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# The inflammatory response of primary epithelial cells of the female genital tract to *Chlamydia trachomatis* infection, and its exacerbation by type-I interferon

Chongfa Tang

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# Sorbonne Université

École doctorale Complexité du Vivant-ED515

*Unité de Biologie Cellulaire de l'infection Microbienne, CNRS-UMR3691*

## **The inflammatory response of primary epithelial cells of the female genital tract to *Chlamydia trachomatis* infection, and its exacerbation by type-I interferon**

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Thèse de doctorat de Microbiologie et Immunologie

Dirigée par Dr. Yongzheng WU

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*Dédicace*

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(From Pengpeng LUO)

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## ABBREVIATIONS

<b>Abbr.</b>	<b>Meaning</b>	<b>Abbr.</b>	<b>Meaning</b>
ALR	absent in melanoma 2 (AIM2)-like receptor	LRR	leucine-rich repeat
AMP	antimicrobial peptide	McCoy	Murine heteroploid fibroblast
aRB	abarrent reticular body	MHC	major histocompatibility complex
BMDM	bone marrow-derived macrophages	MOMP	major outer membrane protein
CDN	cyclic dinucleotide	MTOC	microtubule-organizing center
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate	mTOR	mammalian target of rapamycin
cGAS	cyclic GMP-AMP synthase	MSM	men who have sex with men
CLR	C-type lectin receptors	MyD88	myeloid differentiation factor 88
COMOC	chlamydial outer membrane complex	NK cell	natural killer cell
CPAF	chlamydial protease-like activity factor	NLR	nucleotide binding and oligomerization domain (NOD) class receptor
C .trachomatis	Chlamydia trachomatis	ORF	open reading frames
CTZ	cervical transformation zone	PAMP	pathogen associated molecular pattern
DAMP	danger-associated molecular pattern	PG	peptidoglycan
DC	dendritic cell	Pmp	polymorphic membrane protein
dsRNA	double stranded RNA	Poly(I:C)	polyinosinic-polycytidylicacid
EB	elementary body	PRR	pattern recognition receptor
FGT	female genital tract	PZ	plasticity zone region
EMT	epithelial to mesenchymal transition	RB	reticular body
hBD	human $\beta$ defensin	RIR	RIG-I-like receptors
HSP60	heat shock protein 60	ssRNA	single stranded RNA
IDO1	indoleamine-2,3-dioxygenase 1	STAT	signal transducer and activator of transcription
IKK	I $\kappa$ B kinases	STI	sexually transmitted infection
Inc	inclusion membrane protein	STING	stimulator of interferon gene
ISG	Interferon-stimulated gene	TIR	Toll/IL-1R domain
IFN-I	type I interferon	TLR	Toll like receptor
IFNAR	interferon- $\alpha/\beta$ receptor	TRIF	TIR-domain-containing adapter-inducing interferon- $\beta$
JAK	Janus kinase	Treg	regulatory T cell
JNK	c-Jun N-terminal kinases	T3SS	type 3 secretion system
LGV	lymphogranuloma venereum	3D culture	three-dimensional culture
LPS/LOS	lipopolysaccharide/lipooligosaccharide	WHO	World Health Organization

## ABSTRACT

*Chlamydia trachomatis* is an obligate intracellular bacterium, which grows mainly in epithelial cells of the mucous membranes of the genital tract. Asymptomatic in most cases, infections with this pathogen can lead to pelvic inflammations. The inflammation itself can lead to fibrosis and tubal infertility in women. To better understand the pathological manifestations in the female genital tract (FGT), it is important to study the response of epithelial cells, which constitute the first line of host defense against *C. trachomatis* infection.

To this end, we developed a simplified protocol to isolate a very pure fraction of primary epithelial cells from the FGT of patients undergoing hysterectomy. We observed that these primary epithelial cells were less permissive to *C. trachomatis* infection than the cell line classically used in laboratories, i.e. HeLa cells. We have shown that the difference in culture medium and the addition of serum in HeLa cell cultures explain a large part of these differences. However, when tested in an identical culture medium, primary ectocervical epithelial cells were found to be less permissive than HeLa cells towards *C. trachomatis* infection. Finally, primary epithelial cells expressed overall more pro-inflammatory cytokines, both basally and after *C. trachomatis* infection, than HeLa cells, suggesting a strong capacity of primary epithelial cells to mount an inflammatory response.

We then focused on understanding why type I interferon (IFN-I) acts synergistically with *C. trachomatis* infection towards the pro-inflammatory response of epithelial cells. We demonstrated that IFN-I, but not *C. trachomatis*, increased the expression of several bacterial pattern recognition receptors (PRRs). Expression silencing of TLR3 receptor, or deletion of this gene, prevented synergetic effect between IFN-I and *C. trachomatis*. We also identified the intermediate signaling pathway between IFN-I receptor activation and TLR3 expression, as well as the signaling downstream of TLR3 activation, which results in the expression of the pro-inflammatory cytokines interleukin-6 and 8. Based on these data, we conclude that IFN-I exacerbates the host inflammatory response triggered by *C. trachomatis* infection via increased TLR3 expression, and that this synergetic effect between IFN-I and bacteria on pro-inflammatory signaling in epithelial cells may play a role in the tissue damage that results from infection in some of the patients.

## RÉSUMÉ

*Chlamydia trachomatis* est une bactérie intracellulaire obligatoire, qui se développe principalement dans cellules épithéliales des muqueuses du tractus génital. Asymptomatiques dans la plupart des cas, les infections par ce pathogène peuvent entraîner des inflammations pelviennes. L'inflammation peut elle-même engendrer des fibroses et mener à une infertilité tubaire chez les femmes. Il est important d'étudier la réponse des cellules épithéliales, qui constituent la première ligne de défense contre l'infection par *C. trachomatis*, pour mieux comprendre ses manifestations pathologiques dans le tractus génital féminin (FGT).

À cette fin, nous avons mis au point un protocole simplifié pour isoler une fraction très pure de cellules épithéliales primaires, à partir du FGT de patientes subissant une hystérectomie. Nous avons observé que ces cellules épithéliales primaires étaient moins permissives à l'infection par *C. trachomatis* que la lignée cellulaire classiquement utilisée en laboratoire, *i.e.* la lignée HeLa. Nous avons montré que la différence de milieu de culture et l'ajout de sérum dans les cultures de cellules HeLa, expliquent une grande partie de ces différences. Cependant, testées dans des milieux identiques, les cellules épithéliales primaires issues de l'ectocol se sont révélées moins permissives que les cellules HeLa vis-à-vis du développement de *C. trachomatis*. Enfin, les cellules épithéliales primaires exprimaient globalement davantage de cytokines pro-inflammatoires, au niveau basal et après infection à *C. trachomatis*, que les cellules HeLa, suggérant une forte capacité des cellules épithéliales primaires à monter une réponse inflammatoire.

Nous nous sommes ensuite attachés à comprendre pourquoi l'interféron de type I (IFN-I) agissait-t-il en synergie avec l'infection par *C. trachomatis* vis-à-vis de la réponse pro-inflammatoire des cellules. Nous avons démontré que l'IFN-I, mais pas *C. trachomatis*, augmentait l'expression de plusieurs récepteurs de reconnaissance de motifs bactériens. L'extinction de l'expression du récepteur TLR3, ou la délétion de ce gène, empêche la synergie entre l'IFN-I et *C. trachomatis*. Nous avons identifié la voie de signalisation intermédiaire entre l'activation du récepteur de l'IFN-I et l'expression du TLR3, ainsi que la signalisation en aval de l'activation de TLR3, qui aboutit à l'expression des cytokines pro-inflammatoires interleukines-6 et 8. Ainsi, l'IFN-I exacerbe la réponse inflammatoire de l'hôte déclenchée par l'infection à *C. trachomatis* via une augmentation de l'expression du TLR3. La synergie entre l'IFN-I et les bactéries à stimuler la signalisation pro-inflammatoire des cellules épithéliales pourrait jouer un rôle dans les lésions tissulaires qui résultent de l'infection chez certaines patientes.

## RÉSUMÉ DÉTAILLÉ

Le phylum des *Chlamydiae* regroupe des bactéries intracellulaires obligatoires, qui ont en commun un cycle de développement original, à l'intérieur d'un hôte eucaryote. Cet hôte peut être un protozoaire (amibes par exemple) ou un métazoaire. Le phylum contient un seul Ordre et une seule Classe, mais plusieurs Familles. La famille des *Chlamydiaceae* renferme des espèces infectant les animaux et l'Homme. Deux espèces de *Chlamydiaceae* sont fréquemment retrouvées dans des infections humaines: *Chlamydia trachomatis* et *Chlamydia pneumoniae*. Cette dernière est capable d'infecter des hôtes variés (cheval, koala reptiles...) et cause chez l'Homme des infections de l'appareil respiratoire. *C. trachomatis* se reproduit quant à elle exclusivement au sein de l'espèce humaine. Certaines souches (sérovars A à C) envahissent la muqueuse de la conjonctive de l'œil. Des infections répétées peuvent conduire à des dommages sur la cornée, donnant lieu au trachome, qui représente la première cause de cécité acquise au monde. Le trachome est essentiellement circonscrit à des régions du globe ne disposant pas de conditions d'hygiène adéquates. Les autres sérovars infectent le tractus uro-génital, et ne connaissent pas de barrières géographiques. Les infections génitales par *C. trachomatis* sont généralement asymptomatiques. L'absence de symptôme fait que les infections sont souvent non détectées, et donc non traitées. Elles sont cependant aussi à l'origine d'inflammations pelviennes chroniques, de grossesse extra-utérines, et elles peuvent être causes d'infertilité. De fait, l'infection par *C. trachomatis* représente la première infection sexuellement transmissible d'origine bactérienne. Plus de 100 millions de nouveaux cas sont recensés chaque année.

Une des caractéristiques des *Chlamydiae* est leur cycle de développement « biphasique ». Au cours de ce cycle, la bactérie alterne entre deux formes de morphologie et de métabolisme distincts. La forme « corps élémentaire » (EB) est non répliquative et adaptée à la survie extracellulaire, c'est la seule forme infectieuse. La forme « corps réticulé » (RB) est répliquative et adaptée à la survie intracellulaire, et même intra-vacuolaire puisque la prolifération bactérienne a lieu dans un compartiment vacuolaire, appelé inclusion. L'adhésion d'un EB à la surface d'une cellule initie le cycle infectieux, elle est rapidement suivie de l'internalisation de la bactérie dans l'inclusion en devenir. Rapidement, le chromosome bactérien perd en compaction, permettant l'expression de gènes « précoces ». Ce programme génétique initial accompagne la transformation progressive de la morphologie de la bactérie vers la forme RB. Environ 6 h après l'initiation de l'infection (6 hpi), on observe un doublement de la quantité d'ADN bactérien, témoin de la première division. Il s'en suit une croissance exponentielle, de sorte que chaque inclusion peut contenir plusieurs centaines de bactéries. Dès 20 hpi, une fraction des RBs initie un changement d'expression de gènes et initie leur conversion en EB. Celle-ci a lieu de manière asynchrone. Vers 48 hpi, l'inclusion renferme davantage de EBs que de RBs. Les bactéries sont relarguées de la cellule-hôte soit parce que celle-ci est lysée, soit par un processus d'extrusion de l'inclusion, qui est libérée dans le milieu extracellulaire. Une fois sortis de la cellule ou de l'inclusion, les EBs initient un nouveau cycle de développement dans les cellules épithéliales voisines.

Les *Chlamydiae* ne comptent qu'autour d'un millier de gènes. Elles ont en particulier perdu de nombreux gènes codant pour des fonctions métaboliques essentielles. Cette situation est commune chez les bactéries intracellulaires, et poussée à l'extrême chez intracellulaires obligatoire telles que les *Chlamydiae*. Son corolaire est que les bactéries dépendent de l'hôte pour combler les manques dans des voies métaboliques essentielles, telles que la synthèse de certains acide aminés ou de nucléotides. Les bactéries expriment un transporteur d'ATP, qui leur permet de puiser dans les ressources énergétiques de l'hôte. La croissance de l'inclusion demande également un apport de lipides de l'hôte. Pour faire face à ces besoins, les bactéries sécrètent des protéines dans l'inclusion ou dans le cytoplasme. Le mécanisme majoritaire de sécrétion des *Chlamydiaceae* est connu sous l'appellation système de sécrétion de type 3, il est trouvé chez plusieurs bactéries pathogènes à Gram négatif. Grâce aux protéines effectrices les bactéries manipulent l'hôte et détournent certaines fonctions cellulaires à leur profit. Une des activités attendues de ces protéines est de contrecarrer les défenses naturelles de l'hôte vis-à-vis des microorganismes qui l'agressent. De fait, il a été proposé qu'une des protéines effectrices sécrétée de façon précoce dans le cycle infectieux, TepP, permette une activation locale des phosphoinositide 3-kinases de classe I, et que cette action participe à diminuer l'induction de gènes de réponse à l'interféron de type I tôt dans l'infection.

*C. trachomatis* prolifère essentiellement dans les cellules épithéliales des muqueuses du tractus génital. Ces cellules représentent la première ligne de défense contre l'infection, et possèdent des propriétés antimicrobiennes intrinsèques. Les pathogènes sont en particulier détectés parce qu'ils synthétisent des structures communes appelées pathogen-associated molecular patterns (PAMPs), qui sont reconnus par des récepteurs appelés pattern recognition receptors (PRRs), ce qui déclenche les voies inflammatoires pour signaler l'infection. En utilisant des souris KO pour différents PRRs, il a été montré que les récepteurs Toll-like tels que TLR2, TLR3, TLR4 et NOD1, étaient impliqués dans la détection de la souche adaptée à la souris *Chlamydia muridarum*. La physiologie du tractus génital de Souris est assez différente de celle de la femme, et il est important de confirmer ces résultats dans un modèle impliquant *C. trachomatis* et des cellules humaines.

Pour étudier le cycle de développement des bactéries, des modèles d'infection de cellules en culture ont été établis. Les modèles les plus utilisés reposent sur l'usage de lignées de cellules d'origine cancéreuses telles que les cellules HeLa. Celles-ci ont une certaine légitimité, car elles ont été isolées à partir d'une biopsie de cancer cervical. Cependant, il est connu que leur origine cancéreuse, ainsi que les années de propagation en laboratoire, ont profondément altéré leur génome. Ces cellules présentent une modification du métabolisme basal, se divisent très fréquemment, autant de paramètres susceptibles d'affecter le cycle de développement des bactéries. Plus récemment, le laboratoire d'Alison Quayle a généré la lignée cellulaire A2EN en immortalisant, grâce à l'introduction de gènes codant pour les protéines E6 et E7 du papillomavirus humain, des cellules saines issues de la partie haute du col de l'utérus (endocol). Cette lignée est susceptible à l'infection par *C. trachomatis*, qui effectue un cycle de développement complet en environ 4 jours. Cependant l'étape d'immortalisation introduit elle-

même un biais, comme par exemple la dégradation du suppresseur de tumeur p53, qui pourrait influencer le déroulé de l'infection, et la réponse de la cellule hôte. Pour cette raison il est important d'étudier aussi l'infection dans des cellules dites 'primaires', isolées d'individus sains, mises en culture et utilisées après un petit nombre de divisions, sans étape d'immortalisation. Plusieurs études décrivent la collecte et mise en culture de cellules primaires issues de différentes parties du tractus génital féminin (endocol et exocol, qui correspond à la partie basse du col de l'utérus, endomètre, trompes de Fallope). L'infection par *C. trachomatis* dans ces cellules primaires a cependant fait l'objet de très peu d'études. En particulier, la réponse inflammatoire de ces cellules à l'infection n'a pas été mesurée. Plus récemment, des cultures sous forme d'organoïde de cellules issues d'échantillons d'origine murine ou humaine ont été mises au point. Cette stratégie permet de s'approcher de l'organisation tissulaire, avec par exemple la formation d'un épithélium cilié sécrétant du mucus. La faible quantité de matériel obtenu à partir d'un prélèvement, et la difficulté de reproductibilité, limitent cependant l'usage de ce modèle complexe.

La première partie de ce travail de thèse a consisté à mettre au point un protocole pour l'isolement de cellules épithéliales primaires à partir de fragments d'exocol de patientes ayant subi une intervention chirurgicale nécessitant l'excision de ce tissu. Nous avons atteint une grande pureté d'isolement, évaluée par l'expression de la E-cadhérine et de la cytokératine 14. La descendance infectieuse (progénie) de *C. trachomatis* serovar L2 ou D après un cycle de développement dans ces cellules épithéliales primaires est plus faible que dans les cellules HeLa. Nous avons montré que la différence de milieu de culture et l'ajout de sérum dans les cultures de cellules HeLa, expliquent une grande partie de ces différences. Cependant, testées dans des milieux identiques, les cellules épithéliales primaires issues de l'ectocol se sont révélées moins permissives que les cellules HeLa vis-à-vis du développement de *C. trachomatis* serovar L2 ou D. Enfin, le niveau basal de transcription des gènes codant pour les cytokines pro-inflammatoires était globalement plus élevée dans les cellules épithéliales primaires que dans les cellules HeLa. La transcription de plusieurs gènes pro-inflammatoires est augmentée lors d'une infection par *C. trachomatis*. En conclusion, les cellules épithéliales primaires ont une forte capacité à monter une réponse à l'infection à *C. trachomatis*.

Au cours de ces expériences, nous avons remarqué que la sécrétion des cytokines pro-inflammatoires interleukin-6 (IL-6) et interleukin-8 (IL-8) était plus importantes dans les cellules infectées cultivées en présence d'interféron de type I (IFN-I). L'IFN-I est une molécule de communication entre cellules très importante pour monter une réponse contre une infection, en particulier contre les infections virales. Sa contribution à la résolution des infections bactériennes est moins documentée. Des données fragmentaires indiquent que l'IFN-I a un rôle protecteur contre les infections par *S. pyogenes*, *Legionella pneumophila*, *Bacillus anthracis*, *Shigella flexneri* and *Salmonella typhimurium*, mais pourrait promouvoir la dissémination of *Listeria monocytogenes*. Concernant l'infection par *C. trachomatis*, les données indiquent que le niveau de sécrétion d'IFN-I corrèle avec une progression plus importante des bactéries dans les parties hautes du tractus génital féminin. La sécrétion d'IFN-I pourrait donc être délétère



dans le cas d'une infection par *C. trachomatis*. Utilisé seul, l'IFN-I n'induit pas la synthèse d'IL-6 ou d'IL-8, ni dans les cellules primaires, ni dans les cellules HeLa. La combinaison infection par *C. trachomatis*/IFN-I semblait donc agir en synergie, conduisant à une exacerbation de la réponse pro-inflammatoire des cellules, et nous nous sommes attachés à comprendre les mécanismes moléculaires sous-jacents.

Le traitement des cellules par l'IFN-I conduit à la phosphorylation du facteur STAT-1, et l'extinction de l'expression de STAT-1 conduit à la perte de l'effet synergique entre l'IFN-I et l'infection. En utilisant des molécules inhibitrices de la kinase AKT et de la voie mTOR, nous avons montré que ces molécules étaient également impliquées dans le mécanisme de synergie. Cela est aussi vrai des kinases ERK et p38, en dépit du fait que ces kinases ne sont pas activées par l'IFN-I. Nous avons émis l'hypothèse que l'IFN-I augmente l'expression d'un ou plusieurs PRRs, et ce faisant facilite la détection de l'infection par *C. trachomatis*. Nous avons démontré qu'en effet, l'IFN-I, mais pas l'infection seule, augmente l'expression du PRR TLR3, et que l'extinction du gène correspondant diminue largement l'effet de synergie entre l'IFN-I et l'infection. Dans cellules n'exprimant pas TLR3, l'effet synergique est entièrement perdu. L'extinction de STAT1 ou l'inhibition de AKT ou mTOR atténuent l'induction de l'expression de *tlr3* par l'IFN-I, ce qui peut expliquer leur effet inhibiteur sur la synergie. Enfin, nous avons examiné la voie de signalisation en aval de TLR3, et nos résultats indiquent qu'il s'agisse de la cascade MAPK/ERK. En résumé, nos résultats démontrent que l'IFN-I exacerbe la réponse pro-inflammatoire à l'infection par *C. trachomatis* en facilitant la détection de la présence du pathogène via l'expression de TLR3. L'hyperinflammation qui en résulte pourrait contribuer aux dommages tissulaires observés chez certaines patientes à la suite d'une infection par *C. trachomatis*. Notons que des résultats opposés ont été obtenus dans le modèle d'infection de souris par *C. muridarum*. En effet, une publication rapporte que des souris n'exprimant pas TLR3 présentent une charge bactérienne plus élevée, et des dommages tissulaires plus importants que les souris sauvages.

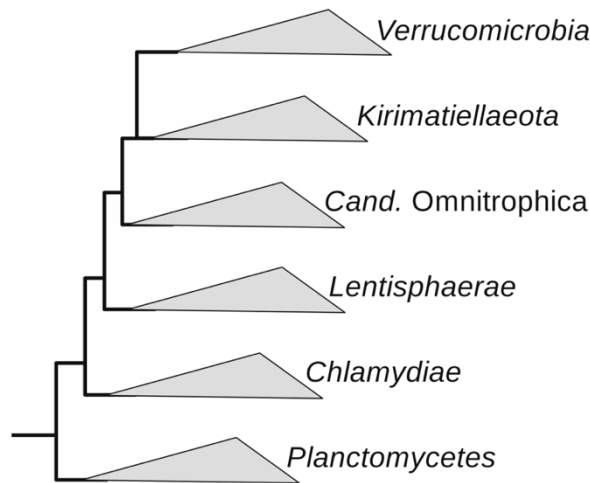
En résumé, nos travaux décrivent un protocole simple d'isolement de cellules épithéliales primaires à partir de prélèvements issus du col de l'utérus, cellules qui constituent la première ligne de défense cellulaire aux infections. Nous avons montré que les cellules HeLa ne reproduisent que partiellement le contexte inflammatoire rencontré par les bactéries au cours d'une infection du tractus génital féminin. Notre protocole d'isolement de cellules primaires va donc faciliter l'étude de la réponse innée à une infection montée par les cellules épithéliales. Nous avons en particulier démontré que l'IFN-I exacerbe la réponse pro-inflammatoire des cellules épithéliales, en stimulant l'expression du senseur TLR3. Ces résultats ouvrent la question de la nature du stimulus d'origine bactérienne détecté par TLR3, qui est jusqu'à présent décrit pour sa capacité de reconnaissance d'ARN double brin viral. La question de savoir si l'augmentation de la réponse pro-inflammatoire favorise chez la femme l'élimination des bactéries, comme cela semble être le cas chez la souris, reste également à explorer. Dans la mesure où les données cliniques suggèrent que chez certaines personnes, l'augmentation de la

sécrétion d'IFN-I augmente le risque de dommages tissulaires irréversibles, la synergie entre l'effet de l'IFN-I et l'infection par *C. trachomatis* pourrait ne pas toujours être bénéfique.

# **INTRODUCTION**

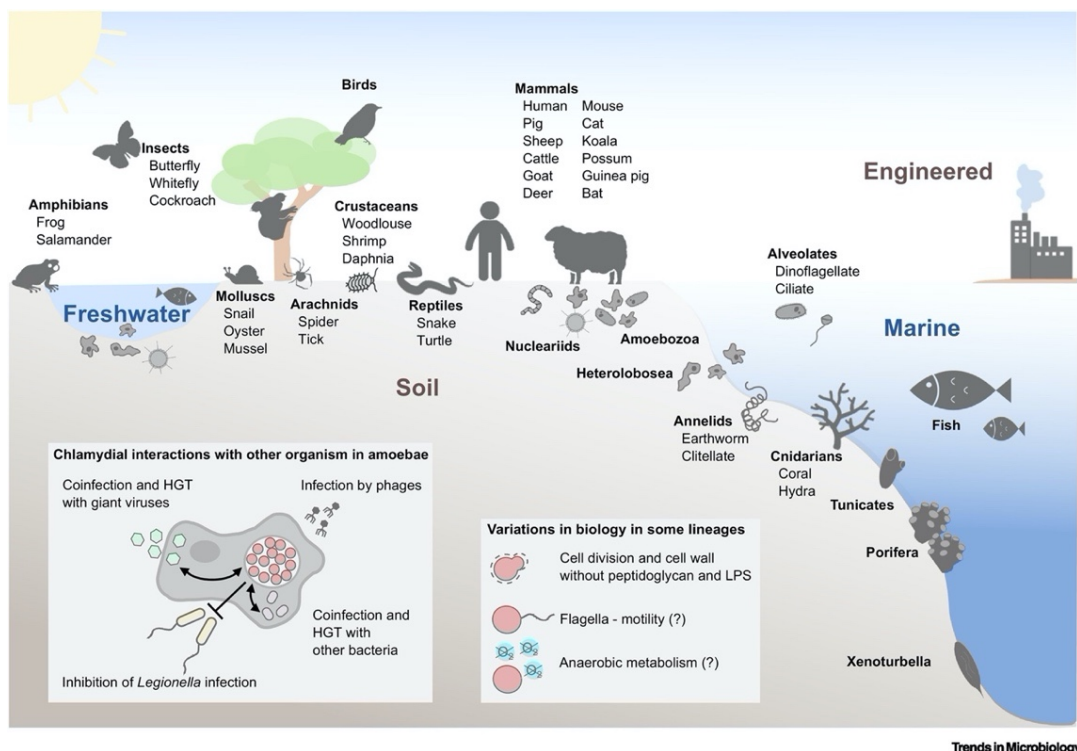
# I. *Chlamydiae* phylum and associated diseases

## 1.1 *Chlamydia* spp.



**Figure 1. Phylogenetic representation of the PVC superphylum.** This was built using 16S rRNA gene sequences and rooted on the *Escherichia coli* K12 sequence. (From Rivas-Marín and Devos, 2018)

*Chlamydia trachomatis* is an obligate intracellular Gram-negative bacterium, which belongs to *Chlamydiae* phylum. *Chlamydiae* is one member of the PVC superphylum that is composed of the bacterial phyla *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and *Kirimatiellaeota* and some uncultured candidate phyla, such as *Candidatus Omnitrophica* (Rivas-Marín et al., 2016; Wagner and Horn, 2006). Based on the analysis of 16S rRNA of PVC, the phylogenetic tree of the PVC is shown in Figure 1 (Rivas-Marín and Devos, 2018; Spring et al., 2016).



**Figure 2. Chlamydial host range and environmental distribution.** (From Collingro et al., 2020)

*Chlamydiae* phylum only contains the *Chlamydiae* class, and two orders: *Chlamydiales* and *Parachlamydiales*. A total of nine families have been identified till now. *Chlamydiaceae* are a chlamydial family of well-known pathogens to humans and some animals (Nunes and Gomes, 2014) and *Chlamydia trachomatis* is one member of *Chlamydiaceae* family. The other families are named *Chlamydia*-like organisms (CLOs), which are also referred to as “environmental *Chlamydiae*”, because most of them are initially isolated from environmental sources (Taylor-Brown et al., 2015). The total number of families for which a name was proposed increased recently from 10 to 17 (Dharamshi et al., 2020; Pillonel et al., 2018), but based on current molecular data, the predicted family-level lineages are nearly 1483 (Collingro et al., 2020). Based on the data from diverse previous studies and current integrated microbial next-generation sequencing analysis, now we know that *Chlamydiae* are abundant in some environments and are found in diverse hosts [Figure 2] (Collingro et al., 2020).

The taxonomy of *Chlamydiae* is shown in Figure 3. The *Chlamydiaceae* family originally consists of the *Chlamydophila* and *Chlamydia* genera, which comprise eight validly named genera and 14 candidate genera (Chen et al., 2019). The genus *Chlamydia* is the most widely studied *Chlamydiae*, which is composed of 15 species including four major human and several animal pathogens (Bayramova et al., 2018a; Taylor-Brown et al., 2015). The well-known human pathogens are *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, *C. abortus* and *C. felis* (Horn, 2008).

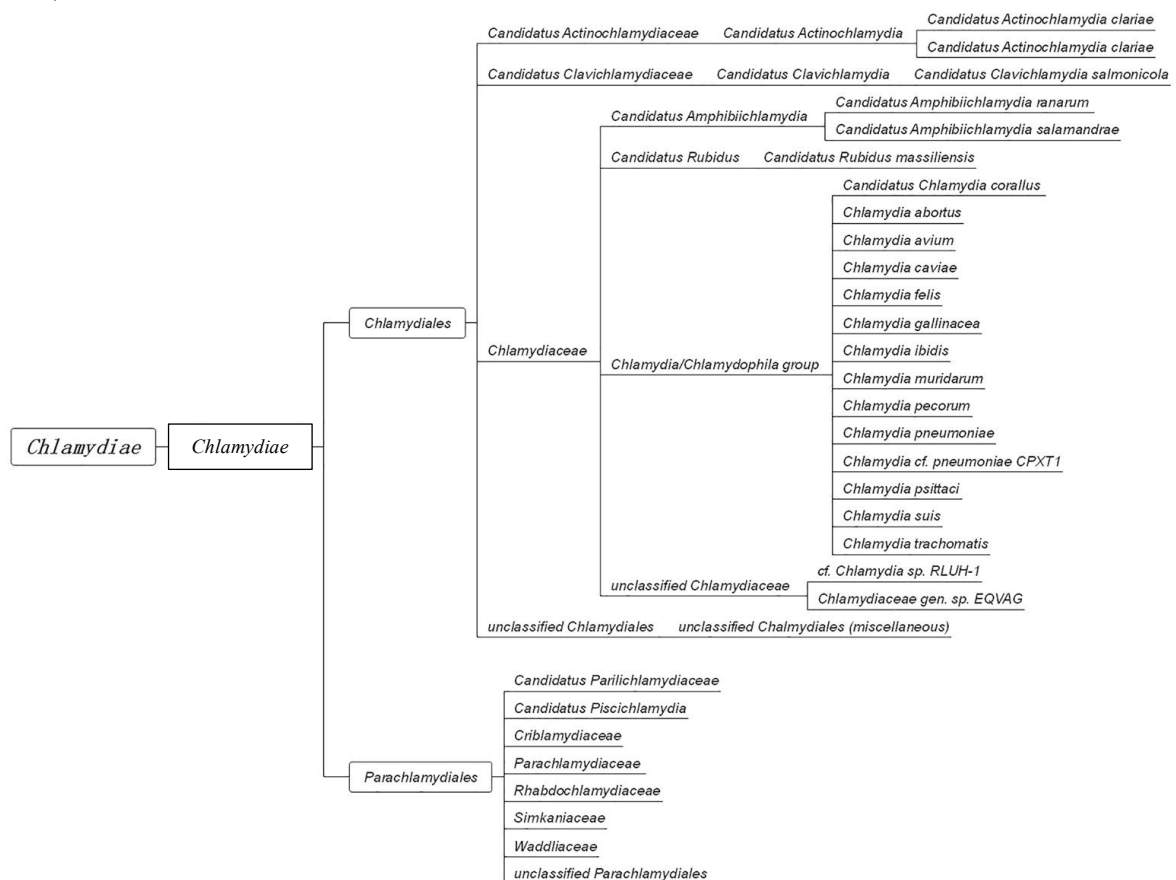


Figure 3. The taxonomy of *Chlamydiae*. (Adapted From Chen et al., 2019)

### 1.1.1 *C. trachomatis*

In 1907, Ludwig Halberstädter and Stanislaus von Prowazek observed intracytoplasmic inclusions in conjunctival scrapings from subjects with trachoma. They suggested the name *Chlamydozoa* (Greek *τράχωμα*, meaning roughness) (Mohammadpour et al., 2016; Taylor-Robinson, 2017). In 1957, *C. trachomatis* was first cultured in the yolk sacs of eggs by Professor TANG Fei-fan and colleagues in Beijing (Tang, 1958). Soon after this breakthrough, *C. trachomatis* was isolated successfully from patients in London using the same method (BR JONES, 1964). One year later, Gordan and Quan made a progress in *C. trachomatis* proliferation in cultured cell (Gordon and Quan, 1965). Despite of the debate among scientists whether *Chlamydiae* belong to protozoa, bacteria, or viruses, with the advent of electron microscopy, *Chlamydiae* were classified as bacteria in 1966, mainly because they possess both DNA and RNA, ribosomes, and have cell walls similar to Gram-negative bacteria (Moulder, 1966).

Based on the primary surface antigen, the major outer membrane protein (MOMP), and its coding gene *ompA*, *C. trachomatis* contains three biovars including trachoma biovar, genital biovar and lymphogranuloma venereum (LGV) biovar. Using specific antibodies against different epitopes of MOMP, *C. trachomatis* can be further sub-typed into serovars. The trachoma biovar (serovars A, B, Ba, C) can infect human and subsequently cause non-congenital blindness in developing nations, if not treated well. The genital tract biovar (serovars D, E, F, G, H, I, J, K) is the most prevalent sexually transmitted bacterium in the world. The LGV biovar (serovars L1, L2 and L3) can induce invasive urogenital or anorectal infection (Cherilyn Elwell, 2016).

More details about *C. trachomatis* will be discussed in section 1.2.

### 1.1.2 *C. pneumoniae*

*C. pneumoniae* was first isolated in 1965 in Taiwan (China), and named *C. psittaci* TWAR (Grayston, 2000), and was identified as the cause of a mild pneumonia epidemic in Finland (Saikku et al., 1985). In 1989, it was reclassified to *C. pneumoniae* (GRAYSTON et al., 1989). Recent studies have revealed that *C. pneumoniae* is not just an acute respiratory pathogen, but also associated with persistent infection in human chronic diseases, such as cardiovascular disease, arthritis, diabetes, Alzheimer's disease and lung cancer (Roulis et al., 2013). Besides human beings, *C. pneumoniae* can also infect domesticated and wild animals [Figure 4] (Roulis et al., 2013). Animal strains of *C. pneumoniae* usually causes vascular and respiratory diseases, the others are not common. It has been demonstrated a wide genetic diversity of animal *C. pneumoniae* strains compared to human strains, and reported at least two separate zoonotic transmissions from animals to human (Mitchell et al., 2010). However, it is still unknown if *C. pneumoniae* can transmit from humans to animals.

Whole genome of human *C. pneumoniae* has been sequenced. It contains 1 073 genes, among which 186 genes are *C. pneumoniae*-specific and have no homologs in any other organism, including other chlamydial species (Kalman et al., 1999).

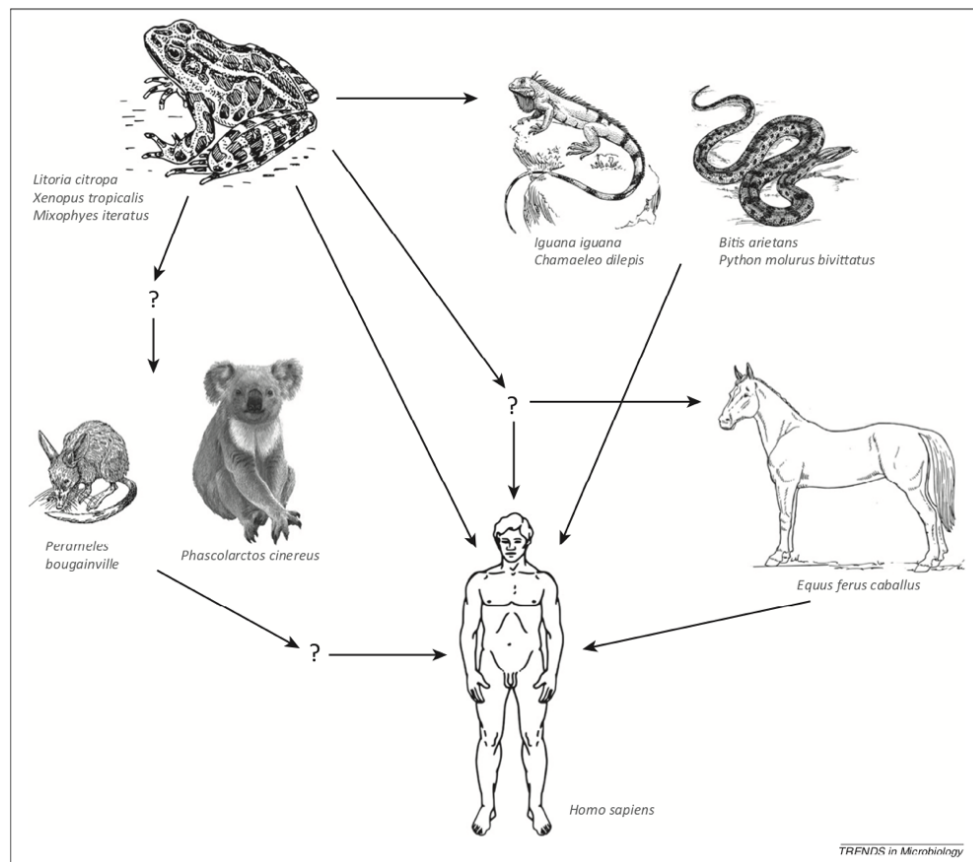


Figure 4. The zoonotic radiation of *C. pneumoniae*. (From Roulis et al., 2013)

### 1.1.3 *C. psittaci*

*C. psittaci* is an economically relevant pathogen in poultry and pet birds. Its infection can cause psittacosis or ornithosis (Harkinezhad et al., 2009). As a zoonotic transmission pathogen, *C. psittaci* can infect human and cause atypical pneumonia, which accounts for approximately 1% of annual community-acquired pneumonia (Hogerwerf et al., 2017). Interestingly, an outbreak of psittacosis in the United States in 1930 contributed to the creation of the National Institutes of Health (Lepore, 2009). *C. psittaci* was also used as a model organism from 1960s to 1970s, which laid the groundwork for modern chlamydial research.

*C. psittaci* can also be detected frequently in non-avian domestic animals, including cattle, swine, horse, goat and cat, even in wild animals and laboratory rodents (Knittler and Sachse, 2015). Whether *C. psittaci* inducing clinic diseases on these non-avian hosts is still not clear.

### 1.1.4 *C. abortus*

*C. abortus* is associated with abortion cases in ruminants, and also infects other animal species such as pigs, cattle, horses, guinea pigs, rabbits, mice and wild ungulates (Wheelhouse and

Longbottom, 2012; Rohde et al., 2010; Salinas et al., 2012; Wheelhouse and Longbottom, 2012). This infection causes a big economic loss for the industry of animal husbandry every year. Similar to other animal *Chlamydia* strains, infected animals can transfer this pathogen to human, leading to serious consequences, such as miscarriage and atypical pneumonia (Ortega et al., 2016).

### 1.1.5 *C. felis*

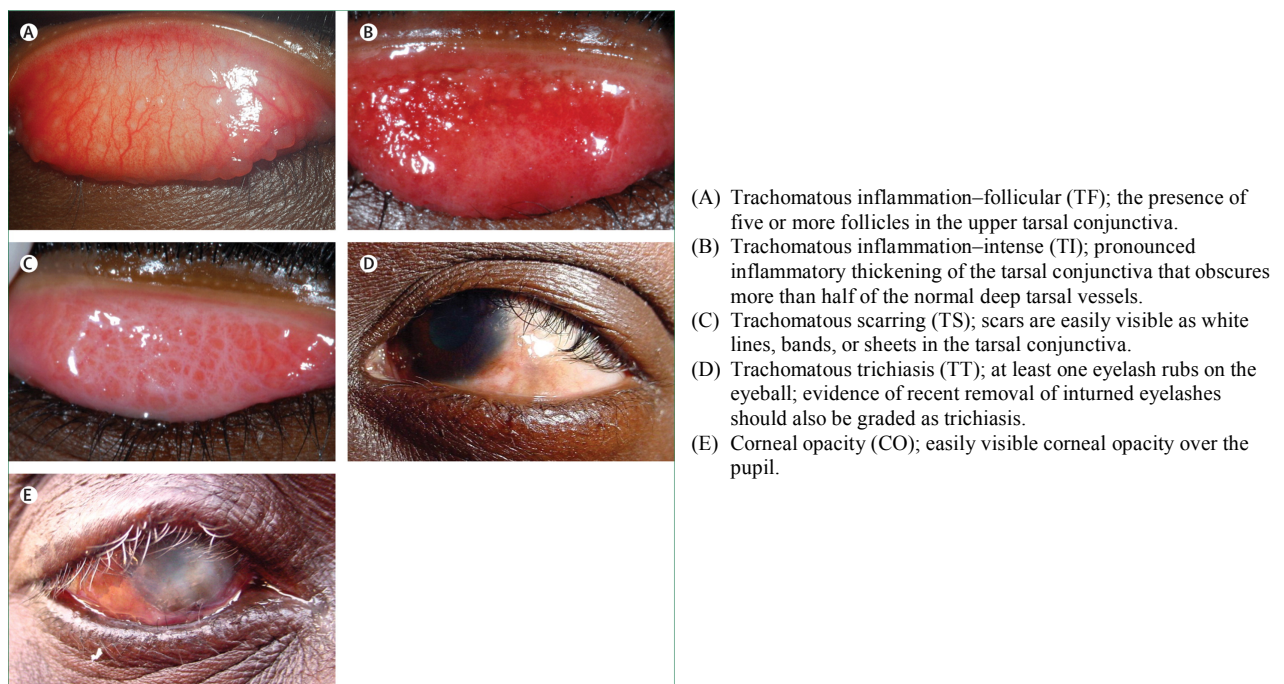
*C. felis* is a common cause of acute and chronic conjunctivitis and pneumonia (“cat flu” syndrome) in felines, and may occasionally cause similar conditions when transmitted to humans (Hartley et al., 2001; Wons et al., 2017). This disease can be cured by systemic use of doxycycline.

## 1.2 Clinical manifestations of *C. trachomatis* infection

*C. trachomatis* infections can be classified into four clinical categories: trachoma, oculogenital disease in adults, perinatal infection, and LGV.

### 1.2.1 Trachoma

#### 1.2.1.1 Trachoma etiology and epidemiology

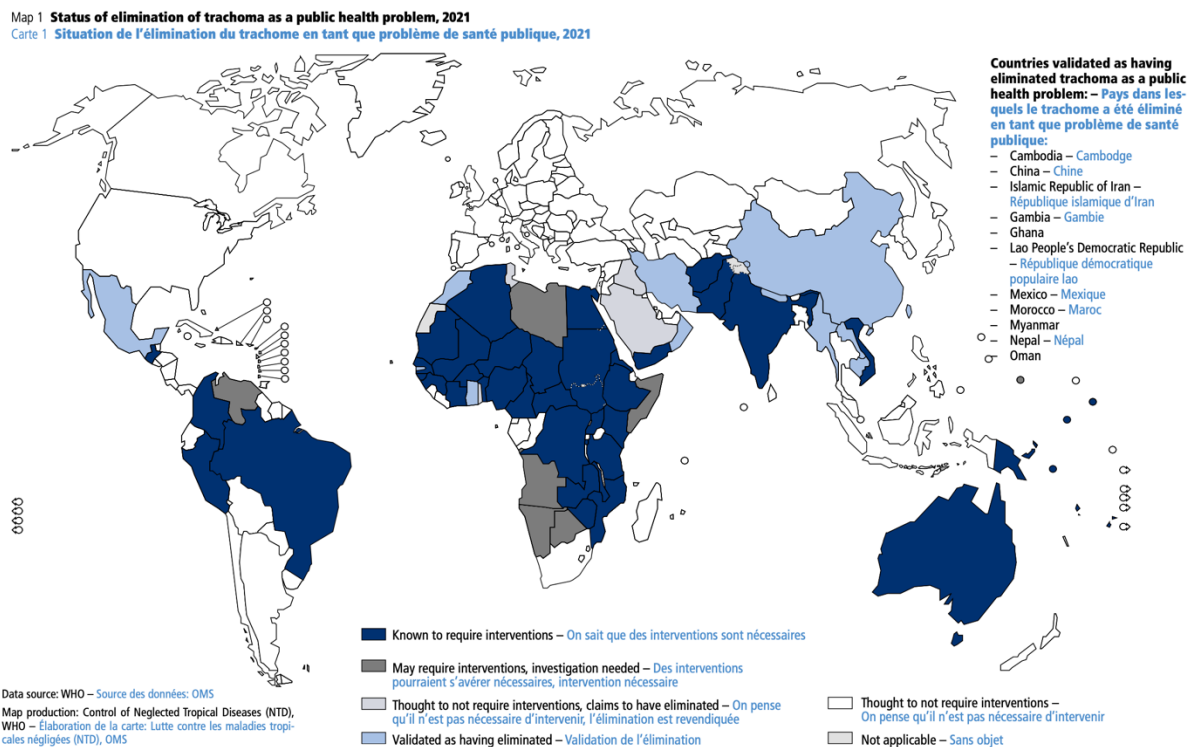


**Figure 5. Clinical features of trachoma and the WHO simplified grading.** (From Taylor et al., 2014)

*C. trachomatis* is the most common bacterial agent of sexually transmitted infection (STI) and the world’s leading cause of preventable blindness (Taylor and Haggerty, 2011; Wright et al., 2007). Trachoma is a keratoconjunctivitis caused by ocular infection with *C. trachomatis*. Serovars A-C and Ba of *C. trachomatis* are the causative pathogen of this disease. The infection is transmitted within ocular and nasal secretions that are passed from person to person on



fingers, fomites and eye-seeking flies (Last et al., 2020; Miller et al., 2004). The repeated or persistent infection by *C. trachomatis* leads to increasingly severe inflammation, triggering scar formation of the upper tarsal conjunctiva. Trichiasis develops when the scarring distorts the upper eyelid sufficiently to cause one or more lashes to abrade the cornea, scarring it in turn and causing blindness (Wright et al., 2007). Till today, trachoma is still endemic in many of the poorest and more remote areas of Africa, Asia, Australia, and the Middle East. The clinical features of trachoma are shown in Figure 5 (Taylor et al., 2014). In populations where trachoma is endemic, the infections are most common in children and are associated with symptoms of active (inflammatory) trachoma. A key sign of active trachoma is "trachoma inflammation-cell", and its prevalence in children aged 1-9 years is used to guide intervention decisions at the district level. The chronic inflammation can lead to immune-mediated conjunctival scarring and may cause inverted eyelashes to scratch the eye, a manifestation of trachoma known as trachoma trichiasis. Eventually, in some cases, vision is lost due to irreversible corneal opacity (Taylor et al., 2014).



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**Figure 6. Status of elimination of trachoma as a public health problem, 2021.** (From World Health Organization, 2021b)

Based on the data in 2020, 137 million people live in trachoma endemic areas and are at risk of trachoma blindness, which is a public health problem in many undeveloped countries, and is responsible for the blindness or visual impairment of about 1.9 million people (World Health Organization, 2021b). Up to 2021, the status of trachoma elimination in countries are listed in Figure 6 (World Health Organization, 2021a). The countries that may not require interventions are not listed, such as all countries in the WHO European region that have no recent history of

trachoma, and countries (Congo and Timor-Leste, etc) that have only recently been surveyed. Eleven countries including Cambodia, China, Gambia, Ghana, Islamic Republic of Iran, Lao People's Democratic Republic, Mexico, Morocco, Myanmar, Nepal and Oman, have been formally recognized in eliminating the trachoma (World Health Organization, 2016). Four other countries (Iraq, Saudi Arabia, Togo and Tunisia) have achieved the goal of eliminating the epidemic. However, the most recent area-level epidemic data in the GET2020 database from 44 countries indicate that trachoma is still a public health problem at least in parts of these countries (World Health Organization, 2021a).

#### 1.2.1.2 Trachoma treatment

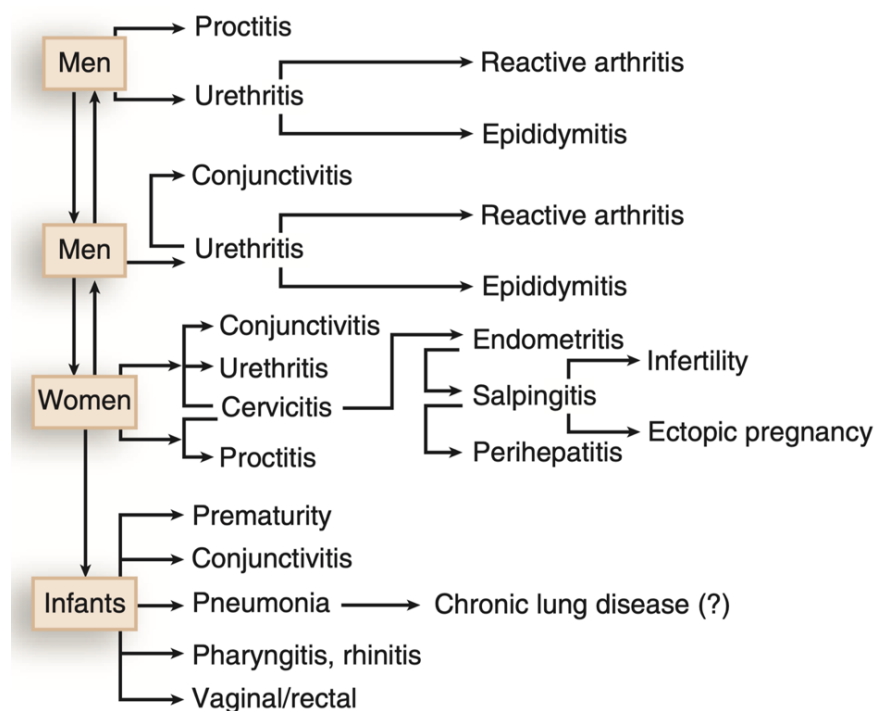
In 1996, WHO set a goal to eliminate trachoma-caused blindness worldwide by 2020, and established the Global Alliance for the Elimination of Trachoma 2020 (GET2020). To this purpose, the "SAFE" strategies (surgery for trichiasis, antibiotic treatment of infection, facial cleanliness to stop the eye infection being passed on, and environmental improvement, in particular clean water and sanitation) have been applied to control this disease (WHO, 2020). Surgery should be offered to any trachomatous trichiasis patient who can benefit from the surgery. Parts of the SAFE strategies is an intervention at public health level to find the active case and to recommend the intervention when the prevalence of trachomatous trichiasis is more than 0.2% in population with age of more than 15 years.

The key criteria for eradicability of trachomatis is that there is an effective treatment for ocular strains of *C. trachomatis* and no known animal reservoir (Melese, 2004). Mathematical models suggest that with repeated and mass treatment, it is possible to eliminate trachoma locally, depending on the level of coverage of the population, the frequency of mass treatment, and the rate of re-entry of the infection into the community after each mass treatment (Melese, 2004). Therefore, the large-scale antibiotic treatment for ocular *Chlamydia* infections has played a key role in WHO's trachoma control program. Indeed, a total of 32.8 million people received antibiotic treatment to eliminate trachoma in calendar year 2020, compared to 95.2 million in 2019 (WHO, 2021b). Current antibiotics used to treat *Chlamydia* infections target 50S and 30S ribosomal subunits, DNA gyrase, and topoisomerase IV, which are the generic drugs for many microorganisms (Sadhasivam and Vetrivel, 2017). The most widely used antibiotic to treat trachoma is azithromycin. It is an azalide subclass of macrolide antibiotics that binds to 50S subunit of the bacterial ribosome, thereby inhibiting mRNA translation (Bakheit et al., 2014). Fourteen studies on children and young people with active trachoma in different regions of the world (Evans et al., 2019), suggest that: (I) trachoma patients receiving antibiotics may have less active trachoma and eye infections at 3 and 12 months post-treatment (low certainty evidence); (II) oral and topical antibiotics may be indistinguishable in active trachoma at 3 and 12 months (low certainty evidence in ocular infection); (III) no serious adverse reactions have been reported. The most common adverse reaction reported is nausea with azithromycin treatment. The other 12 studies conducted in communities where trachoma is common (10 studies in African Region, 1 study in Egypt, and 1 study in Vitetnam ) and treated for the entire community ("mass treatment") (Evans et al., 2019), suggest that: (I) the number of trachoma

(active trachoma and ocular infection) decreases after 12 months of single-dose treatment with azithromycin (moderate established evidence); (II) no strong evidence supports to change the annual mass treatment of affected communities, the currently recommended strategy; (III) the increased risk of antimicrobial resistance appears in treated communities (highly positive evidence). But the poor understanding on the route of *C. trachomatis* transmission and its relative importance have hampered the efforts in eliminating trachoma. An investigation conducted in Orimia Region (Ethiopia) reveal that *C. trachomatis* DNA can be detected on the hands, face and clothes of individuals living in ocular-positive households, suggesting that this may be a route of transmission in *C. trachomatis*-infected households (Last et al., 2020). In addition, *C. trachomatis* is also detected on flies from ocular-positive households and occasionally from ocular-negative households, suggesting that flies may be the vectors within and between *C. trachomatis* infected and uninfected families (Last et al., 2020). Thus, these potential routes of transmission, as well as the antibiotics, are required simultaneously to inhibit trachoma transmission. Considering the different aspects on the goal of elimination of trachoma, the Alliance has changed its due date to 2030 (WHO, 2020).

### 1.2.2 Oculogenital disease in adults

*C. trachomatis* serovars D through K, and occasionally B and Ba, produce a wide variety of oculogenital infections [Figure 7].

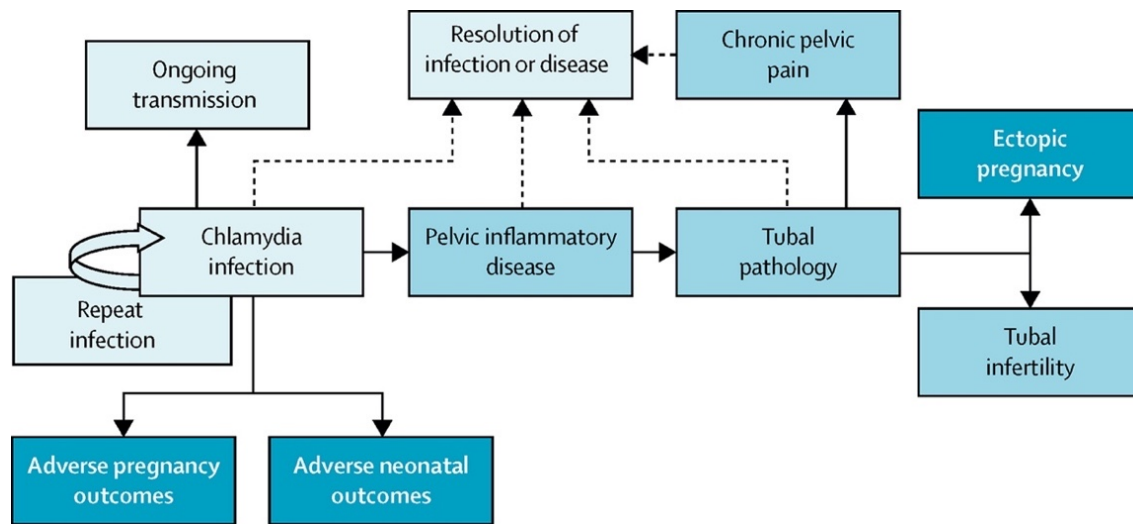


**Figure 7. Clinical manifestations and sequelae of *C. trachomatis* urogenital infections and patterns of transmission.** (From John E. Bennett, Raphael Dolin, 2020)

#### 1.2.2.1 Urogenital infection

The genital tract biovar (serovars D–K) is the most prevalent sexually transmitted bacterium (Elwell et al., 2016). Based on the global STI surveillance by WHO, global estimation of new

*C. trachomatis* cases in 2016 was 127 million (Rowley et al., 2019; WHO, 2018). As a cause of STI, *C. trachomatis* can infect both females and males. In men, 50% of infections do not have any symptoms (Gonzales et al., 2004). While, 70-80% of genital tract infections in women by *Chlamydia* are asymptomatic (Hoenderboom et al., 2017), but 15-40% of infections ascend to the upper genital tract, which can lead to serious sequelae, including pelvic inflammatory disease, infertility and ectopic pregnancy [Figure 8] (Haggerty et al., 2010; Malhotra et al., 2013; Unemo et al., 2017).



**Figure 8. Natural history and sequelae of *C. trachomatis* infection in women.** (From Unemo et al., 2017)

The data from USA CDC show that the rates of reported *Chlamydia* are highest among adolescents and young adults, and that almost two-thirds (61.0%) of all reported cases in 2019 are among populations aged 15-24 years. So, annual *Chlamydia* screening is recommended by CDC for all sexually active women younger than 25 years, whose infection by *C. trachomatis* is three times higher than the general population (USA CDC, 2019). The diagnosed *C. trachomatis* infection of the lower genital tract can be effectively and easily treated with antibiotics. The studies on the natural course of untreated lower genital tract infections by *C. trachomatis* have shown spontaneous clearance rates of 30-50% over the first 2-3 years (Geisler et al., 2013). A pilot study also demonstrates that the treatment for bacterial vaginosis has the potential to modulate the outcome of immunity to *C. trachomatis* (Mott et al., 2021). If *Chlamydia* are not eliminated, it can rise from the lower female genital tract (FGT), thereby affecting uterus, fallopian tubes and ovaries, which can lead to pelvic inflammatory disease (PID), ectopic pregnancy and infertility (Haggerty et al., 2010; Den Heijer et al., 2019). But, the study in primates has shown that a single upper genital tract infection tends to be self-limiting and does not produce tubal scarring (Patton et al., 1987). The prolonged exposure to *Chlamydia*, either chronic persistent infection or frequent reinfection, is considered as a major causative factor for the development of tubal tissue damage (Mårdh, 2004). Interestingly, Land et al report that in addition to behavioral factors and host inheritance, specific chlamydial strains (serotypes) are also thought to influence the infection process (Land et al., 2009). Indeed, about half of the asymptomatic infections of *C. trachomatis* is sustained by serovar E (Bianchi et al., 2016). Serovars E and F also represent up to 60-70% of the urogenital tract infections in

heterosexual populations (Dean et al., 2009; Jurstrand et al., 2001; Labiran et al., 2017). Whereas, G strain seems to be more prevalent in rectal tissues in men who have sex with men (MSM) (Christerson et al., 2012). *C. trachomatis* serovars D-K bacteria also cause inclusion conjunctivitis in adults, as well as perinatal infections, such as neonatal ophthalmia and chlamydial pneumonia in infants (Darville, 2005; Mishra et al., 2011; Numazaki et al., 2003).

