticks *Ixodidae* are involved in numerous tick-borne diseases caused by diverse pathogens such as bacteria, viruses or parasites. The obligate hematophagous ectoparasite, *Ixodes* is vector of bacteria like *Borrelia burgdorferi*, *Rickettsia*, *Anaplasma*, the parasite *Babesia* or the tick-borne encephalitis virus among others (de la Fuente et al. 2008). These pathogens are transmitted when tick attaches to their host through its sophisticated biting pieces, the hypostome and the chelicerae. It finally induces a skin injury that leads to a feeding pool (Sonenshine and Anderson 2014). The blood meal lasts for several days and the hard tick secure its blood meal by the formation of the cement around the biting pieces and by the secretion of its saliva containing pharmacologically and immunologically active molecules (Kazimírová and Stibrániová 2013). At the end of the blood meal, salivary glands degenerate (Alarcon-Chaidez 2014) and the tick detaches from the host to molt into nymphs or adults or die after laying eggs for the female. The tissue damage makes very often a skin necrosis after the tick blood meal (Krause et al. 2009).

Saliva plays a crucial role during the feeding: it allows the tick to overcome host hemostasis (vasoconstriction, platelet aggregation, blood clotting), innate immunity (inflammatory reaction, complement activation) and acquired immunity. Therefore, tick saliva contains a wide range of pharmacologically active molecules (Ribeiro, JM et al. 1985; Hovius et al. 2008c; Francischetti et al. 2009) : anti-clotting (Francischetti et al. 2004; Narasimhan et al. 2004; Prevot et al. 2006; Chmelar et al. 2012), vasodilatory (Dickinson et al. 1976; Bowman et al. 1996) and anti-inflammatory molecules (Ribeiro and Mather 1998; Paesen et al. 1999; Sangamnatdej et al. 2002; Beaufays et al. 2008b). In addition, tick saliva can target the immune system of the vertebrate host like complement cascade (Valenzuela et al. 2000b; Daix et al. 2007b; Schroeder et al. 2007; Couvreur et al. 2008b), antimicrobial peptides (Kern et al. 2011a; Marchal et al. 2011), B-cells (Hannier et al. 2004b) and T-cells (Anguita et al. 2002; Lebouille et al. 2002; Garg et al. 2006). In the context of tick-borne diseases, pathogens take advantage of this cocktail of active molecules to infect more efficiently the host.

The skin of the vertebrate host represents a key interface where the vector bites and inoculates its saliva and the pathogens (Bernard et al. 2014). The epidermis with keratinocytes represents the first barrier encountered by ticks. Then, their biting pieces penetrate deeply into the skin and reach the dermis, interacting with resident cells like dermal dendritic cells, mast cells or fibroblasts. Some pathogens can multiply at dermal level before disseminating. Investigate this initial step is crucial since it is likely essential for a further development of most of these diseases.

We therefore selected Lyme disease, a bacterial infection transmitted by the hard tick, *Ixodes*, to study the transmission of *Borrelia burgdorferi* sensu stricto (ss). In a previous study, we showed that tick saliva has an antialarmin effect on human keratinocytes (Marchal et al. 2011). We also observed that crude salivary gland extract induced a lytic effect on fibroblasts (Schramm et al. 2012). Identify tick molecules responsible of this specific lytic effect could explain the formation of the feeding pool which is essential in the tick blood meal and likely in the transmission of pathogens. To this end, we performed a fractionation of tick salivary gland extract by micro RP-HPLC and tested a potential lytic effect of the chromatographic fractions on human dermal fibroblasts. A tick protein, histone H4, was identified in lytic fractions by enzymatic digestion and nanoLC-MS/MS. This protein was further characterized by mass spectrometry. The biological significance of this protein is discussed in the context of Lyme transmission.

## **MATERIALS AND METHODS**

### **Tick salivary glands extract**

*Ixodes ricinus* originally came from a tick colony in Neuchatel, Switzerland, and was maintained in Bacteriology Institute in Strasbourg, France. For tick salivary gland extract (SGE) isolation, non-infected adult ticks were allowed to feed on rabbit for 3 days. Alternatively, female ticks have also been fed on mice for three days. Then, ticks were removed, washed in ethanol and then in phosphate-buffered saline (PBS). Salivary glands were dissected in PBS under a light microscope. SGE was frozen and thawed three times and centrifuged for 10 min at 10,000 g. The supernatant was kept frozen at -80°C until use.

Adult females *I. ricinus* were also collected in the field, dissected and tested similarly to the ticks maintained in the tick colony. SGE was tested for the presence of endotoxin by the Limulus assay.

### **SGE fractionation**

Tick SGEs were fractionated on a micro-HPLC system U3000 (Thermo Fischer Scientific, San Jose, USA). The solvent system consisted in 0.05% TFA in water (solvent A) and 0.045% TFA in ACN (solvent B). Detection was performed at 214 nm. The system was equipped with a Dionex Acclaim PepMap C18 enrichment column (500 µm x 15 mm, 5 µm) and an Agilent Zorbax 300SB-C18 analytical column (300 µm x 15 cm, 3.5 µm). SGE corresponding to the equivalent of 3 ticks was loaded during 3 min on the enrichment column at a flow rate of 15 µL/min with 0.1% TFA in water. Elution was performed at a flow rate of 4 µL/min with the following step gradient: a 5 min stage at 2% B followed by a 2–9.5% B linear gradient over 15 min, then a 9.5–29.5% B linear gradient over 80 min, then a 29.5–48% B linear gradient over 185 min and a final 5 min stage at 80% B.

### **Measure of the lytic effect of tick SGE on human fibroblasts (FBs)**

Primary human dermal fibroblasts (NHDF, Promocell, Germany) were maintained in FGM2 medium. Cells were used at passage 3 to 5 in 48-well plates. At confluence and one day before lysis assays, FGM2 medium was replaced by FGM medium without fetal calf serum. SGE micro-HPLC fractions were transferred onto FBs and incubated for 24 h. Controls were performed with equivalent H<sub>2</sub>O/0.05% TFA (v/v) and ACN/0.045% TFA (v/v) mixtures. The lytic effect was measured by observation of the cells under inverted microscope.

Alternatively, histone H4 with his-tag (Sigma) (data not shown) or without his-tag (Biolabs) was incubated for 24h at different concentrations (0, 5, 10 or 20 µg/mL) with FBs. Positive control was

performed with crude SGE at 20 µg/mL. The effect of histone H4 was measured by counting trypsinised cells and FBs viability was checked by trypan blue staining.

### **Exact mass measurement**

Intact mass measurements of proteins present in fractions of interest were performed on a hybrid electrospray quadrupole time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Manchester, U. K.) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion Biosciences, Ithaca, U.S.) operating in the positive ion mode. External calibration was performed with the multiply charged ions produced by 2 µM horse heart myoglobin diluted in water/ACN/HCO<sub>2</sub>H (50/50/1) and classical interface tuning parameters of the mass spectrometer ( $V_c = 40$  V,  $P_i = 2.1$  mbar).

### **Trypsin digestion and nanoLC-MS/MS analyses**

Proteins present in fractions of interest were digested by using a liquid trypsin digestion protocol. After evaporation, the fraction was re-suspended in a Tris-HCl 25 mM pH 8.8 buffer. Proteins were reduced 1h at 60°C by adding dithiothreitol to a final concentration of 10 mM. Then alkylation was performed by adding iodoacetamide to a final concentration of 30 mM at room temperature in dark. Digestion was finally performed overnight by adding trypsin (Promega, Madison, WI) to a final concentration of 1.5 ng/µL. After digestion, fractions were evaporated and re-suspended in a 0.1% (v/v) TFA aqueous solution.

Digested peptides were analyzed on an Agilent 1100 series nanoHPLC-Chip system (Agilent Technologies, Palo Alto, USA) hyphenated to an amaZon ion trap (Bruker Daltonics, Bremen, Germany) as described (Schnell et al. 2014) with the following adapted conditions for the analyses of the whole sample. A Zorbax 300SB-C18 analytical column (43 mm×75 µm, 5 µm) was used and proteins were eluted with a 10–30% B linear gradient over 1.5 min, followed by a 30-70% B linear gradient over 7.5 min and a final 4 min stage at 85% B. The voltage applied to the capillary was set to -1 850 V. In MS scanning, the Ion Charge Control was fixed at 100 000. For tandem MS experiments, the six most abundant doubly or triply charged ions were selected to be further isolated and fragmented by CID. Ions were excluded after 1 spectrum and released after 0.12 min. In MS/MS scanning the Ion Charge Control was fixed at 300 000 and a total of 2 scans were averaged to obtain a MS/MS spectrum.



#### Characterization of histone H4

Proteins present in fractions of interest (or recombinant histone H4) were digested by using a liquid AspN digestion protocol. After evaporation, the fraction was re-suspended in 25 mM  $\text{NH}_4\text{HCO}_3$  containing 0.1% RapiGest™, reduced 30 min at 60°C by adding dithiothreitol to a final concentration of 12 mM and alkylated 30 min at room temperature by adding iodoacetamide to a final concentration of 40 mM in the dark. An overnight digestion was performed by adding AspN (Promega, Madison, WI) in a 1/75 enzyme/protein ratio at 37°C. After digestion, trifluoroacetic acid was added to a final concentration of 0.5% (v/v) and the sample was incubated 45 min at 37°C to hydrolyze RapiGest™. After centrifugation (14,000 g, 10 min), the sample was desalted on Sep-Pak C18 cartridges (Waters, Milford, MA) prior to mass spectrometry analyses.

Digested peptides were analyzed on a nanoACQUITY UPLC system coupled to a Q-Exactive Plus (Thermo Fisher Scientific, MA) equipped with a nanospray Flex™ ion source. The peptides were trapped on a nanoAcquity UPLC precolumn (Symmetry C18 Trap, 5  $\mu\text{m}$ , 180  $\mu\text{m}$   $\times$  20 mm, Waters), then separated on a nanoAcquity UPLC column (BEH C18, 1.7  $\mu\text{m}$ , 75  $\mu\text{m}$   $\times$  150 mm, Waters). The solvent system consisted of 0.1%  $\text{HCO}_2\text{H}$  in water (solvent A) and 0.1%  $\text{HCO}_2\text{H}$  in ACN (solvent B). Trapping was performed for 3 min at a flow rate of 5  $\mu\text{L}/\text{min}$ , with 99% of solvent A and 1% of solvent B. Elution was performed at a flow rate of 450 nL/min, using a linear gradient of 1–35% B over 35 min at 45°C, followed by 50% B over 1 min and then 90% B over 5 min. Peptides eluting from the column were analyzed by a top 10 data-dependent MS/MS on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, MA). Resolution was set to 70 000 for MS scans and 17 500 for the data-dependent MS/MS scans. The MS AGC target was set to  $3 \times 10^6$  counts, while MS/MS AGC target was set to  $1 \times 10^5$ . The MS scan range was from 300 to 1200 m/z. MS and MS/MS scans were recorded in profile mode. HCD fragmentation was performed by applying normalized collision energy of 29. Dynamic exclusion was set to 1 min.

#### MS/MS data interpretation

Collected mass data were converted into mgf files using DataAnalysis 4.0 (AmaZon-Bruker Daltonics) or MSConvert 3.0 (Q-Exactive Plus) softwares. The MS/MS data obtained from AmaZon analyses were analyzed using two search engine: the MASCOT 2.3.2 (Matrix Science, London, UK) and OMSSA 2.1.9 algorithms as described (Schnell et al. 2014) against an in-house generated target-decoy protein database composed of protein sequences of *Ixodes* downloaded from National Center for Biotechnology Information non redundant database (NCBI nr) and of common contaminant proteins such as porcine trypsin and human keratins (2 x 21 571 entries on December 16, 2012). Spectra were searched with a

mass tolerance of 0.5 Da for MS and MS/MS data. For the data obtained from AspN digestion and Q-Exactive Plus analyses, spectra were searched with a mass tolerance of 5 ppm for MS and 0.07 Da for MS/MS data, allowing a maximum of one missed cleavage with AspN enzyme and with acetylation, mono-, di- and tri-methylation of both lysine and arginine and oxidation of methionine specified as variable modifications.

#### **Biological activity of histone H4 on bacteria.**

*Borrelia burgdorferi* ss N40 was used at low passage. The spirochetes were grown at 33°C in Barbour-Stoenner-Kelly (BSK) medium supplemented with rabbit serum for 4/5 days. *Staphylococcus epidermidis* ATCC12228, *Micrococcus luteus* and *Escherichia coli* ATCC 25922 were grown in Mueller-Hinton (MH) medium overnight. A secondary culture in fresh MH medium was made for 3 to 4 h. Then, *S. epidermidis*, *M. luteus* or *E. coli* cultures were centrifuged and incubated with different concentrations (from 0 to 100 µg/mL) of gentamycin (Promocell) as positive control and recombinant human histone H4 without his-tag (Biolabs) at 37°C for 3 h. After incubation, bacteria were plated on blood agar plates at 37°C for 24 h. CFU were counted and percentage of survival was calculated according to the negative control. *B. burgdorferi* ss was incubated in microtiter plates for 24 h in serum-free FGM medium with different concentrations of gentamicin (positive control) or histone H4, and then transferred in 1.5 ml-tubes of BSK for an additional 5 days. Then *Borrelia* was counted and percentage of survival was calculated according to the negative control.

## RESULTS

### Isolation of tick SGE fractions with lytic activity

Preliminary micro-HPLC analyses were performed to assess the biological reproducibility and the amount of injected SGE that afford reproducible chromatograms (data not shown). An equivalent of 3 ticks per injection was sufficient to obtain a satisfactory HPLC profile. Elution conditions were optimized with shallow gradients in the zone of lytic activity. Fractions with lytic effect on FBs were observed from 182 to 192 min, and spread along five fractions (fractions 2 to 6 in Figure 1A). The maximum lytic effect was observed in fractions eluted between 184 min and 192 min (fractions 3 to 6 in Figure 1B). No lytic effect was observed before 182 min and after 192 min (fractions 1 and 7 in Figure 1B).

### Histone H4 identification

To identify tick SGE proteins responsible of the lytic effect on FBS, fractions of interest were analyzed by a nanoLC-MS/MS strategy after a tryptic digestion. As many proteins were expected in these fractions, fractions surrounding lytic fractions were also analyzed in order to exclude proteins identified in non-active fractions and eluted along several fractions including active fractions. We identified a total of 11 proteins in lytic fractions (Table 1), among which only histone H4 was present in four (fractions 3 to 6) out of the five active fractions. Moreover this protein was the only one identified in the lytic fraction 5. Histone H4 was identified with a minimum of one peptide in fractions 3, 5 and 6, and with a maximum of 11 peptides (with OMSSA) in the fraction 4. The sequence of the protein is presented in Figure 2. The identified peptides correspond to 66% sequence coverage. The missing part was explained by a high proportion of lysine and arginine, leading to short peptides not detected well during nanoLC-MS/MS. To confirm the lytic effect of histone H4, we checked the activity of the recombinant protein on FBs. The stimulation on FBs showed that the protein induced cell lysis at 24 h. The effect was concentration dependent with an important FB lysis at 20 µg/mL (Figure 3), and was cell specific since human primary keratinocytes were not affected at confluence (data not shown). In addition, we analyzed the recombinant protein by micro-HPLC and we observed a retention time of 187 min in agreement with lytic fractions in SGEs.

However, the effect of recombinant histone H4 at 20 µg/mL was less important than the effect observed with crude SGE at 20 µg/mL (Figure 3). This could be due to the fact than native histone H4 in SGE has a more potent effect than recombinant histone that lacks PTMs, especially on the N-terminal part. Accordingly, the intact mass measurement, using ESI-TOF mass spectrometry, of histone H4 isolated from SGE revealed an average mass of 11306 Da (Figure 4). As compared to the average calculated mass

for sequence without PTM (11236 Da), the difference of 70 Da could correspond to one acetylation and two methylations on the N-terminal part. We tried to further characterize PTMs present on histone H4 using AspN digestion and nanoLC-MS/MS. This confirmed the identification of histone H4 with 77% sequence coverage. Nevertheless, we failed to identify the N-terminal peptide carrying the PTMs.

At this point, we cannot conclude on the precise origin of this histone since the sequence is highly conserved across organisms. In particular, histone H4 sequence is identical in *Ixodes*, human, rabbit and mouse. To be sure that the lytic effect of SGE on FBs was not present only in *Ixodes* tick maintained under laboratory conditions, we tested SGE of adult ticks collected in the fields. These ticks also produced a lytic effect on FBs (data not shown). We also tested two different hosts to control that the effect was not linked to the vertebrate host. We observed a lytic effect of SGE whatever the host used: both rabbit and mouse produced a lytic effect (data not shown).

### **Antimicrobial activity assays**

Histone H4 is known for its antimicrobial activity (Lee et al. 2009b). Since *Ixodes* tick is vector of Lyme borreliosis and it co-inoculates *Borrelia* with tick saliva into the skin, we tested whether the identified histone might affect *Borrelia* and the skin microflora. To this end, we co-incubated *Borrelia* and different bacteria, Gram positive (*M. luteus* and *S. epidermidis*) and Gram negative (*E. coli*) with histone H4 or gentamycin antibiotics (as positive control). Interestingly, histone H4 affected *S. epidermidis*, *M. luteus* and *E. coli* but not the pathogenic bacteria, *B. burgdorferi* ss (Figure 5). However, *S. epidermidis* (Figure 5A) was less affected by histone H4 than *E. coli* and *M. luteus* (Figure 5B-C).

## DISCUSSION

In the context of arthropod borne diseases, tick saliva has been particularly well-studied since efficient vaccines might rely on the use of tick proteins as vaccine candidates blocking the tick blood meal, consequently blocking the transmission of pathogens (Schuijt et al. 2011b). At the skin interface, tick saliva harbors pharmacological as well as immunomodulatory properties (Kazimírová and Stibrániová 2013; Wikel 2013). The effect of tick saliva on different immune cells has been well studied however its effect on skin FBs, the main resident cells of dermis has been poorly investigated. More specifically, the mechanism of the feeding pool formation around the tick biting pieces is not elucidated. Recently, we have shown a lytic effect of *Ixodes ricinus* tick saliva on primary human fibroblasts (Schramm et al. 2012). Since this phenomenon could be responsible of the direct formation of the feeding pool during the tick blood meal, we decided to identify the tick molecule inducing the lysis of dermal fibroblasts. By micro-HPLC and mass spectrometry we identified histone H4 as the lytic molecule for FBs in tick salivary gland. Histones and DNA form the nucleosomes, fold in chromatin fibers. PTMs (acetylation, phosphorylation) occur at the tail domains and are essential for gene transcription and are part of the “histone code” (Gardner et al. 2011). Most common modifications observed for histone H4 are mono-, di- or tri-methylation of lysine 20 (Yang and Mizzen 2009; Jørgensen et al. 2013), histidine phosphorylation (Besant and Attwood 2012), acetylation of lysine 16 (Shia et al. 2006; Shahbazian and Grunstein 2007). We have not been able to identify the PTMs on the purified histone. However, the measured mass in tick salivary gland is the same than the one previously observed by (Kawasaki et al. 2008). These PTMs are important for the various histone activities, among those antimicrobial and lytic activities. Histones are primarily located in the nucleus, but they can also be found in the cytoplasm or on cell surface. They are abundantly synthesized, have a low turnover and constitute 30% of total protein synthesis (Thepparit et al. 2010).

An effect of tick saliva on FBs *in vitro* has been previously described for *Ixodes ricinus* female and *Amblyomma variegatum* female, another hard tick. The effect was attributed to the presence of tick molecules targeting wound healing growth factors such as platelet-derived growth factor (PDGF). This effect would have an immunosuppressive effect on inflammation, allowing the completion of the tick blood meal. These tick saliva factors have not been identified so far (Hajnická et al. 2011). Histone H4 could bind fibroblast growth factors and explain FBs lysis by an alteration of cytoskeletal actin filaments.

At this point, we do not know whether the histone we identified has a vertebrate (mouse or rabbit) or tick origin since they all have an identical amino-acid sequence. This histone could be of tick origin and

be released into saliva during the blood meal while tick salivary glands degenerate (Alarcon-Chaidez 2014). Interestingly, an histone H2B of *Ixodes scapularis* has been shown to interact with *Rickettsia felis*, another bacteria transmitted by tick to facilitate tick cell ISE6 invasion (Thepparit et al. 2010). The histone could also have a mammal origin. The tick chelicerae lacerate mechanically as two blades the host tissue leading to necrotic cells that could release histones. In addition, neutrophils massively infiltrate the bite site and it has been recently shown that neutrophil extracellular trap (NET) form at this site. During this process, nuclear DNA of activated neutrophils are released in the extracellular compartment as well as citrullinated histones (Menten-Dedoyart et al. 2012). In parallel, it has also been shown that tissue injury itself increases blood levels of extracellular histones and nucleic acids that may influence hemostasis and promote inflammation. The feeding pool induced by the tick biting pieces damages the endothelium and facilitates the blood meal uptake. Extracellular histones seem to contribute to the hypocoagulability (Johansson et al. 2013) and would facilitate this process. Since histones, particularly, histone H2B, are receptors for plasminogen, their presence would also enhance all the inflammatory process (Das et al. 2007) that would be further used by pathogens such as *Borrelia burgdorferi* ss to diffuse to the target organs: the joint, the heart and the nervous system. Although, mass spectrometry-based proteomics of whole ticks shows a persistence of certain mammalian host blood components, among those globin chains and histones (Wickramasekara et al. 2008), we hypothesize that the histone identified has more a tick origin. Indeed, this work was done on whole tick and we worked on SGE that were washed in PBS before extraction to remove tick gut and host blood contamination.

Concerning histone antimicrobial activity, different works showed their involvement in innate immunity of different organisms (Kawasaki and Iwamuro 2008; Robb et al. 2014). In invertebrates, truncated forms of histone H4 from the butterfly, *Spodoptera frugiperda* Sf9 cells were reported to be antimicrobial, leading to lysis of *Bacillus megaterium* and *Escherichia coli* (Calles et al. 2005). Several studies demonstrated their effect in the defense of oyster (Dorrington et al. 2011; Poirier et al. 2014). In vertebrates, frog histones H2A, called buforins I and II were identified and showed potent antimicrobial activity (Park et al. 1996). Another study revealed the presence of histone H4 in the skin of the Japanese tree frog, *Hyla japonica* with a lytic effect on red blood cells (Kawasaki et al. 2008). A potential anti-inflammatory function of the rat histone H4 variants was reported as well (Poirier et al. 2006). In that study, they demonstrated that C-terminal peptides transcribed from rat histone H4 variants significantly inhibit the release of prostaglandin E2 and interleukine-8, two pro-inflammatory mediators. In human, histone H4 from sebocytes has been identified to have antimicrobial activity (Lee

et al. 2009b). Interestingly, we showed that when tested on different bacteria, the histone produces different effects. It has a lethal effect on commensal bacteria of the skin (*S. epidermidis* and *M. luteus*) and also on *E. coli*. It has no effect on the pathogenic *Borrelia* that the tick transmits. It really points out that *Borrelia burgdorferi* ss uses its vector extremely efficiently to assure its transmission. The presence of this histone could sterilize the inoculation site and avoid competition between pathogenic bacteria and the commensal bacteria present on the skin.

