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Prevention of pathogenic *Escherichia coli* diseases: immune response to a broad spectrum *E. coli* antigen, and role of nitric oxide in enterohemorrhagic *E. coli* virulence

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To Zakaria. It has been 20 years, my friend, and I have not forgotten what we are fighting for.

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For reasons that the readers of this thesis dissertation will certainly understand, I may unexpectedly switch from English to French, so please bear with me.

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Marc, my husband, my friend, and my teammate…Thank you for standing by my side, every day, near or far, and for racing with me through the finish line. Thank you for believing in me, even when I did not believe in myself.

Prévention des infections à *Escherichia coli* : étude de la réponse immunitaire induite par un antigène d'*E. coli* à large spectre et rôle du monoxyde d'azote dans la virulence des *E. coli* entérohémorragiques

Résumé de la thèse

Les *Escherichia coli* (*E. coli*) pathogènes sont une source de préoccupation grandissante pour la santé publique dans le monde, en raison de leur morbidité et de leur incidence sur la mortalité, en particulier chez les jeunes enfants. En fonction du variant d'*E. coli*, ou pathotype, les maladies vont de la diarrhée aiguë à la septicémie, la méningite ou le syndrome hémolytique et urémique. Les traitements actuels sont insuffisants en raison de l'émergence croissante de la résistance aux antibiotiques, et aucun vaccin n'est encore disponible sur le marché; par conséquent, des efforts sont nécessaires afin d'offrir de futures solutions préventives ou thérapeutiques contre les infections provoquées par *E. coli*. Dans ce contexte, ma thèse de doctorat a eu pour objectif de contribuer à cet effort collectif en développant deux approches différentes. Nous avons d'abord établi un modèle murin d'immunisation avec un antigène à large spectre d'*E. coli*, et montré que ce modèle générait une réponse humorale et cellulaire robuste à la fois dans l'intestin et de façon systémique, sans perturber le microbiote intestinal résident. Un tel modèle d'immunisation pourrait donc potentiellement protéger contre les maladies intestinales et extra-intestinales provoquées par *E. coli*, et apporterait des indications précieuses quant au développement d'un vaccin à large spectre contre ces pathogènes. Dans une seconde approche, nous avons travaillé spécifiquement avec les *E. coli* entérohémorragiques (EHEC) dans le but de déterminer un effet potentiel du monoxyde d'azote (NO) sur la virulence des EHEC. En utilisant des modèles murins d'infection, nous avons montré que la détection de NO par les EHEC est essentielle pour une colonisation efficace du tractus digestif par le pathogène. De plus, l'inhibition de la production de NO par l'hôte diminue l'activité de la Shiga toxine, le principal facteur de virulence des EHEC, alors qu'elle augmente l'adhésion des EHEC au colon. Nos résultats indiquent que le NO, un acteur important de la réponse immunitaire de l'hôte, peut jouer un rôle déterminant lors d'une infection par les EHEC, et pourrait donc faire l'objet de nouvelles stratégies visant à lutter contre ces infections chez l'Homme.

Introduction

Dans le monde de la survie et de l'adaptation, *Escherichia coli* possède de nombreux arguments à faire valoir. Considérant *E. coli* dans sa globalité, nous sommes confrontés à une bactérie qui est passée d'un simple commensal intestinal de l'Homme à plus de neuf variants pathogènes capables de coloniser l'intestin, la vessie, le rein, le sang ou le cerveau, qui sont devenus d'importants agents étiologiques de maladies allant de la diarrhée aux infections récurrentes des voies urinaires et à la méningite néonatale. Pour les enfants de moins de cinq ans, ces maladies présentent des risques importants de décès, en particulier dans les pays en développement où l'assainissement et les antibiotiques ne sont pas nécessairement disponibles. De plus, les soins hospitaliers et les prescriptions d'antibiotiques représentent d'énormes coûts de santé, de même que l'augmentation de la multi-résistance à tous les pathotypes d'*E. coli*. Aujourd'hui, de nombreux efforts sont consacrés à la caractérisation des mécanismes de virulence de ces pathotypes ainsi que la réponse de l'hôte ; ces résultats peuvent à leur tour fournir d'importantes indications dans la recherche de nouvelles solutions thérapeutiques, ou même mieux, pour la conception d'un vaccin ciblant un ou plusieurs variants d'*E. coli* pathogène. Ces perspectives de recherche ont été au centre du programme européen Marie Sklodowska-Curie DISCo, dans lequel quatre projets de doctorat ont été conçus pour travailler sur un vaccin à large spectre contre les *E. coli* pathogènes. Mon objectif en tant que boursière du programme DISCo était double: caractériser la réponse immunitaire murine à un antigène d'*E. coli* hautement prévalent, et étudier l'interaction *in vivo* entre les *E. coli* entérohémorragique (EHEC) de sérotype O157:H7 et une molécule clé de la réponse inflammatoire, le monoxyde d'azote.

Dans une première partie introductive, je mentionnerai brièvement les thèmes auxquels mes travaux sont associés; à savoir l'immunité de la muqueuse intestinale, les *E. coli* pathogènes, et enfin le monoxyde d'azote ainsi que les mécanismes dévelopés par *E. coli* pour détecter cette molécule clé de la réponse immunitaire.

Les résultats de mon mémoire sont consacrés aux deux manuscrits qui reflètent ma recherche de doctorat dans les deux institutions avec lesquelles j'ai travaillé. Mon projet à GSK à Sienne (Italie) était de décrire la réponse immunitaire à l'antigène SslE, un candidat vaccin potentiel contre les *E. coli* pathogènes intestinaux et extra-intestinaux, et l'impact d'une telle immunisation sur le microbiote intestinal murin. Ce projet a été décrit dans une publication qui a été soumise au journal scientifique Vaccine. Mon projet à l'INRA à Saint-Genès-Champanelle, France, était d'étudier l'effet du NO sur la colonisation et l'évolution de l'infection par EHEC O157:H7 chez la souris. Nos résultats sont présentés sous forme d'un

article préliminaire qui sera soumis après la collecte et l'analyse des dernières expériences en cours.

Enfin, je présente une conclusion générale et une discussion de mon travail de doctorat, en élargissant ma recherche à des orientations futures, à court et à long terme.

1. Immunité de la muqueuse intestinale

L'intestin représente la plus grande surface que l'hôte expose au monde extérieur, et ce monde est loin d'être amical. Considérant le fait qu'il traite tout notre apport nutritionnel, fait face à des sécrétions endogènes telles que l'acide chlorhydrique, les sels biliaires et les protéases digestives, tout en hébergeant des milliards de microorganismes, l'intestin est constamment mis à l'épreuve par des entités étrangères. Pour assurer une absorption digestive efficace, maintenir l'homéostasie tissulaire de l'hôte et protéger le corps des envahisseurs microbiens potentiels, la barrière intestinale mucosale a évolué pour devenir une frontière immunitaire hautement dynamique qui surveille attentivement son environnement et adapte sa réponse afin de tolérer antigènes alimentaires ou commensaux, mais répond également rapidement aux microbes nocifs qui sont entrés dans la lumière intestinale et/ou ont franchi la barrière mucosale. Comme on peut s'y attendre, ce processus s'accompagne de mécanismes de détection et de régulation considérables le long de la barrière intestinale mucosale, et implique un dialogue constant entre de nombreuses cellules spécialisées du système immunitaire inné et adaptatif. À cet égard, le microbiote intestinal est un régulateur essentiel de l'homéostasie intestinale et du système immunitaire.

- Les cellules effectrices de la réponse immunitaire adaptative

Les lymphocytes T helper : L'activation et la différenciation des lymphocytes T $CD4^+$, après la rencontre avec leur antigène spécifique, en diverses cellules T helper (T_H) se produit grâce à l'expression induite de cytokines produites par des cellules de l'environnement local. La combinaison de cytokines et de médiateurs détectés par les lymphocytes T conduit à leur polarisation en différents types de cellules T_H, présentées dans la figure 1 ci-dessous.

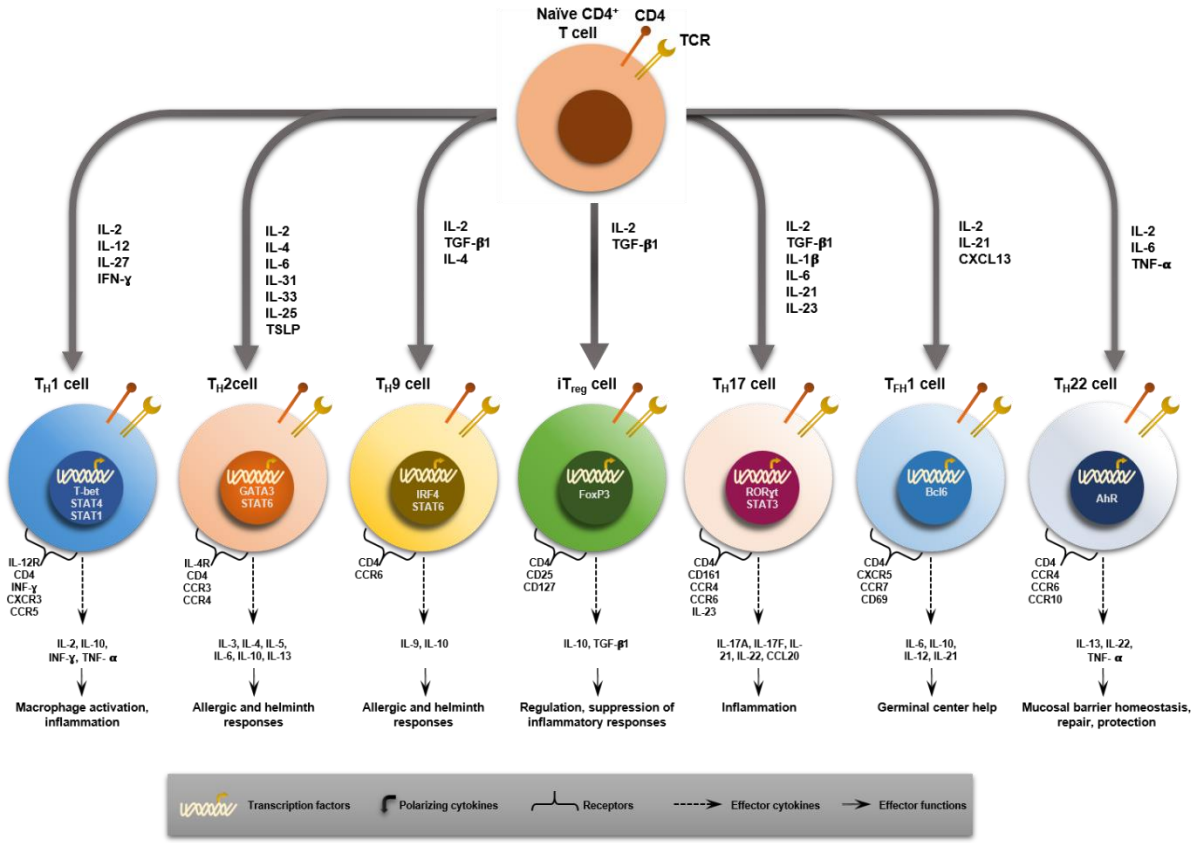

Figure 1. Différenciation et fonctions principales des cellules T helper (T_H). *Figure adaptée de Swain, McKinstry et al. 2012.*

Les lymphocytes B et les immunoglobulines A : Dans l'intestin et les autres surfaces mucosales, les immunoglobulines (Ig) A sécrétoires (SIgAs) sont sans doute le principal type d'immunoglobulines sécrétées par des lymphocytes B différenciées et spécifiques pour un antigène, bien que d'autres immunoglobulines telles que IgG et IgM soient également présentes.

Les SIgAs sont formées après la transcytose des IgAs à travers les cellules épithéliales de la muqueuse intestinale. L'importance des IgAs dans l'immunité protectrice est connue depuis longtemps. En effet, plusieurs études ont révélé l'efficacité de l'immunisation passive chez l'Homme (Hammarstrom, Smith et al., 1993, Tjellstrom, Stenhammar et al., 1993, Corthesy, 2003) et chez la souris (Apter, Lencer et al., 1993, Phalipon, Kaufmann et al. 1995). De même, des études sur des modèles de souris ou sur des patients présentant des déficiences en IgA montrent clairement une protection active par les anticorps SIgAs contre plusieurs agents pathogènes microbiens (Friman, Nowrouzian et al 2002, Langford, Housley et al., 2002).

Les mécanismes à l'origine de la protection immunitaire à médiation par les IgA sont nombreux, et peuvent avoir lieu dans la lumière intestinale et/ou dans la lamina propria. Les SIgAs, dont la plupart sont polyréactifs, permettent principalement l'exclusion immunitaire ; c'est-à-dire en se liant à divers antigènes dans la lumière intestinale, retardant ou prévenant ainsi l'adhésion et/ou la pénétration et/ou l'invasion de la muqueuse intestinale. Un autre effet des SIgAs est de limiter les dommages collatéraux en contrôlant l'inflammation qui pourrait potentiellement être déclenchée par l'invasion de pathogènes.

Les IgAs sériques assurent également une protection supplémentaire contre les agents pathogènes envahissants en engageant plusieurs «nettoyeurs» professionnels. En effet, de multiples cellules myéloïdes telles que les neutrophiles, les éosinophiles, les monocytes et les macrophages expriment à leur surface un récepteur aux IgA. L'opsonisation d'un antigène ou d'un agent pathogène par les IgAs sérique et sa liaison subséquente à ce récepteur permet l'élimination efficace du pathogène ou du complexe immun par phagocytose (par les macrophages ou neutrophiles) ou la dégranulation de composés toxiques pour tuer le pathogène (par les éosinophiles ou basophiles).

- Le microbiote intestinal

Le système immunitaire inné et adaptatif combat les infections microbiennes tout en maintenant microbiote intestinal résident sous contrôle. Cette relation n'est certainement pas unidirectionnelle, et des efforts de recherche approfondis tentent de découvrir les nombreuses voies dans lesquelles le microbiote intervient afin de moduler le développement et la fonction de la réponse immunitaire.

Les microbes colonisent l'intestin immédiatement après la naissance et la composition spatiale du microbiote dépendra en grande partie des besoins en nutriments de chaque espèce, ce qui entraînera une variation importante le long du tractus gastro-intestinal (Mowat et Agace 2014). L’hôte et le microbiote ont co-évolué en une symbiose mutualiste grâce à laquelle les microbes exploitent l'environnement nutritif riche de l’intestin pour prospérer, et en retour fournissent à l'hôte une pléthore de bénéfices physiologiques et immunitaires. Il n'est donc nullement surprenant que le dérèglement de la composition du microbiote affecte de manière dramatique l'homéostasie tissulaire locale et l'immunité de l'hôte à l'infection, mais aussi d'autres aspects physiologiques dans des tissus plus éloignés, voire même de façon systémique.

2. Les *Escherichia coli* pathogènes

La bactérie *Escherichia coli* est un commensal inoffensif présent dans l'intestin des humains et d'autres animaux. En tenant compte de l'ensemble du microbiote intestinal, *E. coli* englobe seulement 1% de la population ; mais en tant qu'anaérobe facultatif, *E. coli* représente environ 80% de la flore aérobie.

Mais il suffit d'une figure pour bien saisir la polyvalence remarquable d'*E. coli* et son potentiel pathogénique chez l'Homme (Figure 2). Grâce à sa capacité à acquérir diverses combinaisons d'éléments génétiques mobiles par transfert de gènes horizontaux, *E. coli* a acquis les outils lui permettant de potentiellement se développer en un agent pathogène humain extrêmement efficace, provoquant diverses formes de maladies diarrhéiques. Certains pathogènes d'*E. coli* ont même évolué un tropisme pour d'autres organes que l'intestin, tels que la vessie, les reins et le cerveau, et sont maintenant tristement célèbres pour la gravité des maladies qu'ils causent, comme la septicémie et la méningite néonatale.

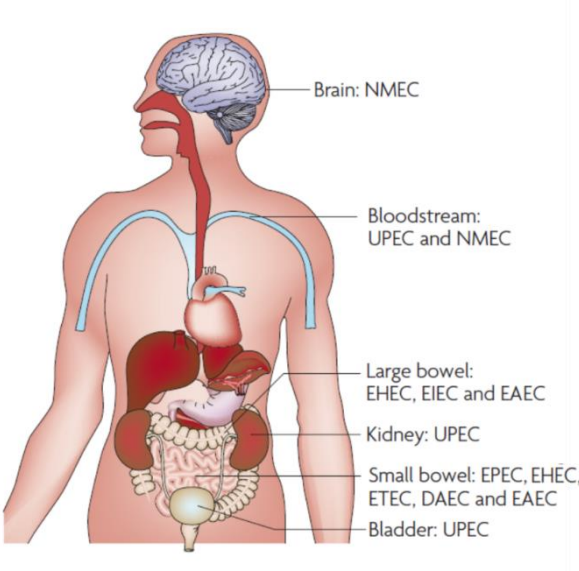

Figure 2. Les pathotypes majeurs des Escherichia coli et leurs sites de colonisation. *Les E. coli entéropathogènes (EPEC), les E. coli entérotoxinogènes (ETEC), et les E. coli adhérents diffus(DAEC) colonisent l'intestin grêle. Les E. coli entéro-invasifs (EIEC) colonisent le colon. Les E. coli entérohémorrhagiques (EHEC) et les E. coli entéroagrégatifs (EAEC) peuvent coloniser l'intestin grêle et le colon. Les E. coli uropathogènes (UPEC) colonisent les voies urinaires et peuvent migrer dans les reins et entrer dans la circulation sanguine. Les E. coli à méningite néonatale (NMEC) peuvent également entrer dans la circulation sanguine et causer une bactériémie, et traverser la barrière hémato-encéphalique pour coloniser le cerveau. Figure de Croxen and Finlay 2010.*

Les divers isolats d'*E. coli* pathogènes connus ont été séparés en deux grands groupes, chacun ayant différents pathotypes, en fonction de leur tropisme organique et du mécanisme de virulence utilisé pour provoquer la maladie. Dans le groupe des *E. coli* pathogènes intestinaux, ou InPEC, beaucoup ont été étudiés depuis longtemps, tels que les *E. coli* entéropathogènes (EPEC), les *E. coli* entérotoxinogènes (ETEC), les *E. coli* entéro-invasifs (EIEC), les *E. coli* entéro-agrégatifs (EAEC), les *E. coli* entérohémorragiques (EHEC), et les *E. coli* à adhérence diffuse (DAEC). Dans le groupe des *E. coli* pathogènes extra-intestinaux (ExPEC), nous trouvons les *E. coli* à méningite néonatale (NMEC) et les *E. coli* uropathogènes (UPEC). À la fin des années 1990, les *E. coli* adhérentes invasives (AIEC), associées à la maladie de Crohn, ont été proposées comme nouveau pathotype d'InPEC (Boudeau, Glasser et al., 1999). De plus, l'épidémie d'*E. coli* notable observée en Europe en 2011 a dévoilé un nouveau pathotype hybride d'InPEC, puisque la souche étiologique était une EAEC qui avait acquis les gènes de la Shiga toxine que l'on trouve normalement chez les EHEC (Rasko, Webster et al., 2011, Karch, Denamur et al., 2012); ce pathotype est ainsi appelé EAHEC pour *E. coli* hémorragique entéro-agrégatif. Enfin, en 2013 en France, un EHEC atypique hybride (*E. coli* O80:H2) porteur d'un plasmide de virulence habituellement observé chez les NMEC provoque une bactériémie chez un adulte, une caractéristique qui n'avait jamais été encore attribuée à une souche EHEC (Mariani-Kurkdjian, Lemaitre et al., 2014, Soysal, Mariani-Kurkdjian et autres 2016). Ces derniers ajouts illustrent certainement le fait que les *E. coli* pathogènes constituent une famille en pleine croissance, et avec eux viennent de nouveaux défis de santé publique. Ces défis prennent de plus en plus d'ampleur alors que nous sommes confrontés à une pénurie de traitements efficaces ; en effet, avec une telle capacité à acquérir et transférer des éléments mobiles, il n'est pas surprenant que les agents pathogènes d'*E. coli* aient rapidement évolué à l'utilisation d'antibiotiques en acquérant un ou plusieurs gènes de résistance aux antibiotiques en plus de leurs gènes de virulence. Comme la découverte d'antibiotiques semble être au point mort, il est primordial de comprendre les mécanismes spécifiques de la virulence et de la pathogénicité de chacun de ces pathotypes dans le but trouver de nouveaux moyens de traitement efficaces.

3. Le monoxyde d'azote

Le monoxyde d'azote (NO) est une petite molécule inorganique composée d'un atome d'oxygène et d'un atome d'azote. Le NO est un radical libre très réactif qui a la capacité d'agir sur de nombreuses molécules des organismes vivants. Physiologiquement, le NO est produit à partir du substrat L-arginine et nécessite l'activité de l'enzyme NOS (nitric oxide synthase). La synthèse de NO se produit dans une grande variété de cellules dans le corps, ce qui explique en partie son rôle crucial dans diverses fonctions biologiques. Ainsi, depuis la

découverte que les macrophages de souris libèrent une grande quantité de NO_2^- (nitrite) et NO_3^- (nitrate) lors de la stimulation avec LPS et IFN-γ, NO est considéré comme un acteur important de l'immunité innée et adaptative, ainsi que de la modulation de l'inflammation.

- NsrR, une protéine spécifique pour la détection de NO chez *E. coli*

NsrR (nitric oxide sensitive repressor) est une protéine homodimèrique contenant un groupe Fe-S. La nature des groupes fer-soufre peut varier en fonction de la souche bactérienne étudiée. Le site de liaison NsrR comprend deux motifs de 11 paires de bases (bp) organisés en répétition inversée et espacés d'une bp, le motif de 11 bp étant lui-même un palindrome (Rodionov, Dubchak et al., 2005, Bodenmiller et Spiro 2006, Partridge, Bodenmiller et al., 2009). NsrR se lie à l'ADN en tant que dimère, vraisemblablement avec un monomère par «demi-site», c'est-à-dire un motif de 11 bp (Tucker, Hicks et al., 2008).

La détection de NO par NsrR dépend strictement de son interaction avec les groupes Fe-S liés à NsrR. En l'absence de NO, NsrR est lié à l'ADN, empêchant ainsi l'accès de l'ARN polymérase et le début de la transcription. En présence de NO, la formation de complexes dinitrosyl-fer dans le groupe Fe-S conduit à la perte de l'activité de liaison à l'ADN NsrR, permettant ainsi la transcription de gènes régulés par NsrR, tels que *hmpA*. NsrR peut également agir comme un activateur transcriptionnel.

Chez *E. coli*, NsrR assume le rôle d'un régulateur global, car il régule un réseau complexe de plus de 60 gènes ; cependant, seuls certains sont directement liés à la détoxification du NO (Partridge, Bodenmiller et al., 2009). Plusieurs gènes impliqués dans l'adhésion, la dégradation des protéines, la motilité, le transport transmembranaire, le métabolisme et la réponse au stress sont régulés par NsrR (Partridge, Bodenmiller et al 2009, Tucker, Le Brun et al 2010).

Dans notre laboratoire, deux études successives examinant l'effet du NO sur les EHEC O157:H7 ont établi que le NO était un inhibiteur de l'expression du gène *stx2* (Vareille, de Sablet et al., 2007) ainsi que de l'expression du LEE (Branchu, Matrat et al., 2014). Les deux inhibitions sont dues à un détachement de NsrR de l'ADNprovoqué par la fixation NO sur NsrR; par conséquent, NsrR agit ici comme un régulateur transcriptionnel positif, qui n'avait pas été décrit auparavant. De manière intéressante, l'inhibition induite par NO de l'expression transcriptionnelle de *stx2* était due à une inhibition, très probablement indirecte, de l'expression transcriptionnelle de *recA*, suggérant ainsi une inhibition médiée par le NO de la transcription de la réponse SOS par NsrR.

Caractérisation de la réponse immunitaire de l'antigène d'*Escherichia coli* SslE et de son impact sur le microbiote intestinal murin

La vaccinologie inverse, provenant de Novartis Vaccines, devenu GSK, a marqué un tournant dans le développement des vaccins. Jusqu'à présent, la recherche sur les vaccins reposait essentiellement sur l'identification des quelques antigènes hautement immunogènes basés sur l'analyse sérique de patients infectés. En revanche, la vaccinologie inverse utilise le génome entier d'un agent pathogène spécifique pour identifier des candidats de vaccin, y compris ceux qui seraient moins immunogènes, tout en conférant un niveau élevé de protection. En utilisant cette technologie, les antigènes exposés à la surface et sécrétés peuvent être sélectionnés et évalués pour leur efficacité protectrice dans le temps. Aujourd'hui, avec le progrès des technologies de séquençage à haut débit, la vaccinologie inverse est utilisée pour réaliser des études génomiques comparatives sur plusieurs isolats de la même espèce bactérienne - une méthode particulièrement efficace afin de couvrir la diversité antigénique présente chez de nombreux pathogènes bactériens. Les *E. coli* pathogènes englobent une énorme diversité, qui est illustrée par l'étendue de leur tropisme d'organe, les facteurs de virulence, et les moyens de colonisation. La sévérité des maladies dues aux *E. coli* pathogènes et l'augmentation de la résistance aux antibiotiques justifient la recherche de nouveaux moyens de prévention, et la vaccinologie inverse représente un outil prometteur pour identifier rapidement de nouveaux candidats susceptibles de se développer en vaccins ciblant plusieurs, voire tous les *E. coli* pathogènes.

Des travaux antérieurs chez GSK ont mené à l'identification de neuf candidats vaccins basés sur une approche de vaccinologie inverse visant les ExPEC. Les neuf antigènes ont montré une protection contre un modèle murin de septicémie avec une souche ExPEC. Avec une efficacité protectrice de 82%, SslE (pour secreted and surface-associated lipoprotein of *E. coli*) était le candidat le plus prometteur. Des modèles supplémentaires ont montré que SslE présentait également une protection croisée contre d'autres souches ExPEC. Des tests fonctionnels ont démontré *in vitro* et *ex vivo* que SslE est une mucinase qui joue un rôle important dans la colonisation et la virulence des *E. coli*.

Dans le cadre du programme DISCo, mon travail chez GSK consistait à caractériser la réponse immunitaire à l'antigène SslE. En particulier, nous voulions déterminer si nous pouvions obtenir à la fois une réponse immunitaire systémique et une réponse immunitaire intestinale à SslE. La protéine SslE étant largement exprimée chez les ExPEC et les InPEC,

l'obtention d'une réponse immunitaire mucosale à SslE dans les intestins en plus d'une réponse immunitaire systémique seraient en faveur de l'utilisation de SslE dans un vaccin à large spectre contre les *E. coli* pathogènes, qui était l'objectif global du programme DISCo. En utilisant divers schémas d'immunisation chez la souris, nous avons recherché les meilleures voies d'immunisation pour obtenir une réponse spécifique robuste à SslE dans les intestins et la circulation (Figure 3). En comparaison, nous avons montré que la combinaison d'une première dose intranasale, suivie de deux doses intramusculaires, était le régime d'immunisation le plus prometteur pour atteindre nos objectifs spécifiques; en effet, les souris ainsi immunisées ont engendré des réponses de cellules B et T robustes, à la fois dans la muqueuse intestinale et de manière systémique. En outre, nous avons cherché à déterminer l'impact potentiel des immunisations avec SslE sur le microbiote intestinal murin. Les analyses avec notre régime d'immunisation le plus prometteur n'ont montré aucun changement significatif dans la richesse ou la composition du microbiote intestinal résident. Ces résultats suggèrent que SslE peut être un composant prometteur d'un vaccin à large spectre contre les *E. coli* pathogènes. Ces travaux ont mené à un article soumis au journal Vaccine le 4 décembre 2017.

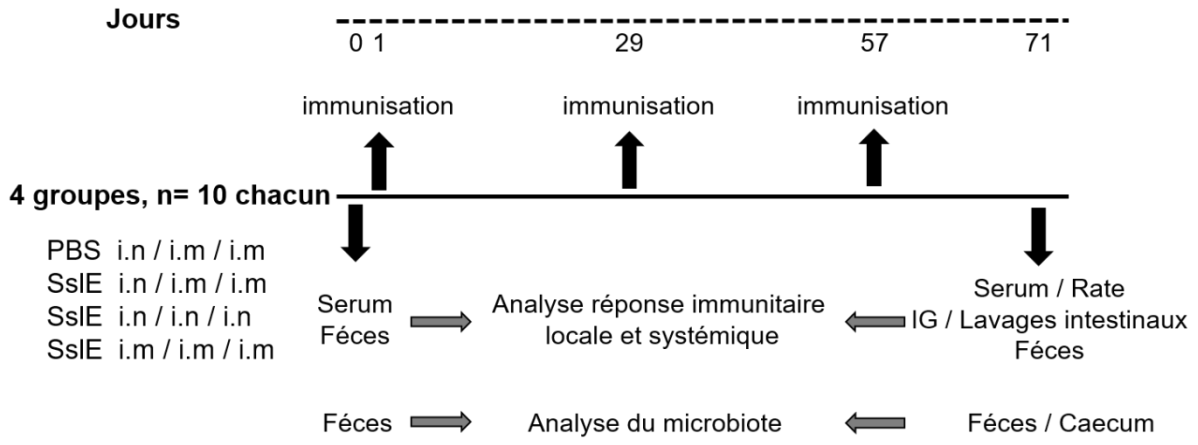

Figure 3. Description de l'étude expérimentale. Pour chaque étude (deux études indépendantes), trois groupes d'immunisation avec l'antigène SslE et un groupe contrôle (PBS) ont été établis (n=10 souris par groupe). i.n / i.m / i.m: immunisation intranasale au jour 1, immunisations intramusculaires aux jours 29 et 57. i.n / i.n / i.n: immunisations intranasales aux jours 1, 29 et 57. i.m / i.m / i.m: immunisations intramusculaires aux jours 1, 29 et 57. Pour chaque groupe, des échantillons ont été prélevés pour analyse au jour 0 (stade pré-immune), ou au jour 71. Pour l'analyse du microbiote, seuls les groupes PBS i.n / i.m / i.m et SslE i.n / i.m / i.m ont été utilisés, avec n=6.

Mixed mucosal-parenteral immunizations with broadly conserved pathogenic *Escherichia coli* antigen SslE induce a robust mucosal and systemic immunity without affecting the murine intestinal microbiota

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L'émergence et la dissémination de la multirésistance chez les *Escherichia coli* pathogènes constituent une menace sérieuse pour la santé publique dans les pays développés et en développement. Dotés d'un répertoire flexible de mécanismes de virulence, les *E. coli* peuvent provoquer de multiples maladies intestinales (InPEC) et extra-intestinales (ExPEC), mais seul un nombre très limité d'antibiotiques reste efficace contre ce pathogène. Par conséquent, un vaccin à large spectre contre les *E. coli* pathogènes pourrait être une alternative prometteuse afin de prévenir ces maladies, en offrant le potentiel d'efficacité contre plusieurs InPEC et ExPEC à la fois. SslE, pour ''secreted and surface-associated lipoprotein of *E. coli*'', est une protéine largement distribuée parmi les InPEC et ExPEC. SslE fonctionne *ex vivo* comme une mucinase capable de dégrader les mucines et d'atteindre la surface des cellules épithéliales productrices de mucus. SslE a été identifié grâce à la méthode de vaccinologie inverse comme un candidat vaccin protecteur contre un modèle murin de septicémie par une ExPEC, et s'est également avéré être efficace contre d'autres modèles d'infection aux ExPEC et InPEC. Dans cette étude, nous avons cherché à mieux comprendre la réponse immunitaire à l'antigène SslE et à identifier une stratégie d'immunisation adaptée pouvant générer une réponse immunitaire mucosale et systémique

robuste. Nous avons montré, en analysant les réponses des lymphocytes T et des anticorps, que les souris immunisées avec SslE par une première dose intranasale suivie de deux doses intramusculaires ont développé une réponse immunitaire globale plus élevée comparée à un régime de trois doses en intranasal ou trois doses en intramusculaire. Nous avons également montré que ce régime d'immunisation n'a pas eu d'impact sur la richesse du microbiote intestinal murin; en effet, les souris avaient une composition microbienne caecale comparable, qu'elles aient été immunisées avec SslE ou avec du PBS. Collectivement, nos résultats confortent l'utilisation potentielle de SslE dans de futures stratégies de vaccination ciblant les InPEC et ExPEC sans perturber le microbiote intestinal résident.

Rôle du monoxyde d'azote dans l'infection des *Escherichia coli* enterohémorrhagiques chez la souris

Les *Escherichia coli* entérohémorragiques (EHEC) représentent une source sérieuse de maladies diarrhéiques d'origine alimentaire, qui peuvent rapidement évoluer vers des affections potentiellement mortelles telles que le purpura thrombocytopénique thrombotique (PTT) et le syndrome hémolytique et urémique (SHU). Les patients qui survivent à un SHU induit par les EHEC peuvent faire face à des séquelles importantes, en particulier dans les reins et le système nerveux central. Les EHEC possède deux facteurs de virulence majeurs: le système de sécrétion de type III (T3SS), nécessaire pour une colonisation efficace par adhésion à la muqueuse intestinale ; et les Shiga toxines, qui induisent la nécrose et l'apoptose des cellules microvasculaires endothéliales, et sont responsables de lésions rénales et cérébrales importantes. Parce que l'expression des Shiga toxines (Stx1 et Stx2) est sous le contrôle de la réponse SOS, le traitement des infections à EHEC avec des antibiotiques, en particulier des agents endommageant l'ADN comme les fluoroquinolones, ne peut être considéré. Les taux de morbidité et de mortalité importants dus aux EHEC, l'absence de traitements et l'apparition de souches hybrides et plus virulentes soulèvent des problèmes de santé publique élevés.

Des travaux antérieurs dans notre laboratoire de l'INRA ont mis en évidence une inhibition *in vitro* de l'expression du T3SS et de Stx2 par le monoxyde d'azote (NO). Dans les deux cas, l'inhibition est médiée par le régulateur bactérien NsrR. Le NO joue un rôle antimicrobien et inflammatoire important dans la défense immunitaire innée de l'hôte contre l'infection; dans l'intestin murin, les cellules épithéliales, les macrophages et les neutrophiles expriment la iNOS (pour inducible nitric oxide synthase), qui est connue pour être fortement induite lors de l'infection par des pathogènes entériques comme *Citrobacter rodentium* et *Salmonella* Typhimurium. Au cours de mon projet doctoral à l'INRA, nous avons cherché à confirmer nos précédents résultats *in vitro* dans un modèle murin d'infection par une souche d'EHEC O157:H7 et à déterminer l'impact potentiel du NO sur les mécanismes de virulence des EHEC ainsi que sur le devenir de l'infection.

Durant ce projet, nous avons établi grâce à l'utilisation d'une souche reportrice, que les EHEC sont capables de détecter le NO dans la lumière intestinale murine. De plus, nous avons utilisé un inhibiteur spécifique des enzymes NOS, le L-NAME, pour déterminer si le NO dans l'intestin murin pouvait influencer l'adhésion des EHEC aux cellules épithéliales intestinales et/ou à la production des Shiga toxines. Le traitement de souris infectées avec du L-NAME a conduit à une augmentation du nombre des EHEC adhérents récupérés dans

le côlon (Figure 4), ce qui est en adéquation avec nos résultats *in vitro* antérieurs. Cependant, le L-NAME a conduit à un niveau d'activité des Shiga toxines plus faible dans les échantillons fécaux par rapport aux témoins infectés non traités (Figure 5). De plus, nous avons observé que le L-NAME empêchait la chute de la gravité spécifique de l'urine, un marqueur de l'insuffisance rénale, observée chez des souris infectées non traitées avec le L-NAME; ces deux résultats suggèrent un effet inducteur du NO sur l'expression des Shiga toxines, contrairement à nos résultats *in vitro*. Des analyses histologiques rénales complèteront ces résultats et indiqueront si le traitement par L-NAME a entraîné une réduction des dommages rénaux par rapport aux souris témoins infectées.

Plusieurs expériences sont encore en cours, mais nos résultats ont été présentés dans cette thèse comme les parties majeures d'un article qui sera bientôt soumis. L'intitulé et le résumé de cet article figurent ci-après.

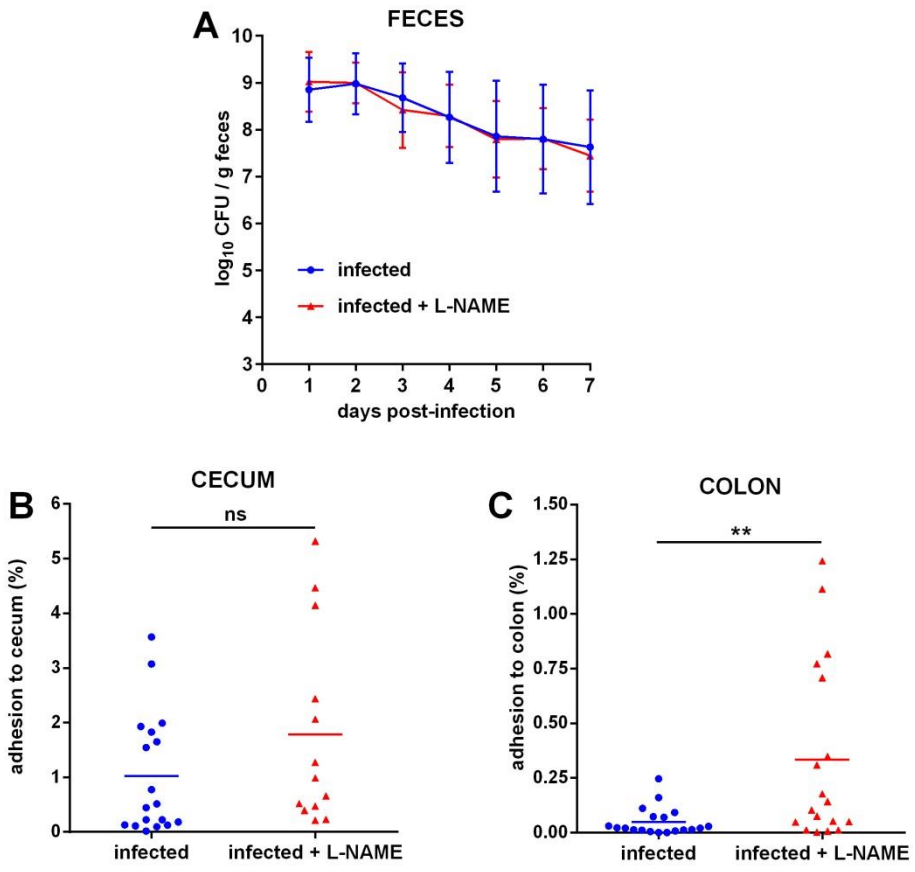

Figure 4. Augmentation de l'adhésion des EHEC a la muqueuse du colon chez les souris infectées et traitées au L-NAME. Les souris, traitées ou non avec le L-NAME, ont été infectées avec la souche EDL933. **(A)** Aux temps indiqués, l'excrétion des EHEC a été quantifiée sur des boites de culture LB + Sm. Les valeurs représentent les moyennes +/- déviation standard. **(B)** and **(C)** Au jour 7 post-infection, les souris ont été euthanasiées et caeca et colons ont été prélevés, laves au PBS, écrasés et étalés sur des boites LB + Sm afin de quantifier les EHEC adhérents à la muqueuse. Les données obtenues sont représentées comme

le pourcentage of de bactéries adhérentes relatif au nombre total d'EHEC quantifiées par échantillon fécal. Chaque point représente 1 souris et la moyenne est indiquée par une ligne. ns: non significatif; ** P<0.01.

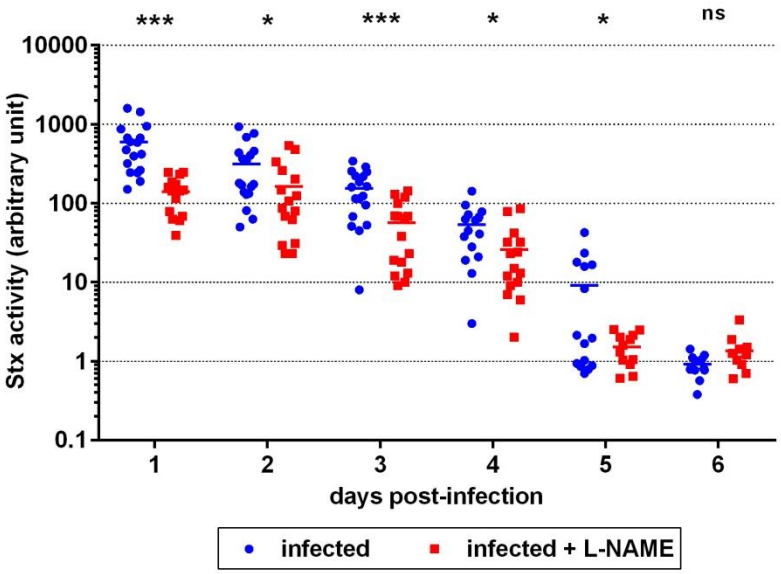

Figure 5. Le traitement au L-NAME limite la toxicité des Shigatoxines dans l'intestin des souris infectées. Les souris, traitées ou non avec le L-NAME, ont été infectées avec la souche EDL933. Aux temps indiques, l'activité Stx obtenue des fèces a été quantifiée en utilisant une lignée Vero-d2EGFP. Chaque point représente 1 souris et la moyenne est indiquée par une ligne. ns: non significatif; * P<0.05; *** P<0.001.

Interplay between enterohemorrhagic *Escherichia coli* and nitric oxide during the infectious process

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Les *Escherichia coli* entérohémorragiques (EHEC) sont des pathogènes bactériens responsables de maladies mortelles chez l'Homme telles que la diarrhée hémorragique et le syndrome hémolytique et urémique. À ce jour, aucune thérapie spécifique n'est disponible et les traitements restent essentiellement symptomatiques. Ces dernières années, nous avons démontré *in vitro* que le monoxyde d'azote (NO), un médiateur majeur de la réponse immunitaire intestinale, réprime fortement la synthèse des deux facteurs de virulence cardinaux des EHEC, à savoir les Shiga toxines (Stx) et le système de sécrétion de type 3, suggérant un fort potentiel du NO à protéger contre l'infection à EHEC. Dans cette étude, nous avons étudié l'interaction entre NO et EHEC *in vivo* en utilisant la souris comme modèle d'infection. En utilisant une souche reportrice, nous avons déterminé que les EHEC détectent le NO dans l'intestin des souris infectées via la protéine NsrR, et que ce processus est essentiel pour une colonisation efficace de l'intestin par le pathogène. Le traitement des souris infectées avec un inhibiteur spécifique de la NOS a augmenté l'adhésion des EHEC à la muqueuse colique mais a diminué de manière inattendue l'activité de Stx dans le tractus gastro-intestinal, protégeant les souris de l'insuffisance rénale. L'ensemble de nos données indiquent que le NO peut à la fois avoir des conséquences bénéfiques et préjudiciables sur le

devenir d'une infection à EHEC et soulignent l'importance des études *in vivo* pour augmenter nos connaissances dans les interactions hôte-pathogène.

Conclusions et perspectives de la thèse

Les *E. coli* pathogènes provoquent une morbidité et une mortalité significatives dans le monde entier. Différentes sous-espèces d'*E. coli* ont en effet évolué pour prospérer en tant qu'agents pathogènes performants: elles peuvent persister à la fois dans l'hôte et dans l'environnement, posséder de multiples résistances aux antibiotiques, diversifier rapidement leur répertoire antigénique, et échapper au système immunitaire. Ces caractéristiques remarquables ont entravé nos efforts pour trouver des thérapeutiques efficaces et/ou à long terme. Le programme Marie Sklodowska-Curie DISCo a recruté quatre boursiers de doctorat, dont je faisais moi-même partie, afin de relever le défi passionnant et ambitieux de mettre en place les bases pour un nouveau vaccin à large spectre prometteur contre les *E. coli* pathogènes. Fruit d'une collaboration entre GSK et l'INRA, les projets développés étaient destinés à identifier de nouveaux antigènes chez les *E. coli* pathogène pour développer un vaccin, caractériser la réponse immunitaire après immunisation avec des antigènes d'*E. coli* pathogènes, et sélectionner des adjuvants pour améliorer efficacement la réponse aux immunisations.

Le premier objectif de mon projet de thèse était de caractériser la réponse immunitaire à l'antigène SslE, une métalloprotéase d'*E. coli* dégradant la mucine et qui avait déjà été identifiée comme un candidat de vaccin prometteur contre diverses souches d'ExPEC. Dans le but d'utiliser éventuellement cet antigène dans un vaccin contre les ExpEC et InPEC, notre stratégie consistait à concevoir un protocole d'immunisation avec SslE qui induirait à la fois une réponse immunitaire intestinale et une réponse immunitaire systémique. Par conséquent, nous nous sommes concentrés sur la recherche de la voie d'immunisation appropriée qui induirait le plus fortement une réponse à la fois mucosale et systémique à notre antigène. À cet effet, j'ai mis en place trois protocoles d'immunisation, comprenant trois immunisations par protocole, que j'ai effectués en parallèle: trois doses intranasales (i.n), trois doses intramusculaires (i.m), ou une dose i.n suivie de deux doses i.m (i.n / i.m / i.m).

De par les divers paramètres immunologiques analysés, nous avons montré que le régime d'immunisation mixte (i.n / i.m / i.m) avec SslE était notre meilleur modèle d'immunisation, comparé aux modèles d'immunisation avec trois doses i.n ou trois doses i.m. En effet, les souris immunisées en i.n / i.m / i.m avec SslE ont développé des réponses immunitaires cellulaires et humorales robustes spécifiques pour SslE, et ce au niveau local et systémique. Spécifiquement, nous avons obtenu: i) des cytokines de type Th dans les lymphocytes de la lamina propria après restimulation avec SslE, ii) des titres d'IgA secrétés (SIgA) anti-SslE dans les lavages intestinaux et les fèces iii) des lymphocytes T $CD4^+$ spécifiques pour SslE

dans les splénocytes, et iv) des titres d'IgG anti-SslE dans les sera post-immunisation. Certaines réponses immunologiques de l'intestin grêle, notamment les cytokines de type Th17 et les SIgAs provenant des lavages intestinaux, étaient comparables entre les modèles d'immunisation i.n / i.m / i.m et i.n / i.n / i.n. Plusieurs études ont observé des réponses Th17 et IgA mucosales post-infection avec divers InPEC (Wenneras, Qadri et al 1999, Atarashi, Tanoue et al., 2015, Mc Arthur, Maciel et al., 2017), résultats qui confirment nos observations pour les modèles d'immunisation mixte et i.n / i.n / i.n. Cela dit, les réponses immunitaires systémiques des cellules B et des lymphocytes T obtenues à partir du régime d'immunisation mixte ont surpassé de manière significative celles provenant du régime i.n ou du régime i.m. Ces derniers résultats ont fortement influencé notre évaluation de l'efficacité des différents systèmes de vaccination; si nous envisagions l'utilisation de l'antigène SslE dans le cadre d'un vaccin à large spectre contre les InPEC et les ExPEC, une réponse immunitaire systémique serait particulièrement importante pour contrer les pathotypes d'*E. coli* capables de se disséminer dans le sang, tels que les NMEC et les UPEC.

Sachant que le gène codant pour SslE est connu pour être présent dans certaines souches commensales d'*E. coli*, nous avons cherché à analyser l'impact potentiel des immunisations SslE sur la composition du microbiote intestinal. En utilisant le régime d'immunisation i.n / i.m / i.m, nous avons montré que ces immunisations avec SslE ne modifiaient pas significativement la richesse ou la composition du microbiote fécal chez les souris immunisées versus naïves, puisque nous avons retrouvé les mêmes familles et genres entre ces deux groupes pré- et post-immunisation. De même, nous n'avons trouvé aucune différence significative entre le microbiote caecal des souris immunisées et les souris naïves post-immunisation.

Dans l'ensemble, le programme d'immunisation i.n / i.m / i.m avec l'antigène SslE a induit une bonne réponse immunitaire intestinale et systémique spécifique pour SslE sans perturber significativement le microbiote résidant dans l'intestin. Ces résultats justifieraient des études supplémentaires afin de démontrer la pertinence de l'utilisation de l'antigène SslE en tant que composant d'un vaccin à large spectre contre les *E. coli* pathogènes.

Une importante prochaine étape de notre étude est de fournir une preuve de protection contre une infection avec *E. coli* après un régime d'immunisation i.n / i.m / i.m avec SslE. SslE a déjà été utilisé dans des études d'immunisation suivies par une infection, que je discuterai ici brièvement. SslE a montré une efficacité protectrice de 82% contre une souche NMEC (IHE3034, la souche à partir de laquelle le gène codant pour SslE a été sélectionné) par immunisation de souris trois fois par voie sous-cutanée (Moriel, Bertoldi et al., 2010).

Une autre étude a utilisé des souris immunisées uniquement par voie intranasale puis soumises à un gavage oral avec une souche ETEC, ce qui a entraîné une diminution de plus de deux log de CFU au niveau du caecum. De plus, des souris immunisées avec SslE (et la toxine cholérique comme adjuvant) et infectées transurétriquement avec une souche UPEC présentaient une réduction significative des CFU dans le rein et la rate par rapport aux souris naïves, bien qu'il n'y ait pas eu de réduction des CFU dans la vessie (Nesta, Valeri et al., 2014). Ces différentes études d'immunisation utilisaient des espèces de souris, des quantités d'antigène SslE, des durées entre les immunisations, ainsi que des voies d'immunisation différentes comparées à notre étude d'immunisation présentée dans ce projet doctoral. Compte tenu de cette variabilité de paramètres, il est difficile de faire des comparaisons spécifiques entre ces études et les nôtres; par conséquent, effectuer des tests d'infection pour valider l'efficacité de notre régime d'immunisation i.n / i.m / i.m avec SslE serait une étape essentielle vers le développement d'un vaccin utilisant SslE.

Parmi les différents pathotypes d'*E. coli* que nous pourrions tester, il semble important de valider une efficacité protectrice sur un modèle d'infection ExPEC ainsi qu'un modèle d'infection InPEC. Des résultats antérieurs obtenus sur un modèle d'infection UPEC pourraient être utilisés comme moyen de comparaison et déterminer un potentiel avantage de protection de notre modèle d'immunisation i.n / i.m / i.m. A cet égard, une réduction de la colonisation de la vessie par rapport aux souris naïves serait particulièrement encourageante. Les infections par des souches d'InPEC chez les souris présentent des défis, car de nombreux pathotypes sont de mauvais colonisateurs de l'intestin murin et ne provoquent pas de maladie. Pour contourner ce problème, des modèles murins d'infections, tels que des modèles EPEC, ETEC ou EHEC, ont été établis chez des souris prétraitées avec de la streptomycine ou chez des souris axéniques (Savkovic, Villanueva et al., 2005, Allen, Randolph et al. 2006, Eaton, Friedman et al., 2008). Compte tenu de l'impact significatif du microbiote sur le développement de la réponse immunitaire intestinale, l'utilisation de souris axéniques ne pourrait être envisagée que dans des expériences d'immunisation passive par transfert de lymphocytes T ou d'anticorps sériques avant infection. Une telle configuration serait intéressante dans le but de déterminer une corrélation de protection, bien qu'elle ne permette malheureusement pas le transfert de l'immunité résidente mucosale, qui représente probablement une composante importante derrière l'efficacité de la protection contre les *E. coli* pathogènes exprimant SslE. Nous utiliserions donc très probablement un modèle d'infection à la streptomycine. L'utilisation d'un modèle EPEC comme modèle d'infection post-immunisation, qui n'a pas encore été réalisée dans notre groupe, pourrait fournir des données complémentaires intéressantes dans notre étude.

Un élément crucial dans l'élaboration des vaccins antigéniques est l'addition d'un adjuvant dans la formulation. Dans notre étude, l'utilisation de la toxine cholérique (CT) comme adjuvant pour les immunisations intranasales a été choisie de par la caractérisation approfondie de la CT en tant qu'adjuvant mucosal performant (Lycke et Holmgren 1986, Mattsson, Schon et al. 2015, Tsai et Wu 2015). Un autre adjuvant mucosal efficace, similaire à la CT, est l'entérotoxine LT des ETEC (Lycke, Tsuji et coll., 1992, Katz, Lu et al., 1997); cependant, la toxicité connue de la CT et de la LT empêche leur utilisation dans les vaccins humains. Plusieurs tentatives ont été faites pour produire des variantes de CT et LT ayant une toxicité atténuée (Freytag et Clements 2005, Norton, Lawson et al., 2012). Une variante de la toxine LT (dmLT) a été largement étudiée quant à son utilisation en tant qu'adjuvant mucosal efficace et non toxique, et les résultats ont jusqu'à présent été très prometteurs (Norton, Lawson et al., 2011). La dmLT possède une double mutation de la sous-unité A, ce qui réduit considérablement la toxicité induite par la LT tout en conservant la forte adjuvanticité de la LT (Norton, Lawson et al., 2011). Dans des modèles animaux, la dmLT a été utilisé en formulation avec plusieurs antigènes, tels que *Helicobacter pylori* et la polio inactivée, et s'est révélée très efficace pour renforcer les réponses immunitaires spécifiques de ces antigènes (Summerton, Welch et al 2010, Norton, Bauer et al., 2015). En outre, la dmLT a été incorporée comme adjuvant pour un vaccin contre une souche d'ETEC dans des études cliniques humaines; le résultat de cette étude a révélé que les formulations étaient bien tolérées et que la dmLT pouvait non seulement améliorer la réponse immunitaire mucosale et l'efficacité du vaccin, mais également diminuer la dose d'antigène, deux paramètres essentiels dans la sélection d'un adjuvant (Lundgren, Bourgeois et al. 2014, Bourgeois, Wierzba et autres 2016). Les travaux actuels sur la dmLT visent à améliorer de futures formulations avec la dmLT pour minimiser la formation d'agrégats et optimiser la stabilité de la formulation au stockage (Toprani, Hickey et al., 2017). Ainsi, comme la dmLT semble effectivement être un adjuvant mucosale et non-nocif et efficace pour une utilisation chez l'Homme, il serait intéressant de tester la dmLT en tant qu'alternative de la CT dans notre régime d'immunisation i.n / i.m / i.m avec SslE. Stratégiquement, l'ajout de la dmLT dans nos formulations pourrait offrir un avantage non seulement en tant qu'adjuvant, mais aussi en tant qu'antigène des ETEC. En effet, les souris immunisées avec la dmLT en tant qu'adjuvant développent également une réponse sérique contre l'entérotoxine LT native (Norton, Lawson et al., 2011). On sait depuis longtemps qu'une réponse immunitaire spécifique à la LT offre une forte protection, bien que de courte durée, contre les ETEC. D'ailleurs, le vaccin anticholérique Dukoral®, qui cible la CT, peut également servir de vaccin à court terme contre les ETEC, étant donné que Dukoral® offre une protection croisée

significative contre les ETEC productrices de LT (Clemens, Sack et al., 1988, Jelinek et Kollaritsch 2008).

Des études de protection croisée avec l'antigène SslE ont montré une efficacité contre des souches d'ExPEC hétérologues, bien qu'il y ait une diminution de l'efficacité protectrice qui est corrélée avec la diminution de l'identité de la séquence d'acides aminés de la souche hétérologue SslE comparée à SslE de la souche NMEC IHE3034 utilisée pour l'immunisation. De même, bien que la protection contre la colonisation par les souches InPEC ait été observée après immunisation avec SslE, il semble probable qu'une protection complète ne puisse être atteinte, même dans le contexte de notre nouveau régime d'immunisation i.n / i.m / i.m. Enfin, bien que l'analyse de la présence et de l'expression du gène codant pour SslE ait révélé une forte prévalence de SslE dans la plupart des pathotypes extraintestinaux et intestinaux, toutes les souches ne possèdent pas SslE, en particulier dans le cas des EHEC. À partir de ces résultats, nous pensons que SslE devrait être utilisé, non pas en tant qu'antigène unique, mais plutôt dans le cadre d'un vaccin à plusieurs composants afin de maximiser la couverture et l'efficacité contre les InPEC et ExPEC. Dans le cadre du programme DISCo, un projet de doctorat a d'ailleurs été entièrement dédié à la recherche d'antigènes distribués à travers les pathotypes InPEC qui seraient exprimés en surface ou sécrétés, par une approche de vaccinologie inverse similaire à celle utilisée pour identifier SslE. Ce projet a mené à l'identification d'au moins un antigène qui s'est avéré conférer une protection contre une souche EHEC O157:H7 chez des souris immunisées versus naïves. Il serait donc tout à fait intéressant de combiner cet antigène avec SslE dans notre régime d'immunisation i.n / i.m / i.m pour évaluer une couverture potentiellement plus large médiée par cette combinaison.

Une autre perspective intéressante, bien qu'audacieuse, consisterait à incorporer un antigène d'*E. coli* présent dans le core génome, c'est-à-dire un antigène présent dans toutes les souches de *E. coli*, qu'elles soient pathogènes ou commensales. Cette idée a été poursuivie par le laboratoire de Mark Schembri, qui a commencé par définir un core génome ainsi qu'un génome accessoire d'*E. coli* à partir de 1700 génomes d'*E. coli* complets ou partiels disponibles (Moriel, Tan et al., 2016). À partir de cette bibliothèque, ils ont identifié un nouvel antigène, appelé YncE, présent dans plus de 99% de tous les génomes d'*E. coli* disponibles (notons que SslE est présent dans 70% des génomes de cette bibliothèque) et sécrété par plusieurs pathotypes d'*E. coli* différents, ainsi que la souche commensale MG1655. Ils ont ensuite utilisé YncE dans un modèle d'immunisation, où YcnE a conféré une protection significative contre un modèle d'infection de bactériémie par une souche d'UPEC. En outre, YncE est connu pour développer une réponse sérique chez l'homme, tel

qu'observé à partir des sérums des patients uroseptiques convalescents (Moriel, Tan et al., 2016). Cette dernière découverte est particulièrement intéressante car ces patients produisent des anticorps ciblant un antigène potentiellement exprimé par des souches d'*E. coli* faisant partie du microbiote intestinal résident. Bien qu'aucune étude n'ait été réalisée pour évaluer si ces anticorps étaient réellement fonctionnels (anticorps neutralisants), cela soulève la question importante de savoir si l'utilisation d'un tel antigène aurait un effet délétère sur la santé humaine. Dans le cas de SslE, qui est exprimé mais ne semble pas être sécrété par les *E. coli* commensales (du moins, dans le cas de MG1655), nous n'avons trouvé aucun changement significatif dans le microbiote intestinal murin dû aux immunisations. De même, une étude utilisant un antigène d'ETEC n'a pas observé de changement significatif dans la composition et la richesse du microbiote après immunisation. Le travail de Moriel et ses collègues ouvre donc des perspectives intéressantes dans les futurs projets de vaccins contre *E. coli*, et nous pensons que les immunisations utilisant des formulations à la fois avec YncE et SslE méritent d'être explorées.

Dans la deuxième partie de mon projet de thèse, nous avons cherché à étudier l'effet *in vivo* du NO, un composant clé de la réponse immunitaire innée de l'hôte, sur les mécanismes de virulence des EHEC. Ce projet est issu de travaux *in vitro* antérieurs dans notre laboratoire, où le NO présentait des effets inhibiteurs sur la synthèse de deux facteurs de virulence majeurs des EHEC, à savoir Stx2 et le T3SS (Vareille, de Sablet et al., 2007, Branchu, Matrat et al., 2014). Ces résultats suggèrent fortement que le NO peut influencer le résultat d'une infection à EHEC chez l'hôte, et nous a incités à mener des expériences *in vivo* pour confirmer cette hypothèse. Nous avons utilisé une souche EHEC reportrice pour la détection de NO pour montrer que les EHEC pouvaient en effet détecter le NO produit dans l'intestin de la souris ; cette même souche reportrice a détecté très peu ou pas de NO, au moins pendant les quatre premiers jours, lorsque les souris ont été traitées avec le L-NAME, un inhibiteur des NOS. La détection de NO par les EHEC peut en effet avoir une importance dans leur capacité à coloniser l'intestin des souris; en effet, la suppression du régulateur-senseur de NO NsrR réduit le fitness des EHEC, comme nous l'avons observé dans une expérience de compétition. Cela pourrait être dû, au moins en partie, à une inhibition de l'adhésion des EHEC aux cellules épithéliales, comme le suggèrent nos résultats *in vitro* antérieurs (Branchu, Matrat et al., 2014). En conséquence, nous avons dénombré plus de cellules EHEC adhérentes dans le côlon de souris qui ont été traitées au L-NAME sept jours après l'infection. Cependant, nous ne pouvons pas encore conclure que cette augmentation de l'adhésion soit effectivement liée à l'absence de NO, car un traitement au L-NAME aurait également pu entraîner une augmentation compensatoire de l'expression de la iNOS (Miller,

Thompson et al., 1996). Nous explorons actuellement cette hypothèse en établissant une cinétique de l'expression de l'ARNm de la *Nos2* à partir de tissus du côlon de souris infectées traitées au L-NAME par rapport aux contrôles infectés. Une induction compensatoire de l'expression de la Nos2 pourrait également expliquer l'augmentation inattendue de la détection de NO par notre souche EHEC reportrice chez certaines souris du jour 5 au jour 7 post-infection. Si cette hypothèse s'avérait être confirmée, plusieurs approches pourraient être envisagées. Premièrement, plusieurs inhibiteurs spécifiques de la iNOS sont connus et ont été utilisés chez la souris. Par exemple, le GW274150 fonctionne comme un inhibiteur à base d'arginine, bien qu'il ait une structure différente de celui du L-NAME (Vitecek, Lojek et al., 2012). Il a été démontré que GW274150 entraînait une diminution significative et constante du nitrite dans les tissus de pattes enflammées chez des rats pendant au moins 72 heures (De Alba, Clayton et al., 2006), bien qu'une inhibition de la iNOS sur des durées plus longues n'ait pas été étudiée. L'aminoguanidine, un autre inhibiteur préférentiel de l'iNOS, a également été utilisé pour montrer une sensibilité accrue chez les souris à une infection de *Salmonella* (MacFarlane, Schwacha et al., 1999). Cependant, une étude a montré que l'aminoguanidine semble provoquer des effets secondaires indépendants de iNOS qui augmentent la sensibilité des souris à l'infection (Zhou, Potoka et autres 2002); ceux-ci pourraient être dus au fait que l'aminoguanidine interfère avec plusieurs systèmes d'enzymes. Pour éviter ces obstacles potentiels, l'utilisation de souris *Nos2*$^{-/-}$ serait pratique afin de déterminer l'implication de la iNOS et du NO dans l'adhésion et la colonisation des EHEC. Alternativement, nous avons validé dans notre étude actuelle l'utilisation de notre souche EHEC reportrice pour la détection du NO; si tant est que cette souche colonise l'intestin de la souris aussi efficacement que la souche EHEC sauvage, nous pourrions utiliser cette souche EHEC comme outil pour évaluer la présence de NO dans l'intestin, tout en testant son effet sur l'adhésion de l'EHEC sur les cellules épithéliales intestinales. Cette mise en place expérimentale peut représenter le moyen le plus précis de corréler le NO à l'adhésion et la colonisation des EHEC. En outre, ce système permettrait également de tester l'efficacité de divers inhibiteurs de NO, tels que ceux décrits ci-dessus, à inhiber la production de NO - un paramètre qui se trouve être extrêmement difficile à déterminer dans l'intestin de la souris.