The recommended therapy for *Chlamydia* genital infection is to orally take 1 gram of azithromycin or 100 mg of doxycycline twice daily for 7 consecutive days. This antibiotic is very popular as a first-line treatment, because its long half-life in tissues makes single-dose therapy possible and addresses concerns about adherence to a multi-day multiple-dose regimen of doxycycline. A meta-analysis shows that no significant difference in the efficacy of the two drugs for chlamydial urogenital tract infection is observed with 97% of the clearance rate of infection for azithromycin and 98% for doxycycline hydrochloride (Lau and Qureshi, 2002). The regimens for *C. trachomatis* treatment are also used for rectal and oropharyngeal infection which are prevalence in MSM and in women with urogenital infection. But, available data suggest that a single dose of azithromycin is not as effective as doxycycline in treating rectal infections in MSM and women (Kong et al., 2014; Páez-Canro et al., 2019). A recent randomized trial for the treatment of rectal chlamydial infections in MSM reports *Chlamydia* clearance rates of 100% for doxycycline and 74% for azithromycin (Dombrowski et al., 2021). Another trial assess the effectiveness of doxycycline and azithromycin in women who are *C. trachomatis* positive for rectal or vaginal or both (Dukers-Muijers et al., 2019). Among 341 rectal infections, microbiological cure rates were 78.5% in women treated with azithromycin and 95.5% with doxycycline. Of the 394 vaginal infections, 93.5% were cured in women treated with azithromycin and 95.4% in women treated with doxycycline (Dukers-Muijers et al., 2019). The reason for the lower efficacy of azithromycin in the treatment of rectal infections is unclear. This should not be due to the development of antibiotic resistance to azithromycin, the kinetics of azithromycin in rectal tissue, or the persistence of *Chlamydia*. Instead, this low efficacy of azithromycin may be related to the higher organism load and local pH in rectal tissue (Hammerschlag and Sharma, 2021). Based on these findings, the USA CDC updated the STI guidelines on the treatment of *C. trachomatis* infection: doxycycline 100 mg twice daily for 7 days is the first choice for chlamydial infection among adolescents and adults; the alternative regimens are azithromycin (1 g orally in a single dose) or levofloxacin (500 mg orally once daily) for 7 days. Erythromycin is no longer recommended, due to its common gastrointestinal side effects that may cause patients not to comply with the instructions (Workowski et al., 2015, 2021). Meanwhile, it is also very important to treat the sexual partner of the patient, which can effectively prevent the patient from re-infection. Indeed, *Chlamydia* re-infection after treatment is common. In a cohort study, more than 20% of young women admitted from general clinics acquired a re-infection within 12 months of treatment (Walker et al., 2012).

Unlike other bacterial pathogens whose resistance genes acquired through lateral gene transfer with subsequent resistant to antimicrobial drugs (Chatterjee et al., 2018), *Chlamydia* is still sensitive to all anti-chlamydial antibiotics. The only exception is *Chlamydia suis*, a species-

specific pathogen in pig, which is resistant to tetracycline (Seth-Smith et al., 2017). Fortunately, there is no horizontal transfer of the resistance gene to other *Chlamydia* species. However, despite the availability of a wide range of effective drugs and the apparent lack of antibiotic resistance mechanisms, *Chlamydia* remains a widespread pathogen. Perhaps due to their long evolutionary history as obligatory intracellular parasites, *Chlamydiae* appear to have evolved unique mechanisms to resist antimicrobial effects triggered by host innate and adaptive immune responses (Panzetta et al., 2018).

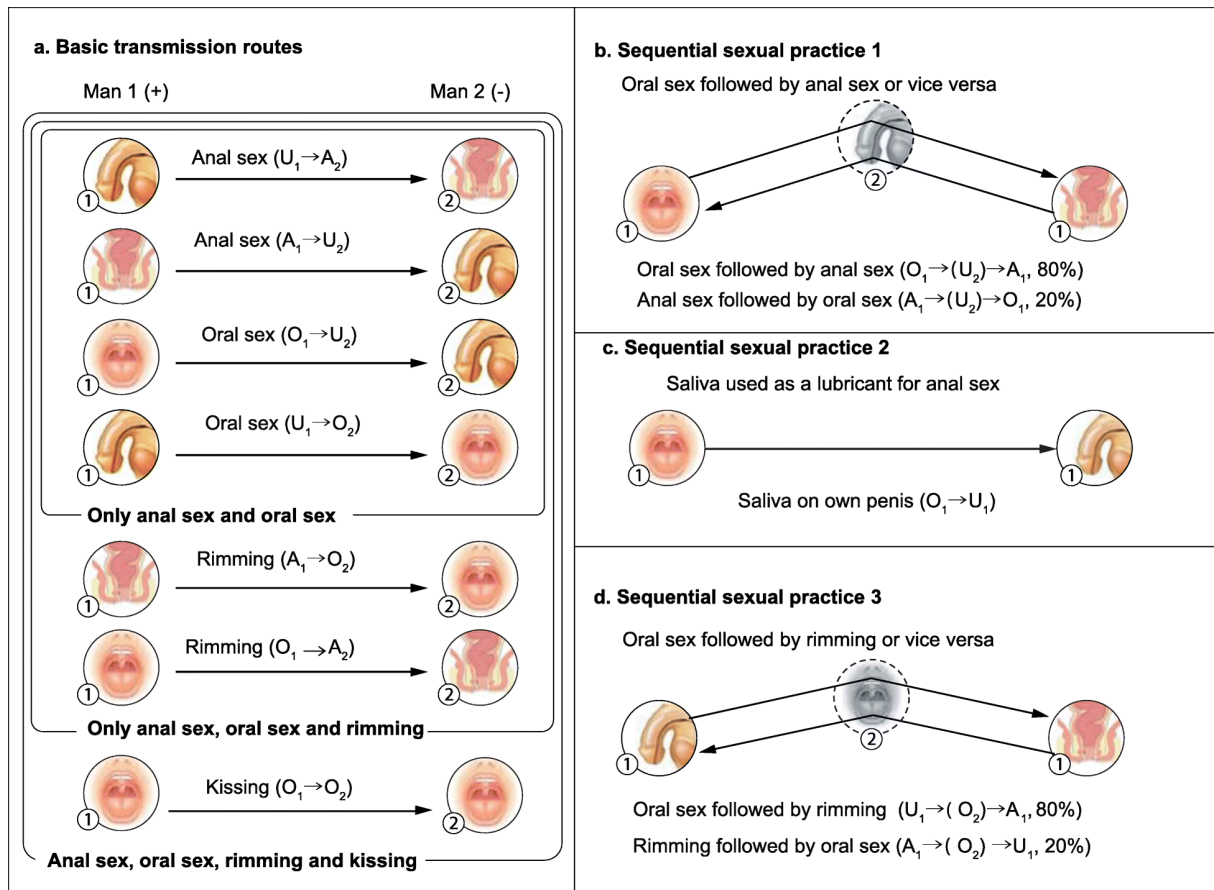
#### 1.2.2.2 Inclusion conjunctivitis

As mentioned previously that *C. trachomatis* serovars D-K also cause inclusion conjunctivitis in adults and in newborns by perinatal infections. If the mother is infected without treatment, 20% to 50% of newborns may develop chlamydial conjunctivitis, and 10% to 20% may develop *C. trachomatis* pneumonia (Rours et al., 2009, 2008). The latter usually occurs at the age of 1~3 months of infants and is a subacute pneumonia, characterized as repeated staccato cough, accompanied by tachypnea and hyperinflammation, and diffuse infiltration on chest radiography (Workowski et al., 2021). Adult chlamydial conjunctivitis can also develop in sexually active populations, with up to 80% of patients having concomitant genital infections (Postema et al., 1996). Analysis of the *C. trachomatis*-positive conjunctival specimens in Hungary shows seven different urogenital chlamydial serotypes: E (16/53), F (15/53), D (6/53), K (6/53), G (4/53), H (4/53) and J (2/53).

#### 1.2.2.3 Extragenital infections

Overall, 53% of chlamydial infections are at non-urethral sites and anorectum infections are commonly found in men who have sex with men. To investigate the prevalence of chlamydial rectal and pharyngeal infection, a study has been conducted in more than 6 000 MSM (Kent et al., 2005). The incidence of chlamydial infection is 7.9% for rectum, 5.2% for urinary tract and 1.4% for pharynx, respectively. In another study, the rectal infection rates of *Chlamydia* in women and MSM was overall 9% summarized from 115 eligible studies between 2000 and 2016 (Dewart et al., 2018). It suggests that rectal rather than urinary tract infections is the most common site of infection among MSM, although approximately 85% of rectal infections are asymptomatic.

Chlamydial transmission among MSM usually occurs at three anatomical sites (oropharynx, urethra, and anus). A large number of potential sexual behaviors may spread the infection, including kissing, licking (oral-anal sex), and the use of saliva as a lubricant for anal sex among MSM (Chow and Fairley, 2019; Cornelisse et al., 2018; Templeton et al., 2008). Using a mathematical model, Xu et al show that besides oral and anal sex, other transmission routes also contribute to the high infection rate of *C. trachomatis* in MSM population (Xu et al., 2020). These widely recognized and presumed routes of chlamydial transmission are shown in Figure 9.



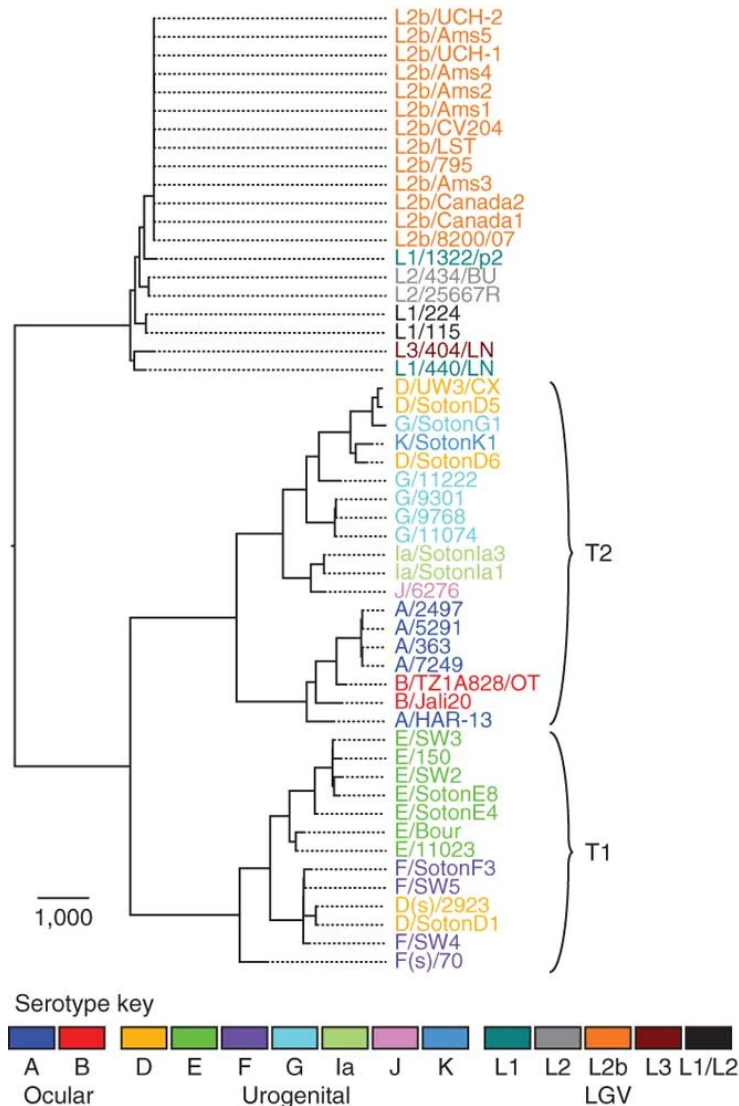
**Figure 9. Baseline transmission routes (a) to which transmission routes 1–3 (b–d) have been added; arrow signifies the direction of transmission. Note: Man 1 (1); Man 2 (2) (From Xu et al., 2020)**

Rectal infection with *C. trachomatis* is also prevalent among women who travel to STI clinics (Van Liere et al., 2017). A meta-analysis demonstrates that the overall prevalence of rectal *Chlamydia* infection is 6% among women working on sexual active workers, and 68% among those who also tested positive for vaginal *Chlamydia* (Chandra et al., 2018). Another survey shows that 33%~83% of women have *C. trachomatis* infection at the anorectal site, and that the test results from these women are not associated with reports of receptive anorectal activity (Dukers-Muijers et al., 2015). Anyway, there are several critical gaps in our understanding of the epidemiology of rectal *Chlamydia* in women, for instance, it is unknown whether rectal *C. trachomatis* circulating among women represents an infection with active bacteria; it is a matter of debate through which route women obtain rectal *C. trachomatis*; finally we also do not know the impact of rectal *C. trachomatis* on the health in these women (Khosropour et al., 2019). It is also hypothesized that women can auto-inoculate *C. trachomatis* from the rectum into the vagina (Bavoil et al., 2017; Rank and Yeruva, 2014, 2015). Fortunately, rectal *C. trachomatis* is usually asymptomatic, and the infection itself is not a morbid condition.

### 1.2.3 Lymphogranuloma venereum (LGV)

LGV is a condition caused by the invasive serovars of *C. trachomatis* (L1, L2, L3 and L2b) (Stoner and Cohen, 2015). If not treated, the infection may lead to long-term complications such as deep tissue abscess formation, strictures, fissures, and chronic pain (McLean et al.,

2007). LGV is reported most frequently in Africa, Southeast Asia, South America and the Caribbean, and is rare in the developed countries (D Mabey, 2002). But, an epidemic of LGV with atypical presentation and symptoms is currently in progress in Europe and North America, primarily affecting MSM, which is caused by infection of L2b serovar (Harris et al., 2012a).

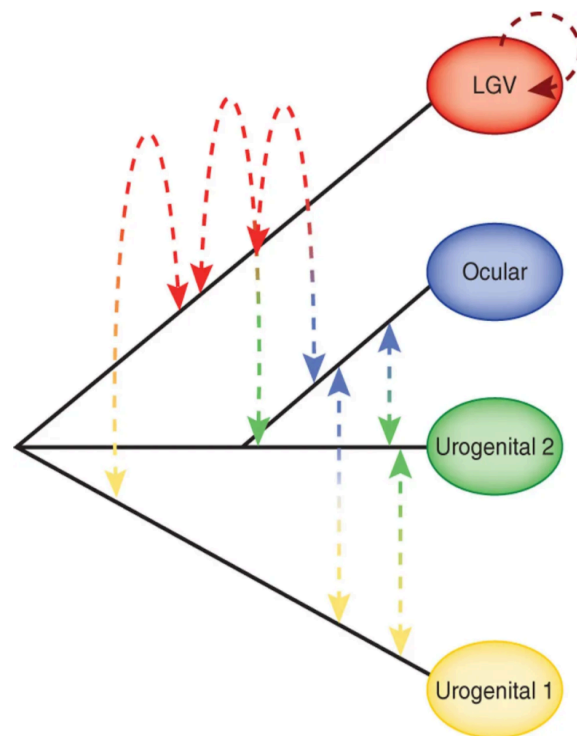


**Figure 10. *C. trachomatis* species phylogeny using the chromosomal sequences of 52 genomes after predicted recombinations have been removed.** (From Harris et al., 2012)

Harris et al analyze the whole genome of diverse *C. trachomatis* strains. They found that *C. trachomatis* comprises three distinct lineages: the species seem to have split early into LGV and urogenital clades, with the urogenital clade itself later splitting into two clades termed T1 and T2 (Harris et al., 2012a). T1 is composed of clinically prevalent urogenital serotypes, whereas T2 contains most of the rarer urogenital serotypes (Suchland et al., 2003). Ocular *Chlamydia* is a younger lineage that, within the limits of their sampling frame, seems to have emerged only once from a urogenital ancestor within T2 (Harris et al., 2012a). Base on this study, a whole-genome phylogenetic tree was constructed as shown in Figure 10. This method



can also be used in tracing gene exchange in the species [Figure 11], which brings new insights into the evolution of this pathogen (Joseph and Read, 2012).



**Figure 11. Phylogenetic reconstruction of the four *C. trachomatis* biovars.** Each biovar (LGV, ocular, and urogenital 1 and 2) is shown in separate colors. Major pathways of recombination within and between biovars are shown using double-headed dotted lines. (From Joseph and Read, 2012)

### 1.3 Cellular biology of *C. trachomatis*

#### 1.3.1 Biphasic life cycle of *C. trachomatis*

The chlamydial developmental cycle was first described by Bedson and Bland (Bedson and Bland, 1932). Due to the well conserved biological characteristics, *Chlamydiae* share a common unique developmental cycle that includes an infectious elementary body (EB, intracellular or extracellular) and an intracellular replicative reticular body (RB) (Bayramova et al., 2018b; Omsland et al., 2014). The EBs are small ( $\sim 0.2 \mu\text{m}$  in diameter) and round, contain a centrally denser nucleoid, and act as a "spore-like" form with a rigid cell wall that allows *Chlamydiae* to survive in the harsh extracellular environment (Cherilyn Elwell, 2016). Historically, the EBs are considered as inert metabolism, but, the active biosynthesis and metabolism of EBs have been observed (Omsland et al., 2014). The EB is the invasive form of *Chlamydiae*. Upon invasion, *C. trachomatis* modifies the phagocytic compartment of host to form a membrane-bound structure, called inclusion. Two to six hours after invasion, the EBs differentiate into the RBs that start the replications within the inclusion. The RB is larger ( $\sim 0.8 \mu\text{m}$  of diameter) than the EB, is structurally flexible, has fragile permeability, is rich in RNA, and also contains diffuse and fibrillar DNA (AbdelRahman and Belland, 2005).

The developmental cycle of *C. trachomatis* is demonstrated in Figure 12 (Elwell et al., 2016). It includes 5 major processes: (i) EBs attach to and enter host cells to form inclusions; (ii) EBs differentiate into RBs in the inclusion; (iii) modification of inclusions and replication of RBs by binary fission (starting 9 ~12 h after infection, RBs divide repeatedly to produce several hundred to a thousand progenies (Lee et al., 2018a)); (iv) inclusion expansion and RB to EB transition; and (v) bacteria are released by host cell lysis or extrusion after 30 ~ 72 hours post-infection (depending on species, strain and host) (Nunes and Gomes, 2014). The *in vitro* evidence also shows that under stressful growth conditions, RBs can remain a non-replicating, but viable persistent state, which is called aberrant RBs (aRBs). More details about this form will be introduced in “*Chlamydia*-Host Interaction” chapter.

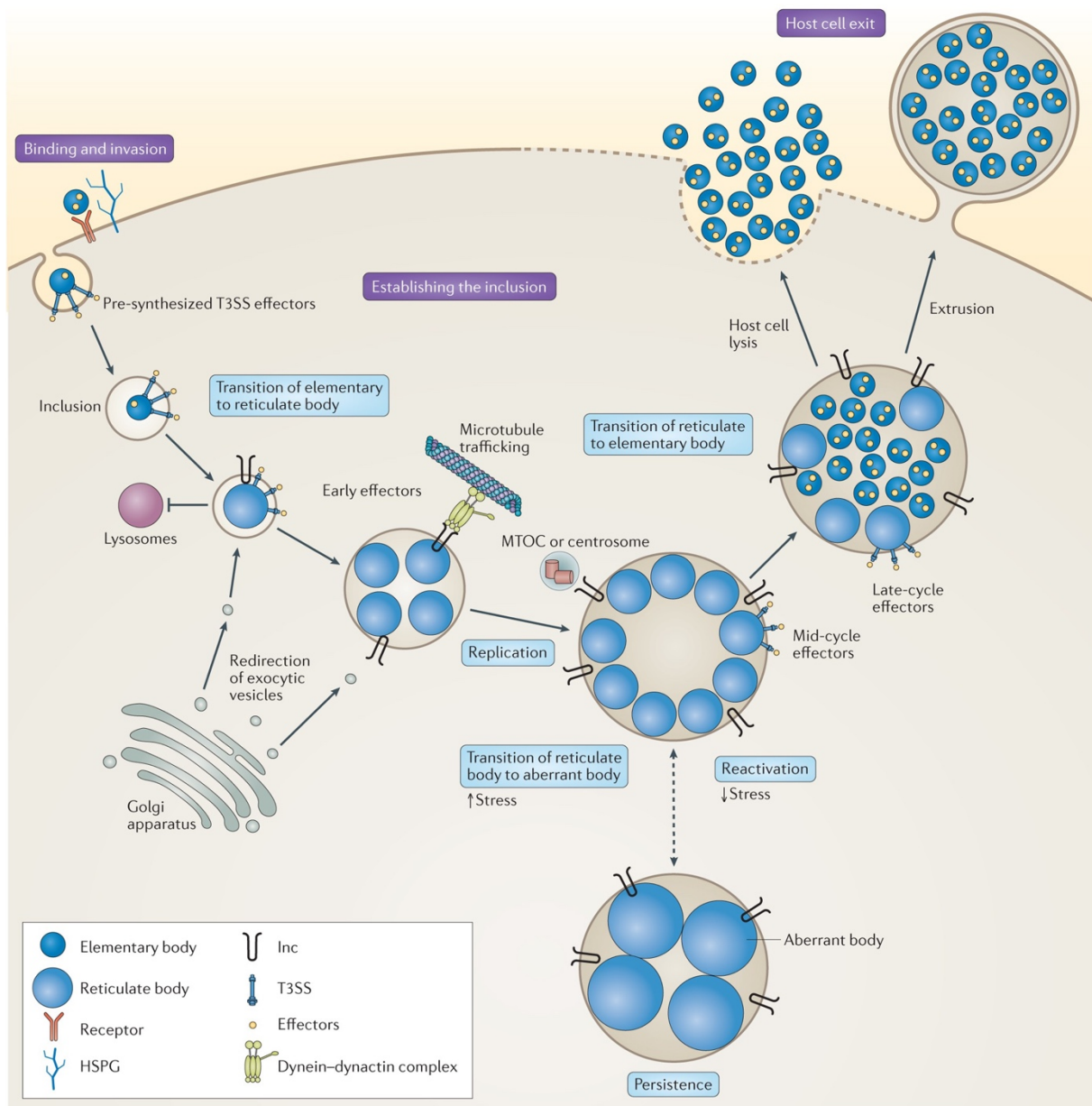
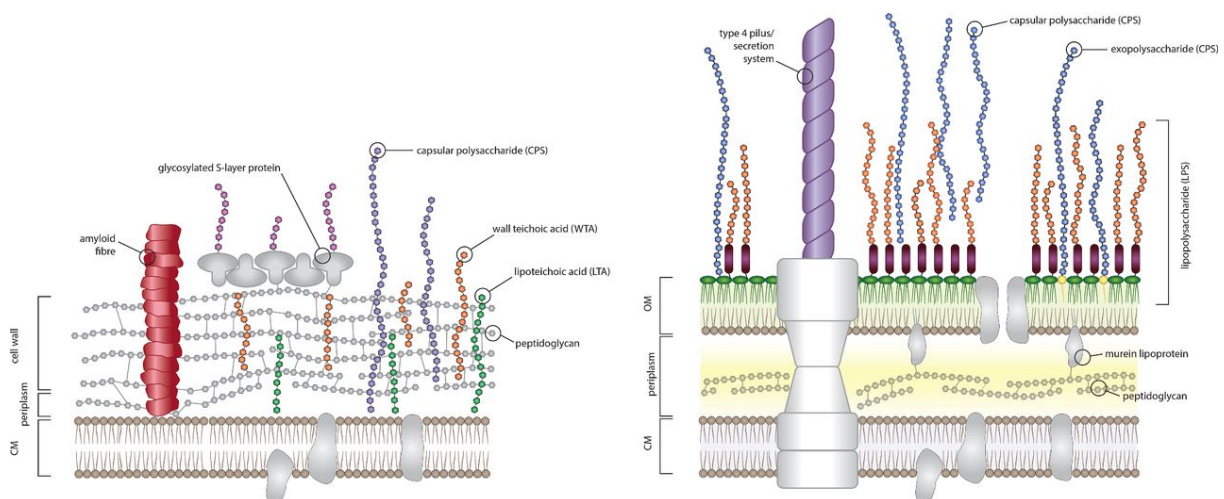


Figure 12. The life cycle of *C. trachomatis*. (From Elwell et al., 2016)

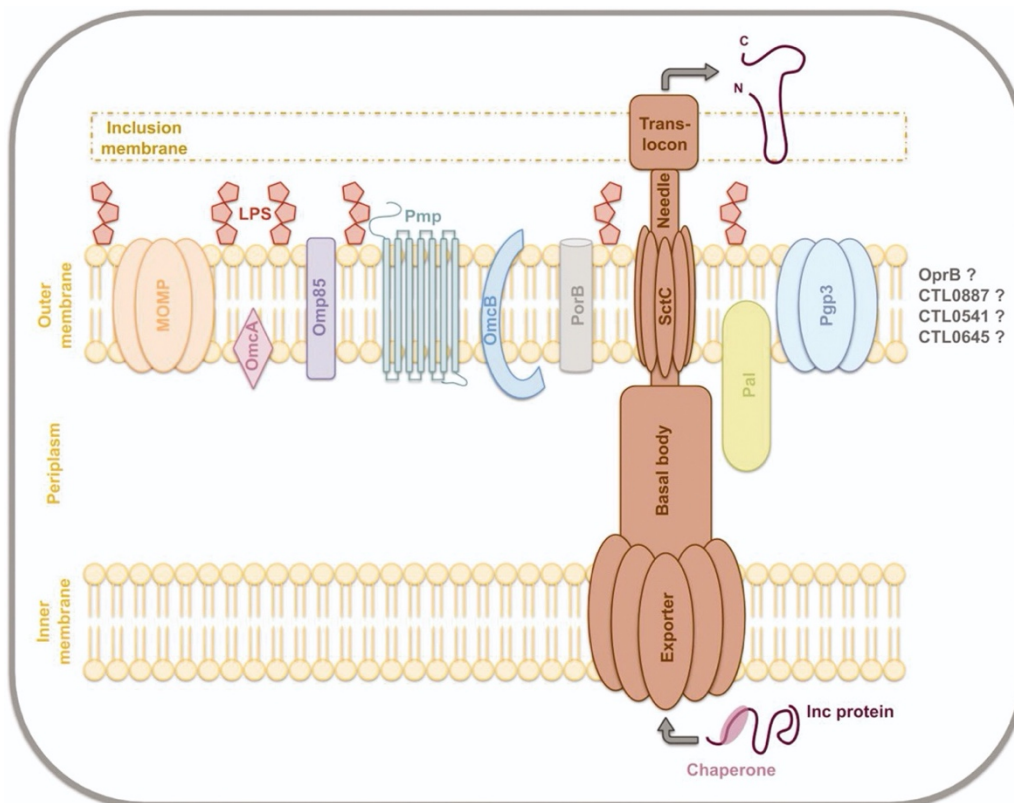
### 1.3.2 The envelope of *C. trachomatis*

Historically, bacteria have been classified based on their ability to retain Gram stain (Gram, 1884). With the development of electron microscopy, the actual structure of the bacterial cell envelope has been revealed. Gram-negative bacteria have a complex cell envelope, including an outer membrane (OM), an inner membrane (IM, or cytoplasmic membrane, CM) and the periplasmic space between both membranes (Vergalli et al., 2020; Zgurskaya et al., 2016). The three essential layers of the Gram-negative cell envelope are chemically and structurally distinct: the IM is an essentially symmetric phospholipid bilayer responsible for various physiological and metabolic functions; the periplasmic space is termed periplasm (Mitchell, 1961) containing a thin layer of peptidoglycan (Braun and Sieglin, 1970); and the OM is the first line of defense, forming a physical and mechanical barrier that protects cells from external aggressive substances such as antibiotics, disinfectants, cationic peptides, and bacitracin (Nikaido, 2003; Silhavy et al., 2010; Zgurskaya et al., 2016). The OM also contains proteins that mediate passive or active uptake of small molecules to promote growth and cellular function (Pagès et al., 2008). The OM also possesses lipopolysaccharides (LPS) in the outer leaflet (Kamio and Nikaido, 1976), which covering ~75% of the bacterial surface (Filloux and Whitfield, 2016). In addition, IM and OM also differ in their integral membrane proteins. The integral IM proteins span the membrane as  $\alpha$ -helices, almost composed of hydrophobic residues, whereas most integral outer membrane proteins (OMPs) are composed of amphipathic  $\beta$ -strands that adopt a  $\beta$ -barrel structure (Fairman et al., 2011; Schulz, 2002). Gram-positive bacteria lack a protective outer membrane compared to Gram-negative bacteria, but the peptidoglycan (PG) layer is many times thicker than that of Gram-negative bacteria (Silhavy et al., 2010; Vollmer et al., 2008). The outline of bacterial envelope is shown in Figure 13.



**Figure 13. Schematic representation of the cell envelope of gram-positive (left) and gram-negative (right) bacteria.** Some elements of the envelope have been labelled. The cytoplasmic membrane (CM) and inner leaflet of the outer membrane (OM) in gram-negative bacterium are made of phospholipids while the outer leaflet of the OM is mainly LPS (core LPS in green). Grey shapes within membranes are mostly integral membrane proteins or lipoproteins. As examples macromolecular complexes have been represented attached to or inserted in the cell envelope such as pili (red in gram-positive) or type III secretion system (needle-like structure in gram-negative). (From Filloux and Whitfield, 2016)

Specifically for *Chlamydiaceae*, as a Gram-negative bacterium, they are surrounded by an outer membrane and a cytoplasmic inner membrane (Tamura et al., 1971), separated by a periplasmic space [Figure 14]. The inner and outer membranes are morphologically and chemically distinct (Glauert and Thornley 1969). Both outer and inner membranes contain phospholipids and proteins. For EB and RB, the chlamydial envelope is the entire membrane on the pathogen surface, which is a lipid mixture typically found in prokaryotes (phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine) and eukaryotes (phosphatidylcholine, phosphatidylinositol, sphingomyelin, and cholesterol). The prokaryotic lipids were synthesized by *C. trachomatis* themselves, while the eukaryotic lipids are acquired from the host cell (Wylie et al., 1997).



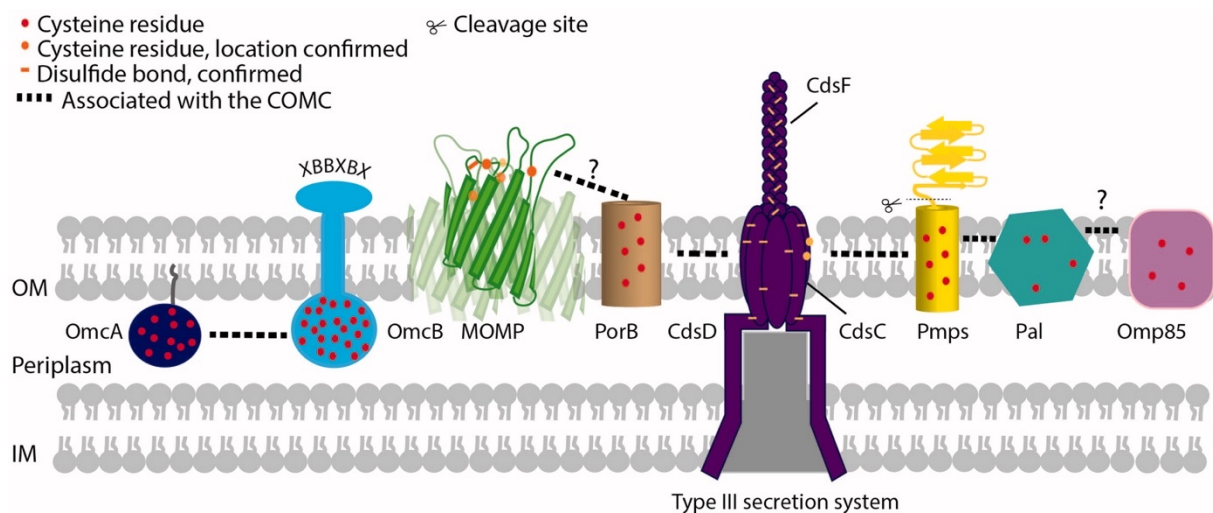
**Figure 14. Model of the envelope of chlamydial EBs.** (From Gitsels et al., 2020)

The specific proteins of the inner membrane have not been identified. EB is encapsulated by the chlamydial outer membrane complex (COMC), which is defined as the insoluble fraction when extracted with the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl buffer). The COMC comprises a protein shell that is cross-linked by intra- and intermolecular disulfide bonds. This dense network confers rigidity and limits maneuverability, leading to EB resistance to physical or osmotic pressure (Hackstadt et al., 1985). Despite of COMC components, prominent appendages of infectious *Chlamydia* is type 3 secretion system (T3SS). The T3SS traverses the inner membrane, periplasm and outer membrane of the chlamydial envelope and even the inclusion membrane. In EB forms, T3SS functions to attach host cell. Once entering the inclusion, *Chlamydia* translocate effectors via T3SS, such as Incs from the cytoplasm of the bacterium to the inclusion membrane or the cytosol of the host cell with the help of chaperones (Gitsels et al., 2020). In addition to proteins, the major component of the outer leaflet of the

outer membrane of gram-negative bacteria is LPS. This is also true in *Chlamydia*, although it is mainly composed of lipooligosaccharide (LOS) (Rund et al., 1999). Furthermore, the presence of peptidoglycan (PG) in *Chlamydiae* has long been debated. Although genetic analysis and antibiotic susceptibility indicate the presence of PG in *Chlamydia* (Moulder et al., 1963; Stephens et al., 1998; Tamura and Manire, 1968), all attempts to detect and purify PG have been unsuccessful, leading to the paradox known as "chlamydial anomaly" (Moulder JW, 1993). Until 2014, Liechti GW et al. demonstrated the presence of PG in *C. trachomatis* by a new metabolic cell wall labeling method (Liechti et al., 2014) and observed that chlamydial PG did not appear to form a saccular structure, but rather existed as a ring structure on a distinct cell division face (Liechti et al., 2016).

### 1.3.2.1 COMC

As mentioned above, through cross-linking by disulfide bonds, the cysteine-rich COMC proteins form a stable network that provides rigidity to the envelope of EBs. Several COMC components are still in RBs but the cysteine residues in the assembly proteins are reduced, so a tight network would not be assembled. The reduction of cross-linked complexes during differentiation of EBs into RBs, provides the fluidity of the membrane required for RB replication (GeorgHäcker, 2018). This simple change in COMC properties is vital for the biology of *C. trachomatis*. The major components of COMC include MOMP, some polymorphic membrane proteins (Pmps), and two cysteine-rich proteins (CRP), OmcA and OmcB (Liu et al., 2010). Other proteins present in COMC are PorB, Omp85, SctC, Pal, CTL0887, CTL0541, CTL0645, and OPRB (Gitsels et al., 2020; Liu et al., 2010).



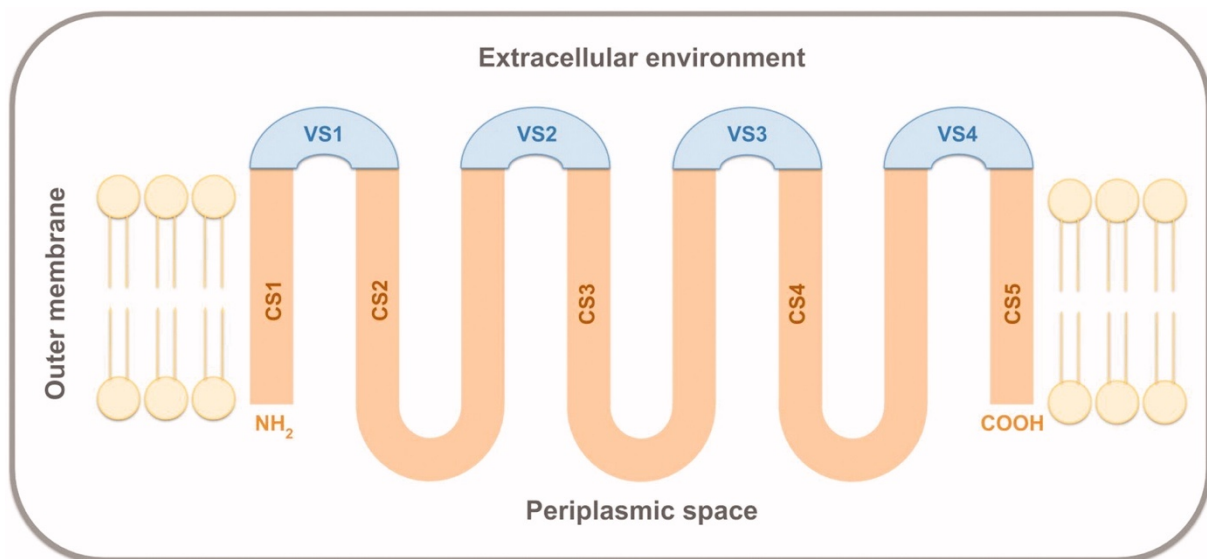
**Figure 15. Composition of the COMC.** (From Christensen et al., 2019)

#### 1.3.2.1.1 MOMP

MOMP was identified and named from purified *C. trachomatis* EBs in 1981, and is the predominant protein in the COMC, which accounts for 60% of the total COMC (Bavoil et al., 1984; Caldwell et al., 1981). The size of MOMP is approximately 39.5 kDa, with little difference in size between species and serotypes (Hatch et al., 1981). As mentioned previously,

MOMPs are extensively cross-linked by disulfide bridges, leading to their insolubility. Depending on species and serovar, MOMP contains seven to ten cysteine residues, seven of which are conserved. Four of them are located in the predicted outer loop and are expected to form intramolecular disulfide bonds (Yen et al., 2005). Its native conformation is a trimer in the outer membrane, mainly 14- or 16-strand  $\beta$ -barrel structure (Feher et al., 2013; Sun et al., 2007; Wyllie et al., 1998). This protein consists of five conserved sequences (CS1-CS5) and four variable sequences (VS1-VS4), which are exposed in the periplasmic and extracellular space, respectively [Figure 16] (Kim and DeMars, 2001). Its function is porin in RB that facilitates the selective passage of nutrients across the chlamydial cell membrane (Bavoil et al., 1984; Jones et al., 2000), and a potential adhesion protein in EB (Su et al., 1990).

MOMP also plays a very important role in stimulating the immune response. MOMP contains genus-, species-, and serovar-specific epitopes (Caldwell et al., 1981). Protective epitopes are located in VS1 and VS2 (Baehr et al., 1988). Serum-specific epitopes are also present in those domains and in VS4, while those specific for genera and species are located in conserved sequences. Some species-specific epitopes are also present in the most conserved region of VS4 (Baehr et al., 1988; Batteiger, 1996). MOMP is located in the outer membrane, is highly immunogenic, and elicits neutralizing antibodies and T-cell responses, making MOMP as an ideal vaccine candidate (Gitsels et al., 2020).



**Figure 16. Schematic representation of chlamydial MOMP.** (From Gitsels et al., 2020)

#### 1.3.2.1.2 Pmps

Pmps are a group of membrane-bound proteins that are present in all chlamydial species. These proteins are grouped together based on a conserved GGA (with I, L, V in the fourth position) and FxxN terrapeptide motifs on their N-terminal part (Tan et al., 2010). Pmps were first found in *C. psittaci* (Longbottom et al., 1996), and subsequent in all other members of the *Chlamydia* (Vasilevsky et al., 2016). The *C. trachomatis* genome encodes nine Pmp proteins called PmpA-I (Grimwood and Stephens, 1999), making the Pmps family becomes the largest protein family of *Chlamydia* (Vandahl et al., 2004).

Pmps have been identified as autotransporter (type V secretion system) proteins based on their cleavable N-terminal signal sequence for transporting across the inner membrane. Their central passenger domain is responsible for the function of the protein, and their C-terminal transport domain forms a  $\beta$ -barrel with phenylalanine at the end of this domain implying outer membrane localization (Dautin and Bernstein, 2007; Henderson and Lam, 2001).

Pmps are immunogenic-like surface-exposed proteins that play an important role not only as adhesions, but also as potent antigenic proteins involved in the immunopathogenesis of *Chlamydia* infection (Vasilevsky et al., 2016). It has been shown that adhesion-competent homomeric and heteromeric Pmp oligomers are important for *C. trachomatis* infection. In addition, adhesion-incompetent fragments may also contribute to the formation of adhesive heteromeric oligomers, supporting the biological function of adhesion-competent Pmps (Favaroni and Hegemann, 2021). Pmps are able to activate innate immune responses in infected cell lines. For instance, Pmp20 and Pmp21 of *C. pneumoniae* induce IL-6 and monocyte chemoattractant protein 1 (MCP-1) secretion by human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner via activating the nuclear factor kappa B (NF- $\kappa$ B) pathway (Niessner et al., 2003). Incubation of *C. trachomatis* recombinant PmpD with a human monocytic cell line (THP-1) also elicits robust IL-8 production in a dose-dependent manner (Wehrl et al., 2004). PmpB, C, D, and I are most commonly identified in clinic and elicit a stronger humoral immune response, while PmpA and E have the lowest recognition rates in sera of women with pelvic inflammatory disease (PID), of adolescent women, and of men. Above evidences suggest that these Pmps may be more frequently exposed or abundantly expressed on the surface of *C. trachomatis* (Tan et al., 2009). Interestingly, there is a gender bias in the recognition of Pmp: PmpB has the highest recognition rate in sera from female patients (82% of sera), while in sera from males, PmpD owns the highest recognition rate (92% of sera). Another study also shows that only sera from *Chlamydia* serovar D, E and G-infected individuals neutralize recombinant PmpC, whereas all infected individuals respond to MOMP (Gomes et al., 2005). Meanwhile, all patient sera positive for chlamydial serovars Ba, E, F and K respond to recombinant PmpD protein. However, patients infected with D, Ia, J, and G strains did not have immunoreactive sera to PmpD. None of the sera from infected individuals responded to recombinant PmpF protein (Nunez et al., 2007). It suggests that Pmps expression is differentially regulated in different chlamydial serovars and may elicit different immune responses to specific serovars. This may also suggest that PmpC and PmpD can elicit particularly strong humoral immune responses in adolescent females.

#### 1.3.2.1.3 OmcA and OmcB

Besides MOMP and Pmps, other major components of COMC are CRPs OmcA (also known as EnvA/small CRP/Omp3) and OmcB (also known as EnvB/large CRP/Omp2) (Liu et al., 2010). OmcA has a cysteine content of ~15% and is the most cysteine-rich chlamydial protein. OmcA is predominantly hydrophilic and has no significant transmembrane component (Everett and Hatch, 1991), which is associated with the membrane via a lipid anchor while its proteinaceous portion faces the periplasmic space (Allen et al., 1990; Raulston, 1995).

OmcB has a cysteine content of 4-6% with no cysteines in the membrane-spanning region and remains constant (70-80%) in *Chlamydia* species, with a highly variable N-terminal region (Newhall, 1987; Watson et al., 1991). OmcB is the primary immunogen for *Chlamydia* infection (Mygind et al., 1998; Sanchez-Campillo et al., 1999). OmcB binds to glycosaminoglycans (GAGs) on the host cell surface, and heparin sulfate on the host cell surface is necessary for infection (Fechtner et al., 2013). Another study has also observed that OmcB is involved in the conversion of RB to EB (Mygind et al., 1998).

#### 1.3.2.2 Peptidoglycan (PG)

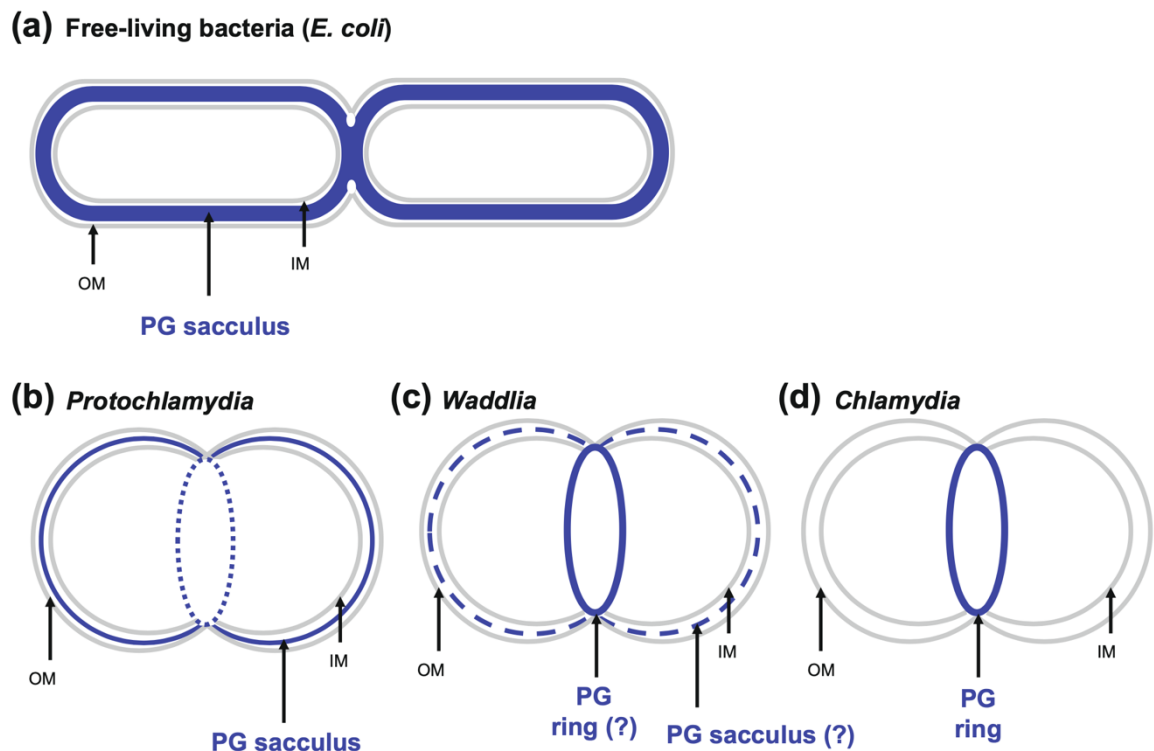
PG, also known as murein, is one of the largest macromolecules in bacterial cells and usually forms a mesh-like structure, named PG sacculus, that encloses the cytoplasmic membrane (Vollmer, 2008). In Gram-negative bacteria, this PG sacculus is located in the periplasm between the cytoplasm and the outer membrane, whereas in Gram-positive species, the PG layer is thicker and connected to other major cell wall polymers such as teichoic acid and cap-shaped glycans (Silhavy et al., 2010; Weidenmaier and Peschel, 2008). In the vast majority of free-living bacteria, PG contributes to cell division, maintains osmotic pressure, and provides a stable anchor for transmembrane complexes and integral membrane proteins (Egan and Vollmer, 2013; Mattei et al., 2010).

The presence of PG in *Chlamydia* has ever been controversial. Genetic analysis and antibiotic susceptibility indicated the potential presence of chlamydial PG (Stephens et al., 1998; Tamura and Manire, 1968). NOD1, receptor for PG, is also activated upon *Chlamydia* infection (Welter-Stahl et al., 2006). The studies on the homologs of PG biosynthetic enzymes of *Chlamydia* also indicate that *Chlamydia* can support a complete PG biosynthetic pathway (Patin et al., 2012). Surprisingly, all efforts to detect or purify chlamydial PG were unsuccessful, resulting in 'chlamydial anomaly' over the past 50 years (Moulder JW, 1993). Until several years ago, the presence of PG in *C. trachomatis* was first detected using the next generation of bioorthogonally labeled D-amino acid dipeptide (DAAD) probes mimicking DA-DA during PG synthesis (Liechti et al., 2014). Later work from the same group, demonstrated that all four pathogenic *Chlamydiae* (*C. trachomatis*, *C. psittaci*, *C. muridarum*, and *C. caviae*) possess a <140 nm wide PG ring limited to the split surface during the replication phase of their developmental cycle (Liechti et al., 2016). The schematic of PG ring was shown in Figure 17 (Klöckner et al., 2016).

The chlamydial genome lacks the gene encoding FtsZ, a cytoskeletal cell division initiation protein that organizes many PG biosynthetic enzymes around the cell division plane in almost all bacteria (Egan and Vollmer, 2013). However, *Chlamydia* possesses an MreB ortholog (Ouellette et al., 2012), an actin-like homolog found in organisms such as *E. coli* and *B. subtilis* (Van den Ent et al., 2001). Ouellette et al hypothesize that MreB may functionally substitute for FtsZ in *Chlamydia*, recruiting the cell division machinery to the division body (Ouellette et al., 2012). Indeed, the PG ring is perturbed in the presence of an inhibitor of MreB, and subsequently blocks chlamydial division at an early step (Liechti et al., 2016). Later, MreB is shown to be localized at the division site by super-resolution microscopy (Lee et al., 2020).



Thus, in contrast to the FtsZ-dependent binary fission process for other bacteria, *Chlamydia* employs an MreB-dependent polarized budding process to divide (Ouellette et al., 2020).



**Figure 17. Model on evolutionary reduction of PG structures in deeply rooting *Chlamydia*-related bacteria and in *Chlamydia*.** IM inner membrane; OM outer membrane. (From Klöckner et al., 2016)

### 1.3.2.3 LPS

LPS is a major component of the outer membrane leaflet of Gram-negative bacteria. It forms a tight permeability barrier that excludes substances such as detergents, proteases, bile salts, and hydrophobic antimicrobials that destroy cells. LPS consists of a lipid A, an unrepeated core oligosaccharide, and a distal polysaccharide (O-antigen) (Raetz et al., 2007). But, chlamydial LPS only consists of a trisaccharide core of 3-deoxy-D-manno-oct-2-ulopyranosic acid (Kdo) linked to penta-acyl lipid A (Rund et al., 1999), thus named as lipooligosaccharide (LOS) (Nguyen et al., 2011). In the absence of LOS, *Chlamydia* remains viable and establishes an inclusion to support robust bacterial replication, however, fails to express selected late-stage proteins and transition to the EBs (Nguyen et al., 2011). *Chlamydiae* LOS has low endotoxin activity, due to the presence of its lipid A molecule with high hydrophobicity, long-chain fatty acids and non-hydroxylated fatty acids connected to the sugar backbone (Brade et al., 1986; Ingalls et al., 1995). It is 10 -100 fold less stimulatory than intestinal bacterial LPS in activating proinflammatory signaling of cultured human epithelial cells (Heine et al., 2007). *C. trachomatis* LOS has been shown to be nontoxic for mice and a poor inducer of both the canonical TLR4 and noncanonical cytosolic caspase-11 inflammatory pathways in mouse bone marrow-derived macrophages (BMDM) (Yang et al., 2019a). A recent study also observes that *C. trachomatis* LOS fails to activate programmed cell death pathways via upregulating certain antiapoptotic proteins (Wang et al., 2020b). These data suggest that chlamydial LOS may help

direct and indirect evasion of pathogens from host innate and adaptive immune responses (Wang et al., 2020b).

### **1.3.3 The inclusion of *C. trachomatis***

After internalized into host cells, the infectious EBs differentiate into RBs and develop within a membrane-bound vacuole, called inclusion (Moulder, 1991). The inclusion membrane serves as a host-pathogen interface that mediates essential interactions with host cell while physically isolating chlamydial organisms from the cytosol (Olson-Wood et al., 2021). *De novo* inclusion arises from eukaryotic vacuole and is extensively modified by chlamydial proteins (Van Ooij et al., 1997). The inclusion membrane is remodeled by insertion of bacterial proteins, mainly a T3SS secreted effector called inclusion membrane (Inc) proteins (Lutter et al., 2012; Subtil et al., 2000), allowing to be rapidly separated from the endosomal pathway and to avoid fusion by lysosomes (Scidmore et al., 1996, 2003). The remodeled inclusion membranes also promote the migration of inclusion along microtubules to the microtubule-organizing center (MTOC), proximity to peri-Golgi regions (Grieshaber et al., 2003), which facilitates interaction with nutrient-rich compartments (Richards et al., 2013). Another study reports *in vitro* that during the developmental cycle of *Chlamydia*, the expansion of inclusion is not related to bacterial replication, but depends on active synthesis of chlamydial proteins, and that blocking the synthesis of chlamydial proteins by chloramphenicol could inhibit the expansion of inclusion (Engström et al., 2015).

### **1.3.4 The genomic of *C. trachomatis***

Due to the specialized intracellular developmental cycle, the difficulty of culturing strains *in vitro*, and the lack of efficient genetic tools for manipulation and transformation, *C. trachomatis* has not been well understood (Wang et al., 2011). Genomic study provides another avenue to understand this bacteria and suggests that *C. trachomatis* evolve through reductive evolution, possibly from a non-pathogenic ancestor to specialization for humans (Borges et al., 2012). In 1998, the genome sequence of *C. trachomatis* serovar D was firstly published (Stephens et al., 1998), followed by sequencing all biovars of *C. trachomatis* subsequently (Carlson et al., 2005; Thomson et al., 2008). *Chlamydia spp.* possess reduced genomes ranging from 1 to 1.5 Mbp (Hooppaw and Fisher, 2016), and 846 of the 889-920 coding sequences are universal to all *C. trachomatis* serovars (Thomson et al., 2008). The chlamydial genome also exhibits a very high coding density (approximate 90%), indicating that the coding capacity of its genome is highly optimized (Albrecht et al., 2009).

Analysis of the chlamydial genome reveals the presence of histone-like proteins retained by *Chlamydia* that are known to complex with chromosomal DNA in EBs, and lack DNA methyltransferases (Hackstadt et al., 1991; Voigt et al., 2012). DNA repair and recombination systems are widespread in the chlamydial genomes, indicating that *Chlamydia* has considerable recombination capacity. All *Chlamydia* have developed and complete T3SS genes, which indicates that the T3SS is an important mechanism for the interaction between *Chlamydia* and

host cells (Hefty and Stephens, 2007). Comparison of all currently sequenced chlamydial species has identified that the plasticity zone region (PZ) is a key region that is different in each *Chlamydia* species (Read, 2000), and contains several virulence factors, such as cytotoxin, MAC/perforin, and phospholipase D enzyme (Nelson et al., 2006; Read et al., 2003; Taylor et al., 2010). The presence or absence of these virulence factors may be associated with host and tissue tropism.

The proteome of *C. trachomatis* serovar D consists of 887 proteins, but the function of 269 of these hypothetical proteins (HPs) remains unknown. Through data mining of the *C. trachomatis* core genome, functional identification of hypothetical proteins can be performed to discover new drug targets and chimeric vaccine candidates. (Aslam et al., 2021a; Turab Naqvi et al., 2017).

### **1.3.5 The plasmid of *C. trachomatis***

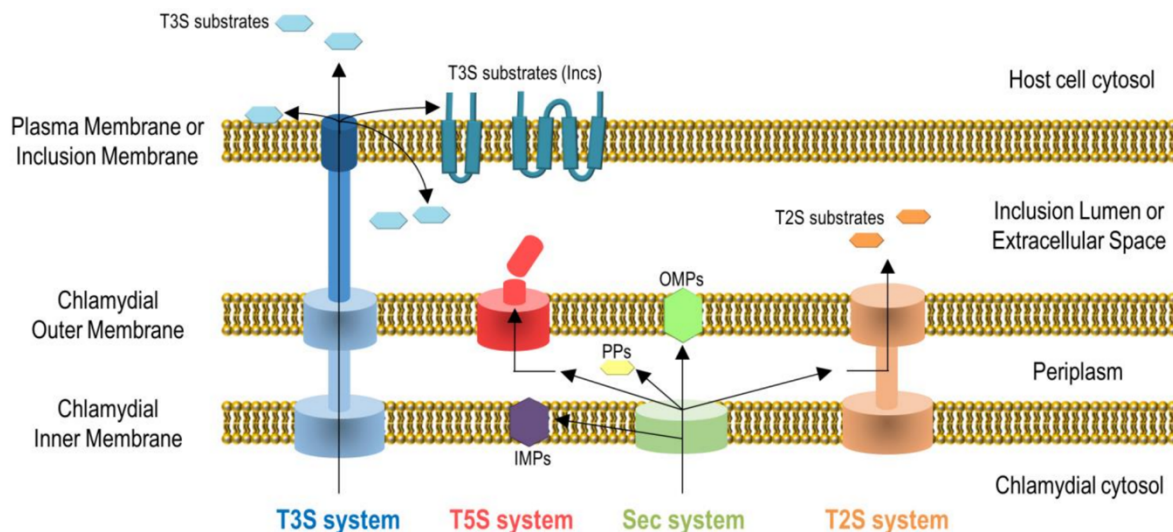
The chlamydial plasmids were discovered from *C. trachomatis* and *C. psittaci* in the mid-1980s (Joseph et al., 1986). The copy number of plasmids is very low, about four to seven times that of chromosomes (Ricci et al., 1993). The cryptic plasmid of *C. trachomatis* is approximately 7.5 kb in size encoding eight open reading frames (ORFs), commonly known as plasmid glycoproteins 1-8 (Pgp1-8) or pORF1-8, and different plasmid proteins play different roles in the life cycle and pathogenic mechanisms of *C. trachomatis* (Li et al., 2008; Zhong, 2017). Pgp1, 2, 6, and 8 are essential for maintaining plasmids, since the stable transformants cannot be obtained if any of these 4 ORFs is removed from the plasmid shuttle. Pgp3, 4, 5, or 7 can be deleted from *C. trachomatis* serovar L2 and *C. muridarum* (Gong et al., 2013; Song et al., 2013). The details of Pgp3 is introduced in the section of 'Pgp3'. Pgp4 is the master positive regulator of plasmid-encoded Pgp3 and chromosomal genes, including GlgA, CT049-CT050, and CT142-CT144 (Song et al., 2013), while Pgp5 is a negative regulator of Pgp4-regulated genes, if deleted, leading to overexpression of several plasmid- and chromosome-encoded genes (Liu et al., 2014a). Pgp5 also contributes to the pathogenesis of *C. muridarum* in urogenital tract, although to a lesser extent than Pgp3 (Huang et al., 2015).

The plasmids of *C. trachomatis* are well conserved (Harris et al., 2012b), together with the near uniqueness of this plasmid among clinical isolates, suggesting that this plasmid has a role in chlamydial infectivity (Jones et al., 2020). Plasmids are usually appendages for bacterial survival, but they can confer a selective advantage, such as carrying antibiotic resistance or virulence-related determinants (Williams and Thomas, 1992). Chlamydial plasmids have no homology to known antibiotic resistance genes, and are dispensable *in vitro* (Peterson et al., 1990). However, *C. trachomatis* plasmids are virulence determinants of natural hosts (Carlson et al., 2008; Sigar et al., 2014). Therefore, there are fewer and fewer naturally occurring plasmid-free variants (E. Lanjouw et al., 2017).