Among the different tick proteins, some targets the pharmacology of the host (coagulation, pain, hemostasis...), other targets the immunity (complement cascade, innate and acquired immunity) (Kazimírová and Štibrániová 2013; Wikel 2013), we show here that histone H4 by its antimicrobial activity and its lytic properties is likely essential in the formation of the feeding pool. This feeding pool could explain the inflammatory process at the bite site such as the skin necrosis observed in certain patients, but also described in animal models (Krause et al. 2009). It could also provide by its antimicrobial activity a sterile environment for the blood meal of the tick. The interaction of FBs with *Borrelia* was described to play a major role in *Borrelia* pathogenicity, by secreting metalloproteases, inflammatory molecules (Zhao et al. 2007a; Marchal et al. 2009; Wu et al. 2011; Lochhead et al. 2012; Schramm et al. 2012) and by internalizing *Borrelia* (Wu et al. 2011). We show here that FBs have a pivotal role during Lyme borreliosis infection, one during the process of transmission as a first target for tick saliva producing a sterile niche for pathogens, then as a potential cell involved in the persistence of *Borrelia* in the skin when the direct effect of tick has disappeared.

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## Conflict of interest

The authors state no conflict of interest.

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## FIGURES LEGENDS

### Figure 1: SGE fractionation and lytic effect measurements on human FBs

(A) UV chromatogram of SGE of *Ixodes ricinus* females, fed on rabbit for three days. Fractions were collected every two min. Fractions whose lytic effect on human fibroblasts was measured are indicated (fractions 1 to 7). Fractions exhibiting a lytic effect on human fibroblasts are indicated by a star (\*).

(B) Images of human FBs co-incubated with *Ixodes ricinus* SGE fractions obtained after microHPLC separation between 180 min and 182 min (1), 182 min and 184 min (2), 184 min and 186 min (3), 186 min and 188 min (4), 188 min and 190 min (5), 190 min and 192 min (6), 192 min and 194 min (7). Images were taken at 63x magnification.

### Figure 2: Histone H4 identification

The sequence of the histone H4 protein and the MS/MS spectra of the peptide **ISGLIYEETR** are presented. The protein was identified with a maximum of 11 peptides (highlighted in bold) representing a sequence coverage of 66%.

### Figure 3: Lytic effect measurements of recombinant histone H4 and crude SGE on human FBs

Images of human FBs co-incubated for 24 hours with 0 (B), 5 (C), 10 (D) or 20 µg/mL (E) of recombinant histone H4 or with crude SGE at 20 µg/mL of proteins (E).

### Figure 4: Intact mass measurement of histone H4 isolated from SGE

The MS spectrum showing the different charge states was obtained by infusion on a Triversa NanoMate/Synapt G2 instrument. A protein with an average mass of  $11305.92 \pm 0.11$  Da was mostly observed.

### Figure 5: Antimicrobial activity of recombinant human H4 histone

*Staphylococcus epidermidis* (A), *Micrococcus luteus* (B) and *Escherichia coli* (C) were incubated for 3 hours in PBS with different concentrations of gentamicin (positive control) or histone H4. *Borrelia burgdorferi* ss (D) was incubated for 24 hours in serum-free KGM medium with different concentrations of gentamicin (positive control) or histone H4. Each experiment was done at least 3 times, and one representative experiment is shown.

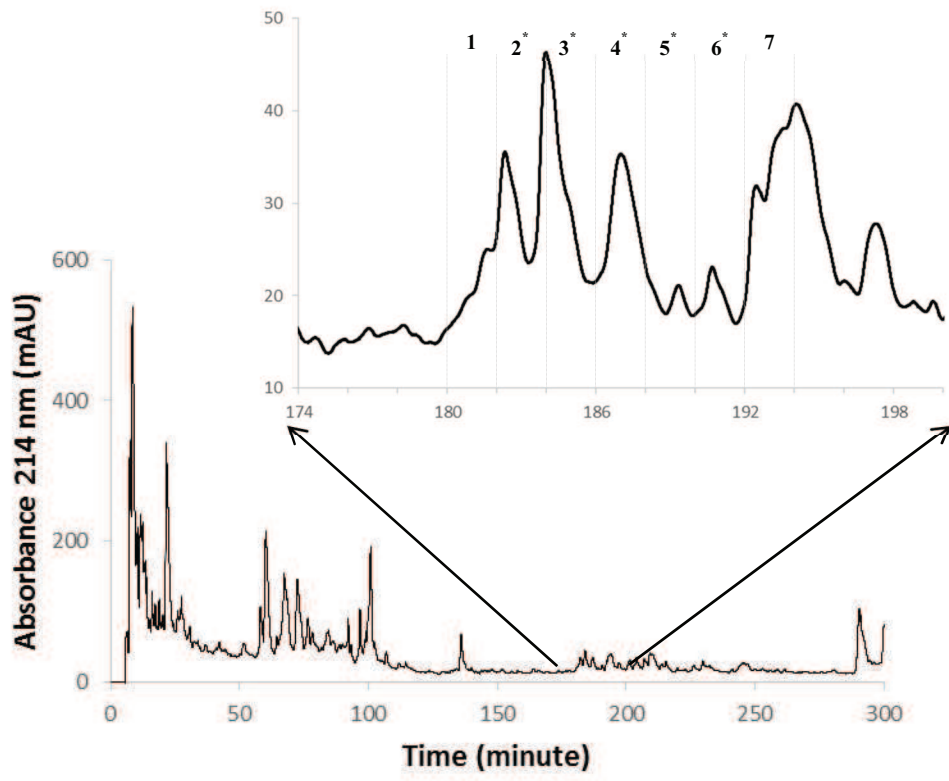
Table 1: Proteins identified in each fraction of SGE of *Ixodes ricinus*

Fraction <sup>1</sup>	Retention time (min)	Identified proteins <sup>2</sup>	Accession number	Lytic activity
1	180-182	cyclophilin A heat shock protein, putative histone H2B, putative calreticulin	gi 67084095 gi 240972667 gi 241568982 gi 39725993	No
2	182-184	small heat shock protein, putative histone H2B, putative	gi 241860225 gi 241568982	Yes
3	184-186	histone H4, putative histone H2B, putative ornithine decarboxylase, putative	gi 241568987 gi 241568982 gi 24200134	Yes
4	186-188	histone H4, putative histone H2B, putative hypothetical protein IscW_ISCW008323	gi 241568987 gi 241568982 gi 241652216	Yes
5	188-190	histone H4, putative	gi 241568987	Yes
6	190-192	cyclophilin A histone H4, putative histone 2A fructose 1,6-bisphosphate aldolase, putative ixoderin B4 hypothetical protein IscW_ISCW008323	gi 67084095 gi 241568987 gi 241251590 gi 241690479 gi 126360866 gi 241652216	Yes
7	192-194	histone H4, putative	gi 241568987	No

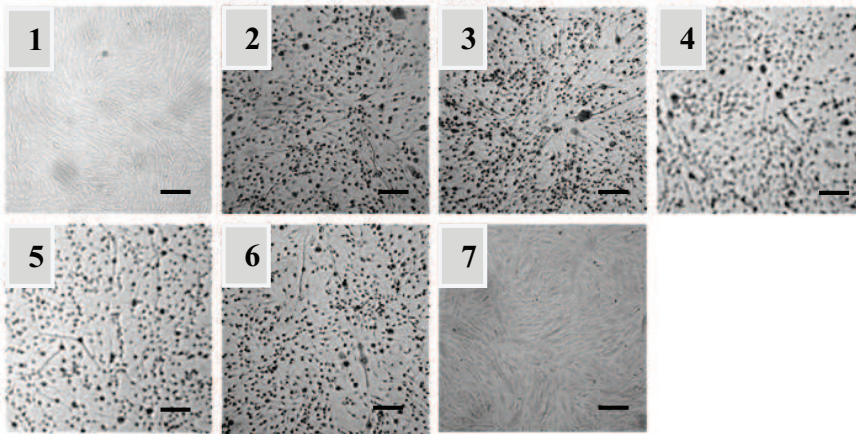
<sup>1</sup>Fractions are described by their number indicated in Figure 1.

<sup>2</sup>NanoLC-MS/MS data obtained after reduction of disulfide bridge, alkylation of cysteines and enzymatic digestion by trypsin were analyzed using MASCOT and OMSSA search engines against an in-house generated target-decoy protein database composed of protein sequences of *Ixodes*.

**a**



**b**



**Figure 1**

MSGRGKGGKGLGKGGAKRHRKVLRLD**NIQGITKPAIR**RLARRGG  
VKR**ISGLIYEETRGVLKVFLENVIRDAVTYTEHAKR**KT**VTAMD**  
VYALKR**QGRTLYGFGG**

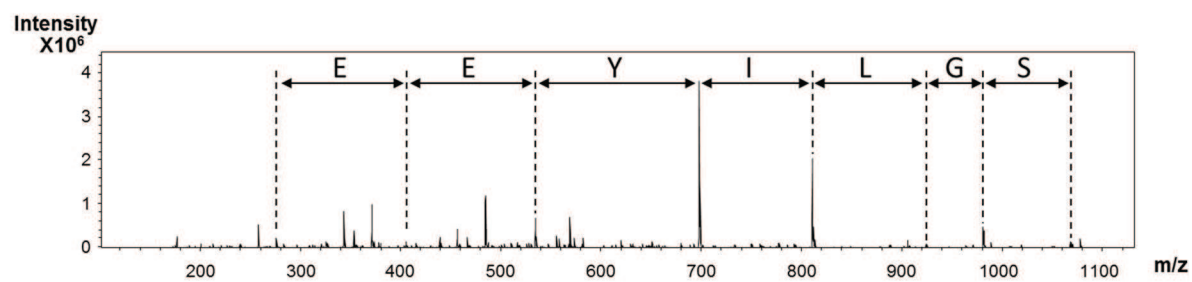
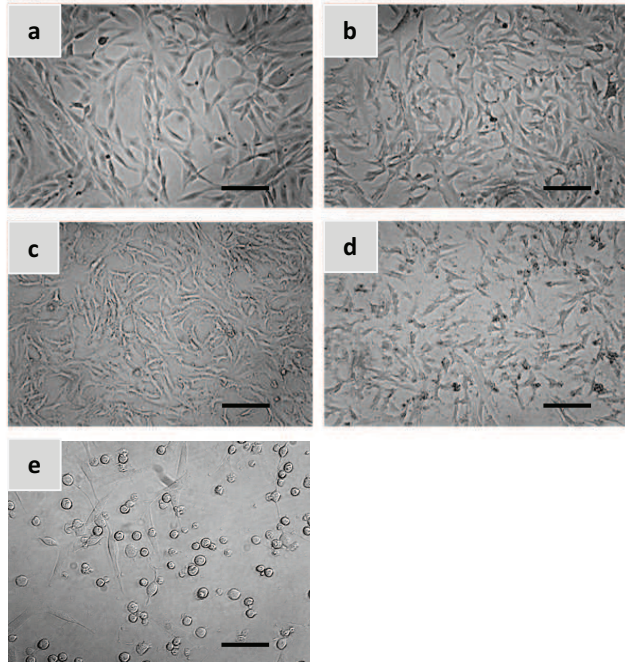


Figure 2





**Figure 3**

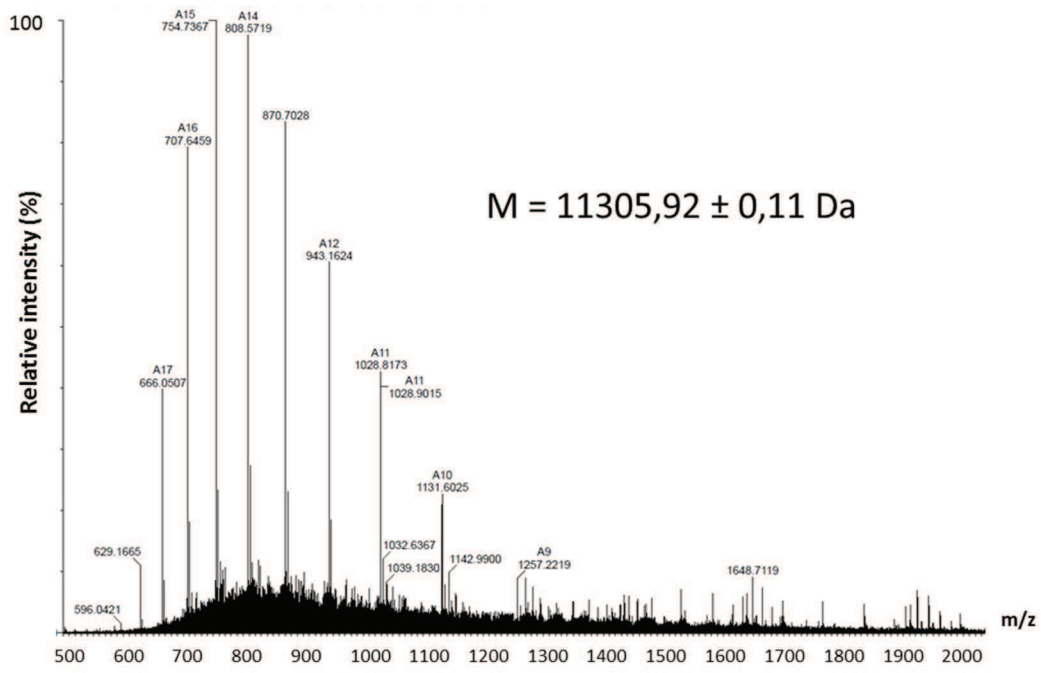


Figure 4

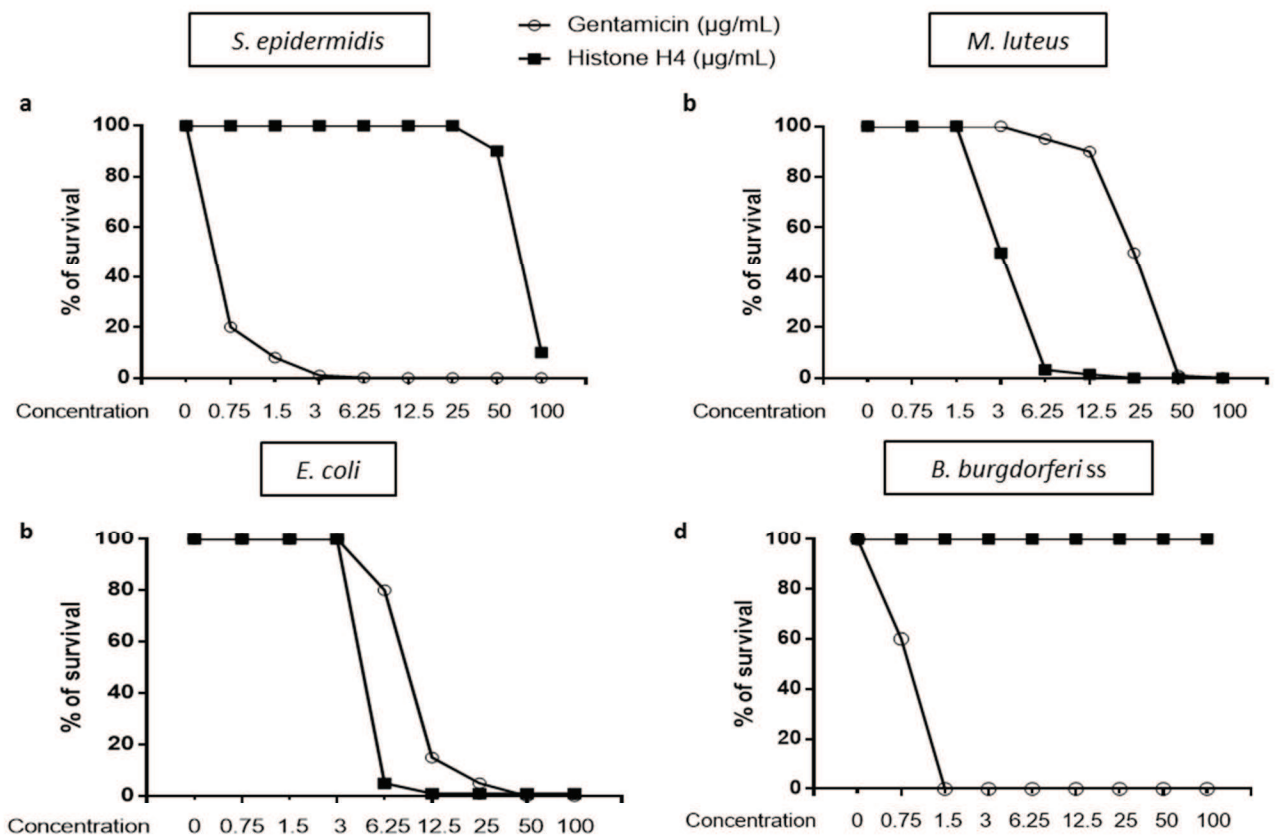


Figure 5

# L'acteur 2: la bactérie



*Borrelia* sp. observée par microscopie électronique à balayage (Centers for Disease Control and Prevention Public Health Image Library (PHIL))

## ARTICLE 3:

Heterogeneity of *Borrelia burgdorferi* sensu stricto population and its involvement in *Borrelia* pathogenicity: study on murine model with specific emphasis on the skin interface.

Aurélie Kern, Gilles Schnell, Quentin Bernard, Amandine Bœuf, Benoît Jaulhac, Elody Collin, Cathy Barthel, Laurence Sabatier, Nathalie Boulanger

Accepté: PLOS ONE, 2015

La peau, en plus de jouer un rôle protecteur contre *Borrelia*, fournit un lieu propice où la bactérie peut organiser sa future dissémination notamment par le biais de son amplification et la sélection des variants génétiques les plus virulents (Troy et al. 2013). De plus, il existe une certaine association entre une espèce de *Borrelia* et l'organotropisme lors de la dissémination, mais le mécanisme sous-jacent à ce phénomène n'est pas connu (Stanek et al 2012). Cette pression de sélection semble s'expliquer en partie par la réponse immunitaire présente au niveau de la peau (Rego et al. 2014; Seifert et al. 2015). Seules certaines populations de spirochètes parmi la totalité de bactéries transmises parviennent à infecter durablement l'hôte vertébré. Afin de comprendre cette sélection, l'inflammation induite par différents isolats cliniques de *Borrelia* chez la souris a été étudiée.

Dans un premier temps, à l'aide de techniques de RT-PCR, nous avons étudié s'il existait des profils d'inflammation et de dissémination associés aux différents isolats cliniques testés. Par la suite, les populations d'une même espèce de *Borrelia* étant hétérogènes, nous avons comparé le développement d'une souche native de *B. burgdorferi* ss, 297, à un clone davantage disséminant de cette même souche, le clone c297/4 (Schnell et al., 2014). Le clone virulent semble induire spécifiquement l'expression de molécules inflammatoires au niveau cutané telles que MCP-1 et mBD14. Ensuite, nous avons comparé l'expression de différents gènes bactériens entre la souche native et le clone c297/4. Pour cela, une approche par électrophorèse et spectrométrie de masse a permis d'identifier la présence de 110 protéines exprimées uniquement par le clone bactérien c297/4 lors de cultures *in vitro*. Parmi ces protéines, 9 protéines correspondantes à des protéines de surface ont été étudiées au niveau expression génique lors de l'infection chez la souris. Parmi ces 9 protéines, seulement deux étaient surexprimées au niveau ARNm par le clone c297/4 comparé à la souche native au niveau de la peau lors de l'infection chez la souris : BB0347 et BB0213.

Globalement, la virulence de la bactérie semble liée (1) à l'expression de molécules inflammatoires spécifiques tels que MCP-1 et mBD14, respectivement une chimiokine et un peptide antimicrobien impliqués tous deux notamment dans la perméabilité vasculaire, et (2) à l'expression de gènes de *Borrelia* tels que *bb0213* et *bb0347* dont le second code une protéine interagissant avec la matrice extracellulaire de l'hôte vertébré. L'augmentation de la perméabilité vasculaire couplée à l'expression de protéines liant la matrice extracellulaire peut expliquer la capacité de certains clones à disséminer de manière plus efficace (Moriarty et al. 2012).

RESEARCH ARTICLE

# Heterogeneity of *Borrelia burgdorferi* Stricto Population and Its Involvement in Lyme Disease Pathogenicity: Study on Murine Model with Specific Emphasis on the Skin-Immune System Interface

Aurélie Kern<sup>1,2</sup>, Gilles Schnell<sup>2</sup>, Quentin Bernard<sup>1</sup>, Amandine Bœuf<sup>2</sup>, Elodie Collin<sup>1</sup>, Cathy Barthel<sup>1</sup>, Laurence Ehret-Sabatier<sup>2\*</sup>, Nathalie Boissier<sup>1</sup>

**1** EA7290: Virulence bactérienne précoce; groupe Borréliose de Lyme, Facultés de pharmacie, Université de Strasbourg, Strasbourg, France, **2** Laboratoire de Spectro-BioOrganique, Institut Pluridisciplinaire Hubert Curien, Université de Strasbourg, CNRS, Strasbourg, France, **3** Division of Geographic Medicine and Infectious Diseases, Tufts University, Boston, Massachusetts, United States of America

\* These authors contributed equally to this work.  
\* [aboutanoer@unistra.fr](mailto:aboutanoer@unistra.fr)



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## Abstract

Lyme disease is a multisystemic disorder caused by *B. burgdorferi* s.l. The involvement of the skin in the development of Lyme disease as the entry site of *B. burgdorferi* in which the spirochete is selected before dissemination. We compared the skin inflammatory responses (cytokines and chemokines) elicited by spirochete population patients presenting different clinical manifestations. Remarkably, these responses induced different inflammatory profiles in the skin of C3H/HeN mice. As the population transmitted into the host skin is heterogeneous, we isolated a clone from a population recovered from a patient with neuroborreliosis and compared it to the parental population. This clone elicited a strong cutaneous inflammation characterized by MCP-1, IL-6 and antimicrobial peptides induction. Moreover, this clone revealed 110 overexpressed proteins when compared with the parental population. We further focused on the expression of nine bacterial surface proteins. A protein that interacts with host fibronectin, allowing bacterial adherence to endothelium and extracellular matrix, was found to be induced in host skin. The gene *bb0213* coding for a hypothetical protein. These findings demonstrate the heterogeneity of the *B. burgdorferi* as population and the complexity of the interaction with the skin.