Les résultats préliminaires que nous avons obtenus montrent que les souris infectées par la souche EHEC O157:H7 peuvent diminuer l'expression de l'ARNm de la *Nos2* dans le côlon au jour 7 post-infection. Ces résultats, s'ils sont validés, contrastent fortement avec la forte induction d'expression de l'ARNm de la *Nos2* chez des souris infectées par d'autres pathogènes entériques, tels que *Citrobacter rodentium* ou *Salmonella* Typhimurium (Cherayil

et Antos 2001, Vallance, Deng et al 2002, Zhou, Potoka et al., 2002). Bien que l'interaction de *C. rodentium* avec les cellules épithéliales intestinales puisse conduire à une inhibition de la production de NO, les cellules non infectées voisines produisent une quantité importante d'iNOS, qui conduirait globalement à un état pro-inflammatoire avec production de NO (Vallance, Deng et al. 2002). D'autre part, les EHEC interviendraient dans l'inhibition anti-inflammatoire de la production d'iNOS; par conséquent, ces résultats préliminaires indiqueraient qu'il n'est pas forcément approprié de comparer ces deux pathogènes, bien que leur T3SS et leur méthode de colonisation de l'intestin soient pourtant très similaires.

Contrairement à nos résultats *in vitro* antérieurs, le NO semble avoir un effet stimulant sur la production de Stx par les EHEC dans l'intestin murin, car un traitement avec le L-NAME chez des souris infectées a entraîné une production de Stx significativement plus faible que chez des souris infectées mais non-traitées, et ce du jour 1 au jour 5 après l'infection. Cette différence peut s'expliquer par les résultats d'une étude récente, qui a montré que le NO augmentait la production à la fois de Stx1 et de Stx2 chez une souche EHEC cultivée dans des conditions anaérobies (Ichimura, Shimizu et al, 2017). Les intestins étant principalement dépourvus d'oxygène, nos résultats *in vivo* semblent corroborer les résultats *in vitro* d'Ichimura *et al*. En outre, nous avons observé que chez les souris infectées avec EHEC O157:H7 et traitées par la ciprofloxacine, le traitement au L-NAME a entraîné le maintien de la gravité spécifique de l'urine, un marqueur de la dysfonction rénale (par exemple, après exposition à Stx). Si ces résultats sont confirmés par nos analyses histologiques rénales (actuellement en cours), nous aurions des preuves solides indiquant que le NO pourrait en fait aggraver le statut d'une infection par les EHEC dans le modèle murin, car il semble provoquer une augmentation de la production de Stx. Il est à noter que, avec l'induction *in vitro* différentielle bien signalée de *Nos2* dans les macrophages murins et humains, nos résultats peuvent ne pas être valides chez l'Homme. Ainsi, nous pensons que la surveillance de la production de NO et la comparaison des niveaux entre patients infectés par une souche EHEC avec ou sans SHU pourrait être très informative, car une augmentation de la production de NO dans un état inflammatoire élevé pourrait avoir un impact sur le développement du SHU. Le caractère pro-inflammatoire du développement du SHU et des lésions rénales a été bien documenté chez les patients atteints du SHU associé aux EHEC. En particulier, la leucocytose, qu'elle soit circulante ou localisée dans les reins, est considérée comme un facteur prédictif du développement de SHU et de mauvais pronostic (Bell, Griffin et al., 1997, Buteau, Proulx et al., 2000). Dans les reins, la réponse immunitaire de l'hôte, due à une lésion rénale induite par l'apoptose, conduit à une sécrétion importante de cytokines et à un afflux de leucocytes par chimiotaxie. Parmi ceux-ci, l'accumulation de

neutrophiles et de macrophages, qui sont des acteurs majeurs au cours des infections EHEC et le développement du SHU, ont été spécifiquement liés à une mortalité accrue (Walters, Matthei et al., 1989, Coad, Marshall et al. et al., 1997). Les cytokines pro-inflammatoires telles que le TNF-α, l'IL-6 et l'IL-8 sont produites dans les reins et récupérées dans l'urine des patients atteints de SHU (Karpman, Andreasson et al., 1995). Le TNF-α est connu pour être impliqué dans la pathogenèse du SHU; en effet, Stx chez la souris induit la sécrétion de TNF-α dans les reins et augmente la sensibilité rénale à la toxicité du TNF-α (Harel, Silva et al., 1993). En conséquence, le traitement par un inhibiteur du TNF diminue à la fois les lésions rénales et cérébrales (Isogai, Isogai et al., 1998). Ainsi, un état inflammatoire élevé semble être intimement lié à l'apparition et à la progression du SHU chez les patients, bien que la cause exacte sous-jacente de cette susceptibilité ne soit pas encore définie. Néanmoins, nous pourrions supposer que chez les patients développant un SHU, un état hyper-inflammatoire pourrait provoquer, en plus de la leucocytose et de la sécrétion de cytokines, une augmentation de la production de NO et, si nos résultats sont comparables chez la souris et l'Homme, ce dernier provoquerait finalement une aggravation du SHU chez ces patients.

En résumé, nos résultats sont importants non seulement pour la compréhension de l'interaction entre l'EHEC et le système immunitaire inné, mais aussi dans les perspectives thérapeutiques futures contre les maladies provoquées par les EHEC. Évidemment, contrairement à ce que nous avions précédemment supposé de nos travaux *in vitro*, l'effet délétère du NO sur le devenir d'une infection à EHEC observé dans notre étude suggère l'utilisation potentielle d'inhibiteurs de NO pour contrer cet effet. À partir de là, nous pourrions même envisager de combiner un inhibiteur de NO à un antibiotique, avec pour objectif d'éliminer le pathogène tout en évitant la production de Stx.

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List of abbreviations

A/E	attaching and effacing lesion
AFSSA	Agence Française de sécurité sanitaire des aliments
AIEC	adherent-invasive *E. coli*
AMP	antimicrobial peptide
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
APC	antigen-presenting cell
ASC	antibody-secreting cell
BBB	blood brain barrier
Bcl	B cell lymphoma
BCR	B cell receptor
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CD	cluster of differentiation
CDK	cyclin-dependent kinase
cGMP	cyclic guanosine monophosphate
CSR	class switch recombination
CTL	cytotoxic T-lymphocyte
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DAEC	diffusely-adherent *E. coli*
DC	dendritic cell
dIg	dimeric immunoglobulin
EAEC	enteroaggregative *E. coli*
EEC	enteroendocrine cell
EFSA	European Food Safety Authority
EHEC	enterohemorragic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E.coli*
ER	endoplasmic reticulum
ETEC	enterotoxigenic *E. coli*
GALT	gut-associated lymphoid tissues
GATA	GATA-binding transcription factor 3
GC	germinal center
GP	glycoprotein
HC	hemorrhagic colitis
HEV	high endothelial venules
HUS	hemolytic uremic syndrome
IBC	intracellular bacterial communities
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
iNOS	inducible nitric oxide synthase
InPEC	intestinal pathogenic *E. coli*
LCMV	lymphocytic choriomeningitis virus
LEE	locus of enterocyte effacement
LFA	lymphocyte function-associated antigen
LP	lamina propria
LPS	lipopolysaccharide
Lti	lymphoid tissue-inducer
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MUC	mucin
NK	natural killer cell
NLR	NOD-like receptor
NMEC	neonatal meningitis *E. coli*
NO	nitric oxide

ExPEC	extra-intestinal pathogenic *E.coli*
FAD	flavin adenine dinucleotide
FAE	follicle-associated epithelium
FDC	follicular dendritic cells
FMN	flavin mononucleotide
FNR	fumarate nitrate reduction
Gad	glutamate decarboxylase
PP	Peyer's patch
PRR	pattern recognition receptor
RA	retinoic acid
REGIIIγ	regenerating islet-derived protein IIIγ
SC	secretory component
SED	subepithelial dome
SFB	segmented filamentous bacteria
SHM	somatic hypermutation
SIgA	secreted IgA
SILT	solitary isolated lymphoid follicle
STAT	signal transducer and activator of transcription
STEC	Shiga toxin-producing *E. coli*
T3SS	type III secretion system
T_{CM}	central memory T cell
NOD	nucleotide binding oligomerization domain
NorR	nitric oxide reductase regulator
NOS	nitric oxide synthase
NsrR	nitric oxide sensitive repressor
OVA	ovalbumin
pIgR	polymeric immunoglobulin receptor
PNAd	peripheral lymph node addressin
TCR	T cell receptor
TCS	two-component system
T_{EM}	effector memory T cell
T_{FH}	follicular T helper cell
TGFβ	tumor growth factor β
T_H	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TTP	thrombotic thrombocytopenic purpura
Treg	regulatory T cell
T_{RM}	resident memory T cell
UPEC	uropathogenic *E. coli*
USDA	United States Department of Agriculture
UTI	urinary tract infection

Introduction

In the world of survival and adaptation, *Escherichia coli* has more than one trick up its sleeve. Considering *E. coli* in its globality, we face a bacterium that has evolved from an innocuous human gut commensal, to nine different pathogenic variants (and counting) capable of colonizing the gut, bladder, kidney, blood, or brain, and have become major etiological agents of diseases ranging from diarrhea to recurrent urinary tract infections and neonatal meningitis. For children under five years of age, these diseases stand significant risks of being fatal, especially in developing countries where sanitation and antibiotics are not necessarily available. Furthermore, hospital care and antibiotic prescriptions amount to tremendous healthcare costs, along with the rise of multidrug resistance spanning all *E. coli* pathotypes. Today, extensive efforts are dedicated to deciphering the mechanisms of virulence of these pathotypes and the response of the host; these findings, in turn, can yield valuable insights in the search for new therapeutics, or even better, for the design of a vaccine targeting one or several variants of pathogenic *E. coli*. These research prospects were at the center of the Marie Sklodowska-Curie program DISCo, in which four PhD projects were designed to work towards a broad spectrum vaccine against pathogenic *E. coli*. My goal as a DISCo PhD fellow was two-fold: to characterize the murine immune response to a highly prevalent *E. coli* antigen, and to investigate the *in vivo* interaction between the enterohemorrhagic *E. coli* O157:H7 and a key molecule of the inflammatory response, nitric oxide.

In a first introductory part, I review in a non-exhaustive fashion the major themes surrounding my PhD thesis project. I begin by describing the immunity of the intestinal mucosa, with a brief overview of the interplay between host immunity and the intestinal microbiota. I then go over the various chemical and physiological properties of nitric oxide, as well as the response of *E. coli* to this immunologically important molecule. Finally, I briefly present the major *E. coli* pathotypes, while placing a particular emphasis on enterohemorrhagic *E. coli* and its virulence factors.

The results' part of my dissertation are dedicated to the two manuscripts reflecting my PhD research from the two institutions I worked with. My project at GSK in Siena, Italy was to describe the immune response to the antigen SslE, a potential vaccine candidate against intestinal and extraintestinal pathogenic *E. coli*, and the impact of such immunization on the murine intestinal microbiota. This project has been described in a publication that was submitted to the journal Vaccine. My project at INRA in Saint-Genès-Champanelle, France, was to investigate the effect of nitric oxide on the colonization and outcome of *E. coli* O157:H7 infection in mice. This project has been described in a publication, which will be submitted after the last results have been collected and analyzed.

Finally, I present a general conclusion and discussion of my PhD work, expanding my research to future directions, both short and long-term.

Bibliographical review

I. Mucosal immunity to intestinal bacterial infections

The gut represents the largest surface that the host exposes to the outside world, and this world is far from being friendly. Considering the fact that it handles all of our nutritional intake, received loads of endogenous secretions such as hydrochloric acid, bile salts and digestive proteases, while hosting trillions of microorganisms, the gut is constantly challenged by foreign entities and subject to potential assault. To ensure efficient digestive absorption, maintain host tissue homeostasis, and protect the body from potential microbial invaders, the intestinal mucosal barrier has evolved to be a highly dynamic immune border that carefully monitors its environment and fine tunes its response to build tolerance for dietary antigens or commensals, but also rapidly respond to harmful microbes that have entered the intestinal lumen and/or breached the mucosal barrier. As we might expect, this comes with tremendous sensing and regulatory mechanisms along the intestinal mucosal barrier, and involves a constant dialogue between many specialized cells of the innate and adaptive immune system. In this regard, the gut microbiota is a critical regulator of both intestinal homeostasis and the immune system.

In this chapter, I will discuss the main physiological and immune functions of the intestinal mucosa, focusing on the small intestine, and emphasizing on the various aspects of innate and adaptive immunity that ensure intestinal integrity and effective protection of the host. Finally, I will discuss the major roles of the gut microbiota in immunity and inflammation.

A. The intestinal mucosa: general overview

As part of the single continuous tube of the gastrointestinal tract, the intestines stretch from the stomach to the anus, along which nutrients, minerals and water are absorbed into the body. To accommodate for these specific aspects of digestion, the intestines are divided into several regions different both in structure and function. The small intestine runs from the pylorus at the base of the stomach, and is divided into the duodenum, going downwards to the jejunum and ileum. Most of the nutrients and metabolites are absorbed in the small intestine, which measures 6-7 meters in length and about 2.5-3.0 centimeters in diameter. From the ileocaecal valve, the large intestine starts with the caecum, then the ascending (or proximal), transverse, and descending (or distal) colon, and finishes with the rectum and anus. Unlike the small intestine, the large intestine is only about 1.5 meters in length but is thicker with a 6-7.5 centimeter diameter. The large intestine handles very little digestive function, and is specialized in water and electrolyte reabsorption. It is also the site where undigested food elements are formed for elimination from the body.

Throughout the entire intestinal tract, the intestinal mucosa is composed of three distinct layers: the intestinal epithelium, the lamina propria (LP), and the muscularis mucosae. The intestinal epithelium is a single layer of columnar epithelial cells for efficient nutrient absorption. It is also a physical barrier against foreign and potentially hazardous agents, and must therefore maintain its integrity in order to protect the host from any assault. Underneath the epithelium lies the LP, a layer of connective tissue where blood vessels, lymph vessels and nerves are located, and where many cells of the innate and adaptive immunity reside. These cells are in constant interaction with cells of the intestinal epithelium to sense microbial stimuli and mount the appropriate response, by either tolerating the foreign agent in the case of resident microbiota, or starting the process of inflammation and subsequent elimination in the case of an invading pathogen. Finally, the muscularis mucosae is a thin layer of muscle cells separating the LP from the submucosa.

B. Functions of the intestinal epithelium

1. A digestive interface

The specific digestive role of each intestinal segment is reflected by their unique epithelial layout. Indeed, the small intestinal epithelium displays up-and-down structures, forming villi and crypts of 0.5-1.5 mm in length. The tips of the villi are composed of absorptive epithelial cells, or enterocytes, which are the main component of intestinal epithelial cells (IECs). Enterocytes also form microvilli on their apical sides towards the lumen, forming a brush border atop the epithelium layer where essential enzymes assume digestion of dietary components. Together, villi and microvilli increase the absorptive surface area of the small intestine by ~600 fold, accounting for a considerable gain in exchange and absorptive surface available in the small intestine. Along the length of the small intestine, villi decrease in length and the brush border decreases in thickness from the duodenum and jejunum to the ileum, which handles much less digestive function. In the large intestine, where little to no digestive function takes effect, villi and microvilli are absent.

Enteroendocrine cells (EECs) are another important component of the digestive machinery in the gut. These highly specialized secretory cells are scattered throughout crypts and villi of the epithelium surface, representing less than 1% of the intestinal epithelium composition. EECs link the central and enteric nervous system, and produce peptide hormones or signaling molecules that regulate digestive functions. EECs are further categorized in different cell types depending on their morphology, localization along the GI tract, and peptide hormone secretion (Gunawardene, Corfe et al. 2011).

2. A highly dividing border

The intestinal epithelium is constantly renewed; in fact, it is the most actively dividing interface of the human body, with a 4-5 days turnover rate (van der Flier and Clevers 2009). Replenishment of epithelial cells is ensured by pluripotent intestinal epithelial stem cells (IESCs) that are located at the base of the crypts (Gordon 1993). IESCs undergo self–renewal and generate highly proliferative daughter cells, which can completely renew the epithelium within a week (Clevers and Bevins 2013). Most of the newly generated cells move up from the highly proliferating transit zone in the crypt to the villus, and will differentiate along the way to any of the IECs that the intestinal epithelium hosts: enterocytes, EECs, as well as goblet cells, tuft cells, and M cells, which will be further described in this chapter. Paneth cells, on the other hand, are long-lived and stay down in the bottom of the crypt interspersed between the IESC pool. Several studies have showed that Paneth cells provide important niche signals for IESCs (Garabedian, Roberts et al. 1997, Sato, van Es et al. 2011). A schematic view of the intestinal mucosa and the IECs is represented on Figure 1.

3. An Immunological barrier

Because the intestinal epithelium is in direct contact with the outside environment, it is of crucial importance to maintain it out of reach from microbial organisms reaching the gut, whether they are commensals or pathogens. Hence, the host has evolved powerful mechanisms to keep microbes at bay, segregated from the epithelial layer and the underlying the mucosa.

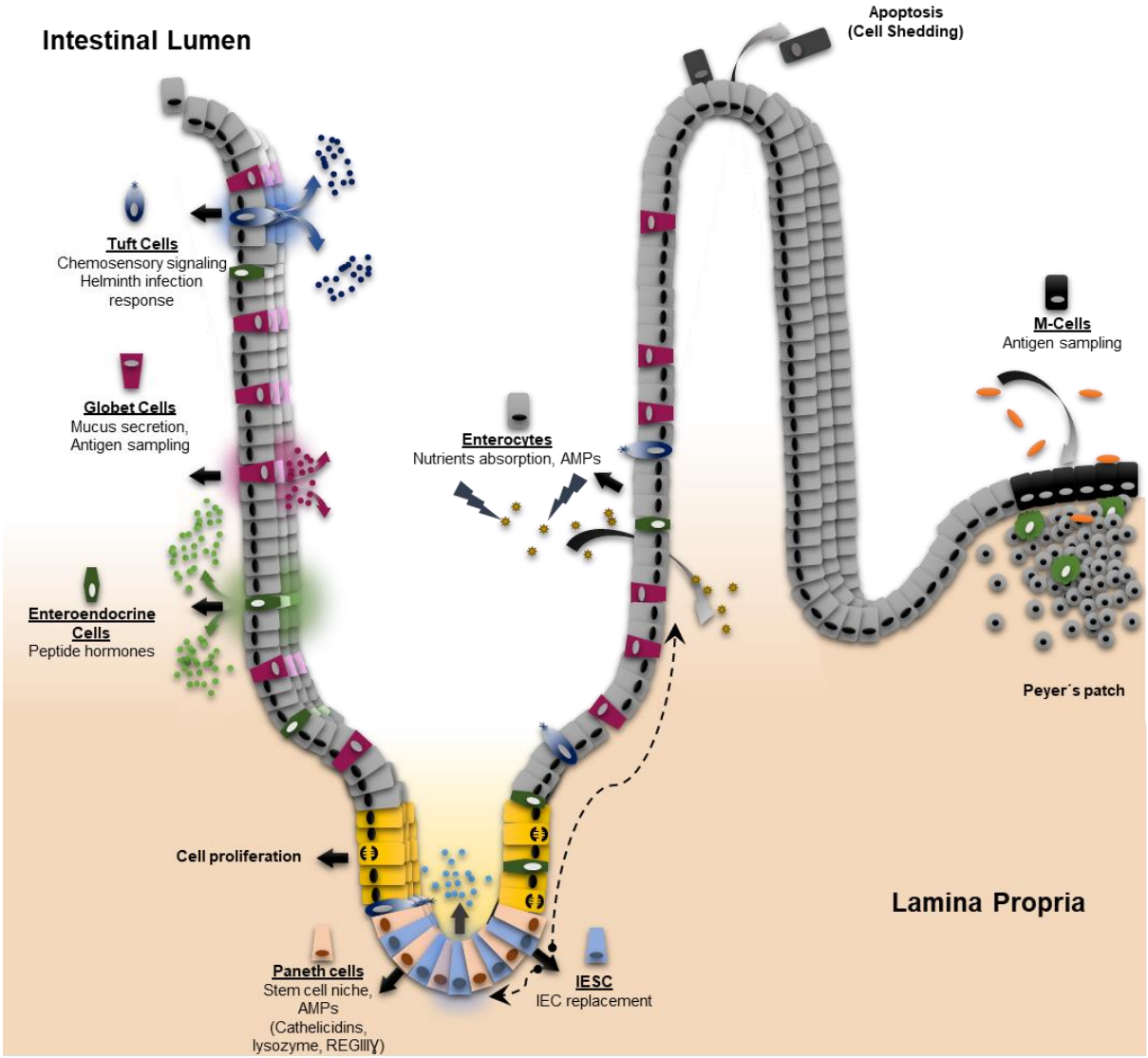

Figure 1. Organization of the intestinal epithelium. *Intestinal epithelial stem cells (IESC) located at the crypt of villi can replenish the entire epithelium with intestinal epithelial cells (IECs) or by differentiating into the many specialized cells composing the intestinal epithelium border. Most cells move up into the cell proliferation zone (highlighted in yellow) to divide, then move up to differentiate to various cell types. Paneth cells are also derived from IESCs but remain down in the crypt. AMPs: antimicrobial peptides; REGIIIγ, regenerating islet-derived protein IIIγ. Adapted from (Gerbe, van Es et al. 2011).*

a) Mucus and mucins

The first physical line of defense that incoming microorganisms will encounter is mucus, a thick matrix covering the entire surface of the intestinal mucosa. The major components of mucus, besides water, are mucins. These glycoproteins are extensively decorated with O-linked glycans, which bind water to make the gel-like consistency of mucus. The bottle brush structure these carbohydrates create a thick matrix, representing a tough layer to cross for microbes.

Mucins are divided into two main groups: gel-forming mucins are secreted into the mucus layer, while transmembrane mucins remain attached to the apical surface of epithelial cells to form a local diffusion barrier called glycocalyx. MUC2 is by far the most abundant secreted mucin in the intestine, while MUC5B is expressed in lower amounts and mostly, if not only, in the colon. Transmembrane mucins include MUC3, MUC4, MUC12, MUC13 and MUC17 that are constitutively expressed, while MUC1 and MUC16 are only upregulated during infection (Johansson and Hansson 2016).

Goblet cells are the professional mucin-secreting IECs in the intestines. These specialized cells are found throughout the epithelial surface, representing about 25% of its composition. Goblet cells constitutively produce MUC2 and MUC4 to replenish mucus and maintain a protective layer between the microbial community and the epithelium. Enterocytes also have an important role in glycocalyx formation. Indeed, they can produce MUC3, MUC12 and MUC17: MUC3 is present throughout the intestine, while MUC12 is more confined in the colon and MUC17 in the small intestine.

Not only are mucins differently expressed between small and large intestine, but the structure of the mucus layer is also different. The colon, which hosts an abundant bacterial community, has two layers of mucus: an inner mucus layer firmly attached to the epithelium, topped by a looser outer mucus layer. The inner layer is densely packed and remains in principle mostly sterile by physically preventing microbial entry (Johansson, Phillipson et al. 2008). Accordingly, the colon has a much higher number of goblet cells present in the epithelium compared to the small intestine. In the small intestine, we only find a single layer of loosely packed mucus, resembling the colonic outer mucus layer. The small intestinal mucus layer is porous enough that it can allow bacterial entry; however, the intestinal epithelium ensures a proper segregation between bacteria and the epithelium surface by the addition of biologically active, non-inflammatory molecules directly targeting the microbiota: antimicrobial peptides, and secretory antibodies.

b) Antimicrobial peptides

The barrier function of the mucus is further enhanced by the secretion by IECs of antimicrobial peptides (AMPs). Paneth cells are the true specialized IECs in the production of AMPs, and ensure a sterile environment at the base of the crypts. AMPs usually target highly conserved structures in bacteria. Defensins (called cryptdins in mice) and cathelicidins are pore-forming AMPs targeting all bacterial membranes. C-type lectins target the peptidoglycan cell wall of Gram-positive bacteria, but are inefficient against Gram-negative due to the inhibitory effect of lipopolysaccharide (LPS) (Mukherjee, Zheng et al. 2014). The importance

of AMPs in the protection of the host is exemplified by the C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ). REGIIIγ was found to be essential to maintain a physical segregation between the microbiota and the host of about 50 µM (Vaishnava, Yamamoto et al. 2011). Additionally, *RegIIIγ*$^{-/-}$ mice showed altered mucus distribution (Loonen, Stolte et al. 2014); as REGIIIγ can bind to mucin-associated glycans, it could indeed have a direct role in the small intestinal mucus distribution. The regulation of REGIIIγ expression and maintenance of this "sterile barrier" is done by a direct sensing of gut commensals by Paneth cells (Cash, Whitham et al. 2006), which respond by modulating REGIIIγ expression (Vaishnava, Behrendt et al. 2008). Furthermore, REGIIIγ is also produced by non-secretory cells of the epithelium: enterocytes and γδ intraepithelial lymphocytes (IELs). γδ IELs, along with αβ IELs, are the only lymphoid adaptive immune cells present on the epithelium surface; their nomenclature reflects the nature of their T-cell receptor or TCR (γδ TCR versus the more classical αβ TCR) that recognize a specific antigen. γδ IELs actively interact with enterocytes and participate in intestinal homeostasis and protection. Their expression of REGIIIγ is an indirect response after bacterial stimulation in neighboring enterocytes (Ismail, Severson et al. 2011).

c) Immunoglobulin A transport

Enterocytes also serve as transport and release of secretory immunoglobulins A (IgAs) from the LP to the intestinal lumen. IgAs are secreted by differentiated B cells (and will be reviewed later in this chapter) in the LP along with a J chain that links two IgA units to form dimeric complexes (dIgAs). dIgAs bind to the polymeric immunoglobulin receptor (pIgR) located at the basolateral surface of enterocytes, which mediate transcytosis to the apical side of enterocytes. Secreted IgAs (SIgAs) are released to the lumen after endoproteolytic cleavage of pIgR near the plasma membrane on the apical surface, forming the secretory component (SC) which remains attached to the SIgAs (Mostov 1994). The outpour of SIgAs into the mucus and the lumen provides an additional mode of protection of the host from microbes. Though IgAs operate via many different mechanisms of action, IgAs mostly promote immune exclusion. For example, SIgAs can entrap microorganisms (or unwanted dietary antigens) in the mucus in a SC-dependent manner (Phalipon, Cardona et al. 2002); additionally, SIgAs can downregulate expression of proinflammatory epitope on the surface of commensals (Peterson, McNulty et al. 2007). A remarkable portion of the gut resident microbiota gets "coated" by SIgAs, which are mostly polyreactive SIgAs, i.e are capable of binding multiple antigens on the bacterial surface of commensals (Bunker, Flynn et al. 2015, Fransen, Zagato et al. 2015, Bunker, Erickson et al. 2017). The low-affinity of microbiota-targeting IgA ensures a more efficient immune exclusion of gut commensal without

overwhelming the immune system by mounting specific IgA for every organism and dietary antigen encountered.

4. A selective “border patrol”

Despite its protective properties, the mucus layer does not completely prevent microorganisms from reaching the epithelial surface. In fact, some members of the gut microbiota manage to thrive in the mucus layer, which constitutes a rich source of carbohydrates for those who have the necessary enzymes to digest them (Sonnenburg, Xu et al. 2005, Ouwerkerk, de Vos et al. 2013). In return, bacteria can produce some key metabolites for the physiology of the host, such as butyrate, acetate and propionate (Donohoe, Garge et al. 2011, Ouwerkerk, de Vos et al. 2013). Furthermore, some bacterial pathogens have evolved mechanisms to breach into the mucus layer and effectively make their way to intestinal epithelial surface; a prime example of such mechanisms is the expression of mucinases, capable of degrading mucins. Thus, whether the bacteria reaching the epithelial surface are friends or foes, it is of crucial important for the epithelium to constantly monitor the presence and composition of the microbiota, and engage a signaling pathway to warn the immune cells of the LP to either tolerate, or mount of proinflammatory response. Additionally, and perhaps counterintuitively, the intestinal epithelium also takes a proactive approach by regularly sampling the luminal content and feed the LP immune cells underneath with information on its inhabitants.

a) Microbial sensing

The host has evolved a very efficient mechanism of innate immune sensing via the expression of Patten Recognition Receptors (PRRs). PRRs recognize microbial components, called Microbe-Associated Molecular Patterns or MAMP (the more traditional Pathogen-Associated Molecular Patterns or PAMP did not consider the interaction between IECs and commensals) that are essential for microorganisms to survive and therefore can hardly be altered. PRRs count Toll-like receptors (TLRs), nucleotide binding oligomerization (NOD)-like receptors (NLRs) and the RNA helicases retinoic acid-inducible gene–I (RIG-I)-like receptors (RLR).

All IECs (as well as immune cells) come equipped with PRRs; based on current research, a list of the known TLRs, NLRs, and RLRs is provided in Table 1. Based on their localization, PRRs will be targeting specific types of microogranisms; RLRs and NLRs are all cytosolic,

and will recognize viral RNA and peptidoglycan fragments from intracellular bacterial pathogens, respectively. TLRs, which count the highest numbers of receptors known, can either be expressed on the surface membrane or on endosomal membranes, as detailed in the table. Of note, not all IECs necessarily carry all listed PRRs; for example, enterocytes express both NOD1 and NOD2, while Paneth cells only express NOD2 (Perez-Lopez, Behnsen et al. 2016). Interestingly, EECs, which were mostly considered to be sensing luminal nutrients, have been shown to express multiple functional TLRs (Bogunovic, Dave et al. 2007) and respond to important bacterial antigens such as lipopolysaccharide (via TLR4) and flagellin (via TLR5) (Selleri, Palazzo et al. 2008).

PRR activation by the appropriate ligand results in a signaling cascade event inside the cell; although different depending on the PRR family, the outcome is most frequently the upregulation of proinflammatory cytokines and other effector molecules (Akira, Uematsu et al. 2006). Cytokines are secreted proteins that play an important role in intercellular communication in immunity, but also in many other physiological or pathological contexts. Here, cytokines are the messengers linking the intestinal epithelium to the innate and/or adaptive immune cells of the LP. Depending on the nature of the cytokine or effector secreted, the epithelium will modulate the immune response from the LP; I will discuss this part further in another section below. Importantly, cytokines and effector molecules triggered by PRRs can also have a direct or indirect effect on IECs themselves, and will enhance protection of the epithelial surface by increasing tight junctions' formation between IECs, upregulating expression of AMPs, or promoting epithelium repair, to cite a few.

In the example of TLRs, all except TLR3 involve the adaptor protein MyD88 in their signaling cascade; studies using MyD88$^{-/-}$ mice in models of infection have shown a significant decrease of inflammation, but resulted in more extensive injury, showing the importance of TLR signaling in epithelium tissue homeostasis (Lebeis, Bommarius et al. 2007, Gibson, Ma et al. 2008).

Table 1. Main studied PRRs with their localization and functions in IECs. *Adapted from (Mowat and Agace 2014).*

PRRs	Expression in IECs Human/mouse	Expression pattern	Ligands	Sources	In vivo effect on IECS
TLRs					
TLR1	+ / ND	Surface membrane	Triacyl lipoproteins	Mycobacteria	
TLR2	+ / +	Surface membrane	Peptidoglycan, lipoteichoic acid	Gram-positive	• IEC injury protection • M cell transport • Mucin crosslinking • Tight junction integrity
TLR3	+ / +	Endosomal membrane	Double-stranded RNA	Viruses	• IEC IL-15 production • IEL cytotoxicity
TLR4	+ / +	Basolateral and endosomal membrane	Lipopolysaccharide, Mannan	Gram-negative bacteria, fungus, virus	• IEC injury protection • M cell transport
TLR5	+ / +	Basolateral membrane, apical membrane?	Flagellin	Flagellated bacteria	• Chemokine/cytokine production • Apoptosis protection • Bacterial overgrowth prevention
TLR6	+ / ND	Surface membrane	Diacyl lipopeptides, Zymosan	Mycoplasma, fungus	
TLR7	+ / ND	Endosomal membrane	Single-stranded RNA	Viruses	
TLR8	+ / ND	Endosomal membrane	Single-stranded RNA	Viruses	
TLR9	+ / +	Endosomal membrane	CpG ODN	Bacteria, viruses	• Paneth cells degranulation • AMP production • IEC injury protection
TLR 10	+ / +	Surface membrane	Not determined	*Listeria monocytogenes*	Inflammation chemokines
TLR11/12	- / +	Endosomal membrane	Profilin	*Toxoplasma gondii*	Activation of NK cells
NLRs					
NOD1	+ / +	Cytoplasm	Meso-lanthionine	Bacteria	• IEC injury protection • Intestinal lymphoid tissue development
NOD2	+ / +	Cytoplasm	Muramyldipeptide	Bacteria	• AMP production • ROS production
RLRs					
RIG-I	+ / +	Cytoplasm	Cytoplasmic double-stranded RNA	Viruses	
MDA5	+ / +	Cytoplasm	Cytoplasmic double-stranded RNA	Viruses	• Interferon production

The necessity to control expression of PRRs and avoid unnecessary inflammation can be exemplified by several studies on TLRs. For example, TLR2 and TLR4 are differentially expressed along the crypt-villus axis; IECs only express high levels of TLR2 and TLR4 at the base of the crypt; their expression decreases as the cell move up from the stem cell to mature towards the villus (Furrie, Macfarlane et al. 2005). The high expression of these TLR could reflect a higher surveillance of the crypt, which should normally be devoid of microbes to protect the stem cell niche; as TLR2 and TLR4 can be highly proinflammatory, a lower expression in the presence of the gut microbiota would be a good strategy to avoid a strong response while interacting with commensals. Nonetheless, both these TLRs remain highly inducible, and have been shown to play a key role in the control of *Salmonella enterica* serovar Typhimurium infection (Sivick, Arpaia et al. 2014). On the contrary, TLR3 expression is highest in mature IECs in the villus, and lowest at crypt; the reason for such expression pattern is not yet fully understood. The case of TLR5 is even trickier. An earlier study had found TLR5 to be expressed only on the basolateral membrane of epithelial cells; since flagellin is expressed by bacteria including commensals, this specific location could have been a strategy to respond to the presence of flagellin only in the case of an invading pathogen crossing the epithelial layer (Gewirtz, Navas et al. 2001). However, in a recent report (Cullender, Chassaing et al. 2013), *Tlr5*$^{-/-}$ mice suffer a great decrease of anti-flagellin antibodies in the gut lumen, and as a consequence show an increase in epithelial breach by bacteria; this finding thus strongly suggests, although it was not proved, that TLR5 is also present on the apical surface of IECs, since it plays a role in sensing the microbiota and responding by the release of anti-flagellin antibodies.

b) Antigen sampling

(1) Microfold cells

Throughout the epithelium border, the presence of specialized cells, called Microfold or M cells, allows for a controlled entry of antigens within the LP (Mabbott, Donaldson et al. 2013). M cells are specially adapted to mediate antigen sampling from the lumen; indeed, they show a reduced glycocalyx and brush border, as well as reduced microvilli. Most importantly, M cells are particularly efficient in transcytosis and phagocytosis of bacteria, viruses, parasites, as well as macromolecules. Bacterial pathogens such as *Vibrio cholera*, *Shigella flexneri*, *Yersinia enterocolitica* and *Campylobacter jejuni* have been shown to interact with M cells (Owen, Pierce et al. 1986, Walker, Schmauder-Chock et al. 1988, Wassef, Keren et al. 1989, Grutzkau, Hanski et al. 1990, Sansonetti and Phalipon 1999). The diversity of microbes and particles M cells are able to transcytose implies a non-specific transcytosis mechanism;

however, M cells also have specific receptors involved in this mechanism. For example, the glycoprotein 2 (GP2) receptor at the apical surface of M cells specifically binds FimH, a protein on the type I pilus tip of bacteria such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Enteritidis, and leads to uptake of those bacteria through the M cell (Hase, Kawano et al. 2009).

Strategically, M cells are predominantly present in the so-called follicle-associated epithelium (FAE), which overlays major sites of priming of the adaptive immune system termed gut-associated lymphoid tissues (GALTs). Thus, M cells are ideally located to transfer antigens or whole microogranisms to immune cells for an efficient priming and launching of an appropriate response. Upon the uptake and transfer of the antigen across the epithelium, the M cells are able to deliver the antigen to dendritic cells (DCs). DCs, as well as macrophages, are professional antigen-presenting cells (APCs), hence they are proficient at uptaking antigens as well as whole organisms. The GALTs contain rich zones of $CD103^+$ DCs, a subset of DCs capable of migrating to perform antigen presentation and prime B and T cells.

(2) Goblet cells

M cells were thought to be the only cells capable of delivering antigens to APCs for a long time. But a recent study using two-photon microscopy demonstrated the ability of goblet cells to uptake soluble luminal antigens via goblet-associated antigen passages (GAPs) and efficiently deliver them to $CD103^+$ DCs; this observation was biologically relevant, since *ex vivo* co-culturing of DCs with OVA-primed T cells showed subsequent proliferation of T cells (McDole, Wheeler et al. 2012). Of note, goblet cells were delivering antigens rather specifically to $CX3CR1^-$ $CD103^+$ DCs, which are known to be involved in tolerance, and are capable of migrating to secondary lymphoid tissues for presentation of the antigenic material they carry (Schulz, Jaensson et al. 2009).

(3) Intraepithelial phagocytes

Another subset of DCs, or what were thought to be, is present in the LP; unlike the more abundant $CX3CXR1^-$ $CD103^+$ DCs, these $CX3CR1^{high}$ DCs do not migrate, and instead remain in close to the intestinal epithelium (Schulz, Jaensson et al. 2009). $CX3CR1^{high}$ DCs were shown to send transepithelial dendrites (TEDs) between epithelial cells (Rescigno, Urbano et al. 2001, Niess, Brand et al. 2005). Furthermore, a functional CX3CR1 on this subset of DCs, which is required for TEDs formation, was demonstrated to be important in the clearance of non-invasive *Salmonella* Typhimurium after oral infection in mice (Niess,

Brand et al. 2005). Since then, with the accumulation of functional data on these cells, and the clear difference in ontogeny of the DC subsets, this CX3CR1^{high} population is now known to be in fact CX3CR1^{high} resident macrophages (Persson, Jaensson et al. 2010, Varol, Zigmond et al. 2010). Moreover, the functional relevance of the antigen sampling of these macrophages are still not entirely clear, since they lack the ability to migrate to secondary lymphoid tissues where antigen presentation takes place. CD103^{+} DCs, on top of their interaction with goblet cells to receive luminal antigens, are also capable of responding to bacterial challenge and crawl into the epithelial layer, extend their dendrites to the lumen, sample antigens (although not soluble proteins) and subsequently activate the adaptive immune system (Farache, Koren et al. 2013). The ability of macrophages to send TEDs for microbial sampling thus seems to only reflect redundancy, though a recent study demonstrated that CX3CR1^{high} resident macrophages are able to transfer soluble antigens they sampled to CD103^{+} DCs via gap junctions formed by connexin proteins present on both cell subsets, and thereby induce DC-mediated antigen tolerance (Mazzini, Massimiliano et al. 2014).

C. Activation at inductive sites

1. Inductive sites of the intestine

a) Mesenteric lymph nodes

Although not a part of the LP, mesenteric lymph nodes (MLNs) are important secondary lymphoid tissues in the adaptive immune response of the intestine, specifically the generation of oral tolerance (Pabst and Mowat 2012). MLNs are classical lymph nodes, formed by a collection of lymphoid lobules that are encapsulated together in one node, drained by lymphatic and blood vessels. Each lobule has its own afferent lymphatic vessel bringing antigen presenting cells (such as CD103^{+} DCs), and a collection of high endothelial venules (HEV) bringing naïve lymphocytes; lymphocytes exit via the efferent lymphatic vessels back to the bloodstream. Each lobule contains several B cell and dendritic cell-rich follicles within which B cells, when activated, will proliferate to form germinal centers (GCs). A rich T cell zone, where T cells are activated, is also present in each lobule (Willard-Mack 2006). MLNs are generally located throughout the mesentery in humans (whereas in mice they only consist of a chain of 4-5 lymph nodes), but do not drain the entire intestine, contrary to general belief. Indeed, the duodenum and transverse colon are drained in the duodenopancreatic lymph nodes, while the descending colon and rectum are drained in the single caudal lymph nodes (Mowat and Agace 2014).

b) Gut-associated lymphoid tissues (GALT)

(1) Peyer's patches

Peyer's patches (PPs) are one of the largest organized lymphoid tissues in the gastrointestinal immune system. There are about 100-200 PPs in the human small intestine, and 6-12 PPs in the mouse small intestine; most of them are concentrated towards the ileum (Cornes 1965, Mowat and Agace 2014). Unlike a classic lymph node, PPs are not encapsulated. PPs consist of several large B-cell follicles and smaller T cell areas flanking the B cell follicles, as well as an overlying follicle-associated epithelium (FAE) and associated subepithelial dome (SED) that lies between the FAE and the follicles. PPs are irrigated by blood vessels as well as efferent lymphatic vessels; the lack of afferent lymphatic vessel is compensated by a direct feed of antigens from the M cells located in the FAE. The SED is, strategically, an area rich in DCs, ready to pick up antigens or organisms exiting from M cells and to present them to T and B cells to launch an antigen-specific immune response. In fact, because of a continuous stimulation of immune cells by luminal antigen sampling, PP constantly have active GCs, where B-cell selection and Ig gene somatic hypermutation takes place; this is not the case in lymph nodes. Of note, similar structures are found in the cecum and colon, called cecal patches and colonic patches, respectively.

(2) Solitary isolated lymphoid follicles

Solitary isolated lymphoid follicles (SILTs) are other organized secondary lymphoid structures dispersed in high numbers throughout the wall of the intestine which, unlike PPs, are only visible microscopically. SILTs can be small, immature cryptopatches, or the more mature isolated lymphoid follicles (ILFs). They are found in high numbers in both mouse and human intestine, but show an increase concentration in distal regions of the colon (Mowat and Agace 2014). Similar to PPs, ILFs contain germinal centers and M cells present in the FAE; however, they do not show clear T cell areas. In further contrast to PPs, ILFs were shown to be the main centers for IgA production in a T-cell independent manner (Tsuji, M. 2008). Interestingly, although structures resembling in mass to ILFs are observed in germ-free animals, their development and maturation to ILFs require colonization of the gut by microbes (Hamada, Hiroi et al. 2002); hence, they are a very dynamic part of the intestinal mucosa, since they are directly dependent on the gut microbiota.

c) Lymphocytes trafficking to secondary lymphoid follicles

For the rare antigen-inexperienced (termed naïve) T cell circulating in the bloodstream to encounter its cognate antigen is no easy task. Therefore, to ensure successful T cell activation, naïve T cells must continuously circulate in and out of secondary lymphoid organs in search for their cognate antigen on the surface of antigen-presenting cells. When entering a lymph node, naïve T cells search for about 8 hours for their cognate antigen (Mempel, Scimone et al. 2004) and, if they do not encounter it, leave the lymph node via the efferent lymph, go back to the bloodstream, and enter a new lymph node to repeat the same search. When they do encounter their cognate antigens, naïve T cells are activated and become differentiated T cells, which still exit the LN via the efferent lymph and into the bloodstream, but can no longer enter a LN. Instead, these differentiated T cells are directed towards peripheral tissues.

Naïve T cells enter the LN by extravasation out of the bloodstream. To facilitate their entry to the LN, specific cell-cell and cell-environment interactions ensure sequential steps of rolling/tethering, arrest, firm adhesion and finally diapedesis that result in T cell extravasation into the LN. HEVs in the LNs constitutively express adhesion molecules that will orchestrate T cell arrest. Hence, the addressin PNAd present on the luminal side of HEVs will contact the L-selectin on the surface of naïve T cells and slow them down without stopping them. Next, CCL19 and CCL21 which are chemokines present on the HEVs, will bind to CCR7 on the surface of T cells, and will provoke a downstream signaling in T cells leading to LFA-1 (an integrin present on the surface of naïve T cells) to adopt a high affinity conformation. LFA-1 binds to ICAMs (ICAM-1, ICAM-2 or ICAM-3) on HEVs, and result in a firm stop of the T cell, and subsequent extravasation. Within the lymph node, T cells are directed towards the T cell zone by chemotaxis with CCL21. This multistep model is not only used to have T cells enter LNs, but also to ensure T cell migration back to the right peripheral tissue to exert their function, as I will discuss later. A recapitulative table showing the main cell adherence molecules and chemokines involved to ensure effective navigation of lymphocytes is shown in table 2 below.

The same multistep model applies for B cells, which preferentially concentrate in the GALTs. Indeed, naïve B cells express high levels of α4β7, allowing them to bind to MadCAM-1 and enter the GALTs. Once they have reached the GALTs or MLNs, B cells are directed towards the B-cell rich follicle region via chemotaxis between B cell-born CXCR5 and CXCL13 (Brandtzaeg and Johansen 2005).

Table 2. Chemokines and cell-cell interactions in lymphocyte and DC trafficking

From blood to lymph nodes

Selectins	**Distribution**
L-Selectin (CD62L)	Näive T cells, myeloid cells, T_{CM} cells

Selectin ligands	**Distribution**
PNAd	HEV of peripheral LNs

Chemokines	**Receptors**	**Function**
CCL19	CCR7	T cell and DC homing to LNs
CCL21	CCR6, CCR7	T cell and DC homing to LNs

Integrins	**Ligand**	**Ligand distribution**
LFA-1	ICAM	Endothelium, DCs, leukocytes

Within lymph nodes

Chemokines	**Receptors**	**Function**
CXCL13	CXCR5	B cell positioning in follicles of LNs and PPs
CCL21	CCR7	T cell and DC positioning in LNs and PPs
CXCL9	CXCR3	Th1 response, NK trafficking
CXCL10		
CXCL11		
CCL1	CCR8	Th2 cell and Treg trafficking
CCL3	CCR1, CCR5	Macrophage and NK cell migration, T cell-DC interaction
CCL4	CCR5	
CCL5	CCR1,CCR3, CCR5	
CCL20	CCR6	Th17 responses, B cell and DC homing to GALT
CCL22	CCR4	Th2 responses, Th2 cell migration, Treg

Gut homing

Integrins	**Integrin ligand**	**Ligand distribution**
α4β7	MAdCAM-1	Intestinal LP endothelium, HEVs of MLNs and PPs

Chemokines	**Receptors**	**Function**
CCL25	CCR9	T cell homing to gut

2. Activation of adaptive immune cells

a) Maturation of DCs

Central in the activation of the intestinal adaptive immune response are DCs and their enhanced ability to pick up and present luminal antigens to B and T cells. Whether they have acquired antigens directly from the lumen, or indirectly via M cells, goblet cells, or $CX3CR1^+$ resident macrophages, the resulting outcome is the activation of intestinal DCs. They upregulate their expression of the major histocompatibility complex (MHC) class II (termed histocompatibility antigen complex for humans) to allow for presentation of the antigen; as well as the CD80/CD86 costimulatory molecules necessary for activating T cells. Additionally, they also upregulate their expression of CCR7, which allows them to effectively reach the MLN where they prime and activate T cells (Forster, Davalos-Misslitz et al. 2008, Saban 2014). The key role of intestinal migratory DCs is highlighted by the fact that mice lacking CCR7 are not capable of mounting a T cell response against oral soluble antigens (Worbs, Bode et al. 2006). Of note, all three major subsets of DCs known ($CD103^+CD11b^+$, $CD103^+CD11b^-$ and $CD103^-$ DCs) are capable of migrating to MLNs (Persson, Jaensson et al. 2010, Bekiaris, Persson et al. 2014), although it is still unclear whether all subsets can effectively activate T cells (but this is more for the gut homing part). Moreover, MLNs are also home of resident DCs coming from circulating DCs, but little is known about their ability to activate T cells.

b) T cell priming

T cells are divided in two important branches of the adaptive response. T cells bearing the CD4 surface marker differentiate in effector T cells called T helper (T_H) cells. As their name suggests, T_H cells essentially help orchestrating the adaptive immune response, making them a central component in both humoral and cellular immune responses. On the other hand, T cells bearing the CD8 surface marker differentiate in cytotoxic T lymphocytes (CTL) which, as will be discussed later, are key effector cells for the clearance of intracellular pathogens such as viruses and obligate intracellular bacteria. For both subsets, the first critical step in the generation of activated effector T cells is the priming and activation by intestinal DC presentation which is facilitated, once again, by chemotaxis to the T cell rich zone.

Priming of $CD4^+$ T cells involves the binding of the T cell receptor (TCR) of the naïve T cell to its specific cognate antigen, presented by DCs on an MHC Class II molecule. Other costimulatory signals are involved, in particular the interaction between CD80/CD86 with CD28 present on the surface of naïve T cells. Both main and costimulatory signals from DCs lead to proliferation and differentiation of T_H cells (Figure 2). $CD8^+$ T cells recognize their cognate antigen presented by DCs on MHC class I molecules. DCs will “load” on MHC class

I antigens resulting from transcription machinery and degradation of cytosolic proteins, including “non-self” proteins coming from an invading pathogen, such as a virus or an intracellular pathogen. Alternatively, DCs can acquire exogenous antigens from the infectious agents and load them on MHC class I molecules, to provide a so-called cross-presentation to $CD8^+$ T cells. Of note, the $CD8\alpha^+$ DC subset is better suited for cross-presentation of foreign antigen to CD8 T cells by upregulation of MHC Class I. On the other hand, $CD103^+$ $CD11b^+$ DCs will upregulate MHC class II for cross-presentation to CD4 T cells. This division is not absolute; importantly, cross-presentation will depend on the way the antigen is presented, more so than the DC subset.

Antigen-activated $CD4^+$ T cells pursue their differentiation by polarizing towards various subsets of T_H cells. In many ways, DCs are a key player in this process. Indeed, DCs essentially convert key signals received by the innate system and from their own antigen sampling into specific output signals that will direct the polarization of the different T_H subtypes. Hence, the polarization of T_H cells is dependent on the sets of cytokines released in the milieu by DCs, as well as environmental cues present in the secondary lymphoid organ (Walsh and Mills 2013). Likewise, in a non-inflammatory, steady state context, DCs express cytokines that will drive the differentiation of activated T-cells to regulatory, tolerogenic T cells, called Treg cells. To date, up to seven different types of differentiated $CD4^+$ T cells are known, and all of them require a unique cocktail of cytokines to allow for differentiation. Accordingly, each different subset has a particular effector, helper, or homeostatic function, as depicted in Figure 2; their specific differentiation and function will be further described in the next section.

For antigen-experienced $CD8^+$ T cells to differentiate into (CTL), $CD8^+$ T cells also need costimulatory signal through CD28, as well as a third signal mediated by cytokines via either interleukin 12 (IL-12), type I interferon (IFN) (Mescher, Curtsinger et al. 2006, Xiao, Casey et al. 2009) or IL-21 (Casey and Mescher 2007).

c) Differentiation of B cells into Ig antibody-secreting cells

Activation of B cells and differentiation into high affinity, antigen specific Ig antibody-secreting cells (ASC), i.e plasmablasts or plasma cells, mostly takes place in the GALT. The direct involvement of MLNs in IgA B cell response is debated, though they clearly indirectly participate as they provide differentiated $CD4^+$ T cells. Unlike in the blood, where the IgG subtype prevails, SIgAs are by far the most prevalent Ig present in the intestines. In fact, B cells in PPs undergo preferential switch from IgM to IgA (Weinstein and Cebra, 1991). Moreover, the GALTs represent the main inductive sites for IgA production (Reboldi and Cyster 2016, Rios, Wood et al. 2016). Thus, for simplicity, I will essentially focus on GALT-mediated induction of IgA response.

(1) Antigen capture and B cell activation

The first step in the B cell, or humoral, response is the recognition and subsequent internalization of the antigen in its native form by naïve B cell-borne surface immunoglobulins, namely IgD and IgM; these immunoglobulins constitute the B cell receptor (BCR). In the GALTs, M cells are the main source of antigen sampling. The SED contains $CD11b^+$ DCs, as well as B and T cells, which are all exposed to antigen uptake from M cells. DCs acquire antigens from M cells, as seen with the example of *Escherichia coli* uptake (Hase, Kawano et al. 2009), and can thus present it to B and T cells directly in the SED. DCs can also migrate to the T cell zone in the follicle and prime $CD4^+$ T cells to become follicular T_H or T_{FH} cells, a specific subset of T cells important for B cell activation. Most activated B cells migrate towards the T cell area where they interact with T_{FH} cells in a cognate fashion; that is, B cell present the cognate peptide on an MHC Class II molecule to interact with the T cell TCR. During cognate interaction, several signals provided by T_{FH} cells activate B cells to start their process of differentiation; notably, interaction of CD40L with B cell-borne CD40 as well as expression of cytokines (notably IL-21, IL-4) are important. Alternatively, B cells can undergo a T cell-independent differentiation path; that is, without cognate interaction with T_{FH} cells or CD40-CD40L interaction. This process is much less frequent in the PPs, and rather occurs in ILFs, in which there is no T cell zone.

A recent study established that, prior to B cell proliferation (called clonal expansion) and subsequent GC formation, activated B cells migrated back to the SED for further critical signaling with DCs (see section below). This process was mediated by upregulation of CCR6 in B cells, which allowed them to move towards the CCL20 rich SED zone. CCR6 upregulation was found to be most efficient via T_{FH} cell-mediated CD40-CD40L interaction. CCR6 was found not be necessary to mount a T cell-independent B cell response; however,

since BCR engagement by itself is sufficient to upregulate CCR6 *in vitro*, B cells could potentially still migrate to the SED despite the lack of CD40 interaction.

(2) Affinity maturation and isotype switch

Within the GC, B cells need to undergo two important processes, namely somatic hypermutation (SHM), and class switch recombination (CSR).

SHM, which occurs during clonal expansion, introduces random point mutations in the antibody variable region (the region recognizing the antigen), leading to selection of high-affinity antibodies; this process is called affinity maturation (Papavasiliou and Schatz 2002). Follicular dendritic cells (FDC), which are important for the formation of GC, also play a critical role during affinity maturation. Indeed, due to its unique ability to retain intact antigen for long periods, FDC are able to present antigen complexes to B cells during SHM, and thus select for high affinity B cells (Heesters, Myers et al. 2014). After selection by the FDC, B cells can either re-enter the process of SHM, or move on to class switch recombination. CSR replaces the heavy chain constant region (C_H) gene from C_μ (IgM) or C_δ (IgD) to C_α (IgA), C_γ (IgG), or C_ε (IgE); thus, rather than changing the affinity of the antibody; CSR leads to production of a new antibody isotype, with unique biological activities due to its associated constant region (Chaudhuri and Alt 2004). T_{FH} cells, which are also important for GC formation ensure cognate interactions with B cells and enable B cell CSR to IgA via important signaling molecules.

Key factors are necessary in these processes. First, expression of activation-induced cytidine deaminase (AID) is required for both CSR and SHM (Muramatsu, Kinoshita et al. 2000). Not surprisingly, AID requires cell division to be functional. Second, the cytokine transforming growth factor β (TGFβ) is required to initiate the specific isotype switch to IgA. B cells are the main producers of TGFβ, which acts in an autocrine fashion to induce CSR. Deficiency of either TGFβ or TGFβ receptor (TGFβRII) on B cells abrogates production of IgA^+ B cells (Stavnezer and Kang 2009). In the PPs, FDCs have been suggested to activate TGFβ, since it is originally released by B cells in a latent form. Interestingly, B cells can also receive TGFβ signaling prior to GC formation. Indeed, activated B cells that have upregulated CCR6 and moved to the SED interact with DCs via αvβ8, which triggers activation of TGFβ in B cells and potentially IgA CSR (Reboldi, Arnon et al. 2016). Moreover, in mice deficient for CD40 and form no GC, the LP contained normal levels of IgA that had undergone CSR (Bergqvist, Gardby et al. 2006). Thus observation, IgA CSR can begin even before B cells form the GC (Lycke and Bemark 2017). These observations question the requirements of T_{FH} signaling to

B cells in GC for IgA CSR. One possibility is that T_{FH} release copious amounts of IL-21, which has been shown to synergize with TGFβ in IgA CSR and production of IgA plasma cells (Dullaers, Li et al. 2009).

Numerous other signals have been shown to be involved in the induction of IgA production. For example, intestinal DCs express the inducible nitric oxide synthase (iNOS), which is responsible for large production of nitric oxide (See “Nitric oxide” chapter); in turn, nitric oxide may induce upregulation of TGFβR, thus enhancing T cell-dependent and T cell-independent IgA production (Tezuka, Abe et al. 2007). Intestinal DC-produced RA was also shown to induce IgA switch, an effect that is enhanced by cytokines IL-5 and/or IL-6 (Mora, Iwata et al. 2006).

Additionally, two DC-derived cytokines, APRIL and BAFF, directly mediate T cell-independent CSR (Litinskiy, Nardelli et al. 2002, Castigli, Wilson et al. 2005). Interestingly, lymphoid tissue-inducer cells, a branch of ILC3s, has also been shown to be critical in the onset of T-independent IgA production in ILFs, although in a different context than in T-cell dependent IgA switch in PPs (Tsuji, Suzuki et al. 2008).

(3) Germinal center recycling in the Peyer’s patches

PPs and SILTs have a unique feature compared to other secondary lymphoid organs, in that they display chronic GCs, due to the constant interaction with dietary antigens and the microbiota. Thus, PPs represent of preformed GCs that B cells could utilize to build an immune response against multiple antigens at the same time. An elegant study showed that, following oral immunization with an antigen, about 3-6 B cell clones had proliferated in response to the antigen and undergone affinity maturation; most importantly, each of these clones was found in multiple PPs in the same mouse (Bergqvist, Stensson et al. 2013). Additional oral immunizations with the same antigen led to the generation of higher affinity B cell clones, which were also found in multiple PPs in the same mouse. Thus, after selection of a high-affinity B cell clone, these B cells leave the GC and migrate towards other PPs to re-utilize preformed GCs, allowing for a synchronization of the gut IgA response throughout the entire small intestine (Lycke and Bemark 2012). Activated B cells exit the gut via the draining lymph to the MLNs, then leave via the thoracic duct to the blood back to the intestine to finish their differentiation into plasma cells (Macpherson, McCoy et al. 2008); this can explain the ability of one B cell clone to seed into multiple PPs and synchronize the gut immune response.

The same principle could potentially be applied to T cell-independent B cell responses. As discussed earlier, mice deficient for CD40 still produce T-cell independent SIgAs (Bergqvist,

Gardby et al. 2006), though these SIgAs show very little SHM due to the lack of GC formation. Thus, the fact that WT type mice exhibit T-cell independent SIgAs with multiple mutations suggests that these B cells most likely go through a GC as well. In accord with this hypothesis, a study looking at IgA-producing plasma cells against *Vibrio cholerae* LPS, which is a typical T-cell independent antigen, found many mutations in those IgAs (Kauffman, Bhuiyan et al. 2016). Lycke and colleagues have thus suggested that B cells activated via a T-cell independent manner probably also exploit preformed GCs in PPs in order to undergo SHM (Lycke and Bemark 2017).

d) Gut homing receptors induction

From the secondary lymphoid tissues, differentiated effector T cells and activated B cells need to find their way back to the small intestine. Navigational directions to effector cells are given through upregulation of both the α4β7 integrin and the CCR9 receptor. HEVs of MLNs and PPs, as well as venules throughout the LP uniquely express mucosal addressin cell-adhesion molecule 1 (MADCAM-1), the receptor of α4β7; moreover, CCL25 is selectively expressed (outside of the thymus) in the small intestinal epithelium and endothelium. Thus, the combination of CCR9 and α4β7 on the surface of effector cells ensures their preferential homing to the unique “zipcode” of the gut.

Intestinal DCs (in MLNs or PPs) are in charge of imprinting on activated B and T cells the expression of α4β7 and CCR9 (Mora, J.R. Science 2006; Mora, J.R. Nature. 2003). This induction requires the generation by intestinal DCs of retinoic acid (RA), a vitamin A metabolite. Vitamin A-deficient mice displayed a markedly reduced number of T_{EM} cells in the gut (Iwata, Hirakiyama et al. 2004) and of differentiated B cells in the PPs (Mora, Iwata et al. 2006). Of note, DCs from other lymphoid tissues do not synthesize RA.

Noteworthy, imprinting of α4β7 and CCR9, although important in the relocalization of B and T cells, is not an absolute requirement. Indeed, β7-integrin-deficient $CD8^+$ effector T cells still manage to reach the small intestine (Lefrancois, Parker et al. 1999), and $CD4^+$ effector T cells also get to the small intestine in *Ccr9*$^{-/-}$ mice (Stenstad, Ericsson et al. 2006).