### 1.3.6 The protein transport systems and the main effectors of *C. trachomatis*

Completion of the entire developmental cycle of *C. trachomatis* involves the secretion of more than 70 *C. trachomatis* proteins into cytoplasm and nucleus of host cells, inclusion membranes and lumen, and even extracellular periphery. These proteins can interfere with the cytoskeleton, vesicular and non-vesicular transport, metabolism and immune signaling of host cells. In general, these proteins promote *C. trachomatis* invasion and escape from host cells, allowing *Chlamydia* to acquire nutrients, and evade innate, humoral, and cellular immunity (Bugalhão and Mota, 2019). The EBs are preloaded with virulence factors including T3SS secreted effectors involved in internalization into host cells, whereas RBs are rich in proteins involved in nutrient absorption, ATP generation, and translation (Saka et al., 2011). Here I briefly introduce the protein transport systems and several effector proteins of *C. trachomatis*, such as CPAF, HSP60, Incs and Pgp3.

#### 1.3.6.1 Protein transport systems in *C. trachomatis*



**Figure 18. Secretion systems used by *C. trachomatis* to transport proteins to different aqueous and membranaceous chlamydial and host cell compartments.** (IMPs, chlamydial inner membrane proteins; PPs, chlamydial periplasmic proteins; OMPs, chlamydial outer membrane proteins.) (From Bugalhão and Mota, 2019)

Gram-negative bacteria use several protein transport systems that are essential for interactions with host cells as well as with extracellular environment (Costa et al., 2015; Tsirigotaki et al., 2017). The genome of *C. trachomatis* also encodes a variety of secretion systems: Sec system, type II secretion system (T2SS), T3SS, Sec export Pmps containing type V secretion system (T5SS)/autotransporter signals, and several other outer membrane proteins [Figure 18] (Bugalhão and Mota, 2019; Stephens and Lammel, 2001; Stephens et al., 1998). These secretion systems can transport chlamydial proteins to different lumen, plasma and membrane regions within bacterial and host cells. The Sec system transports proteins in the periplasm and can be connected to T2SS or T5SS for transporting effectors within the lumen of inclusion. The T3SS spans the inclusion membrane and allows direct secretion of effectors into the host cell cytosol (Bugalhão and Mota, 2019). All *Chlamydia* genomes have a highly conserved, well-developed

and complete T3SS, indicating that the T3SS is an important mechanism for *Chlamydia* interactions with host cell (Hefty and Stephens, 2007). Indeed, the T3SS is necessary for cell invasion and is active in all the developmental life cycle of *Chlamydia* (Betts-Hampikian and Fields, 2010; Betts et al., 2008). Inc proteins is the mainly target substrates, which is introduced in the '1.3.6.4 Incs' part.

#### 1.3.6.2 CPAF

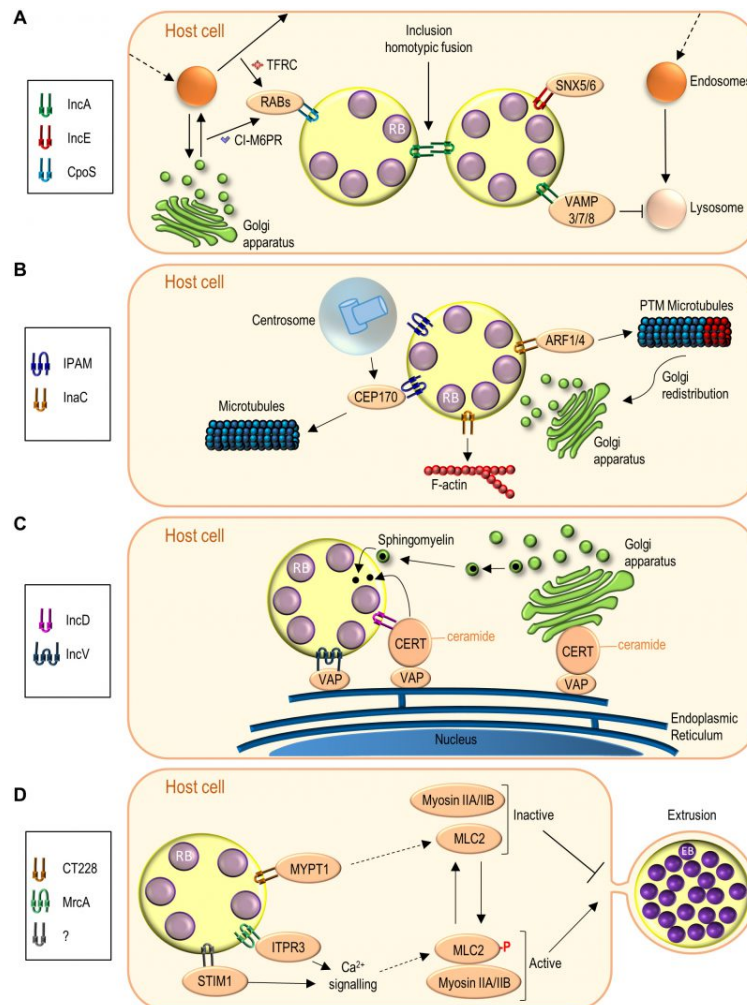
CPAF is a serine protease secreted by *C. trachomatis* during its mid-developmental cycle which enters the cytosol of infected host cells (Zhong et al., 2001). Although the function of CPAF is still unknown, recent data suggest that it may play a role in chlamydial pathogenesis, suppressing the host immune response by degrading host antimicrobial peptides and complement components (Tang et al., 2015; Yang et al., 2016a), and promoting the survival of *Chlamydia* in the lower genital tract of mice (Yang et al., 2016b). CPAF also elicits pro-inflammatory cytokines synthesis in the peripheral blood mononuclear cells from genital *C. trachomatis*-infected patients (Cheong et al., 2019). Through cleaving and releasing formyl peptide receptor 2 (FPR2) from the surface of polymorphic nuclear leukocytes (PMNs), CPAF suppresses the oxidative burst and interferes with chemical-mediated activation of neutrophil extracellular traps, which helps *C. trachomatis* evade the host's innate immune response and survives (Rajeeve et al., 2018a).

#### 1.3.6.3 Heat shock protein 60 (HSP60)

HSP60, a 60 kD mitochondrial chaperone proteins, is structurally highly conserved and abundantly expressed by prokaryotic and eukaryotic cells under stress conditions. Upon stress, *C. trachomatis* enters a persistent phase characterized by cessation of cell division (Wyrick, 2010), accompanied by the production of large amounts of HSP60 (Cappello et al., 2009). HSP60 is then delivered to the cell membrane and subsequently appears on the cell surface, where it acts as a "danger signal" for the host innate and adaptive immunity (Grundtman et al., 2011; Wick et al., 2014). HSP60 has been demonstrated to trigger pro-inflammatory cytokines synthesis in the peripheral blood mononuclear cells from genital *C. trachomatis*-infected patients (Cheong et al., 2019). Since chlamydial HSP60 shares high homology with human HSP60 protein produced by embryonic cells, the immune responses elicited against chlamydial HSP60 are previously thought to harm the developing embryo, resulting in spontaneous abortion. However, clinical data do not support an association between HSP60 antibodies and recurrent miscarriage (Eggert-Kruse et al., 2014; Ozyurek et al., 2018). HSP60 has also been shown to prolong the local inflammatory response (LaRue et al., 2007), and pose a risk for the development of female tubal infertility, since the presence of HSP60 is thought to cause apoptosis of trophoblasts, leading to tubal epithelial cell damage and scar formation (Equils et al., 2006).

### 1.3.6.4 Incs

*Chlamydia*-encoded T3SS is used to secrete bacterial effector proteins. There is a class of secreted effectors, referred to as Incs, that are inserted into the inclusion membrane for mediating critical host-pathogen interactions (Hackstadt et al., 1999). Incs consist of one or more N-terminal bilobed hydrophobic domains of 40 amino acids, consisting of two closely spaced transmembrane regions separated by a short hairpin loop (Bannantine et al., 2000). It is also predicted that their amino and/or carboxyl termini extend into the cytoplasm of the host cell (Bauler and Hackstadt, 2014; Rockey et al., 2002). Furthermore, allowing protein secretion and subsequent insertion into the inclusion membrane is also an identifier of T3SS signals (Bauler and Hackstadt, 2014; Moore and Ouellette, 2014). Based on these features, computational analyses have been applied to predict Inc proteins across different chlamydial species and serovars and 55 *inc* genes in *C. trachomatis*, 68 in *C. felis*, 92 in *C. pneumoniae*, 79 in *C. caviae* and 54 in *C. muridarum* have been predicted. The comparison of these Inc homologs reveals that 23 Incs are highly conserved among these five species (Lutter et al., 2012).



**Figure 19. Functions of *C. trachomatis* Incs effectors.** (A) Incs subvert host cell vesicular transport (IncA, IncE, CpoS). (B) Incs interfere with microfilaments and microtubules (IPAM, InaC), and mediate Golgi redistribution around the inclusion (InaC); PTM, post-translationally modified. (C) Incs participate in ER-inclusion MCSs (IncD, IncV). (D) Incs modulate chlamydial extrusion by subverting Ca<sup>2+</sup> signaling and myosin II function (CT228, MrcA). (From Bugalhão and Mota, 2019)

Inc genes account for approximately 7% to 8% of the chlamydial genome (Weber et al., 2015). The widespread presence and conservation of *inc* genes show that Incs play a key role in intracellular survival of *Chlamydia* (Moore and Ouellette, 2014). In addition, Incs are temporally expressed throughout the *Chlamydia* developmental cycle (Belland et al., 2003), indicating that their expression is related to the function in the inclusion membrane, such as IncF that can be detected early in the developmental cycle (Scidmore-Carlson et al., 1999), and IncA that is not transcribed until later stages (Belland et al., 2003). Furthermore, Incs have been shown to have other functions. For instance, IncA mediates homotypic fusion of *C. trachomatis* inclusions (Cingolani et al., 2019; Weber et al., 2016); certain Incs are involved in the intracellular traffic of inclusion to the MTOC, including IncB (also known as CT232), CT101, CT222, CT192 and CT850 (Mital et al., 2010, 2015); some Incs facilitate the interaction of different host cell compartments to obtain nutrients (Al-Zeer et al., 2014; Tudela et al., 2015); some Inc proteins may play a role in stabilizing inclusion (Gitsels et al., 2019), etc. The known function of Inc effectors is shown in Figure 19 (Bugalhão and Mota, 2019). However, the specific functions of most Incs still remain unclear (Gauliard et al., 2015).

#### 1.3.6.5 Pgp3

As mentioned before, Pgp3, also known as pORF5, is the only plasmid-encoded protein that is secreted into the cytoplasm of infected host cells (Li et al., 2008), and has been identified as one of the key virulence proteins in *C. trachomatis* infection (Liu et al., 2014b). The studies have shown that Pgp3 induces autophagy, involving the up-regulation of high mobility group box 1 (HMGB1) (Lei et al., 2017) and the activation of the unfolded protein response by the MAPK/ERK pathway (Wen et al., 2020). Pgp3 protein can also inhibit cell apoptosis by activating the expression of DJ-1 through the ERK1/2 signaling pathway (Luo et al., 2020) and by the PI3K-AKT-mediated MDM2-p53 axis (Zou et al., 2019). In addition, Pgp3 has been reported to alleviate oxidative stress to promote *C. trachomatis* infectivity by activating the Nrf2/NQO1 signaling pathway (Shu et al., 2021). Furthermore, Pgp3 is also involved in *Chlamydia*-induced oviduct pathology. Intravaginal infection of mice with Pgp3-deficient *C. muridarum* does not induce hydrosalpinx, and with Pgp3-deficient *C. trachomatis* serovar L2 show reduced infectivity (Ramsey et al., 2014). Structural integrity of plasmid-encoded Pgp3 is essential for *C. muridarum* to induce this hydrosalpinx (Huang et al., 2019). Recent study also demonstrates the pivotal role of Pgp3 in establishing *C. trachomatis* persistent infection in the female genital tract (Yang et al., 2020).

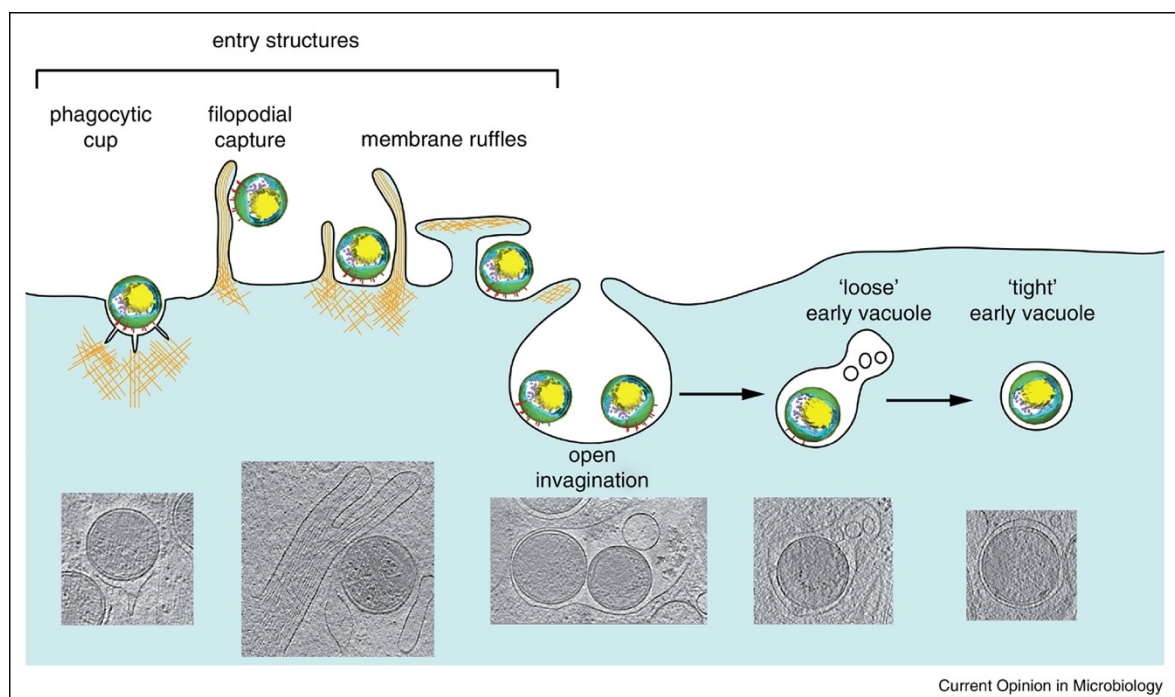
LL-37 is a cathelicidin antimicrobial peptide and activates inflammatory pathways by stimulating epithelial cells to secrete proinflammatory cytokines and by recruiting inflammatory cells (Kahlenberg and Kaplan, 2013). Pgp3 has been shown to prevent LL-37 from inducing cytokine production in human endometrial epithelial cells and from recruiting neutrophils, which may delay the onset of a comprehensive inflammatory response (Hou et al., 2015). Interestingly, although failing to activate epithelial cells, Pgp3 is able to stimulate myeloid cells to secrete inflammatory cytokines (Li et al., 2008), and the proinflammatory activity of Pgp3 on neutrophils is also significantly enhanced by LL-37, which may facilitate

chlamydial transmission. Therefore, it can be speculated that plasmid-encoded Pgp3 may play its virulence role by blocking the anti-chlamydial and proinflammatory activities of LL-37 to promote the survival of *Chlamydia*, and by using LL-37 to enhance its own proinflammatory activity to help chlamydial spread (Hou et al., 2019).

#### 1.4 *Chlamydia*-host interaction

Since *Chlamydia* is a specific intracellular parasitic bacterium, which has the characteristics of a unique biphasic developmental cycle (see details in section of ‘1.3.1 Biphasic life cycle of *C. trachomatis*’), its special interaction with host cells is essential for the survival and expansion of the bacteria. This part will elaborate *Chlamydia* interaction with host cells.

##### 1.4.1 *C. trachomatis* adhesion and invasion

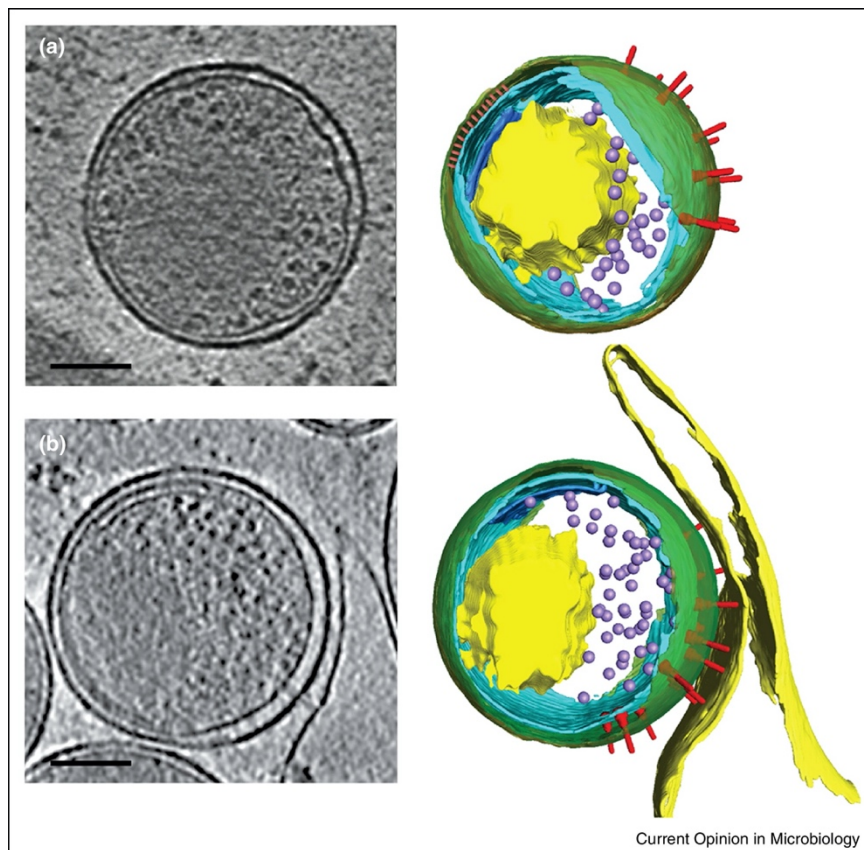


**Figure 20. Early interactions between *C. trachomatis* EBs and host cells identified by cryo-electron tomography.** Schematic representation of the early stages of *C. trachomatis* entry into mammalian cells. Actin accumulation is shown in orange. Corresponding tomogram slices are shown beneath. (From Dumoux et al., 2015a)

The most critical step in the life cycle of an obligatory intracellular bacteria is internalization into host cells. Upon contacting with host epithelial cells, the EBs initially bind to heparan sulfate proteoglycans on the epithelial cells with subsequent interaction with various cell surface receptors including mannose receptor, mannose-6-phosphate receptor, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 2 (FGF2), platelet growth factor receptor, ephrin A2 (EPHA2), protein disulfide isomerase, and  $\beta$ 1 integrin (Cherilyn Elwell, 2016; Dai and Li, 2014; Kim et al., 2011; Subbarayal et al., 2015). This interaction leads to the uptake of EBs via actin-dependent or -independent mechanisms. Actin-dependent mechanisms have been extensively studied, including the emergence of macropinosome, actin-rich filopodia, or phagocytic cups-based internalization (Dumoux et al., 2015a; Nans et al.,



2014) [Figure 20]. Actin-mediated internalization of EBs is initiated by injecting pre-synthesized effectors into the cytoplasm of host cells through T3SS, affecting actin filaments and leading to the typically non-phagocytic uptake of EB (Ferrell and Fields, 2016). The resulting intracellular vesicles containing EBs are termed inclusions as mentioned previously. In addition, it has been shown that the EB of *C. trachomatis* serotype LGV L2 is intrinsically polarized. One pole is characterized by tubular inner membrane invaginations, while the other pole exhibits asymmetric periplasmic expansion to accommodate a range of T3SS. Once internalized by host cells, these EBs lose their polarity [Figure 21] (Dumoux et al., 2015a; Nans et al., 2014).



**Figure 21. Polarized structure of the *C. trachomatis* EB.** (a) Left panel: xy tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram of a representative *C. trachomatis* EB. Scale bar, 100 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of a cryo-electron tomogram. Outer membrane (green), inner membrane (cyan and blue for the inner membrane invagination), T3SS (red), nucleoid (yellow), additional periplasmic complexes (brown) and ribosomes (purple) are shown. (b) Left panel: xy tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram showing a representative *C. trachomatis* EB in contact with a host cell. Scale bar, 110 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of the cryo-electron tomogram. Cellular plasma membrane (yellow), bacterial outer membrane (green), inner membrane (cyan), inner membrane invagination (blue) and T3SS (red) are shown. (From Dumoux et al., 2015b)

An effector protein of *Chlamydia*, known as the translocated actin recruiting phosphoprotein (Tarp), is responsible for driving changes in the cytoskeleton and cell signaling (Clifton et al., 2004; Ghosh et al., 2020; Keb et al., 2021; Shehat et al., 2021). Tarp is a multifunctional chlamydial protein, which has an N-terminal phosphorylation domain and multiple C-terminal actin-binding domains. The latter directly promotes the polymerization of actin and the formation of actin bundles (Jewett et al., 2006; Jiwani et al., 2013). The N-terminal

phosphorylation domain contains six tyrosine-rich repeats, a region that can be phosphorylated by a variety of tyrosine kinases (Src, Abl, Syk) after injection into host cells (Jewett et al., 2008; Mehlitz et al., 2008). Phosphorylated Tarp subsequently activates a complex of host cell signaling molecules, such as Son of sevenless homolog 1 (Sos1), Abelson interactor 1 (Abi1), epidermal growth factor receptor kinase substrate 8 (Esp8) and guanine nucleotide exchange factor VAV2 (Vav2), which are thought to direct cytoskeletal and early anti-apoptotic signals that facilitate *C. trachomatis* invasion and stay in newly infected host cells (Caven and Carabeo, 2020; Lane et al., 2008). Others also show that sorting nexin 9 (SNX9), a protein of SNX-PX-BAR family, is a central host mediator of filopodial capture during early chlamydial entry (Ford et al., 2018; Hänsch et al., 2020).

#### **1.4.2 Inclusion forming and intracellular transportation to the microtubule organizing cente (MTOC)**

The inclusion membrane originates from the host cell membrane and surrounds individual bacteria when they are brought into the cell. This small, membranous vacuole surrounding *Chlamydia* expands, is independent of whether the bacteria replicate or not, but dependent on the active chlamydial protein synthesis (Engström et al., 2015). After forming, inclusion bodies rapidly dissociate from the endocytic pathway and do not acquire markers for endosomes or lysosomes, such as EEA1, Lamp1, or Lamp2 (Fields and Hackstadt, 2002).

As described above, the process of EB internalization and subsequent inclusion formation requires the involvement of secretion of effector proteins (e.g., Incs) by T3SS. Chlamydial Incs, inserted into the inclusion membrane, allow the escape of EB endosomes from the endocytic-lysosomal pathway (Dai and Li, 2014; Nans et al., 2015a). Besides Incs, other chlamydial proteins, such as CPAF and high-temperature requirement A protein (HtrA), are also involved (Bastidas et al., 2013; Cherilyn Elwell, 2016). CPAF is a serine protease that functions in maintaining the integrity of inclusion bodies and promotes bacterial virulence by interfering with several host antimicrobial pathways, such as apoptosis and complement system (Jorgensen et al., 2011; Yang et al., 2016a). HtrA is also a serine protease that has been suggested to be a key factor in the intracellular survival of *Chlamydia* (Wu et al., 2011).

Within a few hours after infection, inclusions wander along microtubules and settle in the perinuclear region near the MTOC by using the minus-end-directed microtubule motor dynein (Grieshaber et al., 2003). This is a pathogen-driven event in which the inclusion protein CT850 is involved through its interaction with dynein light chain DYNLT1 (Mital et al., 2015). Chlamydial inclusions localize to the MTOC, close to the peri-Golgi region, facilitating interaction with nutrient-rich compartments (Richards et al., 2013).

#### **1.4.3 EB transfers to RB**

In the inclusion, EB undergoes primary differentiation into RB. The time from contacting with host cells to the formation of mature inclusions containing replication-competent RBs is around 11 hours (Lee et al., 2018a). Since the studies have detailed transcriptional and proteomic

differences between EBs and RBs (Saka et al., 2011; Skipp et al., 2016), it seems that proteome turnover may play an indispensable role in chlamydial differentiation. For instance, the Clp protease system is well conserved in *Chlamydia* and is important for the turnover of proteins, and ClpX and related ClpP2 have distinct functions in progression and differentiation of the chlamydial developmental cycle (Wood et al., 2020).

The metabolism may also play a role in the EB to RB transition (Grieshaber et al., 2018). EB germination requires the release of the compressed nucleoid structure by the dissociation of histones HctA and HctB. This dissociation is regulated in part by metabolic activity, as an intermediate in the isoprenoid biosynthesis pathway triggers the release of histones from the chromosome (Grieshaber et al., 2006a).

#### **1.4.4 *C. trachomatis* hijack host metabolism for the replication**

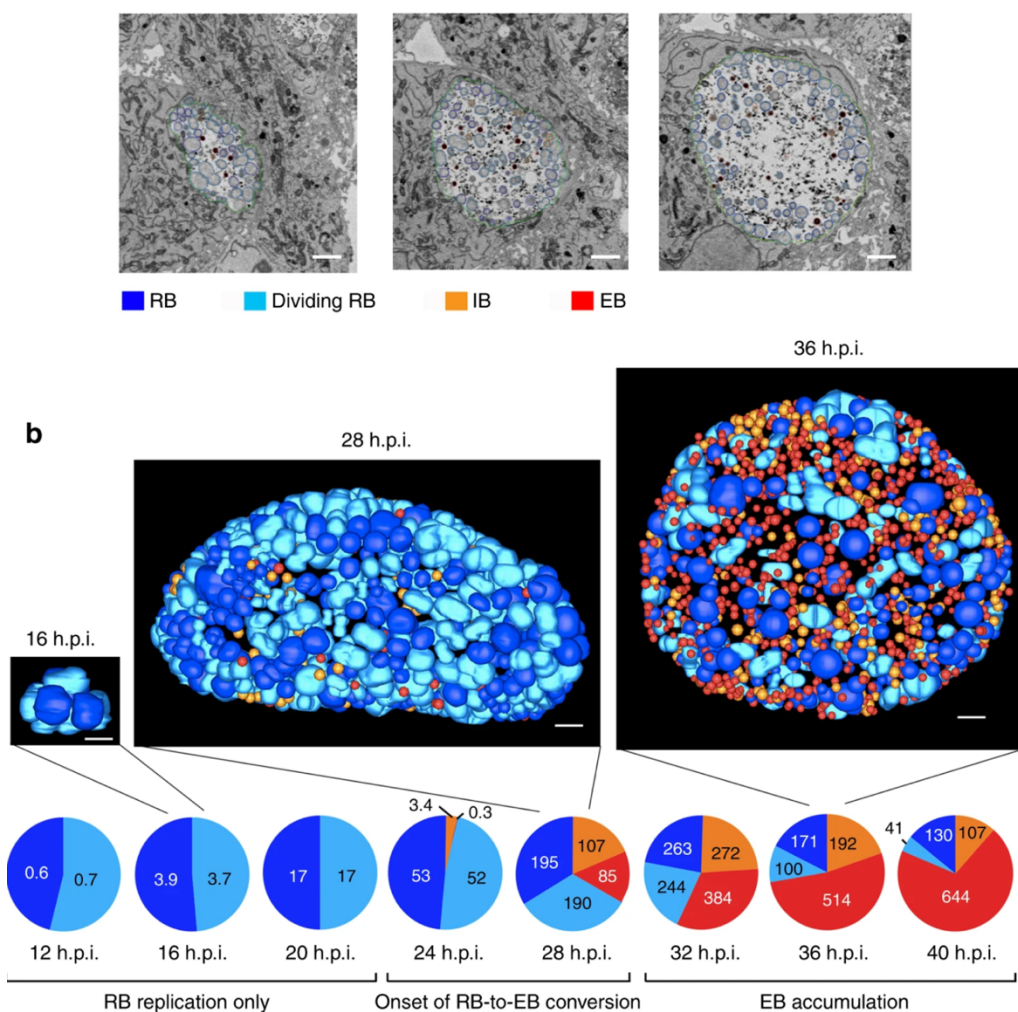
As a professional intracellular bacterium, *Chlamydia* lacks several key metabolic enzymes and must obtain essential nutrients from the host, including amino acids, lipids, glycogen, and ions, while avoiding being detected by the host's innate immune system (Elwell et al., 2016; Finethy and Coers, 2016a; Kellermann et al., 2021; Pokorzynski et al., 2017; Wong et al., 2019). To achieve this goal, chlamydial inclusions maintain close interactions with selected host organelles, e.g. mitochondria (Kurihara et al., 2019; Shima et al., 2021), Golgi (Auer et al., 2020; Faris et al., 2019; Huang et al., 2021), endoplasmic reticulum (Derré, 2015; Stanhope et al., 2017), lysosomes (Ouellette et al., 2011), and endosomes (Paul et al., 2017). However, the detailed mechanisms by which *Chlamydia* control these different interactions are still much unknown.

#### **1.4.5 RB transfers to EB**

The regulation of the conversion of RB to EB is also essential for the life cycle of *Chlamydia*, since *Chlamydia* must balance replication and production of infectious progeny EB. How *Chlamydia* regulates this process is not very clear, and multiple hypotheses have been proposed to explain the control of transformation of RB to EB: 1) RB access to or competes with inclusion membrane contact (Hoare et al., 2008), 2) reduction in RB size (Lee et al., 2018a), or 3) response to changes of nutrient supply (Omsland et al., 2012). As an example, I'll introduce in detail below the hypothesis of the reduction in RB size.

Starting from 9 to 12 h post-infection (hpi), RBs divide repeatedly to produce hundreds to a thousand progenies via a unique process of polarized budding (Abdelrahman et al., 2016). The conversion of RB to EB was first detected after approximately 24 hpi and occurred asynchronously. This unusual developmental cycle ends with the release of EBs to infect new host cells at 40-48 hpi. RB can be divided into two daughter RBs or converted into EBs, making them either as stem cells to produce RB, or as progenitor cells of infectious EB. The signaling and control mechanisms underlying this cell fate decision are unknown. Using serial block-face scanning EM (SBEM), Lee et al. quantitatively analyzes the intracellular chlamydial infection over time, and identify a conversion intermediate, named as intermediate body (IB) [Figure 22].

As a hallmark of RB to EB conversion, IBs are first detected at 24 hpi. At later time points, i.e., after 32 hpi, the number of dividing RBs and IBs decrease proportionally, indicating that both RB replication and RB to EB transition decrease (Lee et al., 2018a). They also observe that the size of RBs gradually decreases before differentiating into EBs. The average RB volume is reduced from  $1.01 \mu\text{m}^3$  at 12 hpi (equivalent to  $1.25 \mu\text{m}$  diameter spheres) to  $0.27 \mu\text{m}^3$  at 28 hpi and  $0.16 \mu\text{m}^3$  at 32 hpi ( $0.67 \mu\text{m}$  in diameter), when the conversion is actively ongoing. Totally, the size of RB reduces six times during its amplifying. Based on these observations, they propose a size control mechanism in which the size of RBs is reduced during replication and can only be converted into EBs when RBs reach a minimum size threshold. According to this model, the time to transition depends on the number of replication cycles needs to become sufficiently small to perform RB to EB conversion (Lee et al., 2018a).



**Figure 22. Temporal analysis of chlamydial developmental forms using a three-dimensional electron microscopy approach.** (From Lee et al., 2018)

### 1.4.6 Exit the Host Cells

Another critical stage in the life cycle of all intracellular pathogens is the exit from the host cell. The process, known as egress, is essential for the transmission and dissemination of pathogens to their new hosts. After the asynchronous conversion of *Chlamydia* from RB to EB, *Chlamydia* is egressed from the cell by one of two separate mechanisms: extrusion of intact chlamydial

inclusion from the host cell, or rupture of inclusion immediately before cell lysis (Hybiske and Stephens, 2007). Extrusion helps *Chlamydia* temporarily evading the host's immune response by enclosure within host membrane and spreading over long distances through delivery of *Chlamydia*-rich vesicles to neighboring cells (Zuck et al., 2017). The extrusion process has been reported *in vitro* for many years (Todd and Caldwell, 1985), but until recently it has been observed *in vivo* in multiple *Chlamydia* serovars (Shaw et al., 2017).

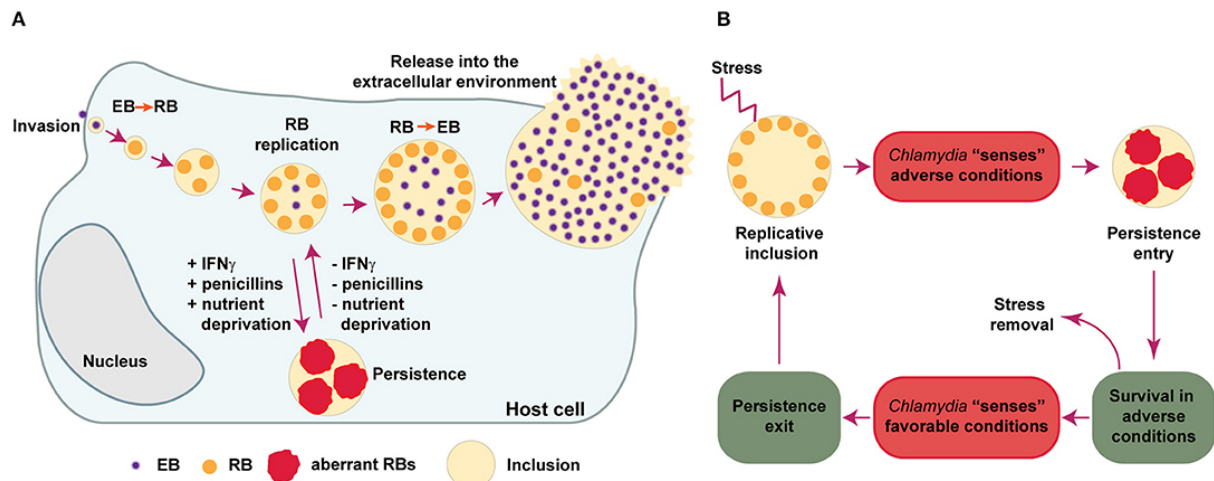
The extrusion mechanism is a result of an actin-dependent polymerization on the cytosolic face of the inclusion process, and has recently been reported to be orchestrated by the actions of myosin phosphatase target subunit 1 (MYPT1), myosin light chain 2 (MLC2), myosin light chain kinase and myosin IIA and IIB based contraction, septin aligning F-actin-containing fibers on *C. trachomatis* inclusion, Rho GTPase and Rho kinase (ROCK) activation, and the chlamydial inclusion membrane protein CT228 (Chin et al., 2012; Grieshaber et al., 2006b; Hybiske and Stephens, 2007; Lutter et al., 2013; Volceanov et al., 2014). It has been shown that extrusion is negatively regulated by the interaction between the *C. trachomatis* envelope protein CT228 and host MYPT1. Whether *Chlamydia* is egress by lysis or extrusion is controlled by the phosphorylation state of MLC2: dephosphorylated MLC2 favors lysis, whereas phosphorylated MLC2 enhances extrusion (Lutter et al., 2013). Mutation CT288 leads to a loss of MYPT1 recruitment *in vitro*, which increases the production of extrusion. Thus, CT228 recruits host MYPT1 into the inclusion, which modulates the mode and extent of host cell egress (Shaw et al., 2018).

The study shows that cysteine protease activity is required during inclusion rupture, and that intracellular calcium is also an important factor in the subsequent lysis of the host membrane and release of *Chlamydia* into the extracellular environment (Hybiske and Stephens, 2007). Pharmacological inhibition of host cell calpains inhibits inclusion rupture, but not subsequent cell lysis (Kerr et al., 2017).

#### **1.4.7 *C. trachomatis* persistent infection**

Under stress during *in vitro* infection, *Chlamydia* are able to enter a viable but non-infectious state characterized by ceased synthesis of structural and membrane proteins and LOS, while continued synthesis of unprocessed 16S rRNA, genomic replication and HSP60, commonly known as the "chlamydial persistence" or "chlamydial stress response" (Bavoil, 2014; Panzetta et al., 2018; Witkin et al., 2017). This persistent state enables bacteria to remain in host cells for long periods of time and is frequently associated with enlarged and morphologically aberrant RBs (aRBs), which maintain their ability to resume normal developmental cycles when the inducer is removed (Wyrick, 2010). Several factors have been shown to induce aRBs through *in vitro* models, including exposure to IFN- $\gamma$  (The role of IFN- $\gamma$  defense against *C. trachomatis* infection will be discussed in chapter "IV. Interferon induction during *C. trachomatis* Infection".) or to antibiotics (e.g., penicillin and amoxicillin), and deprivation of nutrients (e.g., essential amino acids or iron) [Figure 23A] (Panzetta et al., 2018; Di Pietro et al., 2019). A sequential events occur during this persistent infection, including sensing

unfavorable conditions, entering a persistent state, surviving unfavorable conditions, sensing favorable conditions, exiting from a persistent state, and resuming to normal infectious offspring production [Figure 23B] (Panzetta et al., 2018). However, the mechanism and molecular basis of each event remains poorly understood.



**Figure 23.** *Chlamydiae* developmental cycle and its strategy to evade antimicrobial effects triggered during infection *in vitro*. (From Panzetta et al., 2018)

This stressed condition may also occur *in vivo* by immune response or administration of antimicrobial drugs. However, so far the evidence of inducing *Chlamydia* into the persistent state *in vivo* is still not evident (Panzetta et al., 2018). The evidence that persistence may occur *in vivo* comes from direct observation of aRBs in the infected tissues (Lewis et al., 2014; Rank et al., 2011), although this observed aRBs in infected tissues do not indicate the persistent infection.

### 1.5 C. trachomatis alters the host cell transcriptome and proteome

Bacterial interactions with host cells can induce dynamic transcriptional responses in the infected cells, which occurs through bacterial regulation of host cell processes or from innate immune signaling cascades and other cellular responses (Brunham and Rey-Ladino, 2005; Ribet and Cossart, 2015). In addition, bacterial effector proteins that specifically target the nucleus can also affect the physiological structure of cells and directly interfere with transcriptional mechanisms, including chromatin remodeling, DNA replication and repair (Bierne and Cossart, 2012; Niller et al., 2017). Epigenetically mediated transcriptional regulatory changes in host cells, including histone modifications, DNA methylation, chromatin accessibility, RNA splicing, and non-coding RNA expression, may also be arbitrated by bacterial proteins and effectors (Grabiec and Potempa, 2018; Sánchez-Romero and Casadesús, 2020).

Consistent with the interactions between host and other bacterial pathogens, *C. trachomatis* infection also alters host cell transcriptional, translational, and post-translational levels during its developmental cycle, and may also regulate host cell epigenomes (Elwell et al., 2016; Humphrys et al., 2013). For example, NUE (Nuclear Effector), a type III secreted effector of

*C. trachomatis* with methyltransferase activity, enters the host nucleus and methylates eukaryotic histones H2B, H3, and H4 *in vitro* (Pennini et al., 2010), which can alter chromatin structure and subsequent gene expression (Chumduri et al., 2013). Hayward et al. also detect conserved and distinct temporal changes in genome-wide chromatin accessibility related to *C. trachomatis* infection (Hayward et al., 2020).

*C. trachomatis* also induces extensive changes in protein stability, and at least a subset of the altered proteins is required for replication (Olive et al., 2014). For instance, T3SS effectors, the deubiquitinating enzymes ChlaDub1 and ChlaDub2 (also known as CT867) secreted by *C. trachomatis*, can modulate ubiquitination and protein stability in the host (Claessen et al., 2013; Misaghi et al., 2006). Incs may also interact with the host's ubiquitination mechanism, which reveals additional pathway for target protein stability (Mirrashidi et al., 2015).

### **1.6 Pathophysiology of *C. trachomatis* infection**

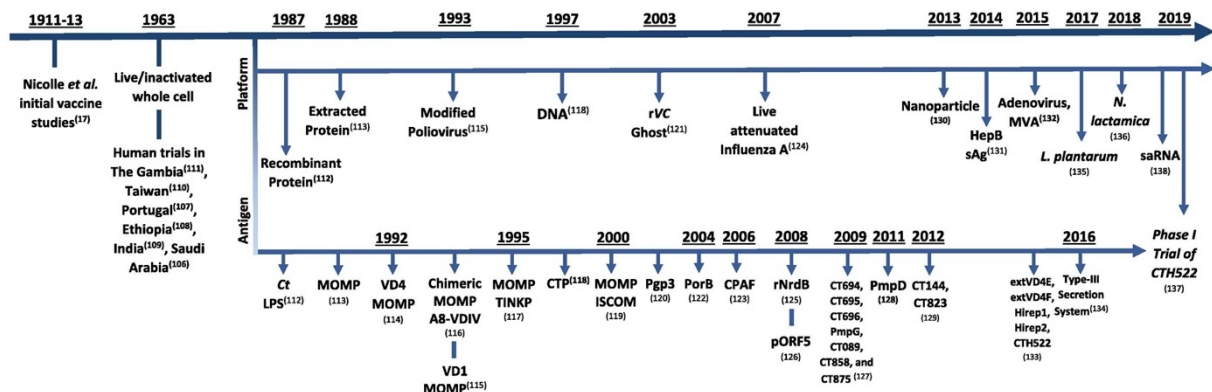
*C. trachomatis* causes a range of infectious diseases, for example, trachoma, cervicitis, pelvic inflammatory disease, scarring, infertility, and association with cervical and ovarian cancer (Zhu et al., 2016). But, elucidating the underlying mechanisms or even the natural progression of infection in the different host tissues remains challenging due to the lack of physiologically relevant infection models (Zadora et al., 2019a). As described previously, *C. trachomatis* reorganizes host actin in various ways to facilitate the invasion, maintains their replication sites, and egress from host epithelial cells. Thus, understanding the function and homeostasis of actin during chlamydial infection may reveal the pathogenic mechanism of *Chlamydia* to the host (Caven and Carabeo, 2020). Indeed, serum response factor (SRF) is clearly activated by regulators of actin dynamics, such as LIM kinase-1, which inhibits thrombin (Geneste et al., 2002). Application of tension force to cardiac membrane-breaking cells *in vitro*, shows tension-dependent expression of  $\alpha$ -smooth muscle actin, a regulatory target of SRF (Zhao et al., 2007). Dysregulation of SRF activity is also associated with the induction of epithelial to mesenchymal transition (EMT), a process by which epithelial cells transform into scar-forming fibroblasts (Zhao et al., 2016, 2014). Interestingly, chlamydial infection has recently been shown to induce EMT, which may promote the pathological changes in scar formation observed in chronic *C. trachomatis* infection (Igietseme et al., 2018; Rajić et al., 2017; Zadora et al., 2019a).

*Chlamydia* has evolved strategies to induce, redirect, or tamper with host signaling pathways to promote the development of productive infections and inclusions, ensuring their survival and spread, and driving pathological outcomes. The main host cell signaling pathways and responses induced by *Chlamydia* during productive infection of epithelial cells are activation of receptor tyrosine kinases (RTKs) and integrin-related signals that stimulate the unfolded protein response (UPR) and induce EMT, all of which require cytoskeletal reorganization to manifest and participate in infectivity and/or pathogenesis (George et al., 2019; Nans et al., 2015b; Stallmann and Hegemann, 2016). *Chlamydia* can up-regulate the expression of TGF- $\beta$  as early as 6 hours after infection of epithelial cells (Igietseme et al., 2020), which cooperates

with other cellular signaling cascades and cytoskeletal remodeling to support optimal inclusion development, EMT induction and the development of complications.

### 1.7 C. trachomatis vaccine: progress and problems

It has taken nearly 100 years for the *C. trachomatis* vaccine development, starting with Nicolle, until 2019, when the Phase I clinical trial for the *Chlamydia* vaccine was completed [Figure 24] (Abraham et al., 2019; Murray and McKay, 2021; Nicolle et al., 1911). Since 1950s, live and attenuated *C. trachomatis* vaccines have been developed, some of which have a protective effect by targeting to specific serotypes, and some cause more serious infectious consequences and an increase number of infections than the placebo group (Sowa et al., 1969; Woolridge et al., 1967). Based on the trial failures of multiple *C. trachomatis* vaccines, a subunit vaccine has been sought since 1980s to prevent infection from genital *C. trachomatis* infection (Carrasco et al., 2011; Swanson et al., 2009). Next, I will describe the results of *Chlamydia* vaccine research in recent years and the problems faced.



**Figure 24. Timeline of *C. trachomatis* vaccination.** Timeline to depict the first use of vaccine platforms and antigens for *C. trachomatis*. Ct = *Chlamydia trachomatis*, LPS = Lipopolysaccharide, MOMP = Major outer membrane protein, VD = Variable domain, CTP = Cytosine triphosphate, ISCOM = Immune stimulating complex, rVC = Recombinant *Vibrio cholerae*. PorB = Porin B, CPAF = chlamydial protease-like activity factor, Pmp = Polymorphic membrane protein, HepB sAg = Hepatitis B Surface Antigen, MVA = Modified vaccinia Ankara, saRNA = Self-amplifying RNA. (From Murray and McKay, 2021)

#### 1.7.1 *C. trachomatis* vaccine candidates: antigens and their immunity response

The test of whole-cell vaccines to protect humans from reproductive tract infections is unlikely. Thus, the research nowadays mainly focus on *C. trachomatis* subunit vaccines. Vaccination against *C. trachomatis* must show sufficient immunogenicity to elicit adaptive immune responses, to guide productive Th1 responses, and to avoid immune mediation that leads to Th17-type inflammatory signals. The latter may worsen disease outcomes from infectious challenges (Su and Caldwell, 1995; Van Voorhis et al., 1997). The main subunit candidate of vaccine antigens are MOMP, Pmp, CPAF, plasmid antigens, OmcB, Tarp, T3SS proteins, CopB, CopD, Cap1, CT584, MIP, IncA, PorB, NrdB, glycolipid antigen-peptide 4, glycogen phosphorylase and multi-subunit vaccines (de la Maza et al., 2021). Combination of core proteomics, immunoinformatics and subtractive proteomics, it has identified the best potential antigenic candidates for the development of multi-epitope vaccines (MEBV): T3SS translocon



subunit CopD2, SctW family T3SS gatekeeper subunit CopN, SycD/LcrH family T3SS chaperone Scc2, CT847 family T3SS effector, hypothetical protein CTDEC\_0668, and CHLPN 76kDa-like protein (Aslam et al., 2021b). The experimental validation is required to prove their effectiveness of these candidates in the future.

To stimulate the host immune system and to mount a coordinated innate and adaptive immune response to antigens, subunit vaccines also require a combination of adjuvants (Nanishi et al., 2020). *C. muridarum* PmpG has ever been used as antigen to test four adjuvant combinations DDAMPL, Montanide ISA 720 VG plus CpG-1826, CAF01 and CAF04, which are experimentally shown to have protective effects (Yu et al., 2012). These adjuvant combinations elicit the highest number of multifunctional CD4<sup>+</sup> T cells co-expressing IFN- $\gamma$  and TNF- $\alpha$ , two cytokines known to be involved in protection against *Chlamydia* infection. Using a combination of aluminum hydroxide and TLR4 agonist E6020 as adjuvant, formulated *C. trachomatis* serum ErMOMP vaccine provoke high levels of neutralizing antibodies and IFN- $\gamma$ -producing T cells in inbred CD-1 mice (Pal et al., 2020). Based on the current human and animal studies, it has been established that the optimal protection requires the induction of both cellular and humoral immune responses. Thus, the use of adjuvant combinations may be required to optimize the protection elicited by chlamydial subunit vaccines (de la Maza et al., 2021).

Several immunization routes have been explored to introduce chlamydial vaccines, such as parenteral route (subcutaneous inoculation, intramuscular inoculation), mucosal route (intranasal route, oral vaccination, sublingual route, colonic vaccination, vaginal route, etc) and combining of parenteral and mucosal routes (de la Maza et al., 2021). It has been shown that the strongest humoral and cell-mediated immune responses are observed in mice immunized by a combination of systemic and mucosal routes (Carmichael et al., 2011; Wern et al., 2017).

### 1.7.2 Problems in vaccine development

Until 2019 the first phase I clinical trial of a *Chlamydia* vaccine was performed (Abraham et al., 2019). This vaccine uses the recombinant protein subunit CTH522 as the antigen and CAF01 liposomes (CTH522:CAF01) or aluminium hydroxide (CTH522:AH) as the adjuvant, and the immunization schedule is 30  $\mu$ g of vaccine administered intramuscularly in the arm for three times (0, 1 and 4 months). Their preliminary results show good safety and humoral immunogenicity of the tested *Chlamydia* vaccines, especially, CTH522:CAF01 has a better immunogenicity profile. As we know, the primary goal of *C. trachomatis* vaccines is to elicit protection against long-term sequelae in women induced by *Chlamydia* infection (de la Maza et al., 2021). The success of phase I clinical trials of *C. trachomatis* vaccines marks a turning point on the long road to control the pandemics caused by this bacterium, though no available experimental data show its protection against the long-term sequelae.

Actually, there are several challenges in *C. trachomatis* vaccine development, and the efforts to address these challenges can accelerate the progress of *C. trachomatis* vaccine development. I) Clinical testing of *C. trachomatis* vaccines is feasible. The choice of clinical trial endpoints

also requires further investigation and discussion. Biomarkers in blood and other novel approaches for identifying upper genital tract infections and inflammation in women will also help to determine endpoints as well as disease burden for the vaccine efficacy studies (Zhong et al., 2019). II) Although the immunological basis for the protection against *C. trachomatis* infection and disease has been well studied, the key issues such as the role of antibodies still need to be clarified. A relative consensus is that a putative *C. trachomatis* vaccine should generate Ct-specific CD4<sup>+</sup> T cells targeting genital epithelial cells, combined with a strong antibody response. III) The utility of different mouse models that can be used to test vaccine candidates is needed. It would be invaluable to unify these models so that vaccine candidates can be compared across laboratories with respect to important clinical endpoints or product indications. IV) While intramuscular immunization is effective in preventing cervical HPV infection, it is unclear whether *C. trachomatis* vaccines can achieve protection in a similar manner given the need for robust local T-cell immunity. Effective *C. trachomatis* vaccines may require the induction of robust transmucosal immunity through resident memory T cells within the genital tract.

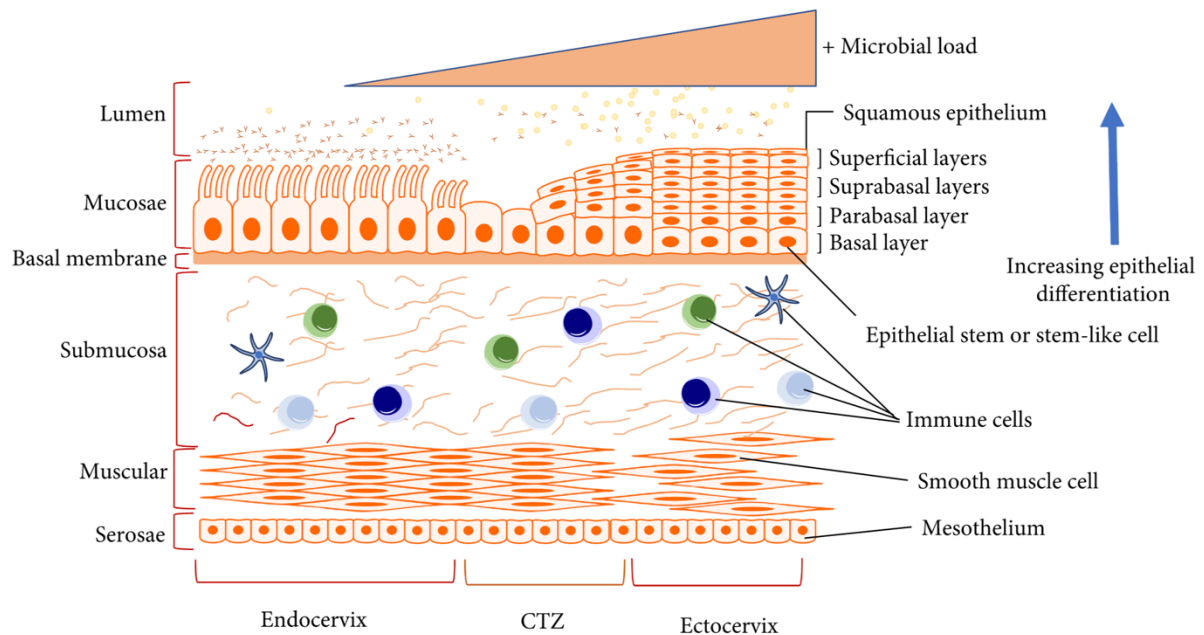
## II. Cell models for *C. trachomatis* infection research

The obligatory intracellular nature of *C. trachomatis*, mainly targets to mucosal epithelial cells, suggests that mammalian cell culture as well as animal models are essential to study the infectious process of this bacteria in the epithelial cells of the reproductive tract of the host. Due to the difficulty of accessibility of human genital tissues for the research purposes as well as the limited passage numbers of primary cells, alternative model systems have been sought to study chlamydial pathogenesis. The epithelial cell lines HeLa (epithelial cells derived from a cervical adenocarcinoma) and McCoy (fibroblast-like cells) have been used in most research surveys aiming at elucidating the mechanism of chlamydial interaction with host cells (FURNESS et al., 1960; KAJIMA et al., 1964). Although these cell lines are sufficiently sensitive to the infection to warrant their use for routine isolation of this organism from clinical samples, neither is representative of the normal targeting epithelium *in vivo* (Moorman et al., 1986). In this part, a brief overview of the female genital tract is introduced, then the cell models and the aspect of their advantage and disadvantage for *Chlamydia* study are described. Finally, cellular 3D cultures, organoid and animal models in *Chlamydia* research are also discussed.

### 2.1 Brief introduction of female genital tract (FGT)

As shown in Figure 27, the FGT of women consists of lower (vagina and ectocervix) and upper (endocervix, uterus, fallopian tubes, and ovary) genital tracts (Lee et al., 2015; Stejskalová et al., 2021). The cervix is divided into the two main compartments, namely, the microbe-laden ectocervix and “sterile” endocervix, which are bordered by the cervical transformation zone (CTZ) and their associated immune cells. Cervical tissue has two main cell types in both the endo- and ecto-cervix, including stromal cells and epithelial cells. These two cell types are

separated by the basal membrane (Prendiville and Sankaranarayanan, 2017). The stromal tissue is mainly composed of fibroblasts, including smooth muscle and sporadic immune cells, embedded in a collagen-rich extracellular matrix, containing hyaluronic acid and proteoglycan (Anton et al., 2017). The inner surface mucosa of the upper genital tract is covered with a single layer of columnar epithelium, while the inner surface mucosa of the lower genital tract is lined with stratified squamous epithelium [Figure 25] (De Tomasi et al., 2019).



**Figure 25. Microscopic illustration of the cervix of women.** The ectocervix has squamous stratified epithelia, while endocervix has simple columnar epithelia. (From De Tomasi et al., 2019)

## 2.2 Traditional cell lines used *in vitro* for *C. trachomatis* infection research and main findings

### 2.2.1 HeLa cells

The HeLa cell line was established in 1951 from cervical cancer cells of a patient, Henrietta Lacks (Lucey et al., 2009), which is the first successful attempt to immortalize human-derived cells *in vitro*. HeLa cells are the most widely used cell model for the study of human cellular and molecular biology. More than 110 000 publications have used or directly referenced HeLa cells. However, HeLa cells contain a very large number of genomic variants due to extensive genomic instability during transmission and transfer between laboratories (Macville et al., 1999; Rutledge, 2014). Currently, the widely used HeLa variants include HeLa CCL2 (the original HeLa cell line), HeLa S3 (also known as HeLa CCL2.2, a third clone isolated from early HeLa cultures), and HeLa Kyoto.

HeLa cell line has been applied to *Chlamydia* study since 1960s (FURNESS et al., 1960), due to its highly sensitive to chlamydial infection, although it is not very representative of primitive, differentiated target epithelial cells (Bose and Goswami, 1986). Indeed, important information about chlamydial attachment and infection obtained from primary cells, differed in several

details from data obtained from non-hormone-responsive, non-polarized, chlamydia-infected HeLa cells (Guseva et al., 2003). For instance, I) the development of chlamydial inclusions in primary human cervical and endometrial epithelial cells is unevenly distributed; II) the level of chlamydial attachment to primary human endometrial epithelial cells and its infectivity are significantly greater in estrogen-dominated cells than in progesterone-dominated cells. III) infectious EBs enter polarized, hormone-responsive primary human endometrial epithelial cells, mainly through lectin-coated pit receptor-mediated endocytosis; IV) chlamydial egresses from primary polarized human epithelial cells is pathogenetically oriented, such as, progeny of human non-invasive *C. trachomatis* serovar E egresses through the apical surface, while progeny of invasive LGV egresses in the basal region.

### **2.2.2 Murine Heteroploid Fibroblast (McCoy)**

The McCoy cell line was originally isolated in 1955 from human synovial fluid of a patient with degenerative arthritis (T. C. Hsu, 1957). Perhaps due to the contamination by mouse cells in the laboratory, the McCoy cells currently used in many laboratories are confirmed in 1993 to be the mouse cells rather than the original cells derived from human (Fong et al., 1994).

McCoy cells are sensitive to different *C. trachomatis* serovars (Gordon and Quan, 1965; KAJIMA et al., 1964; Ripa and Mardh, 1977), and provide a convenient means for isolation of *Chlamydia* and laboratory diagnosis of *C. trachomatis* infection (Hobson et al., 1974). Numerous surveys have subsequently been conducted with various agents in order to improve the sensitivity of cells to the infection with different chlamydial strains. The results show that pretreatment of McCoy cells with diethylaminoethyl-dextran, cycloheximide, cytohalasine B, 5-iodo-2-deoxyuridine, cycloheximide, polybrene or mitomycin C facilitates the successful infection (Draganov et al., 2004). Gradually, using cell culture (usually McCoy) to isolate and replicate *C. trachomatis* has replaced the method of yolk-sac inoculation of embryonic eggs (Taylor-Robinson and Thomas, 1991).

### **2.2.3 Polarized endocervical epithelial cell line: A2EN**

*C. trachomatis* genital biovars mainly infect mucosal epithelial cells of genital tract. In women, chlamydial infection of the lower genital tract (vagina and ectocervix) can cause ascending infection of the upper genital tract (endocervix, endometrium and fallopian tubes) (Taylor and Haggerty, 2011), where mucosal layer is mainly composed of polarized epithelial cells, which have a rich diversity of forms and functions that cannot be easily reproduced in a two-dimensional culture environment (Blaskewicz et al., 2011). Thus, to better understand the overall impact of chlamydial infection on epithelial cell physiology, a new *in vitro* cell model that more accurately reconstructs the organization and complexity of the female reproductive tract is essential (Dolat and Valdivia, 2019).