**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

Lyme disease, a zoonosis caused by the *Borrelia burgdorferi* sensu lato (sl) common arthropod-borne disease in the Northern Hemisphere. Among *B. burgdorferi* sl species, *B. burgdorferi* ss sensu stricto (ss) is a pathogenic species in the United States and Europe. Lyme disease induces a multisystemic disorder characterized first by a skin inflammation, the erythema migrans at the site of *Borrelia* human. After hematogenous dissemination systemic manifestations (eg, neurological and cutaneous) are observed [1].

The factors responsible for *Borrelia* organotropism are not known. In human skin has been shown to constitute a key interface where the spirochetes are migrating to target organs [2]. To better evaluate the role of the vertebrate host in the dissemination, we studied different *B. burgdorferi* isolates recovered from patients with different clinical manifestations. The skin protects itself against infections by innate immunity, an important to maintain homeostasis [3]. Recently the role of the skin has been studied in Lyme borreliosis with specific emphasis on the role of OspC in the transmembrane potential filter in *B. burgdorferi* populations [4]. We selected genes of innate immunity (microbial peptides, chemokines and cytokines) and compared the skin inflammation elicited by human isolates of *B. burgdorferi* in a mouse model, as the immune response in the skin might drive certain *Borrelia* populations towards specific organs.

Spirochetes refer to distinct species and genomic groups and differ in their genetic markers [5]. Two main genetic markers, the outer-surface protein C (ospC) type and intergenic spacer type (RST) can be used to distinguish strains of *B. burgdorferi* [6]. RST strains to correlate the severity of their clinical manifestations [7]. Three RST types are defined: RST1 genotype is correlated with hematogenous dissemination and severe signs, RST3 isolates are considered as non-disseminating strains, and RST2 is considered as intermediate. Within the former marker, 21 OspC groups have been initially defined [8]. Hematogenous dissemination is mainly associated with types A, B, I and J OspC genotypes [9]. These genetic markers are currently the best to define the genetic diversity of *B. burgdorferi* [10]. However, they are not sufficient to determine the infection initiated by a specific *Borrelia* strain and species. The situation is similar in the case of the biodiversity of *B. burgdorferi* strains in ticks and tissues [11] and the genetic diversity in clones isolated from one specific *Borrelia* population [12]. The factors that govern it remain to be identified.

In this study we compared the tissue distribution and the inflammatory response of different clinical isolates (also referred to as pathotypes) belonging to different OspC groups, testing that the skin could act as a filter to select specific clones, we selected a clone (297/4) because of its cerebral tropism. We compared its dissemination pattern to its parental strain. Although these two strains were RST2, they showed different inflammatory profiles in the skin. Proteomic analyses on the clone showed that it was overexpressed compared to the parental population *in vitro*. Among the overexpressed proteins we measured their gene expression in mouse skin after the transmission of the parental 297 strain or in the selected clone 297/4. We tested whether, by virtue of its immunity, constitutes an essential organ that selects *B. burgdorferi* to persist locally or to disseminate. The different factors contributing to this ph

**Table 1. Kinetic of tissue distribution in mouse of *Borrelia burgdorferi* ss strains isolated from skin manifestations.** Tissue strains of *Borrelia burgdorferi* ss. Bacterial distribution was measured by culture (heart, skin at inoculation site and ear) or by PCR (at the inoculation site) and by both methods for the ankle at different time points after intradermal injection of  $10^7$  *B. burgdorferi*. Five mice each time point. RST: rRNA intergenic spacer type; h: hours and d: days. EM: Erythema migrans, MEM: Multiple Erythema Migrans.

	Tissue distribution of strains isolated from skin manifestations								
	Pbre (EM)—RST1					MR726 (MEM)—RST:			
	skin at inoculation site	heart	bladder	ankle	ear	skin at inoculation site	heart	bla	
24h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	
3d	3/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	
5d	5/5	1/5	0/5	1/5	0/5	5/5	2/5	1/5	
7d	5/5	1/5	4/5	4/5	0/5	5/5	5/5	5/5	
15d	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	
30d	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	

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are also possible candidates as factors facilitating tissue persistence or dissemination of *B. burgdorferi*.

## Results

### Transmission and tissue distribution of different pathotypes of *B. burgdorferi* ss in mice

All strains studied exhibited a similar pattern of dissemination. *B. burgdorferi* was first detected at day 3 by PCR or by skin culture at the site of inoculation for all strains. Similarly to the ankle, the heart and the bladder where they were first detected which corresponds to the skin at distance from the inoculation site was then colonized (Tables 1 and 2). We observed for all strains tested a lack of detection within the skin 24 hours after inoculation. We attribute this result to the detection limit. All mice seroconverted to *B. burgdorferi* antigens 15 days after inoculation (data not shown). Weekly assessments of arthritis were performed by measuring the circumference of both ankles with a metric caliper. Ankle swelling provided an indication of arthritis but no significant difference was observed among the strains tested (data not shown). Blood culture was also tested and was positive only transiently at around day 3 but no significant difference between the strains (data not shown).

**Table 2. Kinetic of tissue distribution in mouse of *Borrelia burgdorferi* ss strains isolated from cerebrospinal fluid.** Tissue strains of *Borrelia burgdorferi* ss. Bacterial distribution was measured by culture (heart, skin at inoculation site and ear) or by PCR (at the inoculation site) and by both methods for the ankle at different time points after intradermal injection of  $10^7$  *B. burgdorferi*. Five mice each time point. (CSF): Cerebro-Spinal Fluid, RST: rRNA intergenic spacer type; h: hours and d: days.

	Tissue distribution of strains isolated from cerebrospinal fluid								
	1808/03 – RST1					297 – RST2			
	skin at inoculation site	heart	bladder	ankle	ear	skin at inoculation site	heart	bla	
24h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	
3d	5/5	0/5	0/5	0/5	0/5	5/5	1/5	0/5	
5d	5/5	0/5	1/5	0/5	0/5	5/5	1/5	0/5	



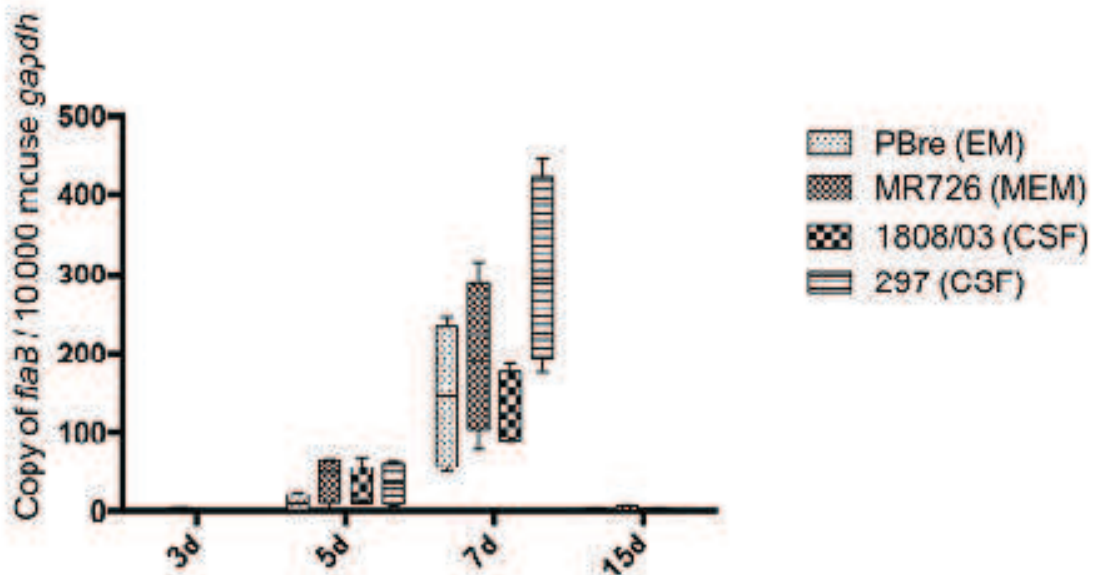
### *B. burgdorferi* pathotype quantification and measure of t in the skin at the inoculation site

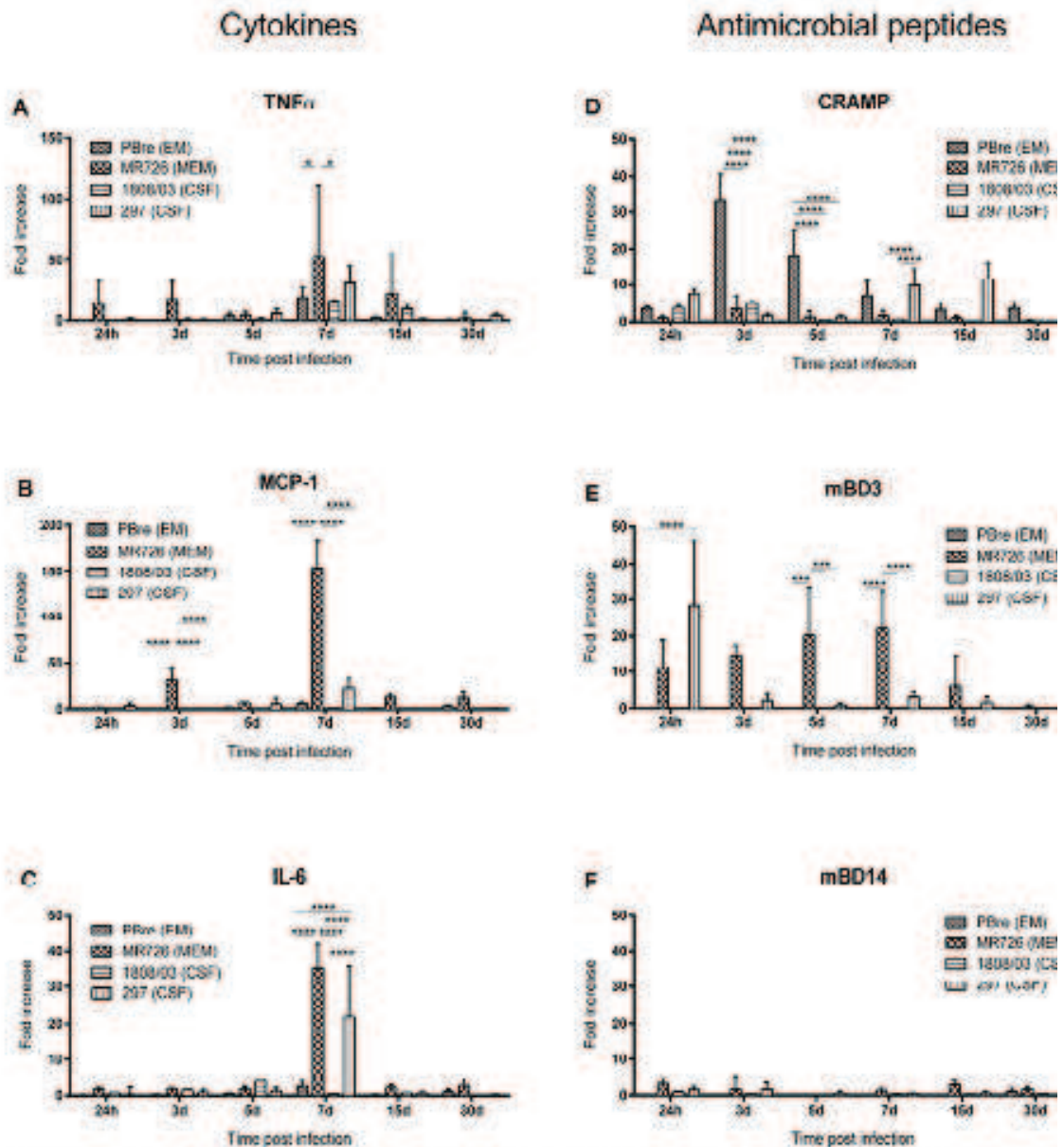
We measured the bacterial load in the skin since it was shown previously of *B. burgdorferi* multiplication [14,15]. The bacteria all multiplied intens infection, with no significant difference in the dissemination kinetics observed strains tested (Fig 1).

We showed previously that *B. burgdorferi* ss N40 elicited different innate immunity in the skin. We tested whether the different pathotypes in the skin. We observed the induction of pro-inflammatory molecules (the chemokine MCP-1. For all of them, a peak induction of TNF- $\alpha$  and/or at day 7 post-infection. Interestingly, MR726 strain isolated from a human the strongest inflammatory profile in mouse skin with a peak of MCP-1 (IL-2B). IL-4, IFN- $\gamma$  and IL-17 were also tested but no significant induction was shown). We then measured the induction of antimicrobial peptides, defensin mBD14) and mouse cathelicidin (CRAMP), as important effectors of skin: [3,16]. PBre strain (EM) induced a significant amount of CRAMP with a p infection (Fig 2D). MR726 (MEM) strain strongly induced the defensin m wild-type strain 297 (CSF) exhibited a peak of mBD-3 at 24h post-infection strain (CSF) induced a negligible amount of all three AMPs tested (Fig 2D).

### Specific analysis of the inflammation induced by *B. burg* parental strain and its clone

Since *B. burgdorferi* infection might be triggered by a heterogeneous population host, we performed the cloning of *B. burgdorferi* ss strain 297 [17].





**Fig 2. Inflammatory profiles of the different strains of *B. burgdorferi* ss.** Levels of transcripts were measured by RT-qPCR from inoculation site. The values were calculated using the 2-delta delta Ct method after normalization with *gapdh*. (h: hours, d: days). Two to analyze the data. At least three mice were analyzed for each time point.

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297/4 because of its cerebral dissemination at day 30 [18]. In addition, the neurological manifestations in a few mice (alteration of behavior and hemorr

**Table 3. Kinetics of tissue distribution of *Borrelia burgdorferi* 297 strain and its clone.** Bacterial distribution was measured by inoculation site, brain and ear) or by PCR (bladder and skin at the inoculation site) and by both methods for the ankle at different time injection of  $10^7$  *B. burgdorferi*. Five mice were analyzed for each time point. h: hours; d: days; wt: wildtype; c: clone.

	Tissue distribution								
	297 wt					c297/4			
	skin at inoculation site	heart	bladder	joint	ear	Brain	skin at inoculation site	heart	bladder
24h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
3d	5/5	1/5	0/5	0/5	0/5	0/5	4/5	1/5	0/5
5d	5/5	1/5	0/5	2/5	0/5	0/5	4/5	1/5	0/5
7d	5/5	4/5	5/5	5/5	2/5	1/5	4/5	4/5	5/5
15d	5/5	5/5	5/5	5/5	5/5	2/5	5/5	5/5	5/5
30d	5/5	5/5	5/5	5/5	5/5	2/5	5/5	5/5	5/5

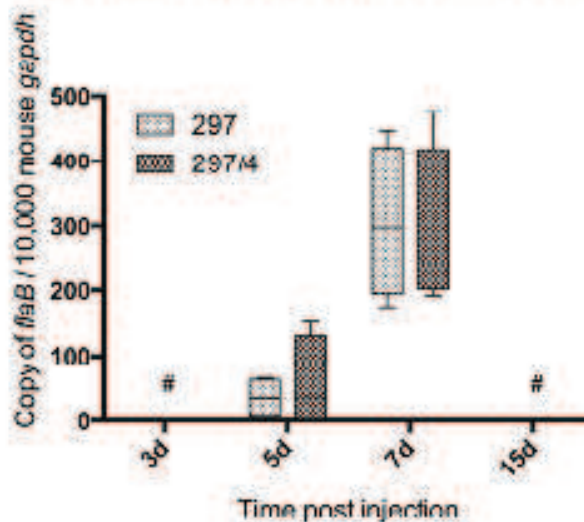
doi:10.1371/journal.pone.0133195.t003

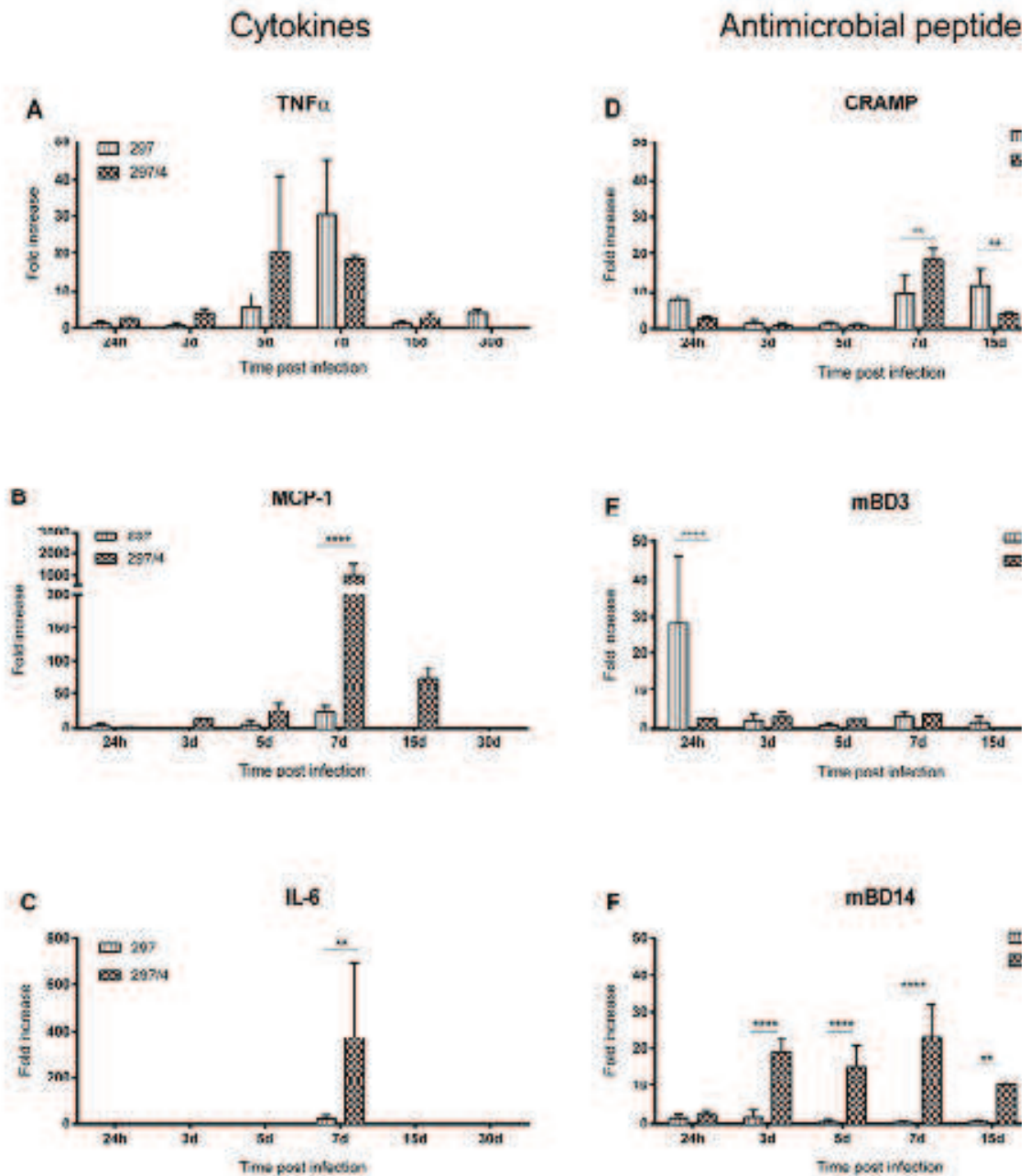
but no significant difference was observed between the clone and the parent strain (Fig 3).

Interestingly, when we compared the inflammatory profiles of the clone strain, clone 297/4 elicited a stronger skin inflammation with a high level of IL-6 (Fig 4). Similarly for the AMPs, we observed a significantly higher expression of mBD-14 and CRAMP for clone 297/4 compared to the parental strain.

### Proteomic characterization of *B. burgdorferi* ss, 297, parent strain versus clone

To determine whether a differential protein expression might explain the inflammatory profile triggered by the clone 297/4, we undertook a global proteomic analysis of the protein profiles of *B. burgdorferi* 297 and the clone 297/4. We used a Gel





**Fig 4. Inflammatory profiles of *B. burgdorferi* ss, parental strain and its clone 297/4.** Levels of transcripts were measured by RT-qPCR at the inoculation site. The values were calculated using the 2<sup>-delta delta</sup> Ct method after normalization with *gapdh*. (h: hours, d: days) used to analyze the data. At least, three mice were analyzed for each time point.

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identified with 848 proteins in 297/4 and 777 proteins in the parental strain (Table). As compared to our previous results [18], we observed a reproducible total proteins identified. Interestingly, in this study, 110 proteins were detected in the clone 297/4. The breakdown of the biological functions of these proteins is shown in Table 1. The number of hypothetical or unknown protein functions (Fig 5B). The genome of *B. burgdorferi* 297/4 contains a single chromosome, 9 circular plasmids, and 12 linear plasmids. The 110 proteins, 91 are localized on the chromosome, 15 on linear plasmids, and 4 on circular plasmids (Fig 5C). However some plasmids present in *B. burgdorferi* 297/4 are not present in the parental strain. Several proteins (39) were detected only in the 297/4 clone. Their under-expression in 297/4 could also contribute to bacterial specificity (Fig 5A).