D. Effectors of the adaptive immune response

1. T helper cells

As described earlier, the differentiation of antigen-experience $CD4^+$ T cells into various T_H cells occurs by induced expression of cytokines produced by DCs, as well as other cells within the local environment, which can be immune cells and/or structural cells like stromal or epithelial cells. The combination of cytokines and mediators sensed by the T cells guide their polarization into T_H cells (Figure 2).

a) T follicular helper cells (T_{FH} cells)

Although the concept of a T_H cell subset helping B cell differentiation in secondary lymphoid tissues is not new, T_{FH} cell characterization as a unique cell lineage took a while to be firmly established (Schaerli, Willimann et al. 2000). Though not directly involved in pathogen clearance or protection, T_{FH} cells play an important role nonetheless, by supporting expansion and differentiation of B cells into high affinity, antigen-specific-producing plasma cells. Thus, instead of going to peripheral tissues upon antigen encounter, activated pre-T_{FH} cells stay within the LNs or GALTs. The DC-mediated upregulation of CXCR5 and CCR7 downregulation (Hardtke, Ohl et al. 2005), allows pre-T_{FH} cells to move from the T cell zone to the B-T cell border in the LNs or GALTs. As discussed earlier, cognate interaction between the pre-T_{FH} cells and B cells allows both proliferation of B cells to form GCs, but it is also induces further differentiation of T_{FH} cells. Several co-stimulatory interactions (CD40L-CD40; ICOS-ICOSL; CD28-CD80) help towards full commitment to the T_{FH} program, and differentiation of B cells to plasma cells. However, it is noteworthy to restate that B cells can differentiate in a T_{FH}-independent manner (mostly in the ILFs); similarly, evidence suggests a T_{FH} differentiation in the absence of B cells, provided naïve T cells get a prolonged exposure to their cognate antigen (Deenick, Chan et al. 2010).

The transcription factor B cell lymphoma 6 (Bcl6) is required for T_{FH} polarization, since *Bcl6*$^{-/-}$ mice show a severe drop (Nurieva, Chung et al. 2009) or complete loss (Yu, Rao et al. 2009) in T_{FH} cells. IL-6 and IL-21 are needed to induce expression of Bcl6; moreover, IL-6 and IL-27 (which requires IL-21) also control regulation of cMaf, a transcription factor important for the upregulation of CXCR5 (Bauquet, Jin et al. 2009).

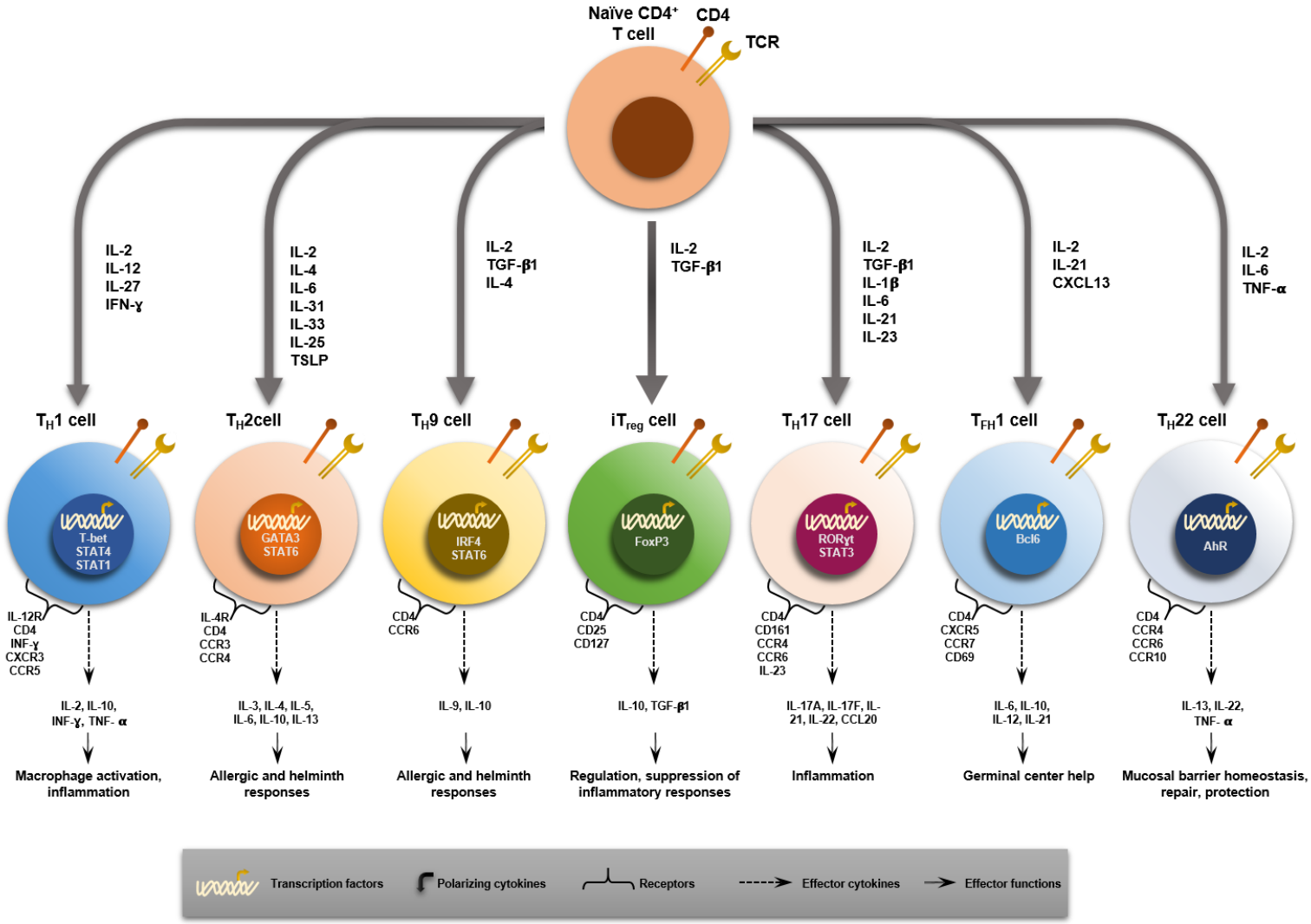

Figure 2. Differentiation and main function of T helper (T_H) cells. *Adapted from a figure in (Swain, McKinstry et al. 2012)*

b) T_H1 cells

T_H1 polarization is characterized by the upregulation of the transcription factor Tbet (for T-box transcription factor expressed in T cells), which is regulated by the cytokines IFN-γ and IL-12. IFN-γ binds to its receptor IFN-γR on the surface of T cells, which upregulate the expression of the Tbet and subsequent upregulation of the receptor of IL-12. IL-12 binding to functional IL-12R will then upregulate the expression of IFN-γ by T_H cells. IL-27, a member of the IL-12 cytokine family, seems also to be involved in induction and/or maintenance of T_H1 cells (Troy, Zaph et al. 2009). Moreover, T_H1 cells are characterized by expression of CXCR3 on their surface, enabling their homing to inflammatory sites.

T_H1 cells generally support cell-mediated immune responses and promote protective immunity against intracellular pathogens, especially those capable of infecting dendritic cells and macrophages, such as *Listeria monocytogenes* and *Toxoplasma gondii*. Their secretions of IFN-γ, as well as Tumor Necrotizing Factor (TNF), induce activation of macrophages and upregulation of inducible nitric oxide synthase (iNOS). The subsequent generation of nitric oxide (NO) by macrophages has direct effects on pathogen replication, such as *Salmonella* (Henard and Vazquez-Torres 2011). IFN-γ is also a major inducer in the activation of CTL which are specially equipped to handle intracellular infections. Finally, T_H1 cells induce preferential production of IgG2 antibodies, a subclass involved in virus neutralization.

c) T_H2 cells

T_H2 cells are characterized by the induction transcription factors STAT6 and GATA3, which are upregulated in the presence of IL-4, as well as IL-25 and IL-33. T_H2 cells participate in the clearance of parasitic helminths, and respond to IL-4 secreted by innate tissue resident cells. IL-4 and IL-13, two signature cytokines of T_H2 cells, drive macrophage-mediated killing of helminths (Allen and Maizels 2011). Additionally, IL-13 acts on goblet cells, favoring production of MUC5A to expel parasites from intestines. T_H2 cells mediate potentiation of allergic responses and asthma by directing, via IL-4, B cell secretions of IgG1 and IgE.

d) T_H17 cells

T_H17 differentiation requires TGFβ in combination with either IL-6 (Mangan, Harrington et al. 2006) or IL-21 (Korn, Bettelli et al. 2007, Zhou, Ivanov et al. 2007). Either one of these combinations induce expression of the transcription factor RORγt, which is necessary for upregulation of the IL-23 cytokine receptor on the surface of T_H17 cells. Subsequent signaling with IL-23 ensures maintenance of the developing T_H17 cells and promotes

production of IL-17A and IL-17F (Zhou, Ivanov et al. 2007), which share important roles in gut.

T_H17 cells are essential in the control of extracellular bacteria and fungi infections. T_H17 cells were shown to control infection in models of *Citrobacter rodentium* (Mangan, Harrington et al. 2006), *Salmonella* Typhimurium (Raffatellu, Santos et al. 2008), *Klebsellia pneumoniae* (Aujla, Chan et al. 2008) and *Candida albicans* (Saijo, Ikeda et al. 2010). T_H17 cells develop in mice upon infection with the enterohemorrhagic *Escherichia coli* strain O157:H7 (Atarashi, Tanoue et al. 2015).

IL-17A and IL-17F are T_H17 cells signature cytokines (Ishigame, Kakuta et al. 2009), promote the recruitment of neutrophils by inducing secretion of chemoattractants (IL-8, CXCL1, CXCL2) by target cells. Neutrophils are abundant leukocytes and essential players in innate and adaptive immunity (Leliefeld, Koenderman et al. 2015). Indeed, they participate in the clearance of many pathogens by phagocytosis, degranulation of antimicrobial compounds and activation of oxidative burst, and release of DNA traps filled with antimicrobial proteins. Moreover, importance of neutrophils in the control of *Salmonella* Typhimurium is highlighted by the fact that patients with primary neutrophil deficiency face dissemination of S. Typhimurium from the gut and subsequent bacteremia (Winkelstein, Marino et al. 2000).

T_H17 cells are also an important source of the cytokine IL-22, a pleiotropic cytokine shared with Th22 cells discussed below, but also with innate lymphoid cells. Interestingly, the combined secretion of IL-17 and IL-22 by T_H17 cells increase the secretion of antimicrobial peptides S1008A and S1009A by skin keratinocytes (Liang, Tan et al. 2006).

e) T_H22 cells

T_H22 cells are a recent addition in the T_H cell lineage, since they were first identified in 2009 in humans (Duhen, Geiger et al. 2009, Trifari, Kaplan et al. 2009), and later in mice (Basu, O'Quinn et al. 2012). Unlike T_H17 cells, T_H22 cells express IL-22 but very little, if any, IL-17. Additionally, T_H22 cells require IL-6, but no TGF-β to differentiate. In fact, TGF-β was found to negatively regulate T_H22 cells (Leung, Davenport et al. 2014). Interestingly, T_H22-secreted IL-22, but not T_H17-secreted IL-22, is important for late phase mouse protection against *C. rodentium* (Basu, O'Quinn et al. 2012), suggesting a functional difference between T_H22 and T_H17 cells. In that same study, T_H22 differentiation was shown to depend on two transcription factors, namely the aryl hydrocarbon receptor (AhR) and T-bet.

f) T_H9 cells

T_H9 cells are also a recently defined lineage in T_H cells, mostly because their signature cytokine IL-9 was originally thought to be secreted by T_H2 cells (Schmitt, Germann et al. 1994, Dardalhon, Awasthi et al. 2008, Veldhoen, Uyttenhove et al. 2008). Indeed, T_H9 polarization requires, just like T_H2, IL-2 and IL-4, but also TGFβ, which is suggested to reprogram T_H2 cells into T_H9 cells. Also similar to T_H2, differentiation involves the transcription factors STAT6 (Goswami, Jabeen et al. 2012) and IRF4 (Staudt, Bothur et al. 2010); GATA3, although less important, is also involved in T_H9 differentiation (Goswami, Jabeen et al. 2012).

Much like T_H2 cells, T_H9 cells are involved in immunity against helminth infection, as was shown for *Trichuris muris* and *Nippostrongylus brasiliensis*. For example, adoptive transfer in T cell deficient mice showed that T_H9 cells, rather than T_H2 cells, were able to reduce the burden of *Nippostrongylus brasiliensis* worm infection by an IL-9-mediated increase in infiltrating eosinophils, basophils and mast cells (Licona-Limon, Henao-Mejia et al. 2013).

g) Regulatory T cells (Treg)

Treg cells are responsible for maintaining self-tolerance and immune homeostasis, and thus hold a key role in keeping inflammation responses from being deleterious. Patients with mutations in the Foxp3 gene, the master transcription factor of the Treg lineage develop a life-threatening severe autoimmune syndrome characterized by, but not limited to, early-onset insulin-dependent diabetes mellitus, enteropathy and eczema (Bennett, Christie et al. 2001). There are two distinct pools of Treg cells (Yuan and Malek 2012, Bollrath and Powrie 2013): natural Treg (nTreg) cells, which develop and differentiate in the thymus, and induced Treg (iTreg) cells, which derive from conventional $CD4^+$ T cells in the periphery. iTreg cells are greatly involved in maintaining tolerance to food antigens and commensal bacteria in the gut. iTreg key cytokines, including IL-10 and TGFβ and, that negatively regulate effector T cells, thus dampening the inflammatory response (Rubtsov, Rasmussen et al. 2008).

As stated above, Treg cells need the transcription factor Foxp3 to differentiate (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). Foxp3 is upregulated in the presence of TGFβ, which makes it a critical component of Treg polarization as well. The concentration of TFGβ is also critical: low TGFβ will, together with IL-6 and IL-21, favor T_H17 differentiation; on the other hand, high TGFβ will repress *IL23r* expression, thus T_H17 polarization, and upregulate Foxp3 to induce Treg differentiation (Zhou, Lopes et al. 2008). Further, RA was found to synergize with TGFβ and promote differentiation of iTreg cells, while repressing

differentiation of T_H17 cells (Mucida, Park et al. 2007). Additionally, co-stimulatory signaling with DCs via CD28 and CTLA-4 are important in iTreg development. Notably, CD28 was shown to promote IL-2 production, an important cytokine in iTreg differentiation (Guo, Iclozan et al. 2008). Interestingly, RA-mediated enhancement of iTreg differentiation was found to be dependent on IL-2 (Mucida, Park et al. 2007).

2. Plasma cells and secretory IgAs (SIgAs)

In the intestines and other mucosal surfaces, secretory IgAs (SIgAs) are undoubtedly the main immunoglobulin type secreted by fully differentiated plasma cells upon antigen encountering, though other immunoglobulins such as IgG and IgM are also found in fair amounts and do hold important roles as well.

As already discussed earlier in this chapter, SIgAs are formed after transcytosis of dIgAs through epithelial cells; dIgAs are released along with a “piece” of the IECs receptor pIgR to which they are bound called secretory component (SC), forming the SIgAs. In the LP, most of the IgAs are found as dIgAs or in aggregates of dIgAs called polymeric IgAs (pIgAs). The importance of SIgAs, dIgAs or pIgAs in protective immunity has long been established; indeed, several studies have revealed the efficacy of passive immunization in both human (Hammarstrom, Smith et al. 1993, Tjellstrom, Stenhammar et al. 1993, Corthesy 2003) and mice (Apter, Lencer et al. 1993, Phalipon, Kaufmann et al. 1995). Similarly, studies in mouse models or on patients with IgA deficiencies clearly show an active protection mediates by SIgA antibodies against several microbial pathogens (Friman, Nowrouzian et al. 2002, Langford, Housley et al. 2002).

The mechanisms behind IgA-mediated immune protection are numerous and can take effect in the intestinal lumen (mostly through SIgAs) and/or in the LP (mostly through dIgAs/pIgAs). SIgAs, most of which are polyreactive, primarily enable immune exclusion; i.e by cross-linking various antigens in the lumen and thereby delay or abolish adherence and/or penetration and/or massive invasion. Another effect of SIgA is to limit collateral damage by controlling inflammation that could potentially be triggered by invading pathogens. For example, SIgAs can deliver directly antigens/pathogens to the M cell for reverse transcytosis and direct handling to $CD103^+$ DCs; the M cell receptor for IgA remained unindentified for several years, but a recent study demonstrated that the immune complex SIgA-p24 (an HIV envelope protein) was transcytosed through M cells and delivered to the LP via binding to Dectin-1 on M cells (Rochereau, Drocourt et al. 2013). A summary and a non-exhaustive list of mechanisms operated by SIgAs/pIgAs are detailed in Table 3 below.

Furthermore, serum IgAs ensure further protection from invading pathogens by hiring several professional “cleaners”. Indeed, multiple myeloid cells such as neutrophils, eosinophils, monocytes and macrophages express on their surface the IgA receptor FcαRI (also called CD89). Opsonization of an antigen or pathogen by serum IgA and subsequent binding to FcαRI allows for effective removal of the pathogen or the immune complex by phagocytosis (by macrophages or neutrophils), or degranulation of toxic compounds to kill the pathogen (by eosinophils or basophils); the IgA-mediated degranulation of eosinophils and killing of schistosomes is a good example (Abu-Ghazaleh, Fujisawa et al. 1989, Dunne, Richardson et al. 1993).

Of note, the presence of a receptor on the surface of B cells and T cells has been suggested in several studies, which could possibly be a receptor for both IgA and IgM (Sakamoto, Shibuya et al. 2001); the function of interactions of B and T cells with IgAs is at this point unknown.

Table 3. Mechanisms of action of IgAs.

Effect	**Mechanism**	***In vitro/ In vivo* example(s)**
Immune exclusion	Agglutination in the lumen	*Salmonella* Enteritidis (Iankov, Petrov et al. 2002, Iankov, Petrov et al. 2004)
	Mucus trapping	*Shigella flexneri* (Phalipon, Cardona et al. 2002)
	Interception, binding and excretion of entering viruses within IECs	Influenza (Mazanec, Coudret et al. 1995) Rotavirus (Corthesy, Benureau et al. 2006) Sendai virus (Fujioka, Emancipator et al. 1998)
	Neutralization of invading pathogens in LP -> transcytosis and clearance of IC	Model *in vitro* using dIgA-antigen (Kaetzel, Robinson et al. 1991) Model *in vivo* using OVA (Robinson, Blanchard et al. 2001)
	Neutralization by free SC of pathogen-derived products	*Clostridium difficile* toxin A, EPEC Intimin (Perrier, Sprenger et al. 2006)
	Toxin/compound neutralization in the lumen	*C. difficile* toxin A (Stubbe, Berdoz et al. 2000) Cholera toxin (Apter, Lencer et al. 1993) Ricin (Mantis, McGuinness et al. 2006)
	Direct suppression of bacterial virulence in the lumen	*S. flexneri* T3SS activity (Forbes, Bumpus et al. 2011) *S*. Typhimurium flagellar motility (Forbes, Eschmann et al. 2008)
Anti-inflammatory properties	M cell transport of IC through Dectin-1/Siglec-5	HIV p24 antigen (Rochereau, Drocourt et al. 2013)
	Downregulation of pro-inflammatory responses -> protection of membrane integrity	Decrease of *S. flexneri* LPS-mediated NF-κB response in IECs (Fernandez, Pedron et al. 2003)

EPEC, Enteropathogenic Escherichia coli; T3SS, type III secretion system

3. Cytotoxic T lymphocytes (CTL)

CTL differentiation is dependent on two transcription factors, namely T-bet and and eomesodermin (Eomes); indeed, compound mutations in both genes results in defect in effector gene response in stimulated $CD8^+$ T cells. Both T-bet and Eomes were found to be necessary for expression of CD122, the receptor of IL-15 (Intlekofer, Takemoto et al. 2005). IL-15 is an important cytokine for the maturation of cytotoxic cells and enhancement of their effector function (Zeng, Spolski et al. 2005). CTLs recognize host cells that are virus-infected, contain intracellular bacteria, or are cancerous, by interaction between the TCR and their cognate foreign antigen carried on an MHC Class I molecule. Should CTLs identify infected or cancerous cells, they mediate killing in two distinct fashions. First, CTLs elaborate and release of granule toxins: perforins that are membrane pore-forming glycoproteins, granzymes that which are serine proteases, and granulysins that are small cationic proteins. As perforin molecules polymerize to form pores in the targeted cell membrane, it allows granzymes and granulysin to enter the cell and induce caspase-dependent (via granzyme B) and independent (via granzyme A) apoptosis. CTLs can also trigger apoptosis through FasL-Fas receptor interactions. Finally, CTLs also secrete large amounts of cytokines such as TNFα and IFN-γ, and TNF is also capable of mediating apoptosis (Chavez-Galan, Arenas-Del Angel et al. 2009). Interestingly, a recent study established that fully activated CTLs via IL-12 stimulation, another important cytokine for differentiation, release exosomes capable to activate resting, bystander $CD8^+$ T cells without the presence of an antigen (Li, Jay et al. 2017).

CTL-mediated killing was shown to be critical in defense against several intracellular pathogens, including *Trypanosoma cruzi* (Martin and Tarleton 2004), and *Listeria monocytogenes* (Pamer 2004).

E. Other cells involved in gut immunity regulation

1. Intra epithelial lymphocytes (IELs)

IELs form a critical branch of intestinal immunity, for an obvious reason: these lymphocytes reside within the epithelium, interspersed between IECs, and thus are in the front line of immune defense against invading pathogens (Cheroutre, Lambolez et al. 2011). IELs form a unique heterogenous population of T cells, and are all antigen-experienced. Natural IELs, which can bare either a conventional TCRαβ or a TCRγδ, develop, differentiate with “self” antigens and acquire their gut homing receptors in the thymus; thus, these IELs are already

present at, or even before birth (Latthe, Terry et al. 1994). On the other hand, induced IELs differentiate, just like conventional $CD4^+$ and $CD8^+$ T cells found in the LP, in the presence of "non-self" antigens. Of note, IELs are mostly $CD8^+$ T cells (Lefrancois 1991), unlike LP T cells that are typically made of a greater percentage of $CD4^+$ T cells than $CD8^+$ T cells.

Much like the rest of the intestinal epithelium resident cells, IELs provide immediate immune protection so as to avoid entry and/or spreading of pathogens. But because IELs are also professional immune cells, they need to be carefully regulated to avoid an excessive and potentially damaging immune response (by harming the resident microbiota or compromising the epithelium integrity).

Natural γδ IELs are important in the maintenance and repair of the epithelium barrier, and have been involved in epithelium growth, clearance of dying cells, and repair of damaged epithelium (Boismenu and Havran 1994, Komano, Fujiura et al. 1995). Probably to accomplish this task, γδ IELs were found to be highly mobile, scanning the epithelium and thereby strongly enhancing their interactions with IECs; γδ IELs migration was found to be dependent on occludin, a tight junction protein, expressed by both γδ IELs and IECs (Edelblum, Shen et al. 2012). Natural γδ IELs also share some similarities with conventional $CD8^+$ T cells, as they are cytotoxic, and can produce TNF and IFN-γ cytokines (Simpson, Hollander et al. 1997). Natural γδ IELs were found to play an important secondary role in induced TCRαβ IEL-mediated protective immunity against *Toxoplasma gondii* in mice, although this protection seemed to be independent from the production of IFN-γ (Lepage, Buzoni-Gatel et al. 1998). Finally, as discussed earlier in this chapter, γδ IELs have the potential of producing AMPs such as REGIIIγ (Ismail, Severson et al. 2011). Intriguingly, no γδ T cell antigen is known to date (Nielsen, Witherden et al. 2017).

The role for natural αβ IELs in protective immunity is still not well understood; they may have a role in early host protection and maintenance of the gut epithelium before the establishment of induced IELs after birth.

Induced TCRαβ IELs contribute, just like their TCRαβ LP T cell counterparts, to protection against invading pathogens, as demonstrated with lymphocytic choriomeningitis virus (LCMV), *Toxoplasma gondii* (Lepage, Buzoni-Gatel et al. 1998) or *Giardia lamblia* (Kanwar, Ganguly et al. 1986).

2. Innate lymphoid cells (ILCs)

ILCs are a recently discovered family of effector cells at the frontier between innate and adaptive immunity. Although some ILC subsets such as natural killer (NK) cells and lymphoid tissue-inducer (LTi) cells have been known and studied for decades, intensive research efforts have now uncovered a much bigger population. ILCs share remarkable similarities with effector T cells, including morphology, transcription factors, and effector cytokines; however, ILCs typically lack an antigen-specific TCR, making ILCs the innate counterparts to many known effector T cells (Klose and Artis 2016, Spits, Bernink et al. 2016). As illustrated in Figure 3 below, group 1 ILCs include the well-known NK cells that are similar to CTL, and ILC1 that are similar to T_H1 cells; group 2 ILC only count ILC2 thus far with similarities to T_H2 cells; group 3 ILCs include LTi cells, as well as two different groups of ILC3s that are comparable to T_H17 and T_H22 cells. Another feature of ILCs is their lack of PRRs; thus, rather than reacting directly to incoming danger, ILCs react indirectly by sensing cytokines or danger signals from myeloid cells or IECs that have been damaged or infected (Klose and Artis 2016).

ILCs are intimately involved in regulation of intestinal tissue development, integrity and homeostasis. ILC1s have been involved in intestinal epithelium protection against *Salmonella enterica*, although these T-bet-dependent ILC1s were originally from RORγt$^+$ ILC3s (Klose, Kiss et al. 2013). ILC2s strongly interact with IECs; under stimulation with IL-33, ILC2s secrete amphiregulin, a cell proliferation regulator that stimulates intestinal epithelium tissue repair (Monticelli, Osborne et al. 2015). Additionally, at steady state, the intestinal epithelium needs continuous signaling from IL-22, largely mediated by group 3 ILC, to maintain barrier integrity and contain commensal microbiota. In fact, ILC3-mediated IL-22 is required for epithelial cell proliferation after chemotherapy-induced intestinal tissue damage (Aparicio-Domingo, Romera-Hernandez et al. 2015). Some ILC3s also express MHC class II molecules, and may have a role in promoting tolerance in mice via interaction with commensal-specific CD4$^+$ T cells in the LP (Hepworth, Monticelli et al. 2013, Hepworth, Fung et al. 2015).

ILCs are also a first line of defense in many infection models (Ebbo, Crinier et al. 2017). For example, ILC1 are important for controlling *Toxoplasma gondii* infection, as seen in T-bet deficient mice lacking ILC1 (Klose, Flach et al. 2014). Also, ILC1s are critical in host defense against *Clostridium difficile* (Abt, Lewis et al. 2015). ILC2 participate in both the expulsion of *Nippostrongylus brasiliensis* and clearance of *Candida albicans* infection (Huang, Guo et al. 2015). Group 3 ILCs have been shown to be the dominant source of IL-22 that controls

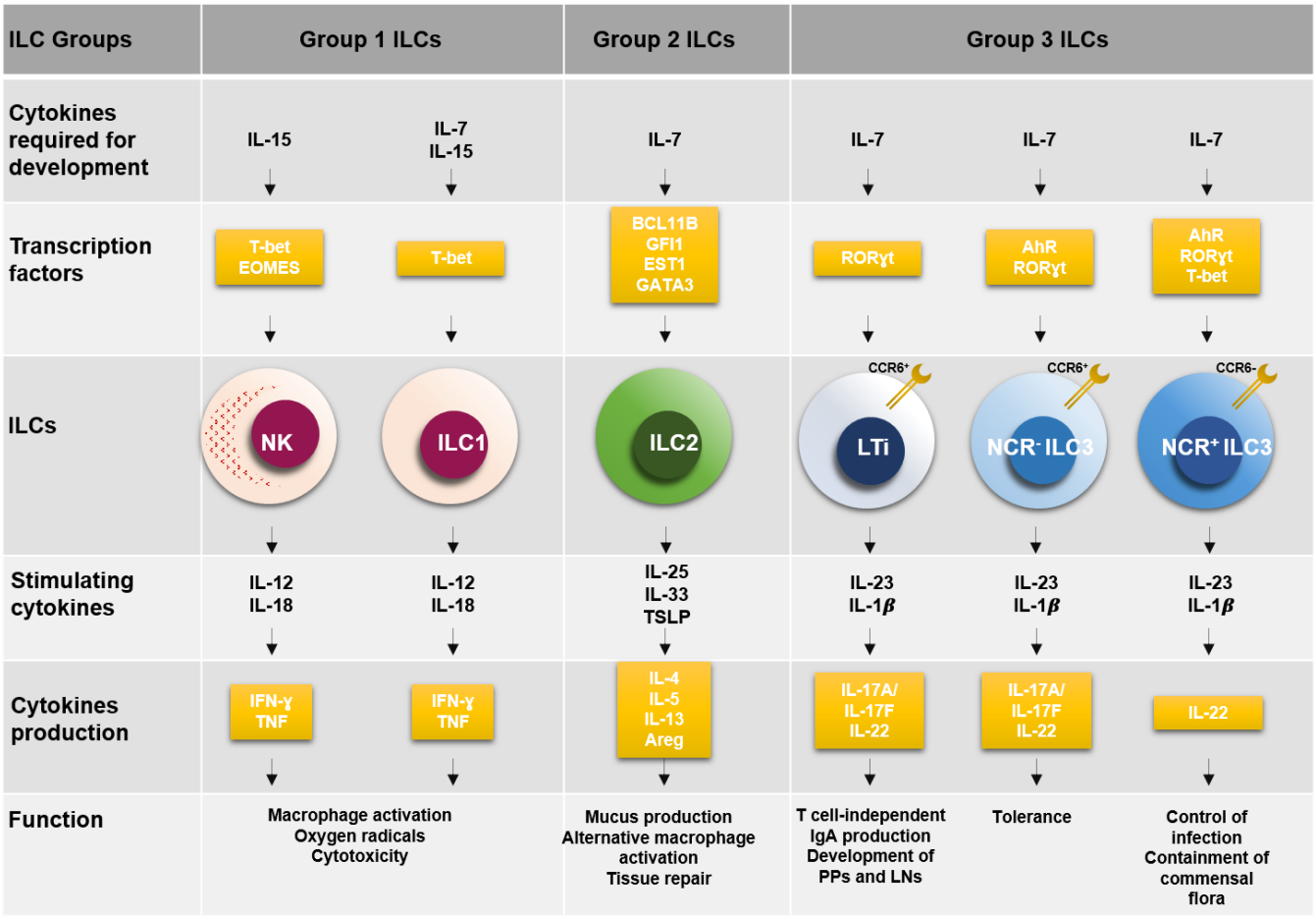

Figure 3. Differentiation and function of Innate Lymphoid cells (ILCs). *Adapted from a figure in (Ebbo, Crinier et al. 2017)*

Citrobacter rodentium during the first week of infection (Zheng, Valdez et al. 2008, Sawa, Lochner et al. 2011, Sonnenberg, Monticelli et al. 2011). LTi cells also have an indirect effect on IgA CSR. Indeed, LTi produce lymphotoxin (LTαβ), which interacts with LTβR on the surface of DCs, thereby ensuring maintenance of DCs in the SED and DC-mediated upregulation of TGFβ in B cells (Reboldi, Arnon et al. 2016).

F. Immune memory

1. Memory T cells

During the course of an infection, T cells evolve through three main phases: the priming phase, the resolution phase, and the memory phase. As discussed earlier in this chapter, during the first phase, naïve $CD8^+$ T cells go through extensive proliferation and differentiate into CTLs. Once the pathogen cleared, CTLs enter the resolution and contraction phase, where most of the CTLs die by apoptosis. Reestablishing homeostasis is essential, considering the metabolic cost generated by rapidly dividing cells, and the potential damage that these cytotoxic cells can cause, such as chronic inflammation or immunopathology. The 5-10% CTLs left persist and enter the memory phase, where their maintenance is ensured by IL-7 and IL-15. In the gut, as in all mucosal sites, memory T cells represent the predominant T cell subset, and a critical arm in infection immunity (Mueller, Gebhardt et al. 2013). Immunological memory allows for enhanced immunosurveillance and rapid onset of highly specific recall responses to reinfection.

Memory $CD8^+$ T cells can be divided in three main groups: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) have been studied for more than a decade, while tissue-resident memory T cells (T_{RM}) are a more recent discovery (Gebhardt, Wakim et al. 2009). T_{CM} cells are primarily located in secondary lymphoid organs; accordingly, these cells express CD62L (L-selectin) and CCR7, which is needed for entry in secondary lymphoid organs. T_{CM} cells have the highest proliferative potential of the three known subsets, which means they can quickly proliferate and differentiate in case of a reinfection. T_{EM} cells, on the other hand, are preferentially located in non-lymphoid tissues, although they can migrate between tissues and secondary lymphoid organs via the afferent lymph; that said, T_{EM} cells do not express CD62L (L-selectin) and CCR7 which would allow them to circulate freely. As their name implies, T_{EM} cells have the ability to exert immediate functions upon rechallenge, by secreting cytokines such as IFN-γ. Hence, T_{EM} cells can be recruited into inflamed tissues and be active within hours to days; on the other hand, T_{CM} cells will take longer but will generate a large wave of secondary effector T cells. A key property of T_{RM} cells is that they

do not re-circulate via the bloodstream; instead, they stay at mucosal sites, and constitute the largest memory cell population in non-lymphoid tissues. To remain in tissues, T_{RM} cells downregulate surface molecules associated with tissue egress (Skon, Lee et al. 2013). T_{RM} cells are characterized by the presence of CD69 and CD103, as well as a downregulation of the gut homing receptor α4β7. Expression of CD103 may explain why T_{RM} cells are found to localize within the epithelial layer, but could have a role in the long persistence of T_{RM} cells in the gut (Casey, Fraser et al. 2012). Although this has not been confirmed or seen yet in the intestines, recent mouse studies in the female reproductive tract tissues have suggested that T_{RM} cells were assuming the role of highly specific cells, yet responding like an innate immune cell. Indeed, as T_{RM} cells sense the pathogen, they quickly secrete IFN-γ, which recruits circulating T and B cells, as well as stimulate NK cells and DCs, thus creating a state of emergency on site and effectively enhancing clearance of the pathogen (Schenkel, Fraser et al. 2014). Noteworthy, T_{RM} cells can express granzyme B, though they have not been shown yet to be effective killer.

$CD4^+$ T cells support in many ways the generation of memory $CD8^+$ T cells (Janssen, Lemmens et al. 2003, Laidlaw, Craft et al. 2016). For example, Treg cell-derived IL-10 facilitates the development of a mature memory $CD8^+$ T cell. Additionally, activated CD4+ T cells are a major source of IL-2, and IL-2 is important during $CD8^+$ T cell priming to imprint memory responsiveness of $CD8^+$ T cells (Williams, Tyznik et al. 2006).

Memory cells belonging to the major T_H subsets have been identified. *Listeria*-specific T_H1_{EM} cells form during infection, as well as $CD4^+$ T_{CM}, for which generation and maintenance is aided by B cells (Pepper, Pagan et al. 2011). Another report identified T_H1 and T_H2 resident memory cells originating after a *Listeria monocytogenes* infection (Marzo, Vezys et al. 2002). Generation of T_H1 memory cells seems to depend on long and stable interactions with the antigen during the primary response (Kim, Wilson et al. 2013). IL-17A-producing $CD4^+$ T_{EM} cells have been shown to increase during acute *Giardia lamblia* infection in humans (Saghaug, Sornes et al. 2015). Memory T_{FH} cells express CXCR5 and remain at the T cell-B cell border in the secondary lymphoid tissues, and get quickly reactivated by B cells to induce expression of the Bcl6 transcription factor (Hale and Ahmed 2015).

2. Memory B cells

While plasma cells are long-lived, their lifespan of about one year is not sufficient to ensure long term protection against enteric pathogens. Memory B cells derive from activated B cells that have undergone SHM and CSR, but maintain their surface BCR. The concept and longevity of intestinal memory B cells after mucosal immunizations was challenged many

times; however, strong evidence is in support of their existence and importance in gut-mediated immunity, as exemplified by the many studies on cholera toxin-induced memory B cells (Vajdy and Lycke 1995, Lycke and Bemark 2010, Lindner, Thomsen et al. 2015).

A recent study (Bemark, Hazanov et al. 2016) demonstrated the development and maintenance of memory B cells after oral immunization, which remains, albeit in low number, present for at least one year in B cell follicles in the gut, but also in the spleen and the MLN. A second immunization showed a sharp increase in antigen specific-IgA plasma cells in the LP. Interestingly, memory B cells and plasma cells shared limited clonal relatedness; on the other hand, memory B cells and newly generated plasma cells one year later post-second immunization were oligoclonal. These observations would suggest that memory B cells would develop and leave early the process of SHM in the GC, and would re-enter the process after antigen recall to generate high affinity IgA plasma cells. This could represent a strategic way by which B cells maintain a fairly large repertoire to prepare for a new encounter against the same pathogen, or a related strain.

G. Immunity and the gut microbiota

I have mainly discussed throughout this chapter the general mechanisms by which the innate and adaptive immune system control a microbial infection, but also keep the gut microbiota under control. This relationship is certainly not unidirectional, and extensive research efforts are undertaken to unravel the many pathways in which the microbiota takes part to influence the development and outcome of the immune response.

Microbes colonize the gut immediately after birth, and the microbiota composition “layout” will largely depend on the nutrient requirement of each microbial member, thus leading to extensive variation along the length of the gastrointestinal tract (Mowat and Agace 2014). Importantly, host and microbiota have co-evolved into a mutualistic symbiosis whereby microbes exploit the rich nutrient environment to thrive, and in return provide the host with a plethora of physiological and immune benefits. Not surprisingly, the dysregulation of the microbiota composition will thus dramatically affect not only the local tissue homeostasis and host immunity to infection, but also other physiological aspects at more distant sites or even systemically. In this section, I will describe the general composition of the gut microbiota in a “healthy” host, and discuss some of the main effects of the microbiota on the host intestinal immunity.

1. Main composition of a healthy gut microbiota

Metagenomics sequencing has allowed considerable progress in determining the composition of the gut microbiota, as it has allowed identification of the many non-culturable microbes, and for functional and sequence-based analysis of this complex microbial community (Riesenfeld, Schloss et al. 2004). The microbiota composition changes dramatically during infancy, it tends to stabilize itself in the healthy adult gut, although inter-individual differences are still very much present and depends on several factors, such as diet and lifestyle. Furthermore, mice still represent a powerful model for functional studies; thus, determining the mouse gut microbiota seems nowadays essential. Two large metagenomics studies have established a genome catalog of the human gut microbiota from 124 European adults (Qin, Li et al. 2010), and of the mouse gut microbiota based on 184 mice with different genetic backgrounds, housing locations, and diet (Xiao, Feng et al. 2015). For both hosts, more than 99% of the cataloged genes were found to be of bacterial origins. The overall composition at the phylum level remains similar between the two hosts. Indeed, as previously established with 16S ribosomal RNA gene analysis, the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria represent more than 70% of the gut microbiota, with a clear dominance of Firmicutes and Bacteroidetes. From these studies emerged core sets of 33 common genera found in the human gut, and 60 in the mouse gut; a list of the 20 top core genera, shown below as table 4, shows significant similarities between mouse and human at the genera level. Although the mouse microbiome seems to be functionally similar compared to the human one, it was interesting to find out that only 4% of the sequences genes were shared between mouse and human (Xiao, Feng et al. 2015).

Considerable variation exists in the microbiota composition depending on the site of the intestine, and exist both longitudinally and transversally. Among physiological parameters at play, nutrients are a source of fierce competition along the intestines, and have drastic effects on the gut microbial landscape, whether in a state of homeostasis or dysbiosis. In healthy mice, for example, the Enterobacteriaceae and Lactobacillales families mostly reside in the small intestine where they can harvest energy sources like disaccharides and amino acids; on the other hand, Bacteroidales and Clostridiales are dominant families in the large intestine, as they express enzymes capable of degrading the complex carbohydrates found in the large intestine. Additionally, the small intestine is characterized by a short transit time, the presence of oxygen, and a high secretion of AMPs in the mucus; thus, this site will be more favorable to facultative anaerobes, which can grow rapidly, adhere to IECs and be resistant to AMPs. Many members of the Firmicutes and Bacteroidetes, as well as *Salmonella* species, decrease the negative charge on their membranes by removing

phosphate groups from their LPS, allowing them to evade cationic AMPs, reach the epithelium border and adhere to IECs (Donaldson, Lee et al. 2016).

Table 4. Top 20 bacterial genera in the mouse and human gut microbiota

Mice top 20 core genera	Human top 20 core genera
Bacteroides (B)	*Bacteroides* (B)
Clostridium (F)	*Clostridium* (F)
Butyrivibrio (F)	*Butyrivibrio* (F)
Prevotella (B)	*Prevotella* (B)
Alistipes (B)	*Alistipes* (B)
Lactobacillus (F)	*Lactobacillus* (F)
Roseburia (F)	*Roseburia* (F)
Ruminococcus (F)	*Ruminococcus* (F)
Eubacterium (F)	*Eubacterium* (F)
Blautia (F)	*Blautia* (F)
Parabacteroides (B)	*Parabacteroides* (B)
Coprococcus (F)	*Coprococcus* (F)
Pseudoflavonifractor (F)	*Streptococcus* (F)
Marvinbryantia (F)	*Oscillibacter* (F)
Enterococcus (F)	*Bifidobacterium* (A)
Desulfovibrio (P)	*Desulfovibrio* (P)
Anaerotruncus (F)	*Klebsiella* (P)
Odoribacter (B)	*Odoribacter* (B)
Coprobacillus (F)	*Coprobacillus* (F)
Faecalibacterium (F)	*Faecalibacterium* (F)

(F) Firmicutes; (B) Bacteroidetes; (P) Proteobacteria; (A) Actinobacteria.

Adapted from a figure in (Xiao, Feng et al. 2015).

2. Role of the microbiota in immunity

a) Colonization resistance

In the healthy gut, gut symbionts form a stable community that, through various means, prevents invasion of incoming non-native microbes and expansion of pathogens; this process is characterized as colonization resistance, and can be observed by the inability of a compromised, antibiotic-treated gut microbiota, to prevent such invasions.

One of the direct means of colonization resistance is by killing competitors in the nearby environment. The gut harbors several bacteria producing bacteriocins. These antibacterial peptides can be either bacteriostatic or bactericidal against close members of the producing species, and can be of various sizes and mechanisms of action (Hammami, Fernandez et al. 2013). Though widespread in nature, bacteriocins seem to be mainly produced by Gram-positive bacteria. In the gut, Bifidobacteria and lactic acid bacteria are common producers of bacteriocins. The potential of bacteriocins to mediate colonization resistance was nicely showed by a study where *Enterococcus faecalis* carrying a plasmid for bacteriocin production was able to outcompete and mediate clearance of a close vancomycin-resistant strain, and a problematic opportunistic pathogen in hospitals (Kommineni, Bretl et al. 2015). Other killing mechanisms are known to shape the gut community. The type VI secretion system (T6SS) is a multi-protein contractile nanomachine that delivers toxin proteins directly into the target cell in a contact-dependent fashion (Alteri and Mobley 2016). The T6SS allows the bacterium to target, via a variety of toxins, other bacteria as well as eukaryotic cells. About a third of Gram-negative bacteria with sequenced genome carry T6SS genes (Durand, Cambillau et al. 2014), among which *V. cholerae*, enteroaggregative *E. coli* and *C. rodentium*; this system can be particularly useful in a crowded environment like the gut lumen, as attackers can effectively kill surrounding cells and have access to precious nutrients and space. Of note, a type VI secretion-related family has been found in Bacteroidetes, which could at least partly explain the successful colonization of the gut by Bacteroidetes (Russell, Wexler et al. 2014).

Additionally, colonization resistance can occur by means of inhibitory metabolic byproducts generated by gut resident bacteria. Short-chain fatty acids are a particularly well-known example, and are produced by anaerobic gut symbionts such as *Bacteroides* and *Clostridia*. Among SCFAs, acetic and butyric acids were shown early on to be inhibitory for *S.* Typhimurium (Bohnhoff, Miller et al. 1964). Similarly, propionic, butyric and acetic acids inhibit growth of *E. coli* O157:H7 (Shin, Suzuki et al. 2002) and *C. difficile* (Rolfe 1984) *in vitro*. Bile acids, produced in the liver and secreted in the intestines, are modified by the gut microbiota into various secondary bile acids with different chemical and biological activities

(Devlin and Fischbach 2015). Antibiotic treatment results in a decrease in generation of secondary bile salts, and a greater susceptibility in *C. difficile* infection in both mice and humans (Buffie, Bucci et al. 2015); this susceptibility was significantly counteracted by implementation of *Clostridium scindens* in mice, which was linked to reestablishment of a particular type of secondary bile acids produced by *C. scindens*. As discussed earlier, Bacteroidales can thrive in the large intestine thanks to their ability to degrade complex carbohydrates. Some of these large carbohydrates come from the mucus glycans, and their degradation by *Bacteroides* give rise to sialic acid and fucose. These byproducts are normally utilized by other members of the gut microbiota; indeed, treatment of mice with antibiotics provokes successful colonization of the intestinal tract by *S*. Typhimurium and *C. difficile*, two pathogens that can feed on these byproducts; this colonization is not seen in mutants of *S*. Typhimurium or *C. difficile* that are unable to utilize sialic acid. Also, reducing the availability of sialic acid by the use of a sialidase-deficient *Bacteroides* reduced colonization of *C. difficile*; dietary addition of free sialic acid reversed this effect (Ng, Ferreyra et al. 2013).

The maintenance and composition of the mucus barrier is also indirectly controlled by the resident microbiota. Indeed, germ-free mice show a compromised inner mucus layer in the colon where bacteria can reside (Johansson, Jakobsson et al. 2015). Members of the healthy gut microbiota modulate the thickness and composition of mucus: for example, by the stimulation of MUC2 production by *Lactobacillus* species, or by the increase of differentiation of goblet cells by *Bacteroides thetaiotaomicron* (Sicard, Le Bihan et al. 2017).

b) Role in immunity development and function

Perhaps the most profound effect of the gut resident microbiota is on development and function of T cells. For example, researchers found that bacterial polysaccharide from *Bacteroides fragilis*, a common gram-negative anaerobe in the mammalian gut, was sufficient to drive the expansion of $CD4^+$ T cells and cytokine production in a DC-dependent fashion, and restored the T_H1/T_H2 imbalance observed in germ-free mice by inducing expression by IFN-γ (Mazmanian, Liu et al. 2005). Moreover, germ-free mice completely lack T_H17 cells in the small intestine and colon (Ivanov, Frutos Rde et al. 2008), this phenotype was reversed after colonization with conventional microbiota, but not with Altered Schaedler Flora, a defined cocktail of bacteria. The generation of T_H17 was later on found to be induced by segmented filamented bacteria (SFB), which are spore-forming Gram-positive bacteria closely related to the genus *Clostridium*, and found in both mice and humans (Ivanov, Atarashi et al. 2009); colonization with SFB in T_H17-deficient mice led to induction of IL-17

and IL-22 expression by $CD4^+$ T cells, whereas colonization with *Bacteroides* or *Clostridium* species did not. This effect of SFB was shown to be due to the upregulation of serum amyloid A (SAA) proteins, which in turn act on DCs to drive T_H17 cell differentiation. SFB, just like the pathogens *C. rodentium* and *E. coli* O157, tightly adhere to the surface of IECs, and all three species promote T_H17 induction, as well as IgA-ASCs, through IEC adherence (Atarashi, Tanoue et al. 2015). Additionally, by selecting in mice colonized by human microbiota for strains enhancing Treg cells, a mixture of seventeen strains belonging to Clostridia were isolated and shown to induce IL-10 and as well as inducible T-cell costimulatory factor, thus strongly driving for Treg differentiation (Atarashi, Tanoue et al. 2013). Mechanistic studies demonstrated that Treg cell enhancement is due to microbiota-derived metabolites, in particular the SCFA butyrate that acts by upregulating Foxp3 and with it Treg differentiation (Arpaia, Campbell et al. 2013, Furusawa, Obata et al. 2013). More generally, the gut microbiota provides a myriad of stimulating ligands, recognized by PRRs on host cells, which act to reinforce the host's immune response to infection. Indeed, antibiotic-treated mice show impaired innate antiviral response to LCMV by macrophages (Abt, Osborne et al. 2012); similarly, germ-free mice failed to induce IFN-γ and NK priming by DCs, after challenge with LPS or virus infection (Ganal, Sanos et al. 2012).

The tremendous influence of SIgAs in controlling the gut microbiota via coating and immune exclusion underlines its importance in the maintenance of gut homeostasis; conversely, the presence and composition of the microbiota is equally important for the production and secretion of SIgAs in the lumen. IgA production can be induced by a variety of microbes, although the amplitude of induction and the level of specificity may differ depending on the source of induction (Lycke and Bemark 2017). For example, SFB strongly stimulates GCs in PPs, but leads to a broadly reactive IgA response; on the other hand, *E. coli* MG1655 does not stimulate GCs nearly as highly, but induces a more specific IgA response (Lecuyer, Rakotobe et al. 2014). Interestingly, a study found the presence of *Alcaligenes* species, which are opportunistic bacteria, specifically inhabiting PPs, and driving antigen-specific mucosal IgA antibodies mediated by stimulation of DCs (Obata, Goto et al. 2010). Norovirus infection in germ-free mice induced IgA as well (Kernbauer, Ding et al. 2014).

II. Pathogenic *Escherichia coli*

A. Introduction: on defining pathogenic *E. coli*

It only seems fair to start this chapter by reminding ourselves that *E. coli* is normally a harmless commensal that occupies some of the available real estate in the gut of humans and other animals. Taking into account the entire gut microbiota, *E. coli* only encompasses 1% of the population; but as a facultative anaerobe, *E. coli* represents about 80% of the aerobic flora.

But it only takes one figure to get a good grasp of *E. coli* remarkable versatility and potential for harm in humans (Figure 4). Thanks to its ability to acquire various combinations of mobile genetic elements by horizontal gene transfer, *E. coli* now has the tools to develop as an extremely efficient human pathogen that cause various forms of diarrheal diseases. Perhaps most impressively, some *E. coli* pathogens have evolved tropism for other organs than the gut, namely the bladder, kidneys, and brain, and are now infamous for the severity of diseases they cause, such as sepsis and neonatal meningitis (Figure 4).

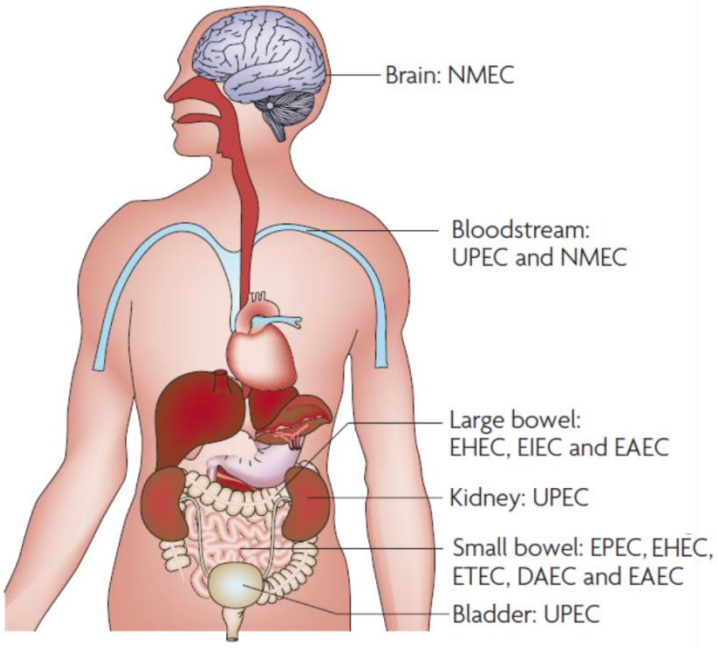

Figure 4. The major E. coli pathotypes and their colonization sites. *Enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), and diffusely adherent E. coli (DAEC) colonize the small bowel. Enteroinvasive E. coli (EIEC) colonizes the large bowel. Enterohemorragic E. coli (EHEC) and enteroaggregative E. coli (EAEC) can colonize both small and large bowels. Uropathogenic E. coli (UPEC) colonizes the urinary tract and can migrate to the kidney and enter the bloodstream. Neonatal meniningitis E. coli (NMEC) can also enter the bloodstream to cause bacteremia, and traverse the blood brain barrier to colonize the brain. From (Croxen and Finlay 2010).*

To account for such diversity, the various pathogenic *E. coli* isolates encountered have been segregated in general groups and pathotypes, depending on their organ tropism and the mechanism of virulence employed to cause disease. Within the group of intestinal pathogenic *E. coli*, or InPEC, many have been studied for long, such as the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), and diffusely-adherent *E. coli* (DAEC). As for the group of extraintestinal pathogenic *E. coli* (ExPEC), we find neonatal meningitis *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC). In the late 90s, the adherent-invasive *E. coli* (AIEC), associated with Crohn's disease, was proposed as a new InPEC pathotype (Boudeau, Glasser et al. 1999). Furthermore, the notable pathogenic *E. coli* outbreak witnessed in Europe in 2011 unraveled a new hybrid InPEC pathotype, since the etiological strain was an EAEC that had acquired the Shiga toxin genes found among EHEC (Rasko, Webster et al. 2011, Karch, Denamur et al. 2012); this pathotype is now termed EAHEC for enteroaggregative hemorrhagic *E. coli*. Lastly, in 2013 in France, a hybrid atypical EHEC (*E. coli* O80:H2) carrying a virulence plasmid usually found in NMEC cause bacteremia in an adult, a characteristic that had never been attributed yet to EHEC (Mariani-Kurkdjian, Lemaitre et al. 2014, Soysal, Mariani-Kurkdjian et al. 2016). These last additions certainly exemplify the fact pathogenic *E. coli* are a growing family, and with them come new public health challenges. These challenges are also growing bigger as we face a scarcity of effective treatments; indeed, with such an ability to pick up and transfer mobile elements, it is not surprising that *E. coli* pathogens have rapidly responded to the use of antibiotics by acquiring one or several antibiotic resistance genes on top of their virulence package. As antibiotic discovery seems to be stalled, understanding the specific mechanisms of virulence and pathogenicity of each of these pathotypes is paramount to come up with new effective means of treatment.

In this chapter, I will briefly introduce each the main players among ExPEC and InPEC, and will particularly focus on the EHEC pathotype, as it constitutes a significant part of my thesis work. It is noteworthy that this choice is not meant to discount the importance of other *E. coli* pathotypes, particularly when it comes to morbidity and mortality in third world countries.

B. Extraintestinal pathogenic *E. coli*

1. Neonatal meningitis *E. coli* (NMEC)

Neonatal meningitis is a severe acute inflammation due to an infection of the central nervous system, including the meninges, the subarachnoid space and brain vasculature. Mortality to neonatal meningitis can be high (up to 58% in developing countries), and neurological

impairment affect 50% of the survivors, half of those with severe disabilities. Early onset meningitis is distinguished from late onset meningitis based on the appearance of signs of infection at ≤ 72 hours and > 72 hours of life, respectively. A systematic study of mortality in children under 5 years of age in 2010 reported that, within the 7.6 million deaths, 40% were neonatal deaths, among which 5% (0.393 million) were caused by sepsis or meningitis (Liu, Johnson et al. 2012). Not surprisingly, the incidence of neonatal meningitis is higher in developing countries compared to developed countries: while incidence is about 0.3 per 1000 live births in developed countries, it rises up to 0.8-6.1 per 1000 live births in developing countries (Ku, Boggess et al. 2015).

NMEC represent the second most common etiological agent of neonatal sepsis and meningitis after group B *Streptococcus* (GBS, *Streptococcus agalactiae*), and the most common cause of mortality. NMEC has been isolated in 30% of all early-onset infections reported from a massive US national study representing a cohort of nearly 400,000 infants (Stoll, Hansen et al. 2011); this study identified NMEC to be the most frequent pathogen in early onset sepsis in pre-term infants. In a US epidemiological study spanning from 2005 to 2014, 1484 cases of early onset sepsis were identified, 25% of which were caused by *E. coli*, which showed a stable rate of infection throughout those 9 years (Schrag, Farley et al. 2016). Low gestational age and birth age was associated with a higher risk of *E. coli* infection compared to GBS.

The pathophysiology of *E. coli*-mediated meningitis is a complex multi-step process. First, NMEC is acquired perinatally from the mother, NMEC colonizes the infant intestinal tract. From there, NMEC enters the bloodstream by transcytosis through the intestinal epithelium border, where NMEC multiplies. Disease progression to meningitis has been linked to a threshold bacteremia level of 10^3 colony-forming units per ml of blood in neonates, which means that survival of NMEC in the blood is a critical step in pathogenesis (Dietzman, Fischer et al. 1974). Once that threshold is attained, *E. coli* penetrates the blood brain barrier (BBB) into the central nervous system. There, NMEC multiplies further and releases pro-inflammatory and toxic compounds; as a result, the BBB becomes permeable to allow entry of white blood cells, which results in meningitis.

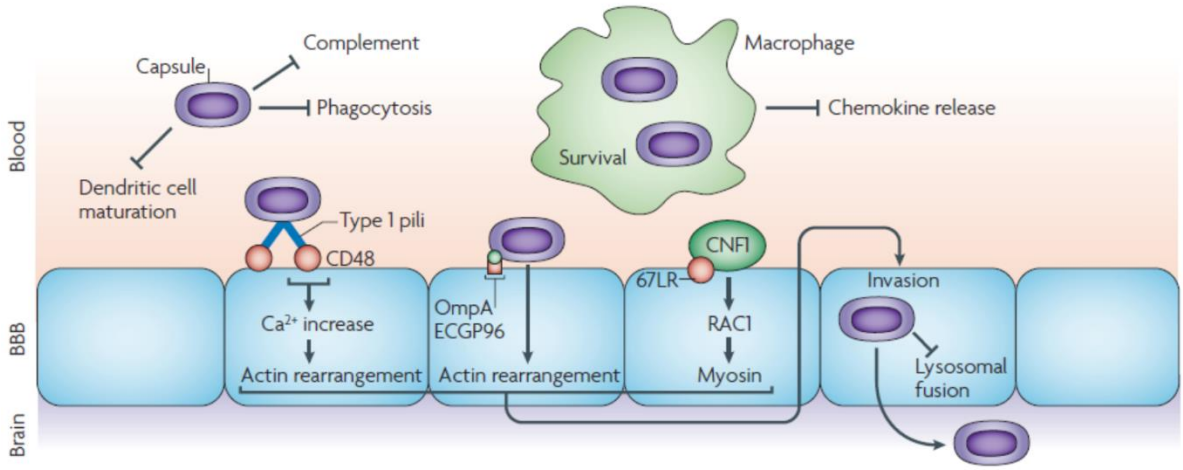

Figure 5. Colonization of the brain by neonatal meningitis E. coli. *Within macrophages, NMEC can replicate while hiding from the immune system and blocking macrophage regular pro-inflammatory functions. Provided NMEC reaches high bacteremia in the blood, NMEC crosses the blood-brain barrier (BBB) to reach the brain. NMEC attaches to BBB cells via the type I pili that binds to CD48, but also via OmpA that binds to ECGP96. The cytotoxic necrotizing factor 1 (CNF1) binds to the laminin receptor 67LR, which along with type 1 pili and OmpA, mediate invasion of BBB cells. From (Croxen and Finlay 2010)*

Several factors have been linked to virulence and pathogenicity of NMEC, especially to allow survival in the bloodstream, and invasion of the BBB cells (Figure 5) (Croxen and Finlay 2010). Survival in the bloodstream is aided by a capsule made of a homopolymer of polysialic acid, providing resistance to phagocytosis, as well as the outer-membrane protein OmpA, which confers resistance to classical complement-mediated killing (Wooster, Maruvada et al. 2006). When invading macrophages and monocytes, NMEC is also able to block apoptosis as well as cytokine release, thus providing the pathogen with a “safe” place to hide and replicate (Sukumaran, Selvaraj et al. 2004, Selvaraj and Prasadarao 2005). At the BBB, multiple proteins are involved in attachment and invasion of brain microvascular endothelial cells. FimH (of the type 1 pili) and OmpA bind to CD48 and ECGP96, respectively, and mediate actin rearrangement within endothelial cells. Furthermore, the cytotoxic necrotizing factor (CNF1) induces myosin rearrangements, which is also needed for NMEC invasion. Importantly, after invasion, the K1 capsule prevents lysosomal fusion within endothelial cells, allowing NMEC to efficiently cross the BBB; this particular feature most likely explain the *E. coli* K1 is the isolate found in 80% of cases of *E. coli* meningitis.

Antibiotics used for treatment against NMEC vary whether the case is an early onset or late onset sepsis. Gentamicin is used for against NMEC involved in an early onset sepsis, while Cefotaxime is preferred for late onset sepsis.

2. Uropathogenic *E. coli* (UPEC)

With 150 million people affected each year worldwide, urinary tract infections (UTIs) are the most common bacterial infections worldwide, and represent a major public health cost. Uncomplicated UTIs are diagnosed in patients who are otherwise healthy, and can be differentiated as lower UTIs if the infection is localized in the bladder (termed cystitis), or upper UTIs if the bladder infection progresses into the kidney (called pyelonephritis). Recurrent cystitis (defined more commonly as recurrent UTIs) is a major health concern; close to a third of women experiencing an acute cystitis will experience a recurrence of the infection within 3 to 4 months (Foxman 2002). Complicated UTIs occur in patients facing a compromised urinary tract or immune system; for example, most cases of complicated UTIs in the US are associated with indwelling catheters (Lo, Nicolle et al. 2014). These catheter-associated UTIs or CAUTIs represent the most common cause of secondary bloodstream infections, which can be fatal, and risks to develop CAUTIs are increased with female genders diabetes, and older age (Chenoweth, Gould et al. 2014).

By itself, UPEC encompasses 75% of uncomplicated UTIs and 65% of complicated UTIs, making it the number one causative agent of UTIs. Other causative pathogens include *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Staphylococcus saprophyticus*.