Wyrick et al. firstly described key differences in chlamydial growth in polarized epithelial cells (Wyrick et al., 1989). Human endometrial epithelial cancer cells polarized on collagen-coated microcarrier beads clearly enhance the growth of *C. trachomatis* serovar E, regardless of EB

attachment efficiency, compared to non-polarized cells. They also revealed differences in chlamydial entry and exit mechanisms, infectious progeny, duration of the developmental cycle, infectivity, duration of persistent growth status, responsiveness to antibiotics, responsiveness to female sex steroid hormones, and innate inflammatory responses. Their study illustrates the importance and precedence of maintaining polarized epithelial cell culture systems at different apical and basal surfaces in order to better mimic the *in vivo* relationship between the host and related infectious organisms.

A2EN cells, a human endocervical epithelial cell line derived from healthy patient samples, has been established by immortalizing using HPV16 retrovirus (Herbst-Kralovetz et al., 2008) and shown to polarize, and appropriately express many of the functional proteins of the endocervical epithelium *in vivo* such as hormone receptors, mucins, anti-microbial peptides, and pro-inflammatory cytokines (Buckner et al., 2011), as well as secretion of mucin and express proinflammatory cytokines during *Chlamydia* infection (Buckner et al., 2013). A2EN cells are susceptible to *C. trachomatis* infection, and the *Chlamydiae* in these cells undergo a normal developmental cycle (Buckner et al., 2013).

#### **2.2.4 Human induced pluripotent stem cells (iPSdMs)**

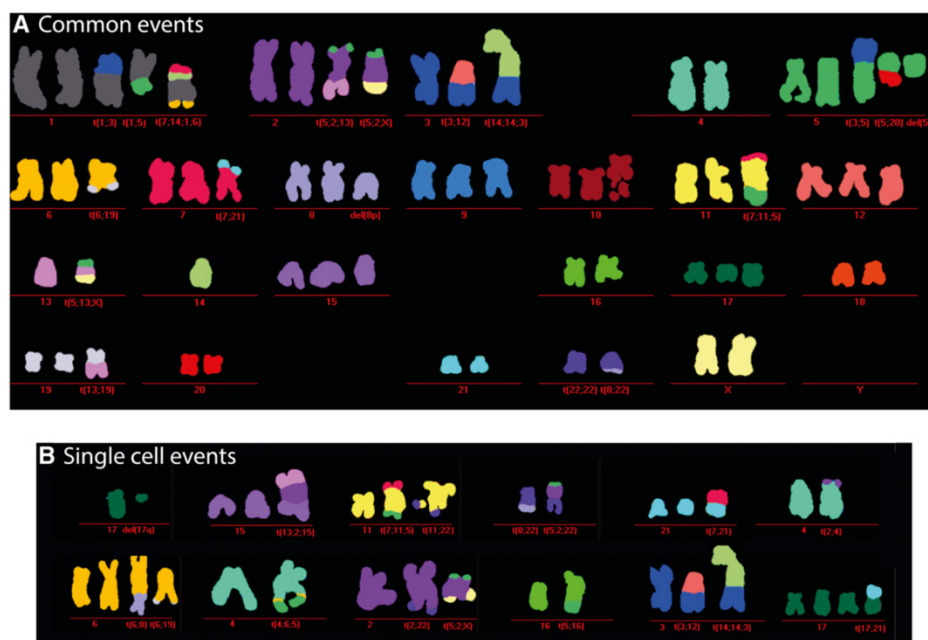
Besides epithelium, the genital mucosa also contains tissue-resident macrophages and monocytes that can come into contact with chlamydial EBs once released from lysed epithelial cells after the end of the developmental cycle (Agrawal et al., 2011). Effective phagocytosis and intracellular killing by macrophages can limit the rise of infection and provide antigen for activating Th1-mediated immune responses in CD4<sup>+</sup> T cells, which is the most critical immune response to eradicate *C. trachomatis* infection (Ziklo et al., 2016). However, if this elimination fails, macrophages may be used as Trojan horses for *Chlamydia* transmission to the lymphatic system for bacterial replication in draining lymph nodes (Lausen et al., 2019). In particular, the LGV biovar is associated with intracellular survival and transmission (De Vries et al., 2009). Additionally, monocytes and macrophages also respond to *C. trachomatis* infection by secreting pro-inflammatory cytokines, causing collateral tissue damage (Prantner et al., 2009). Therefore, study the interaction between macrophages and *C. trachomatis* is essential to understand how protective immunity develops and how immune responses lead to pathological changes.

But most of the *in vitro* models currently used to study *Chlamydia*-macrophage interactions mainly come from macrophages of other mammals, immortalized macrophage cell lines, or primitive monocytes/macrophages (mainly from mice) (Fields et al., 2013; Sun et al., 2012), whose response to bacteria are apparent different to that from humans. In addition, immortalized cell lines are also far from a normal genetic state (Odero et al., 2000). Furthermore, primitive monocytes and macrophages are inherently difficult to genetically manipulate, thus limiting the ability to study how macrophage genetics affects chlamydial pathogenic mechanisms (Yeung et al., 2017). To solve the problem, Yeung ATY et al. induce human pluripotent stem-cell (iPSCs) differentiation into macrophages (iPSdMs) that respond

to *C. trachomatis* similarly to human blood monocyte-derived macrophages by flow cytometry analysis, microscopy, and transcriptional proteome studies (Yeung et al., 2017). These iPScMs can also be genetically mutated by CRISPR/Cas9 approach (Bressan et al., 2017), and generated mutations of candidate genes in functional genomic studies (Yeung et al., 2017).

### 2.2.5 Disadvantage of cell lines

Using different cell lines have facilitated to understand the host-*Chlamydia* interaction. But these immortalized cells are far from the normal genetic state, including harboring extensive genomic rearrangements (Landry et al., 2013). As an example, like many cancer cell lines, HeLa is extensive aneuploidy [Figure 26] (Landry et al., 2013). Liu YS et al. report the substantial heterogeneity between HeLa variants, especially between lines of CCL2 and Kyoto varieties, and observe a gradual divergence in a specific cell line after 50 successive passages (Liu et al., 2019b).



**Figure 26. Colored HeLa karyotype by M-FISH.** M-FISH results of 12 analyzed metaphase spreads identified a hypotriploid karyotype. The karyotype shown in (A) was derived from a single cell in which all aberrations were recurrent except for the one in chromosome 3. Single cell-specific events are shown in (B). (From Landry et al., 2013)

In terms of morphology, expression of innate immune mediators, or responsiveness to TLR agonists, HeLa and McCoy cells commonly used for *in vitro* studies of *C. trachomatis* infection also can not accurately represent target cells *in vivo* such as polarized columnar epithelial cells in endocervix (Buckner et al., 2011; Herbst-Kralovetz et al., 2008). The innate immune signaling pathways have been altered in these cell lines than normal cells as well (Landry et al., 2013; Nair et al., 2003). Hence, the cell lines may not be the best model to study the host-*Chlamydia* interactions.

### **2.3 Primary epithelial cells model and main findings: ectocervical, endocervical, endometrial, fallopian tube epithelial cells**

As an alternative to establish transformed cell lines, some studies have been performed with primary, hormone-responsive polarized human endometrial epithelial cells from women undergoing hysterectomy for benign disease (Moorman et al., 1986; Wyrick et al., 1989). Few *Chlamydia* studies have been performed using primary epithelial cells, particularly, ectocervical epithelial cells, the first line of host defense to encounter invading pathogens in FGT. Acquisition of primary epithelial cells from the female reproductive tract relies on isolation of cells from biopsies. This process is usually performed as part of surgery on diseased organs. Healthy cells can be isolated from organs after prophylactic surgery, such as ovariectomy, salpingectomy, or hysterectomy, usually in premenopausal or menopausal women or during obstetric surgery (Stejskalová et al., 2021). Long-term *in vitro* maintenance and culture of primary epithelial cells has been a significant challenge in this field. One strategy to address this problem is the use of freshly isolated cells, which have the advantage of preserving mixed epithelial cell populations, such as secretory and ciliated epithelial cells (Levanon et al., 2010).

A primary human fallopian tube epithelial cell model has been established recently (McQueen et al., 2020). The primary human fallopian tube epithelial cells are cultured with an air-liquid interface, that stimulates cells differentiate into mucus-secreting and multiciliated polarized cells, recapitulating the architecture of the human fallopian tube epithelium. The apical secretions of cells infected with *C. trachomatis* contain more proteins implicated in chlamydial growth and replication, including transferrin receptor 1, amino acid transporters SLC3A2 and SLC1A5, and T cell chelators CXCL10, CXCL11 and RANTES. The results also show that chlamydial infection induces cell surface expression of T cell homing and activating proteins, including ICAM-1, VCAM-1, HLA I classes and II, and interferon gamma receptors.

However, the limited number of passages greatly limits the available experiments for each donor. Another drawback is the variation in experimental results between different donors. Due to the relatively small surface area of the cervix, it is also difficult to obtain sufficient numbers and high purity of primary epithelial cells. The limitations in establishing an appropriate *in vitro* cervical epithelial cell model may also be exacerbated by more general problems arising from primary and transformed epithelial cell lines derived from FGT tissues, including loss or alteration of hormone receptor expression, hormone responsiveness, and expression of antimicrobial molecules (Isaka et al., 2003; Joly et al., 2009).

### **2.4 Organoid culture or three-dimensional (3D) culture of FGT**

Traditional two-dimensional (2D) cell culture provides an ideal advantage of high throughput and consistency. However, in 2D models, cells are exposed to unphysiological stiffness and unnatural environments, and do not maintain proper cell-cell interactions. Given the complexity of FGT, techniques that enable 3D culture, such as organoid, are more suitable for research in

this field (Stejskalová et al., 2021). The use of organoid culture to study FGT overcomes the limitations of cell lines, partially solves the difficulties associated with the isolation and culture of various cell types for a long time, and can maintain the epithelial cell phenotype (Alzamil et al., 2021).

Organoid constructs from human endometrial epithelial cells were first completed in 1988 (Rinehart et al., 1988), and have been developed from several FGT organs in recent years, especially from endometrium and fallopian tubes. For instance, the isolated human and mouse glandular endometrial epithelial cells cultured in chemically defined media form endometrial organs composed of hormone-responsive polarized epithelial subtypes (e.g., ciliated, secretory) (Boretto et al., 2017; Turco et al., 2017). This model is suitable for screening *C. trachomatis* mutants for the defects in fusion of inclusions, recruitment of intracellular organelles, and inhibition of cell death (Dolat and Valdivia, 2021). The primary immune cell response is also reconstituted by culturing infected epithelial organoids with neutrophils, which show that chlamydial effectors such as CPAF and TepP limit neutrophil recruitment to the infected organoids. Using human fallopian tube organoid, leukemia inhibitory factor (LIF) signaling is activated by acute chlamydial infection, that is involved in salpingitis and increase the risk of ectopic pregnancy (Kessler et al., 2019; Krishnan et al., 2013). The chronic chlamydial infection of this organoid also leads to high methylation of DNA, an indicator of accelerated molecular aging.

Organoids are *in vitro* 3D reconstructions of tissue epithelia that self-develop and self-organize by their stem cells under precise culture conditions (Simian and Bissell, 2017). The main advantage of this approach is to allow long-term cultivation of tissues, thus obtaining biobanks of blasts from a large number of donors, which in turn enables sharing between laboratories (Fassbender et al., 2014; Schutgens and Clevers, 2020). However, the current 3D culture also has certain problems: 1) the vast majority of organoid models currently only represent the epithelial cells, and more complex models still need to be established, such as epithelia-basal layer interactions. 2) the typical organoid composition is in a closed structure, which is not ideal for some research applications. 3) an important issue in organoid culture is the use of relatively undefined, animal-derived, batch-susceptible extracellular matrix sources of Matrigel and basement membrane extracts (Stejskalová et al., 2021). Nevertheless, organoid based models have an environment similar to that of native tissues and the advantages of *in vitro* systems, facilitate more detailed studies of the molecular mechanisms of *Chlamydia*-host interactions.

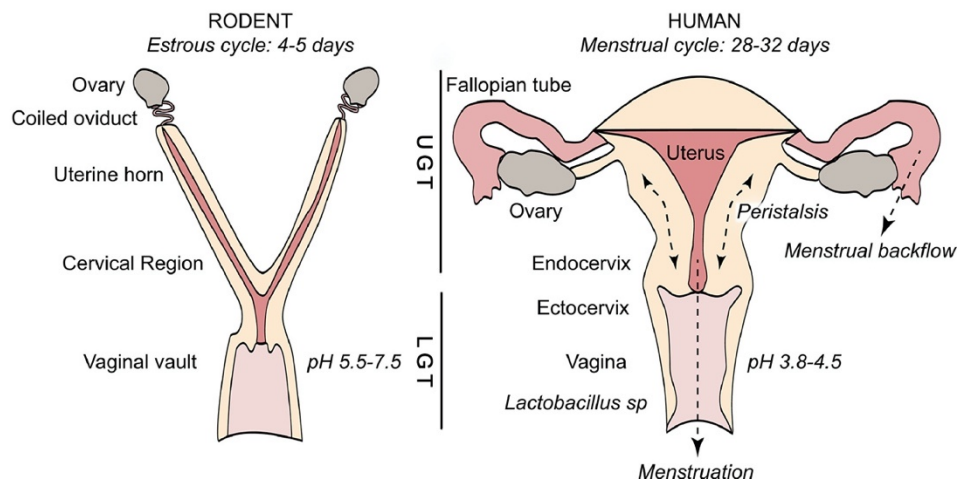
## 2.5 Animal models

*Chlamydia* infection in the FGT of women is characterized by an ascending infection from lower to upper genital tract. To study the pathogenesis and immunity of FGT in this infection process, and to evaluate the effectiveness of vaccine candidates, animal models are frequently used. Mouse model is most common (Barron et al., 1981), but guinea pig (Mount et al., 1973), rat (Kaushic et al., 1998), non-human primate (Patton et al., 1983), rabbit (Patton et al., 1982), and pig models are also used (Vanrompay et al., 2005). Intravaginal inoculation of *C.*



*muridarum*, a mouse-adapted strain, induces ascending infection in FGT, but not human-adapted *C. trachomatis* strain. Intriguingly, trans-cervically introducing *C. trachomatis* to upper FGT of mouse, can trigger a chronic infection and subsequent tissue damage (Gondek et al., 2012). Thus, mice are the first choice to investigate the immune response to chlamydial infection, in term of their small size, ease of experimental manipulation, availability of sufficient numbers, and low cost. Importantly, many well-characterized inbred and knockout mouse strains are also available (Vanrompay et al., 2006).

Although animal studies have historically played an important role in facilitating biological research, the use of animal models to study the female reproductive system has been a challenge to translate into clinically useful insights. This is because the anatomy and physiology of the reproductive system vary greatly between mouse and human [Figure 27] (Stejskalová et al., 2021).



**Figure 27. The rodent and human female reproductive system differ in many aspects.** The rodent uterus undergoes an oestrous cycle in which the endometrium resorbs and is not shed while the female endometrium sheds during menstruation. (From Stejskalová et al., 2021)

### III. Innate immunity of host to *C. trachomatis* infection

Innate immunity, as the first line of host immune response, is ready for an immediate action or is quickly induced when the host is attacked by pathogens, relevant to adaptive immunity that takes couple of days to arise following exposure to antigens. Innate immunity comprises four types of defensive barriers: anatomical (skin and mucosal), physiological (temperature, low pH, and chemical mediators), endocytic and phagocytic, and inflammatory (Marshall et al., 2018). The main anatomical barrier is the epithelial layer on the surface of skin and mucosal tissues (e.g., respiratory tract, digestive tract, genitourinary tract), and the glandular tissues (e.g., sweat glands, salivary glands, sebaceous glands, prostate glands) (Lafrenie, 2011). The chemical barriers of the surfaces include specialized soluble substances, such as antimicrobial peptides (AMPs), reactive oxygen and nitrogen species (Koenderman et al., 2014). All these surface barriers prevent pathogens from entering the host body. Regarding cell-related innate defense,

the host senses invading pathogen by pattern recognition receptors (PRRs) distributed on the cell surface or cytoplasm, that recognize conserved pathogen-associated molecular patterns (PAMPs) presenting on bacteria, viruses, fungi, and parasites, as well as intrinsic danger-associated molecular patterns (DAMPs) caused by cell damage. The main PRRs include C-type lectin receptors (CLRs), toll-like receptors (TLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), nucleotide binding and oligomerization domain (NOD) class receptors (NLRs), RIG-I-like receptors (RLRs), and cGAS/STING system (Brubaker et al., 2015; Yin et al., 2015). These families of PRRs can be divided into two major categories: membrane-bound receptors and non-membrane-bound intracellular receptors (Brubaker et al., 2015). The first category includes TLRs and CLRs, which are present on the cell surface or in the inner cell compartment. These receptors are responsible for monitoring the presence of ligands for microorganisms in the extracellular space and endosomes. NLRs, RLRs, and ALRs constitute a second group, which are located in the cytoplasm and are responsible for monitoring intracellular pathogens.

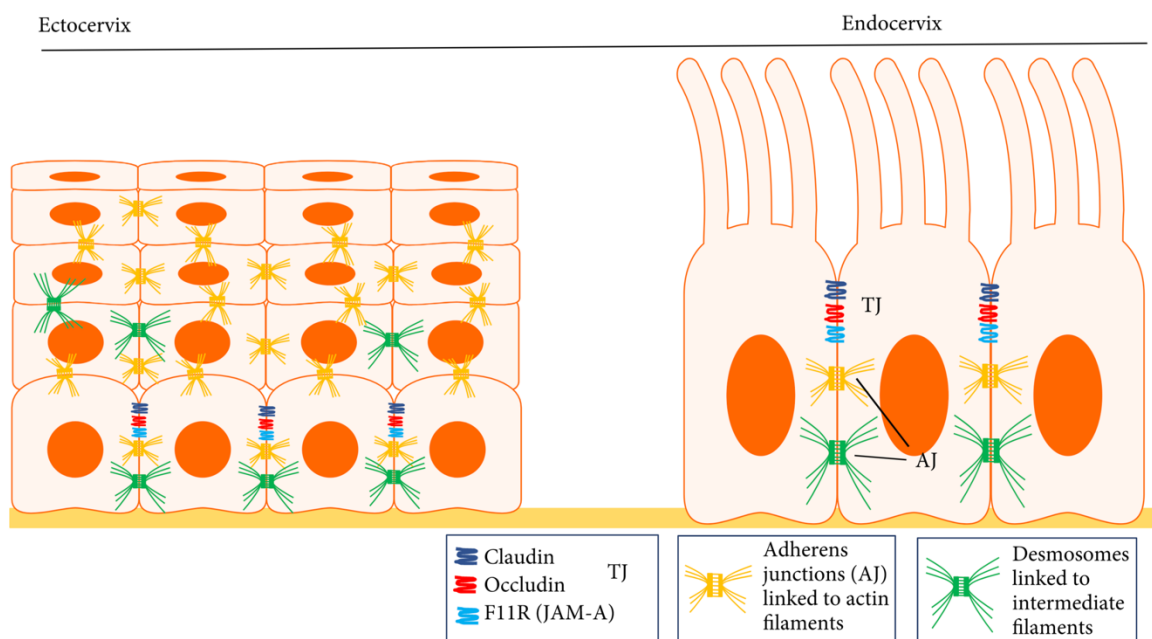
Binding of these receptors to corresponding ligands activates transcription factors such as NF- $\kappa$ B, which in turn trigger inflammatory response. In summary, a major component of the innate immune response induced by PRRs is transcription, which induces the production of proinflammatory cytokines and interferons; these chemokines and cytokines are essential for initiating innate and adaptive immune responses. Activation of PRR also initiates non-transcriptional responses such as induction of phagocytosis, autophagy, cell death, and cytokine processing (Deretic et al., 2013; Lamkanfi and Dixit, 2014).

### **3.1 Anatomical barriers**

As described above, the epithelial surface of multicellular organisms constitutes an interface that forms an anatomical barrier separating the individual from the environment. The junctions between epithelial cells also maintain the integrity and organization of epithelial cells by regulating the communication of molecules and cells and providing an anatomical barrier for pathogen invasion. Three main cell-cell structural adhesions exist between epithelial cells: tight junctions, adherens junctions, and desmosomes (Langbein et al., 2002; Marchiando et al., 2010). The tight junctions are composed of transmembrane proteins that make contact across the space between cells, form a seal, limit the bypass diffusion of molecules on the epithelial sheet, and organize the polarization of epithelial cells (Langbein et al., 2002; Meng and Takeichi, 2009). Adherens junctions connect actin bundles between cells to form a continuous belt of adhesions, usually just beneath tight junctions (Niessen, 2007). The desmosomes link keratin intermediate filaments from the intercellular space to form a structural framework with great tensile strength (Kowalczyk and Green, 2013). The figure 28 shows the profile and intercellular localization of adhesion molecules between ectocervical and endocervical epithelial cells (De Tomasi et al., 2019). It has been shown that columnar epithelial cells in the endocervix contain tight junctions, that are located close to the apical surface, whereas adhesive junctions are just below the tight junctions (Blaskewicz et al., 2011). In contrast, the non-keratinized superficial layer of the ectocervical stratified squamous epithelium does not contain classical cell-cell adhesion and

is permeable to IgG. The suprabasal and basal layers of the ectocervical and vaginal tissues contain the most robust adhesions junctions (Hickey et al., 2011). Its lack of tight junctions and permeability to large molecular weight immune mediators suggests that this region is an important battlefield for host defense against microbial pathogens (Blaskewicz et al., 2011). Desmosomes are the most basal junctions. The structure of tight junctions is influenced by hormones as well as cytokines (Capaldo and Nusrat, 2009; Someya et al., 2013).

The anatomical barriers are altered upon *Chlamydia* infection. In *ex vivo* fallopian tubes infected with *Chlamydia*, infected epithelial cells display loss of apical F-actin and cell-cell junction integrity, indicating that the polarity and barrier function of epithelial cells are compromised during *Chlamydia* invasion (Kessler et al., 2012). *Chlamydia* also target the tight junctions of the mouse oviductal epithelial cell line, altering the expression of tight junction proteins and reducing the transepithelial electrical resistance (Kumar et al., 2019). Chlamydial infection also induce the accumulation of the tight junction protein claudin-4 (CLDN-4) in inclusions, along with a decrease in membrane-bound claudin-4, which can impair epithelial barrier function, and increased susceptibility to co-infection (Amaral et al., 2021).



**Figure 28. Profile of intercellular junctions in the cervix.** (From De Tomasi et al., 2019)

## 3.2 Chemical barriers

### 3.2.1 Mucus

The cervical mucus produced by goblet cells is another barrier against infection in the FGT. Among mucus mixtures, mucins are the main components, which belong to a family of more than 20 members. Mucins are large glycoproteins acting as gels or glues for mucus and therefore able to capture and immobilize pathogens depending on the thickness of mucus (Gipson, 2001). In human, MUC4 and MUC5 (5AC, 5B) as well as MUC6 are the major mucins of endocervical mucus, and their quantity changes during the menstrual cycle (Gipson et al., 1999).

Collectively, cervical mucus acts as a semi-permeable and antimicrobial barrier that prevents pathogens from entering the upper reproductive tract (Martyn et al., 2014).

### 3.2.2 Antimicrobial peptides (AMPs)

The discovery of lysozyme in 1922 (Fleming, 1922), marks the birth of modern innate immunity, followed with the discovery of antibiotics and AMPs. The latter are small proteins (15-150 amino acids) with antibacterial, antiviral, and antifungal activities (Lazzaro et al., 2020). AMPs are ubiquitous in epithelial barriers of eukaryotes. These short peptides have a net positive charge that attracts them to the general negatively charged membrane of bacteria (Lazzaro et al., 2020). Nowadays, a total of 3283 antimicrobial peptides have been recorded in the Antimicrobial Peptide Database, which come from six life kingdoms (Dept of Pathology & Microbiology, 2021). In vertebrates, cathelicidins and defensins are the major families of AMPs.

Defensins and cathelicidin are known to kill *Chlamydia* (Donati et al., 2005; Hou et al., 2015; Yasin et al., 1996). LL-37 is the only member of the human cathelicidin family (Kahlenberg and Kaplan, 2013), and is widely distributed in the urogenital mucosa or glands of the urethra, vagina, and cervix. *C. trachomatis* infection induces LL-37 in the genital tract of women. Besides its antimicrobial activity, LL-37 can also activate inflammatory pathways, stimulate epithelial cells to secrete proinflammatory cytokines and recruit inflammatory cells (Kahlenberg and Kaplan, 2013). On the other hand, bacteria have different strategies to combat AMPs to promote colonization and infection (Law Whyte et al., 2002), as described previously in 'Pgp3' chapter..

Human beta defensins (hBDs) 1, 2, 3, and 4 represent the major group of human AMPs, produced by epithelial cells and immune cells in response to infection (Menendez and Brett Finlay, 2007). It has been shown that women positive for *C. trachomatis* have significantly lower cervicovaginal hBD-1, hBD-2, and hBD-3 compared to those who test negative (Noda-Nicolau et al., 2017).

### 3.2.3 Lactoferrin

Lactoferrin is an 80-kDa iron-binding glycoprotein detected in most body fluids, including vaginal fluid (Valore et al., 2002). It can inhibit bacterial infection rather than iron chelation, and its high positive charge favors binding to microorganisms or host cells, thereby hindering pathogen adhesion and entry into epithelial cells. In addition, lactoferrin also shows potent anti-inflammatory activity by protecting infected host cells from pathological inflammation (Berlutti et al., 2006; Valenti et al., 2011). Indeed, incubation of bovine lactoferrin with cell monolayer before or at the time of *C. trachomatis* infection, inhibits *C. trachomatis* entry into host cells, and downregulates IL-6/IL-8 synthesis by infected cells (Sessa et al., 2017). Pregnant women with asymptomatic *C. trachomatis* infection becomes negative for *C. trachomatis* and show decreased IL-6 levels in cervix after intravaginally given bovine lactoferrin (Sessa et al., 2017). Importantly, after *C. trachomatis* infection, mucosal epithelial cells and neutrophils can release

lactoferrin in the cervico-vaginal tract, as evidenced by higher lactoferrin levels in infected women than in healthy women (Filardo et al., 2019; Sawada et al., 2006; Spear et al., 2011). Thus, lactoferrin is considered as a key component of the first-line defense of the female mucosal reproductive tract (Valenti et al., 2018).

### **3.2.4 Microbiota in the FGT**

Another factor that protects the lower genital tract from pathogen infection is the population of commensal bacteria. The human vaginal microbiome can be divided into five core microbial communities, four of which are dominated by species belonging to the genus *Lactobacillus* (*L. iners*, *L. crispatus*, *L. gasseri*, or *L. jensenii*) and one characterized by higher levels of strict anaerobes, including *Prevotella*, *Gardnerella*, *Dialister* and *Atopobium* (Ma et al., 2012; Ravel et al., 2011). *Lactobacilli* produces lactic acid to establish an acidic environment in vagina (pH 3.5-4.5), which limits colonization by other microorganisms (Boskey et al., 1999; Daniel Johannes Rönnqvist et al., 2006). *Lactobacilli* also produces bacteriostatic compounds to compete with opportunistic pathogens, strives to attach to the vaginal epithelium, and secretes AMPs (Amabebe and Anumba, 2018; Borges et al., 2014; Mijač et al., 2006). Collectively, the vaginal microbiome has emerged as an important regulator of genital tract inflammation.

Women with *Lactobacillus* poor flora has increased susceptibility to sexually transmitted pathogens such as *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis* (Brotman et al., 2010; Ness et al., 2005). The *in vitro* study has shown that propagation of *C. trachomatis* is inhibited by different strains of vaginal lactobacilli independently of alterations in pH (Kohlhoff and Hammerschlag, 2015). Vaginal lactic acid bacteria can inhibit adhesion of EBs to epithelial cells, and replication of intracellular RBs, which is species and dose dependent, suggesting that the species and number of intravaginal lactobacilli are responsible for protection against chlamydial infection.

### **3.3 Pattern recognition receptors (PRRs)**

The cell-related innate immune system is responsible for initial detection of invading pathogens and induction of a rapid cytosolic signaling response. A primary component of this innate immune system is the set of host germline-encoded PRRs that recognize evolutionarily conserved structure of microorganism, named as PAMPs or DAMPs. PAMPs are regularized and highly conserved molecular structures common to pathogenic microorganisms of the same class (Herwald and Egesten, 2016; Zindel and Kubes, 2020), but are absent in host cells such as flagellin, LPS, PG, and DNA or RNA of pathogenic microorganisms (Bianchi, 2007; Saïd-Sadier and Ojcius, 2012). PAMPs are essential not only for establishing the pathogenicity of infection in the host, but also for the survival of the pathogen (Kumar et al., 2013). The host also produces some proteins and metabolites after its own tissue damage, cell necrosis, and other factors, which are called DAMPs (Gong et al., 2020; Pandolfi et al., 2016). Recognition of these PAMPs or DAMPs by corresponding PRRs is key to initiate innate immune responses by activating downstream signaling pathways. The latter have many effects: recruiting and

releasing cytokines, chemokines, hormones, and growth factors; inducing chronic inflammation; forming an inflammatory microenvironment; initiating innate immune killing and subsequent adaptive immune responses, maintaining the balance of host microecology; and eliminating dead or mutated cells (Gajewski et al., 2013; Li and Wu, 2021).

As introduced previously, the diversity of PRRs at different subcellular locations enable the host to thoroughly monitor invading pathogens. In the following sections, several PRRs associated with *Chlamydia* infection are introduced such as TLR2 and NOD1.

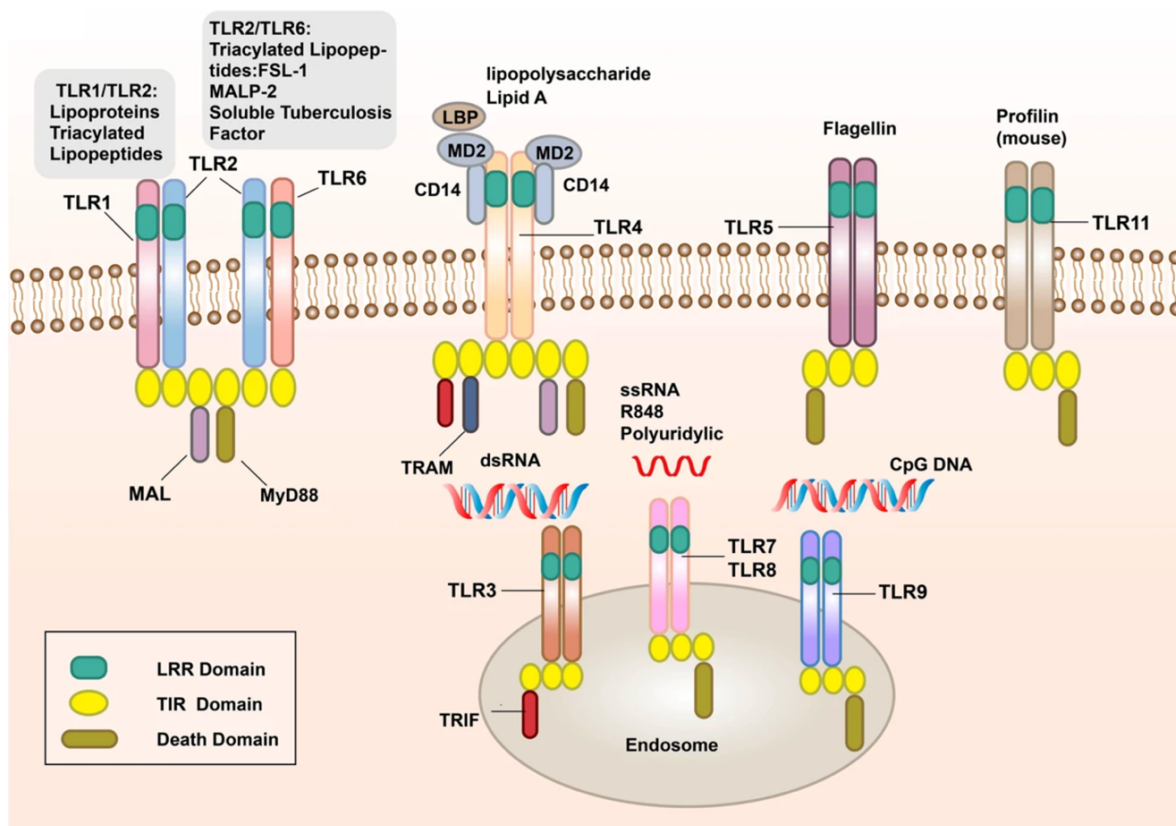
### **3.3.1 Toll like receptors**

#### **3.3.1.1 Brief introduction of TLRs**

One of the best-characterized groups of PRRs is the TLR family. So far, 10 functional TLRs have been identified in humans (TLR1-10) and 12 in mice (TLR1-9 and TLR11-13) (Akira et al., 2006). TLR10 in mice loses its function due to the insertion of a reverse transcriptase (Chuang and Ulevitch, 2001). These transmembrane proteins recognize PAMPs of pathogen at two distinct locations: cell surface or endosomal membranes within the cell. The TLRs localized on the cell surface include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 as homodimer such as TLR4 and TLR5, or heterodimer such as TLR1/2 and TLR2/6. These TLRs recognize a wide-range of bacterial PAMPs, including LPS (TLR4), tri-acylated lipopeptide (TLR1/2), di-acylated lipopeptide (TLR2/6) and flagellin (TLR5) (Chuenchor et al., 2014; Farhat et al., 2008; Takeuchi and Akira, 2010). The intracellular TLRs anchored cytoplasmic organelles membrane, mainly the endosomes, lysosomes, endolysosomes, and endoplasmic reticulum, are TLR3, TLR7, TLR8, and TLR9, which recognize nucleic acids of pathogens (Akira et al., 2006; Thompson et al., 2011). For instance, TLR9 is the first known sensor of bacterial DNA (CpG DNA), and this DNA sensing occurs in endocytic vesicles and cytoplasm (Ahmad-Nejad et al., 2002; Hemmi et al., 2000). TLR3 and TLR7/8 recognize double stranded RNA (dsRNA) and single stranded RNA (ssRNA) respectively, and have largely been studied in the context of viral infections [Figure 29] (Blasius and Beutler, 2010; Li and Wu, 2021).

As type I transmembrane glycoproteins, TLRs consist of an extracellular domain, a transmembrane region, and an intracellular region (Kawai and Akira, 2010). The extracellular region contains leucine-rich repeats (LRRs) and is responsible for recognizing specific ligands and performing extracellular pattern recognition. The intracellular domain contains the same Toll/IL-1R (TIR) domain as IL-1R, which acts in signal transduction. Once recognizing and binding the corresponding PAMPs or DAMPs, the TIR domain of TLRs conducts signals in the cytoplasmic region by binding to different receptor-adapted proteins (Netea et al., 2002; Xu et al., 2000). Depending on the different adaptor proteins, TLRs signaling can be divided into myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (Barton and Medzhitov, 2003; Shimizu, 2017). Four TIR-containing adaptor molecules involving in the MyD88-independent pathway, have been identified, including TIRAP, TRIF, TRAM and SARM (Saikh, 2021). Activation of these adapter molecules results in signaling through NF-

$\kappa$ B and IRF3, 5, and 7 to produce inflammatory cytokines and type I interferons (IFN-I) (Snyder et al., 2017).



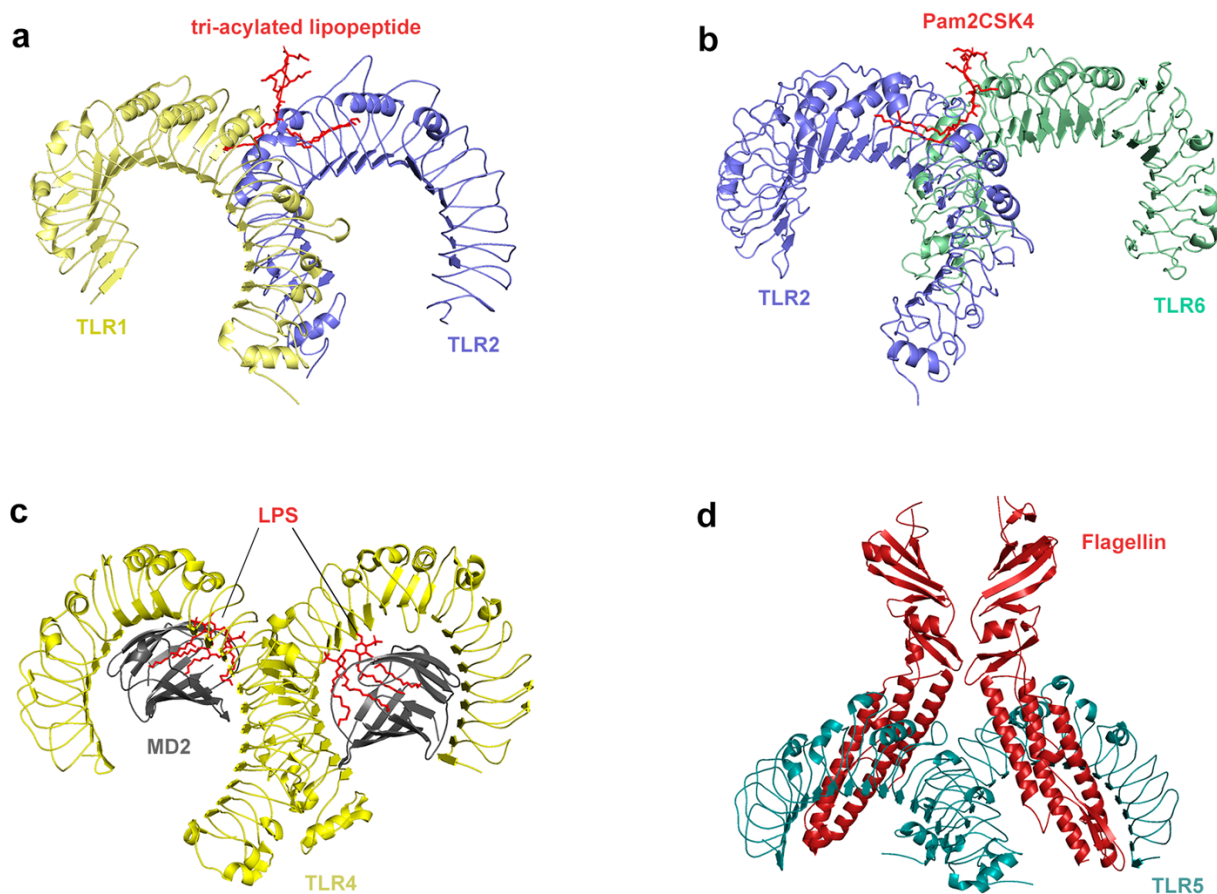
**Figure 29. The signal transduction pathways and structure of TLR-binding ligand complex.** TLRs can recognize one or more PAMPs through LRR domain. They usually dimerize themselves and recruit adaptor molecules with the same TIR domain to transmit signals. (From Li and Wu, 2021)

When sensing the corresponding ligand, TLR2 can form a M-type structure with the extracellular region of TLR1 or TLR6, and this formed pocket structure then can bind to the ligand [Figure 30] (Kaur et al., 2021; Natala et al., 2021). In TLR1/2 heterodimer, there is a prominent gap between the central part and the C-terminal region of TLR2, where two lipid chains of triacylated lipopeptide are inserted into TLR2, and the other lipid chain binds to the lipid-binding channel between the central and C-terminal regions of TLR1 (Jin et al., 2007). The structures of TLR1/6 are highly homologous, but TLR6 lacks a lipid-binding channel, and the degree of acylation of the protein distinguishes TLR2 from TLR1 and TLR6 interactions, resulting in their different performance in recognizing and removing microorganisms (Oliveira-Nascimento et al., 2012; Triantafilou et al., 2006).

Unlike other TLRs that directly recognize ligands, TLR4 binds to two accessory molecules, bone marrow differentiation factor 2 (MD2) and the LRR structural protein CD14, which specifically recognizes LPS (Vidya et al., 2018; Xia et al., 2021). LPS is transported by LPS-binding proteins to CD14 on the cell membrane of monocytes and macrophages, forming a complex that then interacts with TLR4/MD2 (Miyake, 2007). After binding of LPS to the TLR4/MD2 complex, the hydrophobic pocket of MD2 is used to connect two TLR4-MD2-LPS

complexes to form sterically symmetric M-type TLR4-MD2-LPS dimers, and activates downstream signals (Park et al., 2009).

TLR5 is the most conserved and important PRR, that is usually stimulated by the bacterial flagellin (Gewirtz et al., 2001). In the form of homodimers, TLR5 plays an important role in the regulation of primary defense and immune balance in invasive pathogens (Yoon et al., 2012). TLR10, mainly expressed on the cell surface, but also in the endosome (Lee et al., 2018b), is the only member of the human TLRs family that has an inhibitory effect on the induction of innate immune responses and inflammation (Mourits et al., 2020). Recognition of dsRNA by TLR10 activates the recruitment of MyD88 for signal transduction and inhibition of IFN-I-dependent IRF7 production.



**Figure 30. Crystal structure of TLRs with ligands.** (From Li and Wu, 2021)

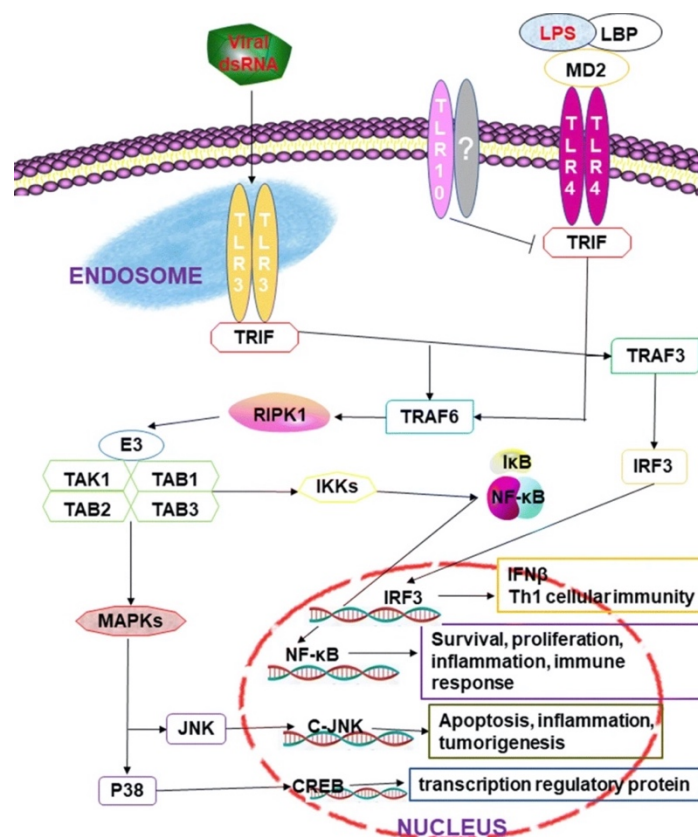
### 3.3.1.2 TLR3 and its signaling pathways

All 10 types of TLRs, as well as MYD88 and accessory molecule CD14, are expressed in the epithelial cells of FGT, including the fallopian tubes, uterine endometrium, cervix, and ectocervix (Carty et al., 2021). As a receptor for the viral replication product dsRNA and the synthetic analog polyinosinic-polycytidylic acid (polyI:C), TLR3 plays an important role in viral infection (Brencicova and Diebold, 2013). Upon binding to dsRNA, TLR3 recruits the adaptor TRIF to the TIR domain, which activates innate immunity, leading to robust induction of IFN-I and inflammatory cytokine expression (Carty et al., 2021). TLR3 can prevent viral



replication and mediate antiviral responses to eliminate pathogens, such as encephalocarditis virus, poliovirus, dengue virus, hepatitis B virus, hepatitis B virus, and herpes simplex virus (Lester and Li, 2014). However, TLR3-mediated inflammatory responses also play a detrimental role in some viral infections. For example, during influenza A virus infection, TLR3-deficient mice instead prolong survival and improve the outcome of acute pneumonia caused by this virus (Le Goffic et al., 2006).

TLR3 binding to TRIF recruits TNF receptor-associated factor 3 (TRAF3) to subsequently activate IRF3 by phosphorylation and dimerization. IRF3 is then translocated into the nucleus where it mediates the expression of IFN-I genes [Figure 31] (Lundberg et al., 2013; Xia et al., 2021). Meanwhile, TRIF also has a binding motif of TRAF6 at N-terminal domain. So, TLR3 can recruit TRAF6 after binding to TRIF, while TRAF6 interacts with receptor-interacting protein 1 (RIP1) through its C-terminal RIP homotypic interaction motif and then forms a complex with transforming growth factor- $\beta$ -activated kinase-1-binding proteins: TAB1, TAB2, and TAB3 (Cusson-Hermance et al., 2005). Formation of this complex activates I $\kappa$ B kinases (IKK) and c-Jun N-terminal kinases (JNK), while IKK leads to phosphorylation and degradation of NF- $\kappa$ B inhibitors I $\kappa$ B and then the translocation of NF- $\kappa$ B to the nucleus. In addition, JNK can activate AP-1 to promote the expression of multiple cytokines and is involved in the process of anti-microbial infection [Figure 31] (Balka and De Nardo, 2019; Mitchell et al., 2016).



**Figure 31.** Activated TLR3 relies on TRIF-dependent signaling pathways. (From Xia et al., 2021)

### 3.3.1.3 TLRs expression in FGT epithelium

All 10 types of TLRs are expressed in the epithelial cells of FGT, including the fallopian tubes, uterine endometrium, cervix, and ectocervix, as well as MYD88 and accessory molecule CD14 (Aflatoonian and Fazeli, 2008; Nasu and Narahara, 2010; Pioli et al., 2004). It has been shown that in reproductive system TLRs are implicated in menstruation, regulation of ovulation, sperm capacitation, fertilization, gestation and parturition, as well as pathological and inflammatory conditions such as endometritis and sexually transmitted infections (Kannaki et al., 2011; Liu et al., 2008; Nasu and Narahara, 2010; Zandieh et al., 2016).

Vaginal epithelial cells express TLR1, 2, 3, 5 and 6, however, the endocervix is rich in TLR1, 2, 3, and 6. The upper FGT is rich in TLR4, while TLR2 is more common in the fallopian tube and the cervix (Krikun et al., 2007). TLR1-9 are abundant in the endometrial epithelial cells, and TLR2 and 4 are abundant in the oviduct and endometrium. In addition, TLRs expression is also menstrual cycle dependent, but which are contradictory depending on the studies (Costello et al., 2007; Krikun et al., 2007). For instance, higher levels of TLR2 and 4 are expressed during the peri-menstrual period, while the secretory phase tends to TLR2-6, 9 and 10. Similar selective expression also appeared during pregnancy, favoring TLR2, 4 and NOD1/2 expression in trophoblasts.

Amjadi F et al. report that fallopian tube epithelial cells express TLRs in a cell type-specific manner (Amjadi et al., 2018). They detect positive immunostainings for all TLRs in the ciliated epithelial cells. However, in non-ciliated cells of the fallopian tube, weak staining for TLR1-8, and no staining for TLR9/10 are observed. Exposing ciliated fallopian tube epithelial cells to agonists of TLR2, TLR3, TLR5, TLR7, or TLR9, induces the secretion of proinflammatory cytokines IL-6 and IL-8. Their study indicates that ciliated cells may play a more effective role than non-ciliated cells in innate immune defense of the fallopian tubes.

#### 3.3.1.4 TLRs function during *Chlamydia* infection

It has been shown that TLR4 plays a key role in *Chlamydia*-mediated host cell activation and pathological processes (Darville et al., 2003). Recognition of chlamydial HSP60 and LPS is mediated by TLR4 (O'Connell et al., 2006). However, *C. trachomatis* LPS is not able to participate in the canonical TLR4/MD-2 or the atypical caspase-11 inflammatory pathway (Nguyen et al., 2011; Yang et al., 2019b), since *C. trachomatis* expresses a LPS-like lipooligosaccharide (LOS) as we described previously, that are more difficult to activate TLR4 (Heine et al., 2007; Wang et al., 2020b).

*C. trachomatis* and *C. muridarum* infections have been shown to induce TLR2-dependent secretion of inflammatory cytokines IL-6, IL-8, and TNF $\alpha$  (Darville et al., 2003; Derbigny et al., 2005; O'Connell et al., 2006). In bone marrow-derived macrophages, recognition of *C. pneumonia* by TLR2 also leads to IL-1 $\beta$  production via MyD88 signaling (Shimada et al., 2011). TLR2-null mice show a reduction in genital tract lesions by *C. muridarum* compared to wild-type mice (Darville et al., 2003). These data indicate the critical role of TLR2 in inflammatory response and bacterial clearance *in vivo*. Indeed, TLR2 colocalizes with the inclusion membrane

in the infected cells and *Chlamydia* MOMP is an effective ligand of TLR2. The interaction of MOMP-TLR2, which leads to the secretion of IL-6 and IL-8 through ERKs and p38/MAPK signaling pathway, helps in clearing *Chlamydia* infection (Massari et al., 2013). Interestingly, exposing cells to plasmid-cured chlamydial strains, dramatically attenuates the activation of TLR2, suggesting that plasmid-encoded gene(s) control either the synthesis or the processing of TLR2-activating molecules (O'Connell et al., 2011). As an example, *Chlamydia* plasmid-encoded protein Pgp4 can control the expression of multiple chromosomal genes of the bacteria, including *glgA* encoding glycogen synthase (Song et al., 2013). This synthase-participated enzymatically synthesized glycogen can activate TLR2 signaling, which offers the intriguing possibility that *Chlamydia*-derived glycogen acts as a major trigger for infection-induced TLR2 activation (Kakutani et al., 2012).

Endosomal TLR3 is also involved in the innate immune response to chlamydial infection, mainly eliciting IFN $\beta$  production (Kawai and Akira, 2010). Chlamydial infection of oviduct epithelial cells displays TLR3-TRIF pathway-mediated IFN $\beta$  synthesis, which regulates the expression of IL-6, CXCL10, CXCL16, and CCL5 (Derbigny et al., 2007, 2012; Lad et al., 2005). The enhanced *C. muridarum* shedding and hydrosalpinx observed in infected TLR3-null mice, also suggest that TLR3 facilitates the host's protective response and bacterial clearance (Carrasco et al., 2018). As a sensor of dsRNA from intracellular viruses (Kawai and Akira, 2010), TLR3 can also recognize dsRNA from bacteria (Kawashima et al., 2013), suggesting that *Chlamydia*-derived dsRNA may function as a TLR3 agonist in epithelial cells, but also in dendritic cells.

### 3.3.2 NOD-like receptors (NLRs)

The life cycle of some pathogenic microorganisms involves infection to the cytoplasm. For example, viral genes are frequently transcribed and translated in the cytoplasm and assemble viral particles there. Some bacteria and parasites also have a range of mechanisms to escape from endosomes, such as punching holes in the phagosome membrane, and then entering the cytoplasm. Therefore, pathogens and their metabolic components, as well as other components produced by infection and injury, which appear in the cytoplasm, require the recognition and intervention by PRRs to protect the host (Motta et al., 2015). NLRs are such a group of intracellular sensors that detect a wide range of PAMPs. The NLRs are defined by their unique structure consisting of three domains: an N-terminal effector domain such as the caspase activation and recruitment domain (CARD) or the pyrin domain (PYD); a central nucleotide-binding and oligomerization domain (NOD), also known as the NACHT domain (the abbreviations of the following four kinds of NLR members: NAIP, CIITA, HETE, TP1), which is shared by the NLR family and is very important for nucleic acid binding and oligomerization of NLRs; and C-terminal leucine-rich repeats (LRRs), which identify the ligands (Brubaker et al., 2015; Fritz et al., 2006; Meunier and Broz, 2017). According to the different N-terminal effector domains, the NLRs family can be divided into five subfamilies: NLRC containing CARDS; NLRP containing PYD; NLRB containing baculoviral inhibitor of apoptosis protein

repeats; NLRA containing acidic activation domains; and NLRX subfamily containing other NLR effector domains (Fritz et al., 2006).

NOD1 and NOD2 (NOD1/2) are two well-known members of NLRC subfamily. NOD1 is widely expressed in various cell and tissue types (Chamaillard et al., 2003; Girardin et al., 2003). Expression of NOD2 is more restricted, though it has still been detected in both hematopoietic and non-hematopoietic cell types (Franchi et al., 2009). NOD1/2 are originally described as receptors to recognize bacterial peptidoglycan, such as diaminopimelic acid iE-DAP of the cell wall of Gram-negative bacteria for NOD1 and muramyl dipeptide (MDP) in all bacterial cell walls for NOD2 (Chamaillard et al., 2003; Girardin et al., 2003; Pashenkov et al., 2018). But, increasing evidences show that host NOD1 and/or NOD2 signals can also be activated upon infection with pathogens lacking peptidoglycan, including viruses (Liu et al., 2019a; Lupfer et al., 2013), parasites (Corbett et al., 2015), and fungi (Gresnigt et al., 2017, 2018). In addition, NOD1 and/or NOD2 are also involved in detecting pathogen-induced processes, such as activation of Rho GTPases, ER stress and unfolded protein response, autophagy and mitophagy, disruption of calcium balance, and cell death (Keestra-Gounder et al., 2016; Keestra et al., 2013).

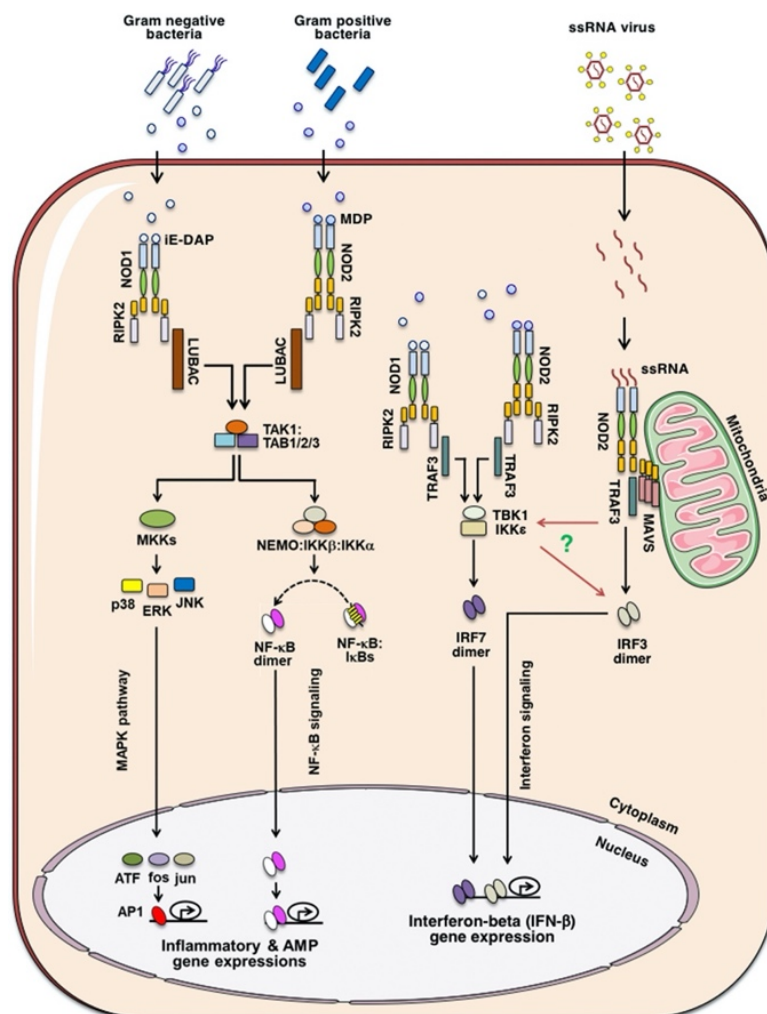


Figure 32. NOD1 and NOD2 induced NF-κB and MAPK signaling pathways. (From Mukherjee et al., 2019)

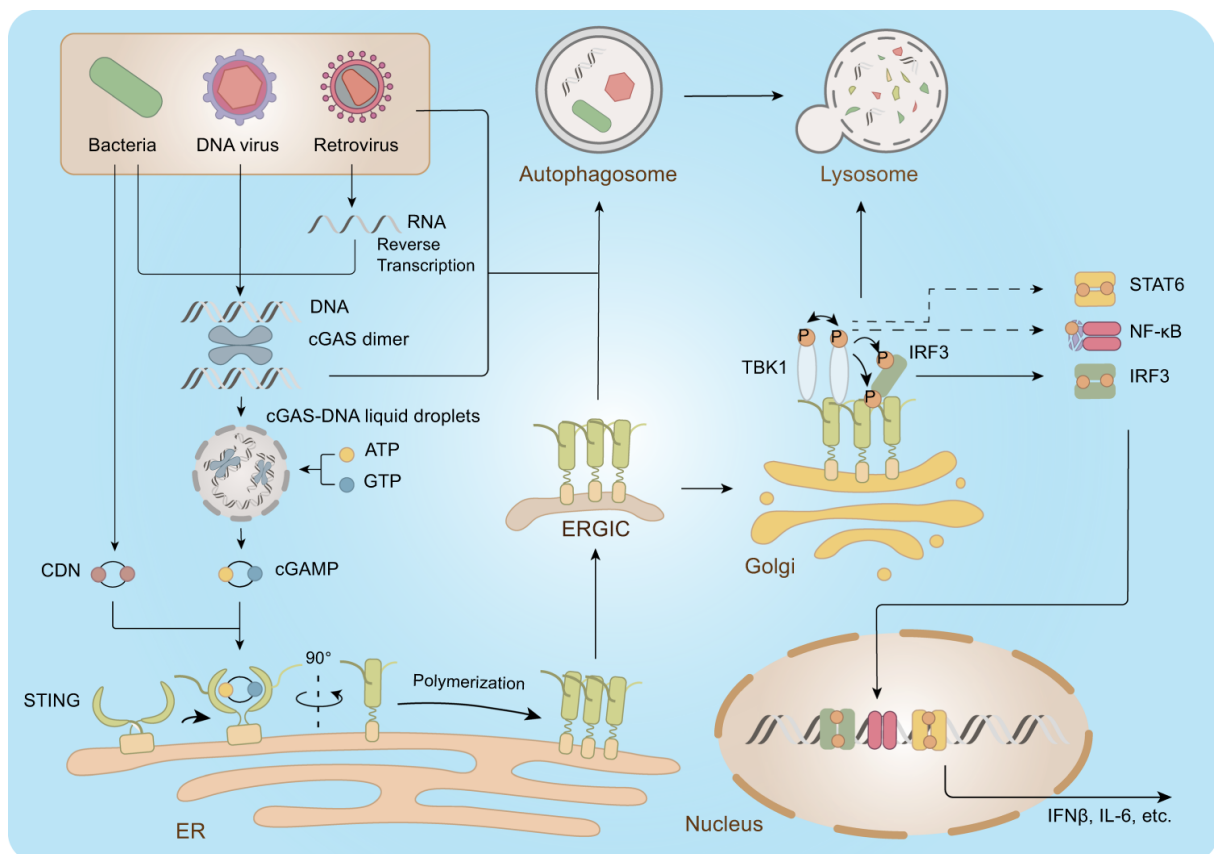
Under the unligand-bound state, the LRR domain of NLR folds to form a U-shaped conformation with the central NACHT domain, which inhibits their multimerization and renders NLR inactive (Hu et al., 2013). Once PAMPs binding to LRRs, the NLR molecule conformation is changed by exposing the NACHT oligomeric domain, which triggers oligomerization and subsequent activation of NLR molecule (Le and Harton, 2013). Meanwhile, the N-terminal effector domain is also exposed, and through homotypic interactions, downstream adaptor molecules and signaling proteins with the same structure are recruited to initiate the corresponding signal transduction (Wang et al., 2019a). The oligomerization and the recruitment of receptor-interacting protein 2 (Rip2) through CARD-CARD interactions then activates NF- $\kappa$ B signaling and induces a robust of pro-inflammatory response [Figure 32] (Mukherjee et al., 2019).