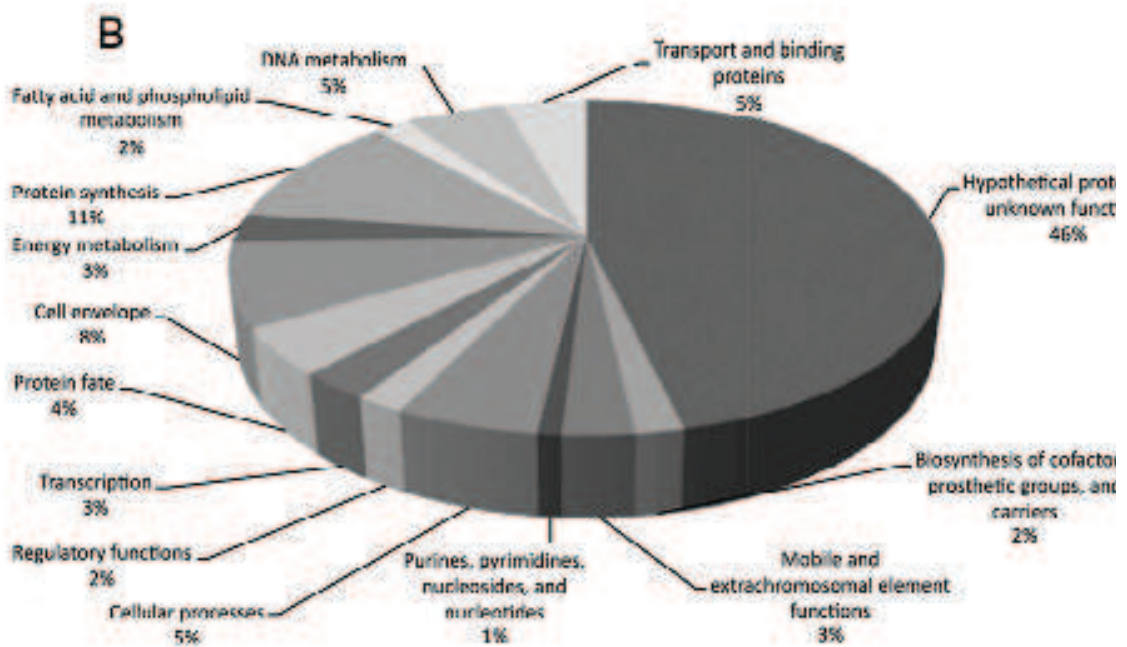
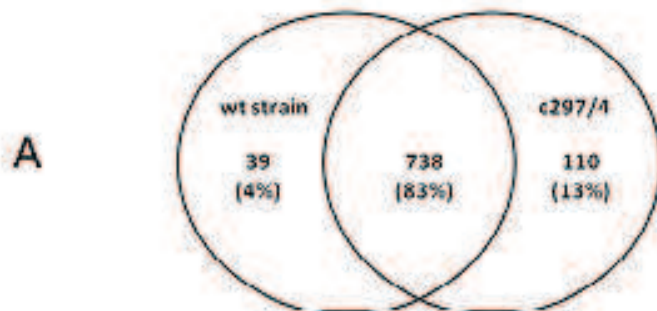
### Comparative induction of specific genes of *B. burgdorferi* versus the parental strain in mouse skin by RT-PCR

Since surface proteins of *B. burgdorferi* also represent candidates for bacterial surface proteins essential in the interaction with the host, we selected among the 110 specific proteins, those assigned to cell envelopes (S2 Table). By RT-PCR, we followed the expression of these genes during the skin inflammation in C3H/HeN mice. Among the 9 selected genes, the mRNA of three of these proteins was significantly induced but without difference between the two bacterial populations: the two well-known genes, *ospC* and *bbk32* are involved in cell wall synthesis (Fig 6). Two genes were highly induced with respect to the parental strain expression at day 5 for the clone 297/4: *bb0213* and *bb0347*. All the other genes (*bb0167*, *bb0160*, *bb0823*, *bb0117* and *bb0718*) were weakly induced.

### Discussion

Considering that the skin of the vertebrate host is the first physical and immunological barrier encountered by the spirochetes, it constitutes an efficient filter for arthropod-borne pathogens. The resident skin cells, keratinocytes and fibroblasts, play a pivotal role in the defense against pathogens via their Toll-like receptors. They also secrete anti-inflammatory molecules and chemokines [19–21]. Recently, the role of the skin has been re-evaluated and the skin now appears to be a critical step in the pathogenesis of certain arthropod-borne diseases, such as leishmaniasis and malaria [22]. The diversity of *B. burgdorferi* ss varies according to its environment: it is greater in tick populations and even less diverse in disseminated sites such as the cerebrospinal fluid of patients [7]. The skin appears to constitute an interface, where among the population of *Borrelia* in ticks, only specific *OspC* alleles are selected to disseminate [8].

To further investigate the role of the skin barrier in Lyme borreliosis, we used a mouse model [2,3] to study the outcome of various human clinical isolates that are different pathotypes. We investigated whether a specific inflammatory profile is characteristic of a pathotype and could explain its organotropism. Because well-known pro-inflammatory molecules are important molecules of skin immunity [3,16] we studied their induction. AMPs are not only antimicrobial molecules but also regulate chemokine production, angiogenesis, and wound healing. We have id

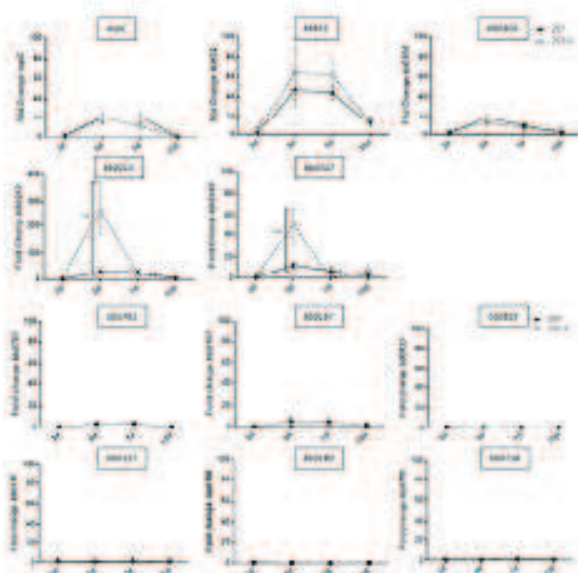


the clone. Categorization was based upon JCVI annotation. The percentages represent the fraction of that category within the proteome of the specific proteins identified in the clone 297/4.

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(an ortholog of human defensin hBD-2). With the human pathotype MR MEM, the mouse exhibited an important perturbation of its skin homeos

We also characterized the RST genotype (data not shown) to obtain information on the potential pathogenicity of the different *Borrelia* strains in t with the *ospC* gene, the RST genotype is defined as a disseminating marks strains can be characterized by their genotype by RFLP as described previ fication determines their invasiveness [9–12]. We investigated whether a associated with a specific inflammatory profile in the skin. PBre and 1808 ble for EM and neuroborreliosis in human respectively, were identified as MR726 strain isolated from MEM lesions as RST3. RST1 is reported as a type while RST3 is considered as a non-invasive genotype [24]. In mice e) by different RSTs of *B. burgdorferi* ss, a clear difference was reported betw isolates. RST1 produces more severe symptoms and a heavier load of spiro organs [25]. In our model, after syringe inoculation of *Borrelia*, the tran significantly between PBre and 1808/03 (RST1) and MR726 (RST3) since inoculation was positive at day 3 in both cases. The quantification in the either between these two RSTs. However, the inflammatory profile in the (PBre and 1808/03) strains was slightly different with an induction of the at day 3 for PBre and TNF- $\alpha$  at day 7 for both strains. The RST3 (MR726) from MEM, gave a strong inflammatory profile in the skin with a strong i defensin mBD-3 and MCP-1. Overall, we showed a clear difference in ski according to the bacterial strain tested, although we cannot correlate a hu



an RST to a specific skin immune response in the mouse. In this sense, *murK* and *OspC* are not sufficient to determine the outcome of an infection with *Borrelia* strain and species due to the heterogeneity and biodiversity exist shown in two recent studies [2,13].

Since the *Borrelia* population is heterogeneous, we decided to generate a well-characterized strain. We selected the strain *B. burgdorferi* 297, similarly disseminating and is defined as RST2 (intermediate in dissemination for its cerebral localization compared to the others we isolated [18]. A recent study also revealed the cerebral localization of a strain of *B. burgdorferi* having the same sequence as *B. burgdorferi* ss 297 [26]. By its cerebral localization, this strain presents features of virulence. The clone and the parental strain both defined as RST2 in the skin at day 7. However, the inflammatory profile induced by the clone was significantly different from the profile observed with the parental strain, although the cytokine profile was identical for both strains. The clone 297/4 induces the defensin  $\alpha$ 1 of human defensin 3 (or hBD3) and more importantly of MCP-1 and IL-6, a chemoattractant for immune cells and also possess angiogenic properties [2] that promotes to better diffuse via the blood to target organs. Recently, mBD-14 has been shown to promote angiogenesis [27]. Similarly, the induction of MCP-1 by the clone 297/4 promotes the diffusion of the bacteria in the host tissues, since it has been described that MCP-1 increases the permeability of vascular endothelium cells in dengue hemorrhagic fever [28]. As reported by Rego et al. [13] confirms our observation concerning the heterogeneity of *B. burgdorferi* clones. They showed that clones behave and persist differently *in vivo*. The clone 297/4 is more virulent than the parental strain *B. burgdorferi* ss B31. Each clone was distinguished by a unique sequence tag. They simultaneously inoculated 7 clones and then conducted a selection. Stochastic selection occurred at the different steps of *Borrelia* acquisition. Interestingly, 9 weeks after the inoculation, not all mice were infected with *B. burgdorferi* and some clones persisted better than others.

Since surface proteins could be specifically involved in the process of dissemination and organotropism of *Borrelia*, we carried out a global proteomic analysis of these two populations. Up to now proteomic approaches, using electrophoretic and mass spectrometry have been described mainly to investigate the immunoreactive proteins [31]. Only few global proteomic approaches have been described for the analysis of *Borrelia* [32–34]. The detection of 110 proteins only in the clone 297/4 by an under-expression in the parental strain and/or an over-expression in the parental strain does not express these proteins, or more likely they are below the limit of detection by GeLC-MS/MS. We then focused on the relative expression of selected surface protein genes *in vivo*, since such proteins have been described as important in the infectivity and potentially in *Borrelia* persistence and dissemination in mouse skin, the two well-known genes, *ospC* and *bbk32* were both well induced in the clone 297/4 but no particular profile was observed. Similar profile was observed for the *murF* enzyme that catalyzes the murein synthesis involved in bacterial cell wall synthesis. In contrast, the clone upregulated two genes at day 5, *bb0347*, related to a fibronectin-binding protein and *bb0213*, a hypothetical protein. It is well established that the interaction with the extracellular matrix is essential for its dissemination and persistence in the host. *bb0347* has recently been described as a ligand for the human fibronectin



bacterial multiplication occurs. Our study demonstrates that the skin is a crucial site for the development of cutaneous immunity against *B. burgdorferi*, and the development of cutaneous immunity might be crucial for the outcome of *B. burgdorferi* in the vertebrate host. We showed that RST genetic markers are essential factors for its dissemination and persistence in the vertebrate host. *Borrelia* inoculum is a bacterium that expresses essential factors for its dissemination and persistence in the vertebrate host, linked to the genetic markers *ospC* and RST, as shown by the data presented here. Some specific factors of both, the bacteria (eg *OspC*, BB0347 and other proteins) and the host (eg AMPs and MCP-1), display a sophisticated interaction that orients the bacterium to specific organs of the vertebrate host. All these factors remain to be identified to understand more clearly the physiopathology of Lyme disease initiated in the skin.

## Materials and Methods

### Mouse and bacterial strains

Three to four week old C3H/HeN pathogen-free mice were purchased from Charles River Laboratories (L'Arbresle, France) and provided food and water *ad libitum*.

Bacterial isolates belonging to *Borrelia burgdorferi* sensu stricto species were obtained from patients with different clinical manifestations and therefore defined as pathogenic. One strain was isolated from a unique *erythema migrans* lesion (EM) in Germany, M1000/03 strain from a multiple *erythema migrans* (MEM) lesion in the United States, 1808/03 strain from nasal fluid in Slovenia and 297 strain from cerebrospinal fluid in the United States. All *B. burgdorferi* ss clones were obtained by culture on solid BSK medium [17]. For comparative study, the clone 297/4 was used in our comparative study with its native strain.

All the strains were cultured in BSK-H complete medium (Sigma) at 37°C. For mouse infection, the clone 297/4 was used in our comparative study with its native strain. Spirochetes were counted and viability was determined by dark field microscopy.

### Follow up of mouse infection and tissue sampling

Mice were infected with  $10^3$  spirochetes in 0.1 mL BSK by intradermal injection in the thoracic area. Serology was performed as previously described [40]. At different times after the beginning of the experiment (0h, 24h, 3d, 5d, 7d, 15d and 30d post-infection) mice were killed by isoflurane gas overdose. About a 1 cm area of mouse skin at the site of the inoculation and stored in Trizol reagent (Invitrogen). The ear, tail and the ankle from each mouse were harvested aseptically and each organ was cut in 2 pieces, for PCR and culture in BSK-H medium. Organs of uninfected mice were collected as a negative control.

### Detection of *B. burgdorferi* by culture and by PCR in mouse organs

Collected organs were placed in 6 ml of BSK-H medium containing 30µg/ml penicillin (BioRad). The tubes were maintained at 33°C and examined weekly for the presence of spirochetes by dark-field microscopy as described previously [17].

For PCR detection, DNA was extracted from the organs of individual mice.

buffer. Ten  $\mu$ L of eluted DNA were used as a template for *B. burgdorferi* DNA amplification, targeting the *flagellin* gene, was carried out as described [14].

### Quantification of spirochete load and measure of inflammation induction in mouse skin by RT-PCR

At the site of inoculation, quantification of the *B. burgdorferi* ss-specific flagellin was carried out on a LightCycler system (Roche Diagnostics, France). The primer and probe for the *fla* gene were those previously described [15].

To measure the inflammation at the inoculation site, total RNA was extracted from mouse skin using Trizol Reagent as recommended by the manufacturer. Samples were treated by DNase (Ambion, USA). Then first-strand cDNA was synthesized from 1  $\mu$ g of total RNA using SuperScript II reverse transcriptase (Invitrogen). *gapdh* quantification was performed as an internal standard. Relative expression was calculated using cDNAs from three uninfected mice as a calibrator. Amplification reactions were performed with an ABI 7000 system with the following thermal protocol: 10 min, 50 cycles of 95°C for 15 s, 60°C for 1 min. Primers for all the genes are listed in [S1 Table](#) or have already been described [15].

### Comparison of the protein profile of *B. burgdorferi* strain and clone, by protein fractionation, in gel digestion and mass spectrometry

Cultures of *B. burgdorferi* 297, parental strain and clone 297/4, were analyzed by SDS-PAGE [18], using 12% SDS-PAGE and a nanoLC-Chip/MS (Agilent Technologies, CA) hyphenated to an ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Data were interpreted using the Mascot 2.3.02 (Matrix Science, London, UK) (Open Mass Spectrometry Search Algorithm, Maryland, USA) algorithm. The search was performed against an in-house generated protein database composed of proteins from *B. burgdorferi* ss B31 and known contaminant proteins such as human keratins. The database was downloaded from NCBI non-redundant database concatenated with reverse sequences (total 2002 entries). This database was used because the *B. burgdorferi* genome has not yet been completely sequenced. The Mascot and OMSSA results were loaded into the Scaffold software (Proteome Software, Portland, OR). This database search allowed us to control the false positive identification rate which

### Dynamics of gene expression of surface proteins of *B. burgdorferi* parental strain and clone 297/4, in mouse skin at the inoculation site

At different time points, skin samples were collected from each mouse at the inoculation site. Total RNA was purified using Trizol reagent according to the manufacturer's instructions. Concentration and purity of extracted RNA were determined by measuring absorbance at 280 nm. Samples were then treated with gDNAse wipeout (QIAGEN) before reverse transcription to avoid contamination. The total extracted RNA was subjected to synthesis of cDNA using reverse transcription (QIAGEN). cDNA was used to quantify the well-known *bbk32* levels as positive controls. For *B. burgdorferi* ss 297/4, genes corresponding to surface proteins ([S2 Table](#)) were selected for the RT-PCR. Relative expression

calculated by comparing cDNA amplification of the native strain to cDNA clone for each day. Then, the curve obtained for the clone was normalized to get a second curve, representative of the native strain, and quantitatively compared to the clone.

### Ethics Statement

The protocols carried out in this study were in accordance and approved by the Committee on the Ethics of Animal Experiments of the University of Strasbourg (Comité Régional d'Ethique en Matière d'Expérimentation Animale Strasbourg—10/39/12/12).

### Statistical analyses

For the different kinetics study, at least two mouse cohorts were used for each time point. For qRT-PCR, at least two to three extractions were made for each time point. For the qRT-PCR results, gene expression relative to control is reported as the mean  $\pm$  SD from at least three independent experiments and each time point corresponds to 2 to 3 mice. The statistical significance of differences was determined by a two-way ANOVA test. A p-value of 0.05 was considered statistically significant. Analyses were performed with Prism 6 software (Graphpad, La Jolla, CA).

### Supporting Information

**S1 Table. Primers designed for this study.**  
(DOCX)

**S2 Table. Proteins specifically detected in *B. burgdorferi* ss, clone c297.** Proteins identified specifically in the clone are given with their gene location and accession number from JCVI annotation. <sup>†</sup> Represents proteins detected by the search engine Mascot. In bold: proteins associated with the clone. (DOCX)

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### Author Contributions

Conceived and designed the experiments: BJ NB LS. Performed the experiments: AB EC CB. Analyzed the data: AK GS QB LS NB. Wrote the paper: AK GS

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# ACTEUR 3 – L'hôte vertébré (La peau)



La tique du genre *Ixodes* attachée sur la peau humaine (WebMD, LLC)

## ARTICLE 4:

*Ixodes* tick saliva modulates mast cell inflammatory response to  
*Borrelia burgdorferi* sensu stricto

Quentin Bernard, Zhenping Wang, Ana diNardo, Richard L. Gallo, Benoît Jaulhac, Nathalie Boulanger

En préparation

La peau constitue la première barrière physique et immunologique que les agents pathogènes transmis par un vecteur doivent traverser. Elle représente une surface d'environ deux mètres carrés et contient plusieurs milliers de cellules. Parmi les cellules résidentes de la peau, on trouve notamment les MCs. Ces cellules, connues pour leur implication dans les phénomènes allergiques sont de plus en plus décrites pour leur importance vis-à-vis de la défense contre les parasites, les virus et les bactéries (Reber and Frossard 2014). Elles sont capables de détecter directement la présence des agents pathogènes à l'aide de nombreux récepteurs PRRs ou indirectement par l'intermédiaire d'anticorps et de ses récepteurs Fc. L'activation de ces cellules conduit à la sécrétion de cytokines, chimiokines et peptides antimicrobiens ainsi qu'au relargage d'autres molécules inflammatoires préformées via le mécanisme de dégranulation. Ces molécules permettent le ciblage direct des agents pathogènes ou l'attraction de cellules immunitaires telles que les neutrophiles ou les cellules T.

Lors de la transmission à l'homme de *B. burgdorferi*, l'agent infectieux de la borréliose de Lyme, les MCs sont donc en première ligne au même titre que les KCs, les FBs et les autres cellules de l'immunité. Cependant, seules deux études se sont intéressées à ces cellules dans le contexte de la borréliose de Lyme (Talkington and Nickell 1999, 2001). Ces études montrent notamment que les cellules de la lignée mastocytaire de souris MC/9 ainsi que les MCs péritonéaux de rats peuvent être activés par *B. burgdorferi*. Il est aussi montré que ce mécanisme d'activation implique les récepteurs Fc $\gamma$ . Nous avons donc décidé d'explorer davantage la réponse des MCs vis-à-vis de *B. burgdorferi* en utilisant des cellules primaires de souris. La lipoprotéine de surface OspC ainsi que la bactérie vivante sont capables d'activer la dégranulation ainsi que la sécrétion d'IL-6 par les MCs. Cependant, la capacité du mutant de *B. burgdorferi* OspC-KO à activer les MCs indique que cette lipoprotéine n'est pas indispensable. De plus, la présence d'extrait de glandes salivaires de tique inhibe clairement la sécrétion d'IL-6 par ces cellules. Nous avons ensuite exploré le rôle *in vivo* des MCs lors de l'infection de la souris par *B. burgdorferi* ss. L'absence de ces cellules n'a aucune influence sur la capacité de la bactérie à se multiplier dans la peau en début d'infection ni sur sa capacité à disséminer par la suite. L'influence de ces cellules sur la sécrétion de molécules inflammatoires nécessite d'être analysée. Il est à souligner ici, que pour le modèle murin, le fond génétique nécessaire est constitué par les souris C3H/HeN (Barthold et al. 1990), ce qui peut expliquer que les souris KO WSH qui ont un fond génétique C57/BL6 n'aient pas constitué le modèle de choix attendu pour étudier le rôle des MCs.

*B. burgdorferi* peut activer la réponse inflammatoire mastocytaire *in vitro*. De plus, la salive de tique est capable d'inhiber efficacement cette réponse. Cependant, le rôle de ces cellules *in vivo* n'est pas clairement identifié dans notre étude. En effet le développement de l'infection chez une souris

déficiente en MCs (souche exacte) est identique à celui chez une souris sauvage C57/BL6 lors des 15 premiers jours. Il n'est cependant pas exclu que ces cellules jouent un rôle tardif dans la défense immunitaire contre *B. burgdorferi*, notamment lors de la mise en place de l'immunité adaptative.



# Ixodes tick saliva modulates mast cell inflammatory response to *Borrelia burgdorferi* sensu stricto

Quentin Bernard<sup>1</sup>, Zhenping Wang<sup>2</sup>, Anna diNardo<sup>2</sup>, Richard L. Gallo<sup>3</sup>, Benoît Jaulhac<sup>1,4</sup>, , Nathalie Boulanger<sup>1,4</sup>

**Key Words:** tick saliva, inflammation, mast cells, *Borrelia*, OspC

<sup>1</sup>EA7290 *Virulence bactérienne précoce: groupe borréliose de Lyme, Fédération de médecine Translationnelle et Faculté de Pharmacie de Strasbourg, Université de Strasbourg, France.* <sup>4</sup>*Department of Medicine, State University of New York, Stony Brook, New York, <sup>4</sup>Centre National de Référence Borrelia, Centre Hospitalier Universitaire, Strasbourg, France.*

**Correspondance:** Nathalie Boulanger, EA7290: Virulence bactérienne précoce: Groupe Borréliose de Lyme, Institut de bactériologie, 3 rue Koeberlé, 67 000 Strasbourg, France. Tel. : 33 3 69 55 14 49 / 33 3 68 85 41 25 ; Fax : 33 3 69 55 16 98 ; e-mail : nboulanger@unistra.fr

**Abbreviations :** Osps : Outer Surface proteins, Interleukin : IL, MC : mast cell,

## ABSTRACT

*Borrelia burgdorferi sensu lato*, the causative agent of Lyme borreliosis is a bacteria transmitted by hard ticks. Bacteria are injected into the skin during the tick blood meal with the tick saliva. There, *Borrelia* and saliva interact together with resident skin cells, keratinocytes and fibroblasts and mast cells. *B. burgdorferi* has been shown to activate mouse mast cell MC/9 line inflammatory response. We report here the bacteria ability to induce mouse primary mast cells inflammatory response. We show that the lipidated OspC protein is not needed for mast cells to be activated. Moreover, the viability of the bacteria is a critical parameter for inflammatory response induction. Additionally, we report that mast cell activation is inhibited in part by the tick salivary gland extract. Finally, we demonstrate with an *in vivo* model of C57Bl/6 mast cell deficient mice that those cells do not influence *B. burgdorferi* multiplication in the skin as well as its dissemination. However, the mice genetic background might influence these results.