Infections with UPEC, much like the rest of pathogens involved in UTIs, usually starts with the contamination of the periurethral area by the pathogen originally residing in the gut (Yamamoto, Tsukamoto et al. 1997, Chen, Wu et al. 2013). From there, successful colonization and ascension from the urinary tract to the bladder and kidneys require complex mechanisms to colonize different organs while evading the host immune response; most importantly, primary colonization of the urinary tract necessarily implies powerful attachment mechanisms to avoid clearance of the pathogen by micturition (Croxen and Finlay 2010, Flores-Mireles, Walker et al. 2015). In this regard, pili are considered a definite virulence factor in UPEC; it is noteworthy to mention that 38 distinct pilus operons have been identified in UPECs, and that a single species can carry as much as 12 of them (Wurpel, Beatson et al. 2013, Flores-Mireles, Walker et al. 2015). One of the most characterized pili, the type 1 pilus, is necessary in the process of adherence to and invasion of the bladder epithelium, or uroepithelium (Figure 6). Indeed, the adhesive tip of the type 1 pilus, FimH, has been shown to bind the uroepithelium via several surface molecules, namely uroplakins and integrins, which coat the surface of umbrella cells (also called facet cells, the first layer of cells of the uroepithelium). Both interactions result in actin rearrangements and bacterial invasion of umbrella cells, thus leading to resistance to the host immune system and antibiotic treatment. Within the host cell cytoplasm, UPEC multiplies by forming biofilm-like structures called

intracellular bacterial communities (IBCs), which upon maturation lead to bacterial dispersion to invade further cells (Anderson, Palermo et al. 2003). The onset of cystitis triggers a massive influx of neutrophils; in an attempt of evasion while transitioning from one IBC to another, UPEC cells can exit the IBCs as filaments rather than rods (by inducing SulA, an inhibitor of cell division), thus preventing neutrophil engulfment (Figure 6) (Justice, Hunstad et al. 2006). Additionally, UPEC secretes the HlyA toxin (for α-hemolysin) that can serve several purposes for UPEC: as HlyA induces cell inflammation, it can trigger cell lysis, thus releasing iron into the milieu, which UPEC can scavenge through siderophores; alternatively, cell inflammation can provoke exfoliation in an attempt to counteract infection (Smith, Rasmussen et al. 2008), allowing UPEC to access and invade the underlying layer of cells in the bladder epithelium, called transitional cells. Within transitional cells, UPEC is likely to form quiescent intracellular reservoirs (QIRs), which consist of a few resting cells within membrane-bound compartments encased in actin (Figure 6). These QIRs can persist for months and allow UPEC to eventually reset an infection process, which could probably explain the high occurrence of recurrent UTIs. Another virulence factor of UPEC, the cytotoxic necrotizing factor (CNF1) induces anti-apoptotic and pro-survival pathways to allow for successful colonization and multiplication of the bacterial population (Miraglia, Travaglione et al. 2007).

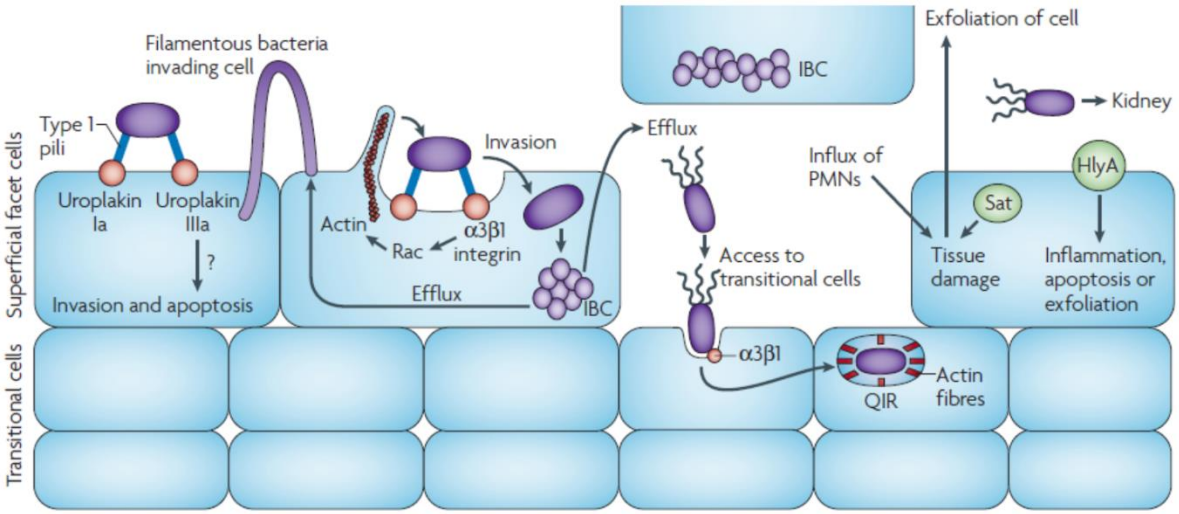

Figure 6. Colonization of the uroepithelium by uropathogenic E. coli (UPEC). *The binding of type 1 pili to urolapkins on the uroepithelium facet cells provokes invasion of UPEC and apoptosis of facet cells. Alternatively, the binding of type 1 pili to α3β1 integrin leads to internalization of UPEC to form intracellular bacterial communities (IBC). UPEC induces exfoliation of facet cells in part by secreting hemolysin A (HlyA), which results in the underlying transitional cells to be exposed. UPEC can further invade transitional cells and establish quiescent intracellular reservoirs (QIR) that can maintain themselves for long periods of time and potentially lead to reinfection of the bladder. From(Croxen and Finlay 2010).*

The infection may progress to pyelonephritis as UPEC makes its way up to the kidneys. Adherence and colonization are aided by the adhesin PapG, localized at the tip of UPEC P pilus, which binds to complex glycolipids called globosides lining the kidney epithelium (Stapleton, Stroud et al. 1998). Interestingly, PapG also interacts with TLR4 at the surface of kidney epithelial cells, leading to the downregulation of the polymeric IgR receptor (pIgR) that is responsible for IgA transport across the kidney epithelium into the urinary space (Rice, Peng et al. 2005); thus, PapG and the P pilus interfere with humoral immune response by inhibiting antibody-mediated opsonization and clearance of UPEC in the kidneys. Eventually, colonization of the kidneys can lead to UPEC entry to the bloodstream via the renal vasculature, and cause potentially serious cases of bacteremia.

Antibiotics constitute the most common line of treatment against UTIs; however, the major increase of antibiotic resistance and the multiple cases of recurrent UTIs have considerably decreased their efficacy. The understanding of UPEC mechanism of pathogenesis unveiled efficient mechanisms to avoid, in many cases, complete clearance by antibiotics, such as by hiding within host cells as quiescent and persistent cells and forming QIRs. Furthermore, although effective antibiotic treatments against UTIs are available, the “blind” prescription of antibiotics without previous characterization of the bacterial species causing the UTI has been a common practice and has led to an increase in antibiotic resistance, and decrease in treatment efficacy.

Multidrug resistance is a common trait in pathogenic *E. coli*; for example, UPEC isolates are now commonly found to carry plasmids encoding extended-spectrum β-lactamases as well as other resistance genes, thus conferring these isolates resistance against a large array of the antibiotics used nowadays to treat UTIs. In Europe, close to 12% and 22% of UPEC isolates are found resistant to third generation cephalosporins and fluoroquinolones, respectively; fluoroquinolone resistance affects more than 30% of UPEC isolates in the US. For women experiencing recurrent UTIs, antimicrobial prophylaxis may be prescribed; though extensive research now focuses on vaccines rather than antibiotic as a prophylactic means to protect patients.

C. Intestinal pathogenic *E. coli*

1. Enterohemorrhagic *E. coli* (EHEC)

EHEC are pathogenic strains that colonize the human ileum and colon, and are responsible for serious diseases such as hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS). Because bovines are healthy carriers of EHEC, EHEC can engender outbreaks by food

contamination. EHEC strains all possess the *stx* genes, encoding Shiga toxins, which are capable of inducing lesions, particularly on the renal and cerebral vascular endothelia (O'Loughlin and Robins-Browne 2001). All strains carrying the *stx* genes are termed Shiga toxin-producing *E. coli* (STEC), while the term EHEC should be reserved for strains that have been isolated from patients. Indeed, not all STEC strains, whether isolated from cattle feces, food or the environment are necessarily pathogenic to humans.

a) EHEC and STEC classifications

(1) General classification of *E. coli* strains, and main EHEC serotypes

The golden standard for identification of *E. coli* strains has been via serotyping three surface antigens: the O polysaccharide antigen, the flagellar H antigen, and the capsular K antigen; thus *E. coli* strains typically have an O:H:K identification card, though the difficulty of serotyping the K antigens often led to a more simplified O:H identification. Up to 186 O antigens and 53 H antigens have so far been characterized, accounting for the wide diversity of *E. coli* strains. Of note, when *E. coli* strains are found to lack flagella, they are referred as O:NM (for non-motile). Nowadays, these phenotype-based typing methods are progressively being replaced by more reliable and discriminatory genotype-based methods that, on top of surface antigens, can also distinguish virulence markers and single-nucleotide polymorphisms (SNPs), which are essential traits to understand the pathogenicity of InPEC and ExPEC (reviewed in (Fratamico, DebRoy et al. 2016)).

In most of the world, the major serotype responsible for HC and HUS is EHEC O157:H7. That said, non-O157 EHEC strains are now also recognized as a source of significant human illness, though the prevalence of these strains differs depending on the geographical location. In France, the “Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail” (ANSES) identified 5 EHECs belonging to serotypes mainly involved in epidemics and human infections. They are referred to as "the top 5" EHECs. The five serotypes are O157:H7, O26:H11, O103:H2, O111:H8 and O145:H28, along with their non-motile derivatives (AFSSA, 2008, ANSES, 2010). All these strains are positive for the *stx* and *eae* (encoding intimin; further discussed below in “The locus of enterocyte attachment”) genes. Additionally, the US Department of Agriculture (USDA) added two other strains, namely O91:H21 and O113:H21, as potentially associated with severe illness in humans; they are termed the “Big 6” (as they are listed in addition to EHEC O157:H7) (USDA, 2012). These strains, along with their seropathotype classification (see below), are listed in table 1.

Table 5. The major enterohemorrhagic E. coli (EHEC) in France and the United States.

O Serogroup	H antigen(s)	Seropathotype	HC	HUS
O157	NM, H7	A	+	+
O26	NM, H11	B	+	+
O45	NM	B	+	-
O145	NM, H25, H28	B	+	+
O103	NM, H2	B	+	+
O111	NM, H8	B	+	+
O121	H19	B	+	+
O113	H21	C	+	+
O91	NM, H21	C	+	+

Each EHEC strain is defined by its O and H antigens, its seropathotype, and its ability to frequently induce hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Adapted from (Croxen, Law et al. 2013).

(2) Seropathotypes

A commonly used classification system, specific to STEC strains, is based on the association and reported occurrence of STEC strains with the incidence and severity of clinical illness (Karmali, Mascarenhas et al. 2003). This system is composed of 5 different seropathotypes, from A to E. Seropathotypes A and B include serotypes associated with outbreaks and HUS, with seropathotype A dedicated so far to STEC O157:H7 strains. Seropathotype C includes STEC strains associated with sporadic HUS cases. Seropathotype D contains STEC strains with little incidence on human illness (usually diarrhea) but no reported case of HUS, and STEC strains of seropathotype E have not been associated with human illness and were only isolated from animals. Of note, following a request from the Austrian Federal Ministry of Health, the European Food Safety Authority (EFSA) issued in 2013 a report where they pointed out the limitations associated with seropathotype classification, as it does not provide an exhaustive list of pathogenic serotypes (European Food Safety Authority Panel on Biological Hazards (BIOHAZ) 2013. EFSA J.).

(3) Lineages

Another EHEC classification, which is actually performed for all pathogenic *E. coli*, involves pooling EHEC strains according to their genetic background. Four phylogenetic groups have thus been characterized: groups A, B1, B2, D and E. Importantly, it should be noted that this particular group labeling does not reflect the seropathotype labeling. Most EHEC strains are present in the phylogenetic groups A and B1, but the serotype O157:H7 strains have been pooled in group E (Escobar-Paramo, Clermont et al. 2004). The most severe pathogens,

which belong to the seropathotype groups A and B, belong respectively to the phylogenetic groups E and B1.

b) Epidemiology

(1) EHEC infection surveillance

Surveillance and control of EHEC infections have become a major goal for public health authorities. Although initially based on the detection of *E. coli* O157:H7 strains, non-O157 serotypes have also been the cause of sporadic cases and epidemics in Europe and the United States. Each country has its own surveillance system.

In France, EHEC infections are not included in the list of notifiable diseases and testing for the presence of these bacteria in the stool is not done routinely in medical laboratories. Surveillance of EHEC infections, which started in 1996, has been based on the surveillance of HUS in children under 15 years of age. This surveillance was set up by the “institut de veille sanitaire” (InVS), now "Santé publique France", in collaboration with hospital services. The latest report from “Santé publique France” reported an annual average of 137 cases of HUS with an average incidence of 0.95 cases per 100,000 children under 15 years of age between 2011 and 2015. Of these, 66% were children under 3 years of age. One death was notified following a case of HUS. The serogroup most frequently involved was O80, followed by O157 and O26.

In Europe, the Food and Waterborne Diseases and Zoonoses (FWD) surveillance system was launched in 2007. It is coordinated by the European Center for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA). In 2015, 5901 cases of EHEC infection were reported in 25 European Union countries including 8 deaths (EFSA. 2016). The incidence was 1.27 cases per 100,000 people. The most common serogroup was O157 (41.7%), followed by O26 and O103. In the United States, the Center for Disease Control and Prevention (CDC) has established a specific program called the Foodborne Diseases Active Surveillance Network (FoodNet), which allows for active surveillance of food-borne illnesses, including EHEC infections. This network was established in 1995 in collaboration with the USDA and the Food and Drug Administration. The latest network report in 2015 showed 465 cases due to O157:H7 (incidence of 0.95 per 100,000 people) and 807 cases associated with non-O157 strains (1.65/100,000). As expected, children under 5 years of age were most affected by EHEC infections (CDC. 2017).

(2) Major EHEC infection epidemics

Although the non-O157 EHEC serotypes are increasingly incriminated, EHEC O157:H7 is currently the most frequently encountered serotype among EHEC involved in epidemics and cases of severe pathologies in the United States and in Europe. Indeed, EHEC O157:H7 was the most common serotype among EHEC responsible for HC and HUS in the 1980s, when EHEC outbreaks began.

(a) The main epidemics

Although EHEC have a global distribution, epidemics related to EHEC infections have been mostly reported in the industrialized countries of the Northern Hemisphere and the Southern Hemisphere. However, EHEC are also found in developing countries such as Argentina, which has the highest incidence of HUS associated with *E. coli* O157:H7 infections in children under 5 years of age (Rivero, Passucci et al. 2010). During major epidemics in Europe and North America, meat products or deli meats were first implicated, though more recently plant products and dairy products have also been at cause (Farrokh, Jordan et al. 2013). Consumption of contaminated drinking water has also led to epidemics.

The first outbreaks of *E. coli* O157:H7 infections occurred in the United States in 1982, in two different states. 47 patients were identified and this epidemic was linked to the consumption of ground beef (Riley, Remis et al. 1983). In Scotland in 1996, an *E. coli* O157:H7 infection outbreak led to 512 pathological cases, of which 34 developed HUS and 17 died. The investigation pointed out the contamination source to be beef and other beef products from the same butcher's shop. This epidemic has been the largest ever reported in the United Kingdom. In Sakai, Japan, an outbreak of *E. coli* O157:H7 occurred in 1996. A total of 9451 cases were identified and 12 deaths ensued. This epidemic has affected several communities, such as daycare centers and retirement homes. The infection was related to the consumption of contaminated and undercooked white radish sprouts from the same producer. However, the origin of the contamination could not be confirmed (Mermin and Griffin 1999). In Canada in 2000, an *E. coli* O157:H7 infection outbreak was reported in a town located near an intensive cattle farming ranch. More than 2000 cases were identified, 27 patients developed HUS and 7 died. Epidemiological investigations have shown that infections are related to the consumption of contaminated municipal drinking water.

Finally, in Germany in 2011, an outbreak caused by *E. coli* O104:H4 was described (Frank, Werber et al. 2011). A total of 3,816 cases were detected among which 845 developed an HUS and 54 died. In addition to Germany, cases of HC and HUS due to *E. coli* O104:H4

were also found in 15 other countries in Europe and North America during the same period. In France, 24 cases were identified among which 7 developed an HUS. Contaminated fenugreek seeds from Egypt appear to be the source of the infection. This epidemic was unusual since the majority of people who developed HUS were adults (89%).

(b) The epidemics in France

In France, the first collective food poisoning related to *E. coli* O157 was detected in December 2000 and was due to undercooked sausages (AFSSA. 2003). The second took place in 2002 and was linked to EHEC O148:H8 serotypes (AFSSA. 2003). The origin of the contamination was traced back to sheep from a family farm. Then, 2005 was marked by the occurrence of two major epidemics. One of the outbreaks was due to EHEC O26:H11 and O80:H2 serotypes, and was related to the consumption of raw milk cheese. The second outbreak was due to EHEC O157:H7 that contaminated frozen ground beef. This was the first outbreak of *E. coli* O157:H7 due to the consumption of beef meat in France. Another outbreak in June 2011 was also linked to the consumption of frozen ground meat and targeted exclusively children; this outbreak was due to EHEC O157:H7 strains (King, Loukiadis et al. 2014). Two outbreaks occurred again in 2012. The first, related to the consumption of ground beef, was due to EHEC O157:H7, while the second was due to person-to-person transmission of EHEC O111 serogroup among children attending the same daycare center. In 2013, an EHEC O157 outbreak was identified, most likely linked to consumption of raw milk cheese. Aside from these particular years, surveillance data since 1996 show that HUS in France is predominantly sporadic. No epidemics were recorded in 2014 and 2015.

(3) Sources and modes of transmission

Worldwide, the main source of infection is foodborne (EFSA & ECDC. 2016). Foods involved in outbreaks all share common characteristics. They are likely to be contaminated by STECs, have physicochemical characteristics allowing the survival of STEC, and are consumed without having been sufficiently cooked. Foods particularly at risk are those of animal origin, which are directly related to the animal reservoir of STEC (mainly bovines and caprines), as well as products in direct or indirect contact with animal feces and consumed either raw or undercooked (Kintz, Brainard et al. 2017). The main foods involved in EHEC infection outbreaks are therefore undercooked ground beef, unpasteurized dairy products, raw or unpasteurized vegetable products, and drinking water. Not all sources of infection causing epidemics or sporadic cases were identified; these are then classified as unknown sources.

Importantly, person-to-person contamination, in close contact with an infected person or persons, is an important mode of transmission (AFSSA. 2003). It has been observed in homes or communities such as daycare centers, retirement homes or medical institutions. Transmission of *E. coli* O157:H7 infections to humans through direct or indirect contact with farm or household animals or their feces has been described in isolated case studies and sporadic case studies, as well as during epidemics. Finally, cases of laboratory contamination have also been described in the literature.

c) Human infections related to EHEC

(1) Pathophysiology of EHEC-related infections

EHEC infections have an estimated infectious dose of less than 50 bacteria (Tilden, Young et al. 1996). Infections are characterized by different symptoms that occur sequentially within 15 days of ingestion of the contaminated food. After an average incubation period of 1 to 9 days post-ingestion, the first symptoms appear as mild watery diarrhea, with severe spasmodic abdominal pain and moderate dehydration. In 90% of cases, diarrhea becomes bloody, which is a characteristic of hemorrhagic colitis (HC). HC lasts 2 to 10 days generally without the appearance of fever, despite evidence of leukocytosis. It can, however, be accompanied by vomiting. In the case of HC, lesions are localized in the ileum and colon, corresponding to edema, hemorrhage and necrosis of the intestinal mucosa.

The association between HC and EHEC was clearly established by O'Brien et al. in 1983 (O'Brien and LaVeck 1983), when the same strain of EHEC was found both in contaminated meat and in the stools of patients.

Life-threatening complications occur in about 10% of cases about one week after the onset of intestinal disorders. These complications result in thrombotic microangiopathy (TMA), characterized by lesions of endothelial cell within the microcirculation followed by cell swelling, platelet adhesion and thrombosis. If the vascular tissue of the kidneys is affected, TMA manifests itself as hemolytic uremic syndrome (HUS), especially in children and elderly patients. If the brain is targeted, TMA manifests itself as thrombotic thrombocytopenic purpura (TTP) in adult patients (Andreoli, Trachtman et al. 2002). As EHEC O157:H7 is the main etiological agent of HUS in the world, most of the clinical symptoms observed are obtained with this serotype.

(2) Clinical Signs of EHEC-Related Infections

- Hemorrhagic colitis

The most common clinical sign of EHEC infections is HC. HC caused by *E. coli* O157:H7 is characterized by abdominal cramps and non-bloody watery diarrhea with 10% of patients recovering. In other patients (90%), non-bloody diarrhea develops into bloody diarrhea after 3 days with stool containing amounts of blood from traces to blood-only stool. Bloody diarrhea persists for 2 to 4 days and 90% of patients often recover after 6 to 8 days (Griffin, Ostroff et al. 1988, Pai, Ahmed et al. 1988, Ostroff, Kobayashi et al. 1989). For about 10% of non-recovering patients, this bloody diarrhea can develop into HUS. For some EHEC O157:H7 infections and the majority of EHEC serotypes other than O157:H7, diarrhea does not often contain visible amounts of blood (Davis, Van De Kar et al. 2014).

- Hemolytic and Uremic Syndrome (HUS)

HUS has been described for the first time in a Swiss patient in 1955, and was associated with EHEC O157:H7 infection for the first time by Karmali and collaborators (Karmali, Steele et al. 1983). It is the leading cause of acute renal failure in infants. HUS is characterized by the following four symptoms:

(i) sudden onset of hemolytic anemia (hemoglobin <80g/L with 2 to 10% fragments of red blood cells or schizocytes);
(ii) thrombocytopenia (platelet count = 40 to 50,000/mm3);
(iii) acute renal failure, i.e high levels of urea and creatinine in the blood (> 60 µmol/L if <2 years,> 70 µmol/L if ≥2 years) (Fong, de Chadarevian et al. 1982, Kaplan and Proesmans 1987);
(iv) involvement of the central nervous system reported in 20% to 50% of cases (convulsions, torpor, stroke, coma) (Steinborn, Leiz et al. 2004, Nathanson, Kwon et al. 2010, Weissenborn, Donnerstag et al. 2012).

The involvement of the nervous system is currently the leading cause of death associated with EHEC infections in France (Decludt, Bouvet et al. 2000). The percentage of patients at risk to develop a HUS after an *E. coli* O157:H7 infection varies by age: it occurs in 10% of children under 10 years of age, and in 10 to 20% of people over 65 years old.

The prognosis of chronic renal sequelae can be predicted by the persistence of oligoanuria (less than 100mL of urine per day) for more than 8 days (Dolislager and Tune 1978, Oakes, Kirkham et al. 2008, Balestracci, Martin et al. 2012).

Of note, EHEC serotypes other than O157:H7 may cause HUS, but the probability of severe renal impairment has been described as low.

- Thrombotic thrombocytopenic purpura (TTP)

TTP was first described in 1925, but only in 1990 was it associated for the first time to EHEC O157:H7 infections (Kovacs, Roddy et al. 1990). It particularly affects adults, and prodromal diarrhea is usually absent (Hofmann 1993).

TTP is characterized, like HUS, by: hemolytic anemia, thrombocytopenia, nerve and renal damage, as well as fever. A major, although not clear-cut, distinction proposed between HUS and TTP is the presence of more frequent and severe renal insufficiency in HUS cases, whereas more severe neurological damages are observed in the case of TTP (Remuzzi 1987).

Clinical manifestations of most TTP in adults are the formation of platelet thrombi (platelet aggregation) and fibrin deposition within arterioles. These fibrin deposits are due to an accumulation in the plasma of molecules with high molecular weight called von Willebrand factor, i.e proteins synthesized by white blood cells and endothelial cells, and which are necessary for platelet aggregation.

TTP can last from a few days to several weeks. Progression of the disease, involvement of the central nervous system and kidneys, as well as neurological symptoms (modification of behavior, confusion, focal impairment, hemiparesis that lead to a coma) are the main causes of death.

(3) Treatment of EHEC-related infections

The course of an EHEC infection is self-limiting and usually resolves after a week; though there is currently no way to actually prevent the onset of HUS. In this regard, the use of antibiotics remains highly controversial for infections involving EHEC as it is likely to lead to bacterial lysis and release of Shiga toxins, thus increasing the risk of patients developing HUS (Wong, Jelacic et al. 2000). To date, treatments are mostly symptomatic, and include rehydration, dialysis, and blood transfusion. Intravenous fluids are strongly recommended to improve renal function.

In recent years, a new treatment based on eculizimab, a terminal complement inhibitor, has been evaluated on HUS patients. The first assay was done on three patients with Shiga toxin-associated HUS with severe neurological involvement, and presenting abnormal levels

of complement (Lapeyraque, Malina et al. 2011). As all three patients showed prompt neurological recovery, eculizumab was subsequently used during the european outbreak in 2011 with EHEC O104:H4-infected patients. In Bordeaux, France, 9 out of 24 infected patients developed HUS and were given eculizumab early on in the disease; again, all 9 patients completely recovered (Delmas, Vendrely et al. 2014). In Germany, 328 patients developing HUS were given eculizumab, but no significant difference was observed in the outcome and recovery of these patients compared to other patients receiving basic supportive care (Kielstein, Beutel et al. 2012). Besides the difference in the number of patients, discrepancies between the results in Germany with the previous ones may come from an administration of eculizumab later in the onset of disease. Strong evidence supporting a beneficial effect of eculizumab is currently lacking due to conflicting results, but the limited number of reports available seems to point to an efficacy of eculizumab when given early in patients developing HUS. This latter point would thus imply the determination of predictors of development of HUS, such as prolonged anuria. Importantly, an ongoing controlled randomized trial phase III trial (NCT02205541) in pediatric patients with Shiga toxins-mediated HUS could shed light on the efficacy of eculizumab.

d) EHEC major virulence mechanisms

(1) Resistance to gastric acidity

After ingestion by the consumer, EHEC passes through the stomach where it has to cope with the host's first defense system, the gastric acidity. EHEC has three mechanisms to respond to this aggression. The glucose-repressed, or oxidative system, is thought to mainly be used by EHEC to survive within acidic food items (Price, Wright et al. 2004). The glutamate-dependent system involves the glutamate decarboxylases (Gad) GadA and GadB, which convert glutamate to γ-aminobutyric acid (GABA). Similarly, in the arginine-dependent system, the arginine decarboxylase AdiA converts arginine to agmatine. For both glutamate and arginine-dependent systems, pH homeostasis occurs by removal and transformation of a carboxyl group, carried by glutamate and arginine, into CO_2 via the consumption of a proton recruited from the environment into the cytoplasm. Antitransporters, namely GadC for glutamate and AdiC for arginine, allow the export of γ-amino butyric acid and agmatine and the import of new amino acids (glutamate and arginine) to restore their intracellular levels.

The Gad system is regulated in a complex manner, and by several environment conditions; indeed, at least 11 regulatory proteins are known to affect the induction of this system. Notably, the general acid stress response regulator is the σ^S factor encoded by the *rpoS* gene (Cheville, Arnold et al. 1996, Castanie-Cornet, Penfound et al. 1999). Additionally, one of the

most important regulators of the Gad system is the central activator GadE. GadE binds a consensus sequence located upstream of the *gadA* and *gadB* transcription sites (Castanie-Cornet, Treffandier et al. 2007). GadE was found to be essential for the expression of *gadA* and *gadBC* and therefore essential for acid resistance.

It is thought that the extent of acid resistance conferred by these various systems may explain the low infectious dose of EHEC (Lim, Yoon et al. 2010), although interestingly, some variability in the degree of acid resistance is observed among the various EHEC serotypes and strains (Large, Walk et al. 2005, Kim, Breidt et al. 2015).

(2) The locus of enterocyte attachment (LEE)

All EHEC strains belonging to seropathotypes A and B carry a type III secretion system (T3SS) that allow them to intimately bind to epithelial cells. T3SS are protein transport nanomachines that are associated with the virulence of many Gram-negative pathogens including, along with EHEC, EPEC and *Citrobacter rodentium*, a natural mouse pathogen. Indeed, the T3SS is used by pathogens to secrete virulence factors directly into the host cell. The resulting disruption of the host target cell signaling and rearrangement of the cytoskeleton provoke an effacement of intestinal microvilli and the appearance of a pedestal under the bacteria (Sherman and Soni 1988, Frankel, Candy et al. 1995). These cellular changes within the target cell are called attachment and effacement (A/E) lesions and lead to an intimate attachment of the pathogen to the epithelial cell surface.

(a) Genetic organization of the T3SS encoding locus

The genes required to form A/E lesions are located on a 43.36-kb pathogenicity island called locus of enterocyte effacement (LEE), located on the bacterial chromosome (Figure 7). For all A/E-proficient bacteria, the LEE is organized into 5 major operons (LEE1, LEE2, LEE3, LEE5 and LEE4), and consists of 41 open reading frames (ORFs; Stevens and Frankel, 2014). Besides the T3SS structural genes, the LEE also contains genes encoding i) several bacterial effector proteins that are injected to the host target cells via the T3SS, ii) for the chaperones of effector proteins, iii) for the intimin adhesin and its translocated receptor Tir and iv) for regulatory elements (McDaniel, Jarvis et al. 1995). The lower GC content of the LEE compared to the rest of the *E. coli* chromosome (38.3% versus 50.8%, respectively) strongly implies that the LEE, like many other virulence-associated elements, was acquired via horizontal gene transfer (Frankel, Phillips et al. 1998).

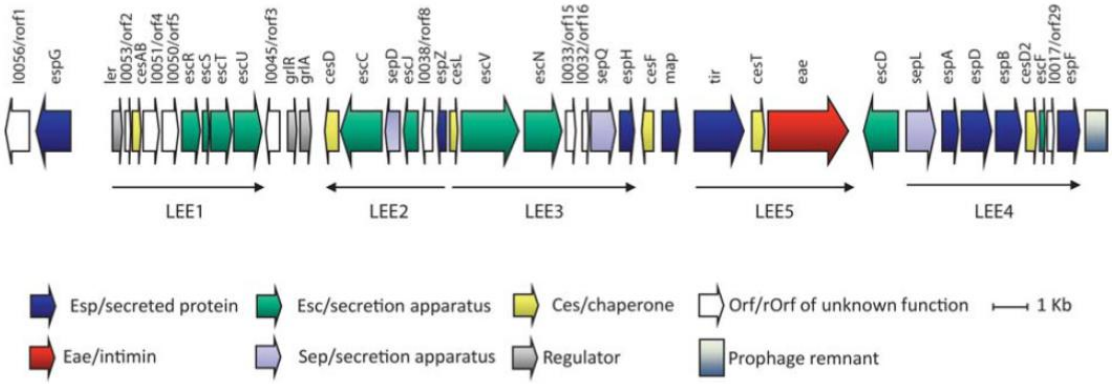

Figure 7. Organization of the locus of enterocyte attachment (LEE) in E. coli O157:H7. *The 41 genes of the LEE are represented by the thick arrows. The prophage present on the LEE of EHEC is represented as the gray rectangle. From (Stevens and Frankel 2014)*

(b) Structure of the T3SS

The T3SS essentially forms “molecular syringe” necessary to translocate effectors into the host target cells. The T3SS is built with three main parts: i) the cytoplasmic components, which include the ATPase complex, ii) the basal body, and iii) extracellular appendages, with the translocation pore, the filament and the needle (Figure 8).

The assembly of the T3SS is sequential (Deng, Marshall et al. 2017). In the early stages of the assembly of T3SS, the formation of the basal body is assisted by the general secretory (Sec) pathway. From that structure, the rest of the structure is assembled in a Sec pathway-independent manner. The transfer of proteins to the host cell requires ATP consumption, and is ensured by the cytoplasmic ATPase complex of the T3SS.

- The basal body

The basal body is composed of oligomerized concentric rings embedded in the inner and outer membranes of bacteria, and connected through a periplasmic rod. EscJ (LEE2) and EscD form the inner membrane rings, while EscC (LEE2) forms the outer membrane ring. At the base of the basal body, the export apparatus is assembled with EscR, EscS, EscT, EscU (LEE1) and EscV (LEE3). The ATPase complex is formed by the ATPase EscN (LEE3), the stalk protein EscO (orf15, LEE3), the stator protein EscL (orf4; LEE1) and the cofactor EscK (orf5; LEE1).

- The needle

The needle is a hollow structure that protrudes from the bacterial surface to reach the host cell and form a channeling system to transfer effector proteins. The needle starts to be assembled on the inner rod, a structure lining the basal body rings. The needle is helically assembled with a single protein, EscF (LEE4); a mutation in *escF* abolishes the secretion of translocon and effector proteins (Sekiya, Ohishi et al. 2001). The needle length is controlled by EscP (orf15; LEE3) and EscU (LEE1).

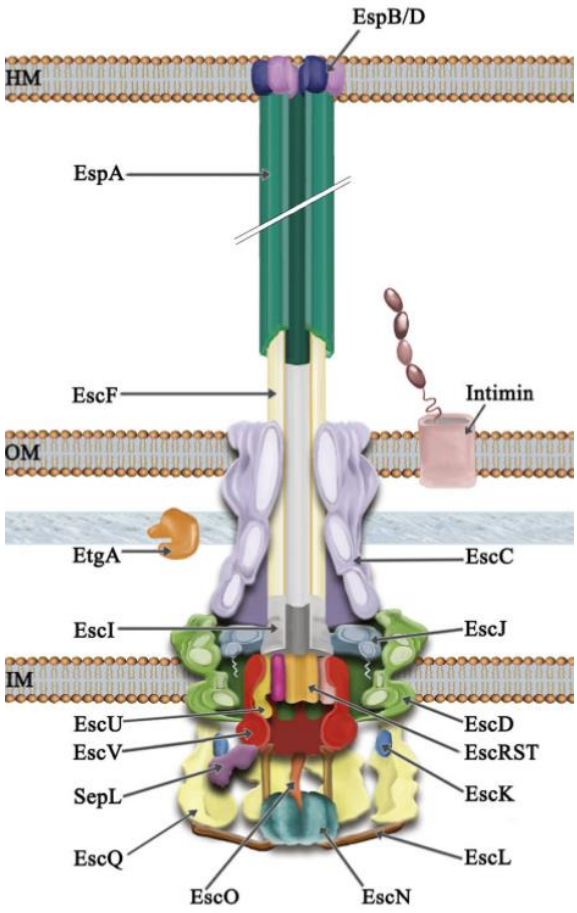

Figure 8. Structure of the type III secretion system (T3SS). *The T3SS is divided in three main parts: the cytoplasmic components where the ATPase complex is located; the basal body that consists of three membrane rings that span the inner (IM) and outer membrane (OM) and are connected by a periplasmic inner rod; and the extracellular appendages, including the needle, the filament and the translocation pore. HM, host membrane. From (Gaytan, Martinez-Santos et al. 2016).*

- The filament and translocation pore

The filament is an extracellular appendage that serves as an adaptor between the needle and the translocation pore inserted in the host target cell membrane. The filament is helically assembled by the polymerization of subunits of EspA (LEE4), forming a "tunnel" between the surface of the host cell and the bacterium which will be used to transfer effector proteins including Tir or EspB (Neves, Knutton et al. 1998).

After being injected into enterocytes, EspB (LEE4), along with EspD are inserted into the host membrane and form translocation pores. The *cesD* gene, also located in the LEE4, encodes a chaperone protein essential for the secretion of EspD and EspB proteins (Wainwright and Kaper 1998). EspB can also be detected in the cytoplasm of target cells; where it regulates the actin network and lead to cell morphology alterations. EspB, via its myosin-binding domain, acts by inhibiting the interaction between myosin and actin; this inhibition leads to microvilli effacement. Additionally, EspB is suggested to suppress NF-κB activation and expression of proinflammatory cytokines.

(c) Translocation of effector proteins

(i) The translocated intimin receptor (Tir)

An important T3SS effector is the protein Tir that, upon translocation by the T3SS, is inserted in the cytoplasmic membrane of the host target cell. As indicated by its name, Tir functions as a receptor for the LEE-encoded intimin (encoded by the *eae* gene), located on the bacterial surface. Tir forms i) a hairpin structure with the C- and N-terminal domains located in the host cell and ii) an extracellular transmembrane region that interacts with intimin. This interaction induces actin rearrangements within the host target cell, forming a pedestal under the bacterium. The binding between intimin and Tir therefore establishes an intimate adhesion to enterocytes (Donnenberg, Tzipori et al. 1993, McKee, Melton-Celsa et al. 1995). An EHEC O157:H7 *Δeae* is unable to colonize the intestinal epithelium, form A/E lesions, or provoke HUS in young rabbits (Ritchie, Wagner et al. 2003).

The *tir* gene is located on the LEE5 operon, along with the *eae* gene. Three α, β and γ variants of the *tir* gene have been described in EPEC and EHEC (China, Jacquemin et al. 1999). To date, 17 variants of the *eae* gene have been identified, where they differ in sequence in the C-terminal cell-binding domain; of note, EHEC O157:H7 carries the Int-γ variant. These variants among EHEC and other A/E-proficient bacteria appear to influence

the tissue tropism and specific colonization site on the host intestinal epithelium. EHEC is known to localize preferentially in the ileum and the colon. Specifically, EHEC is found at the FAE of ileal PPs (Phillips and Frankel 2000, Chong, Fitzhenry et al. 2007, Lewis, Cook et al. 2015). Indeed, an EHEC O157:H7 *Δeae* mutant expressing the Int-α variant of EPEC O127:H7 is now capable, much like EPEC O127:H7, of adhering to explants from several different intestinal sites, instead of remaining localized to the FAE of PPs in the ileum (Phillips, Navabpour et al. 2000, Fitzhenry, Pickard et al. 2002). The reciprocal findings were observed as well (Phillips and Frankel 2000).

Of interest, intimin was also shown to bind to HEp-2 cells via the nucleolin receptor; nucleolin is a protein involved in cell growth regulation (Sinclair and O'Brien 2002). That said, since α, β and γ variants of intimin bind equally well to nucleolin, the distribution of nucleolin along the gut is not likely to influence the preferential colonization site for EHEC.

(ii) Other LEE-encoded effectors

On top of EspB and Tir, five other effectors are encoded by the LEE in EHEC O157:H7, which were reported to be required for full colonization of the intestines of infant rabbits (Ritchie and Waldor 2005).

- **Map**: This protein has 3 distinct functions. Map allows i) to maintain the mitochondrial membrane potential; ii) the formation of the filamentous structures of the T3SS and iii) to induce damage in the intestinal barrier by altering tight junctions. Studies in mice infected with *Citrobacter rodentium* have showed that Map is not essential for colonization and infection development, which somewhat contradicts the findings of Ritchie and colleagues, although the pathogen used was not the same (Deng, Puente et al. 2004).
- **EspF**: EspF is a multifunctional protein that plays a role in altering the intestinal epithelial barrier. Like Map, EspF targets the mitochondria of the host cell and leads to permeabilization of the mitochondrial membrane with cleavage of caspases 9 and 3, indicating that EspF plays a role in apoptosis. Studies using cultures of human intestinal organs have shown that EspF plays a direct role in remodeling the brush border of microvilli (Viswanathan and Hecht 2000).
- **EspG**: EspG interacts with tubulins and stimulates the destabilization of microtubules *in vitro* (Hardwidge, Deng et al. 2005). Additionally, EspG was shown to induce fragmentation of the Golgi apparatus (Clements, Smollett et al. 2011).
- **EspH**: EspH is localized in the host cell membrane and modulates the actin cytoskeleton structure to lead to a pedestal formation.

- **EspZ**: EspZ seems to be involved in the regulation of effectors' translocation. EspZ is injected into the host target cell early after infection, and appears to lead to an arrest of translocation of effectors by a yet unknown mechanism (Berger, Crepin et al. 2012). Mutants lacking the gene *espZ* are more cytotoxic, most likely because of the lack of such effector translocation regulation.

(iii) Non LEE-encoded effectors

In addition, bioinformatics and proteomics analyses that were confirmed experimentally revealed more than 30 more effector proteins secreted via the T3SS in EHEC O157:H7; their functions are not all known yet and require further characterization (Stevens and Frankel 2014).

(d) Regulation of the LEE

Not surprisingly, regulation of attachment and colonization, a central function that pertains to the LEE, is a complex and multi-component mechanism. Transcriptional regulation is not only regulated by members of the locus itself, but also by a myriad of environmental cues, whether physical or chemical, present in the milieu (Connolly, Finlay et al. 2015, Furniss and Clements 2018). I will discuss here the major mechanisms of direct and indirect regulation of LEE expression.

(i) Regulation of the LEE…by the LEE

Transcription of the LEE is predominantly ensured by the master regulator Ler (for LEE-encoded regulator) (LEE1), a 15kD protein that activates transcription of LEE operons as well as non-operonic gene members of the LEE, such as *map*, *espG* and *escD*. Ler is thus crucial in the process of A/E lesion formation and subsequent colonization; indeed, a study showed that an EHEC strain deleted for the *ler* gene was unable to form A/E lesions on HEp-2 cells (Elliott, Sperandio et al. 2000). This study and several others have further demonstrated the role of Ler as a global regulator. Ler induces transcription of non-LEE genes encoding effector proteins that are translocated via the T3SS (Roe, Tysall et al. 2007, Holmes, Lindestam Arlehamn et al. 2012); additionally, StcE, a metalloprotease capable of degrading mucins present in *E. coli* O157:H7 on the pO157 plasmid (which I will discuss later), is also regulated by Ler (Lathem, Grys et al. 2002).

Located between LEE1 and LEE2, the regulators GrlR (global regulator of *ler* repression) and GlrA (global regulator of *ler* activation) modulate the expression of the LEE in a negative and positive manner, respectively (Deng, Puente et al. 2004) (Figure 9). GrlA, by binding the upstream leader sequence of LEE1, induces transcription of the *ler* gene, thus acting as a positive feedback loop by inducing further transcription of the LEE operons (Barba, Bustamante et al. 2005). On the other hand, GrlR binds GrlA and thereby inhibits transcriptional activation of LEE1 (Padavannil, Jobichen et al. 2013). The activity of GrlR is regulated in a growth phase-dependent manner by cleavage by the ClpXP protease, which then frees GrlA to induce transcription of *ler* (Iyoda and Watanabe 2005). The activities of GrlA and Ler are regulated by the CpxAR system, where CpxR induces the Lon protease to degrade both these proteins (De la Cruz, Morgan et al. 2016).

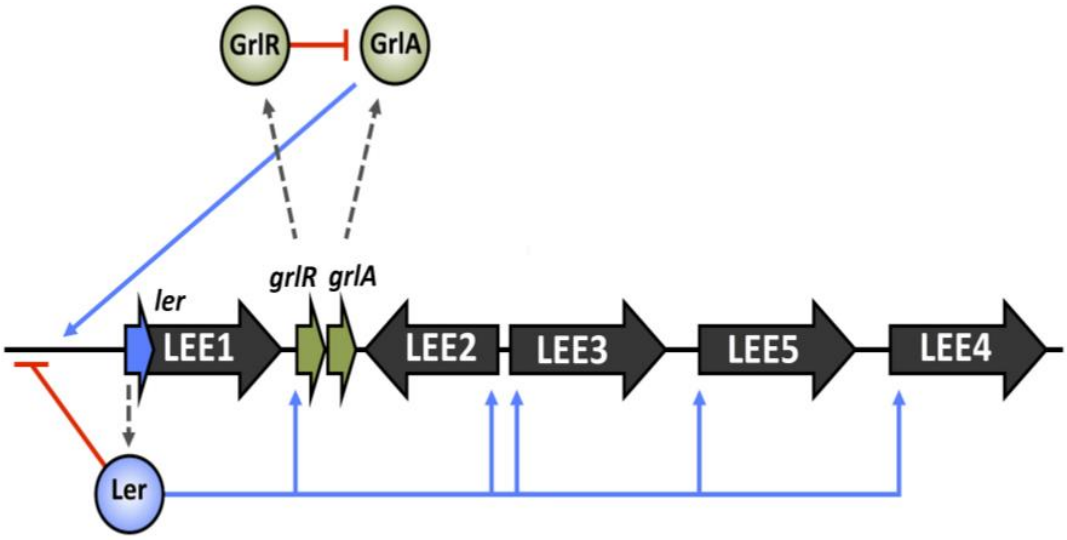

Figure 9. Regulation of the locus of enterocyte attachment (LEE) by internal genes of the LEE. *Ler is the master regulator of LEE genes. GrlR and GrlA also regulate transcription of LEE1, including Ler. GrlR inhibits GrlA, which is a positive regulator of LEE1. From (Connolly, Finlay et al. 2015).*

(ii) Regulation of the LEE by the host environment

- Regulation by quorum sensing molecules

Quorum sensing is an effective mean for bacteria to sense the population level in their environment. When particular sensed molecules reach a specific threshold, they enter bacterial cell in sufficient numbers to activate their sensor protein. This signaling eventually leads to transcriptional regulation of genes involved in response to the stimulus.

In EHEC, the protein SdiA is a transcriptional regulator that can sense acyl-homoserin lactones (AHL) produced by surrounding bacteria, but not from EHEC. AHL are quite abundant in the rumen of cattle, which is not a favorable colonization site for EHEC due to the acidic environment. When sensing AHL, SdiA responds by activating GadX, another protein member of the Gad system, and indirectly represses transcription of LEE1 (Figure 10) (Hughes, Terekhova et al. 2010).

In addition, two other regulatory systems, namely QseEF and QseBC are able to sense the bacterial auto-inducer 3 signaling molecule (AI-3) (Sperandio, Torres et al. 2003), and activate the transcriptional regulator QseA; in turn, QseA activates transcription of the LEE1 operon (Sharp and Sperandio 2007) (Figure 10). QseEF also senses environmental sulfate and phosphate (Reading, Rasko et al. 2009).

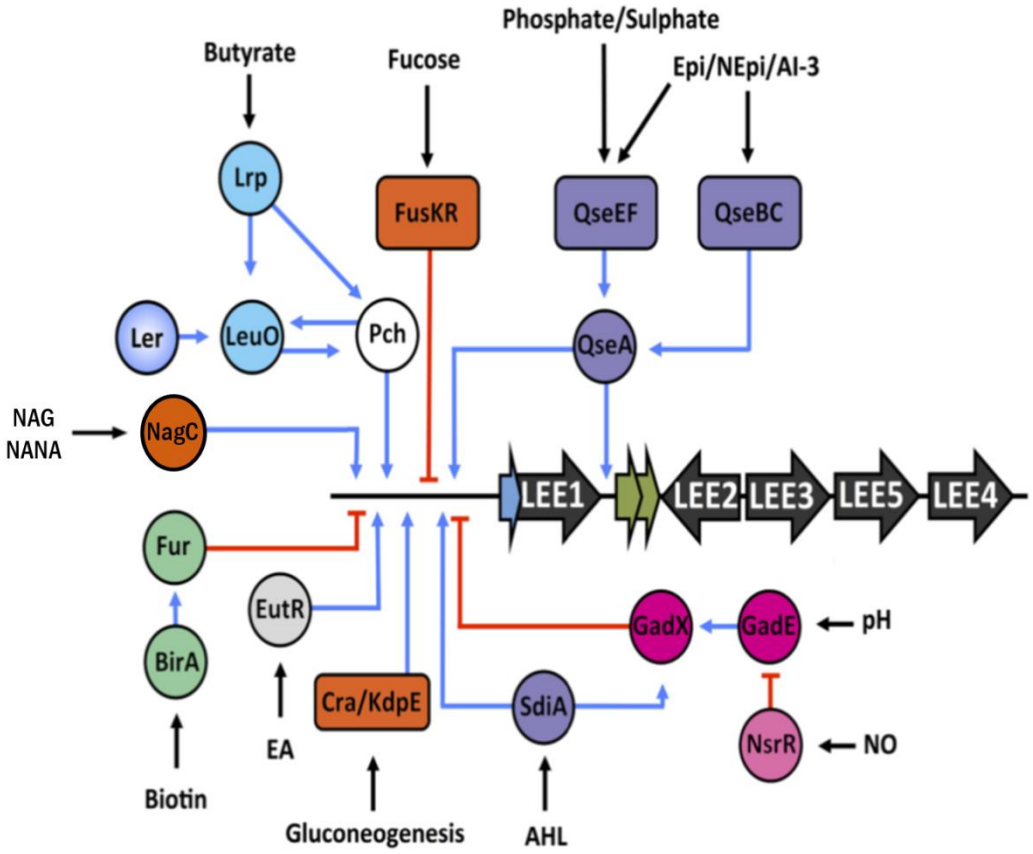

Figure 10. Regulation of the LEE by the host environment. *The stimuli and their sensors are connected by black arrows. Blue arrows indicate a positive regulation, and red blunts indicate a negative regulation. The regulatory pathways are color-coded based on the nature of the stimulus sensed: quorum sensing of hormones and other chemicals in violet, sugar sensing in orange, nitric oxide (NO) sensing in pink, ethanolamine (EA) sensing in gray, butyrate sensing in blue, and biotin sensing in green. Epi, epinephrine; NEpi, Norepinephrine; AI-3, auto-inducer 3 signaling molecule; AHL, homoserin lactones; NAG, N-acetylglucosamine; NANA, N-acetylneuraminic acid. Adapted from (Connolly, Finlay et al. 2015).*

- Regulation by host-derived molecules

In addition to AI-3, the QseEF and QseBC systems can sense epinephrine (Epi) and norepinephrine (NEpi), demonstrating that the LEE can be regulated by host hormones (Clarke, Hughes et al. 2006, Reading, Rasko et al. 2009). This evolution seems appropriate in the context of the gut, where bacteria, whether they are pathogens or commensals, need to compute information from both resident microbiota and the host, and adapt accordingly.

The T3SS is also affected in the presence of nitric oxide (NO), an important mediator of the hot innate immune response (See nitric oxide chapter). This regulation pathway involves the Gad system and the NO sensor regulator NsrR. Indeed, GadE was found to repress the expression of LEE4 and LEE5 genes in a Ler-dependent manner; while GadX repressed LEE1 and activated LEE4 and LEE5 expression (Branchu, Matrat et al. 2014). The presence of NO led to inhibition of LEE1/4/5 gene expression, while inducing transcription of the Gad system. These findings led to the understanding that NO modulated the LEE in a NsrR-dependent fashion via GadE and GadX. Indeed, NsrR bound to the LEE1, LEE4 and LEE5 promoters in the absence of NO, but did not when an NO donor was added (Branchu, Matrat et al. 2014). The observed activation of the LEE by NsrR was suggested to be due to the recruitment of RNA polymerase, and is to my knowledge the first report of a direct activation pathway by NsrR (Branchu, Matrat et al. 2014). In parallel, NsrR indirectly repressed GadE and GadX. Hence, in the presence of NO, NsrR-mediated repression of GadE and GadX is relieved, but so is NsrR-mediated induction of LEE1/4/5 expression. This means that GadE can repress LEE4/5, as well as activate GadX expression, which leads to repression of LEE1 (Branchu, Matrat et al. 2014). This regulatory pathway is biologically relevant since NO was found to decrease adhesion of *E. coli* O157:H7 to epithelial cells.

- Regulation by nutrients

EHEC needs to avoid competition for nutrient sources in the gut to increase its chances to colonize the host and establish its own niche. In this regard, EHEC transiently regulates colonization by using carbon sources from glycolysis and gluconeogenesis (Miranda, Conway et al. 2004). A study found that, while glycolysis byproducts inhibited *ler* expression, gluconeogenesis promoted *ler* expression (Njoroge, Nguyen et al. 2012). This regulation was mediated by two transcription factors, Cra and KdpE. KdpE belongs to the two-component KdpDE system, which responds to osmotic stress, and KdpE was originally known to regulate *ler* expression (Mellies, Barron et al. 2007). Cra (for catabolite repressor/activator) is a transcription factor that regulates transcription using fluctuations in sugars concentrations.

Njoroge et al. demonstrated that Cra and KdpE both bind to and upregulate LEE1 (Figure 11); moreover, they found that Cra was important in A/E lesion formation, while KdpE contributed to Cra-mediated regulation (Njoroge, Nguyen et al. 2012).

Other carbohydrates present in the gut affect gene expression in the LEE in EHEC. Indeed the FusK, which detects fucose, and the regulatory protein NagC, which senses N-acetylglucosamine and sialic acid, affect the expression of LEE operons (Pacheco, Curtis et al. 2012, Le Bihan, Sicard et al. 2017). Interestingly, these sugars are parts of O-glycosylated mucins and gut commensals producing specific glycosidases, such as *Bacteroides thetaiotaomicron*, can release these carbohydrates from exposed mucins (Xu, Bjursell et al. 2003). It has been suggested that EHEC coordinates catabolism of mucin-derived glycosides and T3SS production in a spatio-temporal manner and that such mechanism could contribute to the relocation of the pathogen from the intestinal lumen to the surface of IECs.

Ethanolamine (EA) metabolism is a characteristic that can also provide a competitive advantage for *E. coli* O157:H7 in the host. Indeed, STECs carry the ethanolamine utilization operon (termed *eut* operon) allowing them to metabolize EA (unlike the resident microbiota) and thus gain a competitive advantage during colonization of the gut. EHECs also use EA as a sensing signal to assess the host environment and modulate their mechanism of virulence accordingly (Kendall, Gruber et al. 2012). The transcriptional regulator EutR regulates the *eut* operon by binding to EA and subsequently directly activating the *eut* operon (Luzader, Clark et al. 2013). Interestingly, EutR also directly activate transcription of the LEE by binding to the LEE1 promoter region (Figure 11) (Luzader, Clark et al. 2013).

- Regulation of the LEE by other metabolites

EHEC can also sense the short-chain fatty acid butyrate in the gut. Butyrate is produced by the gut microbiota as an end product from the fermentation of dietary fiber. A study showed that butyrate increased transcription of the LEE in via the plasmid-borne virulence regulator PchA (Nakanishi, Tashiro et al. 2009). This regulation was achieved by the leucine-responsive protein Lrp, which is a global transcription regulator involved in nitrogen metabolism. In addition, a study showed that butyrate induced *pchA* expression, as well as the expression of the transcriptional regulator LeuP, in an Lrp-dependent manner; PchA then activated ler transcription (Figure 11) (Takao, Yen et al. 2014). Both Ler and PchA were found capable of directly activating *leuO* expression, thus implying a positive feedback loop mechanism upon *leuO* transcription activation.

Another mechanism used by EHEC to find the appropriate colonization site is through the concentration of biotin along the intestines. Biotin is an essential cofactor needed for

carboxylases and decarboxylases in all organisms. Unlike microorganisms that can either synthesized it *de novo* or taken it up from the environment; humans cannot synthesize biotin and must absorb it from the gut lumen. Biotin absorption occurs in the small intestine, and a study revealed that biotin concentration was 14 times higher in the small intestine than in the large intestine (Yang, Feng et al. 2015). EHEC senses biotin via the biotin protein ligase BirA, which acts as a negative transcriptional regulator of the biotin synthesis (*bio*) operon; this regulation allows EHEC to stop synthesizing its own biotin if it is found in sufficient concentrations in the environment. EHEC also uses this regulatory pathway to modulate expression of the LEE, and there was an inverse correlation between the biotin concentration and EHEC bacterial counts found in infected mice, that is, EHEC was more present in the large intestines (Yang, Feng et al. 2015). This niche specification is achieved by BirA, which in the presence of biotin binds to the global regulator Fur (See section on Fur, Chapter Nitric oxide); in turn, Fur negatively represses transcription of the LEE operons (Figure 11).

In summary, the differential regulation of the LEE in EHEC clearly has evolved to take into consideration cues from the environmental milieu to adopt a special, temporal, and energy-efficient colonization of the gut.

(3) Shiga toxins

Shiga toxins (encoded by *stx* genes) represent the essential virulence factors of EHEC. Shiga toxins are also termed Verocytotoxins due to the high cytotoxicity they provoke in Vero cells. Their main targets are endothelial cells of the colon, kidneys and brain in humans. Two major types of Stx, namely Stx1 and Stx2, can be distinguished based on toxicity differences *in vitro* and *in vivo*, amino acid sequences or nucleotide sequences. Shiga toxins are produced mainly by *E. coli* isolated from human and non-human sources, by *Shigella dysenteriae* type 1, and sporadically by *Citrobacter freundii*, *Enterobacter cloacae* and *Shigella flexneri*.

(a) Structure / mode of action of Shiga toxins

Shiga toxins are a family of the AB_5 toxins that, along with pertussis toxin and cholera toxin, are all constituted with a catalytic A subunit non-covalently linked to a pentameric B subunit (StxB).

The function of the B subunit (StxB) subunit is to bind to the globotriaosylceramide (Gb3) receptor, which leads to the internalization of the toxin. In Gb3-positive cells, the A subunit (StxA) acts on the ribosomes by halting protein synthesis.

Shiga toxins Stx1 and Stx2 have the same mode of action and they use a retrograde transport to reach the cytoplasm of target cells (Sandvig, Bergan et al. 2010, Melton-Celsa 2014). This retrograde transport of Stx has been demonstrated for the first time in humans on glomerular and mesangial vascular cells from kidneys (Warnier, Romer et al. 2006).

Besides a functional Stx and its association with the Gb3 receptor, the cytotoxicity of Stx was shown to be dependent not only on the amount of Gb3 expressed on the surface of target cells, but also on the association of Gb3 with cholesterol-containing lipid rafts (Falguieres, Mallard et al. 2001, Kovbasnjuk, Edidin et al. 2001, Hanashima, Miyake et al. 2008).

In cells where Gb3 is associated with lipid rafts, the binding of Stx results in the internalization of the toxin-receptor complex (Stx-Gb3) into early endosomes, which then travels to the trans-Golgi network (Figure 11). The StxA subunit is cleaved by furin, a membrane-anchored protease, to generate the active A1 subunit and the A2 fragment (Garred, van Deurs et al. 1995); at this stage, the A1 subunit is still linked to A2 by a disulfide bond. From the Golgi, the Stx-Gb3 complex undergoes a retrograde pathway to the endoplasmic reticulum ER, where StxA1 is freed by reduction of the disulfide bond. Only StxA1 leaves the ER to reach the cytoplasm (Sandvig, Garred et al. 1992). There, it binds to the large ribosomal subunit and cleaves off an adenine from the 28S rRNA, resulting in dissociation of the ribosome from the elongation factor, and inhibition of protein synthesis. The ribotoxic stress response subsequently generated ultimately leads to apoptosis.

On the other hand, in macrophages and dendritic cells, where the Gb3 receptor is not associated with lipid rafts, Stx trafficking is different (Figure 11). After binding Gb3, the toxin is targeted towards the endosomal/lysozomal pathway and gets degraded within lysozomes (Falguieres, Mallard et al. 2001). Thus, Stx cytotoxicity appears to be linked to the retrograde transport of Stx. Of note, this mechanism of resistance to toxicity is similar in bovine lower crypt IECs, at least in the case of Stx1 (Hoey, Sharp et al. 2003).

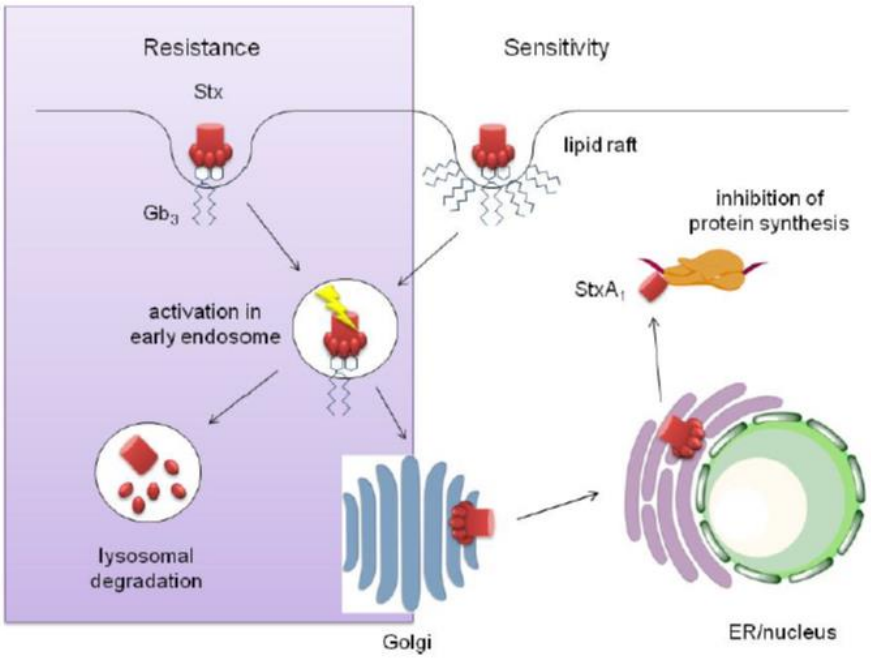

Figure 11. The differential transport pathway of Shiga toxins in Gb3-positive resistant and sensitive cells. *In resistant cells, Gb3 is not associated with lipid rafts; the Shiga toxin-Gb3 complex is internalized into endosomes and degraded in lysozomes. In sensitive cells with lipid raft-associated Gb3, Shiga toxin-Gb3 also fuses with the endosome, then goes to the Golgi and the endoplasmic reticulum by retrograde transport. The cleaved Stx A1 subunit is responsible for a disruption of protein synthesis. Stx, Shiga toxin; ER, endoplasmic reticulum. From (Schuller 2011).*

(b) Major Stx-targeted organs

In humans, the main target organ for Stx toxicity is the kidney. Within glomeruli, the Gb3 receptor is found on endothelial, podocyte, and mesangial cells; in the extra-glomerular regions, Gb3 is expressed by proximal tubules and collecting duct cells (Hughes, Ergonul et al. 2002, Psotka, Obata et al. 2009). In mice, glomerular cells do not express the Stx receptor, but Gb3 is found in proximal tubules and collecting duct cells (the latter expresses more Gb3 than the former) (Rutjes, Binnington et al. 2002).

Other organs are targeted by Stx; notably, Gb3 is also expressed on cerebral microvascular endothelial cells, which explains the complications in the central nervous system (CNS) associated with an EHEC infection. Of interest, a study in mice showed an effect of Stx2 on neurons of the CNS, which expressed Gb3; mice receiving Stx2 suffered hind limb paralysis (Obata, Tohyama et al. 2008).

(c) Stx1 versus Stx2

Stx1 and Stx2 have 99% and 56% homology, respectively, with *S. dysenteriae* toxin (O'Brien, LaVeck et al. 1982, Strockbine, Jackson et al. 1988). Hence, while Stx1 toxins are neutralizable by *S. dysenteriae* anti-Stx antibodies, Stx2 toxins are not (Strockbine, Marques et al. 1986).