Chlamydial inclusion is closely associated with the host's ER, where bacteria acquire host's lipids and other nutrients (Dumoux et al., 2012; Giles and Wyrick, 2008). One potential consequence of this inclusion-ER interaction is to induce an unfolded protein response (UPR), a host response to reduce translation and initiate ER repair. Induction of the UPR has been implicated in the initiation of the inflammatory response and was shown to require NOD1/2 sensing (Keestra-Gounder et al., 2016). Thus, NOD1/2 may initiate ligand-independent induction of UPR-induced inflammation as a consequence of ER stress responses induced by chlamydial infection (Keestra-Gounder and Tsolis, 2017). Indeed, it has been demonstrated that during chlamydial infection *in vitro*, NOD1/NOD2 expression level is increased in response to ER-dependent stress-induced inflammation (Derré, 2015; Packiam et al., 2015; Zou et al., 2016). The *in vivo* study also shows that ER stress-driven inflammation can promote systemic chlamydial clearance (Pham et al., 2020). Furthermore, mice lacking both NOD1/NOD2 or RIP2 show higher bacterial loads after chlamydial infection, as well as NOD1/2-dependent tauroursodeoxycholate (TUDCA, inhibiting the ER stress response)-sensitive inflammatory responses. These studies suggest that RIP2 and NOD1/NOD2 proteins are involved in ER stress caused by *Chlamydia* infection and subsequent induced inflammatory response.

### 3.3.3 The STING pathway

PAMPs from extracellular pathogens are mainly recognized by TLRs, whereas those from intracellular pathogens are mainly sensed by NLRs and RLRs (Pandey et al., 2015). Recent studies have revealed some novel cytosolic surveillance systems, especially the identification of stimulator of interferon genes (STING)-adapted proteins representing an important milestone in nucleotide sensing research (Ishikawa et al., 2009). STING, an ER-resident protein, serves as a sensor for cyclic dinucleotides (CDNs), such as c-di-GMP and c-di-AMP produced by bacteria (Witte et al., 2008), and cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), a second bacterial messenger, synthesized by cyclic GMP-AMP synthase (cGAS) (Li et al., 2013). STING also serves as a link between DNA sensing and cellular activation, allowing STING to play a key role in the host immune response. Since DNA is present in most microorganisms, while CDNs are essential for the metabolism of bacteria, such as biofilm formation and protein function (Römling, 2008; Tamayo et al., 2007). These bacterial

oligonucleotides are regarded as PAMPs. Another most studied CDNs sensor is cGAS (also known as Mb21d1), which belongs to the nucleotidyltransferase family (Sun et al., 2013). In addition to cGAS, other DNA sensors, including IFN $\gamma$ -inducible protein 16 (IFI16) (Unterholzner et al., 2010), DEAD-box helicase 41 (DDX41) (Zhang et al., 2011), DNA-dependent protein kinase (DNA-PK) (Ferguson et al., 2012), and heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) (Wang et al., 2019b), also mediate downstream signaling through STING.



**Figure 33. The cGAS-STING pathway.** (From Cheng et al., 2020)

Upon binding to CDNs, STING undergoes a conformational change, resulting in the release and polymerization of the C-terminal tail (CTT) (Shang et al., 2019; Wang et al., 2014). The polymerized STING is then transferred from ER to Golgi via ER-Golgi intermediate compartment (ERGIC), where STING initiates the autophagic process, which helps clear cell membrane DNA and pathogens (Dobbs et al., 2015; Gui et al., 2019; Srikanth et al., 2019). During translocation, STING also recruits TANK-binding kinase 1 (TBK1) (Zhang et al., 2019; Zhao et al., 2019). The latter undergoes transphosphorylation and then phosphorylates the CTT of STING, followed by IRF3 phosphorylation as well (Liu et al., 2015). In addition to IRF3, TBK1 also activates NF- $\kappa$ B and STAT6 (Chen et al., 2011; Stempel et al., 2019). These activated transcription factors translocate to the nucleus and induce the expression of various immunomodulatory genes, such as IFN $\beta$  and IL-6, leading to the establishment of an anti-pathogen state [Figure 33] (Cheng et al., 2020). With respect to bacteria, activation of STING leads to IFN $\beta$  production that can be beneficial or detrimental to the host, making STING a

friend or foe of bacterial pathogens, depending on the infection (Marinho et al., 2017). After the translocation process, STING can also be targeted to lysosomes for degradation to avoid excessive immunity (Gonugunta et al., 2017; Prabakaran et al., 2018).

*C. trachomatis* is the first Gram-negative bacteria, identified to produce c-di-AMP (Barker et al., 2013). During late stages of chlamydial development, the concentration of bacterial c-di-AMP increases, when replicating RBs differentiate into infectious EBs. Consistent with this phenomenon, c-di-AMP levels in infected cells correlated with the increased production of IFN $\beta$ . Primary mouse lung fibroblasts can produce IFN $\beta$  in response to *C. trachomatis* or c-di-AMP stimulation, while HEK293T cells cannot induce IFN $\beta$  production due to the absence of STING. HEK293T cells lacking cGAS and ectopically expressing STING can produce IFN $\beta$  after c-di-AMP stimulation or chlamydial infection. This evidence suggest that CDNs, potentially secreted by chlamydial T3SS, directly interact with STING and trigger downstream signaling processes. Another possibility is that chlamydial dsDNA is directly recognized by host cGAS outside of the inclusion, and subsequently produces cGAMP to activate STING (Zhang et al., 2014). On the other hand, inactivation of the inclusion membrane protein *Chlamydia* promoter of survival (CpoS) induces rapid apoptosis and necrosis of infected cells (Sixt et al., 2017). This CpoS-null *Chlamydia* induces an exacerbated IFN-I response, that requires the participation of the host cGAS/STING/TBK1/IRF3 signaling pathway. Deficient of STING, but not cGAS or IRF3, attenuates cell death, indicating that STING mediates *Chlamydia*-induced cell death, whereas cell death and IFN responses are independent.

### **3.4 Other innate immune cells**

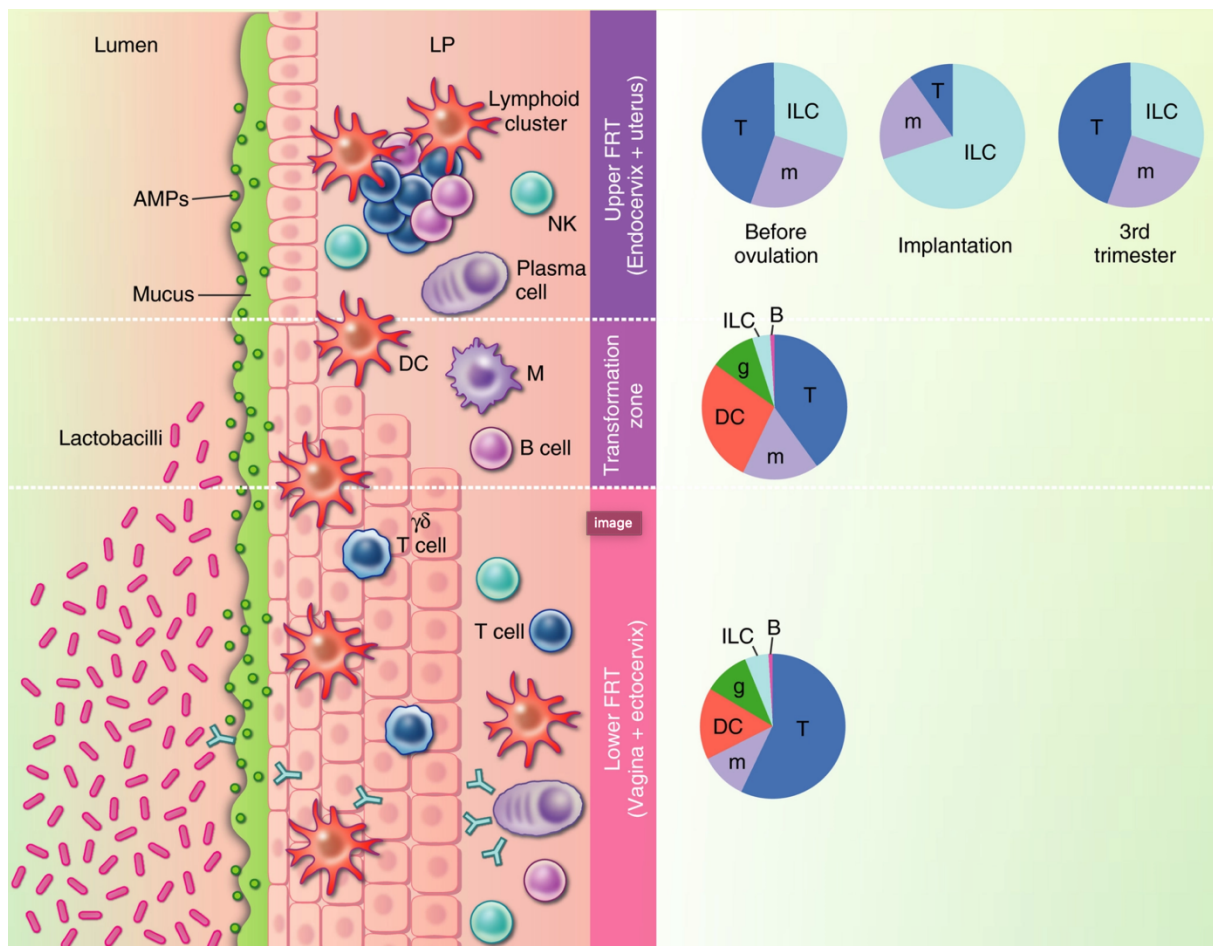
#### **3.4.1 Monocytes and macrophages**

Monocytes or macrophages are present in all tissues and play a role in maintaining tissue balance and in responding to infectious and non-infectious threats by detecting, taking up, and eliminating dead cells, foreign bodies, and other debris (Gordon and Plüddemann, 2017). They are a heterogeneous population that display remarkable plasticity, and their phenotype and function depend largely on the microenvironment of the local tissue (Ning et al., 2016). Macrophages are usually divided into two groups: M1 (stimulated by IFN $\gamma$  or ligands of TLRs, such as LPS) usually associated with a proinflammatory response, and M2 (stimulated by IL-4/IL-13) associated with an anti-inflammatory response (Sica and Mantovani, 2012).

Monocytes and macrophages circulate throughout the FGT and mediate immune recognition and elimination of microorganisms by phagocytosis. These cells comprised 10% of all leukocytes in the FGT and are most numerous in the endometrial stroma and connective tissue but are associated with the menstrual cycle. However, the number of vaginal macrophages remains unchanged and is not affected by hormonal changes [Figure 34] (Monin et al., 2020).

In addition to the brief introduction of the function of macrophages during *C. trachomatis* infection in ‘Part II 2.2.4 Human induced pluripotent stem cells (iPSdMs)’, more details are provided in this chapter. The FGT mucosa contains tissue-resident macrophages and monocytes

that come into contact with chlamydial EBs once released from infected epithelial cells after the completion of their developmental cycle (Agrawal et al., 2011). During early infection stage, epithelial cells secrete some chemokines and pro-inflammatory cytokines, such as CCL2 and MIP-1 $\alpha$ , leading to local inflammation and recruitment of macrophages (Rank et al., 2010; Shi and Pamer, 2011). CD11b positive cells (monocytes/macrophages) are also infiltrated to the mucosa during *C. trachomatis* infection (Morrison and Morrison, 2000). Thus, both resident macrophages and monocyte-derived macrophages recruited from the blood are involved in *C. trachomatis* infection in the FGT mucosa.



**Figure 34. Immune and non-immune barriers in the female reproductive tract.** (From Monin et al., 2020)

The main role of monocytes and macrophages in antimicrobial immunity is achieved by phagocytosis and secretion of proinflammatory cytokines. Indeed, *C. trachomatis* is rapidly degraded in macrophages (Sun et al., 2012). To evade macrophage killing, *C. trachomatis* can induce a persistent state after entering macrophages, within which the bacteria are viable and metabolically active, but do not replicate (Beagley et al., 2009; Datta et al., 2014). This restricted growth pattern is mediated by several mechanisms, including lysosomal trafficking (Al-Zeer et al., 2013; Sun et al., 2012), interaction of perforin-2 (Fields et al., 2013; Johnson and Kerr, 2015), induction of reactive oxygen and nitrogen species (Marangoni et al., 2014), and nutrient starvation (Krausse-Opatz et al., 2007; Lausen et al., 2019).



Antigen presenting cells (APCs) is characterized by their ability to capture, process, and present exogenous antigens to T cells, usually recognized by their constitutive expression of major histocompatibility complex (MHC) II and stimulatory molecules (Gardner et al., 2020). As one of the main populations of APCs, monocytes, particularly macrophages, contain both MHC-I and MHC-II molecules, which give them the potential to induce adaptive immunity (Laupéze et al., 1999). During *C. trachomatis* infection, monocytes upregulate the expression of MHC-II and costimulatory receptors (Johnson and Kerr, 2015; Krausse-Opatz et al., 2007). In addition, *C. trachomatis* infected macrophages express and secrete IFN- $\gamma$  and IL-12, which drive CD4<sup>+</sup> T cells to differentiate towards Th<sub>1</sub> (Mpiga et al., 2006), suggesting *Chlamydia*-infected macrophages tend to direct adaptive responses toward Th<sub>1</sub> immunity. The latter has been shown to be the most predominant adaptive immune response to control and eliminate intracellular bacteria (Karunakaran et al., 2010). Reversely, the activated Th<sub>1</sub> cells secrete IFN- $\gamma$  and TNF- $\alpha$ , which enhance the bactericidal mechanism of macrophages, and inhibit the growth of *Chlamydia* in infected epithelial cells.

*C. trachomatis* infected monocytes may also drive Th<sub>2</sub>-mediated responses (Azenabor and York, 2010; Vicetti Miguel et al., 2013). Cell-mediated immunity against *Chlamydia* may also involve CD8<sup>+</sup> T cells (Nogueira et al., 2015). When activated, these cells differentiate into cytotoxic T cells, which possess efficient killing mechanisms against host cells infected with intracellular pathogens such as *C. trachomatis*. However, the mechanisms involved in *Chlamydia*-induced CD8<sup>+</sup> T cell activation have not been elucidated.

### 3.4.2 Dendritic Cells (DCs)

DCs are the most powerful antigen-presenting cells in the immune system and tightly regulate their inner cellular system to efficiently process and present antigens to MHC molecules (Cabeza-Cabrerizo et al., 2021). Depending on their origin, DCs can be of the myeloid or plasmacytoid (pDCs) (Shortman and Liu, 2002). DCs are present in the subepithelial stromal layer of the endometrium and in the epithelial layer of the vagina ( Monin et al., 2020). Cervical DCs are slightly more numerous than peripheral blood DCs, which account for less than 1% of CD45<sup>+</sup> cells (Trifonova et al., 2014).

During chlamydial infection, DCs and macrophages in the sub-epithelium of the genital tract are observed, and DCs are known to phagocytose bacteria directly across the mucosal epithelial layer (Agrawal et al., 2009; M et al., 2001). Sherrid et al. demonstrate that bone marrow-derived DCs engulf the extrusion, and that encapsulation in the extrusion prolongs the survival of *Chlamydia* in these cells (Sherrid and Hybiske, 2017). A similar phenomenon is observed in macrophages (Zuck et al., 2017). However, extrusion engulfment results in distinct host cell outcomes for DCs and macrophages. The uptake of extrudates by DCs specifically triggers rapid apoptosis, which is inhibited by blocking the activation of caspase-3 and caspase-7. Absorption of the same dose of free *Chlamydia*, on the other hand, had no such effect in macrophages (Sherrid and Hybiske, 2017). Containment in the extrudate also markedly alters

the transcriptional upregulation of biologically relevant cytokines in DC in response to *Chlamydia*.

### 3.4.3 Natural killer (NK) cells

NK cells comprise 10-15% of circulating lymphocytes in the blood (Vivier et al., 2008). NK cells can be divided into NK1 and NK2 according to a cytokine profile, which is similar to Th1/Th2 differentiation of CD4<sup>+</sup> T helper cells. In the presence of IL-12, NK cells produce large amounts of IFN- $\gamma$  upon maturation, whereas NK cells produce IL-5 and IL-13 in the presence of IL-4. The former is defined as NK1 and the latter as NK2 (Peritt et al., 1998). Not only playing a protective role in innate immune responses, limiting viral and bacterial infection before the initiation of adaptive immune responses, NK cells also affect macrophages, DCs and neutrophils, and confer their own regulatory function by influencing subsequent antigen-specific T and B cell responses (Abel et al., 2018; Van Erp et al., 2019; Shegarfi et al., 2009). NK cells can eliminate infected or compressed cells through various effector pathways, including IFN- $\gamma$ , TNF $\alpha$ , cytotoxic granules containing perforin and granzymes, FasL, and TRAIL (Vasilevsky et al., 2014).

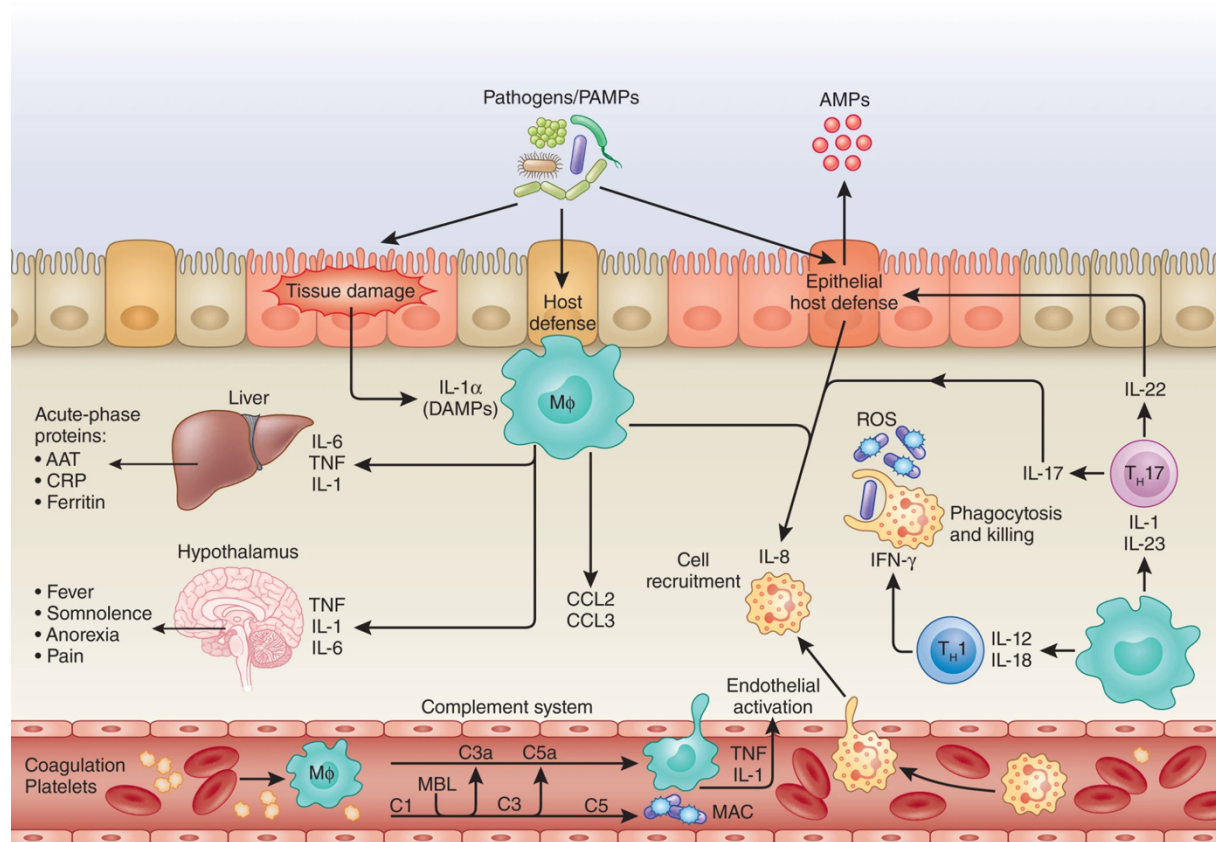
NK cells comprise 2.7% of the total cervical immune CD45<sup>+</sup> cells (Lee et al., 2015). NK cells are an important component of the innate immune response, and can help control chlamydial infection by secreting IFN- $\gamma$ . The activated NK cells can drive CD4<sup>+</sup> T cells towards IFN $\gamma$ -producing Th1 cells, which are key to chlamydial control (Tseng and Rank, 1998). Human epithelial cells and DCs stimulated by *C. trachomatis* produce interleukin-18 (IL-18) and IL-12, respectively, and these cytokines induce NK cells to secrete IFN- $\gamma$  *in vitro* (Hook et al., 2005). In addition, it has been shown that human NK cells are able to lyse epithelial cells infected with *C. trachomatis* (Hook et al., 2004). Scar trachoma is also found to have NK and undetermined CD45<sup>-</sup> cell infiltrates by immunohistochemical analysis, which may play a role in progressive scar (Hu et al., 2016). In the mouse model, depletion of NK cells shows reduced type 1 T cell recall responses, increased IL-4 production by CD4<sup>+</sup> T cells, and markedly increased Tregs response during *C. muridarum* infection. Furthermore, these NK cell-depleted mice also show more bacterial burden and more severe inflammatory pathological changes than control mice. These findings suggest that NK cells contribute to protective memory T cell-associated immunity in chlamydial reinfection by modulating the cytokine pattern of T cells and suppressing Tregs response (Wang et al., 2020a).

## 3.5 Inflammation

Inflammation is a major component of the innate immune system. Its main purpose is to alert, recruit, and activate cells of the immune system, to mobilize the adaptive immune system, to clear infectious pathogens or other pro-inflammatory stimuli, and to ultimately repair tissue damage caused by inflammatory triggers and inflammatory processes (Medzhitov, 2010). Upon PAMPs or DAMPs binding to PRRs, the signal transduction by these receptors enables cells to mobilize a pro-inflammatory cytokines and chemokines genes expression program (Hu and

Shu, 2018; O'Neill, 2008). As a result, the antibacterial mechanism is activated and immune mediators are released, preparing the surrounding tissue for inflammation, leading to the influx of leukocytes from the blood and allowing the recruited cells to adopt an immune activation state (Platanitis and Decker, 2018). But, when released in large quantities, the cytokines are detrimental to the host, such as inducing acute phase proteins in the liver, platelet activation, fever, fatigue, anorexia, and even capillary leak, vasodilatation, and hypotension (Dinarello, 2007).

Cytokine production during innate immunity mobilizes many defense mechanisms throughout the body, but also activates local cellular responses to infection or injury. For instance, IFN- $\gamma$  from Th1 cells activates neutrophils, while the cytokine IL-22 from IL-17-producing helper T cells (Th17 cells) and innate lymphocytes acts on epithelial cells and subsequently stimulates the production and release of AMPs, including defensin (Artis and Spits, 2015; Eyerich et al., 2017). In the blood, activated monocytes and neutrophils also release cytokines, which in turn stimulate the release of prostaglandins that mediate the symptoms and signs of disease (lethargy, fatigue, and fever) (Shattuck and Muehlenbein, 2015). Another important aspect of circulating inflammatory mediators is to activate the complement system, which mediates the killing of microorganisms and produces inflammatory peptides such as C3a and C5a [Figure 35](Netea et al., 2017; Ward, 2010).



**Figure 35. The immunological mechanisms leading to the induction of inflammation during the first stages of host defense against invading pathogens.** (From Netea et al., 2017)

The key inflammatory cytokines released in the early response to bacterial infection are TNF, IL-1 and IL-6. The IL-1 family, such as IL-1 $\beta$ , powerfully induces the expression of adhesion molecules in endothelial cells and promotes the recruitment of neutrophils to the sites of inflammation, as well as monocytes. IL-1 $\beta$  also has a powerful stimulatory effect on phagocytosis, produces chemotaxis on leukocytes, and induces the production of other types of inflammatory mediators as well as other cytokines (Biondo et al., 2014). TNF- $\alpha$  plays an important role by recruiting neutrophils and macrophages, in addition to inducing the expression of pro-inflammatory mediators to the site of infection (Malaviya and Abraham, 2000).

However, uncontrolled inflammation is a key factor in the pathogenesis of countless chronic diseases, including rheumatoid arthritis, systemic lupus erythematosus, and chronic granulomatous disease, resulting in greatly increased sensitivity to severe bacterial and fungal infections, as well as tissue lesion damage (Feehan and Gilroy, 2019; Furman et al., 2019).

Upon *C. trachomatis* infection, cervical epithelial cells can produce several proinflammatory cytokines, including TNF $\alpha$ , IL-1, IL-6, and IL-8, which enhance the inflammatory response of cells, thereby causing direct damage to genital tissues. In addition, IL-8 in turn recruits innate immune cells that are abundant in the genital mucosa and able to further deteriorate chronic inflammation and tissue damage in the reproductive system (Redgrove and McLaughlin, 2014)

It has been shown that lack of TNF- $\alpha$  or application of TNF- $\alpha$  inhibitors has no significant effect on chlamydial clearance *in vivo*, suggesting that TNF- $\alpha$  may not be necessary for chlamydial clearance (Kamalakaran et al., 2013). Several studies have shown that TNF- $\alpha$  is associated with the formation of lesions caused by *Chlamydia* infection. First, TNF- $\alpha$  can not only upregulate adhesion molecules and help leukocytes penetrate into inflammatory sites, but also promote the expression of cytokines such as IL-6 and IL-8, which are associated with tissue necrosis and scar formation (Shen et al., 2019; Zhao et al., 2015). Second, TNF- $\alpha$  accelerates the release of collagenase by stimulating the proliferation of tissue fibroblasts, which leads to histopathological damage. Third, deletion of the specific receptor for TNF- $\alpha$  (TNF- $\alpha$ R), which is essential to mediate its biological function, adding TNF- $\alpha$  antibody, or knockout of TNF- $\alpha$  gene, can significantly reduce the severity of hydrosalpinx induced by chlamydial infection in mice, indicating that TNF- $\alpha$  plays an important role in *Chlamydia*-induced pathological injury (Manam et al., 2015; Zafiratos et al., 2019). Thus, TNF- $\alpha$  is not essential for chlamydial clearance, but it can mediate immunopathological damage caused by *Chlamydia*.

Elevated levels of IL-1, IL-6, and IL-8 can be detected in the cervix of women infected with *C. trachomatis* (Marconi et al., 2014). The IL-1 family may have important functions in immune response and acute/chronic inflammation following chlamydial infection (He et al., 2010; Hvid et al., 2007). IL-1 $\alpha$  can influence IL-8 secretion and maturation during chlamydial infection (Cheng et al., 2008a). Through the activity of IL-8, IL-1 $\alpha$  may play a role in *Chlamydia*-induced inflammation and tissue lesions (Buchholz and Stephens, 2006; Jha et al., 2011). IL-1 $\beta$  assists the host in defense against chlamydial infection by inducing a wide range of inflammatory

cytokines and chemokines, such as IL-6 and IL-8 (Nagarajan et al., 2012; Shimada et al., 2011). In addition, IL-1 $\beta$  is also thought to be associated with exacerbations of lesions in the upper genital tract of mice, during infection with *C. muridarum* (Prantner et al., 2009).

Given that IL-6 null mice are more susceptible to chlamydial infection than wild-type mice, elevated IL-6 levels were found to play a protective role in controlling chlamydial infection (Williams et al., 1998). In the innate immune response, IL-6 induces recruitment of leukocytes and promotes apoptosis of neutrophils, mediating inflammation to control infection (Cunningham et al., 2013; Rose-John, 2018). In the adaptive immune responses, high expression of IL-6 activates Th1-like responses that clear pathogens by modulating IFN- $\gamma$  production, thereby reducing susceptibility to chlamydial infection (Sun et al., 2017). In addition, TNF- $\alpha$ , which is related to the degree of *Chlamydia*-induced tubal obstruction and maintenance of the persistent state of the disease, is inhibited by IL-6 in a dose-dependent manner (Zhao et al., 2015). In addition to its role in host defense, IL-6 also mediates inflammatory lesions. IL-6 is necessary to exacerbate hydrosalpinx caused by low-dose chlamydial infection (Sun et al., 2017). The reason may be that IL-6 increases the inflammatory infiltration of infected tissues, as well as specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells that produce TNF- $\alpha$  under low-dose chlamydial inoculation (Refaat et al., 2016). In conclusion, IL-6 promotes host defense against chlamydial infection by balancing inflammatory and immune responses, but it also exacerbates chlamydial pathogenicity by participating in inflammatory pathology.

IL-8 is a pro-inflammatory chemokine, sometimes referred to as chemokine (C-X-C patterning) ligand 8 or CXCL-8, encoded by the CXCL-8 gene (Bezzetti et al., 2011). It is involved in host defense by recruiting and regulating the activities of immune cells such as leukocytes, basophiles, neutrophils and T lymphocytes (Remick, 2005). IL-8 is produced by many different cell types and also activates the function of neutrophils (Li Jeon et al., 2002). IL-8 may be associated with acute or chronic inflammation following chlamydial infection. For instance, elevated IL-8 has been detected in culture supernatants of cervical and colonic epithelial cells infected with *Chlamydia* (Rasmussen et al., 1997), which is mediated by NF- $\kappa$ B and MAPK/ERK signaling pathways (Buchholz and Stephens, 2007), IL-10-mediated JAK/STAT signaling pathways (Du et al., 2018), or IL-1 $\alpha$ -mediated IL-1RI-independent mechanisms (Cheng et al., 2008b). The up-regulation trend of IL-8 is consistent with the unique developmental cycle of *Chlamydia* (Rasmussen et al., 1997), which might result from the promotion of the lipid metabolism of host cells by IL-8 to provide nutrients, thus enabling the growth and development of *Chlamydia* (Bas et al., 2010). This suggests that IL-8 is also necessary for chlamydial replication and the synthesis of its components. On the other hand, IL-8 produced during chlamydial infection reduces chlamydial sensitivity to azithromycin and other therapeutic agents, and azithromycin concentrations higher than therapeutic serum concentrations are required for effective inhibition of IL-8 (Käding et al., 2021; Matsuo et al., 2019). Another study even show that IL-8 plays a role in preventing chlamydial invasion, although it is ineffective in resolving chlamydial infection, (Rajeeve et al., 2018b).

## IV. Interferon induction during *C. trachomatis* infection

Interferon (IFN) was first discovered in 1957, as a strong interference of the influenza virus life cycle in chicken embryos (Andrewes and March, 1957). Since then, IFNs has been found to provide a strong first line of host innate immunity against pathogen infection. After detecting microorganism via PRRs, many types of immune and non-immune cells produce and secrete IFNs, which subsequently coordinate innate and adaptive immunity through multiple mechanisms (Chen et al., 2017). IFNs are a superfamily of cytokines that are now classified into three subtypes: type I, type II, and type III. All three types of IFNs trigger intracellular signaling cascades through Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathway, leading to different immune functions under biological and pathological conditions. The type III IFN family includes IFN $\lambda$ 1, IFN $\lambda$ 2, IFN $\lambda$ 3 (also known as IL-29, IL-28A, and IL-28B respectively) and IFN $\lambda$ 4 (O'Brien et al., 2014; Prokunina-Olsson et al., 2013), which function similarly to other cytokines of the IFN family but have limited activity because the expression of their receptors is mainly localized on the epithelial cell surface, and immune cells are largely unresponsive to IFN $\lambda$  (Durbin et al., 2013; Witte et al., 2010). So, I mainly introduce below type I and type II interferons, and their roles in the innate immunity to *C. trachomatis* infection.

### 4.1 Type I IFNs and its biological implications during *Chlamydia* infection

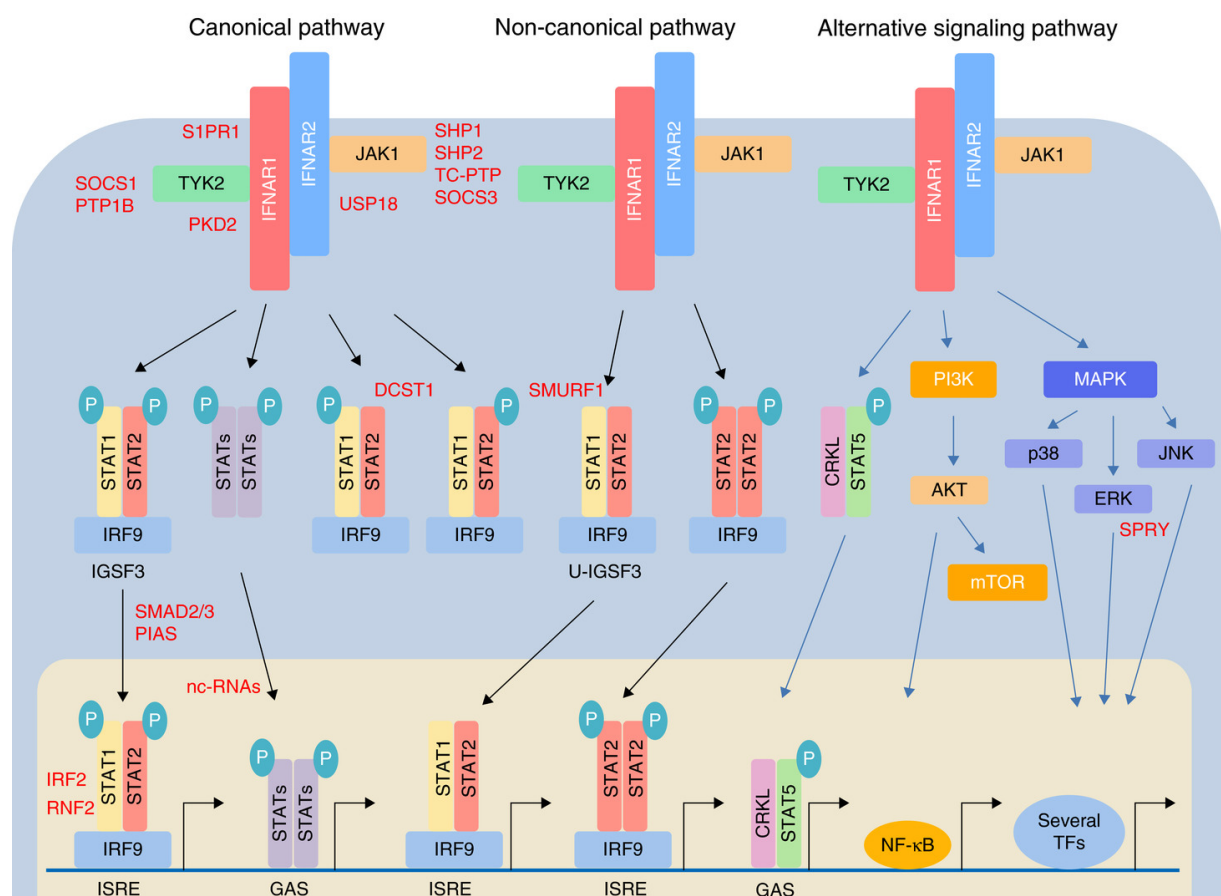
#### 4.1.1 General introduction of type I Interferons (IFN-I)

The IFN-I family is a multigenic cytokine family encoding 13 partially homologous IFN $\alpha$  subtypes in humans (14 in mice), individual IFN $\beta$  and several poorly defined single gene products (IFN $\epsilon$ , IFN $\tau$ , IFN $\kappa$ , IFN $\omega$ , IFN $\delta$  and IFN $\zeta$ ) (Hardy et al., 2004; Pestka et al., 2004). The genes encoding IFN-I clustered on the human 9p chromosome, whereas in mouse, the IFN-I locus is on chromosome 4. IFN-I are essential in host defense because of their role against pathogens and their ability to activate effector cells of innate and adaptive immune responses (Hervas-Stubbs et al., 2011). Indeed, their promoter contains acute response elements, e.g., interferon regulatory factor (IRF) and NF- $\kappa$ B in IFN $\beta$ , through which rapid induction of these IFN genes via the PRR pathway is ensured (Honda et al., 2006; Sato et al., 2000). Except being activated by PAMPs and DAMPs through PRRs (Akira et al., 2006), IFN-I can also be physiologically (not pathologically) induced by TNF (Yarilina and Ivashkiv, 2010), receptor activator of NF- $\kappa$ B ligand (RANKL) (Takayanagi et al., 2002), and M-CSF (Moore et al., 1985). The induced IFN-I can stimulate cellular immune response signaling in an autocrine and paracrine manner.

IFN-I activate subsequent signal through a co-receptor consisting of IFN $\alpha/\beta$  receptor 1 and 2 subunits (IFNAR1 and IFNAR2) (Rusinova et al., 2013). Ligation of IFNAR with IFN-I activates the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). In the canonical pathway of IFN $\alpha/\beta$ -mediated signaling, activated JAK1 and TYK2 phosphorylate STAT1 and STAT2 molecules present in the cytosol, resulting in

dimerization, nuclear translocation, and binding to IRF9 of these molecules to form the ISG factor 3 (ISGF3) complex. This complex then binds to the IFN-stimulated response element in the ISG promoter, leading to the activation of ISG transcription [Figure 36] (Arimoto et al., 2018; Ivashkiv and Donlin, 2014). In addition, there are non-canonical IFN-Is signaling pathways that are phosphorylated in a STAT1-independent manner. Phosphorylated STAT1 transiently increases and decreases over time after IFN stimulation. However, unphosphorylated STAT1 (U-STAT1) increases after IFN-Is treatment and then continues to remain high for several days. U-STAT1, U-STAT2 and IRF9 (U-ISGF3 complex) can also translocate to the nucleus, inducing different expression of ISGs, and prolonging the antiviral state (Cheon and Stark, 2009). Furthermore, IFN-Is can also activate downstream signaling through "alternative" signaling pathways, including CRK-like protein pathway, MAPK pathway, ERK pathway, JNK pathway, and mammalian target of rapamycin (mTOR) pathway (Cao et al., 2008; Du et al., 2007; Plataniias, 2005). These STAT1-independent pathways are thought to be critical for optimal transcriptional activation and mRNA translation of ISGs, as well as various biological activities that ultimately produce IFN.

IFN-Is also induce an antipathogenic state through the up-modulation of hundreds of interferon-stimulated IFN-stimulated genes (ISGs) (Schneider et al., 2014). In addition, they can also recruit monocytes to the site of infection and inhibit pathogenic replication by enhancing the expression of the chemokine CCL2 (Sadler and Williams, 2008).



**Figure 36. Regulation of type I IFN response.** Red letters indicates negative regulators. (From Arimoto et al., 2018)

IFN $\epsilon$  signals through IFNAR1 and IFNAR2 receptors as IFN $\alpha/\beta$  to induce IFN-regulated genes, and it is characterized as type I IFN, although it shares only 30% amino acid identity with IFN $\alpha/\beta$  (Fung et al., 2013; Marks et al., 2019). Expression of the IFN $\epsilon$  gene is not induced upon activation of Toll-like receptors, nor by IRF3, IRF5, or IRF7 (Fung et al., 2013), whereas its expression is regulated by estrogen, seminal plasma (Abdulhaqq et al., 2016; Sharkey et al., 2007), several proinflammatory cytokines, and in particular TNF $\alpha$  that has the strongest response to induce IFN $\epsilon$  in A2EN cells and primary cervical epithelial cells (Courret et al., 2017). In mucosal tissues including female genital mucosa, expression of IFN $\alpha/\beta$  is nearly undetectable and IFN $\epsilon$  is a unique and functionally distinct IFN-I (Hardy et al., 2004; Xi et al., 2012), which is expressed constitutively at the basal level in these tissues (Courret et al., 2017). The protective effect of IFN $\epsilon$  is shown as early as 3 days after the infection of mice FGT with *C. muridarum*. IFN $\epsilon$  is also important at later stages of pathogen clearance since IFN $\epsilon$ -null mice have not cleared the bacteria yet at 30 days post-infection, whereas wild type mice have cleared the bacteria completely (Fung et al., 2013). It has been reported that IFN $\epsilon$  protects host from microbial infection via regulating NK cell numbers in the uterus and activation status (Fung et al., 2013). Indeed, the characteristic of local uterine NK cells is different from those in the blood circulation, and is more cytotoxicity (Bulmer and Lash, 2015). Alternatively, uterine NKs have been demonstrated to be critical for defense against chlamydial infection, by producing IFN $\gamma$  and promoting Th1 cell responses (Cumming and Bourke, 2019; Tseng and Rank, 1998).

#### 4.1.2 Induction of host IFN-Is by some bacteria

Almost all cells in the body can produce IFN $\alpha/\beta$ , termed as ‘classical IFNs’, while macrophages and DCs produce predominantly IFN $\beta$ , and hematopoietic cells and pDCs produce predominantly IFN $\alpha$ . In most IFN-Is *in vitro* studies, in addition to their antiviral effects, IFN-Is also have anti-bacterial properties. For example, IFN-Is limit the infection and replication of several intracellular bacteria, including *B. anthracis* (Walberg et al., 2008), *L. pneumophila* (Naujoks et al., 2016), *H. pylori* (Watanabe et al., 2010), *S. pyogenes* (Castiglia et al., 2016), *C. trachomatis* (Ishihara et al., 2005), and *M. tuberculosis* (Ranjbar et al., 2015). IFN $\alpha/\beta$  can protect macrophages and lung epithelial cells from *L. pneumophila* infection *in vitro*, the causative agent of Legionnaires' disease (Monroe et al., 2009). In addition to promoting restriction of bacterial growth and intracellular bacterial killing, IFN $\alpha/\beta$  may prevent or reduce cellular invasion by invasive intestinal bacteria, such as *S. flexneri* and *S. enterica* subspecies Typhimurium (Helbig et al., 2019; Robinson et al., 2012). Treatment with recombinant IFN $\alpha/\beta$  also increases survival and reduced invasion of intestinal epithelial cells *in vivo*, as well as fibroblast invasion *in vitro* in mice infected with *S. flexneri* or *S. typhimurium* (Bukholm et al., 1984; Niesel et al., 1986). In immune responses to group *B. streptococci*, *E. coli*, and *S. pneumoniae*, IFN $\alpha/\beta$ -mediated signaling contributes to optimal activation of macrophages, in terms of their ability to produce TNF and nitric oxide (Long et al., 2014; Mancuso et al., 2007). IFN $\alpha/\beta$  also contribute to the production of host protective cytokines during *S. typhimurium* infection, inducing strong IFN $\gamma$  production in a manner unrelated to IL-12 (Freudenberg et al., 2002). IFN $\alpha/\beta$ -mediated signaling downstream of NOD1 signaling has been shown to have a



role in protecting intestinal epithelial cells from *H. pylori* infection (Watanabe et al., 2010). Therefore, the induction of cell-intrinsic immunity to kill bacteria or prevent their invasion, as well as the modulation of chemokines, inflammatory cytokines, and phagocytes, are considered the mechanisms by which IFN $\alpha/\beta$  inhibits bacterial infection, the exact mechanisms involved being pathogen dependent (McNab et al., 2015).

However, the role of IFN-Is *in vivo* is more complex, and IFN-Is can lead to beneficial or harmful outcomes to the host, depending on the pathogen and host context. Two of the best examples of the deleterious effects of IFN $\alpha/\beta$  on the host are in infections with *Listeria monocytogenes* and *Mycobacterium tuberculosis*. Animals deficient in IFNAR1 have a long survival time after infection with *L. monocytogenes*, and the load of bacteria in the spleen and liver after infection is lower than in wild-type mice (Auerbuch et al., 2004; Dussurget et al., 2014). Later study reveals that IFNAR1 is essential for *L. monocytogenes* dissemination within infected foci in the livers of infected mice, and for efficient cell-to-cell transmission in macrophages *in vitro*. This IFNAR1 molecule can promote ActA, a cell surface bound virulence protein of *L. monocytogenes*, polarization and actin-based motility in the cytoplasm of host cells (Osborne et al., 2017).

Transcriptome studies in patients with active disease and latently infected individuals or healthy individuals in the United Kingdom and South Africa have revealed for the first time the role of IFN-Is in the pathogenesis of human tuberculosis. Patients with active pulmonary tuberculosis have a prominent IFN $\alpha/\beta$ -induced transcriptional profile that correlates with the extent of lung radiographic disease and diminish with successful treatment (Berry et al., 2010). Integration and meta-analysis of various human tuberculosis datasets confirm the reproducibility of IFN-Is-inducible blood transcriptional signatures in human tuberculosis (Joosten et al., 2013; Singhania et al., 2018). Analysis of purified cells from the blood of patients with active disease reveal that IFN-inducible genes are over-expressed in neutrophils and monocytes, but not in CD4<sup>+</sup> or CD8<sup>+</sup> T cells, suggesting that over-activation of monocytes and neutrophils by IFN-Is during *M. tuberculosis* infection may contribute to disease pathogenesis (Berry et al., 2010; Bloom et al., 2013). Studies on infection with highly toxic *M. tuberculosis* strains in the mouse models have shown a correlation between increased IFN $\alpha/\beta$  levels and increased virulence (Manca et al., 2001, 2005; Ordway et al., 2007). However, the mechanisms mediating IFN $\alpha/\beta$ -driven disease exacerbation are not fully understood but appear to be multifactorial. Survey data on highly toxic *M. tuberculosis* strains initially suggest that suppression of pro-inflammatory cytokines and Th1-type immunity is important. Some evidence from both human cells and mouse models that show that IFN $\alpha/\beta$  inhibit host protective cytokines production after *M. tuberculosis* infection. As an example, the production of IL-1 $\alpha$  and IL-1 $\beta$  is essential for host defense against *M. tuberculosis* (Mayer-Barber et al., 2010), but both cytokines are inhibited by IFN $\alpha/\beta$  either in *in vitro* infected human or in mouse cells and mouse models (Mayer-Barber et al., 2011; Novikov et al., 2011; de Paus et al., 2013). In addition, cell-intrinsic IFN-Is signals have been shown to negatively regulate iNOS production in lung myeloid cells, particularly TNF/iNOS-producing dendritic cells (TIP-DCs) (Mayer-Barber et al., 2011). TPL-

2-ERK1/2 signaling also promotes host resistance against intracellular bacterial infection by negative regulation of IFN-I production. The production of other pro-inflammatory cytokines such as TNF and IL-12 is also negatively affected by IFN-Is (McNab et al., 2013, 2014; de Paus et al., 2013), while the IL-12/IFN- $\gamma$  axis is an important protection mechanism against *M. tuberculosis* infection. Among them, IL-12 is produced by APCs at the early stage of infection and promotes the differentiation of CD4<sup>+</sup> Th1 cells and the production of IFN- $\gamma$ . IFN- $\gamma$  further activates macrophages to produce TNF- $\alpha$  and other protective cytokines, which promote intracellular killing of pathogens by producing reactive oxygen and nitrogen species (Lúcia Moreira-Teixeira et al., 2018; Ravesloot-Chavez et al., 2021).

#### 4.1.3 Induction of IFN-I by *C. trachomatis*

Both human and murine cells induce IFN-Is responses following chlamydial infection (Finethy and Coers, 2016b; Malireddi and Kanneganti, 2013). However, the pathway by which IFN is induced appears to be cell type specific. In murine oviduct epithelium, the *Chlamydia*-induced IFN $\beta$  production shows a unique biphasic expression pattern, and TLR3-dependent pathway is involved in the early stage of infection, while STING-dependent pathway is participated in IFN $\beta$  production in the late stage of *Chlamydia* infection (Hu et al., 2015). Interestingly, unknown chlamydial PAMPs may exist in stimulating TLR3-dependent responses during chlamydial infection since no known dsRNA has been reported from *Chlamydia*. However, *Chlamydia*-induced IFN $\beta$  production by macrophages from mice lacking TLR3, TRIF, TLR7, or TLR9 is not significantly different with that from wild-type animal (Prantner et al., 2010). At the same time, many studies have shown that STING can sense chlamydial infection and induce IFN $\beta$  production in human cells, while the involvement of TLR3 in the response of human cells to *Chlamydia* is rarely reported (Prantner et al., 2010; Zhang et al., 2014). Thus, whether the pathways of IFN-I induction differ in species or tissues needs to be further investigated.

On the other hand, the role of IFN-Is in chlamydial infection could be friend or foe. STING-null mice show a high incidence of chlamydial hydrosalpinx than wild type animal, indicating that the STING pathway is activated during chlamydial infection in animals, and IFN-Is produced by STING dependent pathway have a protective function in host innate immunity (Zhang et al., 2017). In contrast, during *C. muridarum* infection, IFN-Is enhance host susceptibility to the infection (Qiu et al., 2008). In addition, compared to wildtype mice, interferon- $\alpha/\beta$  receptor (IFNAR)-null mice (IFNAR<sup>-/-</sup>) that do not respond to IFN-Is also show significantly reduced bacterial loads, milder pathology, and earlier bacterial clearance. Furthermore, IFN-Is is also shown to exacerbate genital infection with *C. muridarum* by inhibiting *Chlamydia*-specific CD4<sup>+</sup> T cell responses (Nagarajan et al., 2008). These studies also suggest that IFN-Is are deleterious to the host cells during chlamydial infection.

## **4.2 The role of tryptophan-indole-IFN $\gamma$ axis in the innate resistance to infection with *C. trachomatis***

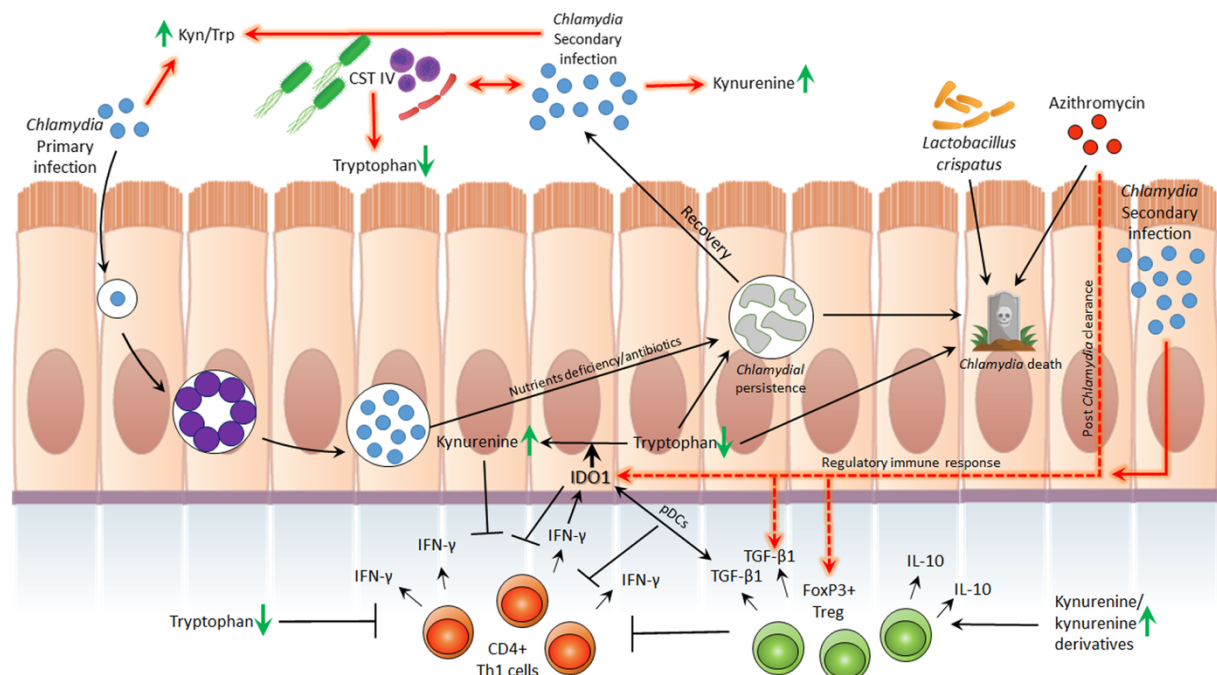
The type II IFN family includes a single gene product, IFN $\gamma$ , which is mainly produced by T cells and NK cells, and can act on a wide range of cell types that express IFN $\gamma$  receptors (IFN $\gamma$ R). It has been shown that IFN $\gamma$  plays an important role in *Chlamydia* clearance against acute infection and in the establishment of persistence. In human epithelial cells, IFN $\gamma$  inhibits *Chlamydia* growth by depletion of tryptophan pool, since tryptophan is an essential amino acid required for normal development in *Chlamydia*, leading to bacterial clearance or entering persistent form named as aberrant bodies. Besides its effect on tryptophan, IFN $\gamma$  also has effect on the inducible NO synthase (iNOS), on the secretion of chemokines and adhesion molecules or on the regulation of T-cell activities (Ramsey et al., 2001).

Several animal studies have shown that host primary defense against *C. trachomatis* is mediated by IFN $\gamma$  secreted by NK cells and T cells in the infectious microenvironment (Gondek et al., 2009; Tseng and Rank, 1998). IFN $\gamma$  induces the expression of indoleamine-2,3-dioxygenase 1 (IDO1), an enzyme that catabolizes tryptophan to N-formylkynurenine via cleavage of the indole ring (Feng and Taylor, 1989; MacChiarulo et al., 2009; Taylor and Feng, 1991). *C. trachomatis* cannot recycle kynurenines, unlike some other chlamydial species (Wood et al., 2004). By depleting tryptophan, activation of IDO1 curbs the growth of *C. trachomatis*, which is a tryptophan auxotrophy (Fehlner-Gardiner et al., 2002). Under conditions of limited tryptophan supply, the life cycle of chlamydial deviates from normal development to persistence (Beatty et al., 1993, 1994). It has been shown that high IFN- $\gamma$  levels are shown to eradicate infection, while lower levels can prompt urogenital biovars of *C. trachomatis* into their persistent form. Aberrant bodies of *C. trachomatis* have also been found in infected women, which are associated with low IFN- $\gamma$  levels in the genital tract.

The primary regulatory response of *C. trachomatis* to tryptophan starvation is mediated by the tryptophan repressor (TrpR) homolog, which binds tryptophan-dependently to cognate promoter elements and represses transcription (Akers et al., 2011; Carlson et al., 2006). In many Gram-negative bacteria, such as *E. coli*, *trpR* is monocistronic and distal to the tryptophan biosynthetic operator. In *C. trachomatis*, TrpR is encoded in an operator *trpRBA* that also contains tryptophan synthase  $\alpha$ - and  $\beta$ -subunits (TrpA and TrpB, respectively) and possesses an intergenic region (IGR) of 348 base pairs that separates TrpR from *trpBA*. TrpBA catalyzes the final step of tryptophan biosynthesis in bacteria: TrpA converts indolyglycerol-3-phosphate (IGP) to indole, which is then condensed with serine by TrpB to form tryptophan. In *C. trachomatis*, TrpA cannot bind to IGP, so *C. trachomatis* requires indole as a substrate to synthesize tryptophan (Fehlner-Gardiner et al., 2002). Genome-wide analysis has shown that *C. trachomatis* from the genital tract biovar can be distinguished from trachoma biovar by specific mutations in the *trpBA* operon (Fehlner-Gardiner et al., 2002). In the genital tract biovar, the *trpBA* operon is intact and encodes a functional tryptophan synthase, while the *trpBA* operon in trachoma biovar contains polymorphisms, which lead to a non-functional tryptophan synthase (Fehlner-Gardiner et al., 2002). There is a strong selective pressure to maintain a

functional tryptophan synthase in genital *C. trachomatis* biovars so that the bacterium is able to synthesize tryptophan from indole, which is provided by the genital tract microbiota (Caldwell et al., 2003).

The IDO1-IFN- $\gamma$  axis is also responsible for downregulating the host's pro-inflammatory response (Babcock and Carlin, 2000; Munn et al., 2005). It has been shown that tryptophan is required for T cell survival and proliferation, and depletion of tryptophan leads to its arrest in the G1 phase of the cell cycle (Munn et al., 1999). Tryptophan depletion also inhibits T cell proliferation through inactivation of the mTOR pathway (Metz et al., 2012). Breakdown of tryptophan into 3-hydroxyphthalic acid or quinolinic acid also can induce T cell apoptosis (Fallarino et al., 2002). Other kynurenine derivatives and kynurenine itself can induce the differentiation of naive T cells into regulatory T cells (Tregs) by activating AhR (Fallarino et al., 2006; Mezrich et al., 2010). IDO1 is inducible in different immune cells as well as mucosal epithelial cells and is regulated by a complex immune signaling cascade (MacKenzie et al., 2007). In addition to IFN- $\gamma$  stimulation, other cytokines can also induce the expression of IDO1, such as IFN- $\alpha$ , IFN- $\beta$ , TGF- $\beta$ , TNF- $\alpha$ , and IL-1 $\beta$  (Babcock and Carlin, 2000; Pallotta et al., 2011). LPS from Gram-negative bacteria is shown to induce IDO1 expression in IFN- $\gamma$ -KO mice (Fujigaki et al., 2001), which is mainly dependent on TNF- $\alpha$ . Furthermore, IDO1 can act as a signaling transducer that contributes to the response of pDCs to the long-term tolerogenic phenotype of the immunomodulatory cytokine TGF- $\beta$  (Pallotta et al., 2011). Ziklo reports that the expression of IDO1 is markedly increased in response to *C. trachomatis* infection by the regulation of TGF- $\beta$ 1 and FoxP3, both *in vivo* and *in vitro*, while the level of IFN- $\gamma$  is not increased. They demonstrate that TGF- $\beta$ 1 produced by host cells stimulated by chlamydial reinfection can induce IDO1 expression independent of IFN- $\gamma$  [Figure 37] (Ziklo et al., 2019).