## INTRODUCTION

*Ixodes* ticks are vectors of several pathogens including viruses (eg tick-borne encephalitis virus), parasites (e.g. babesia) and bacteria (e.g. *Borrelia burgdorferi sensu lato (sl)*) (1). Those are transmitted to the host during the tick blood meal which last several days. Pathogens are inoculated into the skin with the tick saliva. In the case of Lyme borreliosis, caused by *B. burgdorferi sl* infection (2), the bacteria have been shown to stay several days in the skin after the transmission (3). Skin cells, including keratinocytes, fibroblasts, endothelial cells, dendritic/Langerhans cells, T cells and mast cells (MC) are thus of immune importance since they are likely to be the cells encountered by the bacteria (4). However, most of these cell functions have been shown to be inhibited by tick saliva compounds such as sialostatin L and Salp15 during bacteria transmission by *I. scapularis* ticks (5–11). Salp15 protects *B. burgdorferi sl* from the immune system by binding to the bacteria surface lipoprotein: OspC (12). OspC is a major *Borrelia* surface lipoprotein upregulated during the early transmission to the host (13). Moreover, it targets immune cells such as dendritic cells and T cells to inhibit cytokine expression and cell maturation (14,15). The tick saliva also inhibits other mechanisms such as hemostasis, pain and itch to increase tick blood meal efficiency (16). Pathogens have evolved to use these saliva-associated inhibition mechanisms to increase their transmission rate (10).

MCs are widely distributed throughout the body with specific locations at surface epithelia such as the skin, the lung and gastrointestinal and genitourinary tracts (17). These cells are well known for their association with pathological conditions such as asthma, allergy or anaphylaxis. MCs are increasingly studied with new evidence of their involvement in parasitic, bacterial and viral infections. They can directly sense Pathogen-Associated Molecular Patterns (PAMPs) through pattern recognition receptors (PRRs) such as Toll Like Receptors (TLRs) (18). They also can detect antigens through the expression of Fc receptors, able to bind antibodies. MC response to pathogens depends on the PAMPs: some PAMPs induce only cytokine expression (TNF- $\alpha$ , IL-6, IL-13, IL-1 $\beta$ , IL-4, IL-5, IFN- $\gamma$ ), others induce cytokine production as well as degranulation, a mechanism inducing preformed inflammatory mediator release (19). In case of bacterial pathogenesis, the release of TNF, histamine or vascular endothelial growth factor (VEGF) by MCs increases vascular permeability. Chemokines produced by MCs increase the inflammatory cell attraction at the infection site (eosinophils, neutrophils, NK cells) (20), helping certain bacteria to disseminate. MCs can also directly kill pathogens through antimicrobial peptides such as cathelicidins (21). Moreover, their ability to present antigen and to secrete inflammatory mediators allow the recruitment of dendritic cells as well as T cells at the infection site. All the processes give them a central role between innate and adaptive immunity.

MCs have been poorly studied in the context of Lyme Borreliosis. Currently, it is known that murine MCs lines respond to *Borrelia* by secreting TNF- $\alpha$  and by degranulating (22). This activity seems to depend on surface proteins but not on the lipoprotein OspA. An antibody-independent mechanism depending on Fc receptors is also involved (23). Here we analyzed primary MCs response to *B. burgdorferi sensu stricto* (ss) and its lipoprotein OspC, a lipoprotein essential for the bacteria transmission from the tick to the host. We also explored *in vitro* the tick saliva impact on MCs response to *Borrelia*. Finally, we used a murine MC deficient model to study their involvement in the infection process.

## MATERIALS AND METHODS

### ***Borrelia***

*B. burgdorferi* ss strain 297 was cultured in BSK-H medium (Sigma-Aldrich) at 33°C, used at passage 8 in the late-log-phase, centrifuged, and then washed twice (30 min, 5 000 g) with cell culture media. *B. burgdorferi* ss 297, OspC-deficient and complemented mutants, have been described previously (24).

### **Mast cell culture**

Primary MC were generated by extracting bone marrow cells from the femurs of 4- to 8-wk-old C57BL/6 mice and culturing cells in RPMI 1640 (Life Technologies) supplemented with 10% inactivated FCS (Life Technologies), glutamine, non-essential amino acid (NEAA), penicillin/streptomycin, and 2-ME. Recombinant murine IL-3 (1 ng/ml) and recombinant murine stem cell factor (20 ng/ml), both shown to support the *in vitro* growth and differentiation of the MC precursor, were also included. Medium was changed twice a week. After 6 weeks, MC maturation and purity were confirmed by the expression of CD117 and FcεRI measured by flow cytometry.

### **Mast cell degranulation and activation**

*Degranulation.* Degranulation was assessed by measuring the activity of b-hexosaminidase in the supernatants 10<sup>5</sup> MCs in 200 µl Tyrode's buffer (0.1% BSA, 0.1% glucose, 2 mmol/l MgCl<sub>2</sub>, 137.5 mmol/l NaCl, 12 mmol/l NaHCO<sub>3</sub>, 2.6 mmol/l KCl, pH 7.4) incubated for 1 h with different concentrations of live or dead *B. burgdorferi* ss (MOI 50:1 or 100:1) or L-OspC (10 or 50 ng/mL). For each sample assayed, supernatant aliquots (20 µl) were mixed with substrate solution (100 µl), which consisted of 1 mM 4-methylumbelliferyl-2-acetamide-2-deoxy-b-D- glucopyranoside (Calbiochem) in 0.1 M sodium citrate buffer (pH 4.5), and were incubated for 30 min at 37°C. The reaction was then stopped by adding 12 µl of 0.2 M glycine (pH 10.7). The reaction mixtures were excited at 365 nm and measured at 460 nm in a fluorescence plate reader. To determine the total cellular content of this enzyme, an equivalent number of cells were lysed with 1% triton-X-100 (Sigma). Release of b-hexosaminidase was calculated as the percentage of the total enzyme content.

*Activation.* MC were activated with different concentrations of live or dead *B. burgdorferi* ss (MOI 50:1 or 100:1) or L-OspC (10 or 50 ng/mL) for 6 or 24 hours. Cells and supernatants were collected and separated by centrifugation (1500g, 10 min).

### Tick salivary glands

*I. ricinus* salivary gland extract (SGE) was used at 20 µg/ml as described previously (25) when incubated with MCs. SGE was tested by the Limulus assay to check for the presence of endotoxins and were found to contain <0.3 endotoxin units.

### Mice and bacterial challenge

MC-deficient (Kit Wsh<sup>-/-</sup>) mice were kindly provided by Dr. Salah Mécheri (Pasteur Institute, Paris, France). The animals were bred at our facility according. The C.R.E.M.E.A.S approved all animals experiments. *Borrelia* (10<sup>3</sup>/100µL) were intradermally injected into the dorsal thoracic area of mice. With a biopsy punch (Lab Stiefel®), 3 mm skin biopsies were then collected at 3, 5, 7 and 15 days after the inoculation for *Borrelia* quantification by qPCR and inflammatory gene expression by RT-qPCR. Quantification targeting the *fla* gene was performed as described previously (26). Number of *Borrelia* in the skin was normalized to 10<sup>4</sup> *gapdh* DNA copies. Organs (heart, joint and ear tissue) were also collected at each time point post infection and transferred into BSK-H medium to check for the presence of live spirochetes.

### ELISA.

To measure IL-6 secreted by MC, enzyme-linked immunosorbent assays (ELISAs) were performed on cell supernatants. Protocols were based on sandwich techniques, as described by the manufacturer (R&D, France).

### Flow cytometry

MC were processed for staining with PE-labeled rat anti-mouse CD 117 (eBioscience) and APC-labeled american hamster anti-mouse FcεRI (eBioscience). For flow cytometry, a FACScalibur (BD Biosciences) equipped with Flowjo software was used.

### Statistical analyses.

Each experiment was carried out at least three times in independent trials. The most representative experiment is shown. Results represent the mean (+SEM) of at least triplicates of one experiment and were analyzed by two tailed student t test or ANOVA with Tukey post-test using Graphpad software. Differences in values were considered significant if  $p < 0.05$  (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ ).

## RESULTS

### ***B. burgdorferi* ss 297 and its lipoprotein L-OspC induce primary murine mast cell degranulation and cytokine expression**

Primary MC generated by culturing mice bone marrow cells for 6 weeks with IL-3/SCF were preferred over MC cultured for 4 weeks because the cell purity was greater (**Figure S1**). To study the impact of *Borrelia* on MC activation, primary MC were incubated with different concentration of *B. burgdorferi* ss 297 (297 strain) or its surface lipoprotein OspC (**Figure 1**). OspC but not *Borrelia* is able to induce MC degranulation. However, both induce the pro-inflammatory cytokine IL-6 expression by MC.

### **L-OspC is not essential for murine mast cell activation**

OspC is an essential lipoprotein for the transmission of *Borrelia* from the tick to the host. However, to determine whether L-OspC is essential for MC activation, we activated primary MC with an OspC-KO mutant strain of *B. burgdorferi* ss 297 (**Figure 2**). We show here that IL-6 protein expression was similarly induced by wild type, mutant and the complemented strains.

### **Mast cell inflammatory response depends on live *Borrelia***

We then explored more precisely the mechanism of MC activation. To this end, we compared the ability of dead and live *B. burgdorferi* ss 297 to activate primary MC (**Figure 3**). Heating of bacteria strongly decreases the degranulation process as well as the cytokine expression by MC. However, heated L-OspC activates MC similarly to not-heated L-OspC, confirming the fact that L-OspC is not involved in MC activation by the whole bacteria. Also, the MC activation by *B. burgdorferi* ss 297 is rather associated to heat-sensitive antigens.

### **Mast cell inflammatory response is inhibited in part by tick saliva**

Since the *B. burgdorferi* ss 297 transmission occurs in the presence of tick saliva, we explored its effect on the MC inflammatory response (**Figure 4**). Tick salivary gland extract (SGE) was incubated with primary MC in the presence or not of *B. burgdorferi* ss 297. Tick SGE greatly inhibits the IL-6 secreted by *Borrelia*-activated MC, but not totally.

## Mast cells do not alter *B. burgdorferi* ss 297 amplification and dissemination during C57/BL6 mouse infection

We have seen that *B. burgdorferi* ss 297 is able to activate primary MC *in vitro* in an L-OspC-independent way. However, the role of MC *in vivo* during *B. burgdorferi* ss 297 infection has never been investigated so far. To explore the impact of MC during mouse infection by *B. burgdorferi* ss 297, we analyzed the bacteria amplification in the skin as well as its dissemination in wild type versus *Wsh*<sup>-/-</sup> mice (MC-deficient mice) (**Figure 5**). No differences were observed in terms of bacteria amplification and dissemination.



## Discussion

The skin is one of the first defense organ pathogens have to cross to infect the host. Some bacteria, parasites and viruses have evolved to use vectors, such as ticks and mosquitoes, to bypass this physical barrier. However, once pathogens have been injected into the skin by vectors, pathogens have to face a biological barrier made of skin cells and inflammatory molecules. In the case of Lyme borreliosis, bacteria stay in the skin for days, thus first interacting with resident skin cells as well as with attracted immune cells before disseminating. Those cells, including keratinocytes, fibroblasts, macrophages, dendritic cells, neutrophils, T and B cells and NK cells secrete inflammatory molecules such as cytokines, chemokines, adhesins and reactive oxygen species to stop the infection (25,27–33). MCs, which are present in the skin in a large quantity, are also activated *in vitro* by *B. burgdorferi* ss 297. However, MCs studies with *B. burgdorferi* ss 297 have been done with rat peritoneal MCs and murine MC/9 MCs line, and the ability of mice primary MCs has never been investigated so far.

Cell lines are widely used in many types of cancer research as well as in immunological or metabolism studies. However, their behavior may differ from primary cells (34). We thus decided to explore the ability of mouse primary MC to respond to *B. burgdorferi* ss 297 and its lipoprotein OspC, since only mouse MC/9 MCs line has been deeply studied so far in this context (22,23). Primary MC are able to secrete IL-6 and to degranulate in response to *Borrelia*. IL-6 has been essentially associated with late symptoms such as arthritis and neuroborreliosis (35,36), but also with erythema migrans when associated with late symptoms (37). IL-6 is of importance since it has a crucial role during the transition from innate to acquired immunity. Its potential release in the skin by resident MCs might explain early cells attraction such as neutrophils (38). MCs also secrete IL-6 in response to L-OspC. OspC is an essential lipoprotein expressed by the bacteria during the tick blood meal when it reaches the host (39). If its role is not clear, its involvement in the early course of the infection has been demonstrated (40). Also, OspC exhibits a protective role against the innate immune system (41). We have shown that L-OspC is able to induce IL-6 release by MCs. A previous study has determined the inability of the lipoprotein OspA to activate TNF- $\alpha$  secretion by MC/9 MCs (22). The MC activation thus differ between borrelial lipoproteins. However, the difference might also be due to the cell state (line versus primary) or the molecule studied (TNF- $\alpha$  versus IL-6). Our results concerning MC degranulation are slightly different since *Borrelia* do not induce this mediator's release mechanism.

OspC is immunogenic to MCs, but it is not necessarily needed to fully activate those cells. Indeed, OspC-deficient *B. burgdorferi* ss 297 strain activates MCs IL-6 secretion as well as the wild type strain. It might

be due to the presence of other lipoproteins or even other ligands since *B. burgdorferi* ss 297 is able to activate immune cells through many PRRs (42). The ability of *B. burgdorferi* ss 297 to activate MCs might rather depend on heat-sensitive or living-associated ligands, which is not the case of OspC. A similar observation was made with monocytes where elicited inflammation with live spirochetes was greater than inflammation induced with sonicated bacteria (43). Determining the heat-sensitive/living associated ligands responsible for the inflammation might improve the development of vaccines.

During the transmission, the bacteria is injected into the skin with the tick saliva. We have shown here that tick salivary gland extracts are able to significantly but not completely inhibit the IL-6 secretion by MCs. The ability of tick saliva to inhibit inflammatory cytokine secretion was already observed on other cell types such as keratinocytes, dendritic cells and T cells (10). The impact of tick saliva on MCs during *B. burgdorferi* ss 297 transmission has not been well studied. However, the involvement of MCs during non-infected tick bite was analyzed in several studies. Histological analyses of the skin of BALB/c mice after infestation by *Dermacentor variabilis*, another hard tick, revealed an increased number of MCs especially during the secondary or the tertiary infestation (44,45). Similar observations were made with *Ixodes ricinus* infested rabbits (46). However, the role of MCs in acquired tick resistance depends on the tick species involved since MC deficient mice develop normal similar tick resistance as their wild type counterparts after *Dermacentor variabilis* infestation (45), but not after *Haemaphysalis longicornis* infestation (47). The impact of tick saliva on MCs seems to depend on tick species involved. The presence of MCs might have no impact on tick resistance since the saliva is able to inhibit their functions, as our results show at least concerning the IL-6 secretion. The role of saliva on MC degranulation during *B. burgdorferi* ss 297 infection remains to be explored. If the ability of tick saliva to inhibit degranulation is still unknown, it is known that tick saliva contains molecules able to counteract granule-contained mediators after their release. For instance, the saliva contains lipocalins which can bind histamine to inhibit its function, especially itching feelings (48).

The skin is used by the *B. burgdorferi* ss 297 to multiply before efficiently disseminating to other organs such as the heart, the brain or the joint (49). We thus explored the impact of MC deficiency on the multiplication and the dissemination of the bacteria using a MC deficient murine model. This model revealed an important role of MCs during *leishmania* infection, a vector-borne disease transmitted by *phlebotoma* (50,51). However, our results show that MCs have no impact on these phenomena. The impact on inflammatory gene expression remains to be explored. As with tick infestation (44,45), a role of MCs could occur during secondary or tertiary infection by *B. burgdorferi* ss 297. Also, the genetic background of MC deficient mice (C57Bl/6) is not optimal to study *B. burgdorferi* ss 297 infection since

it shows some resistance (52). MCs might have a higher impact on more susceptible mice such as C3H/HeN mice. But no MC deficient mice exist on this background. Moreover, MC might have a role on long term infection, by stimulating the adaptive system (53). Finally, inflammatory cytokines expression in MC deficient mice during early infection remains to be explored.

MCs have the ability to secrete inflammatory cytokines but not to degranulate in response to *B. burgdorferi* ss 297 in our experiments. The degranulation has been associated to the disease severity in another vector-borne disease, the malaria (54). However, the lipoprotein OspC, essential for the early infection, seems to be not involved in this mechanism. Moreover, the inflammation is inhibited in part by the tick saliva, which might increase the infection efficiency *in vivo*. MCs might not be an important cell type to fight the bacteria during early infection since we were unable to find any impact of MC deficiency during *in vivo* infection by *Borrelia*. However, these cells might be important for long term and/or repeated infections.

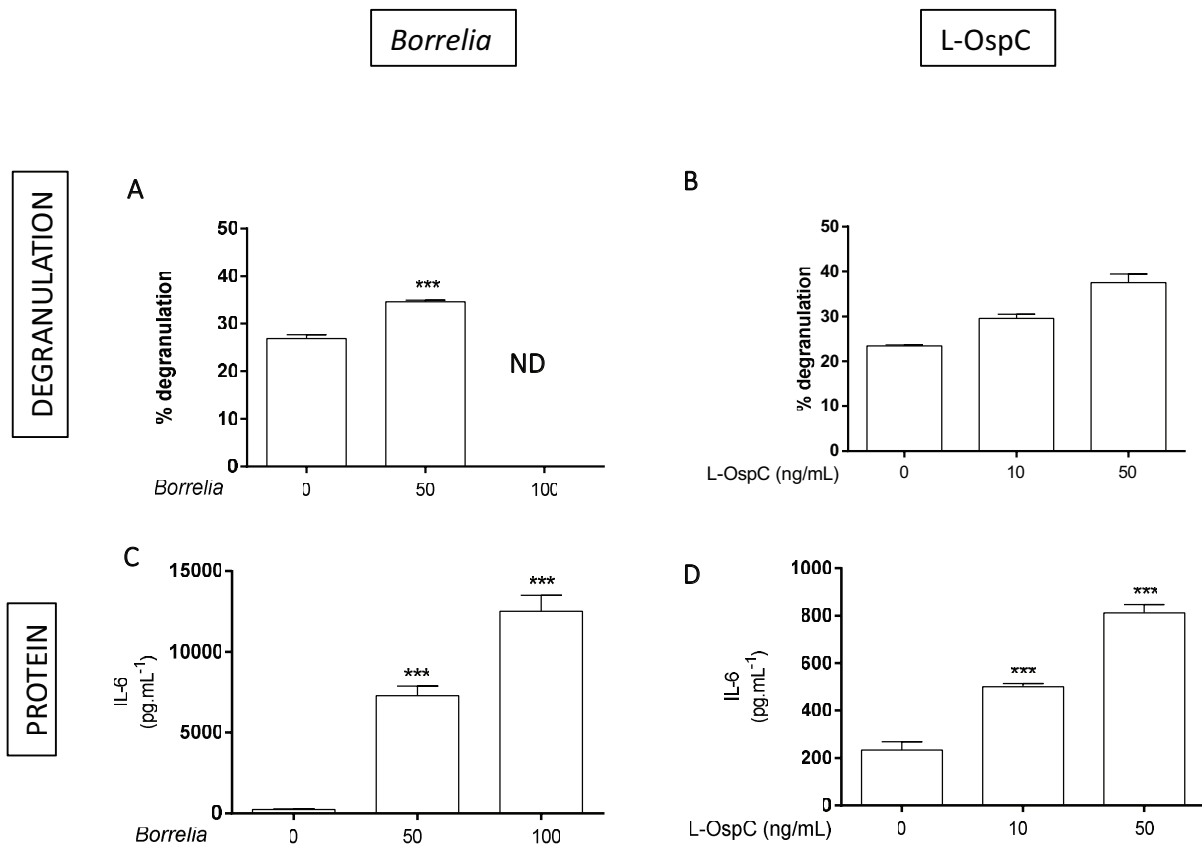
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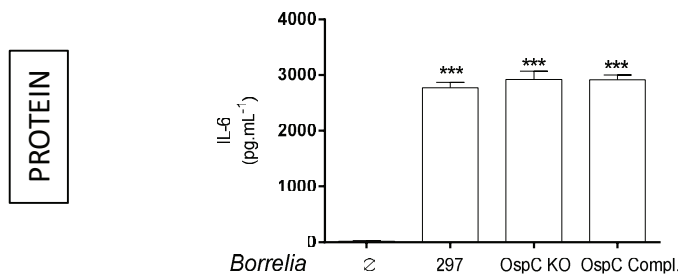
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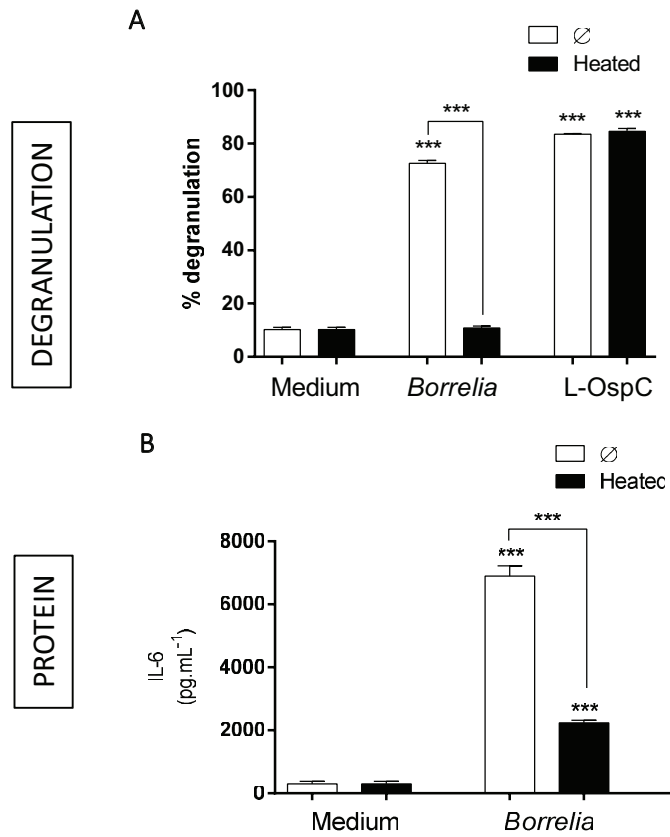


**Figure 1. *Borrelia* and its lipoprotein L-OspC activate murine mast cells inflammatory response.** Primary MCs from C57BL/6 mice were activated with different concentration of *Borrelia burgdorferi* 297 strain (M.O.I 10:1 or 50:1) or L-OspC (10 or 50 ng/mL). After 2 hours, the level of degranulation was evaluated by measuring  $\beta$ -hexosaminidase release and percentage degranulation was calculated (A, B). Or after 24 hours, supernatants were collected to measure IL-6 concentration by ELISA (C, D). Data represent means (SEM) of at least triplicates. Each experiment was done at least 3 times, and one representative experiment is shown.