Although the mechanism of action and the main biochemical properties of Stx1 and Stx2 are similar, it has been shown that Stx1 binds twice better than Stx2 on Gb3 (Nakajima, Kiyokawa et al. 2001 959) and that Stx1 is better internalized in T84 cells (colon carcinoma cells devoid of Gb3 receptors) than Stx2 (Schuller, Frankel et al. 2004). However, *in vitro* studies on renal microvascular endothelial cells (Louise and Obrig 1995) and *in vivo* studies on animal models (Lindgren, Melton et al. 1993) indicate that Stx2 is more toxic than Stx1. These results corroborate epidemiological studies, where Stx2-producing *E. coli* were found to be associated with more severe symptoms than those producing Stx1 (Luna-Gierke, Griffin et al. 2014).

(d) The different *stx* gene variants

There are several Stx variants in both classes. The Stx1 group appears to be more homogeneous. In most cases the *stx1* genes of different strains have the same nucleotide sequence (Zhang, Bielaszewska et al. 2002). Paton *et al.* compared the sequences of 3 STEC strains and showed that the 3 *stx1* genes had 99% sequence identity with the phage sequence that carries *stx1* in the reference strain EDL933 (Paton, Paton et al. 1993, Paton, Beutin et al. 1995). Despite significant homogeneity, several Stx1 variants have been described: Stx1a, Stx1c, Stx1d (Koch, Hertwig 2001; Burk, Dietrich, 2003). Within the Stx2 branch, subtypes are Stx2a, Stx2b, Stx2c, Stx2d (Stx2d activatable), Stx2e, Stx2f, Stx2g. They are distinguished by their differences in biological activity, immunological reactivity, the receptor on target cells, and by their ability to be activated by intestinal mucus elastase (Scheutz, Teel et al. 2012). The Stx2d subtype, which include variants Stx2d1 and Stx2d2, have a cleavage site by elastase present in the intestinal mucus. Elastase cleaves subunit A and thus renders the toxins active (Kokai-Kun, Melton-Celsa et al. 2000); hence, this particular subtype is called Stx2d activatable.

(e) Genetic support of Shiga toxins

Genetic analysis of *E. coli* O26:H11 and O157:H7 strains revealed that the *stx1* and *stx2* genes are encoded by lambdoid phages, that is, bacteriophages that integrate the bacterial genome and maintain themselves by the same mechanism than the well-known

bacteriophage λ (Scotland, Smith et al. 1983, O'Brien, Newland et al. 1984). These phages follow a lysogeny/lysis cycle. In the lysogenic state, the integrated phage DNA remains stable in the chromosome by inhibiting transcription of most of the phage genes, including *stx*. Under stress conditions, the phage enters the lytic cycle, where gene repression is relieved, all phage genes are expressed, and Stx toxins are produced and release upon bacterial lysis.

As with all lambdoid phages, phages bearing the *stx2* genes are inducible and respond to a bacterial stress response system: the SOS system (Figure 12). Bacteria launch the SOS response in the event of accumulation of single-stranded DNA in the cell following damage during UV irradiation, DNA alkylation, or any other exposure with DNA-damaging agents (Sutton, Smith et al. 2000). DNA replication is then stalled, and the SOS response is induced. The master regulator of the SOS response is the RecA protein, present in small but steady amount in the bacterium. RecA binds to single-stranded DNA to form filaments and thereby acquire protease activity. Activated RecA (RecA*) induces the self-cleavage of the repressor LexA, which normally represses expression of genes within the SOS regulon. Among genes of the SOS regulon are *uvr* genes involved in the repair of UV damage, as well as the *sulA* gene which inhibits cell division during DNA repair (Janion 2001).

RecA also induces auto-cleavage of the phage *c*I repressor. The *c*I repressor binds to operators present on the promoters of the early phage genes, P_L and P_R. The *stx* genes are located downstream of the phage late promoter, $P_{R'}$ (Plunkett, Rose et al. 1999, Wagner, Neely et al. 2001). A transcription terminator ensures the silencing of this promoter during lysogeny. On the other hand, when the phage is induced, the *c*I repressor is cleaved, and expression of early genes (in particular those involved in phage replication) is activated. Among these lytic cycle-expressed early genes, the antiterminator Q allows transcription of late phage genes, including *stx* genes and lysis genes, from the $P_{R'}$ promoter (Figure 12).

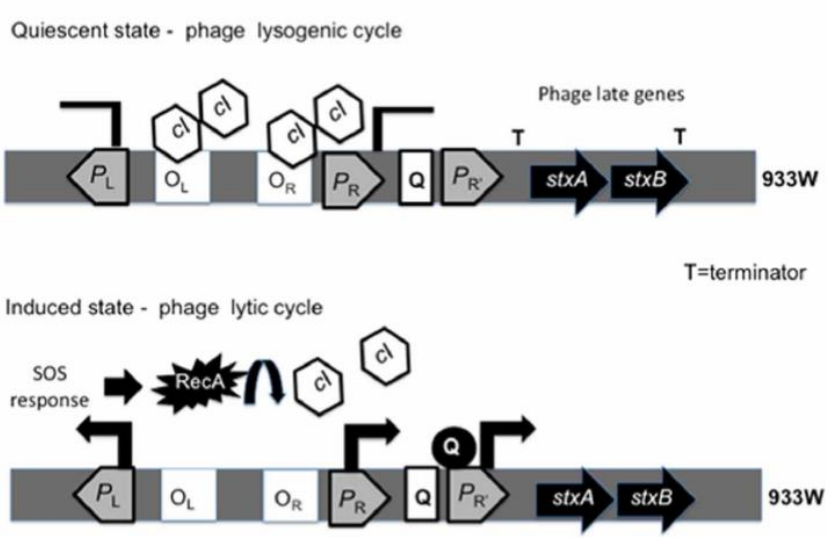

Figure 12. The regulation of Shiga toxin expression in the lambdoid phage. *During the lysogenic cycle, the cI repressor binds to the operators (O_L and O_R) of the phage early genes promoters, P_L and P_R. The terminators (T) downstream of the late phage promoter $P_{R'}$ prevents expression of stx genes. When the SOS response is induced, activated RecA induces cI autocleavage and with it the lytic cycle. The phage early genes are expressed, among which the antiterminator Q that binds to $P_{R'}$ and induces transcription of late phage genes, including Shiga toxins. From (Pacheco and Sperandio 2012).*

(f) Environmental factors modulating *stx* expression

The synthesis of Stx in *S. dysenteriae* is dependent on iron concentration. In 1982, it was shown that Stx1 production in *E. coli* was also repressed in the presence of iron (O'Brien, LaVeck et al. 1982), and that this regulation was under the control of the transcriptional regulator Fur (Calderwood and Mekalanos 1987), which binds upstream of the p_{stx1} promoter. However, since iron concentration is low in the intestines, the p_{stx1} promoter is probably derepressed and it is therefore unlikely that this repression plays an important role *in vivo*. Furthermore, since transcription from p_{stx1} is independent from late phase lysis genes expression, the induction of *stx1* expression does not lead to bacterial lysis; hence, the Stx1 produced remains intracellular, and is found in the bacterial periplasm (Wagner, Livny et al. 2002).

The synthesis of Stx2, on the other hand, is not repressed in the presence of iron (Sung, Jackson et al. 1990). Transcription of *stx2* genes is highly dependent on the phage lytic cycle, since it is mostly governed by the late phage promoter P_R'. Thus, as detailed earlier, any kind of DNA-damaging agents will result in the induction of the SOS response, and of *stx2* genes transcription. It is then not surprising that the absence of RecA in STEC dramatically decreases the production of Stx2 phages, resulting in complete avirulence of the strain *in vivo* (Fuchs, Muhldorfer et al. 1999).

Some antibiotics carry their bacteriostatic or bactericidal activity by the DNA damage they provoke; these antibiotics thus automatically induce the SOS response. Fluoroquinolones, such as norfloxacin and ciprofloxacin, are well-known examples. Fluoroquinolones target gyrase, a topoisomerase responsible for unwinding DNA during replication; this leads to a replication fork stall and collapse, and the generation of double strand DNA breaks (Chen, Malik et al. 1996). Mitomycin C is also commonly used in laboratories for its ability to interfere with DNA replication. Both fluoroquinolones and mitomycin C, as well as others, were shown to increase the production of Shiga toxins (McGannon, Fuller et al. 2010). Hence, these antibiotics are not to be used in the treatment against an EHEC infection.

Many factors produced by the host can also modulate the production of Stx. Exposure of EHEC to hydrogen peroxide (H_2O_2) leads to a dose-dependent Stx production and increase in phage titers. The incubation of EHEC with human neutrophils, which are known to produce H_2O_2, increased Stx expression as well. In a mutant deleted for the Stx phage late promoter $P_{R'}$, the interaction with neutrophils no longer induced the increase of Stx2, suggesting that H_2O_2 induces the production of Stx2 comes from expression of the phage carrying the *stx2* genes (Wagner, Neely et al. 2001).

In addition, it was shown that NO, another molecule generated by the host during inflammation, inhibits the production of Stx2 in EHEC in the presence of mitomycin C (Vareille, de Sablet et al. 2007). The inhibition most likely occurred via an inhibition of *recA* transcription, as the presence of NO led to a decrease in *recA* mRNA (see also Part III.D.1.b and Figure 20). When the authors used cPTIO, a known NO scavenger, *stx2* transcription and Stx2 production were restored (Vareille, de Sablet et al. 2007). Further discussion on NO and its effects will be discussed in the following chapter of my bibliography.

The gut microbiota is also likely to play a significant role in the release of Shiga toxins. For example, probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* were shown to have an inhibitory effect on Stx1 and Stx2 production in mouse infection studies; the reason behind such inhibition is thought to be the production of acetate, thus the decrease of pH, generated by these bacteria (Asahara, Shimizu et al. 2004, Carey, Kostrzynska et al. 2008). Moreover, the human gut microbiota can inhibit Stx production in an EHEC O157:H7 strain. Human fecal flora induces inhibition of Stx2 synthesis and Stx2-phage production. This inhibition is observed when the EHEC O157:H7 strain is cultured in a medium conditioned with *Bacteroides thetaiotaomicron* (de Sablet, Chassard et al. 2009); in fact, a recent study showed that this inhibition is most likely due to the uptake of vitamin B12 by *B. thetaiotaomicron* (Cordonnier, Le Bihan et al. 2016). Interestingly, a study assessed the importance of the commensal *E. coli* present in the human gut microbiota in the outcome of an EHEC-mediated infection and HUS (Gamage, Strasser et al. 2003). They first showed

that *E. coli* cells that were susceptible to the Stx lambdoid phage could get infected and end up producing Stx themselves, unlike *E. coli* cells that were resistant to the phage. To parallel this finding in a physiological context, they performed a screening of human intestinal *E. coli* isolates, and observed that most isolates were resistant to the phage. Nonetheless, 3 isolates were sensitive and ended producing significantly more Stx than was previously incubated in the medium (Gamage, Strasser et al. 2003). This work thus clearly implies that the presence of particular commensal *E. coli* strains may heavily influence the severity of an EHEC infection.

Recently, a study emphasized the role of oxygen on EHEC O157:H7 growth and Stx production (Tran, Billoud et al. 2014). The human gut is generally considered to be anaerobic in the lumen, but microaerobic by the intestinal epithelium, due to oxygen diffusion from enterocytes (Marteyn, Scorza et al. 2011). By using the Stx-resistant T84 cells to mount a microaerobic vertical diffusion chamber, Tran *et al.* showed that microaerobic conditions decreased growth of EHEC O157:H7 as well as Stx1 and Stx2 production (compared to the same aerobic conditions), although Stx2 was the major type produced in that particular strain (strain EDL933). That said, the oxygen concentration had neither effect on Stx2 stability nor on its ability to bind T84 cells (Tran, Billoud et al. 2014). This study was in accord with a previous one showing that EHEC O157:H7 EDL933 showed a lower Stx release in simulated ileal and colonic microaerobic environment media versus aerobic (Polzin, Huber et al. 2013).

In summary, the regulation of *stx* gene expression is dependent on the complexity of the intestinal environment and can respond to a multitude of stimuli exerted on the EHEC by the host, the physico-chemical conditions and the gut microbiota.

(g) How does Stx reach its target organs?

EHEC are not considered to be invasive, although some O157:H7 strains may invade certain cell lines (Oelschlaeger, Barrett et al. 1994) and some LEE-negative O113:H21 strains are able to invade CHO-K1 cells (Luck, Bennett-Wood et al. 2005). Nevertheless, EHEC are never found in the organs or the mesenteric lymph nodes, and do not cause sepsis either (except hybrid clones such as the O80:H2 strains). Currently, there are no good animal models so far to study EHEC pathogenesis, and the passage of Shiga toxins from the intestinal lumen to its target organs has yet to be elucidated. Yet, several hypotheses have been advanced to explain how Stx could traverse the intestinal epithelium barrier (Figure 13), and experimental arguments in favor of each of these hypotheses will be discussed in the next sections.

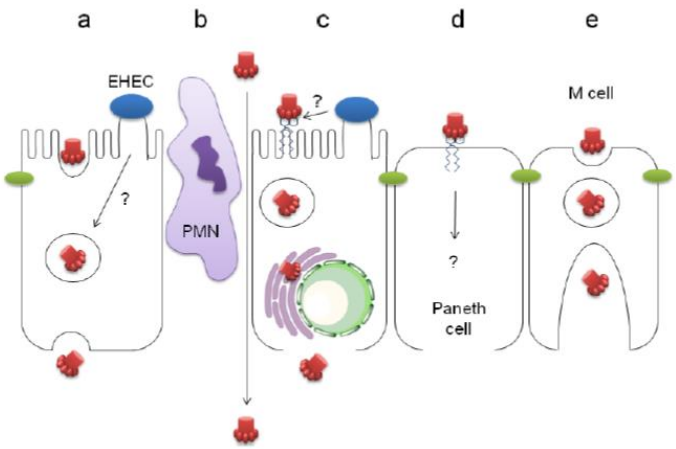

Figure 13. The different models for the translocation of Shiga toxins through the intestinal epithelial border. *Shiga toxin is depicted in red. PMN, polymorphonuclear neutrophils. From (Schuller 2011).*

- Transcytosis across epithelial cells

Stx1 and Stx2 are capable of translocating through T84 cell lines, without affecting cell viability or the epithelial barrier function. In the case of Stx1, the toxin was detected in intracellular compartments such as endosomes, the Golgi and ER, as well as the nuclear membrane; as Stx1 was not associated with tight junctions, transcytosis through IECs seemed more plausible than paracellular transport. Interestingly, retrograde transport is not in play during translocation, as exposure to Brefeldin A, which disrupts the Golgi apparatus, does not affect translocation of Stx1 or Stx2 (Hurley, Jacewicz et al. 1999). Stx1 translocation was also shown to involve actin turnover (Maluykova, Gutsal et al. 2008); interestingly, actin turnover is used in a receptor-independent endocytosis process called macropinocytosis, and it was demonstrated that the stimulation of macropinocytosis in T84 cells enhanced Stx1 transcytosis, thus suggesting that macropinocytosis could be one mode of passage of Stx1 (Malyukova, Murray et al. 2009) (Figure 13, model a). In fact, another study showed that, during an EHEC O157 infection of T84 cells, EHEC soluble factors were actually sufficient to induce macropinocytosis, and that both Stx1 and Stx2 were both uptaken by macropinocytosis (In, Lukyanenko et al. 2013). However, recent work looking at EHEC O157 Stx2 translocation through T84 cells did not report signs of macropinocytosis, and the use of inhibitors of macropinocytosis did not affect Stx2 translocation across T84 cells (Tran, Billoud et al. 2014); the authors explained this discrepancy by significant experimental setup differences.

Another hypothesis regarding Stx transcytosis is the possibility that IECs could express the Gb3 receptor, despite previous findings going against it. Importantly, since it was shown that

Gb3 expression (and with it, Stx cytotoxicity) could be extended in Gb3-positive endothelial cells by treatment with butyrate (found in the intestines), pro-inflammatory cytokines, or LPS, there is a possibility that Gb3 expression could be enhanced on the Gb3-negative IECs (Figure 13, model c). Butyrate did not affect Gb3 expression in T84 cells, but other treatment, such as LPS, has not been put into test yet. Similarly, IECs from normal or inflamed intestinal mucosa did not express Gb3 after exposure to Stx, thus probably ruling out an EHEC-mediated induction of Gb3 expression after damage caused on the intestinal epithelium. Another study showed an apparently high presence of the Gb4 receptor at the surface of normal intestinal epithelial cells obtained from flash–frozen biopsies (Zumbrun, Hanson et al. 2010). Gb4 derives from Gb3 by the activity of Gb4 synthase, and manipulation of a cell line by decreasing levels of Gb4 synthase led to an increase in Gb3 expression and sensitivity of cells to Stx. Thus, one could wonder if an EHEC infection and subsequent manipulation of the host cell machinery could not shut the Gb4 pathway and lead to an accumulation of Gb3 expression at the surface of IECs.

- Translocation via a paracellular pathway

As EHEC affects the intestinal epithelium barrier, notably via redistribution of tight junction proteins, it is possible that these perturbations could open a way for Stx to cross the barrier in a paracellular pathway (Figure 13, model b). Alternatively, the recruitment and subsequent transmigration of neutrophils could be at play. Neutrophil transmigration is known to occur during EHEC infection, as elevated neutrophil levels are found in the stool of infected patients. Neutrophil transmigration was simulated *in vitro* by co-incubating T84 cells with neutrophils on their basal side, while putting a neutrophil chemoattractant on the apical side. Interestingly, as neutrophil transmigration resulted in increased paracellular permeability, an increase in translocation (from the apical to the basal side) of both Stx1 and Stx2 was observed, the extent of which was proportional to the degree of neutrophil transmigration (Hurley, Thorpe et al. 2001).

- Translocation via Paneth or M cells

Paneth cells were shown to express Gb3 at their surface (Figure 13, model d), although it should be noted that, in every patient from which tissue samples were collected, not all Paneth cells expressed Gb3 (Schuller, Heuschkel et al. 2007). Whether binding of Stx leads to cytotoxicity, to effective passage of Stx to the lamina propria (LP), or does not have any physiological relevance remains to be determined.

Another hypothesis that is to be considered is the passage of EHEC, or Stx itself via M cells in PPs (Figure 13, model e). Indeed, EHEC were shown to bind to M cells (Etienne-Mesmin, Chassaing et al. 2011) and that binding occurred via their long polar fimbriae (Cordonnier, Etienne-Mesmin et al. 2017). Furthermore, binding of EHEC to M cells resulted to translocation of EHEC, showing that binding to M cells could be physiologically relevant. The entry of EHEC into the LP, would most certainly trigger the innate immune response to fire up, and many components of the innate immune response (NO, H_2O_2…) are direct triggers of the SOS response in *E. coli* and the release of Stx; we can then envision this possible pathway to explain how Stx is found in target organs during HUS. If this hypothesis holds true, the fact that EHEC has never been found to cause bacteremia would mean that EHEC cells are targeted and destroyed locally, that is within the LP. Alternatively, it remains possible that free Stx in the gut lumen could bind to a non-specific receptor on M cells, as previously observed for cholera toxin (Frey, Giannasca et al. 1996).

(4) Other potential virulence factors of EHEC

(a) Other adhesion factors not encoded by the LEE

In EHEC, many fimbrial and non-fimbrial adhesins contribute to adherence, establishment, and persistence of infection (McWilliams and Torres 2014, Monteiro, Ageorges et al. 2016).

(i) Filamentous structures

Different fimbriae have been described:

- **Type I fimbriae**, capable of binding to mannose, has often been associated with the virulence of *E. coli* strains and appears important for colonization of the intestinal epithelium. However, its role in the pathogenicity of EHEC has never been demonstrated (Iida, Mizunoe et al. 2001). Moreover, as previously mentioned, the majority of EHEC O157:H7 strains are not able to express this fimbriae, in contrast to the O26 and O118 serogroups (Iida, Mizunoe et al. 2001, Roe, Currie et al. 2001, Shaikh, Holt et al. 2007). Indeed, in *E. coli* serotypes O157:H7, there is a deletion of 16 base pairs in the regulatory region that controls the expression of fimbriae.
- **Long polar fimbriae** (Lpf) are known to adhere to extracellular matrix proteins such as fibronectin, laminin and type IV collagen (Farfan, Cantero et al. 2011). As explained above, the interaction and subsequent translocation of EHEC through M cells observed *in vitro* (Etienne-Mesmin, Chassaing et al. 2011) where shown to be both dependent on Lpf (Cordonnier, Etienne-Mesmin et al. 2017). Furthermore, the expression of Lpf in the small

intestine, but not in the colon, would indicate a preferential colonization in early stages of infection of the terminal ileum, where PPs are numerous (Cordonnier, Etienne-Mesmin et al. 2017). A previous study had demonstrated the interaction and uptake of *E. coli* through M cells via the type I fimbriae subunit FimH, which bound to M cell-borne glycoprotein 2 (GP2) (Hase, Kawano et al. 2009). A majority of EHEC O157:H7 strains do not express FimH; therefore, Lpf could in fact bind GP2 and lead to EHEC O157 translocation.

- The **ECP pilus** (*E. coli* common pilus) and the **F9, ELF** (*E. coli* laminin-binding-fimbriae) and **Sfp** (Sorbitol-fermenting fimbriae protein) fimbriae promote EHEC adhesion to epithelial cells *in vitro* (Rendon, Saldana et al. 2007, McWilliams and Torres 2014). In addition, the ECP pilus, the F9 and ELF fimbriae are also able to interact with extracellular matrix proteins (Low, Dziva et al. 2006, McWilliams and Torres 2014). It has also been shown that the ECP pilus and Sfp fimbriae are expressed under low oxygen conditions, which are conditions found in the digestive tract.
- The **type IV pilus HCP** (for hemorrhagic *coli* pilus) is involved in the adhesion of EHEC to many cell types as well as in epithelial cell invasion, hemagglutination of erythrocytes, biofilm formation and pro-inflammatory response. These properties suggest that this pilus contributes not only to virulence, survival and transmission of EHEC but also to inflammation caused by EHEC (Xicohtencatl-Cortes, Monteiro-Neto et al. 2007, Xicohtencatl-Cortes, Sanchez Chacon et al. 2009).

On the other hand, structures such as the curli and flagellum have also been identified as EHEC adhesion factors (McWilliams and Torres 2014).

- **Curli** interacts more specifically with extracellular matrix proteins and its expression is induced by multiple environmental signals such as pH, temperature and nutritional deficiency. Curli also has a role in the adhesion and formation of EHEC biofilms to plants. Under certain conditions, it also has the role of protecting pathogens against chemical antiseptics either by forming a physical barrier around bacteria or by inducing the formation of biofilms (Ryu and Beuchat 2005).
- The **flagellum**, initially associated with bacterial motility, has also been identified as a factor of adhesion to human epithelial cells. After contact with the epithelium, flagellum expression is reduced, suggesting that the flagellum is involved in the early stages of adhesion, at least in the context of bovines (Mahajan, Currie et al. 2009). In addition, the EHEC flagellum is able to interact with a Muc2 mucin protein (Erdem, Avelino et al. 2007).

(ii) Non-organelle adhesins

Non-organelle adhesins include proteins with the ability to be surface-exposed or secreted, called autotransporters.

- The autotransporters **EhaA** and **EhaB** are involved in the formation of biofilms. In addition, EhaA is involved in self-aggregation, while EhaB is involved in adhesion to extracellular matrix proteins such as collagen I and laminin (Wells, Sherlock et al. 2008, Wells, McNeilly et al. 2009).
- Two other autotransporters, the **Saa** (STEC autoagglutination adhesin) and **Sab** (STEC autotransporter contributing to biofilm formation), have been identified in atypical EHEC (that is, EHEC not carrying the LEE) and are involved in adhesion to human epithelial cells and in the formation of biofilms. These two adhesins can thus contribute to the colonization of host cells by EHECs that do not have the ability to form A/E lesions (Paton, Srimanote et al. 2001, Herold, Paton et al. 2009).
- The last described autotransporter, named **Cah** (calcium binding antigen 43 homolog), is involved in autoaggregation and in biofilm formation. However, its role in the pathogenicity of EHEC remains to be demonstrated (Torres, Perna et al. 2002).

Other non-fimbrial adhesins potentially involved in the colonization and/or pathogenesis of EHEC have been identified. This is the case of adhesin Iha, present in strains O157:H7 and absent in other strains but whose role in virulence has not been demonstrated (Tarr, Bilge et al. 2000). OmpA (Outer membrane protein A) protein, present in pathogenic and commensal *E. coli* strains, appears to be important for *in vitro* adhesion of EHEC O157:H7 to human epithelial cells (Torres and Kaper 2003).

Adherence and intestinal colonization as well as Shiga-toxin production are considered essential for the establishment of EHEC infection. However, other factors are involved in the pathogenesis of EHEC.

(b) Cytolethal distending toxin (CDT)

CDT is produced by many Gram-negative bacteria, including numerous strains O157:H7 and O157:H$^-$, as well as non-O157 EHEC strains (O91:H21, O113:H21) (Janka, Bielaszewska et al. 2003, Bezine, Vignard et al. 2014). CDT from EHEC was shown to have an effect on a wide variety of endothelial cell lines as well as human endothelial explants, although CDT from *Campylobacter jejuni* has clearly been shown to induce damage to the epithelial barrier and severe inflammation in the gut (Jain, Prasad et al. 2008). CDT induces a dose-dependent G2/M cell cycle arrest which leads to cell distention, inhibition of proliferation, and ultimately cell death (Bielaszewska, Sinha et al. 2005). There is a positive correlation

between the presence of the CDT toxin and the ability of non-O157 EHEC strains to cause severe pathologies (Bielaszewska, Fell et al. 2004).

(c) The subtilase

A new toxin called subtilase was discovered in 2004 by Paton's group in an O113:H21 strain responsible for an HUS epidemic (Paton, Srimanote et al. 2004) and found in other LEE-negative STECs. This toxin belongs to a new family of AB_5 toxins and has a serine protease activity. It has cytotoxic activity against Vero cells and causes microvascular thrombosis and necrosis of the brain, liver and kidney in the mouse. The subtilase has two activities: a protein synthesis inhibition activity by the SubA subunit, and a vacuolating activity for which only the presence of SubB is necessary (Morinaga, Yahiro et al. 2007). Its production in an *E. coli* K12 strain makes it toxic to all tested cell lines and mice (Paton, Srimanote et al. 2004), suggesting that this toxin is actually involved in the virulence of the strains that possess it.

(d) Plasmid-borne virulence factors

All EHEC O157:H7 strains isolated from clinical cases carry a virulence plasmid called pO157, ranging in size from 93 kb to 104 kb (Figure 14). This plasmid is also present in EHEC O26:H11 strains. It contains about a hundred genes, of which 35 are potentially involved in the virulence of EHEC. The genes carried by the plasmid encode, among others, a hemolysin (HlyA), a catalase peroxidase (KatP), a type II secretion system (Etp), a serine protease (EspP), a putative adhesin (ToxB) and a metalloprotease (StcE) (Levine, Xu et al. 1987, Lim, Yoon et al. 2010).

Figure 14. Map of a virulence plasmid from E. coli O157:H7 (pO157). *Open reading frames (ORFs) of pO157 are categorized with color codes: virulence factors are in red, maintenance and transfer of the plasmid in yellow, insertion sequences (IS) in green, other functions in blue, hypothetical genes in purple, and sequences with no homology to known genes in white. From (Makino, Ishii et al. 1998).*

- The *hlyA* gene of a size of 3.4 Kb, encoding the **HlyA** hemolysin, is carried by pO157. Numerous studies have shown that this hemolysin is conserved among the O157:H7 and non-O157 serotype strains most frequently associated with HUS cases (Lim, Yoon et al. 2010). Hemolysin belongs to the family of RTX toxins (repeats-in-toxin) that are secreted by a type I secretory system and form pores in the membranes of erythrocytes and other target eukaryotic cells. Two types of hemolysin have been described: the free form that has the ability to lyse endothelial cells of the micro-vessels of the human brain and the form associated with membrane vesicles that are internalized in intestinal and endothelial epithelial cells to trigger apoptosis (Bielaszewska, Aldick et al. 2014).
- The **KatP** catalase peroxidase is encoded by the *katP* gene conserved within EHEC O157:H7 strains and other EHEC strains. It is an enzyme located in the cytoplasm and bacterial periplasm that allows bacteria to resist to oxidative stress. KatP functions by degrading hydrogen peroxide, allowing the release of oxygen. Thus, this enzyme seems to favor colonization and survival of EHEC in the digestive tract, an oxygen-poor environment, by allowing the use of oxygen as a substrate in the respiratory chain (Uhlich 2009, Lim, Yoon et al. 2010).

- The **EspP** autotransporter with serine protease activity seems to be involved in EHEC virulence, notably in the development of HC and HUS. Indeed, EspP cleaves and reduces the activity of several coagulation factors, causing coagulation disorders that can facilitate the passage of Shiga toxins in the bloodstream at the site of colonization in the intestinal epithelium. Other roles have been described for the EspP protease. For example, it is able to cleave proteins of the complement, thus protecting pathogens from opsonization (Orth, Ehrlenbach et al. 2010). In addition, it has been shown that EspP monomers can assemble and form a filament (Xicohtencatl-Cortes, Saldana et al. 2010). This filament protects bacteria from antibiotics and detergents, contributes to the formation of biofilms and has adhesion properties to epithelial cells resulting from a cytopathic effect. Thus, EspP seems to promote adhesion and colonization of EHECs to the intestinal epithelium and increase the severity of the infection.
- The **StcE** metalloproteinase is secreted by the type II secretion apparatus Etp. Grys *et al.* demonstrated *in vitro* that StcE promoted intimate adhesion of O157:H7 strains to epithelial cells by cleaving surface glycoproteins such as those found in mucins. Indeed, the mucinase activity of StcE allows pathogens not only to get closer to the intestinal epithelium but also to increase the availability of nutrients (Grys, Siegel et al. 2005). Using mucin-producing LS174T colon carcinoma cells as a model, a recent study showed that EHEC O157:H7 strains were reducing levels of Muc2 in a StcE-dependent fashion; this led to a decrease of the inner mucus layer and an increased access of EHEC to the epithelial cells' surface (Hews, Tran et al. 2017).
- The 9.5 Kb *toxB* gene, encoding the **ToxB** putative adhesin, is carried by pO157. ToxB contributes to the adhesion of EHEC O157:H7 by stimulating the production of proteins secreted by the T3SS, such as EspA, EspB and Tir, required for intimate adhesion of bacteria to enterocytes (Tatsuno, Horie et al. 2001).

2. Other InPEC pathotypes

Besides EHEC, a number of *E. coli* pathotypes have been extensively studied and characterized, like EPEC and ETEC; others, like DAEC and AIEC have been identified more recently, and their mechanism of virulence are still under heavy investigation. These InPEC and their major mechanism of colonization are shown in Figure 15.

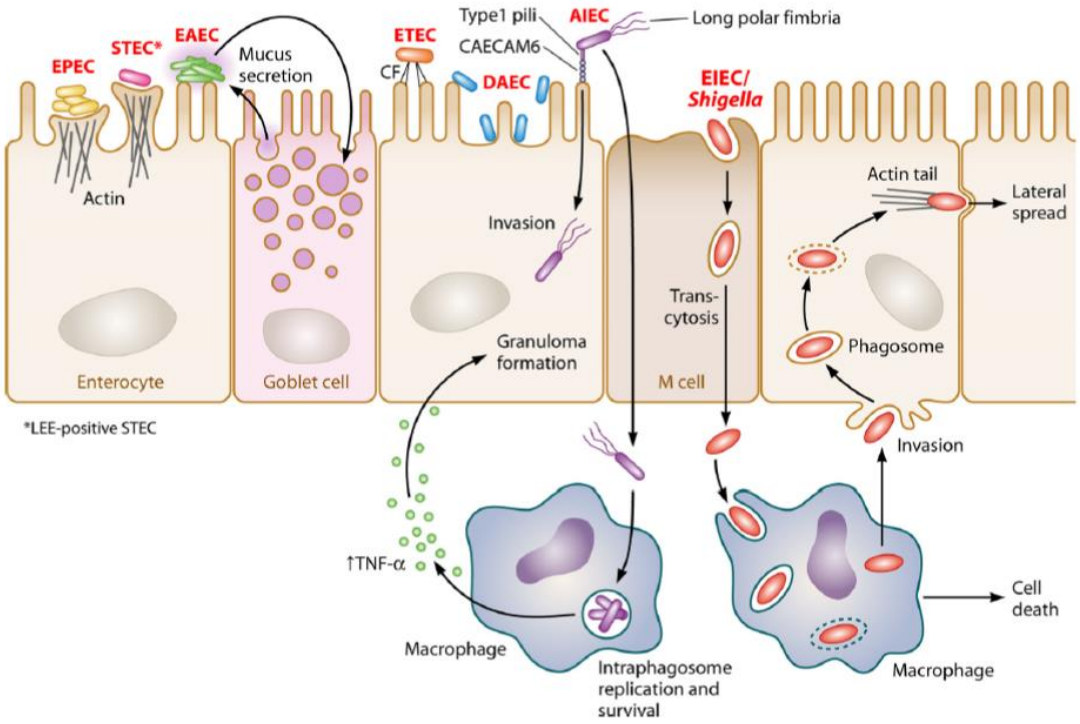

Figure 15. Mechanisms of adherence and colonization of major InPEC. *Enteropathogenic E. coli (EPEC) and Shiga-toxin E. coli (STEC, including enterohemorrhagic E. coli EHEC) provoke attachment and effacement (A/E) lesions by expression of the locus of enterocyte effacement (LEE); these pathogens do not invade enterocytes. Enteroaggregative E. coli (EAEC) forms biofilms on the intestinal mucosa; EAECs adhere to each other and to the surface of the enterocyte to form a characteristic stacked brick pattern. Enterotoxigenic (ETEC) has multiples colonization factors (CF) that mediate attachment to enterocytes. Diffusely adherent E. coli (DAEC) disperses itself throughout the apical surface of the enterocyte, leading to a diffuse adherence. Adherent invasive E. coli (AIEC) uses type I pili to adhere to enterocytes and long polar fimbriae to mediate invasion. Enteroinvasive E. coli (EIEC)/Shigella are intracellular pathogens that traverse the epithelium broder via M cell-mediated transcytosis and invade enterocytes via the basolateral side. They escape macrophage-mediated killing by induction of macrophage cell death. From (Croxen, Law et al. 2013).*

a) Enteropathogenic *E. coli* (EPEC)

Historically, EPEC was the first *E. coli* pathotype to be described. EPEC is a non-invasive pathogen that colonizes the small intestine, and gets transmitted via the oral-fecal route. Since the 1940s, when EPEC was identified, the prevalence of EPEC shifted over the years from developed countries to developing countries. Today, EPEC is a major cause of acute diarrhea and dehydration in children of developing countries. In 2010, EPEC was found to be the second leading cause of foodborne deaths, with children under the age of 5 in developing countries being the most at risk population (Kirk, Pires et al. 2015).

Like LEE-positive STEC, EPEC are characterized by the ability to form A/E lesions on the surface of IECs, owed to acquisition of the LEE. In fact, the atypical EPEC O55:H7 strain is now thought to be the common ancestor from which STEC O157:H7 evolved (Zhou, Li et al.

2010). Furthermore, typical EPEC carry the *E. coli* adherence factor plasmid (pEAF), while atypical EPECs do not.

The bundle-forming pili (BFP) encoded by pEAF are type IV pili responsible for the initial attachment of EPEC (Giron, Ho et al. 1991), tethering bacteria together to form localized adhesions on the host cell surface; this phenotype is not seen in the case of an atypical EPEC infection. After initial attachment, the mechanism of A/E lesion formation is very similar to the one previously discussed with EHEC; however, in EPEC, Tir once in the host cell needs to get phosphorylated by host tyrosine kinases (Deibel, Kramer et al. 1998, Phillips, Hayward et al. 2004); this phosphorylation does not occur for Tir in EHEC (DeVinney, Stein et al. 1999). Although genes encoding the T3SS apparatus keep a high degree of conservation, there are significant variations between genes encoding effector proteins in EPEC versus EHEC.

The exact mechanism that leads to acute diarrhea production is still not fully understood as it most likely involves several factors. Among T3S effector proteins in EPEC, EspF and EspG modulate aquaporin localization (Guttman, Samji et al. 2007), resulting in diarrhea. Additionally, EspF and EspG, along with another effector Map, disrupt tight junctions and probably contribute in the onset of diarrhea in this manner.

Treatment with oral rehydration therapy usually works, although there has been an increase in the failure to respond to it. Persistence is not so uncommon with EPEC infections, in which case the use of antibiotics may be necessary; however, in developing countries, where EPEC infections are more prominent and lethal, access to antibiotics is often very limited. Furthermore, antibiotic-resistant EPECs are now found globally.

b) Enterotoxigenic *E. coli* (ETEC)

ETEC, the main cause of traveler's diarrhea, represents the second etiological agent of diarrheal disease worldwide. In 2010, 240 million cases were recorded, 16% of which occurred in children under 5 years of age; this age category was found to be at higher risk of ETEC-associated mortality (Kirk, Pires et al. 2015). Besides diarrhea, complications exist with ETEC infections. Indeed, a study of children in Bangladesh correlated a prior ETEC infection to malnutrition and stunted growth (Qadri, Saha et al. 2007). Transmission of ETEC occurs via the fecal-oral route, usually by exposure to contaminated food or water. As an example, food acquired from street vendors is one of the common sources of traveler's diarrhea.

ETEC carries a number of colonization factors (CFs), which promote ETEC attachment in the small intestine epithelium. CFs can be non-fimbrial, fimbrial, helical, or fibrillary (Qadri, Svennerholm et al. 2005); of note, different CFs bind different host cell receptors. Additionally, the outer membrane proteins Tia and TibA may mediate further intimate attachment to the host cell (Turner, Scott-Tucker et al. 2006).

A major virulence characteristic in ETEC is the secretion of heat-stable enterotoxins (STs), the heat-labile enterotoxin (LT), or a combination of these; these enterotoxins cause mild to severe non-bloody diarrhea. ST is more frequently found in severe human diseases compared to LT-only ETEC isolates (Qadri, Svennerholm et al. 2005). Because STs mimic the guanylin hormone, they can bind to guanylyl cyclase C receptors in the small intestinal epithelium and stimulate their activity. This results in the accumulation of intracellular cyclic GMP, which both impairs Na^+ absorption and lead to chloride secretion by the cystic fibrosis transmembrane receptor (CFTR) (Turner, Scott-Tucker et al. 2006). LT is an AB_5 toxin very similar to cholera toxin. The B subunit of LT interacts with the receptor GM1 on epithelial cells, leading to the toxin internalization by lipid raft and retrograde transport. The LT A subunit activates adenylyl cyclase, which increases intracellular amounts of cyclic AMP, and ultimately activates CFTR as well (Nataro and Kaper 1998).

Traveler's diarrhea is self-limiting, so the maintenance of fluid and electrolyte balance is usually sufficient. Antibiotics such as fluoroquinolones can also reduce the duration of the disease; however, this practice has generated an increase in antibiotic-resistance isolates.

c) Enteroaggregative *E. coli* (EAEC)

EAEC, is the second causative agent of traveler's diarrhea after ETEC, but it is also known to cause persistent diarrhea in children. EAEC colonizes the small intestine and/or the colon, and provoke inflammatory watery diarrhea that can be accompanied by mucus and blood. Complications from persistent EAEC infections in children of developing countries include chronic inflammation and malnutrition due to nutrient malabsorption, as well as stunted growth (Lima and Guerrant 1992). Transmission occurs through contaminated food, such as salad.

During an EAEC infection, the formation of stack-brick patterns of bacterial cells can be observed at the site of adhesion to the IECs; these are mediated by aggregative adherence fimbriae (AAFs). After adhesion, EAEC can express various toxins, including Pet, which alters the host cytoskeleton, as well as a heat stable enterotoxin EAST-1, and ShET1 (for *Shigella* enterotoxin 1), which induces cyclic AMP and cyclic GMP. In addition, the mucinase Pic, commonly found in *E. coli* pathotypes and *Shigella* (Henderson, Czeczulin et al. 1999), is

likely to be an important colonization factor for EAEC to degrade mucins and establish a niche where it can form a biofilm.

Of importance, the infamous Germany outbreak of 2011, which led to 4321 cases, 900 HUS and more than 50 deaths, was caused by *E. coli* O104:H4, an EAEC strain that had recently acquired the *stx2* gene (Rasko, Webster et al. 2011, Karch, Denamur et al. 2012). Thus, this hybrid strain has been proposed to be included within a new *E. coli* pathotype, called EAHEC (Brzuszkiewicz, Thurmer et al. 2011).

d) Diffusely adherent *E. coli* (DAEC)

DAEC strains are associated with potentially persistent watery diarrhea in children under 5, UTIs, pregnancy complications, but they can also be asymptomatic as part of the gut resident microbiota. The scattered adherence of DAEC throughout the entire surface of IECs is what gave the name to this pathotype. DAEC carries Afa/Dr adhesins, which bind the DAF (for decay-accelerating factor) receptor found on the brush border of IECs in humans (Le Bouguenec and Servin 2006). Binding to DAF results in brush border lesions, expression of pro-inflammatory cytokines, and neutrophil recruitment. In addition, DAEC-secreted Sat toxin mediates rearrangements of tight junctions and an increase in paracellular and transcellular permeability. Currently, the only treatment recommended against DAEC infection is rehydration.

e) Adherent Invasive *E. coli* (AIEC)

AIEC is one of the causative agents of Crohn's disease (CD), which is thought be mediated by a number of causative factors. Indeed, adhesive and invasive *E. coli* were found in more than 30% of patients diagnosed with CD, according to clinical studies. Incidence and prevalence is increasing in developed countries, particularly the US and Europe. Approximately 2.2 million people in Europe and 1.4 million people in the US suffer from CD.

AIEC have the ability to adhere to IECs in the ileum, but also to invade and replicate in IECs and macrophages. AIEC adheres to IECs via the binding of type 1 pili to CEACAM6 (carcinoembryonic antigen related cell adhesion molecule) expressed on the surface of the target cell (Barnich, Carvalho et al. 2007). After adhesion, AIEC use OMVs to fuse with the membrane of IECs (via expression of OmpA) and deliver effector proteins to mediate invasion of AIEC (Rolhion, Barnich et al. 2005). Additionally, AIEC can bind to M cells in the ileum via its long polar fimbriae Lpf (just like EHEC), which mediates transcytosis to the LP (Chassaing, Rolhion et al. 2011), where AIEC invades and replicates within macrophages

while preventing macrophage cell death (Glasser, Boudeau et al. 2001). This results in massive intestinal inflammation, as well as granuloma formation characteristic in CD patients.

Treatments for CD patients currently include the reduction of the intestinal microbiota via intestinal washes and antibiotics, although disturbing microbiota can have important consequences (Wlodarska and Finlay 2010).

f) Enteroinvasive *E. coli* (EIEC) and *Shigella*

EIEC and *Shigella* (which are generally considered to belong to the same pathotype) are quite unique among InPEC in that they are facultative intracellular pathogens carrying neither adherence factors nor flagellum. Although the mechanism of infection is the same between *Shigella* and EIEC, EIEC infection is generally less efficient than *Shigella*, as exemplified by the higher infectious doses observed compared to *Shigella*, and EIEC infections lead to less severe clinical manifestations.

Shigella is associated with up to 50% of bacillary dysentery cases in the world (Pfeiffer, DuPont et al. 2012). In 2010, more than 190 million cases of foodborne disease cause by *Shigella*, among which close to 66,000 deaths were reported; strikingly, 99% of *Shigella* episodes occur in developing countries, and young children are at high risk (Kirk, Pires et al. 2015).

Infection with *Shigella* (or EIEC) in the large intestine is a multistep process (Croxen and Finlay 2010). *Shigella* invades the host via M cell-mediated transcytosis. In the LP, *Shigella* survives macrophage phagocytosis by escaping the phagosome and induces macrophage cell death. From there, *Shigella* invades IECs from the basolateral side, where it replicates while suppressing the host immune response. T3S effectors ensure intra and intercellular movement of *Shigella*, partly by forming an actin tail on the bacterial surface, thereby providing propulsive force for *Shigella* to move. While *Shigella* replicates, effector proteins also ensure survival of the host, until it eventually breaches the epithelium to exit and invade another IEC. These lesions of the intestinal epithelium, along with apoptotic macrophages and recruitment of polymorphonuclear leukocytes lead to the tissue lesions observed in shigellosis. *Shigella* can also carry various toxins. Importantly, Stx present in *S. dysenteriae 1* is very similar to STEC Stx1, and can lead to a more severe and potentially lethal disease; to my knowledge, no EIEC has been found to carry *stx* genes so far.

III. Nitric oxide

Nitric oxide (NO) is a small inorganic molecule composed of one atom of oxygen and one atom of nitrogen. As a free radical, NO is highly reactive and has the ability to act on many molecules in living organisms. Physiologically, NO is produced from the substrate L-arginine and requires one of the several isoforms of the enzyme nitric oxide synthase (NOS). NO synthesis occurs in a great variety of cell types in the body, which partly explains its crucial (pleiotropic) role in diverse biological fonctions. Importantly, since the early discovery that mouse macrophages released large amount of NO_2^- (nitrite) and NO_3^- (nitrate) upon stimulation with LPS and IFN-γ, NO was found to be a critical player in innate and adaptive immunity, and the modulation of inflammation.

A. Chemical properties of NO

The free radical NO (for which the true nomenclature should be $^{\cdot}NO$, but will remain written as NO for the rest of this chapter) has an unpaired electron that it can share with various organic and inorganic molecules. Indeed, we will see in this next section that NO can react with other radicals, but also transition metals, thiols, aromatic rings or lipids. Reactions in which NO participates depend considerably on environmental factors such as oxygen levels and pH. These various molecular targets and chemical reactions underline the important physiological and biochemical properties of NO.

1. Reactions with oxygen and oxygen derivatives

a) NO and oxygen (O_2)

In normoxic (i.e aerobic) aqueous solution, NO gets rapidly oxidized to nitrite (NO_2^-):

$$4\ NO + O_2 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+$$

NO can further be converted to nitrate (NO_3^-), which may require the presence of oxidizing proteins (Ignarro, Fukuto et al. 1993).

b) NO and oxygen derivatives

Oxygen derivatives are generated during normal or pathological metabolic processes by different cells such as macrophages and polynuclear cells. Among these derivatives, superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are major molecules that can react with NO.

(1) NO and superoxide (O_2^-)

The reaction between NO and the free radical O_2^- forms peroxynitrite ($ONOO^-$):

$$NO + O_2^- \rightarrow ONOO^-$$

$ONOO^-$ can also be formed by the reaction between NO_2^- and H_2O_2 or between NO^- and O_2; however, these reactions remain exceptional cases in biological systems.

The protonation of $ONOO^-$ in aqueous solution generates peroxynitrous acid (ONOOH), which can further dissociate to form hydroxyl radical (OH) and nitrite:

$$ONOOH \rightarrow OH + NO_2$$

$ONOO^-$ itself has many biochemical and cytotoxic properties (Radi 2013); for example, $ONOO^-$ is responsible for nitration (e.g of tyrosine residue) and thus inactivation of proteins, as well as lipid peroxidation, causing membrane fatty acid degradation. On the other side, $ONOO^-$ is an efficient mean for O_2^- detoxification (Kroncke, Suschek et al. 2000). In acidic pH, $ONOO^-$ can rapidly get converted to nitrate.

(2) NO and hydrogen peroxide (H_2O_2)

H_2O_2 can directly interact with NO, and such reaction will form singlet oxygen ($^1O_2$) and nitrous oxide (N_2O):

$$2\ NO + H_2O_2 \rightarrow {}^1O_2 + H_2O + N_2O$$

Singlet oxygen is a highly reactive derivative and is known to participate in cellular damage as well as pro-inflammatory reactions upon macrophage activation (Davies 2003, Robinson 2008).

Furthermore, H_2O_2 can also react with NO to form nitrogen dioxide (NO_2):

$$NO + H_2O_2 \rightarrow H_2O + NO_2$$

NO_2 is an S-nitrosylating agent; that is, an agent with which a NO group can be covalently attached to a cysteine residue to create a nitrosothiol group (which I will discuss further in the reaction between NO and thiols).

2. Reactions with transition metals

NO can fix most transition metals, leading to formation of a metal nitrosyl complex. The speed and stability of such complexes depend on the exact transition metal at play in the reaction.

By far, the most common and important metal nitrosyl complex formation is with iron. NO binds reversibly to both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, though it has much greater affinity for ferrous than for ferric iron (Cooper 1999):

$$Fe^{2+} + NO \rightarrow Fe^{2+}NO$$

$$Fe^{3+} + NO \rightarrow Fe^{3+}NO\ (Fe^{2+} - NO^{+})$$

When iron is associated to a protein such as heme (H), the speed and stability of the formed iron nitrosyl complex are enhanced:

$$Fe(II)H + NO \rightarrow Fe(II)HNO$$

The increased kinetic of the latter reaction suggests that heme-containing proteins (or hemoproteins) are important receptors of NO. Indeed, NO efficiently binds to hemoglobin (Hb; (Angelo, Hausladen et al. 2008)) to form the stable nitrosohemoglobin complex NOHb (Ignarro, Fukuto et al. 1993) with a high affinity. The half-life of NOHb in buffered medium is approximately 12 min compared to a few milliseconds for free NO; thus, nitrosohemoglobin represents a stable form of NO transport in the bloodstream and release NO at distant target sites.

Notably, NO is known to bind Fe^{2+} in the heme group of soluble guanylate cyclase (sGC), a mammalian NO sensor; NO binding results in conformational change of sGC which generates cGMP. cGMP is involved in vasodilation (Weissmann, Voswinckel et al. 2000), mitochondria biogenesis (Clementi and Nisoli 2005), as well as platelet aggregation inhibition (Homer and Wanstall 2002). Additionally, NO inhibits mitochondrial respiratory chain by binding to one of the heme groups of cytochrome-c oxidase in competition with oxygen (Brown 2001).

NO has also been reported to bind to copper (Cu^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}), and manganese cations (Mn^{2+}). Notably, I will discuss further the reactions between NO and Fe^{2+} or Zn^{2+} sulfur clusters in a later section.

3. Reactions with aromatic rings

Nitration of aromatic rings is done via transfer of charges between NO in its oxidized state (NO^+, or nitrosonium) and the aromatic group donating an electron, or by reaction with $ONOO^-$. Nitration can occur on an amino acid that is either free or integrated in a polypeptide chain. Notably, peroxinitrite-derived radicals can act on tyrosine to form 3-nitrotyrosine, which is regarded as an important biomarker of inflammation (Radi 2013). Furthermore, although protein tyrosine nitration can occur in healthy cells, the yield is fairly low; accumulation of protein tyrosine nitration can lead to profound conformational and functional changes of the targeted protein, thus promoting altered cell or tissue homeostasis. In mitochondria, the manganese superoxide dismutase (MnSOD) is an important target of nitrosating agents, where a single tyrosine nitration of the protein leads to its inactivation (MacMillan-Crow, Crow et al. 1996); this process is known to be involved in several neurodegenerative and metabolic disease conditions (Castro, Demicheli et al. 2011).

4. Reactions with thiols (-SH)

The covalent addition of a NO group to the thiol group of the amino acid cysteine generates S-nitrosothiols (R-S-NO) in a process termed S-nitrosylation (or S-nitrosation). This post-translational cysteine modification, as well as its reversal by S-denitrosylation, governs many proteins' activation and deactivation, notably in key processes in innate and adaptive immune responses (Hernansanz-Agustin, Izquierdo-Alvarez et al. 2013).

S-Nitrosothiols can be generated via several different reactions (Smith and Marletta 2012). Mainly, NO reacts with NO_2, to form dinitrogen trioxide (N_2O_3), which then reacts with thiol groups, as summarized in the following set of reactions:

$$2\ NO + O_2 \rightarrow 2\ NO_2$$

$$NO_2 + NO \rightarrow N_2O_3$$

$$N_2O_3 + R\text{-}SH \rightarrow R\text{-}SNO + H^+ + NO_2^-$$

Of note, NO_2 can also come from the degradation of $ONOO^-$.

In particular, each Hb β subunit contains thiol residues that can strongly react with NO to generate S-nitroso-Hb (Stamler, Jia et al. 1997). To date, the role of SNO-Hb in red blood cell-dependent hypoxic vasodilation and cardioprotection is a great matter of debate, with published studies going either against (Isbell, Sun et al. 2008) or toward (Zhang, Hess et al. 2015, Zhang, Hess et al. 2016) this hypothesis. It is also not entirely clear whether S-nitroso-proteins do participate in the release of NO under physiological conditions; indeed, previous studies had shown that SNO-Hb and SNO-albumin do not play a measurable role in the maintenance of blood vessel tone, even when *de novo* NO synthesis was prevented (Gladwin, Shelhamer et al. 2000).

5. Reactions with Fe-S and Zn-S clusters

Iron-sulfur clusters are among the primary targets of NO and its derivatives. By binding to iron-sulfur clusters, NO perturbs the spatial configuration of the cluster, which quite often leads to its disruption and subsequent inhibition of the target protein (Figure 16).

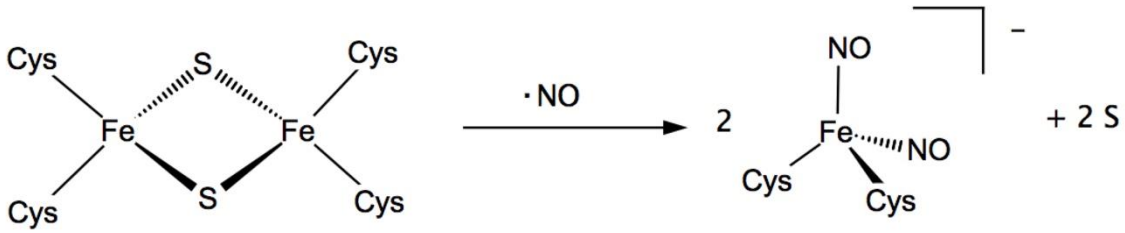

Figure 16. Reaction of nitric oxide with an iron sulfur cluster. *Shown is a hypothetical [2Fe-2S] cluster (left) coordinated by four cysteine (Cys) amino acid residues. Nitric oxide (·NO) can react with the cluster to form two dinitrosyl iron complexes (right) which releases two equivalents of elemental sulfur. Figure adapted from (Fitzpatrick and Kim 2015).*

Among well-known examples, the disruption of the iron-sulfur cluster of mitochondrial aconitase, an enzyme involved in the tricarboxylic acid cycle, seems to be mostly mediated by peroxynitrite, rather than by NO itself (Castro, Rodriguez et al. 1994, Tortora, Quijano et al. 2007). Similarly, NO and peroxynitrite are responsible for *in vitro* inhibition of the iron regulatory protein 1 (IRP1) (Soum and Drapier 2003), although both molecules seem to induce inhibition in different manners (Soum, Brazzolotto et al. 2003); IRPs are key proteins involved in the regulation of iron homeostasis. It is worth noting that NO binding to iron-sulfur clusters does not necessarily lead to the cluster disruption, as shown by a recent study on the effect of NO on the mitochondrial Miner2 protein (Cheng, Landry et al. 2017), whose function is yet unknown but which is highly expressed in several types of cancer.

Besides iron, zinc can also complex with cysteine sulfur ligands and form zinc fingers domains, which are essential domains for specific DNA-binding and found in many eukaryotic transcription factors. Several studies have also demonstrated that NO binds to zinc finger-containing proteins, leading to release of Zn^{2+} and potential destruction of the targeted zinc-sulfur cluster (Kroncke, Fehsel et al. 1994, Spahl, Berendji-Grun et al. 2003). In particular, NO was found to inhibit expression of IL-2 in murine lymphocytes; this inhibition is mediated by interference of NO with the zinc finger domains of Sp1 and EGR-1, which are both IL-2 transcription factors (Berendji, Kolb-Bachofen et al. 1999).

6. Reactions with nucleic acids

Although NO itself is not very reactive with DNA, reactive nitrogen species represent powerful DNA-damaging agents. Several chemical reactions are known to modify DNA bases, notably by deamination, alkylation, or nitration (Sawa and Ohshima 2006). For example, N_2O_3 can induce deamination of 5-methylcytosine and consequently mutation by base substitution from C to T at CpG sites. These mutations are commonly found in the p53 tumor suppressor protein, which could hold a significant impact in human carcinogenesis. Furthermore, peroxynitrous acid can either oxidize or nitrate guanine residues; as an example, the nitration of guanine to 8-nitroguanine can lead to abasic site formation.

7. Reactions with lipids

Similar to nucleic acids, unsaturated lipids can be oxidized (lipid perodixation) or nitrated (nitroalkene formation) by NO and/or NO derivatives (Freeman, Baker et al. 2008). Notably, nitroalkenes (NO_2-fatty acids) are present in detectable quantities and circulate in the plasma, but are also present in tissues and cell membranes. The nitrogen dioxide radical involved can be generated from NO auto-oxidation, but also from NO_2^- as well as $ONOO^-$ and ONOOH can all potentially generate nitroalkenes (Rubbo 2013); although the mechanism for nitroalkene formation *in vivo* is still unknown, several reports confirm their presence and increase in inflammatory models, and have been suggested to have anti-inflammatory properties. For example, nitroarachidonic acid (NO_2-AA) in activated macrophages decreases expression of inducible nitric oxide synthase (iNOS, which I will talk about in details in the next section) as well as expression of proinflammatory cytokines, thereby providing an anti-inflammatory response (Trostchansky, Souza et al. 2007). More generally, nitroalkenes have been shown to inhibit translocation of the transcription factor NF-κB to the nucleus, thus preventing expression of major pro-inflammatory cytokines (Cui, Schopfer et al. 2006, Ferreira, Ferrari et al. 2009). Additionally, nitroalkenes inhibit the NF-κB-mediated

VCAM-1 expression on endothelial cells, which is essential for monocytes rolling, tethering and extravasation into inflamed tissues.

On the other hand, peroxynitrite-mediated oxidation of low density lipoprotein (LDL) is considered to be a key step in the onset of atherogenesis, an artery wall disorder where the arteries' diameter narrow due to the accumulation of pro-inflammatory fatty plaques (Chisolm and Steinberg 2000).

B. Sources of NO in mammalians

1. Mammalian nitric oxide synthases

De novo synthesis of NO occurs via the nitric oxide synthase (NOS), a heme-containing dual flavin protein. The substrate of NOS is L-arginine, which NOS enzymes convert to L-citrulline, water and NO in a complex, two-step oxidoreductase reaction (Figure 17). To catalyze L-arginine conversion, NOS enzymes require the presence of molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), calmodulin, as well as prosthetics groups: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), present in the C-terminal reductase domain, as well as iron protoporphyrin IX (heme) and tetrahydrobiopterin (BH_4), present in the N-terminal oxygenase domain of NOS enzymes (Figure 17). The intermediate N^{ω}-hydroxy-L-arginine (NOHA) is transiently formed and serves as the substrate for NOS to produce NO. Importantly, NOS are only functional as homodimers; NOS monomers can bind neither the cofactor BH_4 nor L-arginine, and therefore cannot catalyze NO production (Schmidt, Werner et al. 1992, Klatt, Pfeiffer et al. 1996).

a) The various NOS isoforms

Three NOS isoforms have thus far been cloned and characterized, and distinguished in two main categories; the constitutive NOS (cNOS) and the inducible NOS (iNOS).

Two isoforms of cNOS are known:

- Neuronal NOS (nNOS, or NOS1) is a 160kD protein whose gene is localized on chromosome 12 in humans.
- Endothelial NOS (eNOS, or NOS3) is a 133kD protein whose gene is localized on chromosome 7 in humans.

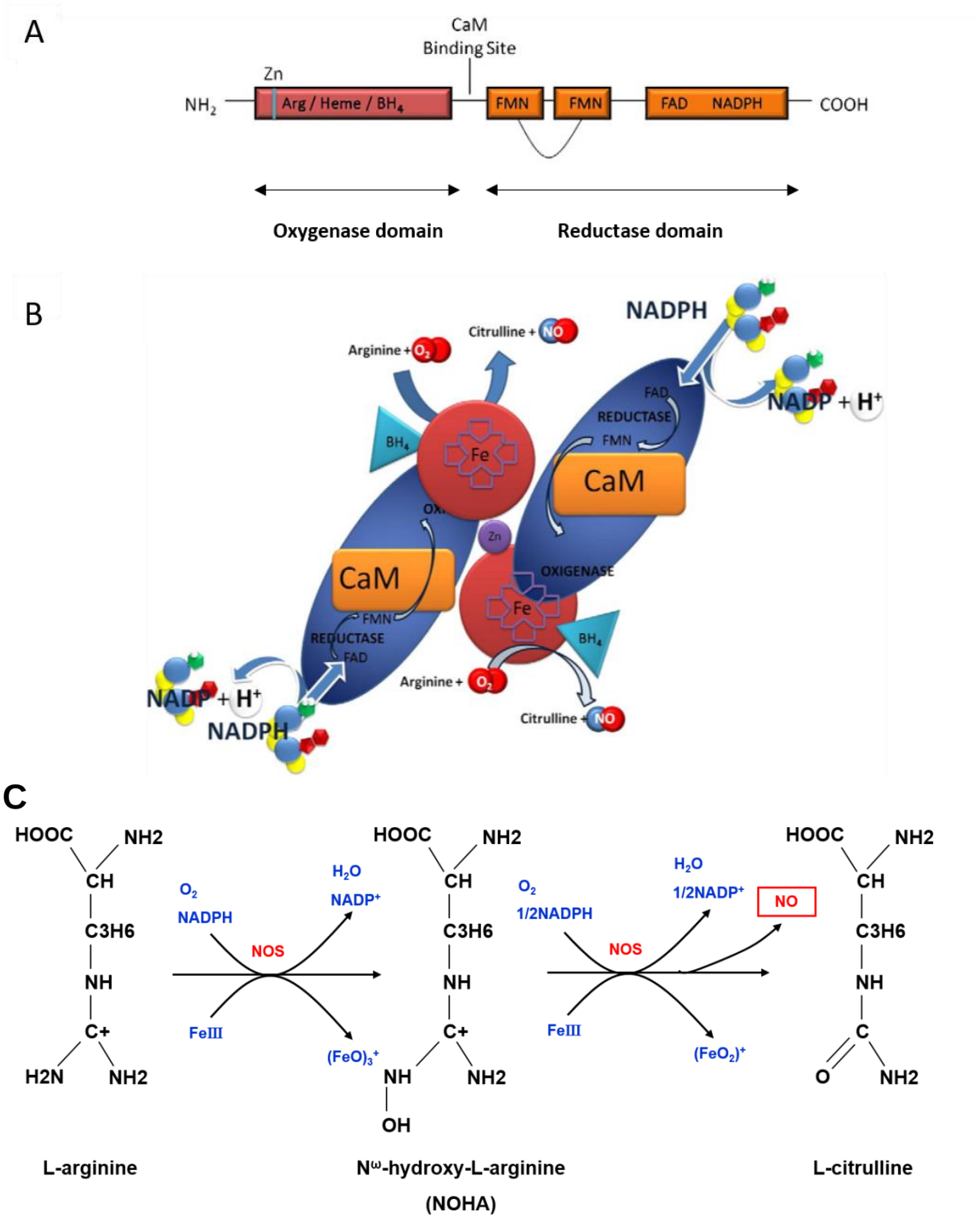

Figure 17. Nitric oxide synthase (NOS): structure and NO generation. *(**A**) Structure of a NOS monomer, with its oxygenase and reductase domains. (**B**) Structure of the functional NOS dimer. In the presence of heme (with Fe in its core), NOS monomers can join into a functional dimer, which can bind the BH_4 cofactor and L-arginine. The Zn ion connects the monomers via their heme groups. The cartoon displays the electron transfer from the reduced NADPH to FAD and FMN, to finally convert arginine and oxygen to the reaction producing citrulline and nitric oxide. (**C**) NO is generated via a two-step reaction. The substrate L-arginine is first converted to NOHA, which is then further converted to L-citrulline and NO. Arg, arginine binding site; Zn, zinc; BH4, tetrahydrobiopterin; NADPH, nicotinamide-adenine-dinucleotide; FAD, flavine-adenine-dinucleotide; FMN, flavin-mononucleotide; CaM, calmodulin; Fe, iron. Adapted from (Gielis, Lin et al. 2011).*

NOS1 and NOS3 were originally named nNOS and eNOS, respectively, based on their predominant tissue distribution. They were also categorized as cNOS as these enzymes are constitutively expressed, though they produce only small quantities of NO. However, several studies have showed that NOS1 and NOS3 are not only expressed in a much greater variety of cells, but also regulated by multiples factors, including cytokines and microbial products (Forstermann, Boissel et al. 1998, Dudzinski, Igarashi et al. 2006). Importantly, cNOS require calcium fluxes in order to bind calmodulin; thus, their activity is largely regulated post-translationally by the available calcium concentration.