**Figure 37. Immunological, bacterial and biochemical factors that are associated with initial or repeated *C. trachomatis* infections in women. (From Ziklo et al., 2019)**

## HYPOTHESIS AND OBJECTIVES

Epithelial cells constitute the main sites of *Chlamydia* proliferation, and the first line of defense from the host. It is therefore essential to understand how these cells respond to *Chlamydia* infection and to which extent *Chlamydia* modulate this level of host defense. Cell lines, including HeLa, McCoy and A2EN cells, either of cancer origin or immortalized by the expression of viral proteins, have been widely used to address these questions. However, these cells, and especially those of tumoral origin, are different from normal somatic epithelial cells of the FGT. In this context, the objectives of my work were:

- To develop a simple protocol for isolating primary epithelial cells from ecto-cervical explants of patients undergoing hysterectomy.
- To examine the susceptibility of these primary epithelial cells and their inflammatory response to *C. trachomatis* infection and compare those with what happened in HeLa cells, a commonly used cervical epithelial cell line.

The anti-viral properties of IFNs, particularly the most studied IFN $\alpha$  and IFN $\beta$  of IFN-I family, have been well studied. However, the role of IFN-I in *C. trachomatis* infection is largely unknown. Recent retrospective studies showed that IFN-inducible protein 10 (IP10), an IFN-dependent protein, was associated with *Chlamydia* infection in patients with pelvic inflammatory disease, and with *Chlamydia* ascending infection in the FGT of women. Using primary epithelial cells, we demonstrated that the inflammatory response of epithelial cells to *Chlamydia* infection was enhanced in the presence of IFN $\beta$ , while IFN $\beta$  alone had no effect. Based on these observations, we postulated that IFN-I might exacerbate *Chlamydia*-induced inflammation in epithelial cells, resulting in hyperinflammation in the FGT and subsequent tissue damage in some women patients. To test this, the second part of my PhD studies aimed to:

- Measure the inflammatory response of primary epithelial cells and HeLa cells in the presence or absence of IFN-I upon *C. trachomatis* infection.
- Identify the key molecule or intermediate by which IFN-I enhances *Chlamydia*-induced inflammation.
- Elucidate the signaling cascades mediating the expression/activation of this key molecule by IFN-I.
- Decipher the mechanism by which this intermediate up-regulates the inflammation induced by *Chlamydia*.

# **RESULTS**

## **Part I. Primary ectocervical epithelial cells display lower permissivity to *Chlamydia trachomatis* than HeLa cells and a globally higher pro-inflammatory profile**

### **Article summary**

*Chlamydia* are obligate intracellular bacteria. Mucosal epithelial cells of the eye conjunctiva and the genital tract are the main targets of *Chlamydia* and their infection can lead to trachoma and STIs, respectively. HeLa cells, originally derived from a patient with cervical cancer, are widely used in the field. However, genomic rearrangements that occurred over several years of culture make these cells largely different from their original parental cancerous cells, and even more so from normal somatic epithelial cells. Therefore, we developed in this study a simple protocol for isolating primary ecto-cervical epithelial cells, from explants of patients undergoing hysterectomy. We measured the susceptibility and inflammatory response of these primary cells upon *C. trachomatis* infection and also used HeLa cells for comparison.

A single dispase II enzyme digestion allowed us to mechanically separate the mucosal epithelial layer from the explant. Trypsin/EDTA treatment of this mucosal epithelial layer dissociated epithelial cells, which were then expanded in keratinocytes serum-free media (K-SFM). A high purity of primary epithelial cells, characterized by the expression of E-cadherin and cytokeratin 14, were obtained using this method. We showed that these primary cells were less permissive to the standard laboratory strain of *C. trachomatis*, serovar L2, than HeLa cells, as well as with serovar D, a serovar commonly infecting the FGT. The distinctive culture medium used to propagate each cell type and the presence of serum in HeLa culture medium largely contributed to the differences between the two cellular backgrounds in respect to *C. trachomatis* infection. In addition, primary epithelial cells globally displayed a higher basal level of transcription of pro-inflammatory cytokine genes (IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$ , CSF, IP10 and CCL5), compared to HeLa cells. Upon *C. trachomatis* infection, the inflammatory responses were stronger in primary epithelial cells than in HeLa cells with both serovars L2 and D.

Overall, the primary epithelial cell model established in this study showed a different capacity to react to *Chlamydia* infection than the commonly used HeLa cells and can be considered as a more accurate model to investigate the *Chlamydia*-epithelia interactions in the future.



OPEN

## Primary ectocervical epithelial cells display lower permissivity to *Chlamydia trachomatis* than HeLa cells and a globally higher pro-inflammatory profile

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The tumoral origin and extensive passaging of HeLa cells, a most commonly used cervical epithelial cell line, raise concerns on their suitability to study the cell responses to infection. The present study was designed to isolate primary epithelial cells from human ectocervix explants and characterize their susceptibility to *C. trachomatis* infection. We achieved a high purity of isolation, assessed by the expression of E-cadherin and cytokeratin 14. The infectious progeny in these primary epithelial cells was lower than in HeLa cells. We showed that the difference in culture medium, and the addition of serum in HeLa cultures, accounted for a large part of these differences. However, all things considered the primary ectocervical epithelial cells remained less permissive than HeLa cells to *C. trachomatis* serovar L2 or D development. Finally, the basal level of transcription of genes coding for pro-inflammatory cytokines was globally higher in primary epithelial cells than in HeLa cells. Transcription of several pro-inflammatory genes was further induced by infection with *C. trachomatis* serovar L2 or serovar D. In conclusion, primary epithelial cells have a strong capacity to mount an inflammatory response to *Chlamydia* infection. Our simplified purification protocol from human explants should facilitate future studies to understand the contribution of this response to limiting the spread of the pathogen to the upper female genital tract.

Infections of the reproductive system are a major global health concern and have become more critical recently due to the increase in incidence of sexually transmitted infections (STIs) with more than 1 million new cases per day worldwide, among which *Chlamydia trachomatis* infection is predominant<sup>1</sup>. Due to its anatomy and hormonal regimen, the female genital tract (FGT) is two to three times more likely than the male's to be infected by certain microorganisms such as *Neisseria gonorrhoea* and *Chlamydia trachomatis*<sup>2,3</sup>. Infections by the latter are asymptomatic in most cases. However, *C. trachomatis* can ascend from the lower to the upper genital tract of females, leading to pelvic inflammatory diseases, tubal infertility, ectopic pregnancy and even cancer<sup>4</sup>.

The surface of the FGT is covered with tightly packed epithelial cells that, together with the mucus that covers the outer surface, constitute a physical barrier between the external environment and the internal tissues. The lower FGT, i.e., the vagina and the ectocervix, is made of multiple layers of stratified epithelia, while a single layer of columnar epithelia covers the different parts of the upper FGT, i.e., the endocervix, uterus, fallopian tubes and ovaries<sup>5</sup>. The epithelial cells in the FGT are exposed to a variety of "foreign" cells: microorganisms of the vaginal flora, sexually transmitted pathogens, sperm, and during pregnancies, they have to tolerate the

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presence of semi-allergic fetus<sup>6</sup>. Together with stromal cells and other antigen presenting cells, epithelial cells present antigens to activate adaptive immunity of the host<sup>7</sup>.

*Chlamydia trachomatis*, an obligate intracellular bacterium, resides and develops mainly within epithelial cells of the FGT. Thus, epithelial cells, especially in the lower FGT, are at the front line of host defense against *C. trachomatis* infection. A better understanding of their intrinsic ability to limit *C. trachomatis* infection is required, and would be complementary to the data obtained from animal models. For this purpose, HeLa cells, an epithelial cell line derived from a cervical cancer, have been widely used. However, HeLa cells are tumoral cells with many chromosomal aberrations that make them very different from normal cells<sup>8,9</sup>. Having been passaged for several dozens of generations they are even very different from the originally isolated cells. Therefore, HeLa cells are not an ideal cell model to study the properties of epithelial cells of the FGT, and normal somatic epithelial cells should be used instead. Primary epithelial cells isolated from the human cervix have been used by different groups<sup>10–12</sup>. They demonstrated, for instance, the expression of Toll-like receptors by these cells<sup>13</sup> and their capacity for antigen-presentation<sup>14</sup>. However, studies on their response to *Chlamydia* infection remain scarce<sup>10,15–19</sup>.

In the present study we isolated and expanded primary epithelial cells from cervical explants of donors undergoing hysterectomies, and compared the host inflammatory response to *C. trachomatis* infection of these primary epithelial cells to that of HeLa cells.

## Results

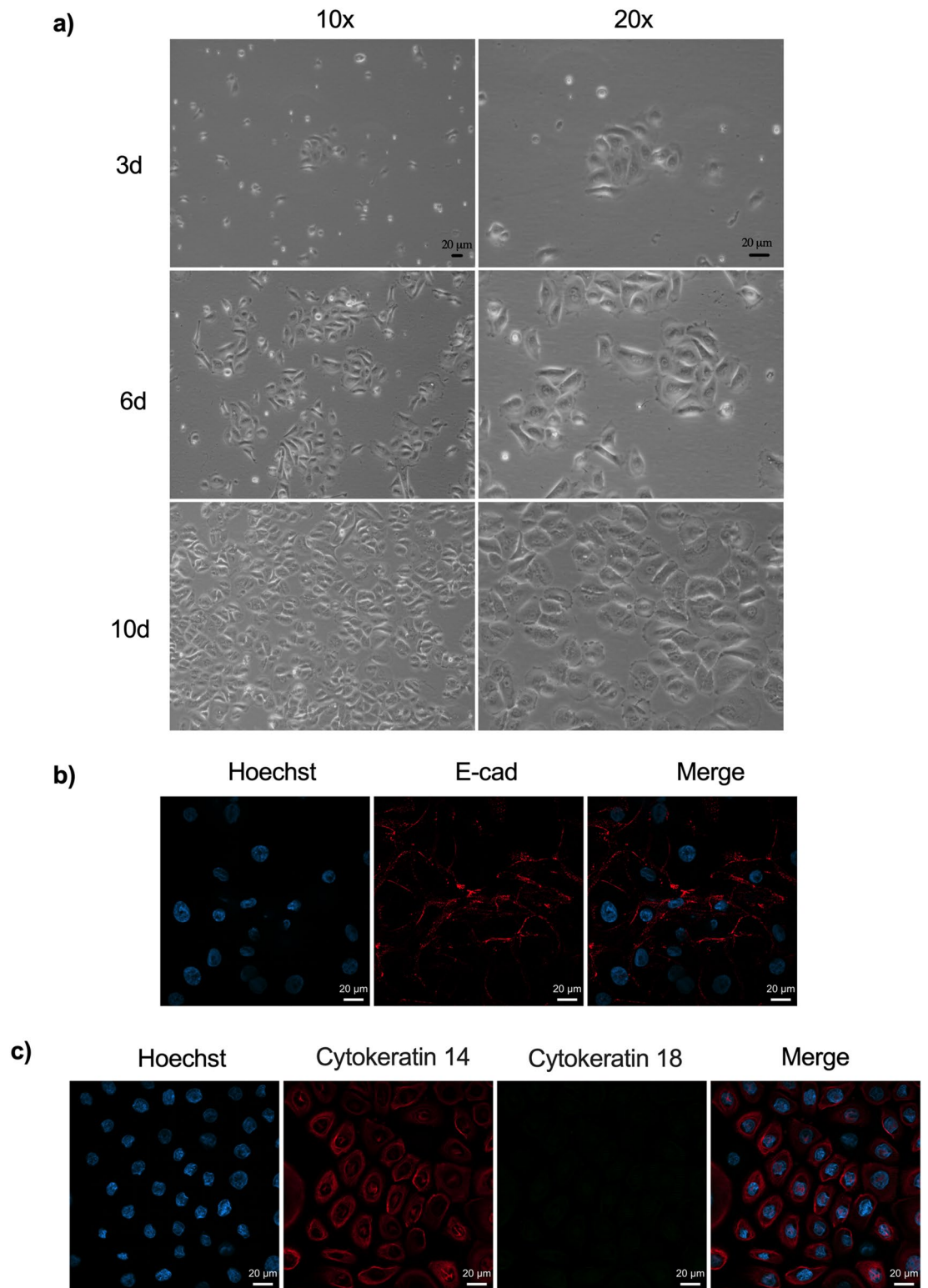
**Isolation and characterization of primary epithelial cells.** Epithelial cells were isolated from ectocervical explants excised after surgery on women undergoing hysterectomy as described in the methodology section and illustrated in Fig. S1. The stromal tissue was removed as much as possible from the explant, and the remaining tissue containing the mucosal layer was cut into small pieces (Fig. S1a), which improved the efficacy of subsequent enzymatic digestion by Dispase II. To limit contamination with fibroblasts, we introduced after digestion a mechanical separation of the mucosal layer, that is made of epithelial cells and mucus, from the stromal tissue (Fig. S1b). Dispase II digestion for either 3–4 h at 37 °C or overnight at 4 °C gave similar results and allowed a good separation of the mucosal layer in > 90% of the cases. The mucosal layers were then treated with trypsin and EDTA to dissociate the epithelial cells (Fig. S1c,d). We observed that a vigorous agitation after the trypsin/EDTA treatment improved cell dissociation. Non-dissociated tissue and debris were removed by filtration (Fig. S1e), and the isolated cells (Fig. S1f) were transferred into K-SFM.

Isolated cells were seeded directly in 75 cm<sup>2</sup> culture flask. Signs of cell divisions were apparent 3 days after seeding, with regular occurrence of small colonies (Fig. 1a). Around 10 days after seeding, the cells (passage 1) showed homogenous morphology, with no detectable contamination by other cell types such as fibroblasts and stromal cells, based on morphological features. We stained the cells with an antibody against E-cadherin, a specific marker of epithelial cells. Observation under a fluorescence microscope showed a plasma membrane localization of E-cadherin on the large majority of the cells, as expected (Fig. 1b). This was confirmed by quantification by flow cytometry, which detected positive E-cadherin signal in > 95% of the cells (Fig. 1d). Primary fibroblasts isolated from the same donor's tissue served as negative control in that experiment (Fig. 1d). Consistent with the observations that ectocervical epithelial cells express cytokeratin 14 but not 18<sup>20,21</sup>, we observed only cytokeratin 14 in the isolated primary cells (Fig. 1c). These results, together with our morphological observations, showed that the large majority of the isolated cells were epithelial cells.

**Culture in 'defined K-SFM' reduced contamination by fibroblast.** Although most passage 1 cultures consisted almost exclusively of epithelial cells, we often observed the emergence of elongated cells, i.e. the typical morphology of fibroblastic cells, upon later passages, or upon thawing cells that had not shown signs of fibroblast contamination at the time of freezing. To solve this problem, we tested a newly commercialized medium designed for the culture of primary epithelial cells, designated here as 'defined K-SFM'. One vial of frozen primary epithelial cells (passage 1 or 2) conserved in liquid nitrogen was thawed and split into two cultures, one in K-SFM containing EGF and BPE (named 'K-SFM'), one in defined K-SFM. Four days after culture, individual fibroblasts appeared in the K-SFM dish, and their relative proportion in the culture increased with time (Fig. S2a, upper panel). In contrast, no elongated cell was observed in the defined K-SFM dish, even 11 days after seeding (Fig. S2a, lower panel), suggesting that this medium inhibited fibroblast growth. To further verify this inhibitory effect on fibroblast proliferation, the sub-confluent cells from day 11 were collected and seeded in duplicate in new wells. Each duplicate well was incubated with either K-SFM or defined K-SFM (Fig. S2b). The two cultures in K-SFM medium showed gradual increase of fibroblasts contamination (Fig. S2c, 1st and 3rd rows). In contrast, the abundance of fibroblasts decreased significantly when K-SFM had been replaced by defined K-SFM (Fig. S2c, 2nd row) and cells continuously cultured in defined K-SFM showed almost no sign of fibroblast contamination (Fig. S2c, 4th row). This experiment showed that defined K-SFM markedly inhibited the growth of fibroblasts in the primary epithelial cell cultures.

## Primary epithelial cells isolated from human cervix were less permissive to *C. trachomatis* development than HeLa cells.

We first checked whether isolated primary epithelial cells were permissive for *C. trachomatis* growth. Bacteria loaded vacuoles, called inclusions, were observed in infected cells and their numbers increased with the infectious dose, with > 90% infected cells at high dose (Fig. 2a). Given that the HeLa cell line is predominantly used by the *Chlamydia* community, we then compared the susceptibility to *C. trachomatis* infection in HeLa and primary epithelial cells. Same number of HeLa cells and primary epithelial cells were infected with serial dilutions of GFP-expressing bacteria (L2<sup>incD</sup>GFP). We observed that 4 to 10 times (depending on the donor sample) more bacteria were needed to reach an equivalent percentage of inclusion-bearing cells 24 h post infection (Fig. 2b). We also observed that the inclusions were smaller in primary epithelial



**Figure 1.** Growth and characterization of primary epithelial cells. **(a)** Contrast phase images were taken 3, 6 and 10 days after seeding the cells. **(b,c)** Cells seeded were fixed and stained with anti-human E-cadherin **(b)** or with anti-human cytokeratin 14 or 18 **(c)** antibodies and fluorochrome-conjugated secondary antibodies. DNA was labeled with Hoechst. **(d)** Epithelial cells and fibroblasts were isolated from one explant and stained (right) or not (left) with FITC-conjugated anti-human E-cadherin antibody, and analyzed by flow cytometry. All experiments were performed using primary epithelial cells isolated from at least 3 donors.

d)

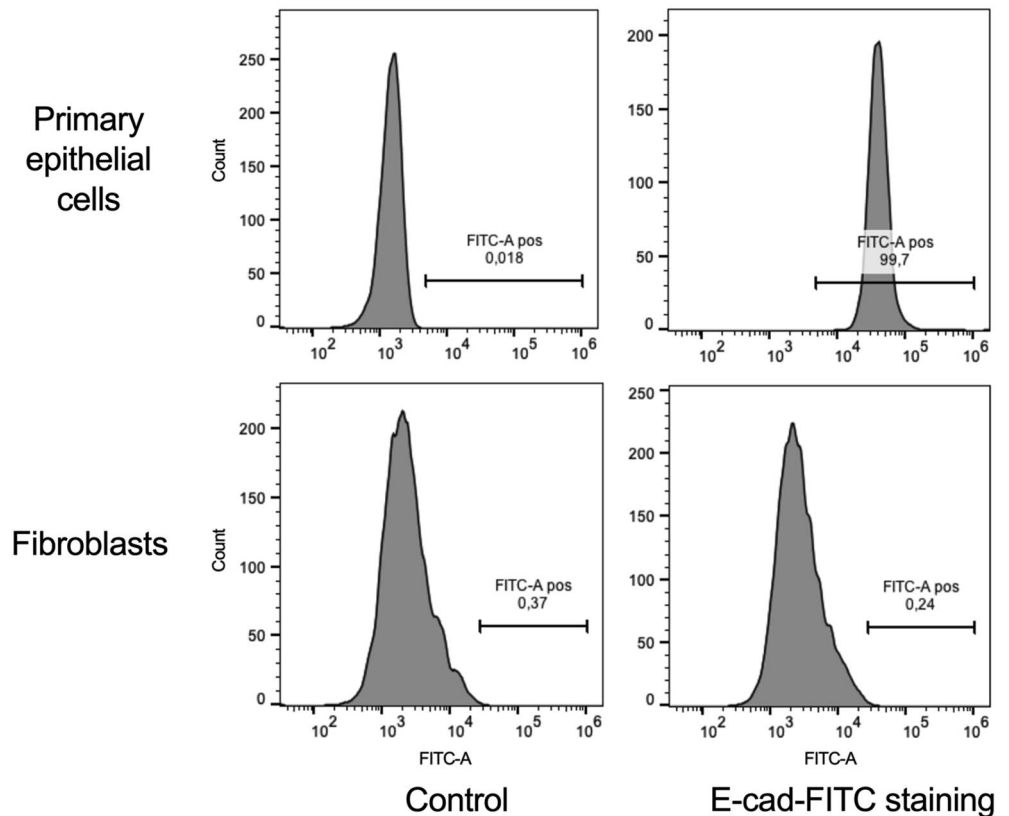
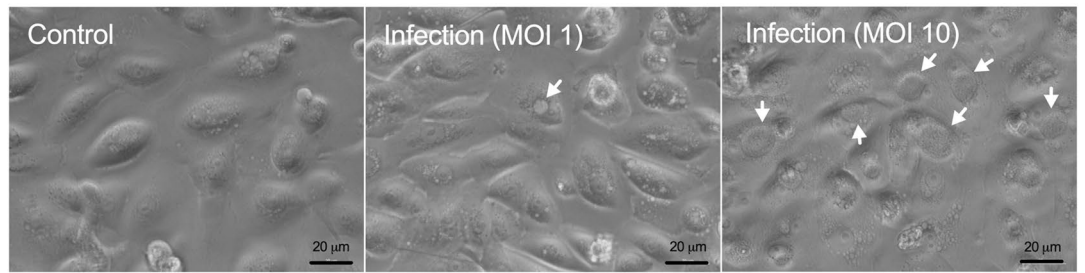


Figure 1. (continued)

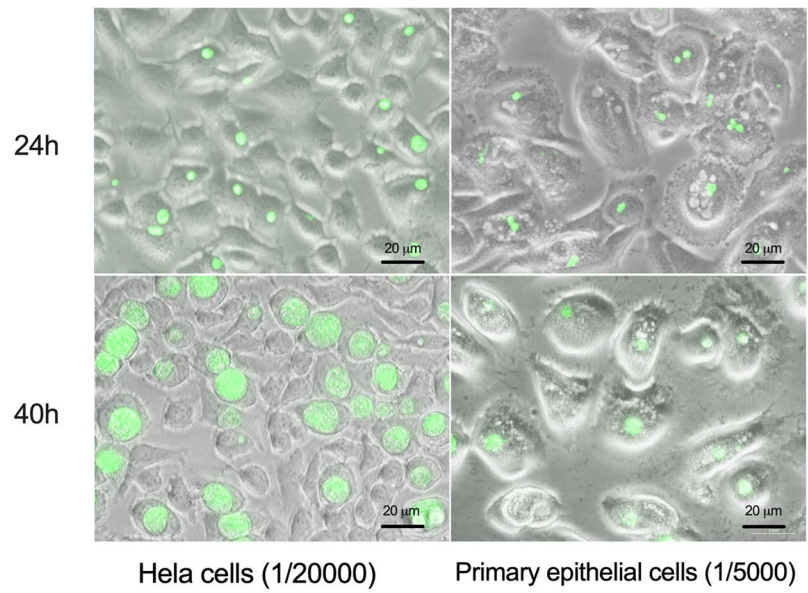
**Figure 2.** Primary epithelial cells are less permissive than HeLa cells to *C. trachomatis* development. (a) Primary epithelial cells were incubated with increasing quantities of bacteria and were observed 48 hpi. (b) Cells were infected with L2<sup>incD</sup>GFP (bacterial stock diluted 1:20,000 for HeLa cells and 1:5000 for primary cells) and observed 24 hpi (top) or 40 hpi (bottom). The picture displays merged images taken with phase contrast and in the green channel. (c) Cells were infected with *Chlamydia* serovar D (same quantity of bacteria used for both cell types) and observed 24 hpi (top) or 40 hpi (bottom). The cells were then fixed and stained with antibody against *Chlamydia* Cap1 and A488-conjugated secondary antibody. The picture displays merged images taken with phase contrast and in the green channel. (d) Quantification of inclusion sizes, each individual point represented one inclusion ( $n > 5$  fields per experiment). (e) Titration of the progeny collected 48 hpi on HeLa cells and primary epithelial cells. (f) Cells were incubated for 4 h at 4 °C with L2<sup>incD</sup>GFP bacteria before measurement of attached bacteria by flow cytometry. (g) Cells were incubated with L2<sup>incD</sup>GFP bacteria for 30 min at 4 °C, before transfer to 37 °C. At the indicated times the cells were fixed and external bacteria were labeled with a red fluorochrome. The graph represented the number of internalized bacteria relative to total cell-associated bacteria. (h) Cells were incubated for 40 h with L2<sup>incD</sup>GFP bacteria. Arrows point to multiple inclusions, only observed in primary epithelial cells. The data were presented as mean  $\pm$  SEM and the non-paired student's t-test was used for two group comparisons. At least 2 independent were performed in (a–c) and (h), and primary cells used in each experiment were from more than one donor. (e–g) were from 3 independent experiments with primary epithelial cells from different donors each time. \*\* $p < 0.01$  and \*\*\* $p < 0.0001$ .

cells compared to HeLa cells, both at 24 and 40 hpi (Fig. 2b,d). Consistent with these observations, a close to two-log difference in the infectious progeny collected 48 hpi (a duration corresponding on average to the completion of one cycle) was observed between the two cellular backgrounds (Fig. 2e). To test whether the binding efficiency of bacteria accounted for the decreased efficiency at establishing an infection observed in Fig. 2b, we measured the binding of L2<sup>incD</sup>GFP to HeLa and primary epithelial cells at 4 °C. No difference was observed (Fig. 2f), indicating that the low rate of infection of the primary epithelial cells was not due to defective binding of the bacteria. We next measured the efficacy of bacterial internalization, and again observed no difference between primary epithelial cells and HeLa cells (Fig. 2g, S3). These results indicate that the decrease in *C. trachomatis* infectivity in primary cells compared to HeLa cells were not due to defects in the attachment nor entry steps, but in failure, for about 75% to 90% of the bacteria, to initiate the development of a viable inclusion. Furthermore, the successful inclusions grew much slower, meaning that the bacteria divided less rapidly. All these observations were achieved using a laboratory standard strain of *C. trachomatis* serovar L2 originally isolated from a man with proctitis. We

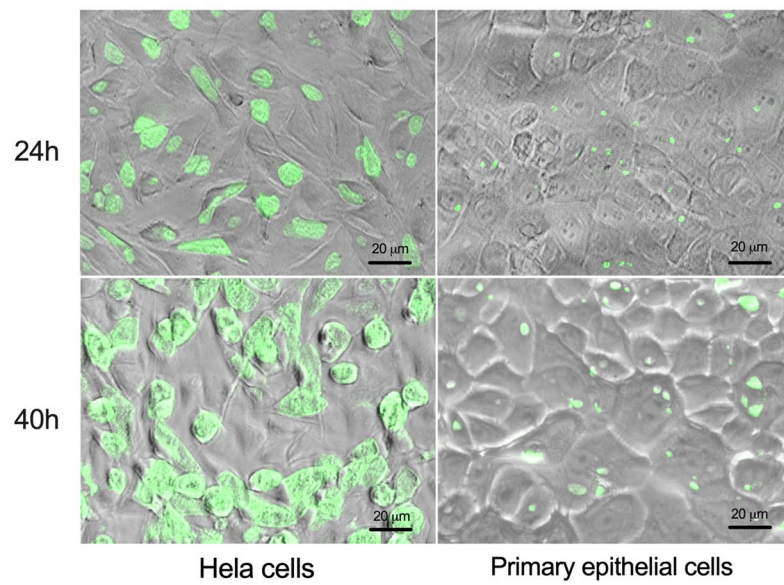
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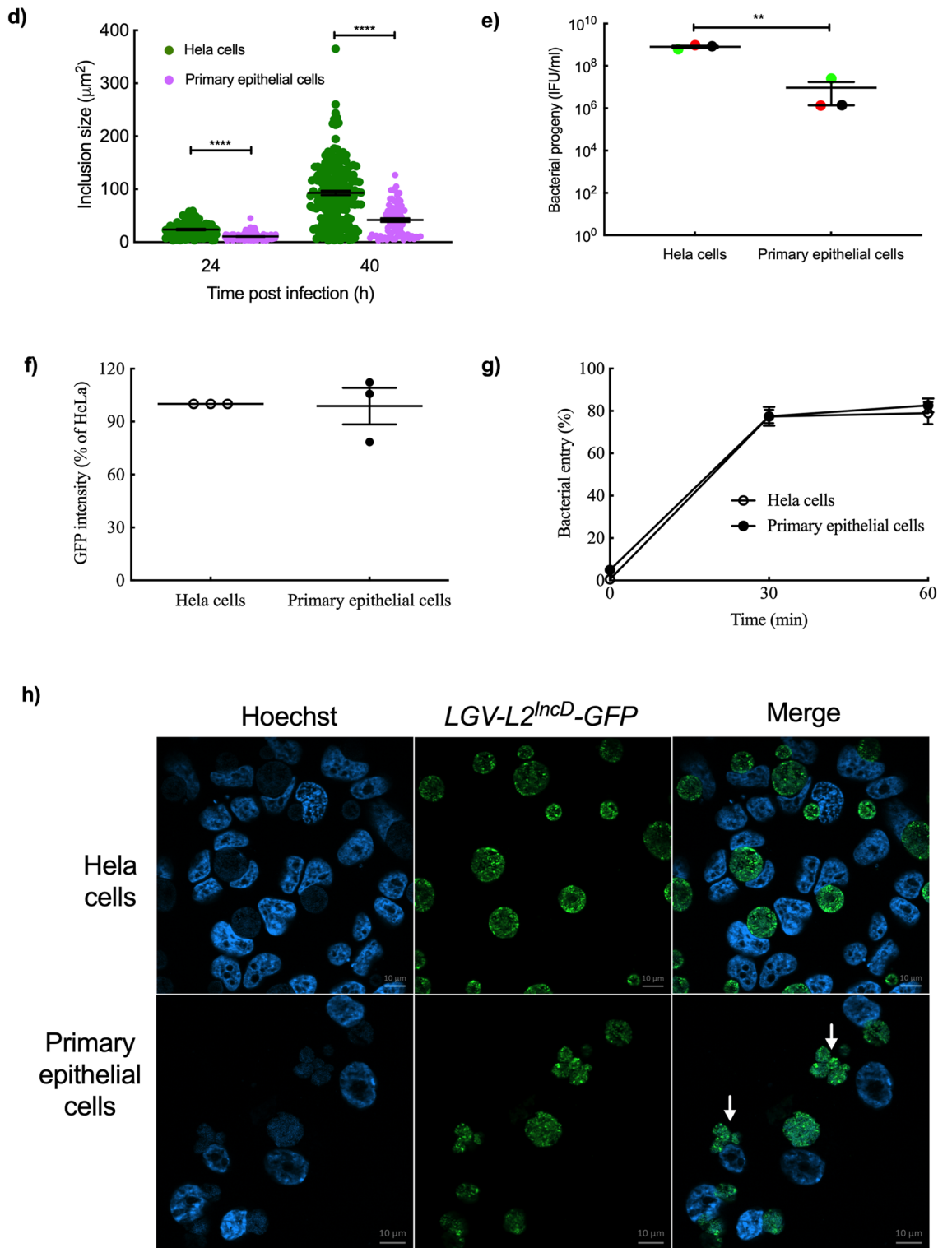


b)



c)





**Figure 2.** (continued)

then tested the susceptibility of primary epithelial cells to a *C. trachomatis* serovar D strain, one of the serovars commonly infecting the FGT. The serovar D strain also showed delayed development in primary epithelial cells compared to HeLa cells (Fig. 2c, S4). Altogether, we concluded that primary epithelial cells were less permissive to *C. trachomatis* development than HeLa cells.

When HeLa cells are infected with *C. trachomatis* at high MOI, multiple inclusions start developing, that then fuse into a single inclusion quickly<sup>22</sup>. Indeed, unique inclusions were observed in most *Chlamydia*-infected HeLa cells (Figs. 2 and 3). Surprisingly, multiple inclusions were regularly observed in *Chlamydia*-infected primary epithelial cells even at low MOI (Fig. 2b,c), and even at late (40 h) time of infection (Fig. 2h). It suggested a delayed inclusion development in primary epithelial cells. One key player in inclusion fusion is IncA, whose absence results in multiple inclusions<sup>22,23</sup>. IncA was detected in primary epithelial cells, even on multiple inclusions (Fig. S5). This observation indicates that another factor(s) than IncA is limiting in this cellular background to ensure efficient inclusion fusion.

### Differences in culture medium partially account for the differences in *C. trachomatis* development in primary epithelial cells and HeLa cells.

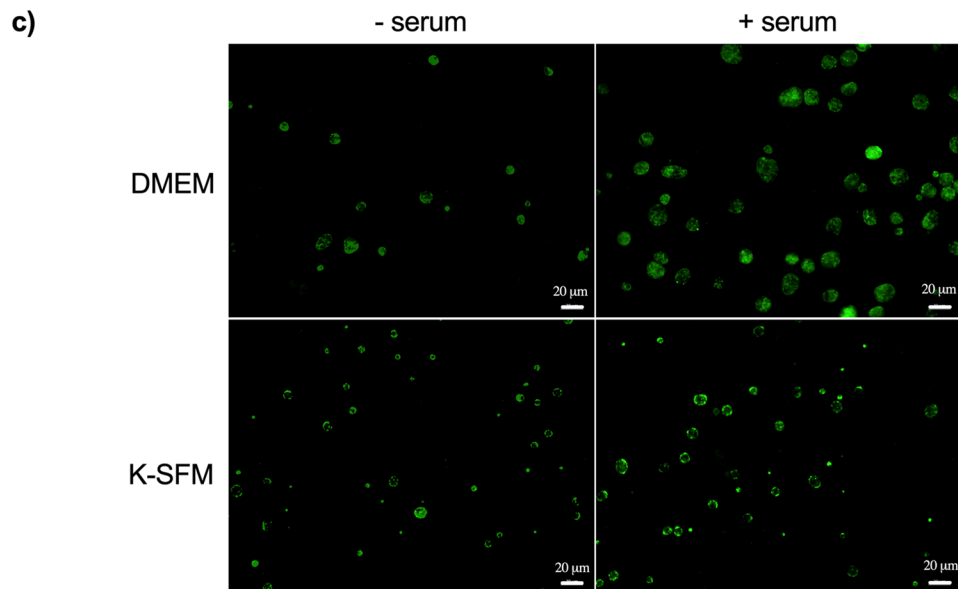
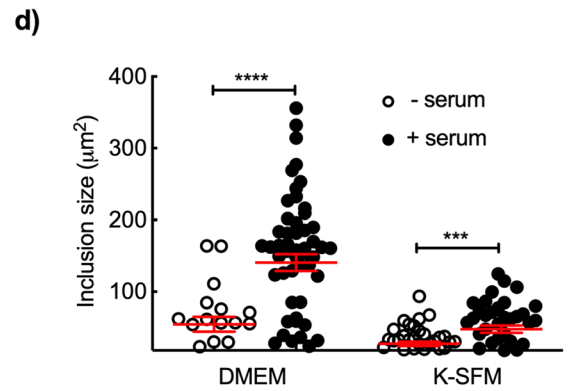
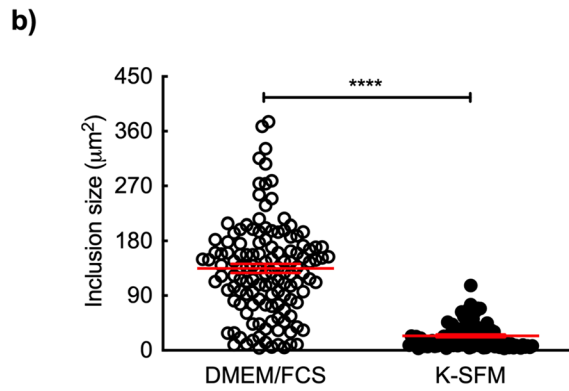
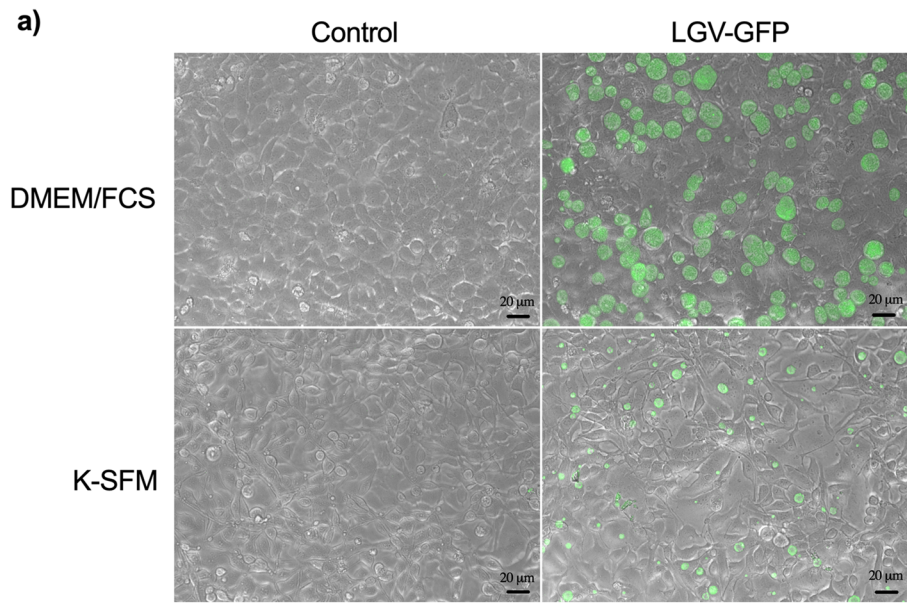
To keep their cellular identity primary epithelial cells have to be grown in K-SFM without serum (it is replaced by EGF and BPE), while HeLa cells are grown in DMEM supplemented with FCS. These differences could result in unequal bacterial access to nutrients, and explain the difference of progeny observed between both cell types. To test this possibility, we first compared the size of inclusions, as a read-out for bacterial load, in HeLa cells grown either in DMEM/FCS or in K-SFM. No apparent difference in cell morphology was observed (Fig. 3a), but inclusions were 6 times larger ( $134 \pm 7$  vs.  $23 \pm 2 \mu\text{m}^2$ ,  $p < 0.0001$ ) in HeLa cells grown in DMEM/FCS compared to K-SFM (Fig. 3a,b). We next tested the contribution of FCS to these differences. Presence of serum in the culture medium markedly increased *Chlamydia* inclusion size in infected HeLa cells especially when cultured in DMEM (Fig. 3c,d), indicating that FCS contributes significantly to the difference of progeny observed previously. In addition, the differences in inclusion size also depend on the nature of the medium, since inclusions in HeLa cells grown in K-SFM were systematically smaller than in DMEM ( $27 \pm 3$  vs.  $54 \pm 10 \mu\text{m}^2$ ,  $p = 0.0017$ ) (Fig. 3c,d).

We next compared the two cell types cultured in DMEM with or without serum. Addition of serum increased the size of inclusions for the two cell types. In contrast, the difference in inclusion size was modest in the absence of serum between the two cellular backgrounds (Fig. 4a,b). These results indicate that medium composition, and, most importantly, addition or not of serum, significantly affect bacterial growth. However, these data are difficult to interpret because transfer from K-SFM to DMEM induced important changes in the morphology of epithelial cells (Fig. 4a), suggesting that they might no longer behave as *bona fide* epithelial cells.

The last condition chosen to compare the two cell types was to incubate them in mixed DMEM/FCS and K-SFM (ratio 1/1, v/v). As observed in DMEM only (Fig. 4a), primary epithelial cells became more elongated, while the morphology of HeLa cells remained unchanged (Fig. 4c). Inclusions were about two-fold smaller ( $80 \pm 14$  vs.  $171 \pm 24 \mu\text{m}^2$ ) in primary epithelial cells than HeLa cells (Fig. 4c,d). However, the percentage of infection was similar between HeLa and primary epithelial cells grown in this mixed medium when the same dose of bacterial was used (data not shown). This result shows that, independently of culture medium, *C. trachomatis* development is slower in primary epithelial cells than in HeLa cells.

### Comparison of the immune response from primary epithelial cells and HeLa cells upon *Chlamydia* infection.

Finally, the inflammatory response of primary epithelial and HeLa cells upon *Chlamydia* infection was examined. Ten times more *C. trachomatis* serovar L2 bacteria were used to infect the primary epithelial cells, to reach 85 to 100% infection in both cellular backgrounds. Transcription levels of four major pro-inflammatory cytokines, *IL6*, *IL8*, *IL1 $\beta$*  and *TNF $\alpha$* , were examined at different times post infection. We observed a robust inflammatory response in primary epithelial cells, with a peak in transcriptional up-regulation around 24–30 hpi for all cytokines, except for *IL1 $\beta$* , that showed very modest induction (Fig. S6). Interestingly, transcriptional up-regulation was stronger in primary epithelial cells than in HeLa cells. Based on these results, we next measured transcription levels of various inflammatory cytokines 30 hpi (Fig. 5a). Primary epithelial cells displayed a higher basal level of transcription of genes for a number of proinflammatory cytokines (*IL1 $\alpha$* , *IL1 $\beta$* , *TNF $\alpha$* , *CSF*, *IP10* and *CCL5*), compared to HeLa cells, with the notable exception of *IL6*. Infection with *C. trachomatis* serovar L2 induced expression of several pro-inflammatory cytokines, including *IL6*, in both HeLa and primary epithelial cells, and the response was stronger in primary cells (Fig. 5a). For instance, a marked increase of the transcripts for the inflammatory cytokines *IL1 $\alpha$* , *IL12p40*, *IP10* and *eotaxin3* was detected in primary epithelial cells upon infection, while, no, or only moderate, increase was observed in the HeLa cell line. We wondered whether the difference could be due to discordant *IL11* response between the two cell types, as this cytokine limits the expression of *TNF $\alpha$* , *IL1 $\beta$* , *IL6*, *IL12p40* and *NO* in LPS-stimulated macrophages<sup>24,25</sup>, and was proposed to play a role in limiting the early response to *C. trachomatis* infection<sup>20</sup>. However, basal *IL11* transcript levels were similar between primary epithelial cells and HeLa cells, and showed similar induction by *Chlamydia* infection in the two cellular backgrounds (Fig. 5a). Thus, the difference in inflammatory profiles between the two cell type does not appear to be linked to *IL11* levels. Of note, several proinflammatory cytokines did not respond to *Chlamydia* infection in both cell types, including *eotaxin1*, *MCP1* and *TGF $\beta$* . Finally, we examined transcripts levels for five inflammatory cytokines, *IL6*, *IL8*, *IL1 $\beta$* , *TNF $\alpha$*  and *CCL5*, upon infection with *C. trachomatis* serovar D. All cytokines were up-regulated in primary epithelial cells except *IL1 $\beta$* , consistent with the observations made with serovar L2 (Fig. 5b). However, the amplitude of the response was lower than in cells infected with serovar L2, an observation consistent with the slower proliferation of the serovar D bacteria compared to serovar L2 (Fig. 2b,c). Altogether, these results revealed that primary epithelial cells had a distinct pattern of inflammatory response, and, overall, displayed a stronger inflammatory response to *C. trachomatis* infection than HeLa



**Figure 3.** Culture medium and serum affect inclusion size in HeLa cells. **(a)** HeLa cells were incubated in DMEM/FCS or in K-SFM medium and infected (right panels) or not (left panels) with L2<sup>incD</sup>GFP at the same multiplicity of infection. Pictures were taken 40 hpi. **(b)** The dot plot shows the mean area of inclusions in the two conditions, each individual point represents one inclusion. **(c)** HeLa cells were infected with L2<sup>incD</sup>GFP in DMEM (upper panels) or K-SFM (bottom panels) in the presence (left panels) or absence (right panels) of serum for 40 h before taking the images. **(d)** The dot plot shows the mean area of inclusions in the four conditions, each individual point represents one inclusion. Two independent experiments, with inclusions from at least  $n > 5$  images per experiment, were shown to measure the sizes of the inclusions. The data were presented as mean  $\pm$  SEM and the non-paired Student's t-test was used for two group comparisons. \*\*\*\* $p < 0.0001$ .

cells. The high basal level of expression of pro-inflammatory cytokines in primary epithelial cells may contribute to this behavior.

## Discussion

The present study establishes a simple protocol for isolating primary epithelial cells from cervical biopsies of donors. The whole procedure can be performed within 24 h or even less, if the biopsy is collected in the morning. The primary epithelial cells can be passaged 4 to 5 times. The cells expand fast during the first 3 passages, then slow down. After 5 passages, the cells stop dividing in general. In our hand, the first 3 passages of primary epithelial cells could be cryopreserved and grew well once thawed, in contrast to one previous study<sup>17</sup>.

Compared to other methods in which enzymatic cocktails were applied to isolate primary epithelial cells from the fallopian tubes and the uterus of human samples<sup>14,26,27</sup>, a single enzyme Dispase<sup>®</sup> II was used. A similar methodology was used by other groups in isolating primary cervical epithelial cells<sup>21,28</sup>. One important step we introduced was the mechanical separation of the mucosal layer from the stromal tissue before dissociating the epithelial cells, which significantly improved the purity of isolated cells.

Avoiding contamination by fibroblasts or other cells is a major challenge for the isolation of primary epithelial cells<sup>29</sup>, as contamination of the culture is a common confounding factor. This was achieved by three complementary strategies. Firstly, the mucosal layer containing the epithelia was separated completely from the stromal tissue at an early stage of the procedure. Secondly, a new defined K-SFM medium was introduced, which significantly slowed down fibroblast growth. Lastly, most of the experiments were performed after the second passage of the primary cells, largely reducing the risk of contamination by other cell types. One other commonly used strategy to expand primary epithelial cells from the cervix is to place the small pieces of explant with mucosal surface down directly into the culture dish<sup>11,17,30</sup>. In our hands, the present protocol was less prone to fibroblast contamination.

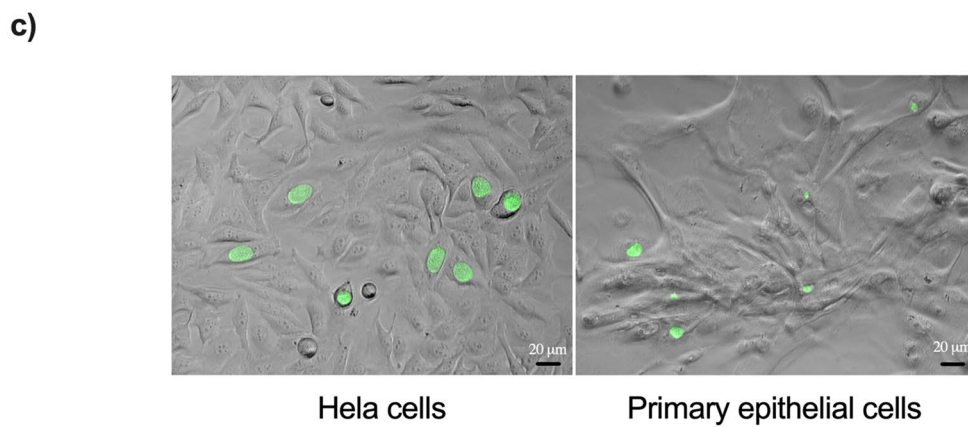
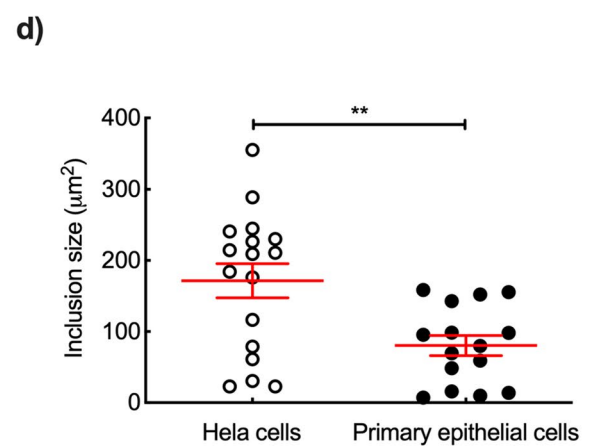
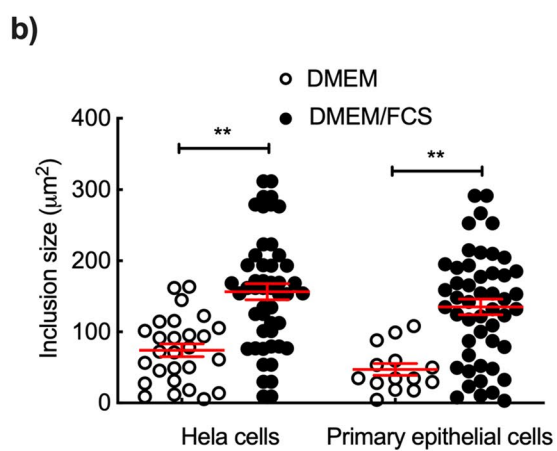
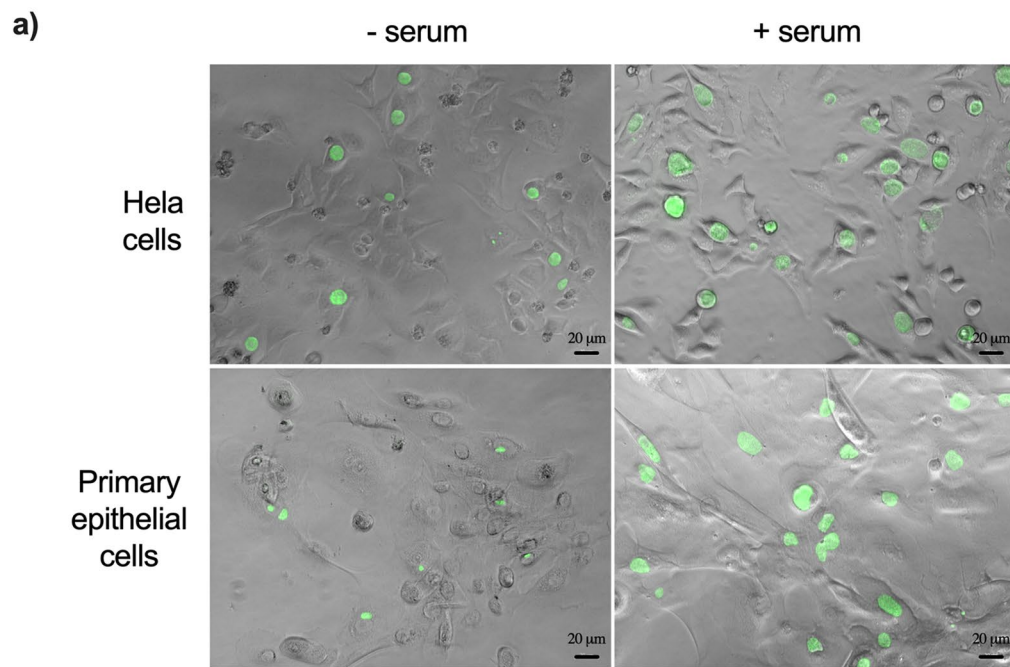
Primary epithelial cells isolated from human FGT have been cultured in a range of different media including DMEM/FCS<sup>10</sup>, DMEM/F12<sup>14,15</sup>, keratinocyte growth medium KGM (Clonetics)<sup>18</sup>, RPMI1640/FCS/KGM<sup>17</sup>, K-SFM (Thermo Fisher Scientific)<sup>13</sup>, EpiGRO Human Epidermal Keratinocyte Complete Media (Millipore)<sup>30</sup> and even lab-made medium<sup>12</sup>. Surprisingly, this is the first report that elongated cell, reminiscent of the morphology of fibroblasts appeared in the culture when primary epithelial cells were switched from K-SFM to DMEM/FCS or to DMEM/FCS mixed with K-SFM. It is unclear whether this reflects a morphological change in the “epithelial” cells, or if contamination became apparent because fibroblasts divide much faster than epithelial cells in this culture medium. Relevant to this observation, serum can promote the growth of fibroblasts and inhibit keratinocytes *in vitro*<sup>31</sup>. Changes in cell morphology and growth upon modification of the culture medium have been also reported, even on cell lines<sup>32</sup>.

Consistent with recent reports<sup>12,17</sup>, *C. trachomatis* was able to infect primary epithelial cells isolated from cervical biopsies. Compared to HeLa cells, we and other<sup>18</sup> observed a reduction in the percentage of infected cells, and in the size of inclusions, indicating that epithelial cells are less permissive to *Chlamydia* infection than HeLa cells. One confounding factor in this comparison is that different culture media need to be used to maintain these two cell types, which likely affects bacterial supply in nutrients. Our experiments showed that indeed, the presence of serum boosts inclusion growth, whatever the cellular background. However, these experiments are difficult to interpret because we observed that the morphology of epithelial cell was very sensitive to the culture conditions, suggesting that replacement of K-SFM with DMEM is sufficient to change their identity, and possibly their ability to respond to infection. Still, when grown in the same mix of DMEM/FCS and K-SFM, primary “epithelial” cells remained less permissive to *C. trachomatis* development than HeLa cells. This observation indicates that primary epithelial cells have intrinsic anti-chlamydial properties that are defective in HeLa cells, and emphasize the need to use primary cells to study the response of the epithelial layer to infection.

Cell-autonomous immune responses play an important part in the host arsenal to eliminate invading pathogens. Transcripts for several pro-inflammatory cytokines were detected in basal conditions (i.e. non-infected cells), and their level was systematically higher in primary epithelial cells than in HeLa cells. Early work on the constitutive secretion of IL-8 and other proinflammatory cytokines by primary epithelial cells of FGT came to the same conclusion<sup>15,18</sup>. This phenomenon may in part reflect the differences in culture conditions. Infection by *C. trachomatis* elicited an increase of transcription of inflammatory genes in primary epithelial cells. This observation is consistent with the increase in secretion of several inflammatory cytokines and chemokines including IL6, IL8, IL10, Eotaxin3, TNF $\alpha$ , MCP1, MIP1, observed in primary endocervical epithelial cells infected with *C. trachomatis*<sup>17</sup>. The range of the transcriptional response to infection was lower in HeLa cells, and was consistent with transcriptomic data collected in this cell type<sup>33</sup>.

The first cells encountered by *C. trachomatis* upon sexual transmission are the epithelia cells from the lower FGT, the vagina and the ecto-cervix. Robust replication of *C. trachomatis* in immortalized human vaginal epithelial cells was reported recently<sup>16</sup>, and we show here that epithelial cells from the ecto-cervix are also





◀ **Figure 4.** Culture medium and serum affect both cell morphology and inclusion size in primary epithelial cells. (a) HeLa and primary epithelial cells were incubated in DMEM medium and infected with L2<sup>incD</sup>GFP at the same multiplicity of infection with (right panels) or without (left panels) serum. Images were taken 40 hpi. (b) The dot plot shows the mean area of inclusions in the two conditions for both cell types, each individual point represents one inclusion. (c) HeLa and primary epithelial cells were incubated in mixed medium of DMEM/FCS and K-SFM (1:1, v/v) and infected with L2<sup>incD</sup>GFP at the same multiplicity of infection. Pictures were taken 40 hpi. (d) The dot plot shows the mean area of inclusions in the two cell types, each individual point represents one inclusion. Three independent experiments, with inclusions from at least  $n > 5$  images per experiment, were performed. The data were presented as mean  $\pm$  SEM and the non-paired Student's t-test was used for two group comparisons. \*\* $p < 0.01$ .

permissive to *C. trachomatis* growth. Other work on primary cells have focused on columnar cells isolated from the endocervix<sup>34</sup>. Interestingly, no significant increase in proinflammatory cytokines including IL6, IL8 and TNF $\alpha$  was observed in this cellular background<sup>34</sup>, in contrast to what we observed in ectocervical primary cells, indicating that the ectocervix might be better equipped to alert the surrounding environment of an intrusion than the endocervix.

In conclusion, the present study demonstrated a slower rate of *Chlamydia* replication in primary epithelial cells isolated from human ectocervical biopsies than in HeLa cells, although this is largely due to difference in culture medium. The most important difference we report here is the stronger ability of ectocervical epithelial cells to deploy an inflammatory response compared to the commonly used HeLa cell line. This support the hypothesis that epithelial cells, while representing the target of *C. trachomatis* replication, also constitute a well-equipped line of defense to counteract the spread of the pathogen. More studies using primary epithelial cells will be required in the future to understand which level of protection this response offer, and why it is sometimes insufficient to contain the ascension of *C. trachomatis* to the upper FGT.

## Methods

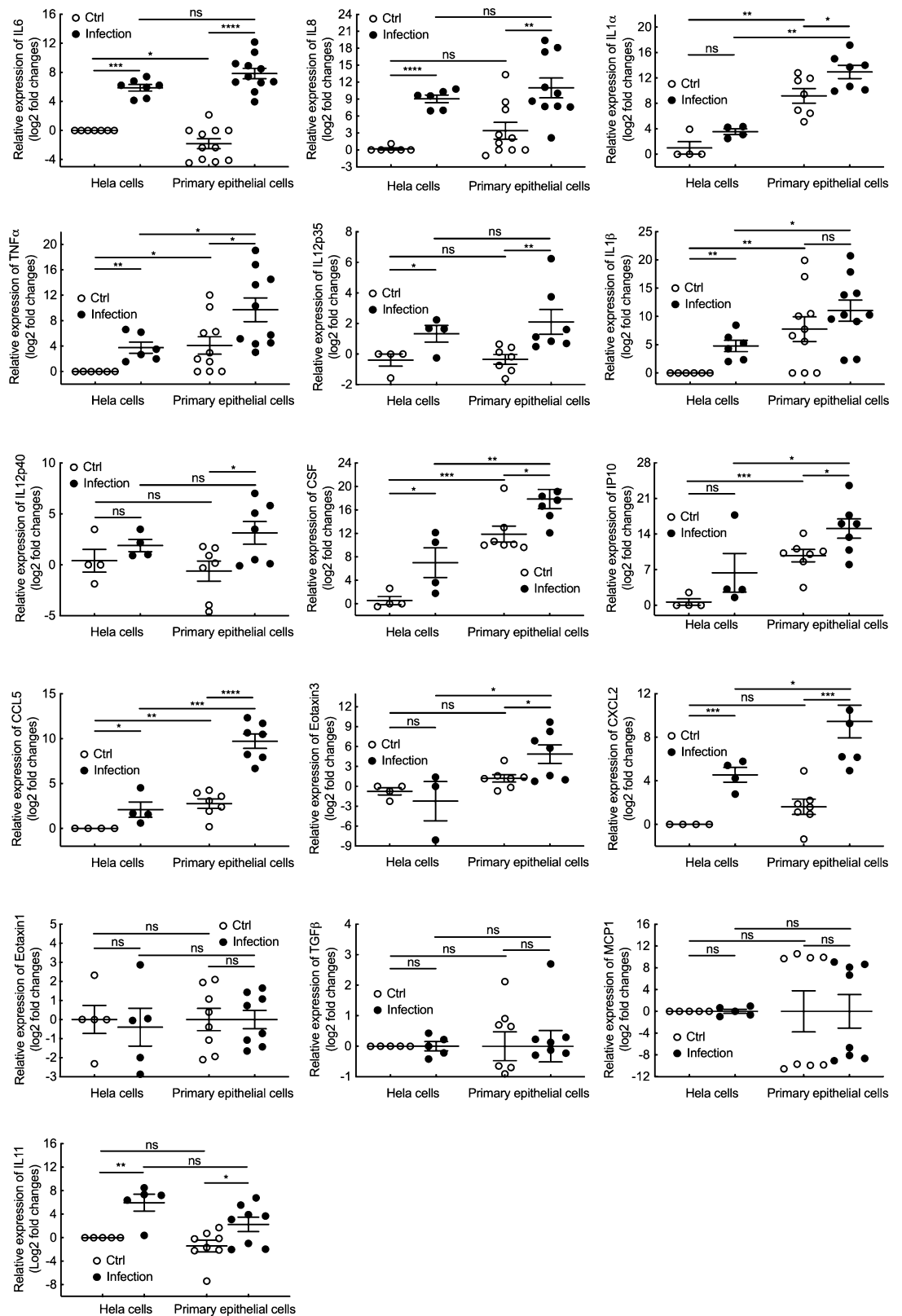
**Collection of samples and ethical approval.** All methods to recruit subjects and collect biological samples were carried out in accordance with relevant guidelines and regulations. All participants (ages ranging from 38 to 55, in pre-menopause), gave written informed consent in accordance with the Declaration of Helsinki principles. Data and samples were collected from women undergoing hysterectomies due to different gynecological problems including uterine fibroma, endometriosis, prolapse of the uterus and adenomyosis. The clinical study was approved by the French Ethical Committee 'CPP Ile de France 1' on May 9, 2016. Approval and authorization of the National Data Protection authority ('Commission Nationale de l'Informatique et des Libertés', CNIL) have been obtained for the research protocol. Ecto-cervical explants distal from the pathological site were rinsed in RPMI 1640 (Thermo Fisher Scientific) containing gentamycin (5  $\mu$ g/ml), and stored in this medium at 4 °C until transport to the laboratory (within 2 to 18 h after surgery).