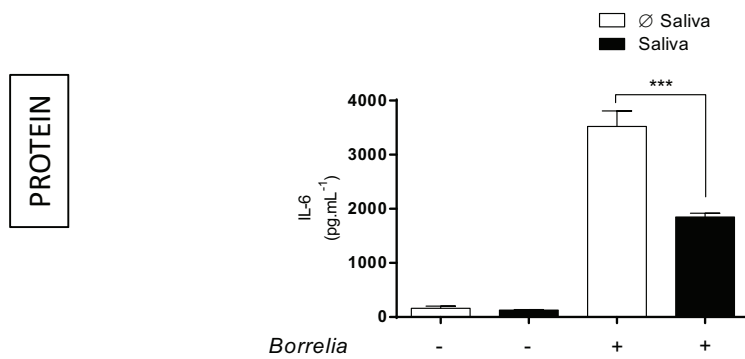




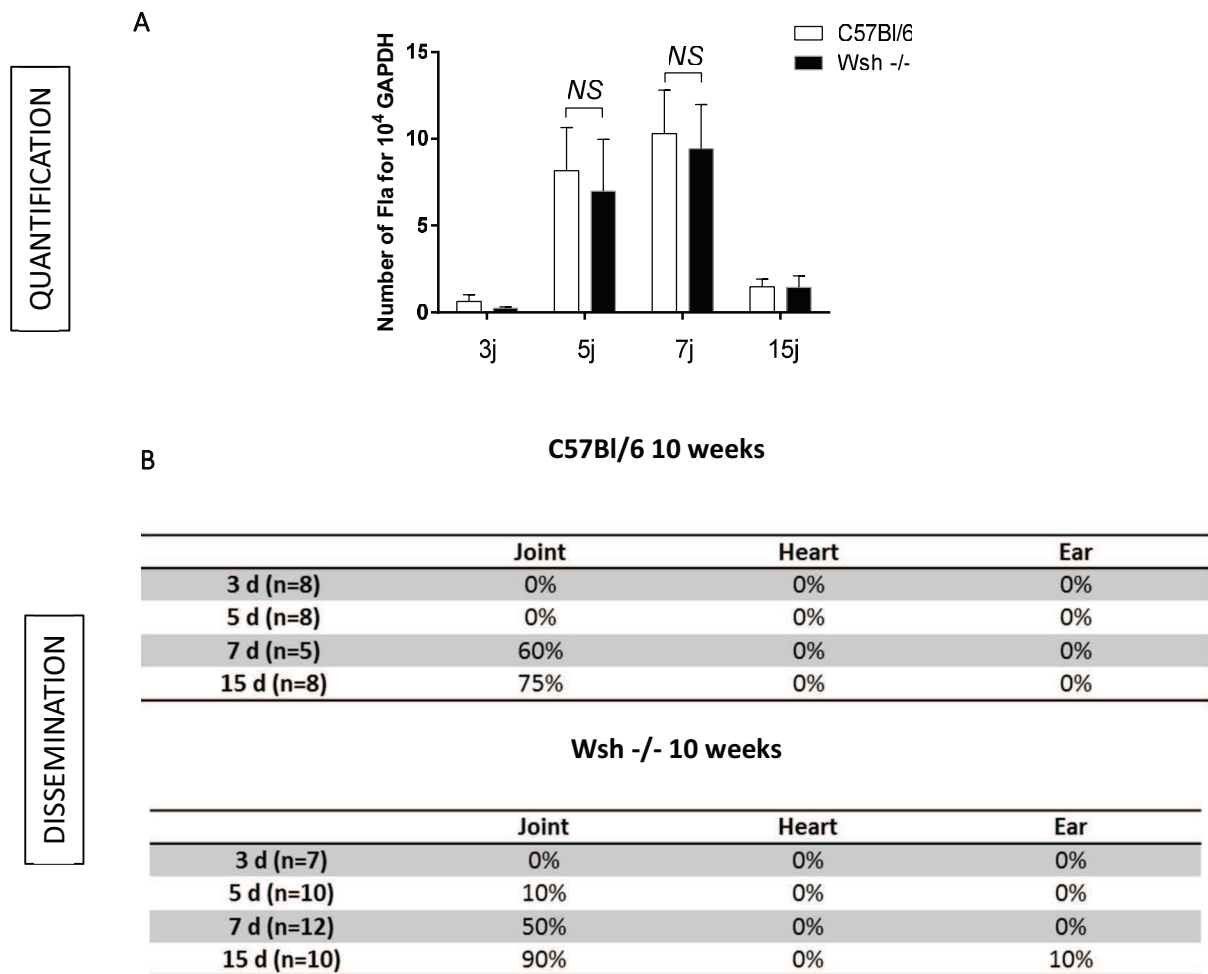
**Figure 2. L-OspC is not essential for murine mast cell activation.** Primary MCs from C57BL/6 mice were activated with *Borrelia burgdorferi* (Bb) 297, Bb OspC-KO or Bb OspC-complemented strains (M.O.I 50:1) for 24 hours. Supernatants were then collected to measure IL-6 concentration by ELISA. Data represent means (SEM) of at least triplicates. Each experiment was done at least 3 times, and one representative experiment is shown.



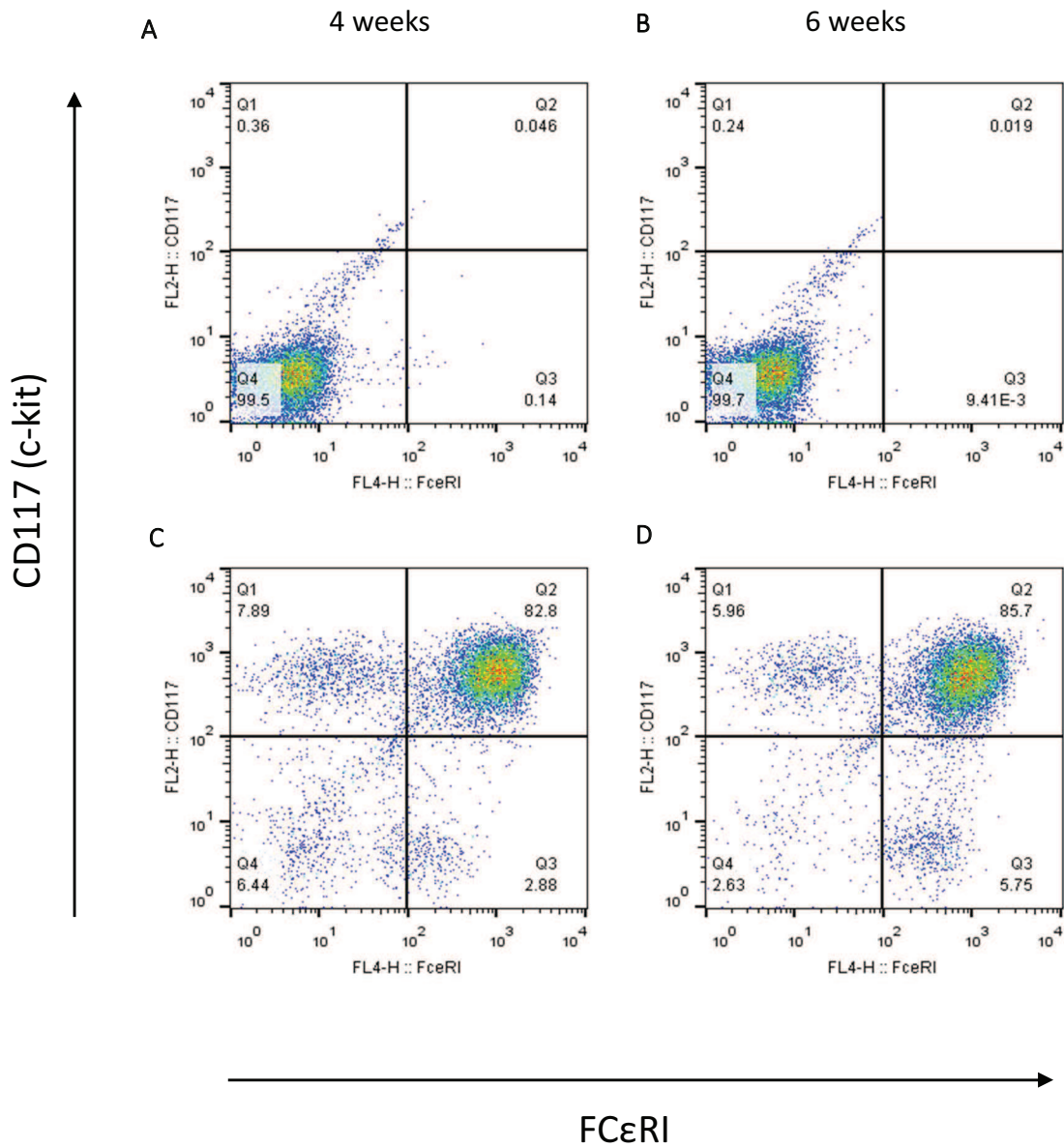
**Figure 3.** The murine mast cell inflammatory response essentially depends on heat-sensitive/living-associated antigens. Primary MCs from C57BL/6 mice were activated with *Borrelia burgdorferi* 297 strain (M.O.I 50:1) or L-OspC (50 ng/mL) previously heated or not. After 2 hours, the level of degranulation was evaluated by measuring  $\beta$ -hexosaminidase release and percentage degranulation was calculated (A). Or after 24 hours, supernatants were collected to measure IL-6 concentration by ELISA (B).



**Figure 4. The tick saliva inhibits the mast cell inflammatory response induced by *Borrelia*.** Primary MCs from C57BL/6 mice were activated with *Borrelia burgdorferi* 297 strain (M.O.I 50:1) in the presence or not of tick salivary gland extract (20 µg/mL). After 24 hours, supernatants were collected to measure IL-6 concentration by ELISA. Data represent means (SEM) of at least triplicates. Each experiment was done at least 3 times, and one representative experiment is shown.



**Figure 5. Role of mast cells upon mouse infection by *Borrelia* (C57Bl/6 vs Wsh<sup>-/-</sup>).** Wild type or MC deficient (Wsh<sup>-/-</sup>) C57Bl/6 mice were infected with *Borrelia burgdorferi* (Bb) N40 strain (10<sup>3</sup> bacteria/ 100  $\mu$ L) by syringe inoculation. Quantification of Bb at the site of inoculation (A) was measured by qPCR at different time points after the infection. The dissemination (B) of Bb was determined by *in vitro* culture (joint and heart) or by PCR (ear).



**Figure S1. Mast cells purity analyzed by flow cytometry.** Primary MCs from C57BL/6 mice were cultured for 4 or 6 weeks in the presence of IL-3 and mSCF. MC were then stained with control antibodies (A, B) or anti-CD117-PE and anti-FCεRI antibodies (C, D) before being analyzed by flow cytometry.

## Discussion et Perspectives

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## I. Le rôle de la piqûre de tique du genre *Ixodes* dans la transmission

La piqûre de la tique effectuée à l'aide de ses chélicères et de son hypostome induit une dilacération des tissus cutanés, provoquant une destruction mécanique des cellules de l'épiderme et du derme. De plus, nous avons observé au sein du laboratoire la capacité de la salive de tique à induire une lyse chimique des FBs, cellules majoritaires au niveau du derme. La lyse physico-chimique de la peau induite par la tique permet la formation d'une poche permettant à la tique de se nourrir plus facilement. Cette poche fournit aussi un lieu propice à la bactérie où elle peut se développer afin de préparer sa dissémination dans les organes distants. La blessure induite par la piqûre de tique fut un aspect central de ce travail de thèse. Nous avons ainsi décidé de décrypter le mécanisme associé à la lyse cellulaire induite par la salive de tique. Avec l'aide d'une équipe de chimistes, nous avons déterminé la présence d'Histone H4 dans les extraits de glandes salivaires de tiques capable de lyser spécifiquement les FBs. Une activité lytique envers les érythrocytes humains associés à une histone H4 modifiée de grenouille avait été démontrée (Kawasaki et al. 2008). La séquence protéique de l'histone H4, très conservée au sein de l'évolution (Mariño-Ramírez et al. 2005), est identique chez l'Homme et la tique. Cependant dans notre étude, la salive possède une activité lytique plus intense que l'histone H4 humaine commerciale. Ceci suggère que l'histone H4 présente dans la salive de tique doit posséder des modifications post-traductionnelles augmentant l'efficacité de l'activité lytique. Nous avons aussi pu associer une activité antimicrobienne à cette histone envers des bactéries commensales de la peau mais pas envers *B. burgdorferi*. L'activité antimicrobienne des histones, notamment de l'histone H4, avait déjà été démontrée. Les histones sont capables de perméabiliser les membranes bactériennes, de pénétrer dans les cellules procaryotes afin de lier l'ADN ou l'ARN bactérien ou encore de former des complexes immunitaires dans le contexte des pièges extracellulaires mis en place par les neutrophiles (NET) (Kawasaki and Iwamuro 2008). Cependant, la résistance de *B. burgdorferi* vis-à-vis de cette activité est une donnée nouvelle indiquant une adaptation de la bactérie à son vecteur. La bactérie pourrait avoir co-évolué avec la tique afin de détourner cette activité à son avantage. De plus, cette résistance peut lui conférer une certaine protection vis-à-vis des mécanismes immunitaires de l'hôte vertébré impliquant les histones. La lyse cellulaire associée à l'effet bactéricide envers les bactéries commensales permet de fournir une niche privilégiée pour le développement de *B. burgdorferi*. Le mécanisme de lyse associé à l'histone H4 n'est cependant pas encore identifié, mais il semble spécifique puisque les KCs notamment ne sont pas affectés par l'effet lytique de la salive de tique. Des modifications post-traductionnelles, nombreuses sur les histones (Jensen 2004), doivent exister afin de permettre la

spécificité de ce mécanisme de lyse dans le temps et l'espace. L'histone H4, comme l'histone H2B, pourrait directement provoquer la nécrose des cellules en formant des pores dans la membrane (Kawasaki and Iwamuro 2008). L'histone H4 pourrait aussi induire l'apoptose en séquestrant les facteurs de croissance nécessaires au développement de ces cellules (Hajnická et al. 2010). Des composés de la salive de tique interagissent avec le PDGF (Platelet derived growth factor) afin d'inhiber la croissance des FBs notamment en disloquant leur squelette d'actine. Il serait intéressant d'explorer le rôle de l'histone H4 dans ce mécanisme. Globalement, l'histone H4 semble être une protéine essentielle au bon déroulement du repas sanguin de la tique. Des expériences d'ARN interférentiels contre l'histone H4, si elles ne sont pas létales pour la tique, pourraient permettre de mettre en évidence l'importance de cette protéine dans le déroulement du repas sanguin.

L'élaboration d'un vaccin anti-histone pourrait donc être une piste intéressante à explorer dans le cadre du développement d'une approche préventive contre la borréliose de Lyme. Cependant, du fait de la séquence hautement conservée de l'histone H4 entre les différentes espèces eucaryotes (Mariño-Ramírez et al. 2005) et de son expression ubiquitaire, la vaccination pourrait poser des problèmes d'auto-immunité. Des anticorps anti-histone ont notamment été décrits dans le cadre de maladies auto-immunes tel que le lupus systémique érythémateux (Shoenfeld and Segol; Pisetsky 2014). Des expériences d'immunisation de souris avec de l'histone H4 commercial vont être prochainement effectuées afin d'analyser son potentiel en tant que candidat vaccin anti-tique. En effet, la tique est une cible de plus en plus étudiée dans le cadre du développement de vaccins dont le but est la mise en place d'une protection la plus universelle possible contre l'ensemble des agents pathogènes transmis par les tiques. Le projet européen qui vise l'élaboration d'un vaccin anti-tique afin de lutter contre la borréliose de Lyme (Sprong et al. 2014), la babéiose et le virus de l'encéphalite à tique en est la preuve. De telles expériences ont permis par le passé de mettre en évidence l'importance de protéines telles que la **subolésine** dans le déroulement du repas sanguin de la tique (de la Fuente and Kocan 2006). De plus, un vaccin contre une protéine de tique située au niveau de son intestin, **Bm86**, a montré une certaine efficacité dans le contrôle des populations de certaines espèces de tiques *Rhipicephalus* en limitant l'infestation des bovins par ces dernières. Le cément et de la salive de tiques sont aussi des sources intéressantes de protéines cibles pour le développement de vaccins. La vaccination contre la protéine du cément **64P** de *R. appendiculatus* confère une protection efficace chez le cochon d'Inde contre cette tique. De plus, ce vaccin réduit la capacité de la tique du genre *Ixodes* à transmettre le virus de l'encéphalite à tique et la bactérie *Anaplasma phagocytophilum* (Sonenshine and Roe 2014b). Une



utilisation pharmacologique de l'histone H4 issue de la salive de tique peut aussi être envisagée dans le cadre de pathologies impliquant une dérégulation des FBs tel que le développement de chéloïdes.

La lyse cellulaire provoque aussi la libération de molécules potentiellement immunogéniques. La présence d'une activité anti-inflammatoire forte associée à la salive de tique permet de moduler l'inflammation induite lors de la transmission (Kazimírová and Štibrániová 2013; Liu and Bonnet 2014). Cette activité anti-inflammatoire est liée à plusieurs protéines dont la plus étudiée est **Salp15**. Une protéine de 15 KDa caractérisée à partir de la salive d'*Ixodes scapularis* qui peut affecter le fonctionnement de différents types cellulaires (DCs, cellules T, KCs) (Ramamoorthi et al. 2005). Cette protéine est surexprimée lorsque la tique est infectée par *B. burgdorferi*, soulignant l'influence importante de la bactérie sur son vecteur. Une influence qui peut être bénéfique aussi bien pour la bactérie que pour le vecteur. D'autres protéines de la salive de tique sont capables d'inhiber l'activité des neutrophiles, des DCs et des cellules T et B. L'un des rôles majeurs de la salive de tique est ainsi la protection de l'arthropode vis-à-vis du système immunitaire de l'hôte vertébré. *Borrelia* sp. a évolué avec la tique du genre *Ixodes* afin d'utiliser ce rôle immunosuppresseur à son avantage. Cependant, la réponse immunitaire qui se développe à l'encontre de la salive à la suite de piqûres répétées peut avoir un effet néfaste sur l'infection. En effet, les patients peuvent développer une hypersensibilité à la salive de tique qui réduit par le biais de l'immunité adaptative le risque de transmission des agents pathogènes, notamment *Borrelia*, transmis par la tique (Burke et al. 2005). Si la salive joue un rôle essentiel dans la transmission précoce de la bactérie, sa persistance dans la peau doit être étudiée afin de déterminer la durée de son action pharmacologique et immuno-suppressive. La rémanence de la salive dans la peau après le détachement de la tique est une donnée inconnue à ce jour qui fait l'objet d'études au sein de notre laboratoire.

## II. La réponse inflammatoire des cellules résidentes de la peau

La peau est le premier organe que les agents pathogènes transmis par un vecteur doivent traverser. L'injection de *B. burgdorferi* dans la peau par la tique met en contact direct les bactéries avec les cellules résidentes telles que les KCs, les FBs et les MCs (**Figure 26**). Ces cellules, en plus de former une barrière physique sont aussi capables de participer à la réponse inflammatoire contre *B. burgdorferi*.

**Les fibroblastes** produisent de l'IL-6, CXCL1 et de l'IL-8 en réponse à *B. burgdorferi*. Ces molécules permettent l'attraction de cellules immunitaires au point de piqûre. Le TNF- $\alpha$  ainsi que les métalloprotéinases telles que MMP-1, -3 et -12 sont aussi induites probablement à l'aide d'un mécanisme dépendant du facteur de transcription NF- $\kappa$ B. Cependant, la protéine OspC ne semble pas impliquée dans ce mécanisme inflammatoire (Ebnet et al. 1997; Zhao et al. 2007b; Schramm et al. 2012). Les MMPs pourraient aider à la dissémination tissulaire de la bactérie. Lorsque la salive n'est pas présente, l'effet lytique de cette dernière n'ayant alors pas lieu, *B. burgdorferi* serait capable d'envahir les FBs *in vitro* à l'aide d'un mécanisme dépendant de l'intégrine  $\beta$ 1 et de la kinase SRC (Wu et al. 2011). Des expériences de microscopie électronique ont permis de mettre en évidence cette capacité de *Borrelia*, pourtant considérée comme une bactérie extra-cellulaire (Chmielewski and Tylewska-Wierzbanska 2010). Ce mécanisme pourrait permettre à la bactérie d'échapper plus efficacement à la réponse immunitaire de l'hôte vertébré. Cependant, cette activité nécessite d'être confirmée *in vivo*. De plus, les FBs étant lysés par la salive de tique, l'importance de connaître le temps de rémanence de cette dernière dans la peau est nécessaire pour juger de la participation des FBs dans la réponse immunitaire contre *Borrelia* et dans la persistance de la bactérie dans les tissus.