In sharp contrast, iNOS (or NOS2) does not require calcium to be expressed, and is not expressed in resting cells. NOS2 is a 130 kD protein, whose gene is present on chromosome 17 in humans. iNOS is induced by immunological stimuli and can produce large amounts of NO for as long as the enzyme is not degraded or runs short on supply. iNOS mainly regulated at the transcriptional and post-transcriptional level, and has been shown to be expressed various cell types including, but not limited to, macrophages, dendritic cells, neutrophils, eosinophils, epithelial cells, endothelial cells, hepatocytes, or astroglia.

b) Regulation of iNOS expression

(1) Transcriptional regulation

The mammalian *Nos2* gene, whether from human, rat or mouse, has a promoter region loaded with binding sites for numerous transcription factors involved in *Nos2* regulation (Pautz, Art et al. 2010). Among these, NF-κB is a key player in iNOS upregulation; in fact, the iNOS gene promoter contains several binding sites for NF-κB, although the importance of each of these binding regions is still a matter of debate. Much work on iNOS has been performed using murine models or cell lines, and sharp differences have been observed in induction of human and mouse iNOS genes, which underlines differential regulation depending on several genetic and environmental parameters.

In the mouse, interferons (e.g IFN-γ) and microbial products (e.g LPS) that engage PRRs are classically used as potent and synergistic inducers of the iNOS transcription in macrophages, and lead to copious amounts of NO produced. An elegant study using mouse macrophages infected with *Listeria monocytogenes* described a sequential and cooperative activation of *Nos2* transcription (Farlik, Reutterer et al. 2010). PRR activation by pathogens or microbial products first leads to NF-κB expression and binding to the *Nos2* promoter region, which recruits the transcription factor TFIIH along with it the cyclin-dependent kinase 7 (CDK7).

PRR activation also leads to expression of IFN-α/β, which in turn causes dimerization of STAT1, expression of interferon-regulatory factor -1 (IRF-1), and formation of the interferon-stimulated gamma factor 3 (ISGF3) complex comprising STAT1, STAT2 and IRF9. ISGF3 binds the *Nos2* promoter region and recruits RNA polymerase II, which gets activated by phosphorylation by NF-κB-recruited CDK7.

Furthermore, cytokines belonging to the IL-1 family have been shown to induce iNOS expression in mouse macrophages, notably IL-1β (Lima-Junior, Costa et al. 2013) and IL-33 (Li, Li et al. 2014), the latter having been involved in antibacterial defense against *Staphylococcus aureus* skin infection. Additionally, negative regulation of iNOS expression by the erythropoietin (EPO) growth factor has been demonstrated, due to EPO-mediated blocking of NF-κB on the *Nos2* gene promoter; thus, EPO was shown to decrease control of *S*. Typhimurium infection *in vivo* (Nairz, Schroll et al. 2011).

On the other hand, induction of *Nos2* in human macrophages *in vitro* is strikingly different compared to their mouse counterparts. Indeed, cytokine and microbial product cocktails only yield little *Nos2* transcription compared to mouse macrophages. Analysis of mouse and human *Nos2* gene loci reveal significant differences, notably in the promoter regions, which could possibly account for the hyporesponsiveness of human macrophages. Notably, the two major regulatory regions for iNOS induction in mouse macrophages are located within the first 1.6 kb of the 5' flanking sequence, while the first 3.7 kb of the 8.3 kb promoter sequence of human iNOS does not show any induction post-stimulation (Chu, Marks-Konczalik et al. 1998). Yet, both promoter regions contain sites for NF-κB, which is known to respond to cytokines like IL-1 and TNF-α, as well as sites for STAT-1α, a component of the JAK-STAT signaling pathway in response to IFN-γ. A recent study that compared *Nos2* induction in human versus mouse macrophages, including primary blood monocytes and alveolar macrophages, found that the human *Nos2* gene was silenced (Gross, Kremens et al. 2014). Indeed, the authors found that human *Nos2* was highly methylated around the transcription, and showed resistance to demethylation; furthermore, human *Nos2*, unlike murine *Nos2*, shows a closed chromatin conformation, suggesting a silenced gene. Despite these findings, the functional iNOS activity in human macrophages is a fact (Weinberg 1998, Fang and Vazquez-Torres 2002). Indeed, many studies have reported the expression of *Nos2*, the production of iNOS and the generation of NO from macrophages in patients infected with *Plasmodium falciparum* or *Mycobacterium tuberculosis*, to cite a few (Nicholson, Bonecini-Almeida Mda et al. 1996, Boutlis, Tjitra et al. 2003, Mattila, Ojo et al. 2013). The exact regulation of human *Nos2* transcription has yet to be fully understood.

(2) Arginine pool availability

Needless to say, the available pool of L-arginine is determinant for NOS activity and NO production. Here, we need to consider both supply and degradation of L-arginine.

L-arginine can be generated via the activity of arginine/lysine- specific metallo-carboxypeptidases or aminopeptidases, which can cleave and release arginine residues from the C terminus or the N terminus of proteins, respectively; such activities have been shown to enhance NOS activity and facilitate activated macrophage function (Hadkar, Sangsree et al. 2004, Goto, Ogawa et al. 2011, Goto, Ogawa et al. 2015). Additionally, the by-product of NOS activity L-citrulline can regenerate L-arginine by the sequential activities of arginosuccinate synthase 1 and arginosuccinate lyase. This recycling process provides macrophages with a fail-safe system in the event of L-arginine starvation, and was shown to be important for pathogen control in a model of *Mycobacterium bovis* macrophage infection (Qualls, Subramanian et al. 2012).

On the other hand, there are three additional pathways, aside from iNOS, for L-arginine degradation. First, cytosolic arginase (Arg1) cleaves L-arginine to convert it to L-ornithine (a precursor for collagen) and urea, and therefore can deplete the arginine pool within cells. Arg1 can be found in activated macrophages, and when both Arg1 and iNOS are expressed, the yield of NO production is in fact null (Gobert, Daulouede et al. 2000). Second, two mitochondrial enzymes are known to use L-arginine: L-arginine decarboxylase (ADC) converts L-arginine to agmatine then further to polyamines, and L-arginine:glycine amidinotransferase (AGAT) leads to formation of creatine. Evidence of the effects of agmatine on iNOS activity has been reported (Regunathan and Piletz 2003, Molderings and Haenisch 2012).

(3) Other levels of regulation

Low oxygen condition (hypoxia) has important, yet contradictory effects on iNOS expression and activity. Indeed, because O_2 is a required component in NOS-mediated conversion from L-arginine to L-citrulline and NO, hypoxic conditions lead to the decrease in NO synthesis by NOS, and such effect was shown to affect the antibacterial activity of macrophages during infection (Mahnke, Meier et al. 2014). However, hypoxia induces stabilization of the transcription factor hypoxia-inducible factor-1α (HIF-1α), which is known to favor transcription of the *Nos2* gene (Robinson, Baumgardner et al. 2011).

There are other means of regulation of *Nos2* expression, such as the regulation of *Nos2* mRNA stability, regulation of iNOS transcription factors by non-coding mRNAs, and the

regulation of iNOS activity by denitrosylation (reviewed in (Pautz, Art et al. 2010, Bogdan 2015)).

2. Other sources of NO

It is noteworthy in the context of my research to consider that, in the gut, commensals and pathogenic bacteria can also generate NO. Indeed, these microorganisms are capable of reducing nitrate or nitrite (either endogenous or dietary) to NO under anaerobic conditions via nitrate respiration. Furthermore, bacterial NOS, which has high similarities with mammalian NOS, but contains no reductase domain (i.e has no FMN, FAD, or NADPH fixation domains), is also known to generate NO; bacterial NOS are mostly present in members of the Firmicutes phylum.

In addition, nitrite can be converted back to NO. In eukaryotes, this can occur without enzymatic action under acidic pH, or by the nitrite reductase activities of enzymes, such as xanthine oxidoreductase, deoxymyoglobin, or deoxyhemoglobin in hypoxic conditions (Lundberg, Weitzberg et al. 2008). Considering that NOS-mediated NO generation is oxygen-dependent, these pathways could be viewed as fail-safe mechanisms to ensure sufficient NO formation in low oxygen conditions.

C. Main physiological functions of NO

1. Main metabolic functions regulated by NO

a) NO and the cardiovascular system

In 1980, seminal work by Furchgott and Zawadski demonstrated the necessary presence of endothelial cells to ensure acetylcholine-mediated relaxation of isolated blood vessels (Furchgott and Zawadzki 1980). From their work, they hypothesized that a secreted factor from endothelial cells may be responsible for the response to acetylcholine and mediate blood vessel relaxation. Such substance, named EDRF for endothelium-derived relaxing factor, was found in 1987 to be in fact NO thanks to the work of Ignarro and colleagues. Endothelial cell-produced NO diffuses out and acts on smooth muscle cells by activating guanylate cyclase; thus, intracellular concentration of cyclic GMP (cGMP) increases within the smooth muscle cell, causing the muscle fibers to relax and induce vasodilation. These studies have granted Robert Furchgott, Louis Ignarro and Ferris Murad with the Nobel Prize in Medicine in 1998 for their pioneer discovery.

Since then, the understanding that NO had a critical role in the cardiovascular and respiratory system opened major avenues in therapies against pathologies of these systems. In many pathologies such as hypertension, neonatal respiratory distress syndrome and chronic obstructive pulmonary disease (COPD), loss of NO-mediated vasodilation is often at play.

Additionally, by mediating the relaxation of the corpus cavernosum smooth muscle, NO and cGMP are also responsible for penile erection. In fact, the proerectile effect of sildenafil (Viagra®) or tadalafil (Cialis®), which are specific phosphodiesterase 5 inhibitors, is due to the fact that they prevent degradation of cGMP by phosphodiesterase 5 in the corpus cavernosum, thereby prolonging the vasodilation effect of NO and cGMP (Turko, Ballard et al. 1999, Rosen and Kostis 2003). Interestingly, sildenafil increases erection duration in wild type and *Nos3*$^{-/-}$ mice, but not in *Nos1*$^{-/-}$, suggesting that NOS1 is necessary for sildenafil-mediated induction of erection (Cashen, MacIntyre et al. 2002). Of interest, since phosphodiesterase 5 is also expressed in pulmonary arteries, the molecules sildenafil and tadalafil are also provided for treatment of pulmonary arterial hypertension under the names of Revatio® and Adcirca®, respectively.

Furthermore, NO and cGMP are also strong inhibitors of platelet aggregation and adhesion to the vascular wall, providing protection from thrombosis (Alheid, Frolich et al. 1987).

b) NO and the central nervous system

In the central nervous system, NO is generated in response to activation of the N-methyl-D-aspartate (NMDA) class of glutamate receptors (Garthwaite, Charles et al. 1988), which then become highly permeable to calcium. Thus, influx of calcium within the cell allows for the calcium/calmodulin-dependent activation of NOS1. A negative feedback loop exists where NOS1-generated NO can block NMDA receptors (Manzoni, Prezeau et al. 1992).

NO functions as a diffusible messenger, and is thought to be involved in modulation of several physiological functions such as neurogenesis, memory, and learning. In particular, NO mediates long-term potentiation; indeed, several studies showed that the use of NO inhibitors impairs learning and memory formation (Holscher and Rose 1992, Bohme, Bon et al. 1993, Majlessi, Choopani et al. 2008).

Overproduction of NO, due to overactivation of NMDA receptors and massive influx of calcium for example, has been involved in cerebrovascular stroke (Lipton, Choi et al. 1993). Additionally, abnormal NO signaling has been linked to neurodegenerative diseases such as multiple sclerosis, Alzheimer's and Parkinson's diseases (Steinert, Chernova et al. 2010).

c) Effect of NO in the gastrointestinal tract

In the gastrointestinal tract, NO acts on the mesenteric plexus as a neurotransmitter to regulate the intestinal smooth muscle fiber relaxation, necessary for peristaltic movement. An *in vitro* study on rabbit colon showed that NO-mediated production of cGMP led to opening of calcium-dependent potassium channels and relaxation of smooth muscle fibers (Benabdallah, Messaoudi et al. 2008).

In addition, NO in the gastrointestinal tract regulates mucus secretion and the maintenance of blood flow. In rat gastric mucosal cells, incubation with NO donors stimulated mucus secretion in a dose-dependent fashion via production of cGMP (Brown, Keates et al. 1993); this finding was further confirmed in *in vivo* experiments (Brown, Hanson et al. 1992). The long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), notably to treat chronic pain, is associated with disruption of gastroprotective mechanism such as mucus secretion and blood flow (Laine, Takeuchi et al. 2008). In the early 2000, research has begun on a new class of agents named cyclo-oxygenase (COX)-inhibiting NO-donating drugs (CINODs) (Lanas 2008). In short, CINODs are NSAIDs to which NO is coupled; thus, this new class of NSAIDs could improve the safety of NSAIDs by preventing gastrointestinal damage. Naproxcinod, a CINODs from the pharmaceutical company NicOx, showed promising results in clinical trials as it was both anti-inflammatory and gastric-safe (of note, applications for Naproxcinod use approval by the FDA and AMM have since then been removed). More recently, NOSH-NSAIDs (NSAIDs coupled with NO and hydrogen sulfide) have been investigated and results show enhanced gastrointestinal safety profiles while remaining potent anti-inflammatories (Kodela, Chattopadhyay et al. 2015).

However, high concentrations of NO have also been involved in several gastroenterological pathologies, including gastric ulcers and gastro-intestinal cancers (Sawa and Ohshima 2006).

2. Is NO our friend or foe?

With the so many cytoprotective, anti-microbial, and anti-tumor effects NO can have, we should not forget that abnormal levels of NO can be just as detrimental to the body, by inducing a pro-inflammatory state, apoptosis, cancers, neurodegenerative diseases, and septic shock, to cite a few. Below are a few examples of these observed paradoxal effects of NO:

NO is a potent anti-proliferative agent against tumor cells (Wei, Richardson et al. 2003); yet NO is also involved in cancer development such as melanoma (Yarlagadda, Hassani et al. 2017), glioblastoma multiform (Altinoz and Elmaci 2017), and breast tumors (Ellies, Fishman et al. 2003). Partly explaining these consequences, NO induces DNA damage via formation of nitrosamines and promotes tumor growth via angiogenesis.

NO is also a major player in apoptosis; pro-apoptotic effects of NO were first seen on macrophages (Albina, Cui et al. 1993), but have since then been documented on many other cell types including endothelial and neuronal cells. NO-mediated apoptosis can be due to downregulation the anti-apoptotic protein Bcl-2 (Brockhaus and Brune 1998), or induction of expression of the tumor suppressor gene p53 (Messmer, Ankarcrona et al. 1994). On the other hand, NO was also shown to have anti-apoptotic effects, for example via S-nitrosylation-mediated inhibition of apoptosis proteins such as caspase 1 (Dimmeler, Haendeler et al. 1997), caspase 8 (Li, Billiar et al. 1997) and caspase 3 (Melino, Bernassola et al. 1997).

Similarly, NO is well-known to be involved in pathogen control and clearance with viruses (Karupiah, Xie et al. 1993), bacteria (Gobert, McGee et al. 2001) and parasites (Gobert, Daulouede et al. 2000). However, excess of NO production is linked with septic shock, a severe condition, usually initiated by bacterial endotoxins, and characterized by large arteriolar vasodilation thus hypotension, hepatotoxicity, microvascular damage, and cardiac dysfunction (MacMicking, Nathan et al. 1995). This is greatly exemplified by the fact that *Nos2*$^{-/-}$ mice become susceptible to *Leishmania* infections, yet resist to LPS-induced septic shock (Wei, Charles et al. 1995). Additionally, high levels of NO in mice infected with *Chlamydia psittaci* are beneficial for the host when the infectious dose is high, but become deleterious by suppressing adaptive immunity in the case of a moderate infection (Huang, DeGraves et al. 2002).

D. The response of *E. coli* to NO

To counter the antiproliferative and cytototoxic potential of NO, bacteria possess mechanisms of sensing of, and resistance to NO; this can be particularly important for enteric invaders such as pathogenic *E. coli* (Brunelli, Crow et al. 1995) and *C. rodentium* (Vallance, Deng et al. 2002). Although these mechanisms indeed help bacteria to counter the effect of exogenous NO released by the mammalian host as a defense mechanism, they most likely evolved as a necessary feature to protect the microorganisms against NO generated as a product of their own metabolism, especially during nitrate and nitrite reduction.

1. NO sensors in *E. coli*

a) Nitric oxide reductase regulator (NorR)

NorR is a σ^{54}–dependent NO sensor belonging to the enhancer-binding protein family, which is activated under both anaerobic and aerobic conditions and in the presence of NO (Hutchings, Mandhana et al. 2002, Mukhopadhyay, Zheng et al. 2004, D'Autreaux, Tucker et al. 2005). Structurally, NorR contains three domains (Bush, Ghosh et al. 2011): (i) a C terminal, helix-turn-helix DNA-binding domain, (ii) a central ATPase-active domain, and (iii) a N terminal regulatory GAF domain containing a non-heme iron center (Figure 18). The GAF module is known to be the NO signal sensing module as deletion of GAF in NorR results *in vivo* in constitutive transcriptional activation by NorR, independently of the presence of NO (D'Autreaux, Tucker et al. 2005). Within the GAF domain, the Fe^{2+} iron center is responsible for binding NO; a mutation in the GAF domain, generating a mutant NorR containing no iron, resulted in loss of function of NorR (D'Autreaux, Tucker et al. 2005).

In the absence of NO, NorR is bound to DNA, but the activity of the central ATPase domain is repressed by direct interaction between the GAF domain and the σ^{54}-interacting region of the central domain, which prevents access to the σ^{54}-RNA polymerase complex (Bush, Ghosh et al. 2010). Reversible binding of NO to iron within the GAF domain results in a conformational change and relieves this repression by freeing the ATPase-active domain and allowing interaction with σ^{54}-RNA polymerase (D'Autreaux, Tucker et al. 2005, D'Autreaux, Tucker et al. 2008) (Figure 18). NorR assembles on the DNA as a pre-activated hexamer, each monomer containing the three domains mentioned below, and the active NorR oligomer is further stabilized by encircling DNA (Bush, Ghosh et al. 2015).

NorR belongs to the *norRVW* gene cluster, and active NorR induces transcription of the *norVW* operon (Gardner, Helmick et al. 2002). The genes *norV* and *norW* encode a flavorubredoxin and its associated reductase, respectively, which together reduce NO to nitrous oxide (see section below).

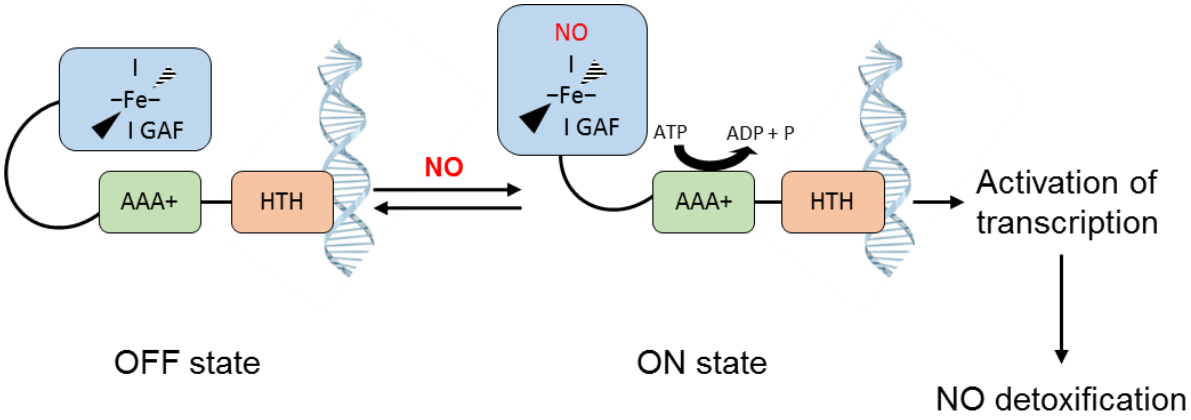

Figure 18. NorR structure and regulation of transcription. *A NorR monomer is shown here with its three domains, the C-terminal helix-turn-helix (HTH) DNA binding-domain (orange), the central ATPase active domain (green) and the N-terminal GAF domain containing a non-heme iron. In the absence of NO, the NorR is bound to DNA and the GAF domain prevents ATPase activity and transcription start. Reversible binding of NO with iron leads to a conformation change and relieves repression of ATPase activity, thus allowing recruitment of σ^{54} RNA polymerase and transcription of genes involved in NO detoxification. Adapted from (D'Autreaux, Tucker et al. 2005).*

b) Nitric oxide sensitive repressor (NsrR)

NsrR was first identified in *Nitrosomonas europaea* as a nitrite-sensitive repressor; indeed, the presence of NO_2^-, just like the deletion of *nsrR*, would result in the expression of the *nirK* gene, coding for a nitrate reductase (Beaumont, Lens et al. 2004). NsrR is now known to be present in a wide variety of bacterial species with diverse ecological niches. Indeed, a detailed computational analysis study, looking into the regulatory network controlling N-oxide metabolism in bacteria, predicted a conserved binding site for NsrR or NsrR homologues in many bacterial species (Rodionov, Dubchak et al. 2005). Using this predicted binding site as a tool to search for NsrR potential targets, a large number of genes were found to be potentially regulated by NsrR. In that same study, *yjeB* was identified in *E. coli* as an orthologue of NsrR. Another work published around the same time (Bodenmiller, D. M. and Spiro, S 2006 J Bac) found *yjeB* to be a transcriptional repressor of genes stimulated by sources of NO (Bodenmiller and Spiro 2006). They also identified a potential binding site for protein YjeB strikingly similar to NsrR binding site, echoing the same findings than Rodionov and colleagues. The *yjeB* gene in *E. coli* is now referred to as *nsrR*.

NsrR is an iron-sulfur cluster-containing homodimeric protein. Several publications were recently released on the structural characterization of NsrR from *Streptomyces coelicolor*, a model organism for the genus *Streptomyces*. NsrR from *S. coelicolor* contains a [4Fe-4S] cluster ligated by three cysteine residues from one monomer and a specific aspartic acid from the other monomer (Crack, Munnoch et al. 2015, Volbeda, Dodd et al. 2017). However, it is important to note that the nature of iron-sulfur clusters can vary depending on the

bacterial strain studied; for example, NsrR from *Neisseiria gonorrhoeae* seems to contain a [2Fe-2S] iron-sulfur cluster (Isabella, Lapek et al. 2009), while *Bacillus subtilis* NsrR contains a [4Fe-4S] cluster (Yukl, Elbaz et al. 2008). To my knowledge, the exact nature of the cluster in *E. coli* NsrR is still unknown.

The NsrR binding site is also subject of interspecies variation. Regardless of the bacterial strain, NsrR binding site is predicted to comprise two 11 base pair (bp) motifs organized in an inverted repeat and spaced by any one bp, with the 11 bp motif itself being a palindrome (Figure 19) (Rodionov, Dubchak et al. 2005, Bodenmiller and Spiro 2006, Partridge, Bodenmiller et al. 2009). However, the DNA sequence of the NsrR binding sites varies between distant related species, as NsrR from *Streptomyces coelicolor* bound only weakly to NsrR binding sites belonging to *E. coli* or *B. subtilis* (Crack, Munnoch et al. 2015). NsrR binds on DNA as a dimer, presumably with one monomer per "half site", that is, one 11bp motif (Tucker, Hicks et al. 2008). Interestingly, a study identified several promoters regulated by NsrR in *E. coli* that only contained half sites (Partridge, Bodenmiller et al. 2009). The regulation mechanism of these promoters by NsrR is still not clear; it was suggested that NsrR would in fact bind to one half site as a dimer, and on both half sites as a dimer of a dimer, but this hypothesis has to date not been verified.

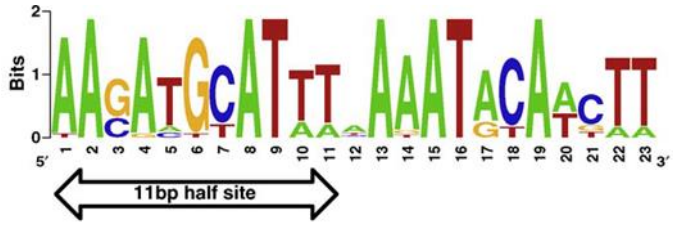

Figure 19. NsrR consensus binding site. *This consensus binding site was obtained based on predicted and experimented validated NsrR binding sites, and generated by the Weblogo program. From (Tucker, Le Brun et al. 2010).*

Sensing of NO by NsrR is strictly dependent on its interaction with NsrR-bound iron-sulfur clusters. In the absence of NO, NsrR is bound to DNA, thus preventing access of the RNA polymerase and transcription start. In the presence of NO, the formation of dinitrosyl iron complexes within the iron-sulfur cluster leads to the loss of NsrR DNA binding activity, thus allowing transcription of genes regulated by NsrR, such as *hmpA* (Figure 20A). Noteworthy, we now know that NsrR can also act as a transcriptional activator, as will be reminded below.

In *E. coli*, NsrR assumes the role of a global regulator, as it regulates a complex network of more than 60 genes; interestingly, only some are directly linked to NO detoxification

(Partridge, Bodenmiller et al. 2009). Genes involved in surface attachment, protein degradation, motility, transmembrane transport, metabolism and stress response are regulated by NsrR (Partridge, Bodenmiller et al. 2009, Tucker, Le Brun et al. 2010).

As an example, in the uropathogenic *E. coli* strain CFT073, NsrR was found to be a positive regulator of CFT073 surface attachment, as addition of NO or deletion of the *nsrR* gene reduced attachment of CFT073 to glass tubes (Partridge, Bodenmiller et al. 2009); whether this effect has physiological relevance is not known. Contrary to this finding, a study looking at the effect of nitrosative stress on the UPEC strain UTI89 found that, although bacteria colonized the mouse bladder more efficiently, pre-exposure to nitrosative stress has no significant impact on the expression of type 1 pilus, the key virulence factor involved in adherence, during infection (Bower, Gordon-Raagas et al. 2009).

Additional regulatory effects of NsrR were discovered in *E. coli*, as already discussed in my Pathogenic *E. coli* chapter. Indeed, two successive studies looking at the effect of NO on EHEC O157:H7 established that NO was a potent inhibitor of *stx2* gene expression (Vareille, de Sablet et al. 2007) and adherence via the inhibition of LEE expression (Branchu, Matrat et al. 2014). Both inhibitions were due to NO-mediated NsrR detachment from DNA; hence, NsrR acts here as a positive transcriptional regulator, which had not been reported before. Interestingly, NO-mediated inhibition of *stx2* transcriptional expression was due to an inhibition, most likely indirect, of *recA* transcriptional expression, thus suggesting an NO-mediated inhibition of the SOS response transcription by NsrR (Figure 20B).

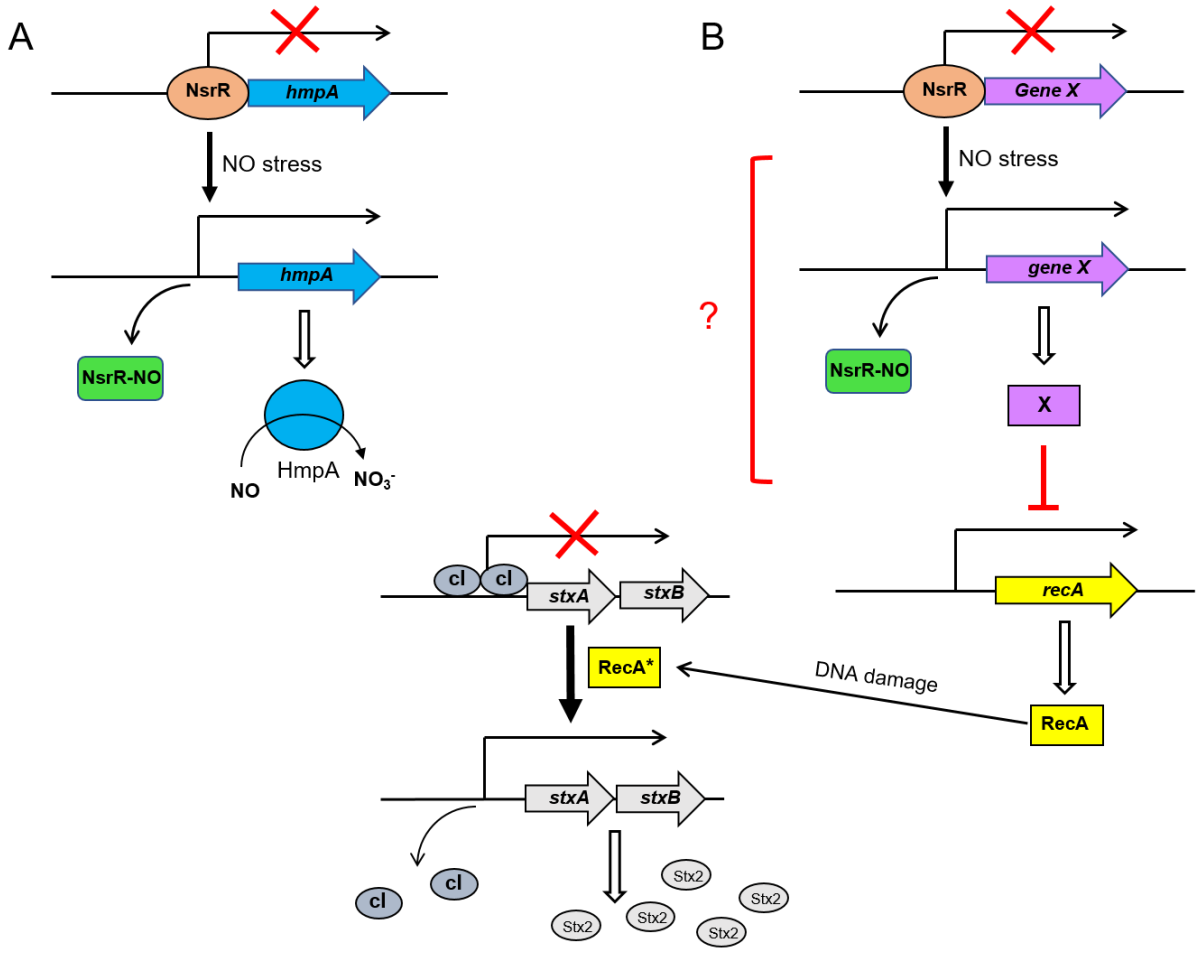

Figure 20. Examples of transcriptional regulation by NsrR. *NsrR is known to function as a transcriptional repressor. NsrR binds to dedicated binding sites upstream of the promoters of genes regulated by NsrR, thereby preventing transcription initiation. In the presence of NO, the formation of nitrosyl iron complexes results in loss of DNA binding from NsrR, which results in upregulation of genes. (**A**) NsrR negatively regulates Hmp; Hmp gets expressed in the presence of NO, and participates to NO detoxification by oxidating NO to NO_3^-. (**B**) NsrR under aerobic and NO stress conditions inhibits EHEC stx2 synthesis, which is most likely due to inhibition of recA transcription, thus downregulation of the SOS regulon, under which the stx2 genes are located. Regulation of recA transcription is most likely indirect and the current (unverified) model is that NsrR most likely works as a repressor of a yet undefined recA transcriptional inhibitor, rather than being a recA transcriptional activator. Adapted from (Tucker, Le Brun et al. 2010).*

c) Other *E. coli* NO sensors

Although NorR and NsrR are the only two regulators truly dedicated to sensing NO, other regulators exist in *E. coli* that are involved in the bacterial response to nitrosative stress, although each of these regulators are specialized in sensing a signal other than NO.

(1) Superoxide response regulator (SoxR)

SoxR is a dimeric transcription factor, which contains a [2Fe-2S] cluster in each of its monomers (Hidalgo, Bollinger et al. 1995). SoxR activation is induced by a reversible one-electron oxidation of its [2Fe-2S] clusters; in this state, SoxR can activate the transcription of its adjacent gene *soxS* (Gaudu and Weiss 1996, Gaudu, Moon et al. 1997). SoxS is itself a transcription factor that can induce expression of more than 100 genes that are part of the SoxRS regulon (Pomposiello, Bennik et al. 2001). When the stressor is removed, the oxidized [2Fe-2S] of SoxR, which remains intact, gets quickly reduced again (Koo, Lee et al. 2003); SoxS gets degraded by proteolysis (Griffith, Shah et al. 2004). While superoxide has traditionally been thought to be the inducer of SoxR (hence its name), Gu and Imlay recently demonstrated that SoxR was directly activated, not by superoxide, but by redox-cycling drugs. Indeed, SoxR could be activated *in vivo* under anoxic conditions and without any presence of superoxide; instead, the [2Fe-2S] clusters of SoxR were readily oxidized *in vitro* by redox-cycling drugs (Gu and Imlay 2011).

SoxR was the first bacterial transcriptional regulator reported to respond to NO in *E. coli*. SoxR was shown to be activated after exposure to NO in the absence of oxygen (Nunoshiba, deRojas-Walker et al. 1993), as well as by iNOS-generated NO inside macrophages (Nunoshiba, DeRojas-Walker et al. 1995). Mechanistically, NO induces *in vitro* and *in vivo* direct nitrosylation of the [Fe-S] clusters within SoxR and activates the transcription factor; the nitrosylated iron-sulfur cluster either gets disassembled or repaired *in vivo* when NO is absent (Ding and Demple 2000). However, following studies made contradictory observations as of NO-mediated activation of SoxR; importantly, in all cases, no expression of SoxS-regulated genes could be observed (Mukhopadhyay, Zheng et al. 2004, Flatley, Barrett et al. 2005, Justino, Vicente et al. 2005). This indicates that, even if NO can activate SoxR, the resulting activation of SoxS may not be to a level sufficient to induce downstream gene expression, as was previously observed in earlier studies (Nunoshiba, deRojas-Walker et al. 1993, Nunoshiba, DeRojas-Walker et al. 1995).

(2) Fumarate nitrate reduction (FNR)

The FNR regulon is an oxygen-sensing, DNA-binding transcription factor that coordinates the switch between aerobic and anaerobic respiration, thus allowing facultative anaerobes to adapt to oxygen deprivation (Spiro 1994, Green and Paget 2004). In *E. coli*, FNR is constituted of two subunits, and has two distinct domains in each of its subunits. The N terminal domain contains a [4Fe-4S] or a [2Fe-2S] cluster via four essential cysteine residues, while the C terminal DNA-binding domain will target specific DNA-binding

sequences of FNR-regulated gene promoters. In the absence of oxygen, FNR binds to one [4Fe-4S] cluster per subunit, which allows its dimerization and enhanced site-specific DNA-binding to target promoters, followed by transcription of genes involved in the regulation of anaerobic respiration (Green, Crack et al. 2009). On the contrary, reaction of FNR with oxygen results in the conversion of its [4Fe-4S] cluster to a [2Fe-2S] cluster; the ensuing conformational change leads to monomerization and loss of high affinity DNA binding. While FNR is primarily a sensor of oxygen, the [4Fe-4S] cluster of FNR can also rapidly react with NO; in fact, the rate constant between FNR and NO is significantly greater than the one between FNR and O_2 (Crack, Stapleton et al. 2013). However, *in vivo* studies suggest that the FNR-mediated transcriptional regulation response is much higher with O_2 than with NO; it is thus possible that FNR serves a backup role, should the NorR and NsrR-mediated responses be insufficient to regulate the amount of NO present. Indeed, in the presence of NO, FNR downregulates the expression of several genes involved in nitrogen metabolism, including the nitrate reductase NarG, which represents the greatest source of endogenous NO production (Vine, Purewal et al. 2011). Furthermore, it is now well established that FNR regulates negatively *hmp*, encoding the NO reductase Hmp (flavohemoglobin, see section below on NO detoxification systems), and has a dedicated binding site upstream of its promoter (Cruz-Ramos, Crack et al. 2002, Constantinidou, Hobman et al. 2006, Myers, Yan et al. 2013). Thus, nitrosylation of FNR could effectively reduce endogenous NO formation, while upregulating an important NO-detoxifying enzyme.

(3) OxyR

OxyR belongs to the LysR type of DNA-binding transcription regulators (Schell 1993), which are characterized by positive regulation of their target genes, and negative regulation of their own expression. OxyR, just like the rest of the LysR family, binds to DNA either as a dimer or as a tetramer (Choi, Kim et al. 2001). OxyR regulates the transcription of genes in defense to increasing hydrogen peroxide within the bacterial cell. However, OxyR has also been shown to be activated by exposure to S-nitrosocysteine (Hausladen, Privalle et al. 1996, Choi, Kim et al. 2001, Kim, Merchant et al. 2002). Interestingly, the S-nitrosylation of a single cysteine within OxyR was sufficient to generate a stable form of OxyR-SNO (nitrosothiol), which had a different structure, and importantly a different DNA-binding affinity, than OxyR-SOH generated by H_2O_2 (Kim, Merchant et al. 2002). More recently, a study elegantly demonstrated that, in fact, S-nitrosylation of OxyR in *E. coli* cells growing anaerobically on nitrate activated OxyR to transcribe a different set of genes than when OxyR is activated by S-oxidation (Seth, Hausladen et al. 2012). In particular, nitrosylated OxyR selectively upregulated *hcp*, encoding a hybrid cluster protein, which was shown to be protective against

nitrosative cells; indeed, *Δhcp E. coli* cells had impaired growth within macrophages, an effect that was reversed when NO production in macrophages was blocked (Seth, Hausladen et al. 2012).

(4) Ferric-uptake regulator (Fur)

In *E. coli*, and other Gram-negative bacteria, the intracellular iron homeostasis is tightly regulated by the Fur protein, which is a homodimer that directly controls 81 genes depending on its state of activation (Seo, Kim et al. 2014). The classical view of Fur is as a positive repressor, whereby the binding of Fe^{2+} to Fur results in DNA-binding of Fur-Fe^{2+} and repression of transcription; in low iron conditions, Fur without Fe^{2+} does not bind to its DNA-binding sites and allows transcription of genes essential for iron scavenging and acquisition (Andrews, Robinson et al. 2003). However, Fur-Fe^{2+} can also be an activator (Delany, Rappuoli et al. 2004, Nandal, Huggins et al. 2010), and Fur without Fe^{2+} has also been shown to function as activator and repressor, notably in pathogenic bacteria (Butcher, Sarvan et al. 2012, Carpenter, Gilbreath et al. 2013). *In vitro*, Fur can bind two NO molecules via its iron center (D'Autreaux, Horner et al. 2004); this ferrous-dinitrosyl form of Fur cannot bind DNA and should theoretically cause derepression of Fur-regulated promoters, and this was shown for at least one promoter *in vivo* (D'Autreaux, Touati et al. 2002). Further studies using microarrays to look at the impact of NO or nitrosative agents in *E. coli* have confirmed the upregulation of Fur-regulated genes (Mukhopadhyay, Zheng et al. 2004, Justino, Vicente et al. 2005). Of note, one study, which findings were contrary to the former works, suggests instead that the physiological impact of NO on Fur and its regulated genes may be very sensitive to the concentration of iron in the environment; in other words, Fur-Fe^{2+} may only be sensitive to nitrosative stress in conditions where iron levels are getting low (Flatley, Barrett et al. 2005). A possible explanation provided by the authors of the latter study is the iron content of the medium used in the various studies, hence the form in which Fur would be found in cells: the LB broth used in the previous studies are likely to be poor in iron; instead, in the defined iron-rich medium used by Flatley *et al*, Fur-Fe^{2+} may be insensitive to the presence of NO.

2. NO detoxification systems in *E. coli*

In *E. coli*, four enzymatic pathways have thus far been characterized for NO detoxification: flavohemoglobin, flavorubredoxin, hybrid cluster protein, and NO_3^-/NO_2^- reductases (Figure 21). These enzymes use NO or NO derivatives as a substrate, thus allowing *E. coli* to resist NO cytotoxic effects, which is especially important during infection.

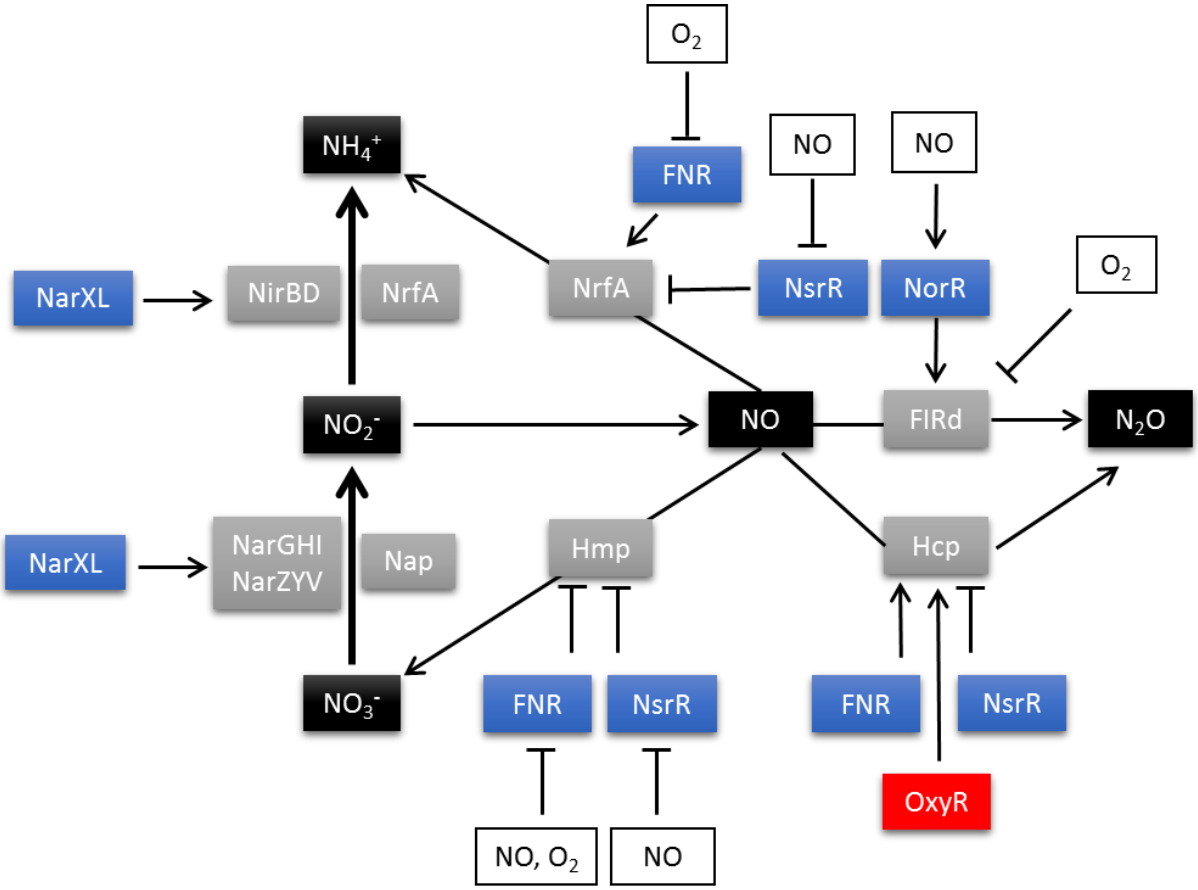

Figure 21. The main NO detoxification systems in E. coli. *NO can be transformed to various of its derivatives as a mean of detoxification, by highly regulated enzymes that are discussed in detail in the text. Nitrite can also be converted back to NO by nitrite reductase (not shown in this figure). Transcription factors are shown in blue except for OxyR, which selectively upregulates Hcp under anaerobic conditions. Positive regulation is shown with arrows, negative regulation is shown with perpendicular lines. NO and its derivatives are shown in black. NO detoxification enzymes are shown in gray. Adapted from (Spiro 2007).*

a) Nitrate and nitrite reductases

Nitrate and nitrite reduction in *E. coli* occur via two different systems and in two different cellular locations. We can distinguish the cytochrome c NO_2^- reductase Nrf that is periplasmic from the NO_2^- reductase Nir that is located in the cytoplasm. Similarly, the NO_3^- reductases Nap and Nar are located in the periplasm and in the cytoplasm, respectively. These enzymes catalyze the reduction of NO_3^- to NO_2^-, then from NO_2^- to NO or ammonium (NH_4^+). Importantly, on top of converting NO_2^- to NH_4^+, NrfA can directly reduce NO to NH_4^+(Poock, Leach et al. 2002); thus, thanks to its location, NrfA may constitute the first line of defense against NO for *E. coli* (van Wonderen, Burlat et al. 2008). Interestingly, nitrate reduction by

NarG generates the most important source of NO in *E. coli*, although alternative pathways also generate NO from nitrite (Vine, Purewal et al. 2011).

The four operons encoding the aforementioned nitrate and nitrite reductases are regulated in enteric bacteria by FNR, and are thus expressed under anaerobic conditions. Additionally, the *nrf* and *nap* operons are negatively regulated by NsrR (Filenko, Spiro et al. 2007).

b) Hmp flavohemoglobin

The Hmp flavohemoglobin was the first NO detoxifying enzyme discovered in *E. coli* (Hausladen, Gow et al. 1998). This inducible NO dioxygenase is expressed by the gene *hmpA* and is induced by NO, but also NO_2^- and NO_3^-. Hmp has an established role in resistance to NO under aerobic conditions, as it quickly catalyzes the oxidation of NO to nitrate (Gardner and Gardner 2002). As such, the importance of Hmp in protection against NO-mediated stress in macrophage model has been demonstrated (Gilberthorpe, Lee et al. 2007). Hmp is negatively regulated by NsrR, but also by FNR. The latter implies that Hmp can be upregulated under microaerobic and anaerobic growth and during exposure to nitrosative stress (Cruz-Ramos, Crack et al. 2002), as oxygen destroys the iron-sulfur cluster of FNR (Khoroshilova, Popescu et al. 1997). Under anoxic conditions, Hmp reduces NO to nitrous oxide, although the reaction rate is low.

c) NorV flavorubredoxin

In *E. coli*, the *norVW* operon encodes a flavorubredoxin or FIRd (*norV*) and its associated NADH:flavorubredoxin reductase (*norW*). The NorV/NorW system represents a nitric oxide reductase, which couples NADH oxidation to NO reduction. The NADH oxidation by NorW leads to an electron transfer to the rubredoxin domain of NorV (Gomes, Giuffre et al. 2002). Electrons reach the catalytic di-iron site of NorV, thereby allowing the reduction of NO to nitrous oxide.

Interestingly, one of two different forms of NorV can be found in EHEC O157:H7 isolates, as we can distinguish the intact *norV* gene from a 204 bp deleted *norV* gene (Gardner, Helmick et al. 2002); the presence of an intact NorV has been suggested to correlate with a greater survival within macrophages, due to a greater ability to reduce the NO concentration in the bacterial cell microenvironment (Shimizu, Tsutsuki et al. 2012).

The NO reductase activity of NorV is oxygen-sensitive, decaying with a half-life of 5 min (Gardner and Gardner 2002); however, it was shown to retain activity, just like Hmp, under

microaerobic conditions; at an oxygen concentration equal or under 5 µM, the deletion of either *hmp* or *norV* did not cause any growth defect in the presence of NO, while deletion of both genes led to a rapid growth arrest (Gardner, Helmick et al. 2002). These results would thus suggest a complete coverage of *E. coli* in NO defense in all oxygen concentration range, with Hmp being mostly efficient under aerobic conditions, and NorV under anaerobic conditions, and both enzymes keeping some efficiency under microaerobic conditions. A very interesting recent study showed, however, that *E. coli* may face trouble in handling NO detoxification under microaerobic conditions (Robinson and Brynildsen 2016). By using a computational model approach paralleled with experimental measurements, Robinson and Brynildsen demonstrated a clear impairment in NO detoxification by *E. coli* at low oxygen concentrations, where the combined activity of Hmp and NorV faced a 60% loss at a 2.8 µM oxygen (versus their activity at 50 µM or 0 µM oxygen). Under anaerobic conditions, NorV accounted for more than 75% of NO detoxification while Hmp was inactive; NorV activity fell sharply after 1 µM O_2, with Hmp taking over the NO detoxification activity, and accounting for more than 50% detoxification activity when O_2 concentrations reaching 17 µM. These results suggest a microaerobic window, between 1 µM and 17 µM oxygen, where *E. coli* experience suboptimal NO detoxification activity via Hmp and NorV, and may thus be more vulnerable to NO toxicity. In the gut, the very low oxygen concentrations combined with the presence of NO could thus be more impactful against invading pathogens.

d) Hybrid cluster protein-reductase (Hcp- Hcr)

The two genes *hcp-hcr* encode a hybrid cluster protein Hcp, and its associated NADH-dependent reductase Hcr. Hybrid cluster proteins contain two different iron-sulfur clusters, a hybrid [4Fe-4S-2O] cluster and a [2Fe-2S] or [4Fe-4S] cluster. In *E. coli*, and under anaerobic conditions, Hcp was shown to be under the negative regulation of NsrR, and the positive regulation of FNR (Filenko, Spiro et al. 2007). In that latter study, the function of Hcp and its potential role in *E. coli* defense against nitrosative stress was unknown. More recently, a study provided evidence that Hcp is in fact a high affinity NO reductase, i.e that is activated by concentrations of NO much lower than NorV under anaerobic growth conditions; hence, Hcp and NorV would work complimentarily by ensuring efficient NO detoxification under various levels of nitrosative stress (Wang, Vine et al. 2016). Additionally, as previously mentioned, Hcp in *E. coli* is also upregulated by OxyR under anaerobic growth, and its importance in protection against nitrosative stress generated by macrophages was demonstrated (Seth, Hausladen et al. 2012).

Results

Mixed mucosal-parenteral immunizations with broadly conserved pathogenic *Escherichia coli* antigen SslE induce a robust mucosal and systemic immunity without affecting the murine intestinal microbiota

Background and summary

Reverse vaccinology, originating at Novartis Vaccines, now GSK, has marked a turning point in vaccine development. Thus far, vaccine research had essentially relied on the identification of the few highly immunogenic antigens based on serum analysis of infected patients. In contrast, reverse vaccinology makes use of the entire genome of a specific pathogen to identify vaccine candidates, including those that would be less immunogenic, yet still confer a high level of protection. Using this technology, surface-exposed and secreted antigens can be selected and evaluated for their protective efficacy in a time-efficient manner. Today, with the advance of high-throughput sequencing technologies, reverse vaccinology is employed to perform comparative genomic studies with multiple isolates of the same bacterial species – an extremely valuable setup to cover the antigenic diversity present in many, if not all, bacterial pathogens. Pathogenic *E. coli* encompass an enormous diversity, which is exemplified by the extent of their organ tropism, virulence factors, and means of colonization. The severity of the diseases from pathogenic *E. coli*, along with the rise of antibiotic resistance, warrant the search for new means of prevention, and reverse vaccinology represents a promising tool to rapidly identify novel candidates suitable for potential development into vaccines that target several, maybe all, pathogenic *E. coli*.

Previous work at GSK led to the identification of nine vaccine candidates from a reverse vaccinology approach aimed toward extraintestinal pathogenic *E. coli*. All nine antigens showed protection against a murine sepsis model with an ExPEC strain. With 82% protective efficacy, SslE (secreted and surface-associated lipoprotein of *E. coli*) was the most promising candidate. Additional models showed SslE to also be cross-protective against other ExPEC strains. Functional assays have demonstrated *in vitro* and *ex vivo* that SslE is a mucinase which plays an important role in colonization and virulence of *E. coli*.

Within the DISCo program, my work at GSK was to characterize the immune response to the antigen SslE. In particular, we wanted to determine whether we could obtain both a systemic immune response and an intestinal immune response to SslE. Since SslE was found to be broadly expressed by both extraintestinal and intestinal pathogenic *E. coli*, obtaining a mucosal immune response to SslE in the intestines on top of a systemic immune response would support the use of SslE in a broad-spectrum vaccine against pathogenic *E. coli*, which was the overall goal of the DISCo program. Using various mouse immunization regimens, we looked for the best routes of immunization to obtain a robust, specific response to SslE in both the intestines and the circulation. By comparison, we identified that the combination of an intranasal prime, followed by two intramuscular boosts, was the most promising immunization regimen to reach our specific goals; indeed, mice immunized with this regimen

mounted robust B and T cell responses, both in the gut mucosa and systemically. In addition, we sought to determine the potential impact of immunizations with SslE on the murine intestinal microbiota. Analyses with our most promising immunization regimen showed no significant changes in the richness or the composition of the intestinal resident microbiota. These results promote SslE as a safe and promising component of a broad spectrum vaccine against pathogenic *E. coli*.

This work led to a manuscript, presented herein, which was submitted to the peer reviewed journal Vaccine on December 4th, 2017.

Manuscript

Mixed mucosal-parenteral immunizations with broadly conserved pathogenic *Escherichia coli* antigen SslE induce a robust mucosal and systemic immunity without affecting the murine intestinal microbiota

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Abstract

Emergence and dissemination of multidrug resistance among pathogenic *Escherichia coli* has posed a serious threat to public health across developing and developed countries. In combination with a flexible repertoire of virulence mechanisms, *E. coli* can cause a vast range of intestinal (InPEC) and extraintestinal (ExPEC) diseases but only a very limited number of antibiotics still remains effective against this pathogen. Hence, a broad spectrum *E. coli* vaccine could be a promising alternative to prevent the burden of such diseases, while offering the potential for covering against several InPEC and ExPEC at once. SslE, the Secreted and Surface-associated Lipoprotein of *E. coli*, is a widely distributed protein among InPEC and ExPEC. SslE functions *ex vivo* as a mucinase capable of degrading mucins and reaching the surface of mucus-producing epithelial cells. SslE was identified by reverse vaccinology as a protective vaccine candidate against an ExPEC murine model of sepsis, and further shown to be cross-effective against other ExPEC and InPEC models of infection. In this study, we aimed to gain insight into the immune response to antigen SslE and identify an immunization strategy suited to generate robust mucosal and systemic immune responses. We showed, by analyzing T-cell and antibodies responses, that mice immunized with SslE via an intranasal prime followed by two intramuscular boosts developed an enhanced overall immune response compared to either intranasal-only or intramuscular-only protocols. Importantly, we also report that this regimen of immunization did not impact the richness of the murine gut microbiota, and mice had a comparable cecal microbial composition, whether immunized with SslE or PBS. Collectively, our findings further support the use of SslE in future vaccination strategies to effectively target both InPEC and ExPEC while not perturbing the resident gut microbiota.

Keywords: immunization, intranasal, intramuscular, SslE, pathogenic *E. coli*, gut microbiota

Introduction

Escherichia coli is a multifaceted Gram-negative bacterial organism. More than a laboratory workhorse, *E. coli* also colonizes and thrives within the healthy mammalian gut microbiota as a harmless facultative anaerobe. However, the remarkable ability of *E. coli* to allow for acquisition and loss of genetic material by horizontal gene transfer has driven the appearance of multiple pathogenic variants of *E. coli* via successful combinations of virulence factors. Today, at least nine different pathogenic variants, or pathotypes, account for the high versatility of *E. coli*. These pathotypes cause a wide range of human diseases, which bare the potential to be lethal, and carry a significant economic and public health burden.

The six commonly studied pathotypes within intestinal pathogenic *E. coli* (InPEC) are responsible for diarrheal diseases of various severity along the gastrointestinal (GI) tract, and differ by their mechanism of colonization and virulence, as well the clinical symptoms they provoke [1-3]. A recently published comprehensive study by the World Health Organization (WHO) on the global burden of foodborne diseases estimated that in 2010, InPEC were responsible for over 324 million cases of diarrheal diseases, with more than one third of it affecting children under 5 years of age [4]. Among these cases, enteropathogenic *E. coli* (EPEC) was the second leading cause of deaths from diarrheal diseases in the world. Extraintestinal pathogenic *E. coli* (ExPEC) can invade distant sites like the urinary tract, the bloodstream or the central nervous system [2]. Among ExPEC, uropathogenic *E. coli* (UPEC) is the main etiological agent of urinary tract infections (UTIs), accounting for 75% of all uncomplicated UTIs [5]. Recurrent UTIs are a common problem for young women, causing significant morbidity and care-associated cost [6, 7]. The neonatal meningitis-associated *E. coli* (NMEC), is one of the leading causes of early- and late-onset neonatal meningitis and sepsis [8, 9].

Resistance to commonly used antibiotics such as fluoroquinolones, tetracycline and cephalosporins is now widespread in both InPEC and ExPEC [1, 5, 7, 10, 11]. The extent of multidrug resistance (MDR), including to last-line antibiotics such as carbapenems, tigecyclin

and colistin [12-14], is becoming a growing concern, especially in the developing world, where treatment options are limited. For many in the scientific and clinical community, vaccines represent one of the most promising approaches to address the ever-increasing antibiotic resistance in pathogenic *E. coli*. The usual suspects such as toxins, adhesins, and siderophores, all important for colonization and virulence of the pathogen, have been targeted as potential vaccine antigens and explored in vivo for their immunological and protective properties [7, 15-18]. Despite some positive leads, few have gone through clinical trials, and results have had limited success. Among the many difficulties, the high antigenic diversity and virulence factor redundancy of *E. coli* has undoubtedly hampered the identification of a vaccine protective against various members of a specific pathotype. Instead, reverse vaccinology [19] has offered the opportunity to search and rapidly identify, using bioinformatics, potential vaccine candidates that would be secreted or present on the cell surface of multiple *E. coli* pathogenic strains.

Using this strategy on several ExPEC strains, our group has identified protein SslE (for Secreted and Surface-associated Lipoprotein of *E. coli*), also known as ECOK1_3385 or YghJ, as a protective vaccine candidate in a murine sepsis model with NMEC [20]. Additional immunization studies showed SslE to also be protective against other ExPEC strains in different animal models [20, 21], as well as an InPEC strain. Furthermore, analysis of human sera of convalescent patients from urosepsis [22] or ETEC infections [23, 24] revealed the presence of SslE-specific antibodies, confirming the immunogenicity of SslE. Functionally, SslE was characterized as a mucin-degrading metallopeptidase ([25]), and shown *in vitro* to degrade several mucins including MUC2, the most common intestinal mucin [21, 25]. A series of assays has demonstrated that SslE mucinase activity helps *E. coli* penetration through the mucus layer, a step that could favor colonization by allowing bacteria to better reach the epithelial layer [25, 26]. SslE has also been associated with biofilm formation in EPEC [27] and, more recently, with significant tissue damage and hemorrhage in mouse ilea [28], further supporting the role of SslE as an important virulence factor. The SslE-encoding

gene is widely distributed in the *E. coli* phylogeny, with a higher presence in intestinal and extraintestinal pathogenic isolates (between 70% and 83%) compared to commensal isolates (59%) [20]. In a recent study, SslE was found to be present in 70% of all strains of an *E. coli* data set containing 1700 complete or draft genome sequences spanning commensals and pathotypes from human and animal origins [22].

Overall, SslE represents a very promising component for a broad-protective vaccine, as it is immunogenic, protective, conserved among different pathotypes, and could play a key role in pathogenic *E. coli* virulence and disease. Yet, very little is known about the mechanism behind SslE protective efficacy. In this study, we sought to further characterize the immune response profile of antigen SslE in mice, at both systemic and mucosal levels. Because the response generated mucosally and systemically can be heavily influenced by the route of immunization chosen, we wanted to take a comparative approach by looking at mucosal-only (intranasal, or i.n), parenteral-only (intramuscular, or i.m), as well as a mixed immunization regimens. It is now well-established that mixing mucosal and parenteral immunizations can have significant benefits in the immune response generated versus mucosal-only or parenteral-only [29-33]; we therefore hypothesized that using a mixed immunization approach would induce a greater immune response, both at the mucosa and systemically. Here, we indeed show that immunizations with SslE using as priming an i.n dose, followed by two i.m boosts (referred as i.n / i.m / i.m), provided the most robust cellular and humoral responses in both the small intestine lamina propria (LP) and the systemic environment, compared to either i.n-only or i.m-only immunizations. Additionally, considering the known presence of antigen SslE in several *E. coli* commensals, we were particularly interested at investigating, besides the systemic immune response to SslE, the effect of an intestinal immune response to SslE on the resident gut microbiota. The influence of the gut microbiota on human health is now well-acknowledged; its disruption has a critical impact on the development and maintenance of the intestinal immune system, and on the onset of severe diseases [34]. Thus, ensuring that an immunization effect on the gut immune response does

not perturb the microbiota is paramount. Analysis of the microbial population from both fecal and cecal contents of mice immunized i.n / i.m / i.m reveals no changes to a significant level of the overall composition at the phylum level, and the composition profiles at both family and genus levels appear undisturbed in the cecum before and after immunizations. Together, these results provide the first in-depth immunological profile associated with antigen SslE, and set important groundwork for the use of SslE in future vaccine and clinical studies.