**Isolation of primary epithelial cells and fibroblasts.** The explant was rinsed several times with pre-chilled PBS containing gentamycin (5  $\mu$ g/ml) to remove the blood. The non-mucosal tissues, such as muscle and stromal tissue, were removed as much as possible under a binocular microscope. The remaining part containing the mucosal layer (1–1.5 cm<sup>2</sup>) was cut into small pieces (around 3  $\times$  3 mm<sup>2</sup>) and placed, mucosal surface face-down, in a 6-well culture dish containing Dispase II (Roche) solution (2.4 units/mL in RPMI 1640).

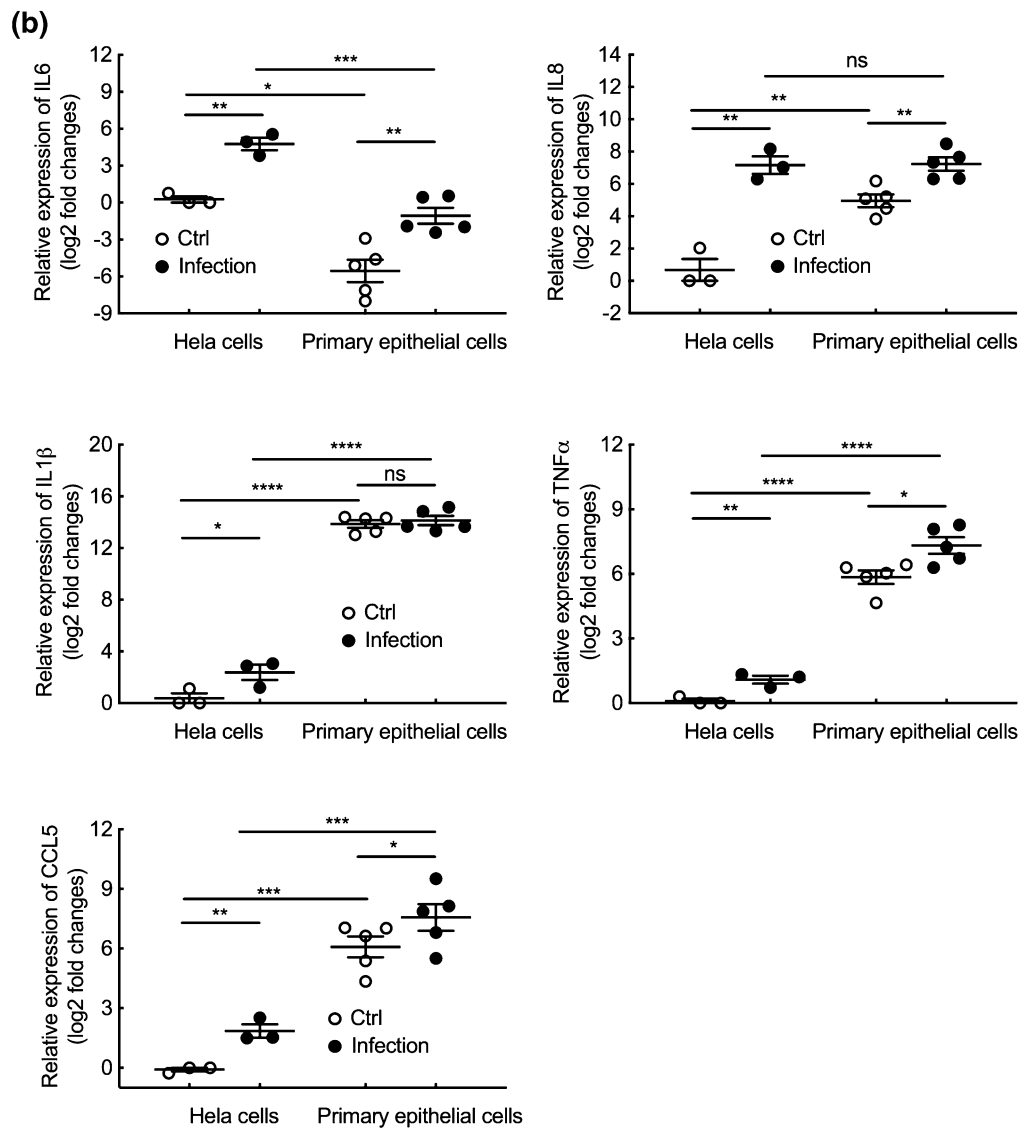
Dispase II digestion was performed either at 4 °C overnight or at 37 °C for 3–4 h. After the digestion, the mucosal layers were separated from the stromal tissue using forceps, and pooled together into a 15 ml centrifugation tube containing RPMI 1640. The mucosal layers were pelleted by centrifugation (500 $\times$ g for 5 min at room temperature) and re-suspended with 0.25% Trypsin, 0.02% EDTA solution in Hanks' Balanced Salt Solution (Sigma-Aldrich) and digested further for 10 min at 37 °C. Digestion by trypsin was stopped by adding fetal calf serum (FCS). After vigorous vortex, the suspension was passed through a 40  $\mu$ m mesh to get rid of the non-digested debris. The collected cell suspension was pelleted by centrifugation (2000 $\times$ g for 15 min at 4 °C). The pellet was resuspended into Gibco™ keratinocyte serum-free media supplemented with 5  $\mu$ g/L of human recombinant epidermal growth factor (EGF) and 50 mg/L bovine pituitary extract (BPE) (K-SFM, Thermo Fisher Scientific, #17005075), and seeded in 75 cm<sup>2</sup> culture flasks. The culture medium was changed every 2–3 days until cell reached confluency (10–15 days depending on the mucosal size of the explant). Upon confluency cells were rinsed twice by 0.5 mM EDTA in PBS, detached by incubation with 1 mL of Trypsin/EDTA at 37 °C for 5 min. The cell suspension and rinsing medium were pooled together and centrifuged again. The pellet was resuspended in K-SFM and re-plated in 150 cm<sup>2</sup> dishes for further expansion (dilution 1:2 to 1:4). The cells at passage 2–3 were used for the experiments or cryo-frozen in K-SFM and DMSO suspension (1:1, v:v) containing around 1  $\times$  10<sup>6</sup> cells per vial. The data presented in Fig. S1 were obtained using a novel culture medium, the Gibco™ defined K-SFM (Thermo Fisher Scientific, #10744019). The rest of the data were obtained with early passage (P2) epithelial cells cultured in K-SFM.

For isolation of fibroblasts, the cells isolated as described above were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM, Invitrogen) containing 10% fetal calf serum (FCS). After 2–3 passages, the large majority of cells were fibroblasts and either used for the experiment or cryo-frozen. HeLa cells (ATCC) were cultured in DMEM medium containing 10% FCS.

(a)



◀ **Figure 5.** Inflammatory response of HeLa and primary epithelial cells upon *Chlamydia* infection. (a) HeLa and primary epithelial cells were infected with *C. trachomatis* serovar L2 (MOI=1 for HeLa cells and MOI=10 for primary epithelial cells, respectively). Thirty hours post infection, RNAs were extracted from cell lysates and the transcripts for the indicated genes were measured by real-time quantitative PCR and normalized to house-keeping gene  $\beta$ -actin following the  $2^{-\Delta\Delta Ct}$  method. (b) HeLa and primary epithelial cells were infected with *Chlamydia* serovar D (bacterial stock diluted 1:500 for HeLa cells and 1:50 for primary epithelial cells). Forty-eight hours post infection, the samples were processed as in (a). The data are presented as Log2 fold changes of mRNA compared to uninfected HeLa cells. Each plot displays data obtained from at least 3 independent experiments and each individual point in the primary cell group represents the results of cells isolated from a different donor. The data were presented as mean  $\pm$  SEM and the non-paired Student's t-test was used for two group comparisons. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns, not significant.



**Figure 5.** (continued)

**Bacterial preparation and infection.** *Chlamydia trachomatis* serovar L2 (434/Bu) and serovar D (UW-3/CX) strains were propagated in HeLa cells. In certain experiments, a L2 strain stably expressing the green fluorescent protein under the control of the IncD promoter (L2<sup>IncD</sup>GFP) was used<sup>35,36</sup>. The purified elementary bodies (EBs) of *Chlamydia* were stored in sucrose-phosphate-glutamic acid buffer (SPG) at  $-80^{\circ}\text{C}$ .

For infection, adhered cells ( $0.2 \times 10^6$  cells/well in 12-well plates) were washed twice in serum-free medium and incubated with bacteria for the indicated time before collecting the samples. For serovar D infection, the plates were centrifuged at  $1200 \times g$  for 30 min at  $30^{\circ}\text{C}$  to facilitate the infection.

**Adhesion assay.** Adhesion assays were performed as previous description<sup>36</sup>. Briefly, HeLa or primary epithelial cells seeded in a 24-well plate the day before ( $0.1 \times 10^6$  cells/well) were pre-cooled 30 min at  $4^{\circ}\text{C}$  and then incubated for 4 h at  $4^{\circ}\text{C}$  with L2<sup>IncD</sup>GFP (MOI = 10). The bacteria were sonicated prior infection to disrupt bacterial aggregates. After incubation, cells were washed gently with PBS 3 times and detached using 0.5 mM EDTA in PBS. The pelleted samples were fixed in 4% (w/v) paraformaldehyde (PFA) 4% (w/v) sucrose in PBS for 30 min, washed with PBS and analyzed using flow cytometry.

**Bacterial entry assessment.** Entry experiments were performed as described previously<sup>36</sup>. In brief, HeLa or primary epithelial cells seeded on coverslips in a 24-well culture dish the day before ( $0.1 \times 10^6$  cells/well) were pre-cooled 30 min at  $4^{\circ}\text{C}$  and then incubated for 45 min at  $4^{\circ}\text{C}$  with L2<sup>IncD</sup>GFP bacteria (MOI = 10). The bacteria were sonicated before infection to disrupt bacterial aggregates. After incubation, cells were rinsed with pre-chilled PBS. Then pre-warmed medium was added and incubated at  $37^{\circ}\text{C}$  before being fixed at different time points in 4% PFA 4% sucrose in PBS for 20 min. Extracellular bacteria were stained with a mouse anti-MOMP-LPS antibody (Argene #11–114) followed with Cy5-conjugated anti-mouse secondary antibody (Amersham Biosciences). The dilutions were made in PBS containing 3% of BSA. DNA was stained using 0.5  $\mu\text{g}/\text{mL}$  of Hoechst 33342 (Thermo Fisher Scientific) added in the secondary antibody solution. Images were acquired on a DeltaVision Elite microscopy system (GE Healthcare Life Science), equipped with an Olympus IX71 inverted microscope (Olympus, Japan). Images were taken with a CoolSnap HQ2 cooled CCD camera using the software Zen. The bacterial entry was presented as ratio between intracellular bacteria (single GFP staining) and total bacteria (all GFP positive staining including GFP/Cy5 double stained bacteria).

**Flow cytometry.** After infection or treatment, the cells were detached with 0.5 mM EDTA in PBS and transferred to a Falcon™ 96-well U-bottom microplate (Corning, USA) for centrifugation ( $500 \times g$  for 5 min). The pellets were resuspended in 4% PFA 4% sucrose in PBS for 20 min fixation at room temperature. The cells were washed with PBS and incubated with 50 mM  $\text{NH}_4\text{Cl}$  to quench the residual PFA. After blocking in 1% BSA in PBS for 15 min, the samples were incubated in PBS, 0.1% BSA with or without 1/200 dilution of FITC conjugated anti-human E-cadherin (BD Sciences, #612130) for 1 h. After washes, the cells were resuspended in PBS and analyzed by CytoFLEX flow cytometer (Beckman Coulter). For experiments using L2<sup>IncD</sup>GFP, the cells were fixed and analyzed by flow cytometry directly. The data were analyzed using FlowJo (version 10.0.7).

**Cell imaging and measurement of inclusion size.** The cells ( $0.1 \times 10^6$  cells/well) seeded on coverslips in a 24-well culture dish were infected with L2<sup>IncD</sup>GFP strain or with *C. trachomatis* serovar D strain. Twenty-four to 40 h later, the cells were fixed with 4% PFA 4% sucrose for 20 min at room temperature. After fixation, the cells were washed in PBS and incubated for 10 min in 0.05% (w/v) saponin, 0.1% (w/v) BSA and 0.5  $\mu\text{g}/\text{mL}$  of Hoechst 33342 (Thermo Fisher Scientific) to enable cell permeabilization and DNA staining. Coverslips were mounted on slides in a Mowiol solution. Serovar D-infected cells were stained with antibody against *Chlamydia* Cap1 and A488-conjugated secondary antibody. Images were acquired on an Axio observer Z1 microscope equipped with an ApoTomemodule (Zeiss, Germany) and a  $63 \times$  Apochromat lens. Images were taken with an ORCAflash4.OLT camera (Hamamatsu, Japan) using the software Zen. For characterizing epithelial cells, the fixed primary cells were incubated for 1 h in PBS, 0.1% (w/v) BSA and anti-human E-cadherin (1/200), followed with Cy5 conjugated anti-rat (1/500) secondary antibody, or anti-human cytokeratin 14 (GenTex, #GTX104124) and 18 (Invitrogen, #MA5-12104) followed with corresponding Cy5- and A488-conjugated secondary antibodies for an additional hour. DNA was labeled with 0.5  $\mu\text{g}/\text{mL}$  of Hoechst 33342 (Thermo Fisher Scientific). IncA was stained with rabbit anti-IncA antibodies (kind gift from T. Hackstadt, Rocky Mountain Laboratories). In certain experiment, the cells were fixed directly in the culture dish and observed under an Axio Observer microscope (Zeiss, Germany). The size of inclusions ( $\mu\text{m}^2$  in area) was measured using ImageJ software. All inclusions from at least 5 fields were counted.

**Progeny assay.** One hundred thousand cells were seeded in a 24-well plate. The next day cells were infected with L2<sup>IncD</sup>GFP bacteria at a MOI = 0.15 for HeLa cells, and with ten to twenty times more bacteria for primary epithelial cells, in order to reach about 15% infected cells in both cell types (this factor was later considered for the calculation of the progeny). Forty-eight hpi, cells were detached, lysed using glass beads and the supernatant was used to infect HeLa cells plated the day before ( $0.1 \times 10^6$  cells/well in a 24-well plate), in serial dilutions. Twenty-four hours later, cells with an infection lower than 30% (checked by microscopy) were detached and fixed as described above, before analysis by flow cytometry and determination of the bacterial titer.

**RT-PCR and quantitative PCR.** The adhered cells ( $0.1 \times 10^6$  cells/well) in 24-well plate were infected or not with *C. trachomatis* serovar L2 or D (MOI = 1 for HeLa cells and MOI = 10 (titrated in HeLa cells) for primary epithelial cells). Total RNAs were isolated 6 to 40 hpi for serovar L2 infection and 48 hpi for serovar D infection

with the RNeasy Mini Kit (Qiagen) and RNA concentrations were determined with a spectrophotometer NanoDrop (Thermo Fisher Scientific). Reverse transcription (RT) was performed using the M-MLV Reverse Transcriptase (Promega) and real-time quantitative PCR (qPCR) undertaken in triplicates on the complementary DNA (cDNA) with LightCycler 480 system (Roche) using SYBR Green Master I (Roche). Data were analyzed using the  $2^{-\Delta\Delta Ct}$  method with *actin* as a control gene and results were presented as fold changes compared to non-infected control. The primers were purchased from Eurogentec and are shown in Table S1.

**Statistical analysis.** The experimental data were recorded and analyzed using Prism8 (GraphPad). The non-paired Student's t-test was used for two group comparisons. P-values less than 0.05 were considered as significant.

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## Author contributions

C.F.T., C.L., B.M., B.N., T.V. and J.H. performed the experiments, H.C., A.K., A.C.D., Y.L.Z., E.V. and C.L.S. collected the clinical explants, A.S. and Y.Z.W. designed the study and wrote the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-021-85123-7>.

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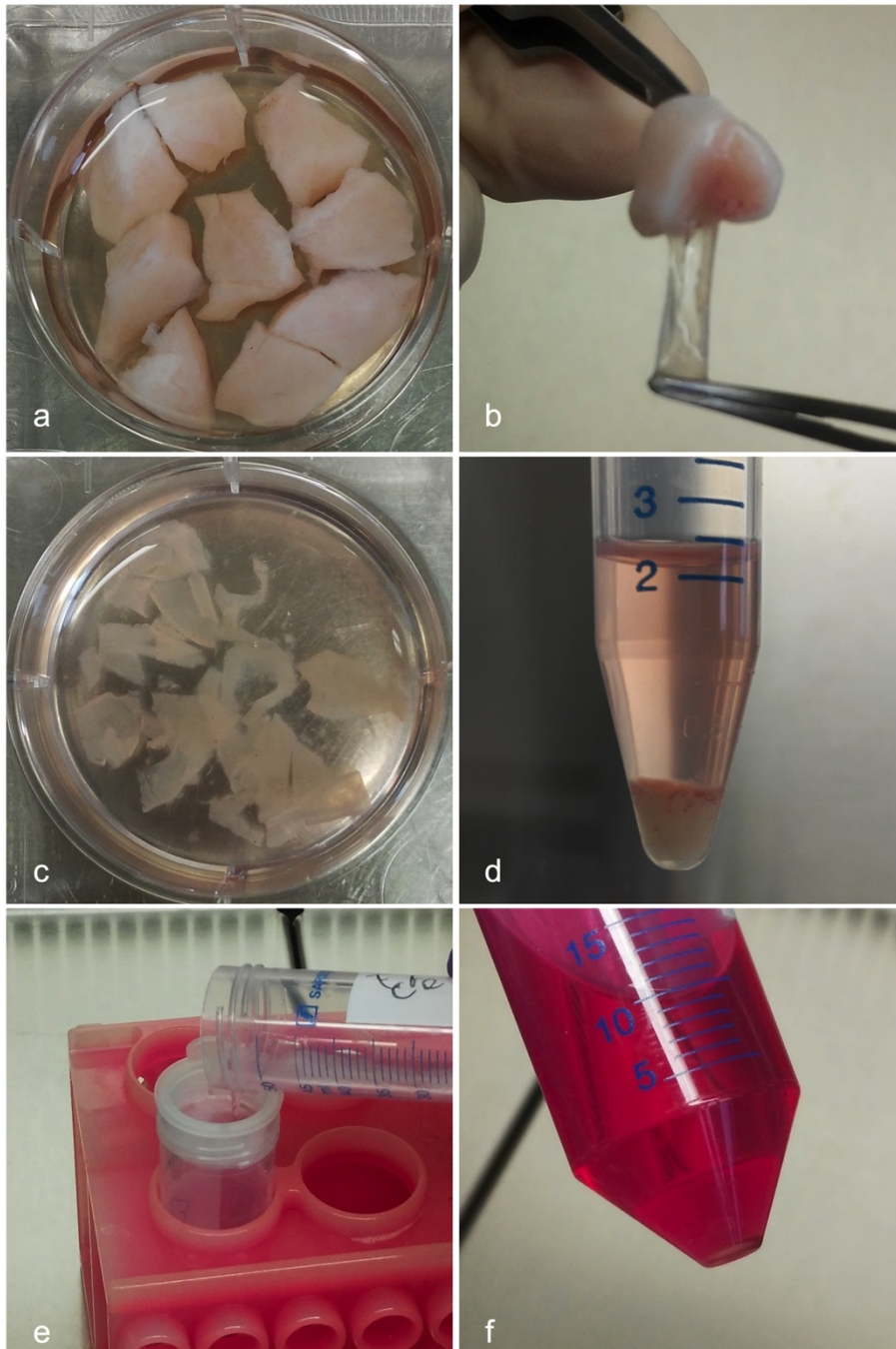
**Table S1: Primers used for real-time quantitative PCR**

		sense	anti-sense
1	actin	GGACTTCGAGCAAGAGATGG	GCAGTGATCTCCTTCTGCATC
2	IL-1 $\alpha$	AGATGCCTGAGATACCCAAAACC	CCAAGCACACCCAGTAGTCT
3	IL-1 $\beta$	CCTCTCTCTAATCAGCCCTCTG	GTCGGAGATTCGTAGCTGGA
4	IL-6	CACACAGACAGCCACTCACC	CATCCATCTTTTTCAGCCATC
5	IL-8	AGCCTTCCTGATTTCTGC	GCCCTCTCAAAAACCTTCTC
6	GM-CSF	TCCTGAACCTGAGTAGAGACAC	TGCTGCTTGTAGTGGCTGG
7	TNF $\alpha$	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
8	TGF $\beta$ 1	CTAATGGTGGAAACCCACAACG	TATCGCCAGGAATTGTTGCTG
9	MCP1	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
10	CCL5	CCAGCAGTCGTCTTTGTCAC	CTCTGGGTTGGCACACACTT
11	CXCL2	GGCAGAAAGCTTGTCTCAACCC	CTCCTTCAGGAACAGCCACCAA
12	IL-12p35	CCTTGCACTTCTGAAGAGATTGA	ACAGGGCCATCATAAAAAGAGGT
13	IL-12p40	TGACACCCCTGAAGAAGATG	TTTTGCCAGAGCCTAAGACC
14	IP10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
15	Eotaxin1	CTGAGGGGGACAAAGATGG	GGGAATAGAGGGCTGGAATG
16	Eotaxin3	ATCAGGCAGGAGGAGTTTG	AGCAGCAGGTCTTGGATATG
17	IL-11	GACCTACTGTCCTACCTGCG	AGTCTTCAGCAGCAGCAGTC



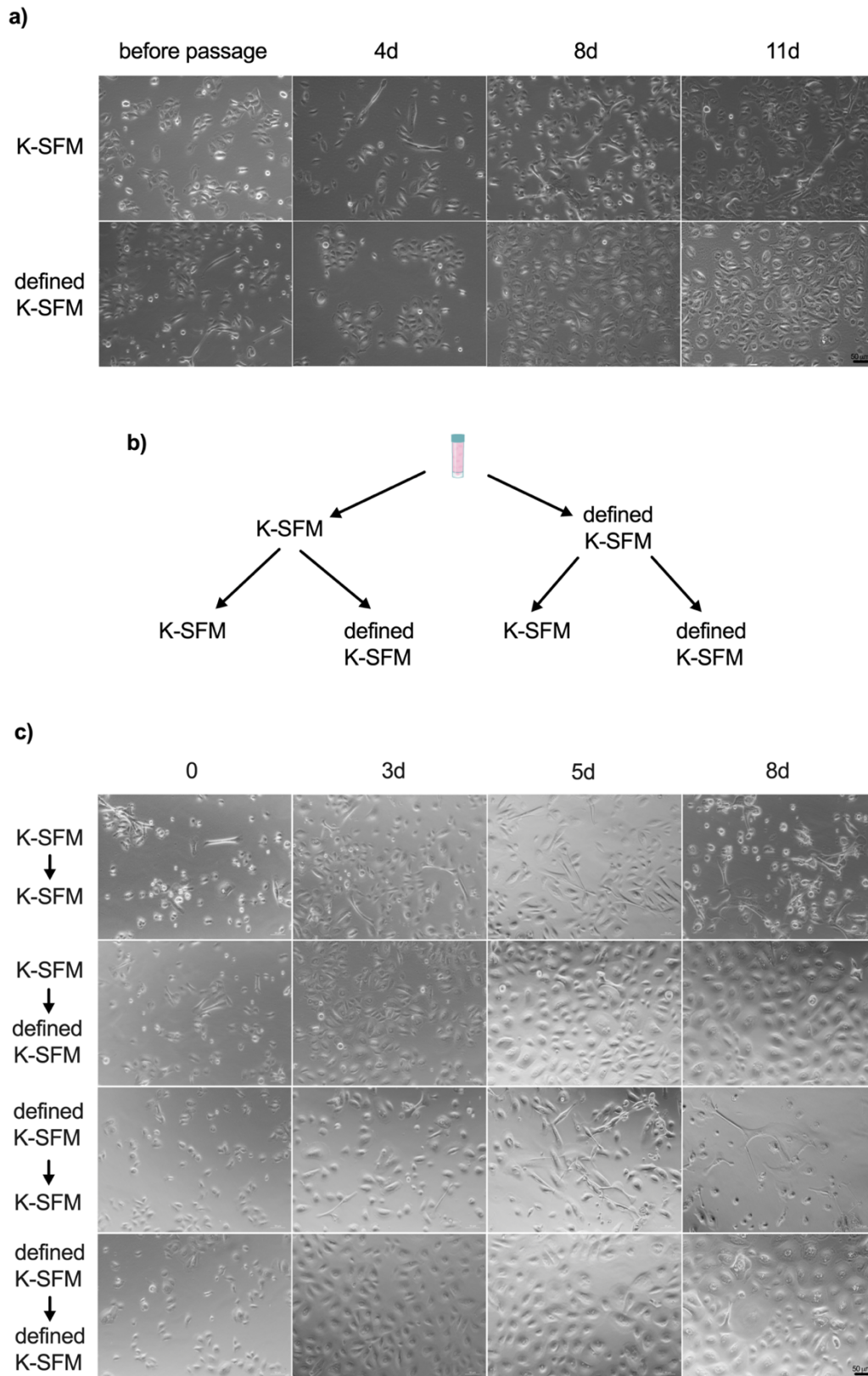
## Legends to the supplementary figures

**Fig S1**



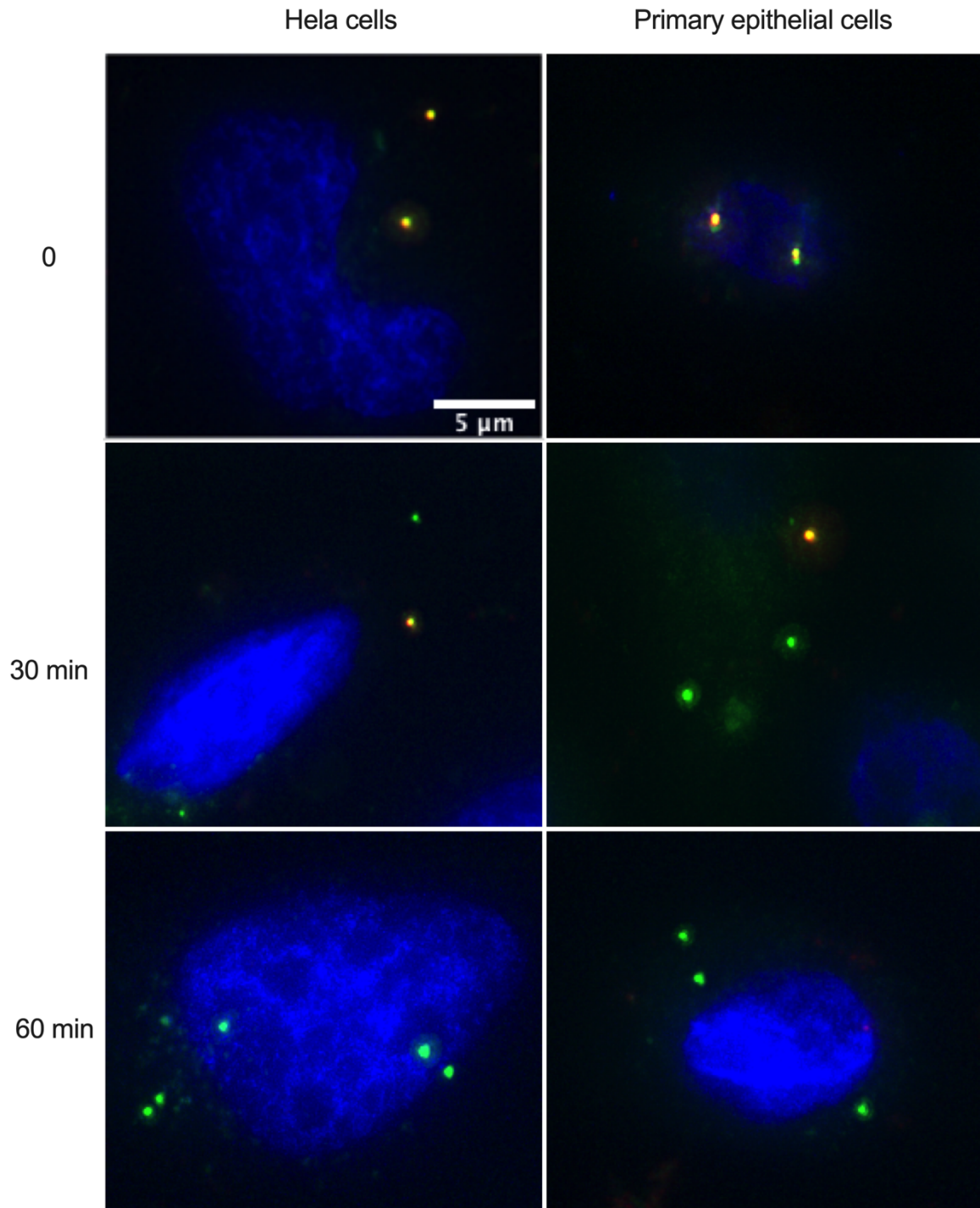
**Fig. S1. Key steps in the isolation of primary epithelial cells.** a) A cervical explant was cut into small pieces and digested with Dispase II. b) The mucosal layer was mechanically teased off after enzymatic digestion. c-d) Mucosal layers were pooled together and pelleted by centrifugation. e) Trypsin/EDTA-treated cell suspension was passed through a 40  $\mu\text{m}$  cell strainer to remove debris. f) Cell pellet containing epithelial cells.

**Fig. S2**



**Fig S2. Defined K-SFM medium inhibits culture contamination by fibroblasts.** a) One thawed vial of primary epithelial cells was split into two parts and grown either in K-SFM or in defined K-SFM. b-c) Eleven days after culture, these cells were then re-plated in the indicated culture medium. The morphology of the cells for each culture condition was monitored at regular intervals over 8 days. Two independent experiments were performed.

**Fig. S3**



**Fig. S3. Representative example of raw data for bacterial entry experiments.** Cells were incubated with L2<sup>incD</sup>GFP bacteria for 30 min at 4°C, before transfer to 37°C. At the indicated times the cells were fixed and external bacteria were labeled with antibody against *Chlamydia* MOMP and Cy5-conjugated secondary antibody. Internalized bacteria (GFP positive) and extracellular bacteria (GFP and Cy5 double positive) appear in green and yellow, respectively.

Fig. S4

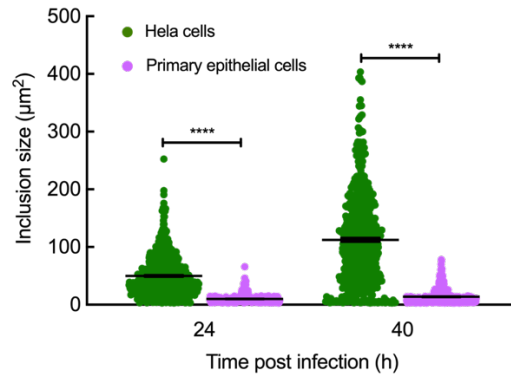


Fig. S4. Quantification of the inclusion sizes on primary epithelial cells infected with *C. trachomatis* serovar D and stained as in Fig. 2c. Two independent experiments were performed using primary epithelial cells from different donors. Each individual point represents one inclusion ( $n > 5$  fields per experiment). \*\*\*\* $p < 0.0001$ .

Fig. S5

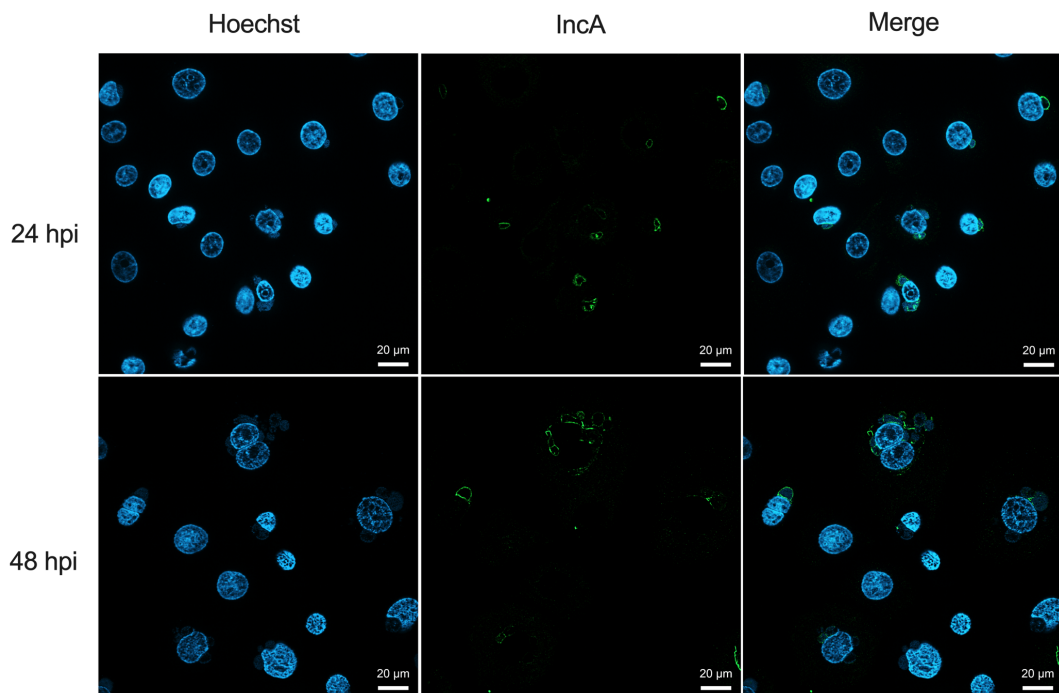
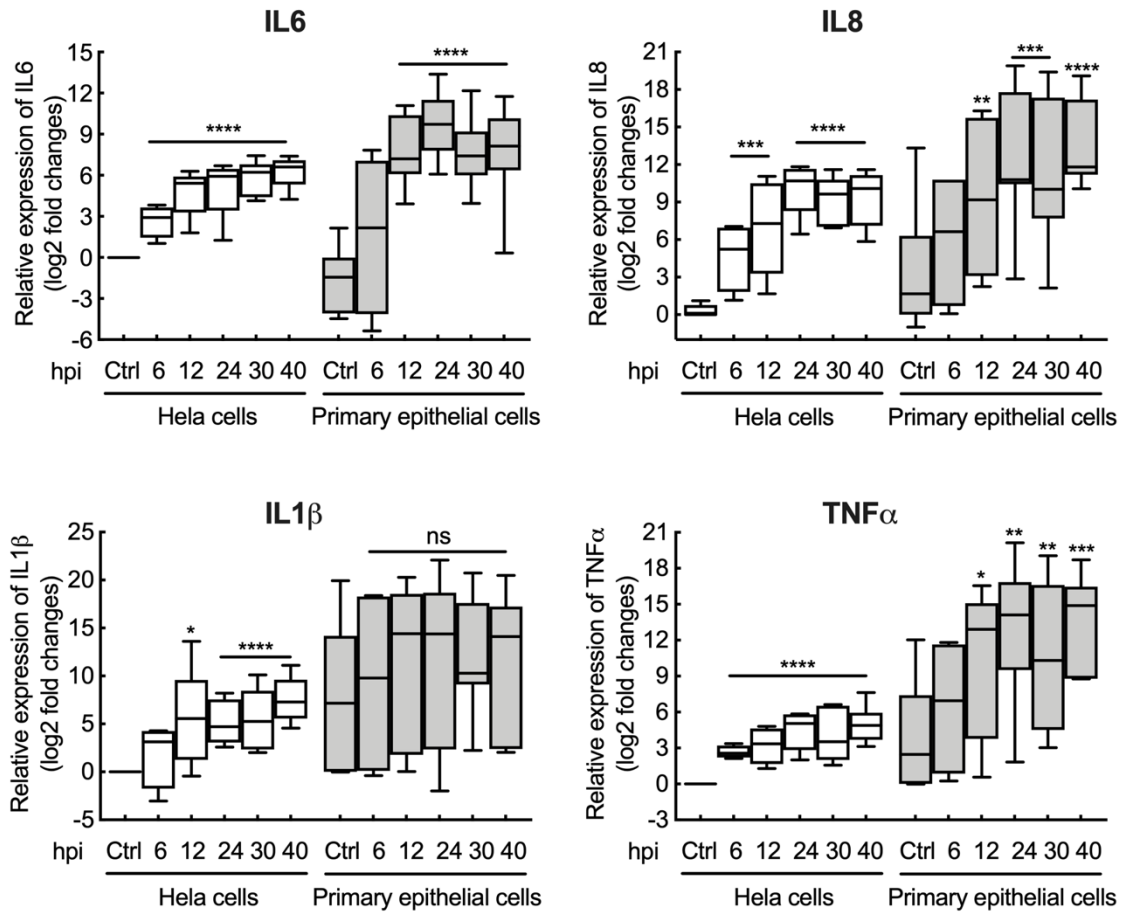


Fig. S5. Expression of IncA in primary epithelial cells. Primary epithelial cells were infected with *C. trachomatis* serovar L2. At the indicated times post infection, cells were fixed and stained with antibody against the bacterial inclusion protein IncA and A488-conjugated secondary antibody. DNA was labeled with Hoechst. The images are representative of observations made using cells from 4 different donors.

**Fig. S6**



**Fig. S6. Kinetics of *Chlamydia*-induced inflammatory response in HeLa and primary epithelial cells.** HeLa and primary epithelial cells were infected with *C. trachomatis* serovar L2 (MOI= 1 for HeLa cells and MOI= 10 for primary epithelial cells, respectively). At the indicated times post infection, RNAs were extracted from cell lysates and the transcripts for the indicated genes were measured by real-time quantitative PCR and normalized to  $\beta$ -actin following the  $2^{-\Delta\Delta C_t}$  method. The data were presented as Whiskers boxplot in Log<sub>2</sub> fold changes of mRNA compared to uninfected HeLa cells. Each plot displays data obtained in at least 3 independent experiments and each individual time-point in the primary cell group represents the results in cells isolated from at least 4 donors. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 and \*\*\*\*p<0.0001, compared to corresponding control. ns, not significant.

## **Part II. IFN-I exacerbates the pro-inflammatory response of epithelial cells to *C. trachomatis* infection**

### **Manuscript summary**

The inflammation in the FGT induced by *Chlamydia trachomatis* infection can cause scar formation and tissue damage with subsequent consequences such as tubal factor infertility. The anti-viral property of IFN-I has been well studied, but the consequence of its production during *Chlamydia* infection are poorly understood. A higher capacity to clear *Chlamydia* and less tissue damage have been reported in IFN receptor -deficient mice compared to wildtype animals, as well as in *Chlamydia*-infected mice treated with IFN-I neutralizing antibodies. Intriguingly, clinical studies showed that IFN-inducible protein 10 (IP10), a cytokine specifically related to the IFN response, was associated with ascending *C. trachomatis* infection in the FGT of women and with *Chlamydia* infection in patients with pelvic inflammatory disease. Based on these observations we hypothesized that IFN-I production during *Chlamydia* infection favored bacterial dissemination and inflammatory damage in the FGT. The present study aimed at monitoring the consequence of IFN-I treatment in *C. trachomatis*-induced inflammation by epithelial cells, and to reveal the signaling pathways implicated.

Using the primary epithelial cell model established in the first part of my thesis, we showed that the presence of IFN $\alpha$  or IFN $\beta$  enhanced the host inflammatory response to *Chlamydia* infection compared to *Chlamydia* alone, while IFN $\alpha$  or IFN $\beta$  alone had no effect. These observations suggested IFN-I exacerbated *Chlamydia*-induced inflammation. Similar results were observed in HeLa cells that were then used to examine this synergetic process between IFN-I and *Chlamydia* at a mechanistic level. Given that IFN-I alone did not trigger inflammation in our cellular model and that pathogen recognition receptor(s) (PRRs) are essential to initiate pathogen-induced inflammation, we hypothesized that IFN-I may up-regulate the expression of pathogen recognition receptor(s) (PRRs), therefore promoting *Chlamydia* detection and subsequent inflammation. Small interfering RNA (siRNA) screen of PRRs associated with *Chlamydia* infection identified IFN-induced TLR3 as a key mediator in the synergy of IFN-I and bacteria, which was further confirmed in TLR3-deficient HeLa cells. Using siRNA and pharmacological inhibitors, we showed that the IFN-I signaling cascades STAT1 and PI3K/AKT-mTOR mediated IFN-induced TLR3 expression. We also investigated how TLR3 modulated the host inflammatory response to *Chlamydia* infection and found that ERK signaling was involved.

In summary, we demonstrated that IFN-I exacerbated *Chlamydia*-triggered host inflammatory response by increasing TLR3 expression. These finding give a molecular basis to the detrimental role of IFN-I in *Chlamydia* infection process. This cytokine, and the TLR3 intermediate, can be considered as therapeutic target to alleviate inflammatory tissue damage in the FGT of patients.

# **IFN-I exacerbates the proinflammatory response of epithelial cells to *Chlamydia trachomatis* infection by enhancing TLR3 expression**

Chongfa Tang, Béatrice Niragire, Zhi Li, Sandra Pelligrini, Agathe Subtil, Yongzheng Wu

## **Abstract**

The inflammation induced by the infection of the female genital tract (FGT) by the bacterium *Chlamydia trachomatis* can cause severe consequences. Recent studies in patients and mice suggested that type I interferon (IFN-I) may hamper bacterial clearing and be associated with susceptibility to *C. trachomatis* infection. This study aimed at elucidating the consequence of elevated IFN-I levels in *C. trachomatis*-induced inflammation by epithelial cells, and to decipher the molecular pathway(s) involved. We showed that combination of IFN-I and *Chlamydia* infection resulted in a much stronger induction of inflammation than *Chlamydia* alone, while IFN-I alone had no effect, indicating that IFN-I exacerbated *Chlamydia*-induced inflammation. IFN-I elicited phosphorylation of STAT1. Silencing STAT1 or inhibiting AKT and mTOR significantly attenuated the synergetic effect of IFN-I and bacteria on inflammation. Interestingly, inhibition of ERK and p38 also blocked this synergistic effect, although ERK and p38 were not activated by IFN-I. We hypothesized that IFN-I enhanced the expression of pathogen recognition receptor(s) of the host, thereby potentiating bacterial detection. Supporting this hypothesis, we demonstrated that IFN-I, but not *C. trachomatis*, increased the expression of Toll-like receptor 3 (TLR3). Silencing or knocking-out TLR3 expression prevented the synergetic effect between infection and IFN-I. Consistently, silencing STAT1 or inhibiting AKT and mTOR also attenuated TLR3 expression induced by IFN-I, and strongly attenuated the synergetic effect between infection and IFN-I on inflammation. Finally, we examined how TLR3 modulated *Chlamydia*-induced inflammation, and our data pointed to the TLR3-induced MAPK/ERK signaling cascade. Taken together, our data indicate that IFN-I exacerbates the host inflammatory response triggered by *C. trachomatis* infection by increasing TLR3 expression and subsequent ERK activation, leading to hyperinflammation. A strong IFN-I response to infection might therefore favor deleterious tissue damage in the FGT.

## **Introduction**

Infection by the obligate intracellular bacterium *Chlamydia trachomatis* is the leading cause of sexually transmitted infections (STIs) of bacterial origin (World Health Organization, 2019). The bacteria multiply principally in epithelial cells of the reproductive tract of men and women. In women, the bacteria can ascend from the vagina to the cervix, the endometrium and the fallopian tubes. Epithelial cells from these tissues constitute the first line of defense against *C. trachomatis* infection. In particular, their sensing of the *Chlamydia* by the potential pattern

recognition receptors (PRRs) such as TLR2, TLR3, TLR4 and NOD1 (Darville et al., 2003; Derbigny et al., 2012; Rodriguez et al., 2006; Welter-Stahl et al., 2006a), leads to a pro-inflammatory response, with the secretion of cytokines and chemokines, that will contribute to bacterial clearance by immune cells. In some women, the inflammation induced by infection can result in tissue damage and scar formation, eventually causing severe consequences, such as pelvic inflammatory disease and tubal infertility.

Type I Interferon (IFN-I) belongs to a group of cytokines, that can activate the signaling cascades in a variety of cell types, including JAK1/TYK2 and downstream Stat1, MAPKK/Erk, PI3K/AKT/p38 and PI3K/AKT/mTOR, therefore, inducing the expression of interferon-stimulated genes (Chen et al., 2017). As the well-known members of this family, IFN $\alpha$  and  $\beta$  exert an important role in the infectious diseases. While their protective properties against viral infections are extremely well documented, the effects on bacterial infections are less studied (Carrero, 2013; McNab et al., 2015). IFN-I production appears to be beneficial to the host during infections by *S. pyogenes* (Castiglia et al., 2016), *Legionella pneumophila* (Opitz et al., 2006; Schiavoni et al., 2004), *Bacillus anthracis* (Gold et al., 2004), *Shigella flexneri* and *Salmonella typhimurium* (Bukholm et al., 1984; Niesel et al., 1986).

Regarding the effect of IFN-I on *Chlamydia* infection, early works have reported an inhibitory role of IFN-I on *C. trachomatis* and *C. pneumoniae* replication in epithelial cells (Ishihara et al., 2005; Rothfuchs et al., 2006). However, mice with IFN receptor gene knocked out (IFNAR KO mice) or deficient for IFN-I signaling cleared chlamydial infection earlier than control mice and developed less oviduct pathology (Nagarajan et al., 2008). In addition, anti-IFN-I neutralizing antibodies also inhibited bacterial replication and subsequent tissue damage in the FGT of *Chlamydia*-infected mice (Prantner et al., 2011). These data indicate that IFN-I production favors bacterial proliferation and tissue damage in a mouse model of *Chlamydia* infection. Furthermore a recent retrospective study showed that ascending *C. trachomatis* infection in the FGT of patients correlated with increased levels of interferon-inducible protein 10 (IP10), a cytokine specifically related to the IFN-I response (Poston et al., 2019; Zheng et al., 2018). IFN-I is synthesized by many cell types, including immune cells. It is in particular secreted by epithelial cells of the FGT upon *C. trachomatis* infection both in humans and mice, including in a organoid model of infection (Derbigny et al., 2007; Lad et al., 2005; Prantner et al., 2011; Xia et al., 2003).

We recently described a simplified protocol for isolating primary epithelial cells from ectocervical specimen from women undergoing surgery. Upon investigation of their innate response to *C. trachomatis* infection we observed that the pro-inflammatory response, e.g. interleukin 6 (IL-6) and interleukin 8 (IL-8) production, was largely enhanced in the presence of IFN-I, while the cytokine had no effect when applied alone. In this paper, we characterize the molecular mechanism underlying the synergistic effect between IFN-I and *C. trachomatis* infection on the pro-inflammatory response of epithelial cells to infection, and reveal the central role of the PRR TLR3 in this process.



## Material and methods

### *Cell line and culture*

The human cervical epithelial cell line HeLa was from ATCC (Virginia, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutaMAX<sup>®</sup> (Gibco, France) and 10% heat-inactivated fetal calf serum (FCS). For the experiments, cells ( $0.3 \times 10^6$  cells/ml) were seeded in 12-well plate. After 4 h to overnight culture, cells were rinsed twice with pre-warmed DMEM to remove non-adhered cells prior to *Chlamydia* infection or IFN treatment. After infection and/or treatment, the culture media were collected, and cells were wash twice using PBS. The cell-free culture media after centrifugation (5 000 rpm at 4 °C for 15 min) were aliquoted and frozen at -20 °C for later ELISA assay. The cells were either detached by incubating with 0.5 mM EDTA in PBS for 5 min at 37 °C before fixation with 2% paraformaldehyde for the flow cytometry analysis, or lysed in urea buffer (30 mM Tris, 150 mM NaCl, 8 M urea, 1% SDS, pH= 8.0) or ALT buffer (Qiagen) for protein and transcriptional analysis, respectively. In certain experiments, the cells were pre-treated with pharmacological inhibitors for 1 h before bacterial infection and/or IFN treatment.

TLR3-KO HeLa cells were established as described previously (Ran et al., 2013). In brief, 0.5 µg of pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (Addgene #62988) containing guide RNA (gRNA) duplex sequence of human TLR3 (F: 5'-CACCGTACCAGCCGCCAACTTCACA-3'; R: 5'-AAACTGTGAAGTTGGCGGCTGGTAC-3') inserted with BbsI enzyme site, was transfected into pre-adhered HeLa cells ( $0.15 \times 10^6$  cells/well, 24-well plate) using 1 µl of jetPRIM<sup>®</sup> (Polyplus). Four hours later, fresh medium was replaced containing puromycin (2 µg/ml) and cells were incubated for 24 h. The surviving cells were then detached, counted and serially diluted to collect individual clones in 96-well plates. After expanding the individual clones, genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) and sent for sequencing using primer 5'-CTCAGGTACCCGATGATCTACC-3'.

### *Isolation of primary epithelial cells from clinical samples*

Ecto-cervical explants from patients undergoing hysterectomy were collected and primary epithelial cells were isolated as described (Tang et al., 2021a). Briefly, after digested the explant with Dispase<sup>®</sup> II (Roche) solution (2.4 units/mL in RPMI 1640) overnight at 4 °C, the mucosal layer was separated mechanically from the tissue, pooled together and further treated with 0.25% Trypsin, 0.02% EDTA solution (Sigma-Aldrich) for 10 min at 37 °C to dissociate epithelial cells. The cell suspension was passed through a 40 µm mesh to get rid of the non-digested debris. The pelleted cells by centrifugation ( $2\ 000 \times g$  for 15 min at 4 °C) were resuspended into keratinocyte serum-free media supplemented with 5 µg/l of human recombinant epidermal growth factor (EGF) and 50 mg/l bovine pituitary extract (BPE) (K-SFM, Thermo Fisher Scientific), and expanded in 75 cm<sup>2</sup> culture flask for 10-15 days till confluency before using for the experiments. This protocol was approved by the French Ethical Committee 'CPP Ile de France 1' on May 9, 2016. Approval and authorization of the National Data Protection authority

(‘Commission Nationale de l’Informatique et des Libertés’ CNIL) have been obtained for the research protocol. All participants gave written informed consent in accordance with the Declaration of Helsinki principles.

#### *Bacterial preparation and infection*

C. LGV-L2 (434/Bu/ATCC) strain was propagated in HeLa cells and purified as described (Scidmore, 2005). The purified elementary bodies (EBs) of *Chlamydia* were stored in 220 mM sucrose, 10 mM sodium phosphate (8 mM Na<sub>2</sub>HPO<sub>4</sub>- 2 mM NaH<sub>2</sub>PO<sub>4</sub>), 0.50 mM l-glutamic acid) (SPG buffer) at -80 °C.

For infection, adhered cells were incubated with bacteria suspended in DMEM containing 10% FCS for different times (depending on the experiments) before harvesting the samples.

#### *Flow cytometry*

Twenty-four hours post IFN treatment and/or *Chlamydia* infection, the cells were incubated with 5 µg/ml brefeldin A solution (Biolegend, #420601) for 6 h to block cytokine secretion. Cells were then detached with 0.5 mM EDTA in PBS. The detached cells were fixed by 4% PFA 4% sucrose in PBS for 20 min at room temperature, followed with quenching by 50 mM NH<sub>4</sub>Cl in PBS for 10 min. The cells were then permeabilized with 0.3% (v/v) Triton X-100 in PBS for 10 min and blocked in 1% BSA in PBS for 1 h, following with incubation in PBS, 1 % BSA with or without 1/40 dilution of PE/Cyanine7-conjugated anti-human IL-6 (Biolegend, #501119) or 1/80 dilution of PE-conjugated anti-human IL-8 (Biolegend, #511408) for 3 h. After washes, the cells were resuspended in PBS and analyzed by CytoFLEX flow cytometer (Beckman Coulter). The data were analyzed using FlowJo (version 10.0.7).

#### *siRNA treatment*

One and half microliter siRNA (10 µM stock) was mixed with 0.5 µl Lipofectamine iMax (Invitrogen) in 50 µl of DMEM media (Gibco®) for 10 min at room temperature. The mixture was added into pre-seeded cells in culture dish (0.15×10<sup>6</sup> cells/well for 24-well plate) suspended in 0.45 ml DMEM/10%FCS medium, mixed and incubated for 24 h before treatment or infection.

#### *Immunofluorescence*

p65-GFP expressing HeLa cells (Connor et al., 2021) seeded on coverslips in a 24-well culture dish (0.1×10<sup>6</sup> cells/well) were treated with recombinant human IL-1β (10 ng/ml) for 30 min, or with recombination of IFNβ (2.5 ng/ml) and LGV-L2 strain (MOI = 1) from 4 to 36 h. The cells were then fixed with 4% PFA and 4% sucrose for 20 min at room temperature, followed with quenching with 50 mM NH<sub>4</sub>Cl in PBS for 10 min. The cells were then permeabilized with 0.3% (v/v) Triton X-100, 1% (w/v) BSA. DNA was stained using 0.5 µg/ml Hoechst 33342 (Thermo Fisher Scientific, #H3570) suspended in PBS containing 1 % of BSA. Coverslips were mounted

on slides in a Mowiol solution. Images were acquired on an Axio observer Z1 microscope equipped with an ApoTome module (Zeiss, Germany) and a 63× Apochromat lens. Images were taken with an ORCA-flash 4.0 LT camera (Hamamatsu, Japan) using the software Zen.

#### *RT-PCR and quantitative PCR*

Adhered cells ( $0.15 \times 10^6$  cells/well) in 24-well plate were treated with the indicated concentration of recombinant IFN-I, or infected with *C. trachomatis* at MOI = 1 for HeLa cells and MOI = 10 for primary epithelial cells. At these doses of bacteria, majority of cells (>85%) were infected (Tang et al., 2021a). Total RNAs were isolated 24 h post IFN-I treatment or 40 hpi with the RNeasy Mini Kit (Qiagen) and RNA concentrations were determined with a spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific). Reverse transcription (RT) was performed using the M-MLV Reverse Transcriptase (Promega) and quantitative PCR (qPCR) undertaken on the complementary DNA (cDNA) with LightCycler 480 system using SYBR Green Master I (Roche). Data were analyzed using the  $2^{-\Delta\Delta C_t}$  method with the *actin* gene as a control gene and results were presented as relative quantity (RQ) compared to uninfected control cells.

The primers used are shown in Table 1.

**Table 1. The sequence of primers used for real-time quantitative PCR**

		sense	anti-sense
1	actin	GGACTTCGAGCAAGAGATGG	GCAGTGATCTCCTTCTGCATC
2	IL-6	CACACAGACAGCCACTCACC	CATCCATCTTTTTTCAGCCATC
3	IL-8	AGCCTTCCTGATTTCTGC	GCCCTCTTCAAAAACCTTCTC
4	TLR2	ATCCTCCAATCAGGCTTCTCT	GGACAGGTCAAGGCTTTTTTACA
5	TLR3	GTGATGCTTTCTCTTGTTG	GTTGGCTATGTTGTTGTTGC
6	TLR4	TTTGGACAGTTCCACATTGA	AAGCATTCCCACCTTTGTTGG
7	NOD1	ACTGAAAAGCAATCGGGAAGCTT	CACACACAATCTCCGCATCTT
8	STAT1	ACAAGGTGGCAGGATGTCTC	AAACGGATGGTGGCAAATG

#### *Western blot and ELISA assay*

Equal volumes of cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with the proper primary antibodies diluted in PBS containing 5% (w/v) fat-free milk and 0.01% (v/v) Tween-20. Primary antibodies used were rabbit anti-human STAT1, rabbit anti-human phosphorylated STAT1, ERK and p38, which were purchased from Cell Signaling Technology, homemade rabbit antibody against the heat shock protein 60 of *Chlamydia*, and mouse anti-human  $\beta$ -actin (Sigma, #A5441).

Immunoblots were analyzed using horseradish peroxidase secondary antibodies (Abliance), and chemiluminescence was analyzed on a ChemiDoc Touch Imaging System (Bio-Rad).

The DuoSet ELISA kit (R&D systems) was used to measure cytokine secretion in the culture medium according to the manual instruction.