**Les kératinocytes** ne sont pas sensibles à l'effet lytique de la salive de tique. Mais tout comme les FBs, ils peuvent initier une réponse inflammatoire en présence de *B. burgdorferi*. Ces cellules peuvent sécréter des chimiokines (IL-8, MCP-1), des peptides antimicrobiens (hBD-2, hBD-3) et aussi la métalloprotéinase MMP-9 en réponse à la bactérie (Gebbia et al. 2001; Marchal et al. 2011). Cette réponse inflammatoire est en partie médiée par la protéine de surface OspC de *B. burgdorferi*. Cependant, la présence de la salive de tique inhibe fortement cette réponse inflammatoire. Plus encore, la salive de tique modifie le profil de sécrétion des cellules épidermiques afin de favoriser le développement d'une réponse immunitaire de type Th2, favorable à la persistance de la bactérie (Skallová et al. 2008). Lors de la transmission de *B. burgdorferi*, les dégâts tissulaires induits par la piqûre de tique et par la lyse des FBs sont susceptibles de provoquer la libération de molécules pouvant activer une réponse inflammatoire. Les cellules nécrotiques libèrent de l'acide urique, des « heat shock proteins » (HSP), des « High-mobility group box 1 proteins » (HMGB1) ainsi que des acides nucléiques (Miyake and Yamasaki 2012). Parmi les acides nucléiques, les ARNs sous forme double brins peuvent activer une réponse inflammation par l'intermédiaire de TLR3 lors d'une lésion cutanée (Karikó et al. 2004a; Cavassani et al. 2008; Amarante et al. 2011). Nous avons montré dans ce travail de thèse que l'inflammation lié aux dégâts tissulaires augmentait la réponse inflammatoire des KCs envers *B. burgdorferi*. Le mécanisme dépend des voies de l'inflammation impliquant TRIF et MyD88 et des

récepteurs TLR2 et TLR3. Une coopération entre TLR2 et TLR3 avait déjà été observé dans le contexte d'activation des cellules NK par les DCs (Vanhoutte et al. 2008). Cependant, l'extrait de glandes salivaires de tique inhibe très largement cette inflammation et n'est que partiellement observable chez la souris. Ces résultats nous permettent d'établir l'hypothèse suivante : lorsque la tique est présente, la salive injectée au point de piqûre inhibe l'inflammation induite par la blessure et la bactérie ; puis lorsque celle-ci se détache et que la bactérie commence à disséminer, la coopération entre TLR3 et TLR2 pourrait en partie expliquer l'apparition de manifestations inflammatoires telles que l'EM. L'absence d'EM pourrait alors correspondre à une sensibilité moindre envers la bactérie due à un polymorphisme de TLR2 favorisant une inflammation plus faible, ou à une implication altérée des différents types cellulaires. *In vivo*, la coopération entre TLR2 et TLR3 augmente le nombre de cellules attirées au point de piqûre. Un fois au point de piqûre, ces cellules sont inhibées par la salive de tique. Ce mécanisme pourrait ainsi provoquer une certaine tolérance vis-à-vis des antigènes présents au point de piqûre, notamment ceux de *B. burgdorferi*. L'absence du synergisme entre TLR2 et TLR3 chez la souris au niveau de l'expression des gènes inflammatoires pourrait en partie expliquer l'absence d'EM chez ce modèle. Ces travaux soulignent les limites du modèle murin chez qui les symptômes sont en partie différents de chez l'homme. Ce type de travaux permet de mettre en évidence le rôle de la peau au-delà de la niche cellulaire qu'elle représente ainsi que l'implication du vecteur au-delà de son rôle de seringue. Les dégâts tissulaires font partie de l'environnement dans lequel est transmis *B. burgdorferi*. Il serait intéressant de transposer cette étude dans le contexte de la transmission de *Plasmodium* qui est capable de provoquer la mort des cellules de la peau en traversant leurs membranes plasmiques (Formaglio et al. 2014). L'environnement de la transmission de *Borrelia* se caractérise aussi par la présence de bactéries commensales. Ces dernières peuvent pénétrer au sein de la peau une fois que la tique a effectué une brèche au sein de l'épiderme. Les bactéries commensales, notamment *S. epidermidis*, sont capable de réguler l'inflammation lors d'une lésion cutanée par le biais d'un mécanisme impliquant TLR2 et TLR3 (Lai et al. 2009). Le microbiome de la tique et son implication dans la transmission de *Borrelia* commencent à être étudiés (Narasimhan and Fikrig 2015). Cependant, aucune étude n'existe concernant le rôle de la flore commensale cutanée humaine lors de la transmission de *Borrelia*. Une étude sur l'impact de cette flore commensale sur l'inflammation des KCs envers *B. burgdorferi* a été initiée récemment au sein de notre laboratoire.

**Les mastocytes**, dont le rôle a été exploré dans ce travail, n'ont pas été beaucoup étudiés dans le cadre de la borréliose de Lyme. Jusqu'à présent, il a été démontré que les MCs murins appartenant à la lignée MC/9 sont capables de dégranuler et de sécréter des molécules inflammatoires (IL-6 et TNF-

$\alpha$ ) en réponse à *B. burgdorferi*. Ce mécanisme semble en partie dépendre d'un mécanisme lié aux récepteurs Fc $\gamma$  (Talkington and Nickell 1999, 2001). De plus, lorsque les tiques ne sont pas infectées par la bactérie, il a été montré que les MCs jouaient un rôle dans l'immunité envers les arthropodes uniquement lors d'une infestation secondaire voire tertiaire (Brossard and Fivaz 1982; Matsuda et al. 1985; Steeves and Allen 1991). Une corrélation entre la dégranulation des MCs dans la peau et la parasitémie et la sévérité du paludisme a récemment été mise en évidence (Wilainam et al. 2015). Le rôle des MCs dans l'activation des cellules T (Th1 et Th17) au cours de l'infection par les parasites du genre *Leishmania*, un parasite transmis par le phlébotome, a aussi été démontré (Dudeck et al. 2011; Rodríguez and Wilson 2014). Nous avons observé la capacité des MCs primaires à répondre *in vitro* à la présence de *B. burgdorferi* en sécrétant des molécules pro-inflammatoires telles que l'IL-6. Ce mécanisme d'activation dépend de la présence d'antigènes sensibles à la chaleur dont OspC ne fait pas partie. Les antigènes tels que les acides nucléiques de la bactérie pourraient être impliqués puisqu'ils sont capables d'activer l'inflammation notamment par l'intermédiaire de TLR7, 8 et 9 (Petzke et al. 2009; Cervantes et al. 2013). La salive est capable d'inhiber partiellement la sécrétion d'IL-6. Le mécanisme et les molécules de la salive impliqués restent inconnus à l'heure actuelle. De plus, la capacité de la salive à activer ou inhiber la dégranulation des MCs en présence de *Borrelia* doit être analysée. Le modèle *in vivo* utilisé dans notre étude ne permet pas de mettre en évidence l'implication des MCs lors de l'infection de la souris par *Borrelia*. En effet, le fond génétique des souris déficientes en MCs est un fond C57Bl/6. Ces souris possèdent une résistance naturelle à l'infection par *B. burgdorferi* contrairement aux souris C3H/HeN (Ma et al. 1998). L'absence de résultats significatifs entre les souris sauvages et les souris déficientes en MCs pourraient provenir du fond génétique non optimal pour l'infection par *Borrelia*. Tout comme l'implication des MCs dans la résistance contre les tiques lors des infestations secondaires, ces cellules pourraient davantage jouer un rôle protecteur lors d'infections secondaires par *Borrelia*. Enfin, les MCs pourraient ne pas jouer un rôle fondamental dans le contrôle de l'infection par *Borrelia* chez la souris.

**La peau** semble être un site de persistance privilégié pour la plupart des agents pathogènes transmis par un vecteur. *Borrelia* est capable de persister plusieurs semaines dans la peau afin de contaminer une nouvelle tique qui viendrait piquer l'hôte vertébré infecté. Les follicules pileux pourraient être la structure immuno-privilégiée permettant à la bactérie de persister. En effet, ces structures ont déjà été incriminées dans la persistance de *Plasmodium* au niveau cutané après sa transmission par le moustique *Anopheles* (Gueirard et al. 2010). De plus, les cellules T régulatrices (Treg), essentielles pour développer une réponse immunitaire efficace et présentes dans la peau, pourraient

être manipulées par *Borrelia* ou la salive de tique afin d'induire une tolérance vis-à-vis de la bactérie. Ce genre de mécanisme a été observé pour *Plasmodium*, où le phénotype des Treg est altéré par le parasite (da Silva et al. 2012). Dans le cadre de la leishmaniose, les Treg ont un rôle protecteur et la diminution du nombre de ces dernières exacerbe les symptômes inflammatoires précoces (Barth et al. 2012). Les cellules de la peau sont en première ligne. Certains agents pathogènes, comme *Borrelia* avec l'aide de la salive de tique, préfèrent inhiber leur activation. D'autres agents pathogènes comme le virus de la dengue ont plutôt évolué afin de pouvoir infecter ces cellules et ainsi accélérer son processus d'infection (Bustos-Arriaga et al. 2011; Surasombatpattana et al. 2011). La peau est l'endroit où est initiée la réponse immunitaire dans le contexte de ces maladies vectorielles. Il est essentiel de décrypter les mécanismes immunitaires mis en place dans cet organe afin d'optimiser le développement de vaccins.

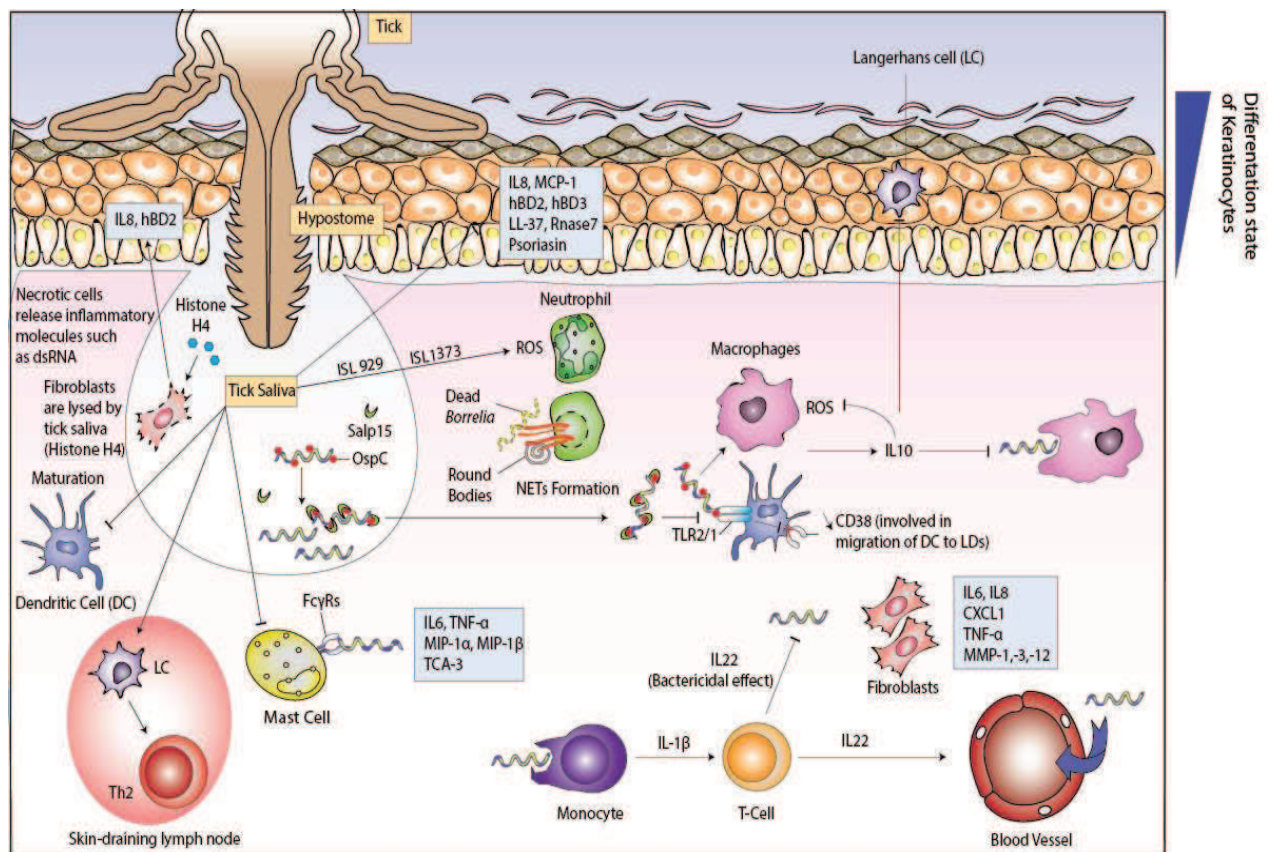


Figure 22. Schéma récapitulatif des événements inflammatoires cutanés lors de l'infection précoce par *B. burgdorferi*

### III. La virulence de *B. burgdorferi* dans la peau

Les mécanismes responsables de l'organotropisme chez l'Homme des différentes espèces de *Borrelia* ne sont pas élucidés à ce jour. La peau en tant que site initial de l'infection permet de filtrer la population hétérogène de *Borrelia* injectée lors de la piqûre de tiques infectées. Seules les bactéries qui possèdent des allèles spécifiques d'OspC peuvent disséminer dans l'hôte vertébré (Brisson et al. 2011). Dans le but d'identifier les paramètres responsables de cet organotropisme, une étude a été menée au laboratoire. Deux points majeurs ont été explorés : la réponse immunitaire cutanée induite par différents pathotypes, et l'expression de gènes de virulence par la souche 297 de *B. burgdorferi* ss et un clone 297/4 de la même souche disséminant au niveau du cerveau. Le profil inflammatoire cutané diffère selon l'espèce de *Borrelia* considérée, mais n'est pas suffisant pour associer ces espèces à des manifestations cliniques spécifiques. Chez l'Homme, l'hétérogénéité des *Borrelia* lors d'une inoculation unique associée à l'hétérogénéité génétique des hôtes vertébrés peut expliquer la difficulté à corréler positivement un profil inflammatoire donné à un pathotype spécifique (Troy et al. 2013; Seifert et al. 2015). La capacité de dissémination du clone 297/4 s'explique à la fois par l'induction de molécules vasodilatatrices chez l'hôte vertébré telles que MPC-1 et par l'expression de protéines de surface telles que BB0347 spécialisées dans l'interaction avec la matrice extracellulaire (Gaultney et al. 2013). De nombreux gènes de virulence chez *Borrelia* ont une expression régulée par des facteurs de transcription (facteurs sigma) alternatifs. Ces facteurs sigma alternatifs (RpoN-RpoS) gouvernent notamment l'expression de la protéine OspC et Bbk32, très importantes lors de l'infection de l'hôte vertébré (Ouyang et al. 2008). Globalement, la protéine Rrp2 est phosphorylée par une histidine kinase, interagit avec RpoN afin de transcrire RpoS qui à son tour permet la transcription des gènes de virulence. Déterminer l'implication de la voie de transcription RpoN-RpoS dans l'expression de **bb0347 et bb0213**, les deux gènes exprimés par le clone 297/4, nécessite d'être analysée. L'expression de différentes protéines capables d'interagir avec la matrice extra-cellulaire de l'hôte vertébré pourrait expliquer en partie l'organotropisme de la bactérie. Par exemple, l'expression d'une protéine capable d'interagir spécifiquement avec la matrice extra-cellulaire de l'articulation pourrait permettre à la bactérie de s'implanter plus efficacement. L'expression de gènes de virulence spécifiques par la bactérie pourrait permettre à certaines populations seulement de *Borrelia* de résister à la pression immunitaire de l'hôte vertébré appliquée au niveau de la peau (Rego et al. 2014). Il est à noter que parmi l'ensemble des protéines exprimées *in vitro* et mise en évidence par protéomique, certaines avaient une expression très faible *in vivo*. Cela souligne également la possibilité d'expression artéfactuelle de gènes associée à

la culture de la bactérie *in vitro* dans un milieu riche par rapport à ce qui se passe dans un milieu plus restrictif tel que l'hôte vertébré. Cela pose également le problème des modèles d'étude pour certains agents pathogènes. Par exemple, l'Homme développe un EM mais pas la souris soulignant la spécificité de l'interaction Homme-*Borrelia*. Dans la borréliose de Lyme, qui est une zoonose, l'Homme est un hôte vertébré accidentel, ce qui pourrait expliquer la particularité des manifestations cliniques et de l'organotropisme de la bactérie chez celui-ci.

# Conclusion

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Les maladies vectorielles, transmises par des arthropodes tels que les insectes et les tiques, sont un réel problème de santé publique. Le paludisme, provoqué par *Plasmodium*, un parasite transmis par un moustique du genre *Anopheles* provoque plus de 600 000 décès par an. Le virus de la dengue, transmis par un moustique du genre *Aedes*, infecterait environ 390 millions de personnes par an. La borréliose de Lyme quant à elle est la plus fréquente des maladies vectorielles transmises par les tiques, avec environ 85 000 personnes touchées chaque année (Hubálek 2009). Le vecteur et l'agent pathogène interagissent finement ensemble afin d'améliorer l'efficacité de transmission au niveau de la peau (**Skin and arthropods : an effective interaction used by pathogens in vector-borne diseases, Figure 1**). Le caractère essentiel de la peau démontré au cours de cette thèse vient renforcer les observations similaires faites lors de l'étude sur le paludisme et sur la leishmaniose (Ehrchen et al. 2010; Gueirard et al. 2010; Kautz-Neu et al. 2011; da Silva et al. 2012; Guilbride et al. 2012; Sinnis and Zavala 2012).

Les agents pathogènes utilisent le vecteur de différentes manières, et non pas seulement comme une simple seringue. En effet, l'évolution de l'infection par *B. burgdorferi* chez la souris varie selon que la bactérie a été injectée par une seringue en intraveineux ou en intradermique ou par l'intermédiaire de la tique. Si la piqûre de la tique permet à la bactérie de traverser la barrière physique que constitue la peau, elle permet aussi de protéger la bactérie via l'injection concomitante de sa salive aux activités notamment anti-inflammatoires. En plus de la salive, les dégâts mécaniques induits sur les tissus par les pièces piqueuses des arthropodes, notamment par la tique, semblent jouer un rôle notable dans l'inflammation précoce, comme montré dans ce travail. Cependant dans le cas des moustiques ou du phlébotome, de par la nature fugace de la piqure, dit de type solénophage, les dégâts tissulaires alors induits ont sûrement un rôle moindre à jouer dans l'inflammation.

La peau ne constitue pas seulement un élément protecteur vis-à-vis des agents pathogènes transmis par un vecteur, mais aussi un environnement propice à leur développement. C'est un organe dans lequel ils peuvent persister, certainement à l'aide des structures immunoprivilégiées comme les follicules pileux. C'est aussi un organe clé de l'immunité dans lequel les agents pathogènes pourraient induire une immunotolérance. La peau constitue aussi une niche écologique dans laquelle les agents pathogènes peuvent se multiplier. Cependant, ces niches écologiques sont aussi convoitées par une flore commensale dont le rôle dans la transmission des agents pathogènes par des vecteurs doit encore être défini. Enfin, la peau permet une sélection des populations des agents pathogènes les plus virulents lors de l'infection, améliorant l'efficacité de persistance de ces derniers dans l'ensemble de l'organisme. Tous ces paramètres méritent d'être analysés dans le contexte des maladies vectorielles afin d'appréhender au mieux les mécanismes précoces d'infection et orienter le développement de vaccins.

Quentin BERNARD<sup>1</sup>  
Benoît JAULHAC<sup>1,2</sup>  
Nathalie BOULANGER<sup>1,2</sup>

<sup>1</sup> EA7290 : Virulence bactérienne précoce :  
groupe borréliose de Lyme,  
université de Strasbourg, France  
<sup>2</sup> Centre national de référence *Borrelia*,  
centre hospitalier Universitaire,  
Strasbourg, France

Reprints: Q. Bernard  
<nboulanger@unistra.fr>

## Skin and arthropods: an effective interaction used by pathogens in vector-borne diseases

In the last years, the skin has been described as a major interface in arthropod borne diseases. Although it constitutes an efficient immune and physical barrier, pathogens have developed effective strategies to thwart the host. In this process, the arthropod plays a major role. For mosquitoes, the quick blood meal is made through an efficient inoculation process directly into the blood vessel. For the long lasting blood meal of hard ticks, the sophisticated biting pieces and the tick saliva provide potent tools to help pathogen transmission. Lyme borreliosis and leishmaniasis have been particularly well investigated in this context.

**Key words:** keratinocytes, fibroblasts, dendritic cell, arthropod saliva, vector-borne diseases, leishmaniasis, Lyme borreliosis

**V**ector-borne diseases (VBDs) rely on complex interactions between three actors: the vertebrate host, the arthropod and the pathogen (*figure 1*). First, arthropods are attracted to the vertebrate host through their olfaction. Volatile products are detected such as lactic acid, sweat products modified by skin resident bacteria [1]. Then, facing the skin barrier, the arthropod punctures the skin by its biting pieces, either through a solenophage or telmophage bite. Pathogens are inoculated into the skin, where they are going to interact with different skin cells [2]. In this process, arthropod saliva plays a major role. First it targets the coagulation cascade very efficiently, then the different immune cells or immune pathways (complement pathway) allowing the pathogen to escape, hide or multiply in the skin. Besides, it is now well documented that arthropods react to pathogens they transmit by mounting an innate immunity [3-5], but within the vector, microorganisms seem to be not affected by this immune response. In this review, we detailed some of these processes that make VBDs an example of fascinating interactions for pathogen survival. Lyme borreliosis and leishmaniasis are VBDs that illustrate the best this triad of interactions.

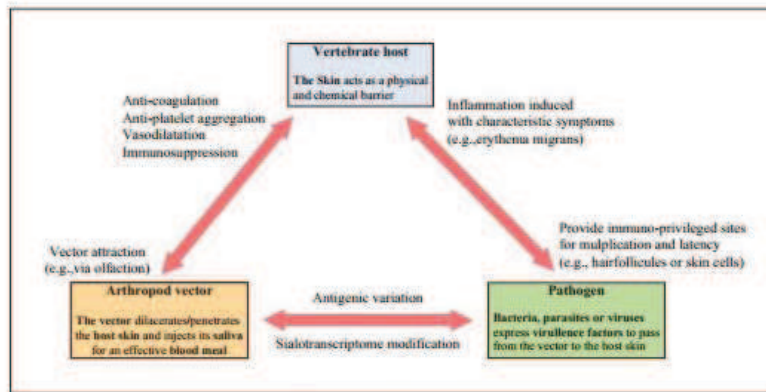
### The host skin

The skin constitutes a physical and immune barrier. Upon an injury, pharmacological processes (coagulation, platelet aggregation, vasoconstriction), and immune processes are activated. All this occurs in the epidermis, the outermost layer mainly composed of keratinocytes, and in the dermis where resident fibroblasts and more specific immune cells, especially dendritic cells are present. All these cells sense self and non-self danger signals through membrane receptors, the Toll-like receptors (Toll-like receptors or TLRs) and other recognition receptors (PRR: Pattern Recognition Receptors) leading to the secretion of inflammatory cytokines, chemokines and antimicrobial peptides [6]. Pathogens have evolved strategies to circumvent this efficient barrier. Through the biting pieces of arthropod,

they are inoculated efficiently into the skin. Mosquitoes puncture the skin by their solenophage bite, introducing directly pathogens into the blood vessel. More sophisticated, the bite of hard ticks is particularly complex. By its chelicerae and hypostome, the skin is cut and dilacerated, forming a feeding pool around the biting pieces [7]. In addition, a cement acting as a glue is secreted around them, to anchor efficiently the hard tick for several days. Clinically, the bite itself can induce hypersensitivity with skin lesions [8], even necrosis in animal models or patients [9]. When pathogens are inoculated, they target resident skin cells, FBs or KCs or more specific immune cells like dendritic cells to hide or immunosuppress the host immune system [2]. The essential role played by the skin is clearly visualized for some VBDs, by the presence of various inflammatory skin manifestations: erythema migrans in Lyme disease, skin lesions more or less widespread in leishmaniasis, trypanome in trypanosomiasis, eschar for Rickettsiae, to name a few. Elucidate the mechanism of these skin manifestations, understand the development of protective immunity and how pathogens persist, require further investigation.