Materials and Methods

Formulation with antigen SslE:

Cloning, expression and purification of the NMEC IHE3034 strain SslE recombinant protein were performed as previously described [21]. The batch of recombinant SslE used to perform our experiments was determined to have a purity of 94%, and an endotoxin level below 0.07 EU/µg. Before each immunization, antigen SslE was freshly dialyzed using a Slide-A-Lyser dialysis cassette 10K MWCO (ThermoFisher Scientific) overnight in PBS 1X in order to remove the glycerol used (40%) to store the antigen at - 20°C. The antigen was then concentrated using a 15 ml centrifugal filter unit 50K MWCO (Millipore); the final antigen concentration was determined by performing the Pierce BCA ProteinAssay Kit (ThermoFisher Scientific) following manufacturer's recommendations.

For i.n immunizations, we used cholera toxin (CT, Sigma) as adjuvant; we combined 10 µg of recombinant SslE with 3 µg of CT in formulations for a final volume of 10 µl per mouse. For i.m immunizations, we used an oil-in-water emulsion adjuvant, named SEA for Self-Emulsifying-Adjuvant, which was manufactured as previously described [35]; we combined 10 µg of antigen SslE in a 1:1 ratio with SEA in formulations for a final volume of 20 µl per mouse. Each formulation was freshly prepared the day of immunization, using PBS 10X (Ambion) diluted with Water For Injection (WFI). Each formulation batch was inspected for pH (range of 7.4 ± 0.5) and osmolality (range of 300 ± 60 mOsm/kg). Antigen and adjuvant characterizations were also performed by running each formulation by SDS-PAGE to verify the presence and integrity of each component upon formulation. In particular, for intramuscular formulations, the soluble part of the formulation (the subnatant) was isolated by ultracentrifugation at 60000 rpm and used in the SDS PAGE to confirm the antigen stability in the formulation.

Mice and immunizations:

Protocols were approved by the Italian Ministry of Health (authorization number 689/2015-PR). All mice were housed under specific pathogen-free (SPF) conditions at the GSK Vaccines Animal Resource Center, which is an AAALAC (Association of Assessment and Accreditation of Laboratory Animal Care) accredited facility. Four groups of ten eight-week old BALB/c mice were immunized with SslE or PBS three times, four weeks apart, at days 1, 29 and 57 (Fig 1): an i.n-only group i.n / i.n / i.n, an i.m-only group i.m / i.m / i.m, a mixed immunization group i.n / i.m / i.m, and a naïve group receiving PBS pH=7.4 i.n / i.m / i.m. Immunizations were given as 5 µl per nostril on anesthetized animals when done intranasally, and in the quadriceps when done intramuscularly.

In vitro restimulation of antigen-specific $CD4^+$ T cells and intracellular cytokines staining:

To measure $CD4^+$ T-cell responses, spleens were harvested and single-cell suspensions were prepared. Splenocytes were plated at 2 x 10^6 cells/well in 96-well U-bottom plates in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 25 mM HEPES (Gibco-Life Technologies), 10% heat inactivated FBS (low endotoxin; HyClone, Logan, UT), 1X Pen/Strep/Glut (100X; Gibco-Life Technologies) and 50 µM B-mercaptoethanol (Sigma), and stimulated with 10 µg/ml SslE for 2 hours before the addition of 5 µg/ml of Brefeldin A (Sigma) for 4 hours at 37°C + 5% CO_2. Cells were then washed and stained with Live/Dead Fixable Near-IR viability marker (Molecular Probes-Life Technologies). Cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed with Perm-wash buffer (BD Biosciences) and stained with the following antibodies: BV605-labelled anti-CD3 (BD Biosciences), PE-CF594-labelled anti-CD8, BV510-labelled anti-CD4 (Biolegend), V450-labelled CD44, A-488-labelled anti-TNFα (BD Biosciences), BV785-labelled anti-IFNγ (Biolegend), PE-Cy5-labelled anti-IL-2 (Biolegend), PerCP eFluor710-labelled IL-4 and IL-14 (eBioscience), PE-Cy7-labelled anti-IL-17 (eBioscience), all resuspended in Perm-wash 1X solution. Cells were acquired on an LSR II SOS1 flow cytometer (BD Bioscences) and

analyzed with FLOWJO software (TreeStar). Double-positive cells for CD44 and CD4 were gated on IL-2, IFNγ, IL-17, IL-4 and IL-13.

ELISA assays:

For all ELISA assays, we used 96 well Nunc-Immuno MicroWell MaxiSorp flat bottom plates (Thermofisher Scientific). Plates were coated with 100 µl of antigen SslE at 1 µg/ml in PBS pH 7.4 overnight at 4°C. Plates were blocked for 1 hour at 37°C + 5% CO_2 with PBS + 0.05% Tween 20 + 2% BSA heat shock fraction (Sigma-Aldrich). Plates were then incubated with diluted serum, intestinal washes, or fecal pellet supernatants (in series of 2X dilutions) in PBS + 2% BSA for 2 hours at 37°C + 5% CO_2. For each assay, 100 µl of goat anti-mouse secondary antibody coupled to alkaline phosphatase was added at the appropriate dilution in PBS + 2% BSA and incubated for 2 hours at 37°C + 5% CO_2: anti IgG (H+L) at 1:2000, anti-IgA at a dilution of 1:1000, anti-IgG1, anti-IgG2a and anti-IgG2b at a dilution of 1:2000 (all antibodies from SouthernBiotech). Between each of these steps, plates were washed three times with PBS-0.05% Tween20. Plates were then incubated with 100 µl of p-nitrophenyl-phosphate liquid substrate (Sigma-Aldrich) for 30 min at room temperature then immediately read on a SpectraMax microplate reader (Molecular Devices) at 405 nm. The linear part of the curve was used for calculating titers at a cutoff value of 0.1 (for sera) or 0.5 (for intestinal washes and fecal pellets).

Lamina propria cells (LPC) cytokine quantification:

Purification of LPC from mouse small intestine (SI) was adapted from a previously described protocol [36]. At the end of the immunization schedule, whole SI tissue was cut and incubated twice in PBS 1X (Gibco-Life Technologies) supplemented with 10% heat inactivated FBS (low endotoxin; HyClone, Logan, UT), 5 mM EDTA (Sigma) and 1 M HEPES

(Gibco-Life Technologies) for 20 min at 37°C, shaking. The precipitate, containing the LPC, was further cut before being digested in RPMI supplemented with 10% FBS, 100 U/ml collagenase type VIII (Sigma) for 20 min. Cells were then washed, passed through a 70 µm nylon cell strainer and resuspended in 100% Percoll (GE Healthcare Life Sciences), which was overlayered with 40% percoll, and centrifuged for 20 min at 2100 rpm with no break. LPC were recovered from the interface layer. Viability was verified to be > 80% using the Nucleocounter NC-250™ (Chemometec) and staining cells with a solution of acridine orange and DAPI (Solution 18; Chemometec). LPC were plated at 2 x 10^6 cells/well in 96-well U-bottom plates in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 25 mM HEPES (Gibco-Life Technologies), 10% heat inactivated FBS (low endotoxin; HyClone), 1X Pen/Strep/Glut (100X; Gibco-Life Technologies) and 50 µM B-mercaptoethanol (Sigma), and stimulated with 10 µg/ml SslE for 24 hours at 37°C + 5% CO_2. Plates were then centrifuged for 10 min at 2000 rpm and supernatants were collected and placed at -80°C until ready to process.

Each supernatant was quantified for IL-1β, IL-2, IL-4, IL-5, IL-13, TNFα, IFNγ, IL-17A, IL-17F, IL-21 and IL-22 using a custom made Meso Scale Discovery U-PLEX mouse Biomarker Group 1 Assays (Meso Scale Discovery) according to the manufacturer's instructions. Plates were read using the QuickPlex SQ 120 imager (Meso Scale Discovery).

Genomic DNA extraction and microbiota composition analysis:

Fecal pellets were collected at day 0 and day 71, along with cecal contents at day 71 (Fig 1) for six mice in PBS-immunized group and six mice of the i.n / i.m / i.m group (three mice per cage for each group). All samples were immediately stored at - 80°C until ready to process. Genomic DNA from all samples was isolated based on a previously published protocol [37]. Samples were sent for Illumina sequencing and analysis of bacterial population at Life Sequencing (Valencia, Spain), using the capture of the hyper-variable regions V3-V4 of the

ribosomal gene 16s according to previous literature [38]. On average, 75000 sequences (62794 - 97213 sequences) were recovered from our samples.

Statistics:

All statistics were made using GraphPad Prism (San Diego, California). Statistical significance (for all assays but the microbiota analysis) was calculated using analysis of variance (ANOVA) with Dunnett's post test. A Mann-Whitney two-tailed test was used to compare microbiota relative abundance between PBS-immunized and SslE-immunized mice. Significance is calculated as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; ns, not significant ($P\geq0.05$).

Results

Effect of various SslE immunization regimens on the gut immune response

We first investigated the effect of i.n-only, i.m-only, and i.n / i.m / i.m immunizations on the mucosal immune response. Lamina propria cells (LPC) were isolated from small intestines at day 71, and the levels of secreted cytokines released in the supernatant after 24 hours of *in vitro* re-stimulation with antigen SslE were measured using MSD technology (Fig 2). When comparing the i.n / i.m / i.m and i.n / i.n / i.n routes, our results showed that LPC produced cytokines associated with Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A, IL-17F, IL-21 and IL-22) responses in both these groups. Interestingly, although the levels of Th1 cytokines produced were nearly identical in both protocols; we observed a statistically significant increase in Th2 cytokines when mice were immunized through the i.n / i.m / i.m routes, notably with a > 5 fold increase of IL-13. The i.n-only immunizations led, on the other hand, to a 2.5 fold increase of IL-17A compared to the i.n / i.m / i.m routes. The levels of recovered cytokine were similar between i.n-only and mixed immunization groups for IL-17F, as well as for IL-22, for which we roughly obtained a 12 fold increase compared to naïve mice. As for i.m / i.m / i.m immunizations, this regimen only gave a very limited cytokine response in the small intestine (SI), at least for the panel of cytokines that we investigated.

These results showed that, within the protocols tested, an i.n prime immunization is important to induce a Th1, Th2 and Th17 response in the SI. The i.m boosts seem to result in a greater induction of Th2 cytokines, while i.n boosts increase production of IL-17A after antigen recall. Overall, both i.n / i.n / i.n and i.n / i.m / i.m immunization protocols appear to be efficient at inducing a robust intestinal T cell response to antigen SslE.

The gut humoral immune response often plays a critical role in protective immunity against enteric pathogens [39], so we looked at the effects of each of the various immunization protocols on the release of antigen-specific secreted immunoglobulin A (SIgA) antibodies. To

do so, we quantified by ELISA SslE-specific SIgA titers recovered in both intestinal washes and fecal pellets at day 71 (Fig 3). Similarly to the cellular response in the small intestine LP, results obtained with i.m / i.m / i.m immunizations were limited, with virtually no detectable levels of SIgAs. On the other hand, i.n / i.n / i.n or i.n / i.m / i.m regimens were able to induce detectable SIgA titers which were of the same magnitude when measured in intestinal washes; however, the mean SIgA titer recovered from fecal pellets was 4 times higher with i.n / i.m / i.m than with i.n / i.n / i.n immunizations. Thus, the mixed i.n / i.m / i.m protocol generates just as high, if not more, levels of SslE-specific SIgAs versus a mucosal-only, i.n / i.n / i.n protocol.

Effect of various SslE immunization regimens on the systemic immune response

Since we consider SslE to be a strong candidate as part of a universal vaccine against both InPEC and ExPEC strains, we wanted to assess not only the intestinal immune response, but also the systemic immune response to antigen SslE. To look at and compare the cellular immune response to antigen SslE after our different immunization protocols, splenocytes were isolated at day 71 and re-stimulated *in vitro* with antigen SslE for 6 hours (with the last 4 hours with Brefeldin A). We then performed an intracellular staining in order to quantify by flow cytometry the cytokine response of effector memory T cells after antigen recall, which we present as frequency of the total amount of $CD4^+/CD44^+$ T cells recovered (Fig 4). For all the cytokines tested, the i.n / i.m / i.m immunization protocol led to a greater percentage of cytokine-positive cells compared to either i.n / i.n / i.n or i.m / i.m / i.m regimens. Indeed, when compared to the i.n-only immunizations, i.n / i.m / i.m immunizations led to at least 4 times more TNF-α and IL-2- positive $CD4^+/CD44^+$ T cells, as well as nearly 3 times more IL-17-positive $CD4^+/CD44^+$ T cells. Noteworthy, we recovered IFN-γ and IL-4/IL-13- positive cells only in splenocytes from i.n / i.m / i.m immunized mice. On the other hand, very few cells recovered from mice immunized with the i.m-only protocol were positive for Th1

cytokines (IFN-γ, TNF-α and IL-2), and none were apparently positive for the Th2 or Th17 cytokines tested.

To look into the serum antibody response, we performed ELISA assays using sera from mice at day 71, which we compared to the pre-immune sera. The total IgG titers (Fig 5A) showed that mice immunized via i.n / i.m / i.m routes produced higher amounts of IgGs in the sera compared to either i.n-only or i.m-only routes, for which mice produced similar levels of IgGs, approximately 3 times lower than the mixed immunization protocol. We also sought to look into various IgG subtypes to determine the IgG subclass dominance for each immunization protocol (Fig 5B). All three immunization regimens led to a predominant production of IgG1 antibodies, while IgG2a levels were weaker, and IgG2b even lower. For all IgG subclasses tested, mice immunized with SslE via the i.n / i.m / i.m routes showed higher titers versus i.n-only or i.m-only routes. The ratio IgG2/IgG1 calculated for each of these regimens was lower than 0.5 in all cases, confirming a strong Th2 response for all three regimens (Supplemental fig 1). However, we did note that both i.n / i.m / i.m and i.n / i.n / i.n regimens had a statistically significant higher ratio value compared to the i.m / i.m / i.m regimen, indicating a bias, although small, towards a Th1 response with both regimens using i.n injections.

Overall, mice immunized with SslE via the i.n / i.m / i.m routes showed a stronger systemic immune response than those immunized via i.n-only or i.m-only routes, both at the cellular level with higher Th1, Th2 and Th17 cytokines, and at the humoral level with greater amounts of IgG antibodies, in any of the IgG subclasses tested.

Effect of a mixed i.n / i.m / i.m immunization with antigen SslE on the gut microbiota of conventional mice.

With the gut being a prime example, it is now well established that mucosal immunity and microbiota constantly and dramatically shape the outcome of one another [40-42]. In particular, the immune response to an enteric pathogen has been shown to alter the

microbiota, as seen with Salmonella or *Citrobacter rodentium* [43, 44]. Thus, a vaccine-induced strong inflammatory milieu in the gut could have the potential to perturb the resident microbiota. To address this hypothesis, we focused on the i.n / i.m / i.m regimen, which gave us the strongest overall immune response locally and systemically, to investigate whether immunizations with antigen SslE would lead to an alteration of the mouse gut microbiota. The question was twofold: to determine whether these immunizations would provoke any sort of dysbiosis in the gut microbiota, and to specifically find out whether immunizations with antigen SslE, which is present in pathogenic as well as commensal *E. coli* [20], would affect the commensal population to significant, perhaps detrimental levels.

We collected fecal pellets from mice immunized with antigen SslE versus PBS using the i.n / i.m / i.m regimen at days 0 and 71, analyzed their genomic DNA content and compared the resulting data. After immunizations with PBS or SslE, we recovered the same phyla, and in similar proportions, than prior to immunizations (Fig 6A): the Firmicutes and Bacteroidetes represented the vast majority of classified sequences, while the Actinobacteria and Proteobacteria were present in much lower amounts. We did notice a small increase of the Proteobaceria phylum in SslE-immunized mice at day 71 compared to PBS-immunized mice.

The Shannon diversity index, which accounts for abundance and evenness, remained high at roughly 1.5 at the family level (Fig 6B), and 2.1 at the genus level (data not shown) in both PBS and SslE-immunized mice compared to pre-immunization. We looked more closely at the microbiota composition at the family level in the fecal pellets pre- and post-immunizations (Fig 6C, upper panels). We recovered the same families before and after immunizations, but observed some fluctuations of the relative abundance over time. Notably, the relative abundance of the Firmicutes Lactobacillaceae and Lachnospiraceae changed significantly from pre- to post-immunizations. All pre-immunized mice showed a relative abundance of Lactobacillaceae of roughly 35% and a lower abundance of Lachnospiraceae at roughly 17%. In PBS-immunized mice, we recovered 24% of Lactobacillaceae and 30% of Lachnospiraceae in fecal pellets; in SslE-immunized mice, we obtained 10% of

Lactobacillaceae and 41% of Lachnospiraceae. Interestingly, when we looked at the microbiota composition from cecal contents post-immunization (Fig 6C, lower panels), we did not observe such fluctuations between control and immunized mice at the family level; instead, we obtained a similar distribution for both PBS-treated and SslE-immunized mice which was comparable to the one recovered from fecal pellets of SslE-immunized mice from the fecal pellets at day 71, with about 7% of Lactobacillaceae and 44% of Lachnospiraceae. Additionally, the small raise in the relative abundance of Proteobacteria appeared to be the result of an increase in the family Desulfovibrionaceae (Fig 6C), and more specifically the genus Desulfovibrio (data not shown), which was noticeable in both fecal and cecal contents post-immunization. The relative abundance of Desulfovibrio in the cecum, where the difference was the highest, increased from 0.06 ± 0.01 in naïve mice to 1.97 ± 0.8 in immunized mice; however, statistical analysis with a Mann-Whitney two-tailed test revealed no significance in this difference. To the extent of our analysis, we did neither recover the genus *Escherichia*, nor the Enterobacteriacea family, within the Proteobacteria phylum, from our classified sequences prior or post-immunizations, whether in the fecal pellets or the cecum. Thus, we could not assess whether immunizations with antigen SslE would affect the commensal *E. coli* in the gut.

Overall, mice immunized with antigen SslE using the regimen i.n / i.m / i.m maintained the same richness of the microbiota. SslE-immunized and PBS-immunized mice showed some disparities in the fecal relative abundance of Lachnospiraceae and Lactobacillaceae, but maintained a strikingly similar distribution in the cecal relative abundance of families.

Discussion

In this study, we described the immune response to the ExPEC antigen SslE and the influence of immunization with SslE on the gut microbiota. SslE is a mucin-degrading metalloprotease widely distributed among the many *E. coli* pathotypes, with a highly conserved zinc-metalloprotease core motif [20, 21, 45]. Functional studies have demonstrated that SslE is capable of degrading major intestinal mucins such as MUC2 and MUC3 [25]. When *E. coli* is in contact with adherent enterocytes, secretion of SslE is increased, which is thought to facilitate colonization by degrading mucus and allowing bacteria to reach the surface of enterocytes [26]. SslE immunizations confer protection, although to various extent, in murine models of ExPEC and InPEC infections, including sepsis, UTI, and intestinal colonization with enterotoxigenic *E. coli* (ETEC) [20, 21]. Sera from patients recovering from UTIs or an ETEC infection contain anti-SslE antibodies [22-24]. Thus, SslE is conserved in sequence, widely distributed, secreted during infection, and immunogenic, all of which are important characteristics with respect to a potential vaccine candidate.

We sought to better explore the opportunity and investigate the immune response generated against SslE following various methods of immunization. Our goal was to define the immunization regimen that would deliver the highest mucosal and systemic immune responses, hence bare the potential to be cross-effective against multiple *E. coli* pathotypes. Based on previous studies demonstrating the benefits of combining mucosal with parenteral immunizations [29-33], we set up immunization regimens with either i.n immunizations only, i.m immunizations only, or an i.n prime followed by two i.m boosts. We conclusively showed that mice immunized i.n / i.m / i.m with SslE mounted a greater overall immune response than i.n / i.n / i.n or i.m / i.m / i.m.

In the SI, both mixed immunizations and i.n-only immunizations led to a significant cytokine response after *in vitro* antigen restimulation compared to i.m-only immunizations. It is very likely that these responses are antigen-specific $CD4^+$ T cell responses, though we cannot

rule out, due to our experimental set up, a non-specific response of other LP lymphoid cells. Notably, for both regimens, we observed a strong induction of the IL-17A, IL-17F and IL-22 cytokines. IL-17A can be secreted by several cells of the LP, including Th17 cells and group 3 innate lymphoid cells (ILC) [46]. IL-17A and IL-17F, who are thought to share similar biological activities, are important in the initial control of pathogen proliferation through the release of antimicrobial peptides and induction of neutrophil recruitment [47, 48]. Deficiency in either one of these cytokines leads to full susceptibility of mice to *C. rodentium* [49], suggesting that these two cytokines are not absolutely redundant and may have some unique biological activities in early gut pathogen control. IL-22, also in part secreted by Th17 cells and ILC3, can act synergistically or additively with IL17-A and IL-17F by increasing antimicrobial peptide secretion and intestinal protection. Previous studies showed that Th17 cells developed in mice after infection with EHEC O157:H7 [50], or immunization with ETEC $F4^+$ fimbriae [51]. Th17 cells are also known to be important for the induction of an antigen-specific IgA response by B cells in the germinal center of Peyer's patches in the small intestine [52]. Thus, the presence of these cytokines in the LP after recall with SslE suggests that IL-17 and IL-22-secreting cells are specifically induced *in vivo* after SslE immunization, and may contribute to protective immunity against InPEC strains expressing SslE.

While we observed a bias towards a higher IL-17A secretion with i.n / i.n / i.n, the i.n / i.m / i.m regimen led to a significantly greater release of Th2-like cytokines. These differences may be attributed to an adjuvant effect. CT, the adjuvant used in i.n immunizations, has been recently shown to induce a balanced Th1/Th2/Th17 response, whether injected mucosally or parenterally [53]; the i.n-only regimen could thus potentially lead to more efficient priming by CT of Th17 cells at each immunization. Likewise, the enhanced Th2-like cytokine levels seen with i.n / i.m / i.m may possibly be attributed to the use of SEA, which has been suggested to behave like most delivery system type of adjuvants by priming Th2 $CD4^+$ T cells and enhancing the antibody response.

Besides these important mechanistic questions, our results made evident that the mucosal route of immunization, more than the adjuvant itself, was an essential element in mounting the immune response to SslE in the SI. Indeed, i.m-only immunizations, regardless of the use of SEA, performed very poorly in the mucosa, with little to no cytokine response recovered in the SI. Similarly, both mixed immunizations and i.n-only immunizations promoted the secretion of anti-SslE IgAs in the gut, while we could not observe any detectable levels of SIgAs after i.m-only immunizations. Interestingly, the SIgA titers recovered from intestinal lavages were fairly similar between i.n-only and mixed immunizations, yet titers in fecal pellets seemed to be significantly higher in mixed immunizations. Secretions of IgA antibodies in the intestinal lumen represent a crucial element in the immune response against gut bacterial pathogens. In experimental ETEC challenges, infected volunteers all produced mucosal IgA upon challenge, [54], suggesting that IgA antibodies are indeed an effective arm in the control and/or clearance of the ETEC infection.

Thus, with antigen SslE, i.n-priming seemed to be an essential element in the priming of B and T cells in the gut, and sufficient to induce an immune response of the same magnitude regardless of whether boost immunizations were performed i.n or i.m. It would be interesting to investigate whether the timing of the mucosal immunization is also a critical element for an effective mucosal immune response, by comparing i.n / i.m / i.m immunizations to i.m / i.m / i.n immunizations for example; however, previous studies on immunization against influenza showed that priming with a mucosal immunization was essential at generating a mucosal immune response [32]. Additionally, the choice of i.n versus another mucosal route could be an important factor in the gut immune response obtained: preliminary results from an equivalent experiment study with SslE using the sublingual route for mucosal immunizations showed no particular benefit in using of the mixed immunization regimen, which instead led to a smaller cellular and humoral immune response in the SI compared to sublingual-only immunizations (unpublished data).

The differences between i.n / i.m / i.m and i.n-only immunizations were more evident when we quantified the systemic immune response to antigen SslE. The $CD4^+$ T cell response obtained from splenocytes showed a consistently higher percentage of Th1/Th2/Th17 cytokine-positive $CD4^+$ T cells when mice were immunized i.n / i.m / i.m versus i.n-only. These results were paralleled by the serum IgG titers recovered post-immunizations, where i.n / i.m / i.m led to titers of all IgG1, IgG2a and IgG2b far higher than i.n-only immunizations. The i.m-only regimen showed very little systemic T cell response and a much lower IgG antibody titer compared to the mixed immunizations, regardless of the antibody subtype assessed. This finding suggests that an i.n prime immunization with SslE is critical not only for a mucosal immune response, but also for a systemic immune response. Further, the important difference in both B and T cell responses observed between i.n / i.m / i.m and i.n / i.n / i.n indicates that boosting with parenteral immunizations ensured an efficient mounting of the systemic immune response, at a magnitude not attained with i.n-only immunizations.

In the consideration of a broad-spectrum vaccine against both InPEC and ExPEC, the robust systemic immune response obtained with i.n / i.m / i.m is of particular interest. Indeed, studies have showed that UTI infection with UPEC resulted in the proliferation of antigen-specific splenic T cells and increase of serum IgG titers, and transfer of either T cells or serum led to protection of naïve mice from infection [55]. There may therefore be great benefits associated with a mixed immunization by effectively targeting multiple pathotypes of *E. coli*.

Mucosal immunizations, particularly with an antigen present on the surface of commensals [20, 56], must raise the question of the impact of immunizations on the gut microbiota. Choosing i.n / i.m / i.m immunizations to address this possibility, we did not observe any significant change in the overall richness of the microbiota between PBS and SslE-immunized mice all the way down to the genus level, as indicated by the Shannon diversity index. Accordingly, we recovered the same families and genera between the two groups pre-

and post-immunizations, which was an important indicator that immunizations with SslE did not significantly perturb the overall composition of the gut microbiota.

Within Firmicutes, the relative abundance of the families Lachnospiraceae and Lactospiraceae was markedly different in the fecal pellets of pre- versus post-immunized mice. The high abundance of Lactobacillaceae and lower abundance of Lachnospiraceae in pre-immunized mice was rather surprising, as Lactobacillaceae tend to be in high abundance up only until the ileum, while dropping below 10% in the cecum, colon and feces; as for the Lachnospiraceae, they normally constitute the largest part of the Firmicutes, with a relative abundance at 30% at least [57, 58]. However, at day 71, we observed a shift in these two families, whose relative abundance became quite similar to previously reported results [57]; this was the case in fecal and cecal contents of SslE-immunized mice, and in the cecal content of PBS-immunized mice. This shift could have been due to the transfer of mice from the vendor to our facility. The length of our experimental study could also be at play: immunizations were performed and followed between weeks eight and eighteen; considering the significant age-dependent change, even in an SPF environment, in the gut microbiota of laboratory mice [59, 60], this factor could explain at least part of the changes observed between pre- and post-immunized mice. Additionally, we noticed a different profile of these two families between the fecal content of mock-immunized and SslE-immunized mice. While we cannot rule out that this difference is influenced by immunizations with SslE, we believe it is unlikely, as this difference was not perceived in the comparison of the cecal content between these two groups.

The Proteobacteria phylum represents a small component of the microbiota composition, and *E. coli* is generally a poor colonizer of the mouse GI tract [61]. To the extent and depth of our study, we did not find the Enterobacteriaceae family and thus the Escherichia genus in our sequence analysis of pre- and post-immunized mice, whether in the feces or the cecum. Although this result was to be expected, assessing whether immunization with SslE would

affect resident *E. coli* commensals still remains an important question to be addressed in the context of the human gut microbiota.

In summary, we have described an efficient, mucosal-parenteral immunization schedule with antigen SslE to induce a robust intestinal and systemic immune response against SslE, while not significantly disturbing the resident gut microbiota. SslE thus appears as a potential key component of a broad spectrum vaccine against pathogenic *E. coli*. Although widespread and protective against different *E. coli* pathotypes, SslE does not cover all known strains [20]. Using the same reverse vaccinology approach against InPEC, our group is currently characterizing some promising vaccine candidates that have showed protective efficacy against an intestinal enterohaemorrhagic *E. coli* model of infection (Rojas-Lopez, M. et al.; manuscript in preparation). Another approach is to use reverse vaccinology to identify antigens present in the *E. coli* core genome from all pathogenic and non-pathogenic strains. A recent study has identified antigen YncE, present in >99% of all *E. coli* genomes available, which shows protection against a bacteremia model of infection, and is recognized by antibodies present in the sera of convalescent urosepsis patients [22]. We and others have showed that immunizations with conserved *E. coli* antigens do not perturb to significant extent the murine gut microbiota [62]. Although *E. coli* is a prevalent member of our microflora, it only accounts for about 0.1% of its total composition. The idea of a multi-component broad-spectrum vaccine including candidates such as SslE and YncE thus deserves to be considered in future vaccine research strategies.

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Author contributions

IN, UD and CB conceived the study; IN, CB and BCB designed the experiments; IN and JV performed the experiments; IN, CB, AB-D and GJ analyzed the data; IN wrote the manuscript; all authors reviewed and approved the manuscript.

Disclosures

CB, UD, BCB and MP are current permanent employees at GSK. JV is a former GSK employee and was supported by the People Programme (Marie Sklodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013 under the REA Grant agreement 317057 HOMIN-ITN. Ilham Naili is a former GSK employer and a Marie Sklodowska-Curie PhD Research Fellow granted by ITN EID DISCo, whose work was performed at both GSK Vaccines and INRA. All other authors declare no conflict of interest.

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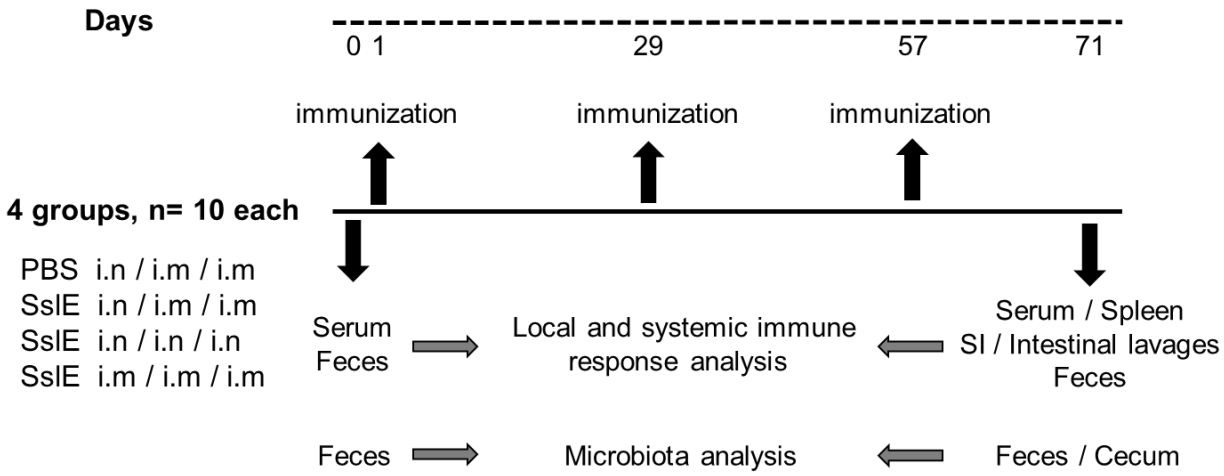


Figure 1. Study design for the animal experiments. For each experiment (two independent experiments), three immunization groups with SslE and one control (PBS) group were used (n=10 mice per group). i.n / i.m / i.m: intranasal immunization at day 1, intramuscular immunizations at days 29 and 57. i.n / i.n / i.n: intranasal immunizations at days 1, 29 and 57. i.m / i.m / i.m: intramuscular immunizations at days 1, 29, 57. For each group, samples were taken for analysis at day 0 (preimmune stage), or day 71. For microbiota analysis, only PBS i.n / i.m / i.m and SslE i.n / i.m / i.m were used, with n=6.

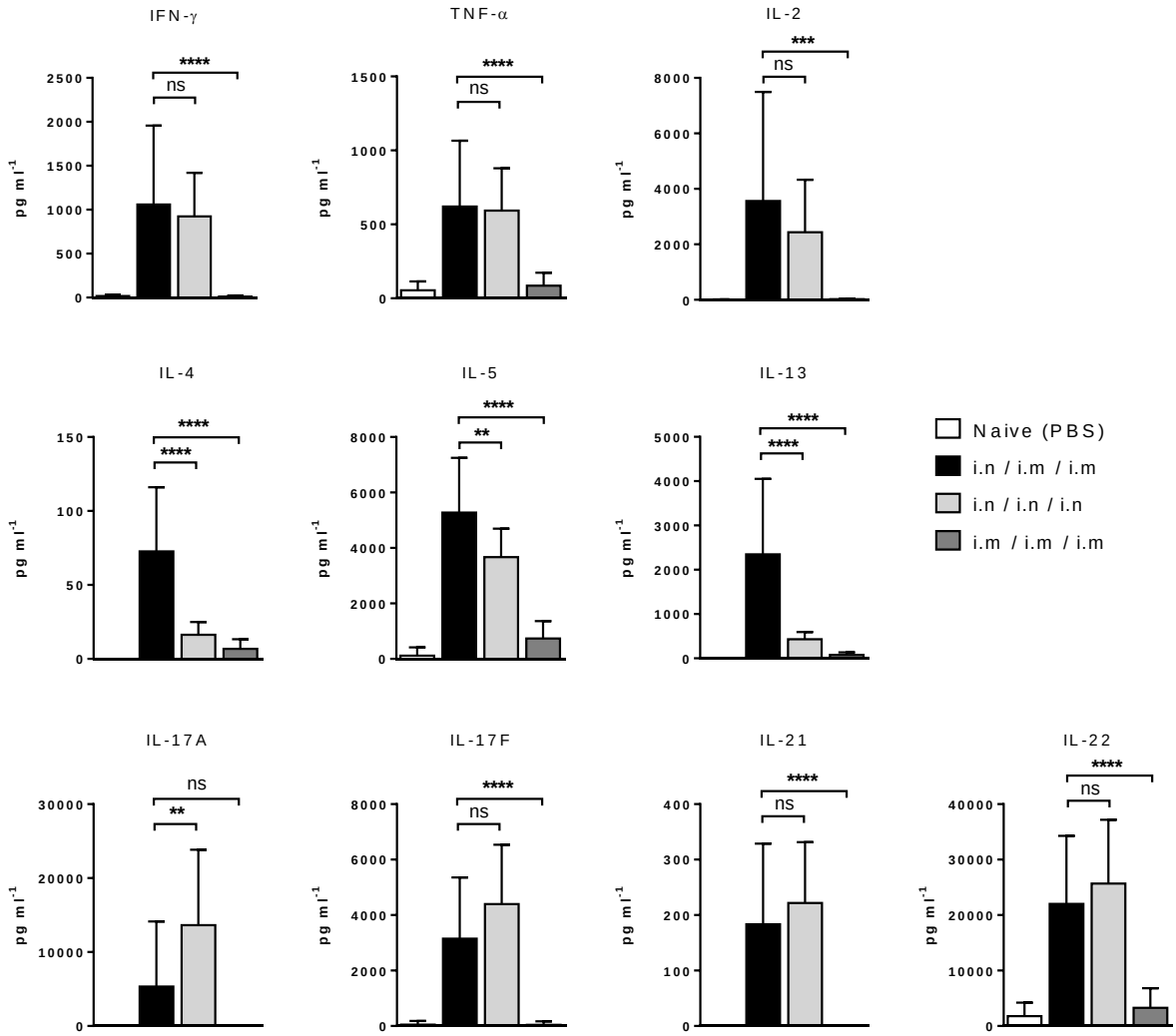


Figure 2. Cytokine quantification of murine lamina propria cells (LPC) after immunizations. Balb/C mice were immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive), and LPC were harvested and purified at day 71. Purified LPC were seeded at 10^6 cells/well and cultured with recall antigen SslE (10 µg/ml final concentration) for 24 hours at 37°C, and incubated for 24 hours at 37°C. Supernatants were collected and analyzed for cytokine quantification using the MesoScale Discovery U-PLEX mouse cytokine assay kits. Data are from two independent experiments with ten mice per group, and shown as mean ± standard deviation. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin.

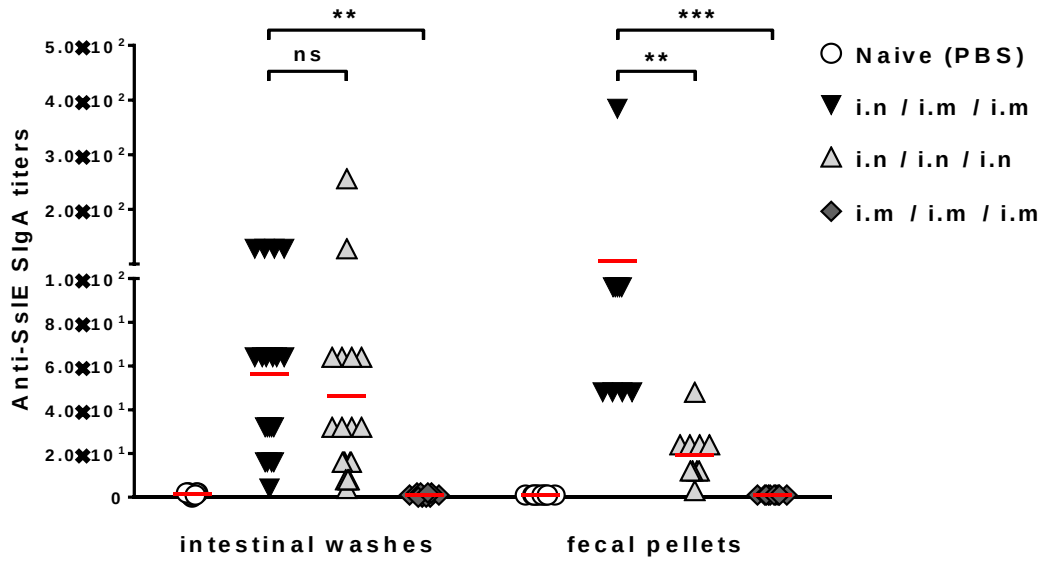


Figure 3. Quantification of secreted IgA (SIgA) after immunizations. Intestinal washes and fecal pellets were harvested at day 71 from Balb/C mice immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive). Specific SIgA antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Data are from one (fecal pellets) or two (intestinal washes) independent experiment(s), where each dot represents one mouse.

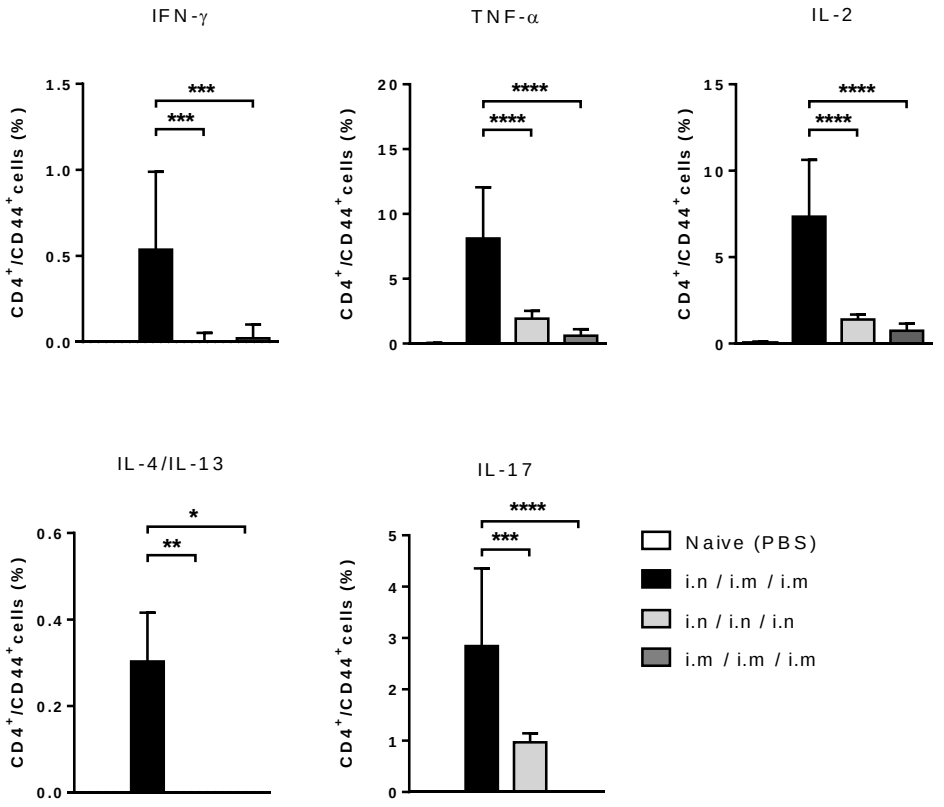


Figure 4. Cytokine quantification of murine splenocytes after immunizations. Balb/C mice were immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive), and splenocytes were harvested and purified at day 71. Splenocytes were seeded at 10^6 cells/well, cultured with recall antigen SslE (10 µg/ml final concentration) and were incubated for 6 hours at 37°C, including 4 hours with Brefeldin A. Cells were stained with Live/Dead, fixed and permeabilized, then stained with fluorescent antibodies for flow cytometry acquisition. Data are from two independent experiments with ten mice per group, and shown as mean ± standard deviation. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin.

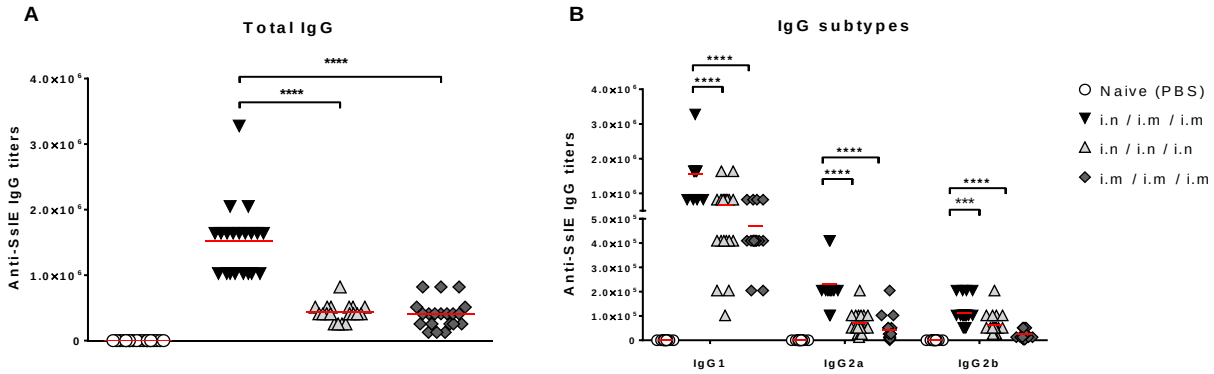


Figure 5. Quantification of IgGs after immunizations. Sera from Balb/C mice immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive) were taken at day 71. Specific (A) total IgG antibody titers, or (B) IgG1, IgG2a and IgG2b were determined by enzyme-linked immunosorbent assay (ELISA). Data are from two independent experiments, where each dot represents one mouse.

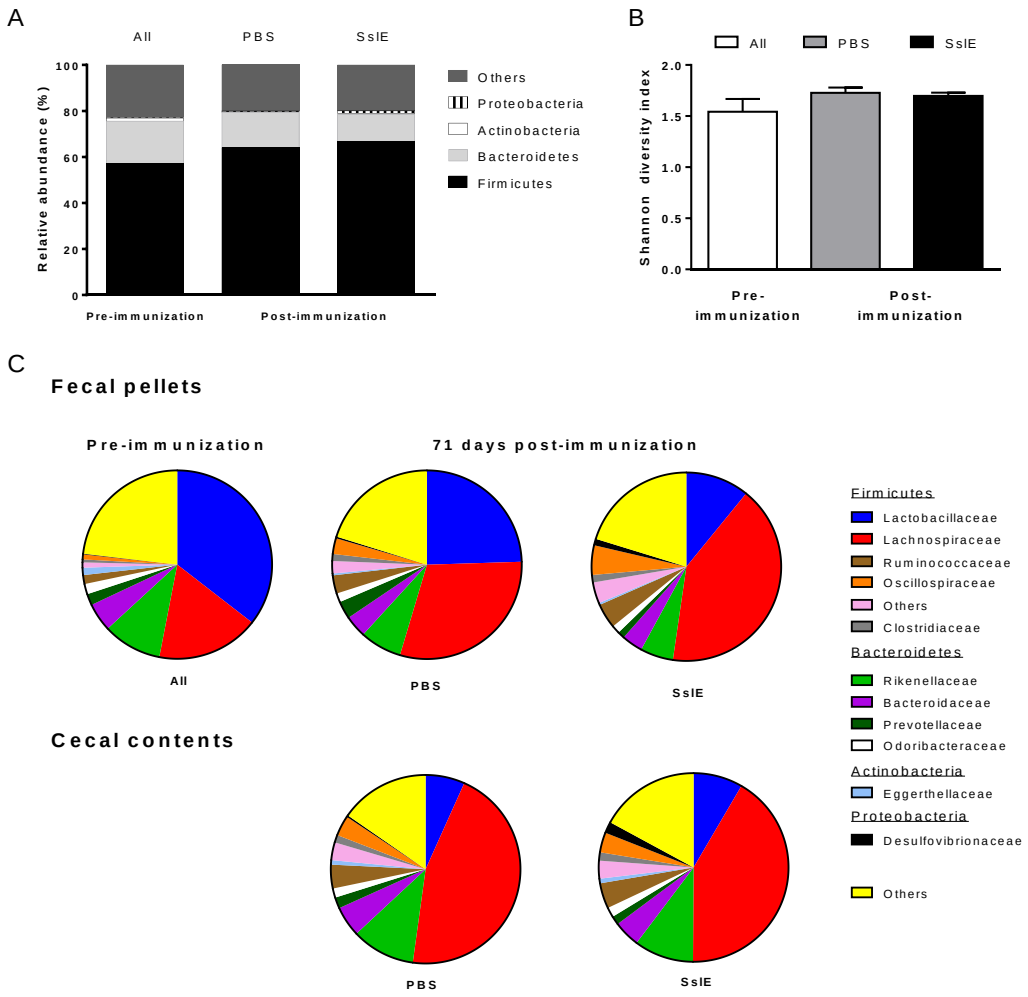
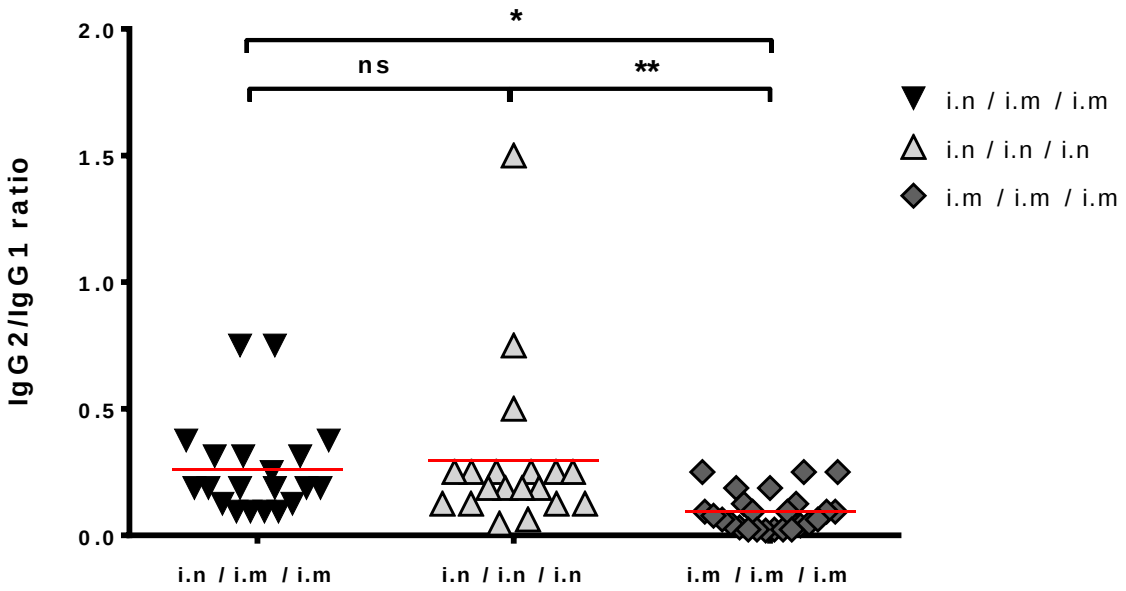


Figure 6. Microbiota composition before and after immunization with SslE. (A) Normalized Phyla relative abundance obtained from fecal pellets of PBS-immunized or SslE-immunized mice (i.n / i.m / i.m) at days 0 (pre-immunization) and 71 (post-immunization); n=6 for each group. (B) Shannon diversity index obtained at the family level for each group. (C) Microbiota composition (normalized to 100%) at the family level obtained from fecal pellets (upper panels) pre and post-immunizations or from cecal contents (lower panels) obtained post-immunizations. “Others” represents sequences in clusters where no association has been found.



Supplementary figure 1. Ratio IgG2/IgG1 for all immunization groups with SslE. Data are from two independent experiments, where each dot represents one mouse.

Interplay between enterohemorrhagic *Escherichia coli* and nitric oxide during the infectious process

Background and summary

Enterohemorrhagic *Escherichia coli* (EHEC) represent a serious source of foodborne diarrheal illness, which can rapidly develop into life-threatening conditions such as thrombotic thrombocytopenic purpura (TTP) and the hemolytic and uremic syndrome (HUS). Patients surviving to an EHEC-mediated HUS can face important sequelae, particularly in the kidneys and central nervous system. EHEC possess two major virulence factors: the type III secretion system (T3SS), which is required for efficient colonization by adherence to the intestinal mucosa; and Shiga toxins, which induce necrosis and apoptosis of microvascular endothelial cells, and are responsible for important renal and brain lesions. Because the expression of Shiga toxins (both Stx1 and Stx2 variants) is under the control of the bacterial SOS response, treatment of EHEC infections with antibiotics, especially DNA-damaging agents like fluoroquinolones, cannot be considered. The significant morbidity and mortality rate due to EHEC, the lack of safe treatments available, and the appearance of hybrid and more virulent strains, raise high public health concerns.

Previous work in our laboratory at INRA established an *in vitro* inhibition of the expression of both the T3SS and Stx2 by nitric oxide (NO). In both cases, the inhibition was mediated by the bacterial regulator NsrR (NO sensitive repressor). NO plays an important antimicrobial and inflammatory role in the innate immune defense of the host to infection; in the murine intestine, epithelial cells, macrophages and neutrophils express the inducible NO synthase (iNOS), which is known to be highly induced upon infection with enteric pathogens like *Citrobacter rodentium* and *Salmonella* Typhimurium. During my work at INRA, we aimed at translating our previous *in vitro* findings in a mouse model of infection with EHEC O157:H7, and determine the potential impact of NO on the virulence mechanisms of EHEC and the outcome of the infection.

We established that EHEC are capable of detecting NO in the murine intestinal lumen by using a NO-sensing reporter strain. Furthermore, we used the NOS inhibitor L-NAME to determine whether NO in the murine gut could influence the adherence of EHEC to intestinal epithelial cells and/or the production of Shiga toxins. The treatment of infected mice with L-NAME led to an increase of the number of adherent-EHEC recovered in the colon, which aligns with our previous *in vitro* findings. However, L-NAME led to a consistently lower level of Shiga toxin activity found in fecal samples as compared to infected controls. Further, we found L-NAME to prevent the drop in urine specific gravity, a marker of renal failure, observed in infected mice untreated with L-NAME; both these results suggest an inducing effect of NO on the expression of Shiga toxins, unlike our *in vitro* results. Histology analyses

of the kidneys will complete these findings and indicate whether L-NAME treatment resulted in reduced renal damage compared to infected controls.

This work awaits upcoming results, but our findings are presented herein as the core parts of a manuscript to be submitted.

Manuscript

Interplay between enterohemorrhagic *Escherichia coli* and nitric oxide during the infectious process

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) are a bacterial pathogen responsible for life-threatening diseases in humans such as bloody diarrhea and the hemolytic and uremic syndrome. To date, no specific therapy is available and treatments remain essentially symptomatic. In recent years, we demonstrated *in vitro* that nitric oxide (NO), a major mediator of the intestinal immune response, strongly represses the synthesis of the two cardinal virulence factors in EHEC, namely Shiga toxins (Stx) and type 3 secretion system, suggesting NO has a great potential to protect against EHEC infection. In this study, we investigated the interplay between NO and EHEC *in vivo* using a mouse-model of infection. Using a NO-sensing strain, we determined that EHEC sense NO in the gut of infected mice via the NO-sensing protein NsrR, which is essential for an efficient gut colonization by the pathogen. Treatment of infected mice with a specific NOS inhibitor increased EHEC adhesion to colonic mucosa but unexpectedly decreased Stx activity in the gastrointestinal tract, protecting mice from renal failure. Taken together, our data indicate that NO can have both beneficial and detrimental consequences on the outcome of an EHEC infection and underline the importance of *in vivo* studies to increase our knowledge in host-pathogen interactions.

INTRODUCTION

A wide range of gastrointestinal diseases in humans are caused by bacterial infections. Invading pathogens have the capacity to adapt to the environment of the digestive tract, to resist to multiple stresses, to grow and colonize the mucosa, and finally to produce virulence factors including toxins, leading to the development of disease. Among the harmful conditions imposed by the gastrointestinal tract, pathogens must survive and adapt to oxidative and nitrosative stresses. These include exposure to reactive oxygen species (ROS) and reactive nitrogen species (RNS) liberated by mucosal immune cells in response to infection. A key component in RNS synthesis by the host is nitric oxide (NO), a highly reactive inorganic free radical. NO is produced from L-arginine by NO synthases (NOS) and particularly by the inducible NOS (iNOS) isoform under pathophysiological conditions such as infection [1]. NO can react with a large spectrum of molecules such as inorganic elements, various DNA structures, proteins and lipids, thereby carrying a strong antimicrobial activity [2]. To counter such activity, intestinal pathogens such as pathogenic *Escherichia coli*, *Salmonella* spp. and *Campylobacter jejunii*, have developed numerous mechanisms involved in NO detoxification [3]. These include globins which oxidize NO into nitrate, and reductases which reduce NO into nitrous oxide. Inactivation of these proteins leads to mutants with higher susceptibility to NO, impaired survival within macrophages and reduced virulence capacity in their respective host.

Enterohemorrhagic *E. coli* (EHEC), especially those belonging to the O157:H7 serotype, are foodborne pathogens responsible for intestinal disorders that may ultimately evolve to life-threatening diseases such as the hemolytic uremic syndrome (HUS) or the thrombotic thrombocytopenic purpura (TTP). To date, no specific therapy is available to fight EHEC infections in humans. Treatments remain essentially symptomatic and most patients require prolonged clinical and follow-up outpatient care [4]. Healthcare costs associated with such disease are thus very significant [5]. The use of antibiotics is usually not recommended since they may induce release of Shiga toxins (Stx), the main virulence factor of EHEC. Once produced in the gut lumen, Stx translocates across the intestinal epithelium, reaches the bloodstream and targets the glycolipid globotriaosylceramide-3 (Gb3) receptors of endothelial cells. The internalized Stx alters ribosomal function and induces necrosis or apoptosis of vascular endothelial cells, leading to the development of disease symptoms such as HUS and TTP [6]. Stx is a heteropolymer consisting of a catalytic A subunit and five B subunits responsible for the binding to Gb3. Two antigenically distinct forms of Stx, Stx1 and Stx2, can be produced alone or in combination by EHEC strains. Epidemiological studies have shown that Stx2-producing *E. coli* strains are more likely to cause HUS than strains that produce only Stx1 [7]. Stx-encoding genes are located in lambdoid phages integrated into the bacterial chromosome and their expression is mainly driven by activation of the SOS response [8]. Another major virulence factor in typical EHEC, in addition to Stx, is the production of type III secretion system (T3SS) implicated in the formation of characteristic attaching and effacing lesions at the surface of the intestinal epithelium [9]. The T3SS is

a protein-based complex resembling a molecular syringe that allows injection of bacterial effectors into epithelial cells where they subvert specific cell signaling pathways [10]. Among them, the protein Tir becomes inserted into the host cell membrane and acts as a receptor for the intimin protein (encoded by the *eae* gene) localized at the bacterial surface, leading to an intimate adhesion between bacteria and enterocytes. Genes encoding the T3SS are gathered into five major operons located into a pathogenicity island called the locus of enterocyte effacement (LEE).

We have previously demonstrated that subinhibitory concentrations of NO inhibit the synthesis of Stx2 by EHEC and the release of Stx2 phages via inhibition of the SOS system *in vitro* [11]. We also have recently shown that NO represses the expression of several LEE genes and, consequently, drastically reduces EHEC adhesion to epithelial cells *in vitro* [12]. Both effects are mediated by the NO responsive regulatory protein NsrR [11,12]. By repressing the production of the two major virulence factors in EHEC, combined with its typical antimicrobial effect, NO has therefore a great potential to protect against EHEC infection. As a way to counteract the host defense and the potentially deleterious impact of host-produced NO, EHEC can modulate the production of NO by eukaryotic cells. Indeed, it has been reported that EHEC inhibit iNOS expression in IFN-γ-activated human enterocytes [13]. Crosstalk occurring between EHEC and intestinal cells therefore appears to be of importance and may determine the outcome of an infection. In this context, the aim of this work was to decipher the role of NO in the control of EHEC *in vivo* using mousemodels of infection. We determined that EHEC sense NO in the gut of infected mice via the NO sensing protein NsrR, which is essential for efficient gut colonization by the pathogen. Using a specific NOS inhibitor, we demonstrated that host-produced NO inhibits EHEC adhesion to colonic mucosa but increases Stx toxicity in the gastrointestinal tract and consequently affects urine specific gravity, a marker of renal dysfunction. Taken together, our data indicate that NO can have both beneficial and detrimental consequences in the course of an EHEC infection.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table S1. When required, antibiotics were used at the following concentrations: 25 µg.ml^{-1} kanamycin; 50 µg.ml^{-1} ampicillin; 25 µg.ml^{-1} chloramphenicol; 15 µg.ml^{-1} gentamycin; 50 µg.ml^{-1} streptomycin (Sm). A streptomycin resistant derivative of the prototype EHEC strain EDL933 was used throughout the study. *nsrR* deletion was obtained by using the one-step PCR-based method [14] with primers nsrRmut-f and nsrRmut-R (Table S2). Chromosomal complementation of Δ*nsrR* mutant was obtained by using the mini-Tn7 method as described in Crepin *et al.* [15]. Briefly, *nsrR* coding sequence and its promoter region were amplified from EDL933 genomic DNA using primers nsrR+prom-F and nsrR+prom-R (Table S2), digested with *Apa*I and *Sac*I, and inserted into the corresponding sites of pGP-Tn7-Gm. The construct was then mobilized into EDL933 Sm^R Δ*nsrR* harboring pSTNSK-Cm vector (grown at 30°C) through a mating experiment. The correct clones were selected by plating on LB + gentamycin and chromosomal integration was verified by PCR using primers glmS-F and z5225-R (Table S2). All strains were grown in LB medium at 37°C unless otherwise indicated.

Construction of a NO sensing EHEC reporter strain

In order to monitor NO sensing by EHEC, we constructed a reporter strain using the TnpR recombinase-based *in vivo* expression technology (RIVET) [16]. To this end, the promoter region of gene *ytfE*, known to be highly induced by NO in *E. coli* [17], was amplified from genomic DNA of EDL933 using primers 2F10-F and 2F10-R (Table S2), which contain *Bgl*II restriction sites in their 5' ends. The PCR product and vector pGOA1193 were digested with *Bgl*II and ligated to yield plasmid p1193-*ytfE* carrying a P_{ytfE}-*tnpR* transcriptional fusion. Correct orientation of *ytfE* promoter was validated by PCR and sequencing. The plasmid was mobilized through a mating experiment into EDL-RES, an EDL933 strain harboring the RES-flanked marker cassette which contains a gene confering kanamycin resistance and a gene conferring sucrose sensitivity [18]. Plasmid insertion into the *ytfE* locus was verified by PCR and sequencing.

Resolution assays

To validate the ability of our reporter strain to detect NO, EDL-RES P_{ytfE}-*tnpR* strain was grown for 6 h in LB supplemented or not with increasing concentrations of NOR-4, a NO donor (Enzo Life Sciences). Expression of TnpR was monitored by calculating the percentage of bacteria that have lost the RES marker cassette. This percentage, termed resolution, is calculated for individual samples by dividing the titer obtained on LB without NaCl agar plates with Sm and 4 % sucrose (resolved bacteria) by the titer obtained on LB agar with Sm (total bacteria). Resolutions were also calculated

from fecal samples of mice infected with EDL-RES P_{ytfE}-*tnpR* to evaluate the sensing of NO by EHEC *in vivo* during infection.