### The arthropod: a major actor

Two major groups of arthropods are involved in VBDs: insects and ticks. Insects and soft ticks (*Argasidae*) have a short blood meal, while hard ticks have a long lasting blood meal of several days. Vector saliva is a critical parameter for the efficient transmission of the pathogen in VBDs. It contains anti-hemostatic molecules, anti-inflammatory and immunomodulatory factors to facilitate the uptake of the blood meal, essential to their survival [10, 11]. In VBDs, some pathogens use arthropod saliva to facilitate their transmission. Studies of Lyme borreliosis and leishmaniasis revealed a major contribution from the saliva of *Ixodes* ticks and sandflies in the transmission of the bacteria [12] and the parasite [13] respectively. The vectors are not simple syringes that inoculate pathogens; they play an essential role as reservoir for certain pathogens, as virulence factor



**Figure 1.** Schematic presentations of the complex interactions between the triad in VBDs: vertebrate host, arthropod vector, pathogen.

and facilitating agent for pathogen transmission. Indeed, within the arthropod the pathogens are subject to major antigenic modifications that increase their virulence and in the presence of vector saliva, fewer pathogens are necessary to induce an infection in the vertebrate host.

Transcriptomics and proteomics analyses of arthropod salivary glands, infected and uninfected, have shown that certain pathogens increase arthropod proteins to facilitate their transmission to the vertebrate host [14]. Some proteins have been well characterized and have been the subject of vaccine trials in laboratory animals. For example, a peptide called maxadilan has been identified as a facilitating molecule in the sandfly saliva [10, 15]. Similar studies have been made in tick vectors. Because of their long blood meal (3-10 days), the hard tick must develop different strategies to remain attached efficiently. This probably explains the high number of molecules with pharmacological and immunomodulatory properties found in hard ticks [16, 17]. In Lyme borreliosis, bacteria specifically upregulate a tick salivary protein, Salp15. Its allows *Borrelia* to escape the immune system in the vertebrate host [18] thereby improving the transmission of the bacterium.

Arthropod saliva is therefore a potential target for vaccine development [10, 15, 19]. For some VBDs including malaria, analyzing the effects of saliva deserves more study since its initial role in pathogenesis is unclear or controversial [20].

## The pathogens

Some pathogens have evolved strategies to efficiently infect vertebrate hosts by taking advantage of the injury produced in the skin barrier via the bite of the arthropods, insects or mites. These VBDs have a major impact on public health by their morbidity and mortality. During the infectious bite, pathogens use arthropod saliva to circumvent the pharmacology and the immunity of the vertebrate host. Consequently, the vector transmission becomes an extremely efficient process and requires very little inoculation of pathogens [2]. Once in the skin, pathogens use this immune-privileged organ as an amplification and/or persistence site [21]. Some host-pathogen interactions are particularly well studied (e.g. leishmaniasis and Lyme bor-

reliosis). Recent technologies such as intravital imaging and the development of fluorescent pathogens (gfp: green fluorescent protein) showed new developmental mechanisms in the skin for some VBDs.

## Insect borne diseases

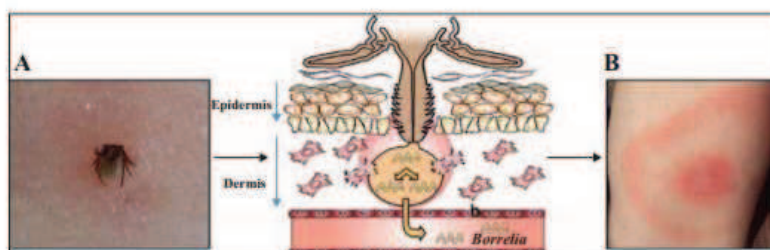
Insects are major vectors of VBDs. Mosquitoes are the most important as they transmit a large variety of pathogens, mainly parasites (malaria, filariasis) and viruses (dengue, yellow fever, West Nile virus...). The bite is very short in time. According to the insect, the long proboscis catheterizes the blood vessel (mosquitoes) or the small cutting mouthparts lacerate the skin tissues, inducing hemorrhages of surface capillaries (sandfly, blackfly). The physiopathological processes involved in the transmission of *Leishmania* has been the most studied so far.

### Leishmaniases

This parasitic infection is caused by a trypanosomidae parasite, *Leishmania*, transmitted by a small diptera, the sandfly *Phlebotomus* (Old World) or *Lutzomia* (New World). The parasite can remain localized in the skin or diffuse in the body according to the parasite species involved. The first clinical manifestation is a skin lesion that may persist for several weeks. For decades, it was thought that the promastigote forms only infected macrophages where it turned into amastigote forms. By altering the pH of the phagolysosome, they were able to escape the host immune response. Recently, it was shown that the cell first targeted by the parasite is in fact the neutrophil. These phagocytic cells are among the first to migrate at the site of infection. A study by fluorescence microscopy showed that *Leishmania* first locates in neutrophils and then only after within macrophages [22]. Parasitized neutrophils become apoptotic and are then preferentially internalized by macrophages [23].

### Malaria

It is a parasitic infection transmitted by *Anopheles* mosquito. *Plasmodium* is a protist Apicomplexa causing approximately 1 million deaths per year in tropical countries. The mosquito inoculates sporozoites after a probing time without causing visible damage to the skin. The



**Figure 2.** Skin manifestations during the transmission of Lyme disease by *Ixodes* tick. Tick biting pieces first dilacerate the epidermis, then the dermis where a feeding pit is formed upon fibroblast lysis by tick saliva. After around 24 hours, *Borrelia* are inoculated into the dermis, multiply locally, then migrate via the blood vessel to the target organs (joint, nervous system, distant skin). The infective blood meal of the nymph (tick stage the most involved in the transmission to human) lasts around 5 days. Seven days to weeks later, an erythema migrans can appear at the site of *Borrelia* inoculation. **A)** tick bite; **B)** Erythema migrans. (Photos: commons.wikimedia.org)

mouthparts are introduced directly into host blood vessel according a solenophage mode. The bite lasts for a few minutes. The effect of mosquito saliva on *Plasmodium* development and infectivity is still poorly understood. Invasive forms, sporozoites are inoculated in the dermis by the female anopheles mosquito. These sporozoites invade hepatocytes, where intense multiplication occurs, then the red cells. For decades, parasitologists thought sporozoites injected by mosquitoes quickly leave the skin in a few minutes to reach the liver without specific cellular interactions in the skin. Recent studies show that some sporozoites remain in the skin and initiate an immune response. In fact, 60% stay in the skin, where they can invade and develop inside skin cells, 15% actively reach and remain in the proximal draining lymph node and only the remaining 25% reach the blood and the liver [24]. Into the skin, sporozoites develop into exoerythrocytic forms and survive for several weeks in the hair follicles [25]. These skin forms may be responsible for immune tolerance [21] and are more and more investigated for vaccine development [26].

### Dengue

Among VBDs, dengue threatens 2.5 billion people, mainly in tropical countries and may be responsible for fatal hemorrhagic fevers. The pathogen is a flavivirus with four serotypes (DENV- 1 to 4). In the skin, the virus initially infects resident dendritic cells by binding to DC-SIGN molecules [27]. More recently, it has been shown that resident skin cells, keratinocytes are also susceptible to infection [28] as well as fibroblasts [29]. Given the high proportion of these cells in the skin, it gives the virus an opportunity to grow rapidly before activating a strong immune response by specific immune cells. The role of mosquito saliva is more investigated. It decreases the innate immunity of keratinocytes by inhibiting the secretion of antimicrobial peptides, facilitating the virus multiplication [28]. The virus downregulates the secretion of a mosquito saliva protein, aegyptin involved in the host clotting, increasing locally the virus titer and facilitating the transmission of the flavivirus [30].

### Tick-borne diseases

Ticks have a worldwide distribution - from the hottest areas of the globe to the coldest regions. They transmit many

infectious agents - bacteria, viruses and parasites - some of them have major health impact. Ticks are among the most important vectors in human and veterinary medicine. Two major groups of ticks, hard ticks (Ixodidae) and soft ticks (Argasidae) may parasitize humans, domestic animals and a wide range of wild hosts. Their bites exert direct effects on host (blood loss, anemia, toxin injection). In contrast to soft ticks, hard ticks have a long lasting blood meal (3 to 10 days), therefore to succeed their saliva needs to counteract the host response efficiently by pharmacologic and immunologic processes.

### Lyme borreliosis

As the first VBD of the northern hemisphere, Lyme borreliosis has been particularly well studied. The bacterium is a spirochete, *Borrelia burgdorferi sensu lato* transmitted by a hard tick, *Ixodes* sp. The initial infection is usually characterized by a skin inflammation, the erythema migrans, followed by a multi-systemic symptoms affecting the nervous system, joints and skin [31]. In this infection, the skin is an amplification site where the bacteria multiply intensely [32] and appears to be a "filter" where different bacterial clones among the heterogeneous population of *Borrelia* is selected to further disseminate in the vertebrate host [33]. The mechanism of this selection is unknown and merits further investigation. Clearly, the resident skin cells, keratinocytes [34] and fibroblasts [35], are involved in the transmission process. After laceration of the epidermis, the tick biting pieces penetrate the dermis where fibroblasts are lysed [35], inducing a feeding pool. There, *Borrelia* are inoculated, multiply and disseminate via the blood to target organs. After the infected tick has detached, most of the time a skin inflammation appears, the erythema migrans [36] (figure 2).

### Tick-borne encephalitis

It is a viral infection due to a Flavivirus responsible of thousands cases in Europe and Asia and transmitted by the hard tick, *Ixodes* [37]. Three virus subtypes have been described with different virulence according to the geographic distribution: the European, the Siberian and the Far Eastern subtype [38]. They multiply mainly in dendritic cells where tick saliva increases the rate of infection and decreases type I interferon response [39]. For this pathology, an interesting approach has been tested to control the virus transmission.

A tick protein 64TRP, involved in the cement formation, has been used to vaccinate animals. A reduction of the tick feeding and virus transmission has been observed and constitutes an interesting approach to control this infection [40].

## Conclusions

A better understanding of the triad interaction is essential to establish efficient vaccines against VBDs. So far, only four vaccines are available in humans against: yellow fever transmitted by *Aedes* mosquito, Japanese encephalitis transmitted by *Culex* mosquito, Rift valley fever by *Aedes* mosquito and tick borne encephalitis transmitted by *Ixodes* hard tick [41]. The skin interface should be the organ of choice to develop vaccine studies because it constitutes the first interface encountered by the pathogens and the arthropod. There, pathogens multiply and hide, and arthropod saliva facilitates the transmission by different pharmacologic and immunologic processes. As long as these different interactions occurring in the skin will not be understood, no efficient vaccines will be available for VBDs. The failure of many vaccine trials in the field of VBDs highlights gaps in the understanding of their initial transmission. Although many studies have been performed on the effects of saliva on the host immunity, additional data are missing to lead to an efficient tick vaccine based on tick saliva. Many advances have been made in recent years with intravital microscopy techniques to track fluorescent pathogens in the skin. It remains to be understood how pathogens escape host immunity and why they persist in the skin within immunoprivileged sites. In absence of protective vaccines against VBDs, the best is yet to disturb arthropod olfaction by repellents that prevent infective bite and thus the transmission of pathogens into the skin. In addition, the presence of cutaneous skin microbiome is likely to interfere with pathogen transmission since it seems to influence the skin homeostasis by regulating the host immunity [42]. Very few studies have been performed to investigate its role except for the anthropophilic mosquito, *Anopheles gambiae* and malaria [43]. A large area of investigation remains to be explored. ■

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# Curriculum Vitae

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# Quentin Bernard

45 Grand Rue  
Strasbourg, France  
French / English

[quentin.bernard67@gmail.com](mailto:quentin.bernard67@gmail.com)   
+33 650674915 

## Experience

2012 – 2015, **PhD student in immunology**, *Institut de bactériologie* (Strasbourg, **France**), Nathalie Boulanger Lab, *EA7290 Virulence bactérienne précoce, groupe borréliose de Lyme*, Molecular analysis during the early transmission of Lyme disease

2012, **Internship**, *Department of Medecine* (San Diego, **USA**) *UCSD Gallo Lab, Innate mechanisms through which the skin protects against microbial invasion*, The role of Toll like receptors during the early transmission of Lyme disease

2011, **Internship**, *Institut de bactériologie* (Strasbourg, **France**), Benoit Jaulhac Lab, *EA7290 Virulence bactérienne précoce, groupe borréliose de Lyme*, The role of commensal bacteria during the Lyme disease transmission

2010, **Intership**, *Faculté de pharmacie* (Strasbourg, **France**), Dominique Wachsmann Lab, *EA3432 Laboratoire de Physiopathologie des Arthrites*, Targeting study of ASK1 and TAK1 messenger RNAs by the miR-20a

2009, **Internship**, *Centre de biophysique moléculaire* (Orléans, **France**), Bertrand Castaing Lab, The functional role of the tyrosine 222 within the formamidopyrimidin-DNA glycosylase. Centre de Biophysique Moléculaire UPR 4301, CNRS

## Education

2010 – 2012, **Master of science** in Molecular and Cellular Biochemistry with immunology qualification, *Université de Strasbourg, France*

## Academic Experience

2013 - 2014, **Teaching Assistant** in biochemistry and molecular biology, *Faculté de médecine de Strasbourg, France*

## Publications

**Bernard, Q.**, Jaulhac, B., & Boulanger, N. (2014). Smuggling across the Border: How Arthropod-Borne Pathogens Evade and Exploit the Host Defense System of the Skin. *The Journal of Investigative Dermatology*, 134(5), 1211–9

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Kern, A., Schnell, G., **Bernard, Q.**, Bœuf, A., Jaulhac, B., Collin, E., Barthel, C., Sabatier, L., Boulanger, N.. Skin interface: the golden gate to investigate virulence factors involved in lyme borreliosis : **in Revision, *Plos One***

## Oral presentations

**Bernard, Q.**, Gallo, RL., Jaulhac, B., Lipsker, D., Nakatsuji, T., Luft, B., Yang, X., Boulanger, N. Potential role of the crosstalk TLR2/TLR3 during the early transmission of Lyme borreliosis. 21 november 2014, the Skin Physiology International Meeting (Vichy, France) – **1<sup>st</sup> Price**

**Bernard, Q.**, Nakatsuji, T., Gallo, RL., Jaulhac B., Boulanger, N. Molecular and Cellular analysis of the early transmission of the Lyme Disease. 2 july 2014, Fédération de médecine translationnelle de Strasbourg (**Strasbourg, France**) - **1<sup>st</sup>Price**

**Bernard, Q.**, Nakatsuji, T., Gallo, RL., Jaulhac B., Boulanger, N. The role of the skin injury during the early transmission of Lyme Disease. 10 february 2014, Réseau Ecologie des Interactions Durables (**Montpellier, France**)

**Bernard, Q.**, Nakatsuji, T., Gallo, RL., Jaulhac B., Boulanger, N. The role of Toll like receptors during the early transmission of Lyme disease. 11 April 2013, Séminaire de Microbiologie de Strasbourg (**Strasbourg, France**)

## Poster presentation

**Bernard, Q.**, Nakatsuji, T., Gallo, R.L., Jaulhac B., Boulanger, N. Poster: The crosstalk TLR2/TLR3 during the early transmission of Lyme disease. 19 february 2014, Doctoral School Day (**Strasbourg**)

## Awards

Fédération de médecine translationnelle de Strasbourg (**Strasbourg, France**). Best Immunology presentation (€500 prize), 2 July 2014

The SPIM Junior Scientist 2014 award (**Vichy, France**), A €7,500 prize rewarded the best oral presentation, 21 November 2014

### References Contact Information:

Pr. Richard. L. Gallo, University of California San Diego : rgallo1997@gmail.com

Pr. Benoit Jaulhac, University of Strasbourg : jaulhac@unistra.fr

Dr. Nathalie Boulanger, University of Strasbourg : nboulanger@unistra.fr

## Cellular and molecular analysis of the early transmission of Lyme borreliosis: the role of the skin interface

### Abstract

Vector-borne diseases account for seventeen percent of world-wide infectious diseases. They are a major threat to public health. Lyme borreliosis is the first vector-borne disease of the northern hemisphere. It is caused by a bacteria, *Borrelia burgdorferi* sensu lato, inoculated by a hard tick belonging to the *Ixodes* genus. The first contact between the vertebrate host and the tick, and so between the vertebrate host and the bacteria, occurs at the skin interface. The skin is then of major importance for the early development of the immune response against *Borrelia*.

The tick bite induces a skin injury owing to its biting pieces, the hypostome and two chelicerae. The tick saliva also creates a feeding pool allowing the tick to feed efficiently. This process also facilitates *Borrelia* transmission. We have characterized a tick saliva protein which might participate to the formation of the feeding pool: histone H4. This protein lyses fibroblasts and harbors bactericidal properties against commensal bacteria. These two activities might help *Borrelia* to infect the vertebrate host by sustaining its development in the skin. Once bacteria have been injected into the skin, they interact with resident skin cells, keratinocytes and fibroblasts, and immune cells. We have shown that the inflammation induced by the tick bite increases the keratinocyte inflammatory response against *Borrelia*. However, the saliva inhibits this cross-talk which depends on TLR3/TRIF and TLR2/MyD88 pathways. Once the tick has detached and the saliva has disappeared, the cross-talk might explained the inflammation observed during the erythema migrans.

Other skin cells than keratinocytes and fibroblasts are involved in the early inflammatory response against *Borrelia* such as dendritic cells, macrophages and neutrophils. We have explored the involvement of another poorly-studied cell-type: mast cells. We have shown that these cells can secrete IL-6 and degranulate in response to *Borrelia*. Bacteria antigens responsible of the activation might depends on the living state of *Borrelia*. The tick saliva is able to negatively control the secretion of IL-6, but not to completely inhibit it. At this point, we cannot conclude in a WSH mouse model deficient in mast cells, to a major role of these cells in the inflammatory response against *Borrelia*.

While in the skin, *Borrelia* expresses many genes which will facilitate the dissemination across the vertebrate host, to reach different target organs (brain, joint, distant skin). We have characterized two genes potentially involved in the dissemination of a virulent clone of *B. burgdorferi* sensu stricto: *bb0347* and *bb0213*. *bb0347* encodes for an adhesion which can specifically interact with the extracellular matrix of the skin while the role of *bb0213* is unknown. *bb0347* might help the bacteria to migrate through skin tissues and then increases the infection rate.

Keywords; *Borrelia*, tick, skin, injury, saliva, virulence, innate immunity, Toll receptors



## Analyse cellulaire et moléculaire de la transmission précoce de la borréliose de Lyme: rôle de l'interface cutanée

### Résumé

Les maladies à transmission vectorielle, qui représentent dix-sept pour cent de l'ensemble des maladies infectieuses, sont un réel problème de santé publique. Parmi elles, la borréliose de Lyme est la maladie vectorielle la plus répandue de l'hémisphère nord. Elle est causée par la transmission d'une bactérie, *Borrelia burgdorferi* sensu lato, par une tique dure du genre *Ixodes*. Le premier contact entre l'hôte vertébré et la tique, et donc *a fortiori* entre l'hôte vertébré et la bactérie, se fait au niveau cutané. La peau est donc une interface essentielle dans le développement précoce de l'immunité envers *Borrelia*.

La tique effectue un repas de plusieurs jours qui induit une lésion cutanée par ses pièces piqueuses et sa salive, créant une cavité lui permettant de se nourrir. Ce processus facilite le repas sanguin, mais aussi la transmission de *Borrelia*. Nous avons caractérisé une protéine présente dans la salive de tique responsable de la formation de la cavité : l'histone H4. Celle-ci lyse les fibroblastes et possède une activité bactéricide envers les bactéries commensales de la peau. Ces deux activités pourraient être exploitées par *Borrelia* afin de favoriser sa transmission et son développement dans la peau. Une fois injectées, *Borrelia* et la salive interagissent avec les cellules de la peau telles que les kératinocytes de l'épiderme, les fibroblastes et les cellules immunitaires du derme. Nous avons montré que l'inflammation induite par la lésion augmente la réponse inflammatoire des kératinocytes contre *Borrelia*. Cependant, la salive de tique inhibe efficacement cette coopération inflammatoire dont la mise en place dépend des voies inflammatoires TLR3/TRIF et TLR2/MyD88. Une fois la tique détachée de l'hôte vertébré et la salive de tique disparue, la coopération inflammatoire ne serait plus inhibée et pourrait expliquer en partie l'apparition de l'érythème migrans.

L'inflammation cutanée précoce implique d'autres cellules que les kératinocytes et les fibroblastes telles que les macrophages, les cellules dendritiques ou encore les neutrophiles. Nous avons étudié l'implication d'un autre type cellulaire peu exploré dans le contexte de la borréliose de Lyme : les mastocytes. Ces cellules sont capables de répondre efficacement contre *Borrelia* par la sécrétion d'IL-6 et la dégranulation. Les antigènes bactériens nécessaires à l'activation des mastocytes semblent liés à la bactérie intacte et vivante. La salive de tique réduit la sécrétion d'IL-6 induite par *Borrelia*, mais ne l'inhibe pas complètement. A ce stade de nos études, les mastocytes ne semblent pas jouer un rôle majeur.

Lors de la transmission, *Borrelia* exprime différents gènes qui lui permettront notamment de disséminer vers différents organes cibles de l'hôte vertébré : le système nerveux, l'articulation et la peau à distance. Nous avons identifié chez un clone de *B. burgdorferi* sensu stricto aux propriétés disséminatrices importantes, l'expression de deux gènes, *bb0347* et *bb0213*, qui semblent associés à cette virulence. *bb0347* code une protéine d'adhésion à la matrice extracellulaire, ce qui pourrait favoriser la migration de la bactérie à travers les tissus cutanés de l'hôte vertébré et donc sa dissémination. Le rôle de *bb0213* n'est pas connu à ce jour.

Mot clés : *Borrelia*, tique, peau, blessure, salive, virulence, immunité innée, récepteurs Toll