Mouse infection

All animal experiments were reviewed and approved by the Auvergne Committee for Animal Experimentation C2EA (Agreement N°7289-2016093010075533). C57BL/6 female mice 5 weeks old with SPF status were purchased from Janvier Labs (Le-Genest-St-Isle, France) and housed in cages of no more than five mice per cage. Mouse experiments were performed with 5-10 mice per group and repeated on (at least) two separate occasions. Mice were given drinking water containing 5 g/L of streptomycin sulfate (Sigma-Aldrich) throughout the experiment. For some groups, drinking water was also supplemented with 1g/L of N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; Enzo Life Science) and changed daily over the course of the experiment. On day 1 following the addition of streptomycin, each mouse was infected intragastrically with 100 µl of PBS containing 10^7 bacterial cells of EDL933 Sm^R grown in LB for 6 h. Uninfected mice were given PBS only. At indicated time points, fecal samples were collected, homogenized in PBS and subsequently diluted before plating on LB + streptomycin agar plates or Stx activity quantification. To quantify adherent bacteria to the gut mucosa, a piece of both cecum and colon were taken at day 7, cut longitudinally and washed extensively in PBS before dilution and plating. Adhesion was expressed as a ratio between adherent bacteria and the total number of EHEC detected in fecal samples at the same time point. For co-infection experiments, mice were treated with a mix containing 10^7 each of EDL933 Sm^R and EDL933 Δ*nsrR* strains. Following plating of fecal samples on LB agar plates supplemented with streptomycin or with streptomycin + kanamycin, competitive indices were obtained by dividing the output ratio by the input ratio (WT / Δ*nsrR*). For the lethal infection model, mice also received an intraperitoneal dose of 40 µg of ciprofloxacin (prepared in 500 µl PBS) on 1, 2 and 3 days post-infection (DPI) (adapted from[19]). Body weight and clinical signs of mice were monitored daily to evaluate the severity of infection. Mice presenting weight loss > 15% compared to body weight at day 0, or presenting severe clinical symptoms such as ataxia and lethargy, were immediately eithanized. Urine was collected daily for determination of the specific gravity using a refractometer, as well as urea concentration using the QuantiChrom Urea assay kit (BioAssay Systems)

Quantification of Stx activity

The Stx activity from different samples was monitored using the Vero-d2EGFP cell line [20]. The cell line was maintained and propagated routinely at 37 °C with 5% CO_2 under humidified conditions in a complete medium made of DMEM supplemented with 10% fetal bovine serum (Gibco), Zell Shield (Minerva Biolabs) and 200 $\mu g.ml^{-1}$ Geneticin (Gibco). For the assays, Vero-d2EGFP cells were seeded in black 96-well plates with clear bottoms at 3.10^4 cells per well and incubated for 3 days to reach 80-90 % confluence. Samples to be tested as well as purified Stx2 (Toxin Technology) used as an internal

standard were two-fold diluted in complete medium and then transferred to Vero-d2EGFP-containing plates. After a 16 h period of incubation in a CO_2 incubator, samples were removed and 100 µl of PBS was added to each well before GFP quantification in a Spark microplate reader (Tecan) with excitation at 485 ± 20 nm and emission at 530 ± 20 nm. Stx activity was expressed as an arbitrary unit by comparing fluorescence values from samples with the standard curve obtained with purified Stx2.

Statistical analyses

All statistics were made using GraphPad Prism software (San Diego, California). Unpaired two-tailed student's *t* test was used to determine significant differences between two groups. ANOVA with the Holm-Sidak test was used to analyze differences among multiple groups. The Log-rank (Mantel-Cox) test was used to compare survival curves; $P < 0.05$ was considered significant.

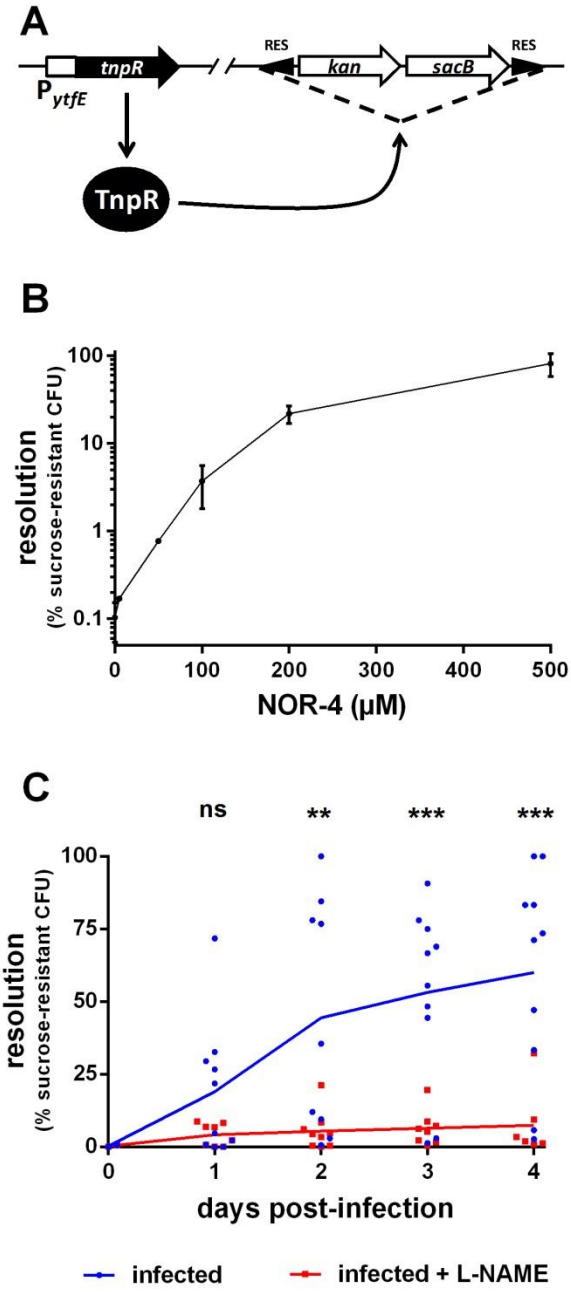

Figure 1. EHEC sense NO in the gut of infected mice. (A) Schematic representation of the NO sensing reporter system. The EHEC EDL933 strain carries a RES-flanked marker cassette and a P_{ytfE}-*tnpR* transcriptional fusion. In the presence of NO, P_{ytfE} is activated, leading to the expression of TnpR recombinase and excision of the cassette. The strain becomes kanamycin sensitive and sucrose resistant. **(B)** The NO reporter strain was grown for 6 h in LB supplemented with various concentrations of NOR-4. Resolutions (percentage of bacteria that have lost the RES marker cassette) were calculated following bacterial numeration on plates with or without sucrose. Values represent mean +/- standard deviation. **(C)** Mice, treated or not with the NOS inhibitor L-NAME, were infected with an EHEC NO reporter strain. At the indicated time points, resolutions were calculated from fecal samples. Each dot represents one mouse and curves represent mean values. A two-tailed unpaired t-test was applied to compare both groups. ns: non significant; ** P<0.01; *** P<0.001.

RESULTS

EHEC sense NO in the gut of infected mice

Before investigating the role of NO in the control of EHEC infection, we first wanted to evaluate if EHEC are able to sense NO in the gut of infected mice. To this end, we constructed an EHEC EDL933 strain that reports the detection of NO using elements from the RIVET system [16], a RES cassette containing two selective markers, and the reporter gene *tnpR*, which encodes a recombinase that specifically cleaves the marker cassette (Fig. 1A and see M&M). The reporter gene was placed under the control of the promoter region of gene *ytfE*, whose expression is known to be highly upregulated in the presence of NO through the release of repressing activity from NsrR, a major NO-sensing regulatory protein in *E. coli* [17,21]. To validate the efficiency of our reporter strain to detect NO, the strain was grown in LB with various concentrations of NOR-4, a NO donor (Fig. 1B). As expected, the resolution (i.e. the percentage of bacteria that have lost the RES cassette) from a bacterial culture without NOR-4 was very low, indicating that the P_{ytfE}-*tnpR* fusion was not expressed in the absence of NO. In contrast, the addition of increasing concentrations of NOR-4 led to a strong increase of the resolution, reaching more than 80% with 500 µM of NOR-4 (Fig. 1B). This data demonstrates that our reporter strain efficiently detects the presence of NO in the surrounding environment. Next, we infected mice with this reporter strain and monitored the resolution status of EHEC recovered from feces of animals over time. As shown in Figure 1C, the resolution was very low at the beginning of infection and increased gradually at 1, 2, 3 and 4 days post-infection (DPI). As a control, we also infected mice treated with L-NAME, a specific inhibitor of NO synthase (NOS) activity. The resolution level did not increase over time and was significantly different from the resolution obtained from untreated mice at days 2, 3 and 4. This result clearly indicates that EDL933 detects host-produced NO in the gut during mouse infection. Furthermore, we examined whether the NO-sensing protein NsrR plays a role in EHEC fitness during mouse infection. Mice were co-infected with an equal ratio of wild-type (WT) and Δ*nsrR* strains of EDL933 and both populations were monitored over the course of infection. As shown in Figure 2A, the WT strain was detected in significantly higher concentrations than the Δ*nsrR* strain in feces at 3 DPI. This competitive advantage was specific to *nsrR* as shown by a competition assay between Δ*nsrR* and a Δ*nsrR* complemented strain (Fig. S1). Determination of the competitive index for individual animals confirmed that the WT strain outcompetes the Δ*nsrR* mutant *in vivo* (Fig. 2B). Taken together, these data demonstrate that EHEC detect NO in the gut of infected mice and that the NO-sensing transcription factor NsrR is required for the pathogen to efficiently colonize the mouse gut.

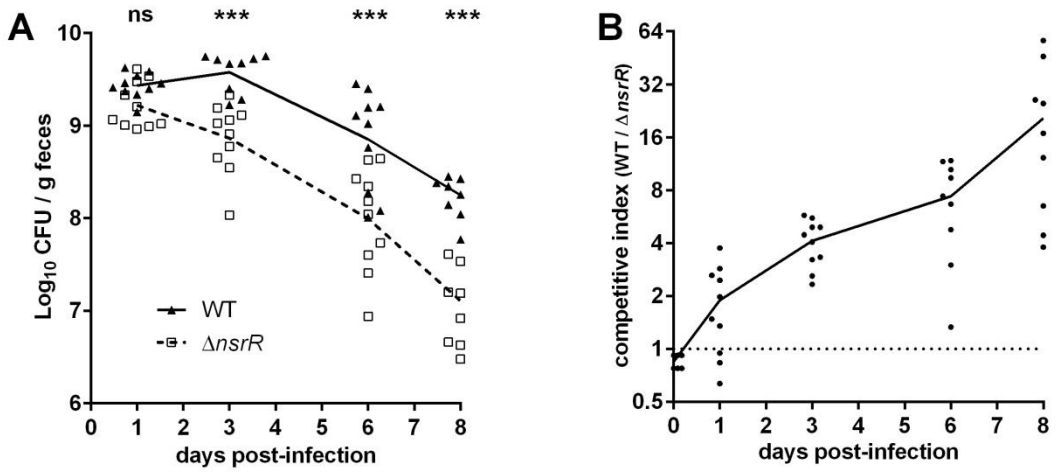

Figure 2. NsrR is required for an efficient colonization of the mouse gut by EDL933. Mice were co-infected at a 1:1 ratio with wild-type (WT) and Δ*nsrR* EDL933 strains. At the indicated time points, concentrations of each strain in feces **(A)** and corresponding competitive indices **(B)** were determined by plating samples on LB + specific antibiotic plates. Each dot represents one mouse and curves or lines represent mean values. A multiple two-tailed unpaired t-test was applied to compare both groups each day. ns: non significant; *** P<0.001.

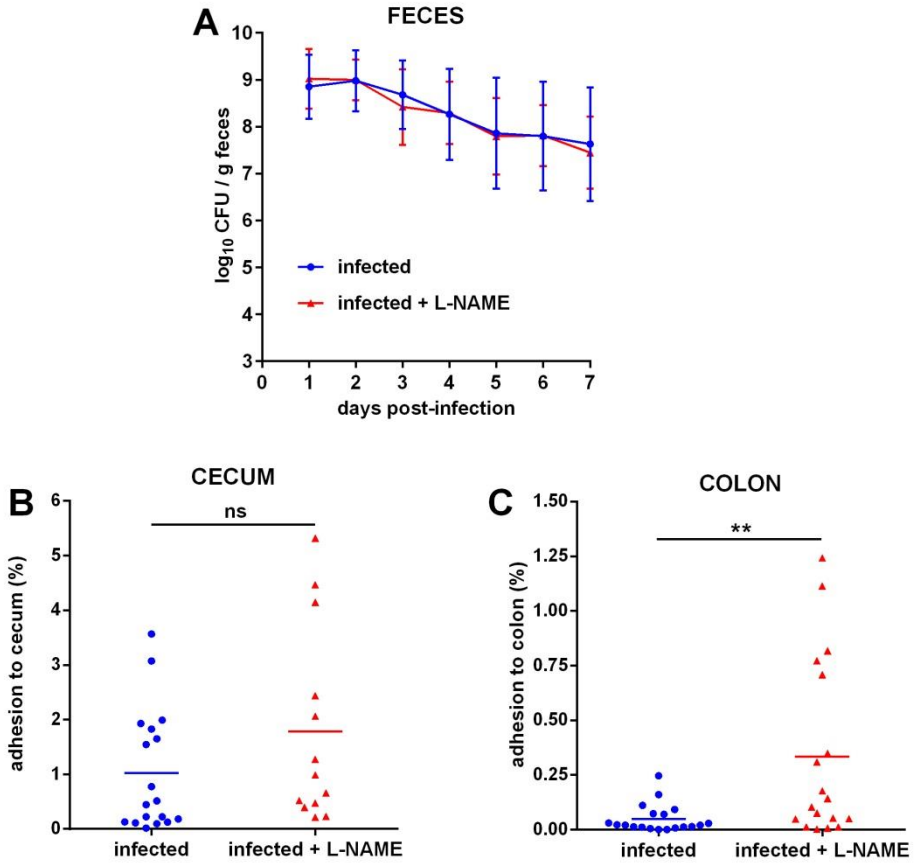

Figure 3. L-NAME treatment of infected mice increases EHEC adhesion to the colonic mucosa. Mice, treated or not with L-NAME, were infected with EDL933. **(A)** At the indicated time points, EHEC shedding was quantified by plating fecal samples on LB + Sm plates. Values represent mean +/- standard deviation. **(B)** and **(C)** At day 7 post-infection, mice were euthanized and cecum and colon were sampled, washed in PBS, crushed and then plated on LB + Sm plates in order to quantify mucosa-associated EHEC. Data are represented as the percentage of adherent bacteria relative to the total number of EHEC quantified in fecal samples. Each dot represents one mouse and mean are indicated as a line. A two-tailed unpaired t-test was applied to compare both groups. ns: non significant; ** P<0.01.

NOS inhibition favors EHEC adhesion to the colonic mucosa

We have previously shown that NO inhibits EHEC adhesion to epithelial cells *in vitro* [12]. To assess if NO affects the ability of EHEC to adhere to the gut epithelium *in vivo*, mice treated or not with L-NAME were infected with EDL933. As previously observed [22], EHEC infection of streptomycin-treated mice allows a stable establishment of the pathogen within the gastrointestinal tract without weight loss or development of clinical symptoms. Addition of L-NAME in the drinking water of animals affected neither these parameters nor the excretion level of EDL933 in feces (Fig. 3A and data not shown). No difference was observed between the two groups in terms of bacterial adherence to the cecal tissue at 7 DPI either (Fig. 3B). In contrast, the proportion of EHEC adherent to the colon was significantly increased by a factor 7 in mice treated with L-NAME (Fig. 3C). These data demonstrate that alteration of NOS activity in infected mice limits EHEC adherence to colonic epithelium.

NO enhances Stx toxicity in the gut lumen

Previous work from our laboratory has determined that NO also inhibits the production of Stx2 during *in vitro* growth of EDL933 [11]. We therefore investigated whether mouse treatment with the NOS inhibitor L-NAME affects the level of Stx activity in the gut of EDL933-infected mice. Following infection, fecal samples were collected daily and Stx activity was quantified using Vero-d2EGFP, a Stx-sensitive cell line harboring a destabilized variant of GFP used to monitor protein synthesis inhibition [20]. Unexpectedly, Stx activity was significantly lower in the gut of infected mice treated with L-NAME versus untreated mice (Fig. 4). Indeed, the toxin activity was reduced from 1 to 5 DPI with a decrease fold change ranging from 2 to 6. No Stx activity was detected in urine (day 6) or serum (day 7) of infected mice from either group (data not shown). These data indicate that NO produced by the host during infection increases the cytotoxicity associated to Stx.

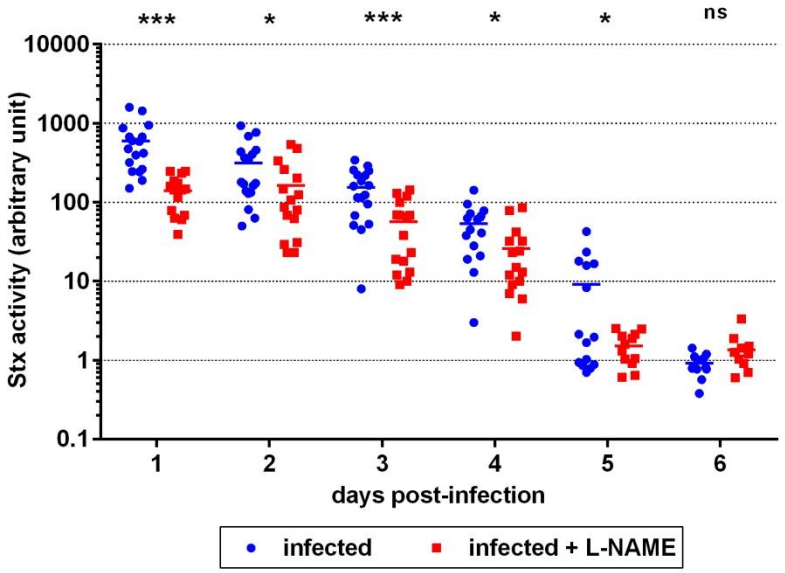

Figure 4. L-NAME treatment of infected mice limits Stx toxicity in the gut. Mice, treated or not with L-NAME, were infected with EDL933. At the indicated time points, Stx activity from feces was quantified using Vero-d2EGFP cell line. Each dot represents one mouse and means are indicated as a line. A multiple two-tailed unpaired t-test was applied to compare both groups every day. ns: non significant; * P<0.05; *** P<0.001.

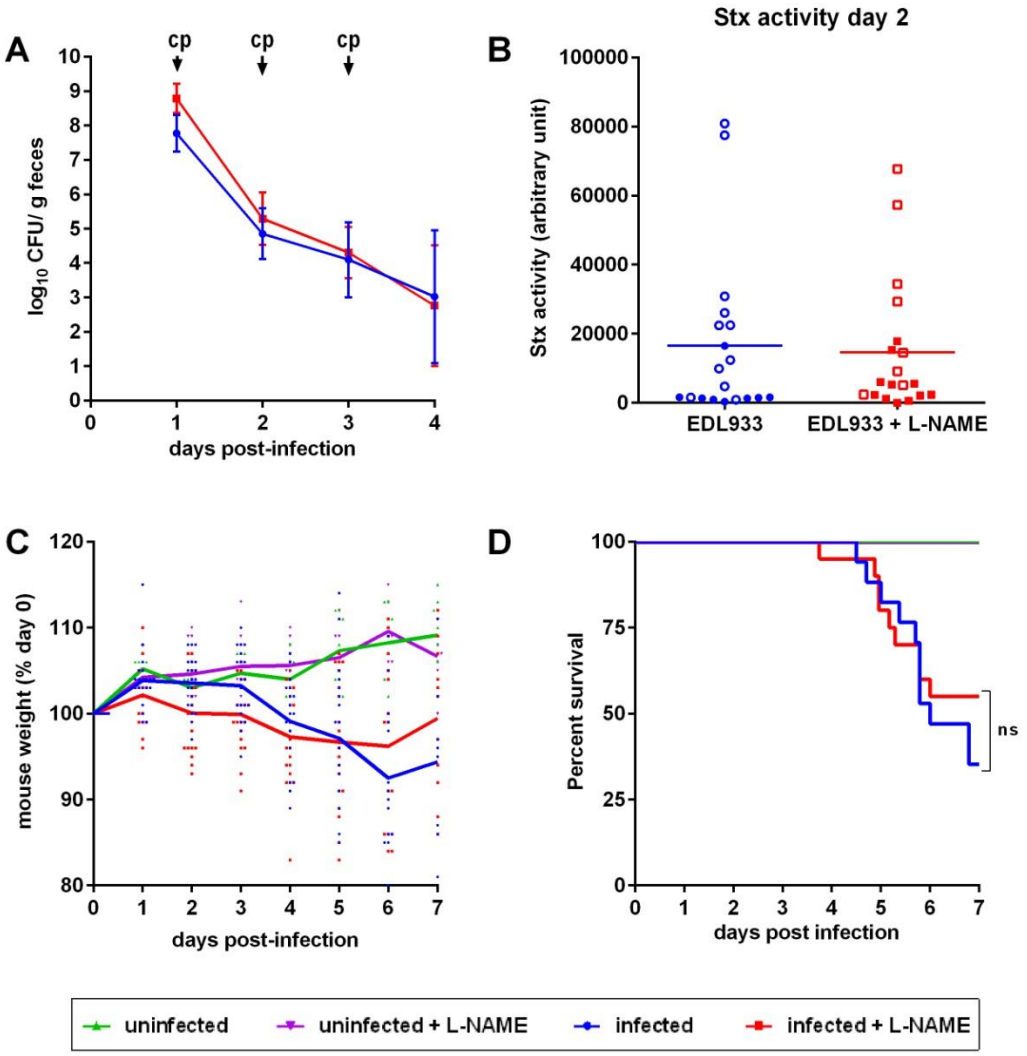

Figure 5. Role of NO in a lethal EHEC infection model. Mice, treated or not with L-NAME, were left uninfected or were infected with EDL933. At days 1, 2 and 3 post-infection, each mouse was injected 40 µg of ciprofloxacin (Cp) intraperitoneally to induce Stx production and release. **(A)** At the indicated time points, EHEC shedding was quantified by plating fecal samples on LB + Sm plates. Values represent mean +/- standard deviation. **(B)** At day 2 post-infection, Stx activity from feces was quantified using Vero-d2EGFP cells. Each dot represents one mouse and means are indicated as a line. Opened and closed symbols indicate died and alive mice 7 days post-infection, respectively. **(C)** Mouse weight was determined every day post-infection and weight curves are presented as the percentage relative to animal weight at the day of infection (day 0). Each dot represents one mouse and curves represent mean values. **(D)** The survival time and rate were recorded for 7 days after infection. No significant difference was observed between infected and infected + L-NAME groups by Log-rank (Mantel-Cox) test (n= 20 per group).

NO exacerbates renal failure following a lethal EHEC challenge

We next investigated the role of NO in a lethal EHEC infection model. Following infection of mice treated or not with L-NAME, intraperitoneal injections of ciprofloxacin were performed in order to boost Stx production (see M&M). As a consequence of Stx prophage lytic cycle induction, ciprofloxacin treatment led to a massive decrease of EDL933 in feces as well as a high level of toxin produced as determined by the measurement of Stx activity from feces (Fig. 5A and compare Fig. 4 and 5B). When compared to control mice, mice infected with EDL933 significantly lost weight, developed clinical signs of illness and eventually died (Fig. 5C and 5D). As expected, the development of disease symptoms was strongly associated with Stx production since death occurred for mice that had the highest Stx activity levels (Fig. 5B, opened symbols). Addition of L-NAME to the drinking water of mice did not significantly affect these parameters (Fig. 5). We next determined the urine specific gravity (USG), a consistent marker of renal disease in EHEC-infected mice [23,24]. Whereas the USG was stable overtime for control mice, the USG decreased for EDL933-infected mice, starting from 3 DPI (Fig. 6). Interestingly, the USG did not change for infected mice treated with L-NAME and values were significantly different compared to those from untreated infected mice at 4 to 7 DPI (Fig. 6). Lower USG values recorded from infected mice were correlated with a decrease of urea concentration in urine (Fig S2), strongly suggesting an alteration of renal function in these animals. Altogether, these data suggest that inhibition of NOS activity by L-NAME reduces renal damages provoked by Stx in EHEC-infected mice.

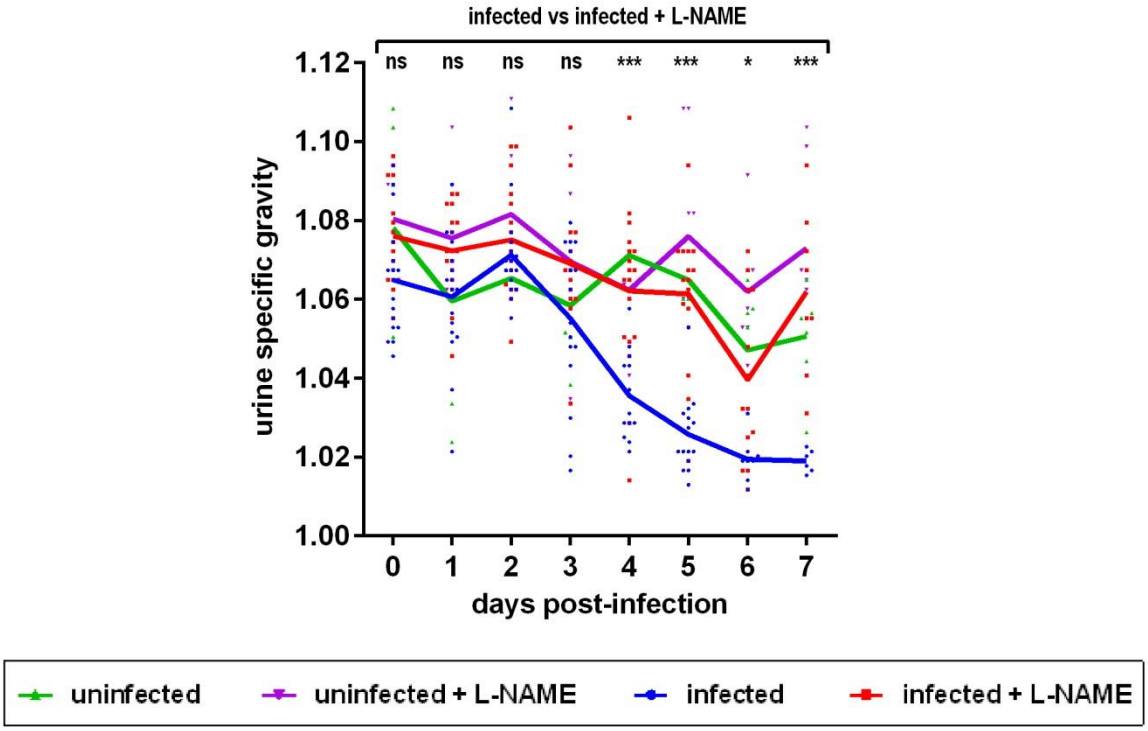

Figure 6. L-NAME treatment limits renal failure in EDL933-infected mice. Mice, treated or not with L-NAME, were left uninfected or were infected with EDL933. At the indicated time points, urine was collected

from each animal and urine specific gravity was quantified using a refractometer. Each dot represents one mouse and curves represent mean values. An ANOVA with the Holm-Sidak test was applied to compare all groups each day. ns: non-significant; * $P<0.05$; *** $P<0.001$.

DISCUSSION

During the past decades, NO has been recognized as an important player of the immune system, participating notably to host defense against infectious agents [1]. Indeed, NO exerts a potent antimicrobial activity against many bacteria, viruses, fungi and parasites as demonstrated by *in vitro* studies [25,26]. NO also alters the synthesis of virulence factors in some pathogens through inactivation of virulence-associated transcription factors, especially iron-containing proteins [27]. In EHEC, we have demonstrated *in vitro* that NO inhibits the synthesis of two critical virulence factors, Stx2 and T3SS, via the NO-sensing regulator NsrR [11,12]. Expression of others virulence determinants is probably also affected by NO since they are controlled by regulatory circuits that can be disturbed following NO exposure [28-30]. The aim of this study was to evaluate the potential role of NO in the control of an EHEC infection. We first determined that EHEC sense NO in the mouse gut as early as the first day of infection using an *in vivo* reporter system based on the expression of NsrR-dependent gene *ytfE*. To our knowledge, this is the first time that detection of NO by an intestinal extracellular pathogen has been directly demonstrated. When mice were treated with the NOS inhibitor L-NAME, expression of *ytfE* was abrogated during the first 4 days following infection, validating the inhibition of NOS activity by L-NAME in our *in vivo* model. However, the NO-sensing strain resolved in some animals between 5 and 7 DPI (data not shown). A study has shown that administration of L-NAME to rats does not necessarily result in a sustained suppression of NO synthesis 7 days post-treatment because of a compensatory expression of the *Nos2* gene [31]. Such mechanism might explain why EHEC have detected NO in some individuals after day 5. Otherwise, detected NO might come from the synthesis by some bacterial species [3] potentially present in the mouse gut microbiota. We also showed that the NO-sensing regulator NsrR is essential for efficient fitness of EHEC in the gastrointestinal tract. This demonstrates that appropriate expression of NsrR-dependent genes is required for EHEC to manage its gut colonization process.

The physiological consequences of NOS inhibition in EHEC-infected mice was then evaluated at several scales. While no difference was observed in bacterial shedding, the proportion of mucosa-adherent EHEC was significantly increased in the colon of mice treated with L-NAME at 7 DPI. However, this result should be interpreted with caution as it can be the consequence of either NO suppression by L-NAME treatment or compensatory increase of NO synthesis [31]. Nevertheless, this anti-adhesion property of NO is in accordance with a previous study which determined that chemical or cellular sources of NO inhibits EHEC adhesion to intestinal epithelial cells grown *in vitro* [12]. In addition, NO seems to have general anti-bacterial adhesion properties as determined by its efficiency to limit adhesion of several Gram-positive and Gram-negative bacteria to abiotic surfaces [32].

We also quantified the impact of NOS inhibition on Stx production in the gut of infected mice. Until day 5, mouse treatment with L-NAME led to a significant decrease of Stx cytotoxic activity recorded from fecal samples. These results were unexpected since our team demonstrated that NO

inhibits Stx2 synthesis during standard *in vitro* growth conditions [11]. Differences in the quantification methods (Stx1 and Stx2 activities using VERO cells in this study vs Stx2 quantity by ELISA in Vareille et *al.*) are probably not responsible for this discrepancy. In addition, a recent study demonstrated that NO enhances the production of Stx1 and Stx2 in EHEC grown under *in vitro* anaerobic conditions in a RecA-and Fur-dependent way [33]. Because the gastrointestinal tract is an anaerobic milieu, it may thus not be surprising that NO would increase the synthesis of Stx1 and Stx2 in the gut of infected mice, which would explain why we observed an inhibition of Stx toxicity following L-NAME treatment. Furthermore, to define the potential role of NO in EHEC-associated symptoms, we used a lethal model of infection consisting of ciprofloxacin injections to EHEC-infected mice in order to boost Stx phage induction and toxin release. In this model, the renal function was seriously affected in EHEC-infected mice, as determined by USG values. Interestingly, L-NAME treatment seems to prevent renal failure in infected mice, suggesting that NO production in response to infection can be detrimental to the host. As observed in the non-lethal mouse model of infection, NO might have increased Stx synthesis in the gut to levels sufficient to provoke a renal failure. However, we were not able to reproduce this statement at a significant level in infected-mice treated with ciprofloxacin, probably as a result of heterogeneous animal response to antibiotic injections. Another possible explanation could be linked to a better translocation of Stx across the gut mucosa in the presence of NO. Indeed, it has been reported that NO contributes to an intestinal barrier dysfunction in rodents via an increase of intestinal permeability [34,35]. Because NO has pleiotropic effects in the organism, we cannot exclude that NO worsens the alteration of renal function in the presence of Stx. NO is a well-known player acting on blood pressure as well as on renal excretory functions. In particular, Inhibition of NO synthesis has been shown to decrease renal blood flow and sodium excretion in the urine [36,37].

Altogether, our results demonstrate that the production of NO in response to EHEC infection can be detrimental to the host, in particular to the renal function. Despite the protective properties attributed to NO against many infectious agents from *in vitro* studies, the effect of NO in controlling pathogens *in vivo* is however more complex, as exemplified by our study. While an iNOS-dependent production of NO has been shown to protect mice from several pathogens such as *Listeria monocytogenes* or *Leishmania major* [38,39], iNOS expression has no discernible effect or even worsens the disease symptoms for other infectious agents. In *Salmonella* infections, the iNOS gene expression is highly induced in tissue of infected mice [40] but the NO subsequently produced appears to have contradictory effects. Whereas NO limits the colonization of Peyer's patches by the pathogen as well as its replication in the spleen and liver [41,42], NO and the ensuing proinflammatory cascade also promote the fitness of *Salmonella* and allow the pathogen to outcompete the intestinal microbiota [43,44]. In neonatal meningitis caused by *E. coli*, iNOS-dependent NO has serious detrimental impact on the outcome of infection, since it promotes development of bacteremia as well as disruption of the blood-brain barrier [45,46]. As in the case of *E. coli*-associated meningitis, our study showed that

production of NO by the host may exacerbate the severity of symptoms caused by an EHEC infection. This work pushes forward an essential role of an NO-EHEC interplay in the outcome of an infection and may assist future works to evaluate the efficiency of novel therapeutic strategies based on the modulation of NO concentration either by synthesis, delivery or scavenging.

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SUPPLEMENTARY FIGURES AND TABLES

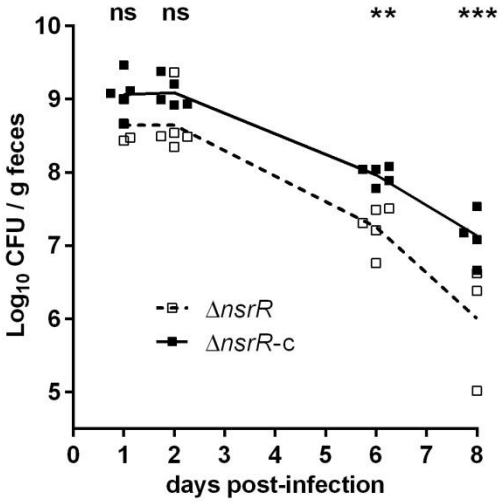

Figure S1: Mice were co-infected at a 1:1 ratio with Δ*nsrR* and Δ*nsrR*-c EDL933strains. At the indicated time points, concentrations of each strain in feces were determined by plating samples on LB + specific antibiotic plates. Each dot represents one mouse and curves represent mean values. A multiple two-tailed unpaired t-test was applied to compare both groups each day. ns: non significant; ** P<0.01; *** P<0.001.

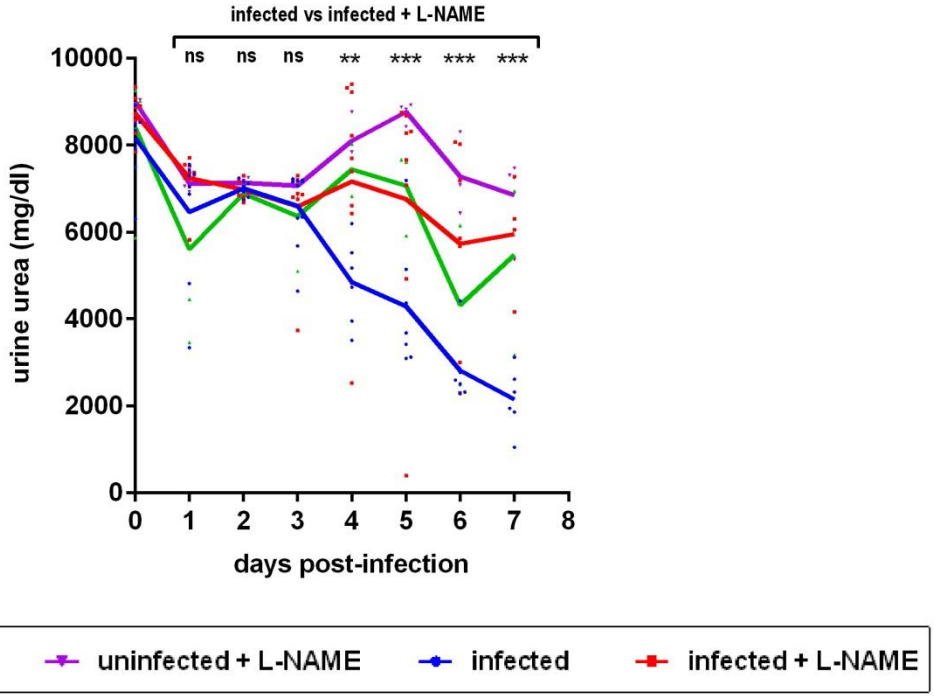

Figure S2: Mice, treated or not with L-NAME, were left uninfected or were infected with EDL933. At the indicated time points, urine was collected from each animal and urine urea was quantified. Each dot represents one mouse and curves represent mean values. An ANOVA with the Holm-Sidak test was applied to compare all groups each day. ns: non-significant; ** P<0.01; *** P<0.001.

Table S1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
EDL933 Sm^R	Streptomycin-resistant derivative of EDL933	[18]
EDL933 Δ*nsrR*	EDL933 Sm^R Δ*nsrR*, Sm^R, Kan^R	this study
EDL933 Δ*nsrR*-c	EDL Sm^R Δ*nsrR att*Tn7::*nsrR*, Sm^R, Kan^R, Gm^R	this study
EDL-RES	EDL933-Sm^R containing *res-kan-sacB-res* cassette, Sm^R, Kan^R, Suc^S	[18]
EDL-RES P_{ytfE}-*tnpR*	EDL-RES carrying a P_{ytfE}-*tnpR* fusion; Sm^R Kan^R Gm^R Amp^R	this study
Plasmids		
pSTNSK-Cm	*oriSC101*(Ts) *tnsABCD*; Km^R, Cm^R	[15]
pGP-Tn7-Gm	*oriR6K mobRP4 Tn7-Gm;* Ap^R Gm^R	[15]
pGOA1193	*oriR6K mobRP4 lacZ tnpR*; Ap^R	[16]
p1193-*ytfE*	pGOA1193 P_{ytfE}-*tnpR*; Ap^R	this study

Table S2: Primers used in this study.

Name	Sequence 5' – 3'
nsrRmut-F	GTGCAGTTAACGAGTTTCACTGATTACGGATTACGTGCGCTGATCTACATGTGTAGGCTGGAGCTGCTTC
nsrRmut-R	GCAAGCGTGTAGTTATCCAGTTCCGTAAGAAAACTTTGCACGGCCTTAGACATATGAATATCCTCCTTAGTTCC
nsrR+prom-F	GATCGGGCCCTTCGACGCGTAATTCTGG
nsrR+prom-R	AAGCTGGAGCTCGAAGATTTTCGTCACTCCACCAGC
glmS-F	CACCAATCTTCTACACCGTTCCGC
z5225-R	TCCACAACTATGAATTCGCGTAGA
2F10-F	CTGCAGGAAGATCTGTGGTCATCGCGGTTAGAGC
2F10-R	GATCGTGAAGATCTCGATAAGCCATAGCTGATACCTCATTC

General discussion

Pathogenic *E. coli* cause significant morbidity and mortality worldwide. *E. coli* subspecies have indeed evolved to thrive as successful pathogens: they can persist in both the host and the environment, display resistance to treatment with multiple antibiotics, rapidly diversify their antigenic repertoire, and escape the immune system. These remarkable features have hampered our efforts to find efficient and/or long-term therapeutics. The Marie Sklodowska-Curie program DISCo enlisted four PhD fellows, myself included, to tackle the exciting and challenging task of laying a foundation for a novel, promising broad-spectrum vaccine against pathogenic *E. coli*. In a collaborative effort between GSK and INRA, the projects developed were destined to identify new antigens in pathogenic *E. coli* to develop a vaccine, to characterize the immune response after immunization with pathogenic *E. coli* antigens, and to select adjuvants to effectively enhance the potency of immunizations.

The first aim of my PhD project was to characterize the immune response to antigen SslE, an *E. coli* mucin-degrading metalloprotease that had been previously identified as a strong vaccine candidate against various ExPEC strains. With the goal of eventually using this antigen as part of a vaccine against both ExpEC and InPEC, our strategy was to design an immunization protocol with SslE that would induce both an intestinal and systemic immune responses. Therefore, we placed a particular emphasis on aiming for the appropriate route of immunization that would best induce the onset of both mucosal and systemic responses to our antigen. To this end, I set up three immunization protocols, with three immunizations per protocol, that I performed in parallel: an intranasal (i.n) only regimen, an intramuscular (i.m) only regimen, and an i.n prime followed by two i.m boosts (referred to as the mixed immunization regimen, or i.n / i.m / i.m).

Through the various immunological readouts analyzed, we have shown that the mixed immunization regimen (i.n / i.m / i.m) with SslE was our best model of immunization, when compared with the i.n-only or i.m-only immunization regimens. Indeed, mice immunized i.n / i.m / i.m with SslE mounted robust cellular and humoral immune responses specific to SslE, both locally and systemically. Specifically, we recovered: i) Th-like cytokines in lamina propria lymphocytes after recall with SslE, ii) anti-SslE SIgA titers in intestinal washes and fecal pellets iii) $CD4^+$ T cells specific to SslE in splenocytes, and iv) anti-SslE IgG titers in post-immunization sera. Some immunological readouts from the small intestines, notably Th17-like cytokines and SIgAs from intestinal washes, were comparable between the i.n / i.m / i.m and the i.n-only regimens. Several studies have observed Th17 and mucosal IgA responses post-infection with various InPEC (Wenneras, Qadri et al. 1999, Atarashi, Tanoue et al. 2015, McArthur, Maciel et al. 2017), results which lend credence to our findings from the i.n-only and the mixed immunization regimens. That said, the systemic B and T-cell immune responses obtained from the mixed immunization regimen significantly surpassed those from

the i.n-only or the i.m-only regimen. These latter results strongly influenced our evaluation of the various immunization schemes' efficiency; should we envision antigen SslE as part of a potentially broad-spectrum vaccine against InPEC and ExPEC, a systemic immune response would be particularly valuable in countering *E. coli* pathotypes capable of disseminating in the bloodstream, such as NMEC and UPEC.

As the gene encoding SslE is known to be present in some commensal *E. coli* strains, we sought to analyze the potential impact of SslE immunizations on the composition of the gut microbiota. Using the i.n / i.m / i.m immunization regimen, we showed that immunizations with SslE did not significantly change the richness or the composition of the fecal microbiota in conventional immunized versus naïve mice, as we recovered the same families and genera between these two groups pre- and post-immunization. In accord, we found no significant differences between the cecal microbiota of immunized mice and naïve mice post-immunizations.

Overall, the i.n / i.m / i.m immunization schedule with antigen SslE induced a good intestinal and systemic immune response against SslE without significantly perturbing the gut resident microbiota. These results certainly warrant further investigation on the suitability of antigen SslE as a component of a pathogenic *E. coli* broad spectrum vaccine.

An important follow up to our work is certainly to carry out infection challenges to establish a proof of protection after an i.n / i.m / i.m immunization regimen with SslE. SslE was previously used in immunization and challenge studies, which I will discuss briefly. SslE was shown to provide an 82% protective efficacy against an NMEC strain (IHE3034, the strain from which the gene encoding SslE was selected) by immunizing mice three times subcutaneously (Moriel, Bertoldi et al. 2010). Another study used i.n-only immunized mice and challenged them with an ETEC oral gavage, resulting in a greater than two-log decrease of CFU from the cecum. Additionally, mice immunized i.n with SslE (with cholera toxin as an adjuvant) and infected transurethrally with a UPEC strain had a significant reduction of CFU in the kidney and spleen compared to naïve mice, though notably, there was no reduction of CFU in the bladder (Nesta, Valeri et al. 2014). All of these various immunization schedules used different mice, quantities of antigen SslE, durations between immunizations, and routes of immunizations compared to our i.n / i.m / i.m immunization regimen. Given this variability, it is difficult to make specific comparisons between these studies and ours; hence, carrying out infection challenges to validate efficacy of our i.n / i.m / i.m immunization regimen with SslE would be an essential step toward the development of SslE as a vaccine.

Among the various *E. coli* pathotypes that we could choose from, an ExPEC and an InPEC challenge experiment would be an important proof-of-concept starting point. Of interest, the

previous results we have obtained from the UPEC challenge could be used as a means of comparison and determine the potential benefit of our i.n / i.m / i.m immunization model. In this regard, a reduction of bladder colonization compared to naïve mice would be particularly encouraging. Infections with InPEC strains in mice come with challenges, as many pathotypes are poor colonizers of the murine gut and do not cause disease; to circumvent this problem, mouse models of infections, such as for EPEC, ETEC, or EHEC, have been established in mice pre-treated with streptomycin or in germ-free mice (Savkovic, Villanueva et al. 2005, Allen, Randolph et al. 2006, Eaton, Friedman et al. 2008). Considering the significant impact of the microbiota on the development of the gut immune response, germ-free mice could only be considered in passive immunization experiments by transfer of either T cells or serum antibodies before infection challenge. Such a set up would be informative in determining a particular correlate of protection, though it would unfortunately not allow for transfer of the mucosal resident immunity, which most likely represents an important arm behind protection efficacy to SslE-expressing *E. coli* pathogens. We would therefore most likely use a streptomycin model of infection. Keeping that in mind, the use of EPEC as a challenge model, which has not yet been performed, could provide interesting data in our study.

A crucial component in the elaboration of antigen vaccines is the addition of an adjuvant in the formulation. In our study, the use of CT as an adjuvant for intranasal immunizations was guided by the extensive characterization of CT as a potent mucosal adjuvant (Lycke and Holmgren 1986, Mattsson, Schon et al. 2015, Tsai and Wu 2015). Another strong mucosal adjuvant, similar to CT, is the ETEC LT enterotoxin (Lycke, Tsuji et al. 1992, Katz, Lu et al. 1997); however, the toxicity of both CT and LT prevents their use in human vaccines. Several attempts have been made to produce variants CT and LT with attenuated toxicity (Freytag and Clements 2005, Norton, Lawson et al. 2012). One of the latest attempts is a double mutant of LT (dmLT) that has been extensively studied for its suitability as a safe and potent mucosal adjuvant, and results have so far been very promising (Norton, Lawson et al. 2011). dmLT carries a double mutation in the A subunit, which considerably reduces LT-mediated toxicity while retaining the potent adjuvanticity of LT (Norton, Lawson et al. 2011). In animal models, dmLT has been used in formulation with several antigens, such as *Helicobacter pylori* and inactivated polio, and has proved very efficient at enhancing immune responses (Summerton, Welch et al. 2010, Norton, Bauer et al. 2015). Furthermore, dmLT has been incorporated as an adjuvant for an ETEC vaccine in human clinical studies; the outcome of that study revealed that the formulations were well tolerated, and that dmLT could not only improve mucosal immune response and vaccine efficacy, but also lower the antigen dose, two essential parameters in the selection of an adjuvant (Lundgren, Bourgeois et al. 2014,

Bourgeois, Wierzba et al. 2016). Current work on dmLT aims to improve further formulations with dmLT to minimize formation of aggregates and optimize the formulation stability in storage (Toprani, Hickey et al. 2017). Thus, as dmLT appears to indeed be a safe and potent mucosal adjuvant for human use, it would be interesting to test dmLT as a CT alternative in our i.n / i.m / i.m immunization regimen with SslE. Strategically, the addition of dmLT in our formulations could offer a benefit not only as an adjuvant, but also as an ETEC antigen. Indeed, mice immunized with dmLT as an adjuvant mount antibodies against the native LT enterotoxin (Norton, Lawson et al. 2011). It has long been known that an immune response specific to LT offers strong protection, although short term, against ETEC. In fact, the cholera vaccine Dukoral®, which targets CT, can also serve as a short-term vaccine against ETEC, as Dukoral® affords significant cross-protection against LT-producing ETEC (Clemens, Sack et al. 1988, Jelinek and Kollaritsch 2008).

Cross-protection studies with antigen SslE have shown efficacy against heterologous ExPEC strains, though there is a decrease in the protective efficacy that correlates with diminishing amino acid identity of the heterologous strain SslE compared to NMEC IHE3034 SslE used in immunization. Similarly, while protection from colonization by InPEC strains has been observed after immunization with SslE, it seems likely that full protection will not be attained, even in the context of our new i.n / i.m / i.m immunization regimen. Finally, although analysis of the presence and expression of the SslE-encoding gene revealed a high prevalence of SslE in most extraintestinal and intestinal pathotypes, the coverage does not encompass all strains, especially in the case of EHEC. From these findings, we believe that SslE should be used, rather than in standalone formulations, as part of a multi-component vaccine in order to maximize coverage and efficacy against both InPEC and ExPEC. With this strategy in mind, the DISCo program had an entire PhD project dedicated to the search of antigens distributed throughout InPEC pathotypes that would be surface-expressed or secreted, via a reverse vaccinology approach similar to the one used to identify SslE. This project led to the identification of at least one antigen that proved to confer protection upon challenge with an EHEC O157:H7 strain in immunized versus naïve mice. Certainly, an exciting future step would be to combine both this antigen and SslE in our i.n / i.m / i.m immunization regimen to evaluate a potential broader coverage mediated by this combination.

Another interesting, though bold prospect would be to incorporate an *E. coli* antigen present in the core genome, that is, one that is present in all *E. coli* strains, pathogenic and commensal alike. This idea was actually pursued by the laboratory of Mark Schembri, where they started by defining a core and accessory *E. coli* genome from 1700 draft and complete genomes of *E. coli* available (Moriel, Tan et al. 2016). From this library, they identified a novel core antigen, named YncE, present in more than 99% of all *E. coli* genomes available

(of note, SslE is present in 70% of all genomes throughout the library), and secreted by at least several different *E. coli* pathotypes as well as the commensal strain MG1655. They went on to use YncE in an immunization study, where YcnE conferred significant protection against a UPEC bacteremia model of infection. Additionally, YncE is known to mount an antibody response in humans, as observed from the sera of convalescent urosepsis patients (Moriel, Tan et al. 2016). This latter finding is particularly interesting since these patients produce antibodies targeting an antigen potentially expressed by *E. coli* members of the resident gut microbiota. Though no study was done to assess whether these antibodies were actually functional (that is, neutralizing antibodies), this raises the important question of whether targeting the commensal *E. coli* population would have any deleterious impact in the overall health in humans. In the case of SslE, which is expressed but does not appear to be secreted by commensal *E. coli* (at least in the case of MG1655), we found no significant changes in the murine gut microbiota due to immunizations. Similarly, a study using an ETEC antigen did not observe any significant change in the composition and richness of the microbiota post-immunization. The work from Moriel and colleagues thus opens interesting perspectives in future *E. coli* vaccine designs, and we certainly believe immunizations using formulations with both YncE and SslE are worth exploring.

In the second part of my PhD project, we aimed to investigate the *in vivo* effect of nitric oxide, a key component of the host innate immune response, on the virulence mechanisms of enterohemorrhagic *E. coli* O157:H7. This project spun out from previous *in vitro* work in our laboratory, where NO was shown to carry inhibitory effects on the synthesis of two major virulence factors in EHEC, namely Stx2 and the T3SS (Vareille, de Sablet et al. 2007, Branchu, Matrat et al. 2014). These findings strongly suggested that NO may shape the outcome of an EHEC infection in the host, and prompted us to conduct *in vivo* experiments to confirm this hypothesis. We used a NO-sensing EHEC reporter strain to show that EHEC could indeed sense NO produced in the mouse gut; the same reporter strain detected very little to no NO, at least for the first four days, when mice were treated with the NOS inhibitor L-NAME. NO-sensing may in fact hold importance in the colonization of EHEC in the mouse gastrointestinal tract, as the deletion of the NO-sensing regulator NsrR reduced the fitness of EHEC in our competition experiment. This could be due, at least in part, to an inhibition of EHEC adherence onto epithelial cells, as suggested by our previous *in vitro* findings (Branchu, Matrat et al. 2014). Accordingly, we found more adherent EHEC cells in the colon of mice that were treated with L-NAME on day 7 post-infection; however, we cannot yet conclude that this increase of adherence was in fact due to the absence of NO, as an L-NAME treatment could also have led to a compensatory increase of iNOS expression (Miller, Thompson et al. 1996). We are currently addressing this possibility by quantifying the *Nos2*

mRNA expression over time in preserved colon tissues from infected mice treated with L-NAME versus infected controls. A compensatory induction of *Nos2* expression could also explain the unexpected increase in NO-sensing by our EHEC reporter strain in some mice from day 5 to day 7 post-infection. Should this be the case, several approaches could be considered. First, several specific iNOS inhibitors are known and have been used in mice. For example, GW274150 functions as an arginine-based inhibitor, though it uses a different scaffold than L-NAME (Vitecek, Lojek et al. 2012). GW274150 was shown to lead to a significant and maintained decrease of nitrite in tissues of rats' inflamed paws up for at least 72 hours (De Alba, Clayton et al. 2006), though evidence of iNOS has not been studied for longer periods. Aminoguanidine, another preferential inhibitor of iNOS, has also been used to show increased susceptibility in mice to Salmonella infection (MacFarlane, Schwacha et al. 1999). However, a study has called for caution with these results, as aminoguanidine seems to provoke iNOS-independent secondary effects that increase susceptible to the infection (Zhou, Potoka et al. 2002); these could be due to the fact that aminoguanidine interferes with several enzymes system. To avoid these potential hurdles, the availability of *Nos2*$^{-/-}$ mice would be useful to determine the involvement of iNOS and NO in EHEC adherence and colonization. Alternatively, we did validate in our current study the use of our EHEC reporter strain to sense NO; provided it colonizes the mouse gastrointestinal tract just as efficiently as the WT EHEC strain, we could use this EHEC reporter strain as a tool to monitor NO presence in the gut, while simultaneously testing its effect on adherence of EHEC on intestinal epithelial cells over time. This experimental set up may represent the most accurate way to correlate NO to adherence and colonization of EHEC. Further, this system would also allow for testing of the efficacy of various NO inhibitors, such as those described above, at inhibiting production of NO – a parameter that in fact is extremely difficult to effectively monitor in the mouse gut.

Of interest, preliminary results we obtained show that EHEC-infected mice may decrease *Nos2* mRNA in the colon at day 7 post-infection. These results, if validated, stand in sharp contrast compared to the high induction of *Nos2* mRNA in mice infected with other enteric pathogens, such as *Citrobacter rodentium* or *Salmonella* Typhimurium (Cherayil and Antos 2001, Vallance, Deng et al. 2002, Zhou, Potoka et al. 2002). Although the interaction of *C. rodentium* with intestinal epithelial cells may lead to an inhibition of NO production, the neighboring uninfected cells produce substantial amount of iNOS, which overall would lead to a pro-inflammatory state with production of NO (Vallance, Deng et al. 2002). On the other hand, EHEC would mediate an anti-inflammatory inhibition of iNOS production; thus, we may need to be cautious on comparing these two pathogens, especially since their T3SS-mediated adherence and colonization of the gut is very similar.

Contrary to our previous *in vitro* findings, NO appears to have a stimulating effect on the production of Stx by EHEC in the murine gut, as treatment with L-NAME in infected mice led to a significantly lower production of Stx compared to untreated EHEC-infected mice from day 1 to day 5 post-infection. This difference may be explained by the findings of a recent study, which showed that NO enhanced the production of both Stx1 and Stx2 from EHEC cells grown under anaerobic conditions. As the intestines are mainly devoid of oxygen, our *in vivo* results seem to corroborate the *in vitro* ones from Ichimura *et al.* Furthermore, we found that in mice infected with EHEC and treated with ciprofloxacin, treatment with L-NAME led to a maintenance of the urine specific gravity, a marker of renal dysfunction (such as after exposure to Stx), to the same levels than uninfected mice. If these findings are confirmed by our ongoing kidney histology analyses, we would have strong evidence indicating that NO may in fact worsen the outcome of an EHEC infection in the mouse model, as it appears to provoke an increase in the production of Stx. It is noteworthy that, with the well-reported differential *in vitro* induction of *Nos2* in murine and human macrophages, our findings may not hold true in humans. Thus, we believe that monitoring NO production and comparing levels between EHEC-infected patients with and without HUS could be greatly informative, as an increase in NO production under a high inflammatory state could have an impact on the development of HUS. The proinflammatory character of the development of HUS and renal damage has been well-documented in patients with EHEC-associated HUS. In particular, leukocytosis, whether circulating or localized in the kidneys, is considered a predictor of HUS development and poor outcome (Bell, Griffin et al. 1997, Buteau, Proulx et al. 2000). In the kidneys, the immune response of the host, due to apoptosis-mediated renal injury, leads to an important secretion of cytokines and an influx of leukocytes by chemotaxis. Among these, the accumulation of neutrophils and macrophages, which are major actors during EHEC infections and development of HUS, have been specifically linked to an increased mortality (Walters, Matthei et al. 1989, Coad, Marshall et al. 1991, Inward, Howie et al. 1997). Proinflammatory cytokines such as TNF-α, IL-6 and IL-8 are produced in the kidneys and recovered in the urine of HUS patients (Karpman, Andreasson et al. 1995). TNF-α is known to be involved in the pathogenesis of HUS; indeed, Stx in mice induces the secretion of TNF-α in kidneys and increases the renal sensitivity to the toxicity of TNF-α (Harel, Silva et al. 1993). Accordingly, treatment with a TNF inhibitor decreases both renal and brain damage (Isogai, Isogai et al. 1998). Thus, a high inflammatory state seems to be intimately linked to the onset and progression of HUS in patients, though the exact cause underlying this susceptibility is yet undefined. Nevertheless, we could speculate that in patients developing HUS, a hyperinflammatory state could provoke, on top of leukocytosis and cytokine secretion, an increase of NO production and, should our results be comparable in mice and

humans, an increase in Stx. The latter would ultimately provoke an aggravation of HUS in these patients.

In summary, our findings hold importance not only in the understanding of the interplay between EHEC and the innate immune system, but also in future therapeutic prospects against EHEC-mediated disease. Evidently, unlike what we had previously assumed from our *in vitro* work, a NO-mediated aggravation of the disease caused by EHEC and Stx implies that the use of NO inhibitors could potentially counter that effect. From there, we could even envision combining an NO inhibitor to an antibiotic, which would presumably carry a bactericidal effect while avoiding Stx production.

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Scientific contributions

Publications

Ilham Naili, Juliette Vinot, Barbara C. Baudner, Annick Bernalier-Donadille, Mariagrazia Pizza, Mickaël Desvaux, Grégory Jubelin, Ugo D'Oro, Cecilia Buonsanti. Mixed mucosal-parenteral immunizations with broadly conserved pathogenic *Escherichia coli* antigen SslE induce a robust mucosal and systemic immunity without affecting the murine intestinal microbiota. ***Manuscript in review at Vaccine.***

Ilham Naili, Marion Gardette, Annie Garrivier, Julien Daniel, Valérie Rousseau, Mickaël Desvaux, Mariagrazzia Pizza, Alain Gobert, Thierry Marchal, Estelle Loukiadis, Grégory Jubelin. Interplay between enterohemorrhagic *Escherichia coli* and nitric oxide during the infectious process. ***Manuscript in preparation.***

Oral presentations

"*In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **GSK PhD workshop**, November 23rd and 24th 2016 at GSK Vaccines in Siena, Italy.

Poster presentations

Ilham Naili, Cecilia Buonsanti, Miguela Vieru, Federica Corrente, Alain Gobert, Valérie Livrelli, Barbara Baudner, Elisabetta Affabbris and Ugo D'Oro. *In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **GSK PhD workshop,** November 26th and 27th 2015, at GSK Vaccines in Siena, Italy**.**

Ilham Naili, Cecilia Buonsanti, Marco Soriani, Mickaël Desvaux, Grégory Jubelin, Miguela Vieru, Barbara Baudner, Ugo D'Oro. *In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **American Association of Immunologists Annual Meeting**, May13th-May 17th 2016, Seattle, USA.

Ilham Naili, Juliette Vinot, Barbara Baudner, Annick Bernalier-Donadille, Mariagrazia Pizza, Mickael Desvaux, Ugo D'Oro, Grégory Jubelin, Cecilia Buonsanti. *In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **American Association of Immunologists Annual Meeting**, May 12th-16th 2017, Washington D.C., USA.

Ilham Naili, Juliette Vinot, Barbara Baudner, Annick Bernalier-Donadille, Mariagrazia Pizza, Mickael Desvaux, Ugo D'Oro, Grégory Jubelin, Cecilia Buonsanti. *In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **Doctoral School Days at Université Clermont Auvergne,** May 18th-19th 2017, Clermont-Ferrand.

Ilham Naili, Juliette Vinot, Barbara Baudner, Annick Bernalier-Donadille, Mariagrazia Pizza, Mickaël Desvaux, Ugo D'Oro, Grégory Jubelin, Cecilia Buonsanti. *In vivo* characterization of the mucosal immune response towards pathogenic *Escherichia coli* antigens and modulation of the intestinal microbiota. **International Congress of Mucosal Immunology,** July 19th-22nd 2017, Washington D.C., USA.

Abstract

Pathogenic *Escherichia coli* are a source of growing public health concern worldwide, due to their morbidity and mortality incidence, particularly in young children. Depending on the particular variant, or pathotype, diseases range from acute diarrhea to sepsis, meningitis and hemorrhagic uremic syndrome. Treatments are falling short due to the increasing emergence of antibiotic resistance, and no vaccine is yet on the market; hence, efforts are needed to support future preventive or therapeutic solutions against *E. coli*-mediated diseases. In this PhD thesis project, we joined these efforts, and tackled this challenge by taking two different approaches. We first established a mouse model of immunization with a broad spectrum antigen of *E. coli*, and showed that this model generated a robust humoral and cellular response both in the intestine and systemically, while not disturbing the resident gut microbiota. Such model of immunization could thus potentially be protective against both intestinal and extraintestinal diseases of *E. coli*, and brings valuable insights for the definition and development of a broad vaccine against pathogenic *E. coli*. In a second approach, we worked specifically with the enterohemorrhagic *E. coli* (EHEC) and explored whether nitric oxide (NO) had a particular role in EHEC virulence. Using a mouse model of infection, we showed that detection of NO is critical for the pathogen to efficiently colonize the gastrointestinal tract, Moreover, inhibiting NO production by the host decreased the activity of Shiga toxin, the main virulence factor of EHEC, while increasing colonic adherence of EHEC. Our results indicate that NO, an important actor of the host immune response, can play a determinant role on the outcome of an EHEC infection, and may influence future strategies aimed against EHEC infection in humans.

Keywords: *Escherichia coli*, vaccine, mucosal immunity, nitric oxide, enterohemorrhagic *E. coli.*