### *Statistical analysis*

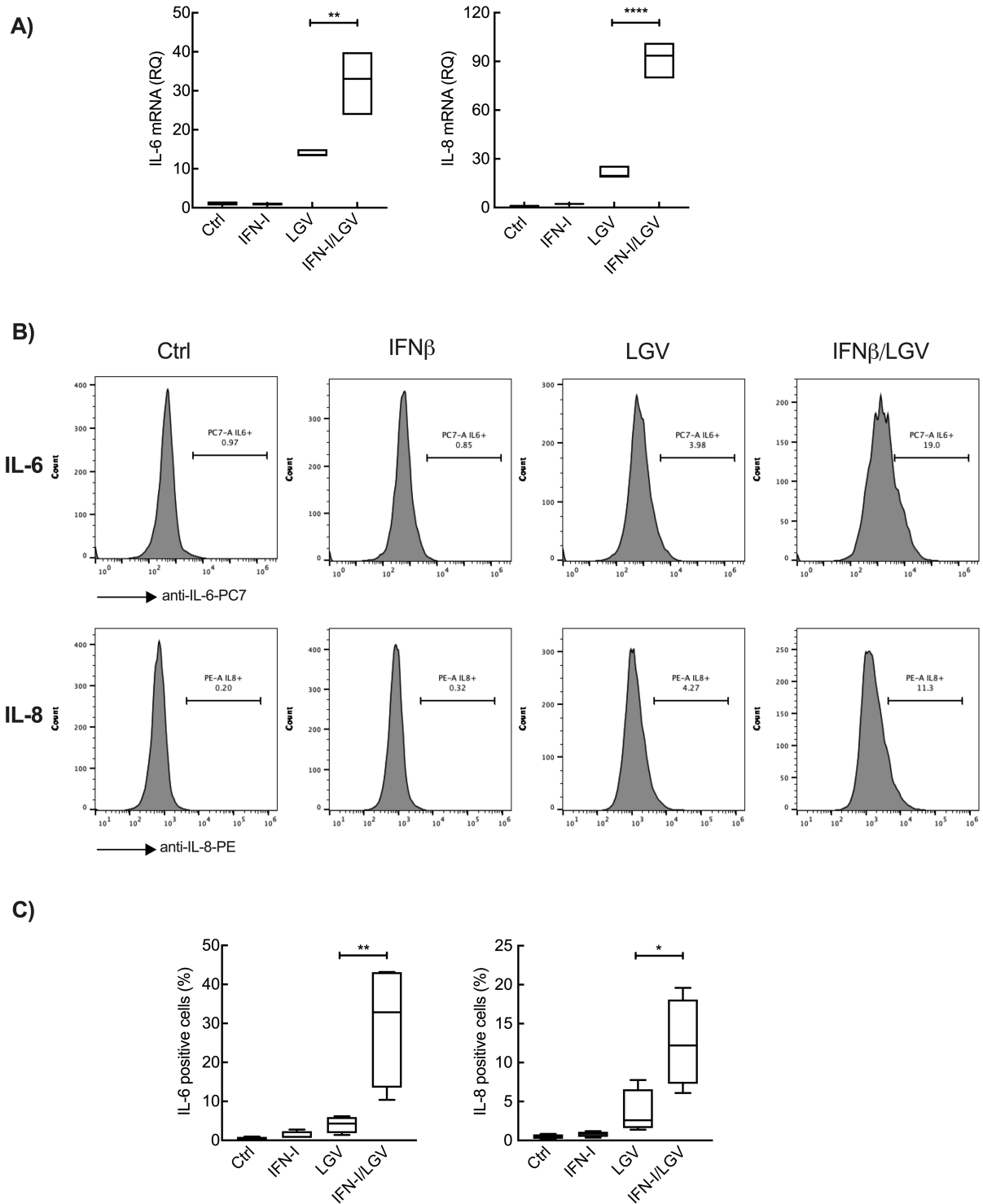
The experimental data were recorded and analyzed using Prism9 (GraphPad). Non-paired t-tests and one-way ANOVA were used for two group and multiple group comparisons, respectively.

## **Results**

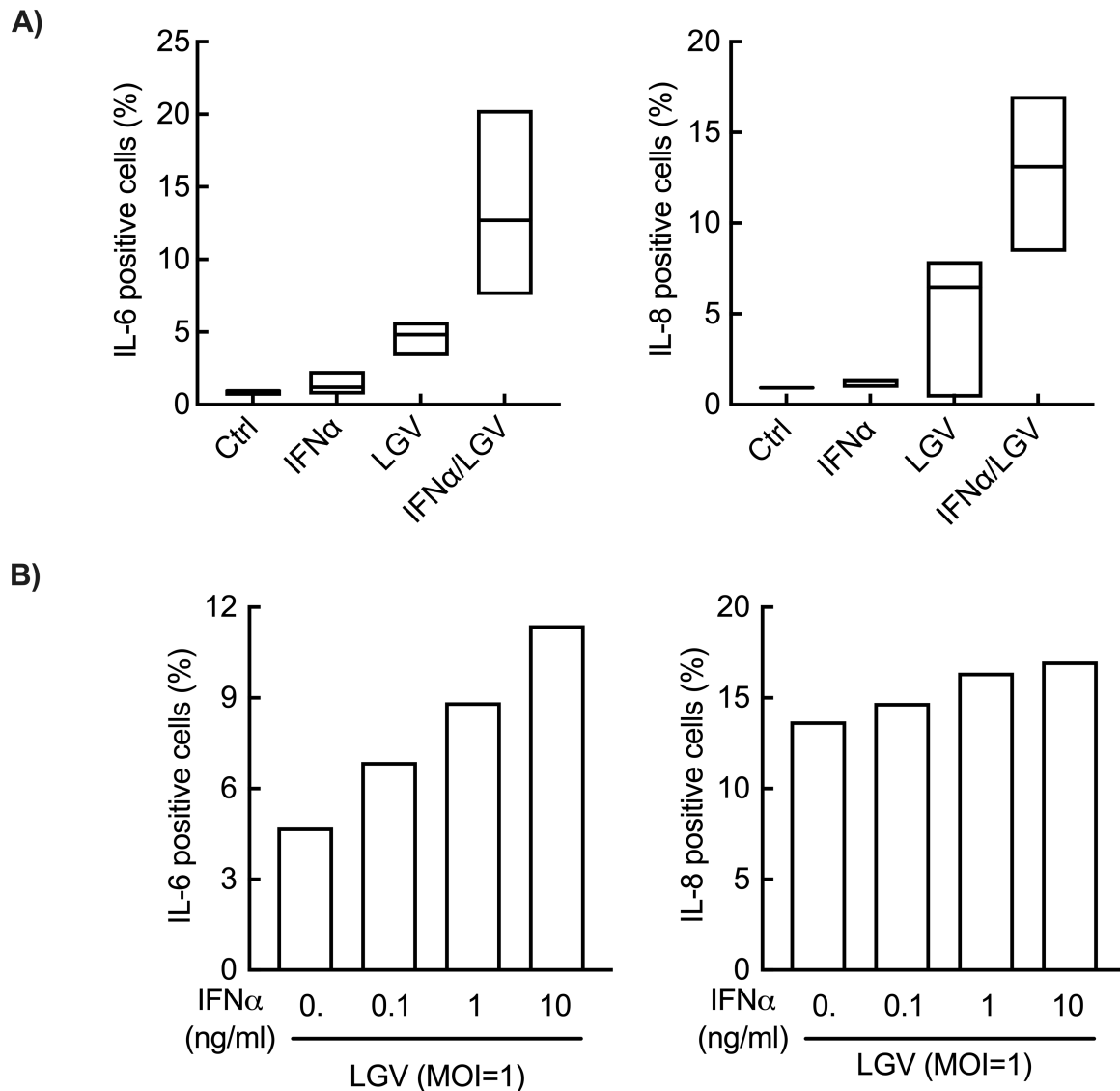
### I. IFN-I enhances the inflammatory response to *C. trachomatis* infection

To determine the effect of IFN-I on the inflammatory response to infection, we infected primary epithelial cells with *C. trachomatis*, in the presence or absence of recombinant human IFN $\beta$ , and measured the transcripts level for two prominent proinflammatory cytokines in *C. trachomatis* infection, interleukin 6 (IL-6) and 8 (IL-8) (Cunningham et al., 2013; Rasmussen et al., 1997; Tang et al., 2021a). For both cases transcription was strongly enhanced by IFN $\beta$  treatment, when IFN $\beta$  alone had no effect (Fig. 38A). To test whether this increase in transcription correlates with increase in cytokine levels we used flow cytometry to detect IL-6 and IL-8. This experiment requires higher cell numbers and was therefore conducted in HeLa cells. Infected cells were treated or not with IFN $\beta$  for 30 h. In the last 6 h of infection brefeldin A was added to the culture medium to block protein secretion. The cells were then fixed, permeabilized, and stained with antibodies against IL-6 or IL-8 coupled to a fluorochrome for analysis by flow cytometry. We observed addition of IFN $\beta$  increased the percentage of cells positive for IL-6 and IL-8, compared to infection alone (Fig. 38B, 38C). Again, IFN $\beta$  alone did not elicit IL-6 nor IL-8 production in HeLa cells (Fig. 38B, 38C). Similar results were obtained using IFN $\alpha$  (Fig. 39A), and the response was dose-dependent (Fig. 39B). These data suggested a synergetic effect of IFN $\alpha$  and IFN $\beta$  on the *Chlamydia*-induced inflammation. IFN $\beta$  was then used in the rest of the study.

Altogether, these data indicate that IFN-I exacerbates the inflammation triggered by *C. trachomatis* infection in primary epithelial cells as well as in HeLa cells.



**Figure 38. IFN-I enhances the inflammatory response of epithelial cells to *C. trachomatis* infection.** (A) Primary cervical epithelial cells isolated from patients were incubated with IFN $\beta$  (2.5 ng/ml) for 24 h, or with *C. trachomatis* (MOI = 10) for 40 h in the absence or presence of IFN $\beta$ . The expression of inflammatory cytokines IL-6 & IL-8 was detected by quantitative PCR. (B-C) Adhered HeLa cells were incubated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for 24 h prior to adding brefeldin A (5  $\mu$ g/ml) for additional 6 h incubation. After the treatment, the intracellular IL-6 and IL-8 was examined by flow cytometry using anti-human IL-6-PC7 and IL-8-PE antibodies, respectively. Histogramy was used as representative of the experiments (B) and the quantification of results from 4 experiments was presented as box and whiskers plot (C).

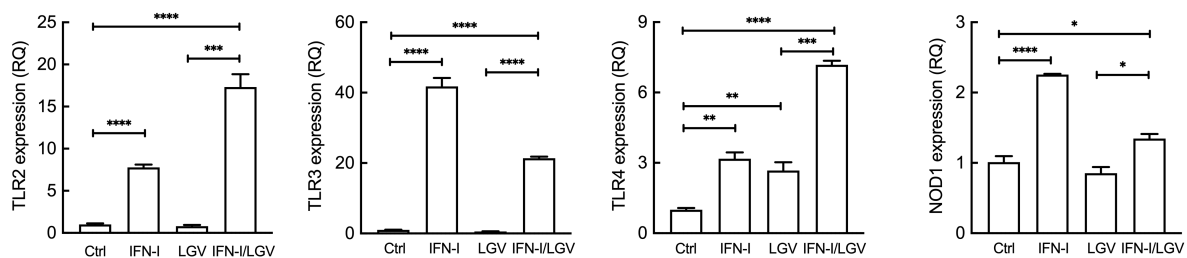


**Figure 39. IFN $\alpha$  exacerbates *Chlamydia*-induced inflammation by HeLa cells.** (A) The cells were incubated with IFN $\alpha$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for 24 h before adding brefeldin A (5  $\mu$ g/ml) for additional 6 h. The stained cells with anti-human IL-6-PC7 and IL-8-PE were analyzed to check the synthesis of IL-6 & IL-8. (B) The same as in A, except for the IFN $\alpha$  treatment at the indicated concentration.

## II. The synergy between IFN-I and *Chlamydia* infection is mediated by the upregulation of TLR3 expression

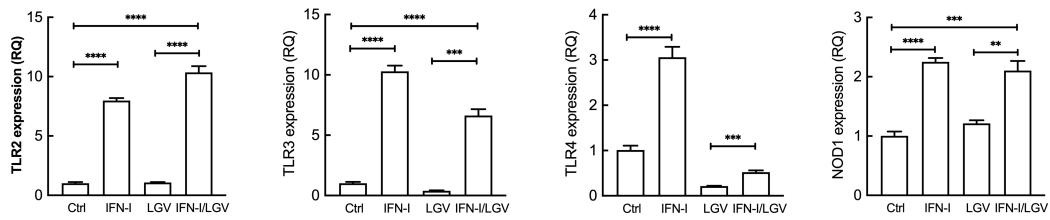
IFN-I itself did not elicit IL-6 nor IL-8 synthesis, while *Chlamydia* infection alone did (Fig. 38). Upon infection, host cells sense the invading microorganisms by recognition of pathogen associated molecular patterns (PAMPs), via specific PRRs distributed either on their surface (e.g. TLR2, 4 and 6), in the cytoplasm (e.g. NODs) or in endosomes (e.g. TLR3, 7, 9) (Janeway, 1989, 1992). Activation of these PRRs triggers signaling cascades, that eventually lead to expression of genes involved in innate immunity such as pro-inflammatory cytokines. We hypothesized that IFN-I might facilitate the detection of the bacteria, and thereby enhance the inflammatory response to the infection. To test this hypothesis, we first screened the expression of PRRs TLR2/3/4 and NOD1, that were previously implicated in the detection of *C.*

*trachomatis* (Darville et al., 2003; Derbigny et al., 2012; Rodriguez et al., 2006; Welter-Stahl et al., 2006b). IFN-I stimulated the expression of TLR2, TLR3, TLR4 and NOD1 by primary epithelial cells, compared to non-treatment and infection alone (Fig. 41A). Similar results were obtained in HeLa cells that the transcription of *TLR2/3/4* and *NOD1* were significantly upregulated by IFN-I compared to infection alone (Fig. 40). Silencing *TLR2*, *TLR4* and *NOD1* in HeLa cells did not affect the level of IL-6 or IL-8 production upon *Chlamydia* infection in the presence of IFN $\beta$  (data not shown), while silencing *TLR3* did (Fig. 41B). Interestingly, *Chlamydia* infection alone did not trigger the expression of TLR3, it actually tended to repress its transcription (Fig. 40, 41A). To confirm these results, we mutagenized the *TLR3* gene in HeLa cell using CRISPR-Cas9. Two clones were obtained, that displayed a deletion of 5 (clone 59) and 6 (clone 23) base pairs within exon 2 of the *TLR3* gene, respectively (Fig. 41C). In both clones, the production of IL-6 (Fig. 41D) and IL-8 (Fig. 41E) upon *Chlamydia* infection in the presence of IFN-I was similar to its level in untreated infected cells, indicating that the loss of TLR3 expression abrogates the synergetic effect between IFN-I and infection. These data strongly support the hypothesis that IFN-I acts by facilitating *Chlamydia* detection through enhancing TLR3 expression.

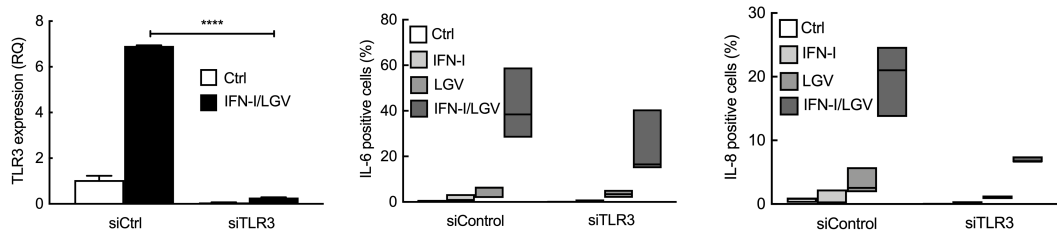


**Figure 40. IFN $\beta$  up-regulates the expression of pattern recognition receptors (PRRs) in HeLa cells.** HeLa cells were treated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for 24 h. After treatment, the total RNA was extracted from cells for screening the expression of PRRs.

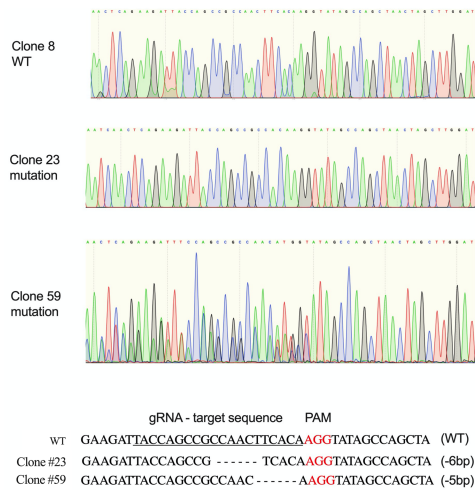
A)



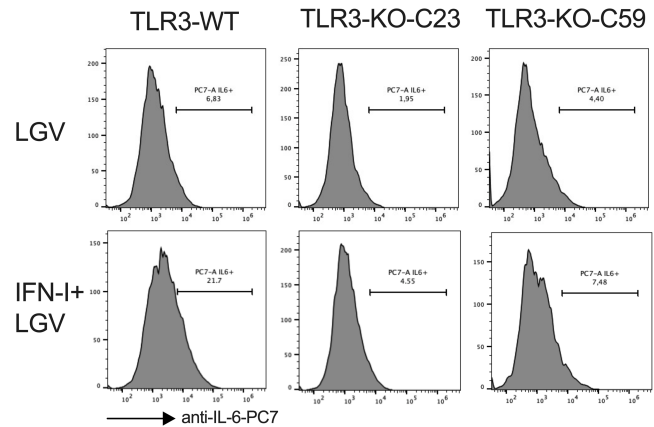
B)



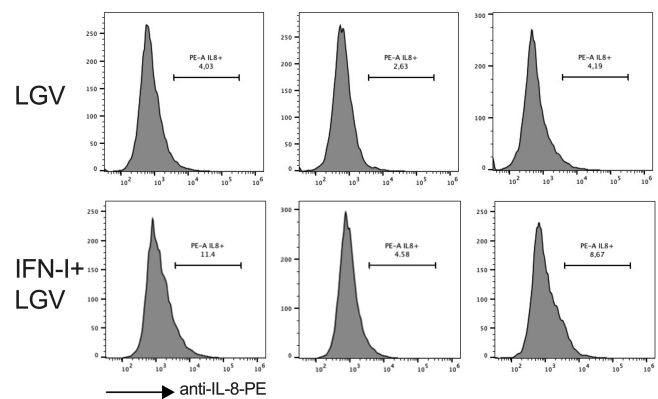
C)



D)



E)



**Figure 41. TLR3 mediates the synergistic process between IFN $\beta$  and *C. trachomatis* on the inflammatory response of primary cervical epithelial cells.** (A) Primary cervical epithelial cells isolated from patients were incubated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 10). Twenty-four hours later, the expression of pattern recognition receptors (PRRs) was checked by quantitative PCR. (B) siRNA against human TLR3 and irrelevant oligonucleotide (final concentration 30 nM) were incubated with HeLa cells for 24 h. Cells were then



treated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for additional 24 h before determining expression of TLR3 (left panel) and IL-6/IL-8 as indicated in the methodology (middle and right panels). (C) Sequencing results of TLR3-WT and two knock-out clones generated by CRISPR-Cas9. (D-E) Two clones of TLR3-KO cells or parental HeLa cells were infected with *C. trachomatis* (MOI = 1) in the presence or absence of IFN $\beta$  (2.5 ng/ml) for 30 h before examining the synthesis of IL-6 (D) and IL-8 (E) in these cells.

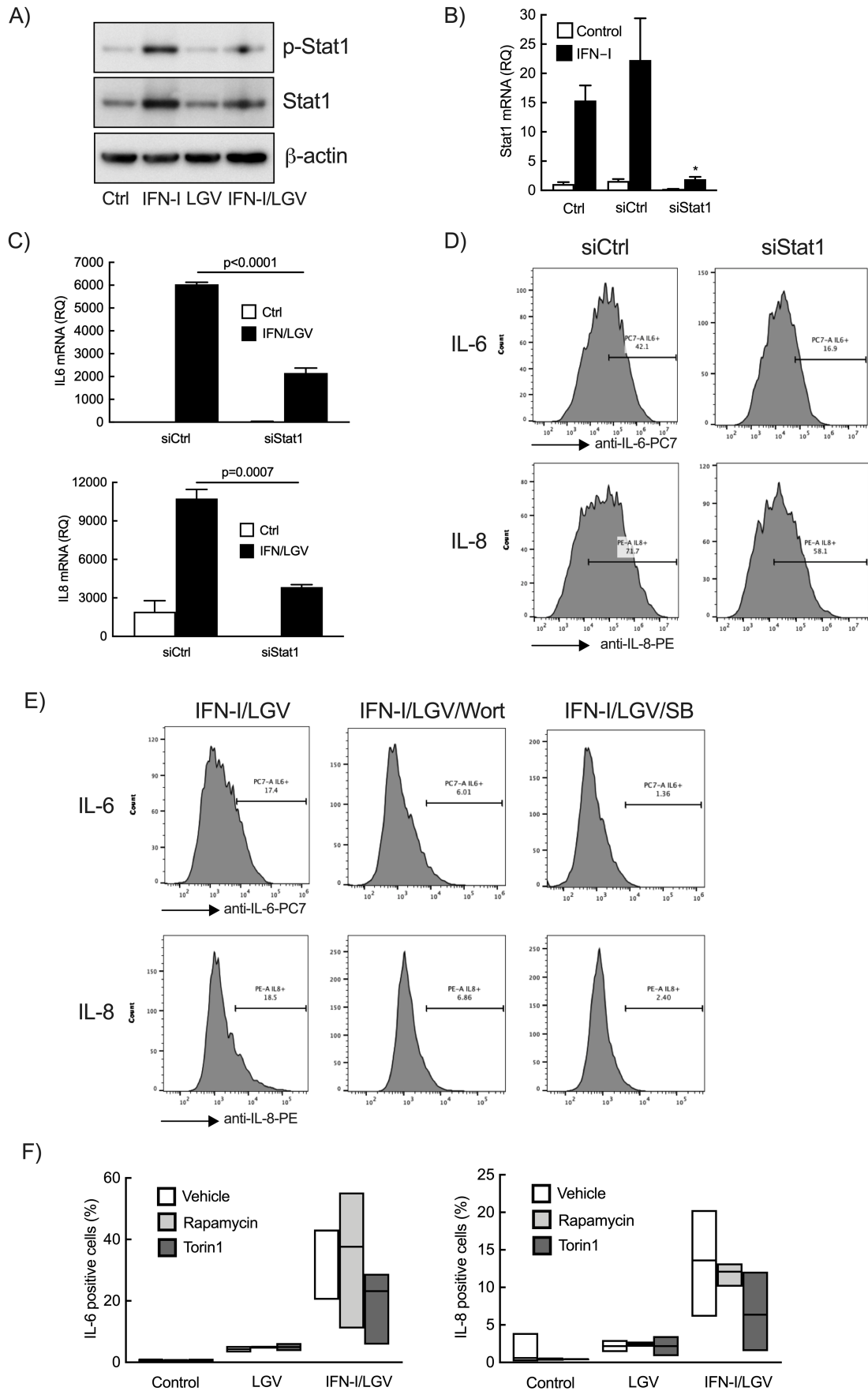
### III. The synergy between IFN-I and infection requires the STAT1 and PI3K/AKT/mTOR signaling pathways

Before deciphering the mechanism by which IFN-I up-regulated TLR3 expression, we determined which IFN-I signaling cascades contributed to the synergetic response to IFN-I and *C. trachomatis* infection. We first looked at the expression level, and phosphorylation status, of the protein signal transducer and activator of transcription 1 (STAT1). Expression and phosphorylation of STAT1 were increased in cells treated with IFN-I (Fig. 42A). Our preliminary result showed that silencing STAT1 decreased the synthesis of IL-6 and IL-8 by infected cells treated with IFN-I, both at the transcriptional and protein level (Fig. 42B, C, D). These data suggested that STAT1 contributed to the synergy between IFN-I and *C. trachomatis* on pro-inflammatory response. However, these data are preliminary and need further validation.

Next, wortmannin, an irreversible inhibitor of phosphatidylinositol 3-kinases (PI3Ks), was used to test whether PI3Ks were involved in the synergy between IFN-I and infection. Pre-treatment of cells with wortmannin decreased IL-6 and IL-8 production upon infection in the presence of IFN-I (Fig. 42E), indicating that the PI3Ks are part of the implicated signaling cascade(s). It led us to investigate the activation of its downstream signal MAP kinase p38. Incubation of cells with SB203580, a commonly used inhibitor of protein kinase B (PKB or AKT) and p38, prevented IL-6 and IL-8 synthesis upon treatment with IFN $\beta$  and *Chlamydia* (Fig. 42E). However, p38 activation was not observed by IFN $\beta$  or *Chlamydia* (Fig. 43A). As positive control, recombinant human TNF $\alpha$  induced phosphorylation of p38 (Fig. 43B). These results suggest that AKT, but not p38, is involved in this synergetic process of inflammation, supporting a role of a signaling cascade involving PI3K and AKT.

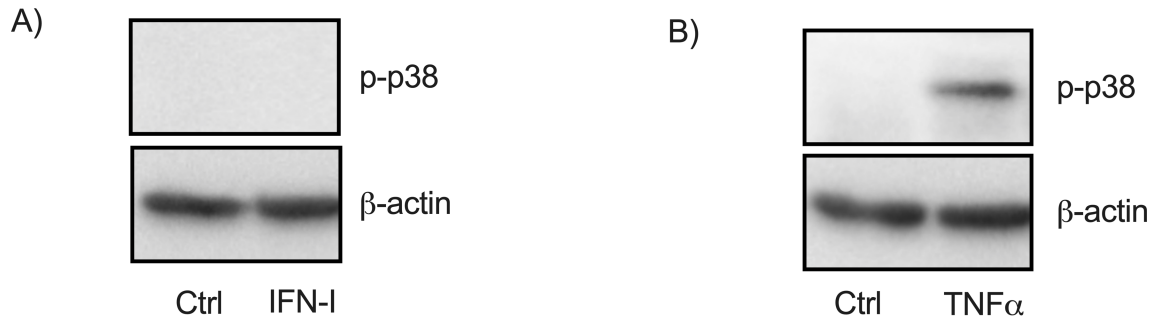
We also explored the implication of the kinase mammalian target of rapamycin (mTOR) in the signaling cascade(s) linking IFN-I and *C. trachomatis* infection to cytokine secretion. Our results showed that rapamycin, a specific inhibitor of mTOR complex 1 (mTORC1) (Thoreen et al., 2009), had marginal effect on cytokine synthesis. In contrast, Torin1, which blocks both mTORC1 and mTORC2, markedly decreased IFN-I/*Chlamydia*-induced inflammation at transcriptomic (Fig. 44) and protein level (Fig. 42F). These data indicate that in HeLa cells, mTORC2 is required for the synergetic response to IFN-I and *C. trachomatis* infection.

Based on all these data, we concluded that the canonical IFN-I signaling STAT1 and uncanonical PI3K/AKT/mTOR contributed to the exacerbation of inflammation by IFN $\beta$  upon *C. trachomatis* infection in epithelial cells.

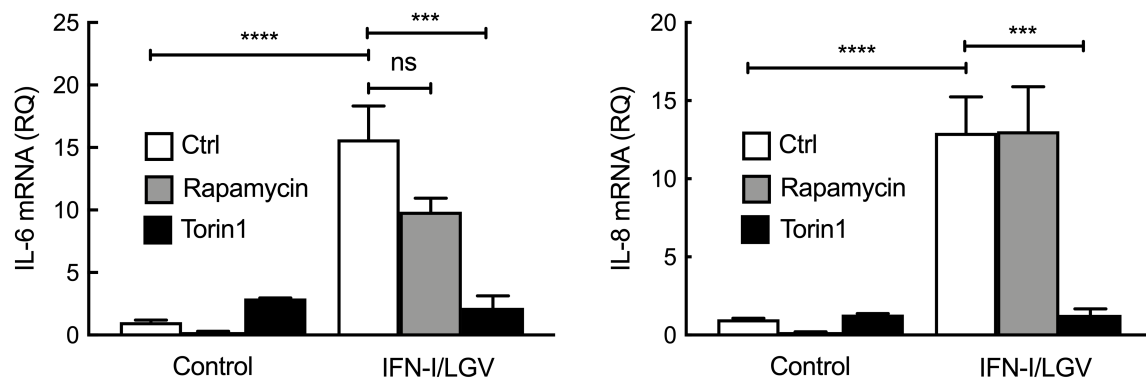


**Figure 42. STAT1 and PI3K/AKT-mTOR signaling pathways of IFN-I are involved in synergistic increase of the inflammation by IFN-I and *C. trachomatis*.** (A) HeLa cells were incubated with IFNβ (2.5 ng/ml) and/or

*C. trachomatis* (MOI = 1) for 24 h followed by the detection of activation and expression of STAT1. (B-D) siRNA against STAT1 or irrelevant oligonucleotides (final concentration = 30 nM) were transfected into HeLa cells for 24 h prior to incubation with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1). The transcriptional level of STAT1 (B) and IL-6/IL-8 (C), and the translational level of both cytokines (D) were determined 24 h (B) and 30 h (C-D) post treatment, respectively. (E) HeLa cells were pre-incubated with pharmacological inhibitors wortmannin (5  $\mu$ M) or SB203580 (10  $\mu$ M) for 1 h. Cells were then treated with IFN-I and *C. trachomatis* for 24 h in the presence of the inhibitors prior to add brefeldin A for additional 6h. After treatment, IL-6 and IL-8 expression was analyzed. (F) The similar protocol was used as in D using mTOR inhibitors, rapamycin and torin1 (1  $\mu$ M). One experiment has been performed for B-D; 3 experiments for E and F.

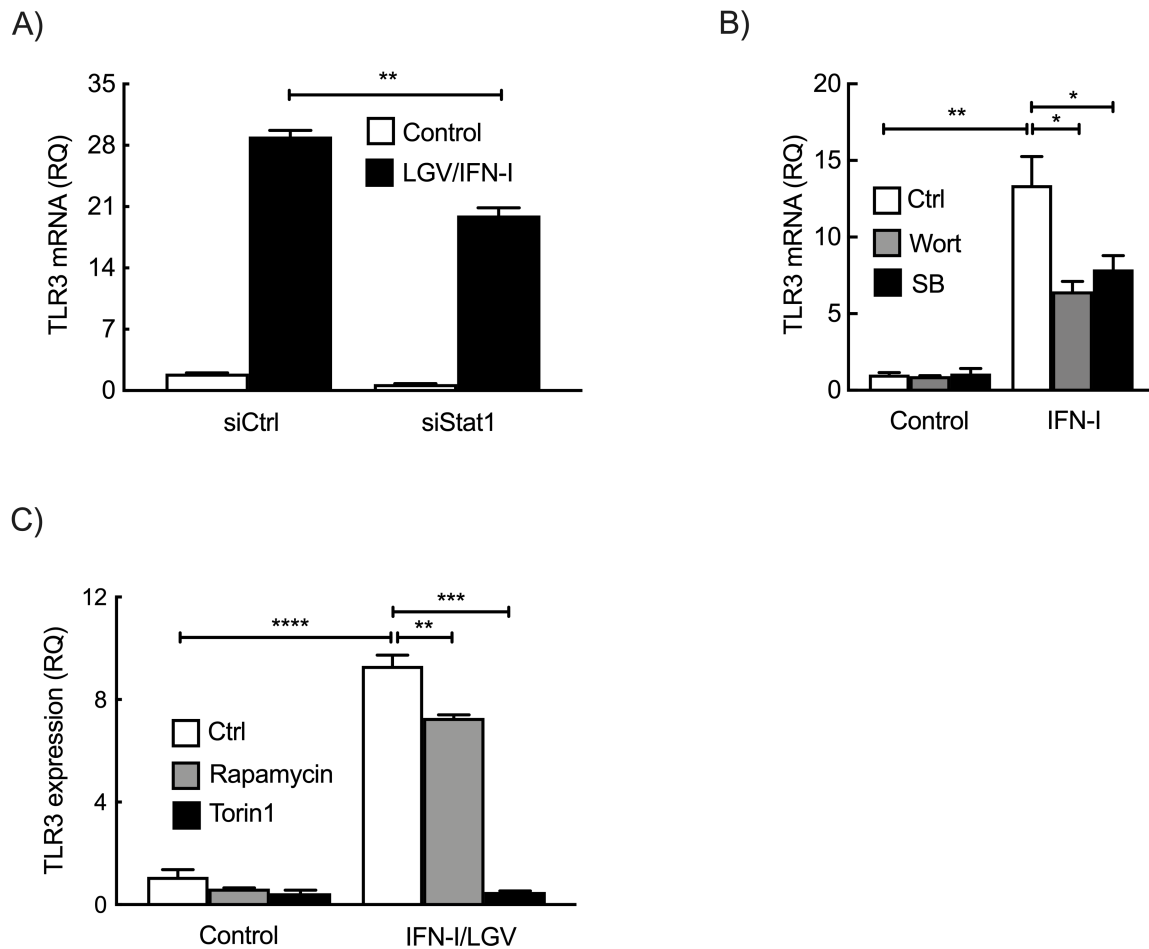


**Figure 43. IFN $\beta$  does not activate MAPK/ERK and p38 in HeLa cells.** (A) HeLa cells were treated with IFN $\beta$  (2.5 ng/ml). Twenty-four hours later, phosphorylation of ERK and p38 was detected by Immunoblotting. (B) As positive control, HeLa cells were incubated with recombinant human TNF $\alpha$  (10 ng/ml) for 24 h followed by p38 phosphorylation detection.



**Figure 44. mTOR complex 2, but not complex 1, is implicated in the synergistical effect between IFN-I and *Chlamydia* on the inflammation.** HeLa cells were pre-incubated with inhibitors rapamycin (1  $\mu$ M) or torin1 (1  $\mu$ M) for 1 h before treated with IFN $\beta$  (2.5 ng/ml) and *C. trachomatis* (MOI = 1) for 40 h in the presence of the inhibitors. After treatment, IL-6 and IL-8 expression was checked by quantitative PCR.

To validate our model, we measured the effect of modulating these signaling cascades on TLR3 expression. We showed that silencing STAT1 significantly decreased TLR3 synthesis induced by IFN $\beta$  in HeLa cells compared to siRNA control (Fig. 45A). Pretreatment of epithelial cells with wortmannin or SB203580 also attenuated IFN-induced TLR3 (Fig. 45B). A similar TLR3 inhibition was also detected when using Rapamycin and IFN-induced TLR3 was abolished when using Torin1 (Fig. 45C). These results further supported our conclusion that IFN-I modulates the inflammatory response to *Chlamydia* infection by enhancing TLR3 expression through both the STAT1 and PI3K/AKT/mTOR signaling pathways.



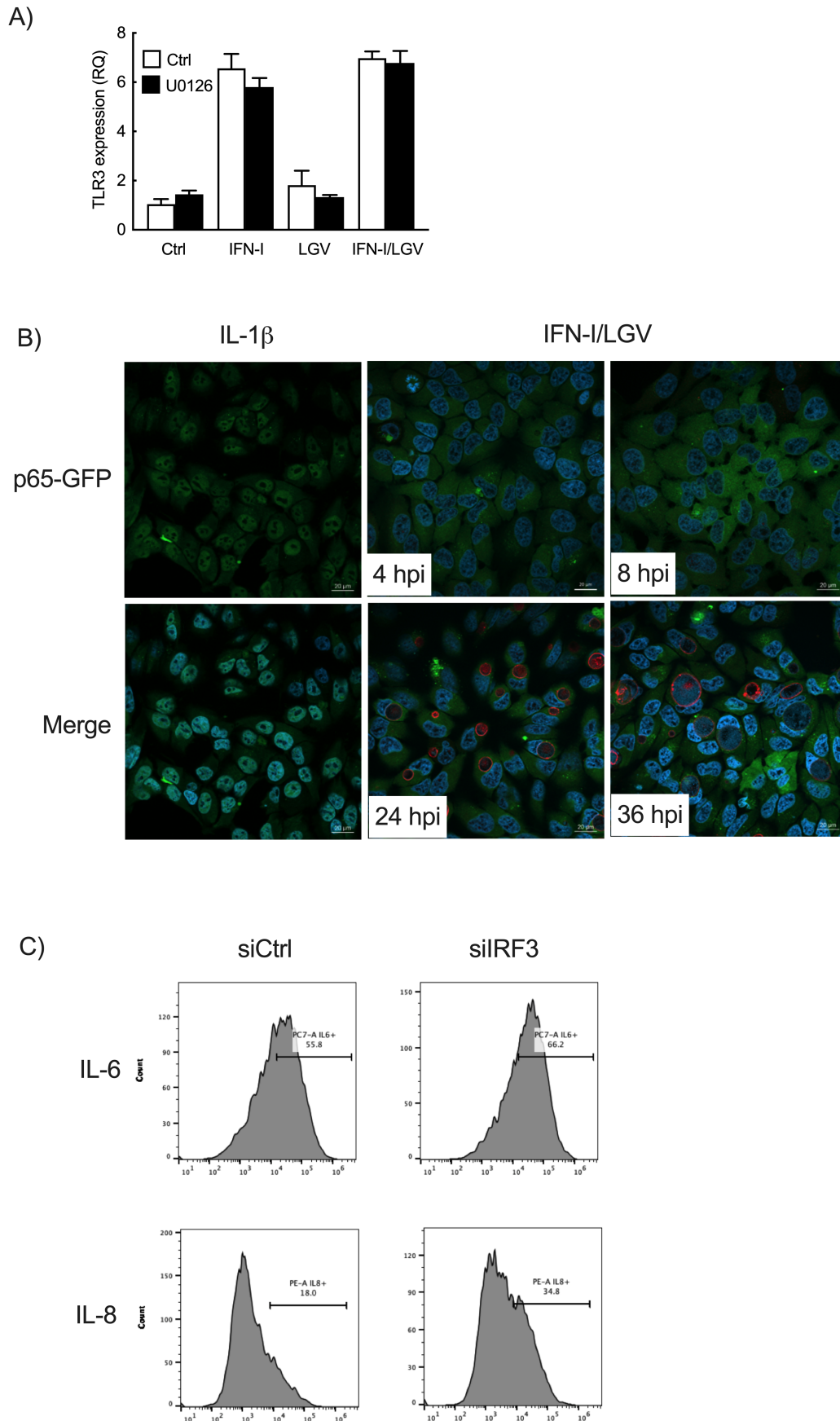
**Figure 45. Silencing or inhibiting IFN-I signaling cascades dampens IFN $\beta$ -induced TLR3 expression by HeLa cells.** (A) The cells were incubated with siRNA against human TLR3 and irrelevant oligonucleotide (final concentration 30 nM) for 24 h before IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) treatment for additional 24 h. The TLR3 expression was examined by qPCR. (B-C) HeLa cells were incubated with wortmannin (5  $\mu$ M), SB203580 (10  $\mu$ M), rapamycin (1  $\mu$ M) or torin1 (1  $\mu$ M) for 1 h prior to be treated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1). Twenty-four hours later, the expression of TLR3 was checked.

#### IV. TLR3 modulated *Chlamydia*-induced inflammation by ERK pathway

Finally, we investigated the signaling intermediates between TLR3 activation and cytokine expression. TLR3 signaling generally triggers MAPKs, NF- $\kappa$ B and IRF3 to induce the expression of inflammatory cytokines (Kawai and Akira, 2006; Muccioli et al., 2012). NF- $\kappa$ B is a transcription factor playing a central role in inflammation. Using HeLa cells stably transformed to express a fusion between p65 and the green fluorescent protein (Connor et al., 2021), we tested whether NF- $\kappa$ B was activated by *Chlamydia* infection upon IFN-I treatment. As positive control, we used recombinant human IL-1 $\beta$ , which induced the translocation of GFP-p65 into cell nuclei (Fig. 46B, left panel). In contrast, no nuclear translocation of GFP-p65 was observed in *Chlamydia* infected cells, in the presence of IFN-I (Fig. 46B, or in its absence (data not shown). These data indicated that NF- $\kappa$ B was not implicated in the proinflammatory response to *Chlamydia* infection, even in the presence of IFN-I. In addition,

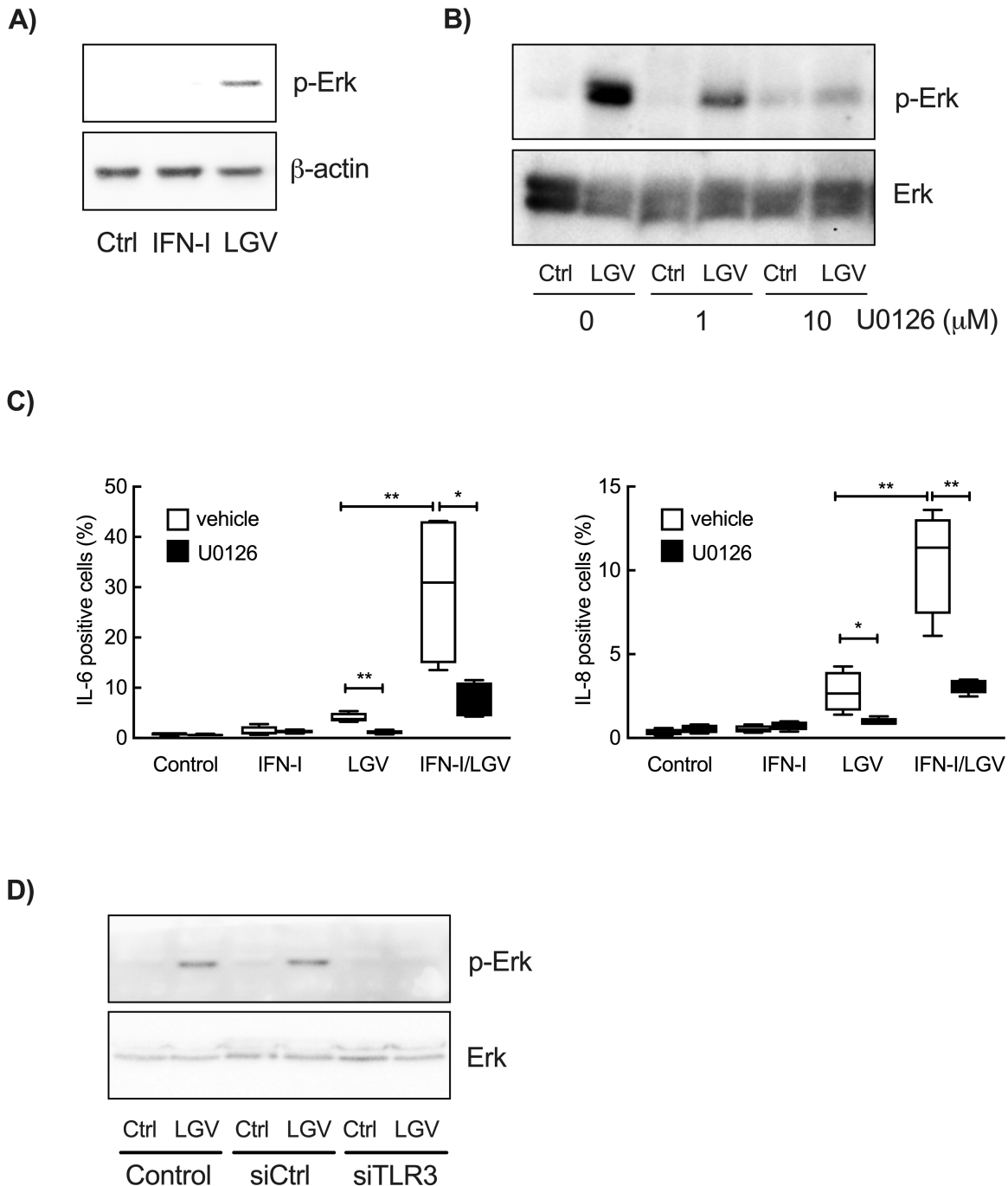
silencing IRF3 failed to prevent IL-6 and IL-8 expression upon infection in the presence of IFN-I, indicating that the TLR3-IRF3 cascade was also not involved (Fig. 46C).

*Chlamydia* infection activated MAPK/ERK by inducing phosphorylation of this kinase (Fig. 47A), but not MAPK p38 (data not shown). Application of U0126, an inhibitor of MEK/ERK, reduced ERK phosphorylation induced by *Chlamydia* infection, in a dose-dependent manner (Fig. 47B). Pre-incubation HeLa cells with this inhibitor also alleviated the synergetic inflammation by IFN-I and infection (Fig. 47C), without blocking *TLR3* expression (Fig. 46A). In addition, compared to irrelevant siRNA, silencing TLR3 blocked ERK activation upon the infection (Fig. 47D). These data suggested that TLR3 modulated host inflammatory response to bacterial infection by tuning the activation of MAPK/ERK.



**Figure 46. TLR3 signaling cascade NF- $\kappa$ B and IRF3 did not implicated in the synergetic process between IFN $\beta$  and *C. trachomatis* infection.** (A) HeLa cells were pre-incubated with inhibitor U0126 (10  $\mu$ M) for 1 h before treated with IFN $\beta$  (2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for 24 h in the presence of the inhibitors.

After treatment, TLR3 expression was checked by quantitative PCR. (B) p65-GFP expressing HeLa cells were seeded on coverslip and incubated with recombinant human IL-1 $\beta$  (10 ng/ml) for 30 min (left panel), or with IFN $\beta$  (2.5 ng/ml) and *C. trachomatis* (MOI = 1) for different time as indicated (middle & right panels). The NF- $\kappa$ B activation was detected by checking the nuclear translocation of p65-GFP using Immunostaining. (C) HeLa cells were incubated with siIRF3 or irrelevant oligonucleotide (final concentration 30 nM) for 24 h. The cells were then treated with IFN $\beta$  (2.5 ng/ml) and *C. trachomatis* (MOI = 1) for additional 30 h for analysis of the synthesis of IL-6 and IL-8.

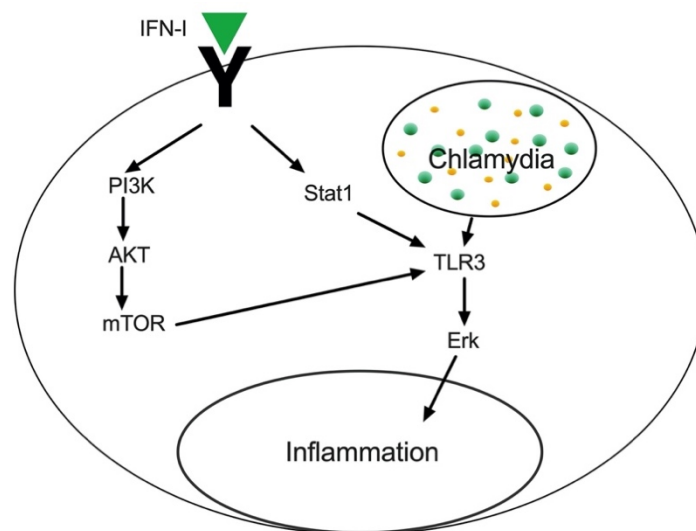


**Figure 47. TLR3 modulates the host inflammatory response to the infection by tuning activation of ERK induced by *C. trachomatis* infection.** (A) HeLa cells were treated with IFN $\beta$  (2.5 ng/ml) or *C. trachomatis* (MOI = 1) for 24 h. The activation of ERK was determined by Immunoblot. (B) The cells were pre-treated with U0126 (10  $\mu$ M), an inhibitor of MEK/ERK. One hour later, HeLa cells were infected with *C. trachomatis* (MOI = 1) for 24 h in the presence of U0126 at the different dose as indicated, followed by the detection of the ERK phosphorylation. (C) After one-hour pre-treatment with U0126 (10  $\mu$ M), HeLa cells were incubated with IFN $\beta$

(2.5 ng/ml) and/or *C. trachomatis* (MOI = 1) for 24 h in the presence of U0126, prior to adding brefeldin A for additional 6 h. Then, the synthesis of IL-6 and IL-8 was assayed by flow cytometry. (D) HeLa cells were incubated with siRNA against human TLR3 or irrelevant oligonucleotide for 24 h before *C. trachomatis* infection for additional 24 h to examine the activation of ERK.

## Discussion

IFN-I has been extensively studied for its anti-viral properties, but appears to have contrasted effects on bacterial infections. We characterized the effect of IFN-I on the inflammatory response of epithelial cells to *Chlamydia* infection. Our results showed that incubation of primary cervical epithelial cells isolated from patients and cervical HeLa epithelial cells of cancer origin with IFN-I strongly increased the synthesis of the pro-inflammatory cytokines IL-6 and IL-8 upon *C. trachomatis* infection. We provided evidence that this synergetic effect is accounted for by an increase in the expression of the PRR TLR3 upon IFN-I treatment, which facilitates *Chlamydia* detection, and we identified the signaling cascades upstream and downstream of TLR3 expression [Figure 48].



**Figure 48. IFN-I exacerbates pro-inflammatory response of host to *C. trachomatis* infection through enhancing TLR3 expression.**

In line with our observations, a increase of bacterial clearance and reduction of IFNR-KO duration of infection were demonstrated in the lower genital tract of IFNR-deficient mice upon *C. muridarum* infection, compared to wildtype animals (Nagarajan et al., 2008). Higher level of IFN-inducible protein 10 (IP10), a cytokine related to IFN-I and IFN-II response, was also detected in cervical secretions of patients with ascending *C. trachomatis* genital tract infection by or with pelvic inflammatory disease and *Chlamydia* infection (Poston et al., 2019; Zheng et al., 2018). In addition, treatment of mice with neutralizing antibodies against murine IFN-I significantly decreased *C. muridarum* dissemination and alleviated the tissue damage (Prantner et al., 2011). These observations are consistent with our findings, and collectively suggest that high IFN-I levels play a deleterious effect during *Chlamydia* infection. Interestingly, IFNR mutated mice were 1000-fold more resistant to infection with *Listeria monocytogenes* than wildtype mice (Auerbuch et al., 2004), and IFNR was required for *L. monocytogenes*



dissemination (Osborne et al., 2017). Also, a cohort study showed that IFN-inducible gene profile was discriminated active infection by *M. tuberculosis* in patients from other infectious diseases (Berry et al., 2010). It might be that IFN-I is more prone to negative effect in the case of infections by intracellular bacteria, since *Chlamydia*, *Listeria* and *Mycobacteria* are all intracellular bacteria. This hypothesis should however be considered cautiously, as counter examples exist (Bukholm et al., 1984; Niesel et al., 1986; Opitz et al., 2006; Schiavoni et al., 2004).

Many cell types are able to produce IFN-I, and part of the IFN-I signaling triggered in epithelial cells during *C. trachomatis* infection could correspond to autocrine signaling. Indeed, IFN $\beta$  secretion has been detected upon *C. trachomatis* infection of oviduct epithelial cells isolated from mice (Derbigny et al., 2007; Lad et al., 2005; Xia et al., 2003), as well as from infected murine FGT (Derbigny et al., 2007; Prantner et al., 2011). Robust IFN $\beta$  signaling was the most prominent hallmark of acute infection by *C. trachomatis* serovar D in an human fallopian tube-derived organoid model of infection (Kessler et al., 2019). Immune cells attracted to the site of infection could also contribute to increase locally the level of IFN-I. Although the significant increase of IFN-I production in *C. trachomatis*-infected patients is not detected in clinic. One possible explanation is that the half-life of IFN-I is relatively short (~1-3 h) (Shechter et al., 2001), and therefore difficult to detect in the serum or cervical secretion of patients. Also, the production might be local, restricted to the site of infection, and undetectable in the serum. In addition, the time at which samples are collected might not correspond to the time of acute infection when IFN-I is being produced.

When investigating the IFN-I signaling cascades involved in the synergetic pro-inflammatory process between IFN-I and *Chlamydia*, particularly the MAPKs p38, we observed that the inhibitor of the MAPK p38 SB203580 attenuated the synergy of IFN-I and bacteria as well as the expression of TLR3, indicating that p38 was involved in the cascade leading to TLR3 expression. However, IFN $\beta$  did not activate p38. We need to repeat these experiments with lower concentrations of SB203580, since it was shown that this compound inhibited the p38 MAPK kinase at IC<sub>50</sub> ~0.3-0.5  $\mu$ M, and PKB (AKT), a known downstream signal of PI3K, at 10-fold higher concentrations (IC<sub>50</sub>~3-5  $\mu$ M) (Lali et al., 2000). The concentration we had chosen (10  $\mu$ M) should inhibit both AKT and p38. It is therefore possible that AKT, and not p38, mediates the increase of TLR3 expression upon IFN-I stimulation in infected cells.

IFN-I increased the expression of several PRRs in primary human epithelial cells, including TLR2, TLR3, TLR4 and NOD1. Silencing of TLR3, but not of other PRRs, attenuated the synergetic inflammation to IFN-I and infection. This indicated that TLR3 might mediate the synergy of the cell response to IFN-I and *Chlamydia* infection, a hypothesis confirmed by the use of cells knocked out of TLR3. ERK activation, which is needed for the synergy between IFN-I and infection, was also lost upon TLR3 silencing, confirming the central role of TLR3 in the synergy between IFN-I and *C. trachomatis* infection in regards to the pro-inflammatory response of epithelial cells. The importance of TLR3 signaling in the response to *Chlamydia*

infection was largely documented by the Derbigny team, who showed that the deficiency of TLR3 or its receptor altered the innate immune response of oviduct epithelial cells of mice and humans to *Chlamydia* infection (Derbigny et al., 2012; Kumar and Derbigny, 2018; Xu et al., 2019). The integrity of the epithelial barrier and bacterial clearance, as well as multiple cellular processes, such as cellular matrix and small molecule transport, apoptosis and antigen-presentation, were also altered in TLR3-deficient models of *Chlamydia* infection (Carrasco et al., 2018; Kumar and Derbigny, 2018; Kumar et al., 2019). These data demonstrated that TLR3 was a key feature of the innate response to *Chlamydia* infection.

Our results confirm the induction of TLR3 expression by epithelial cells upon IFN-I treatment (Tissari et al., 2005). IFN $\gamma$ , which plays a crucial role in the immune response to *C. trachomatis* infection, also enhanced TLR3 expression in keratinocytes (Kajita et al., 2015). However, we did not observe a synergetic effect between *Chlamydia* and IFN $\gamma$  on IL-6 or IL-8 production (not shown), possibly because IFN $\gamma$  restricts bacterial growth (Nelson et al., 2005). It is interesting to note that TLR3 expression is induced by IFN-I, the latter is one of the cytokines induced by TLR3 stimulation. The positive transcriptional feedback loops between the two molecules might contribute to the synergetic effect observed at the level of IL-6 and IL-8 synthesis.

One important question raised by our findings is the nature of the TLR3 activator in the case of *Chlamydia* infection. TLR3 is mainly known for its ability to recognize double-stranded RNA (dsRNA) associated with viral infection. Recognition of poly(I:C), a synthetic mimic of dsRNA (Kawai and Akira, 2010), by TLR3 triggers a strong induction of inflammatory cytokines by macrophages via activation of the NF- $\kappa$ B cascade (Alexopoulou et al., 2001). However, there is no evidence that dsRNA is released upon *C. trachomatis* infection. Interestingly, the signaling cascade downstream of TLR3 activation in the context of *Chlamydia* infection does not appear to involve NF- $\kappa$ B. Bacteriophages were also shown to activate TLR3 cascade (Sweere et al., 2019), however, while phages have been identified in several *Chlamydiae* lineage (Everson et al., 2002; Hsia et al., 2000; Karunakaran et al., 2002), *C. trachomatis* appears to be devoid of such element (Śliwa-Dominiak et al., 2013). Short interfering RNA or mRNA have also been reported as potential ligand of TLR3 (Karikó et al., 2004b, 2004a), and the possibility that such molecules are produced during *Chlamydia* infection will need further investigation. Last but not least, activation of other TLRs cascade by IFN-I as we observed in the present study, might also engage the activation of TLR3 signaling pathway. All these possibilities have to be further verified in the future.

Altogether, we conclude that IFN-I produced during *Chlamydia* infection exacerbates the pro-inflammatory response of epithelial cells. In the context of symptomatic infections by *C. trachomatis*, the use of anti-IFN-I therapy or of TLR3 antagonists as therapeutic strategies might allow to alleviate the hyperinflammation-induced tissue damage and to improve the clinical outcome of infection.

# GENERAL DISCUSSION AND PERSPECTIVES

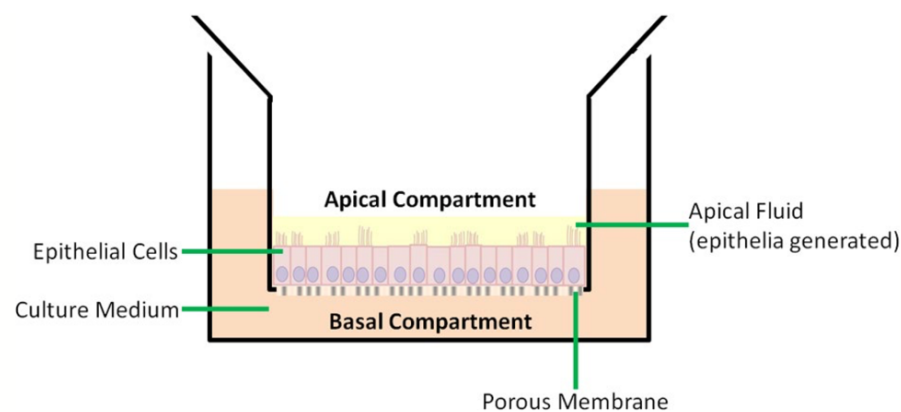
## I. *Chlamydia* infection, a major public health concern

*C. trachomatis* infection has become a major concern for public health with the rapid increase of sexually transmitted infections (STIs) in the world. WHO estimates among the main causative agents for STIs, e.g. *syphilis*, *gonorrhoea* and *trichomoniasis*, *Chlamydia* infection comes first with approximate 129 millions cases for 374 millions new STIs in 2020 ([https://www.who.int/en/news-room/fact-sheets/detail/sexually-transmitted-infections-\(stis\)](https://www.who.int/en/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis))), particularly in young women population at 14-25 years. Beyond the immediate impact of the infection itself, asymptomatic *Chlamydia* infections can result in severe consequences, particularly in women. However, the pathogenesis of *Chlamydia*-induced tissue damages is largely unknown, and no vaccine is available. Thus, it is urgent and critical to better understand the interplay between *Chlamydia* and its host, particularly in the FGT of women, a need that drove my doctoral work.

## II. The cell models in *Chlamydia* research

The FGT is structurally dynamic, and each part has unique structural and immunological properties. The lower genital tract (vagina and ectocervix) are gatekeepers to prevent the host from microbial infection. The stratified squamous epithelia cover the surface of the lower genital tract, facing a variety of microorganisms including *Chlamydia*. The HeLa cell line, originally derived from a patient with cervical cancer, has been widely used for *C. trachomatis* research. This model has advantages, mainly its easy amplification and genetic tractability. However, in the introduction I explained the limitations of this cell model, that largely differ from normal somatic epithelial cells. Thus, we first established a simple protocol for isolating primary ectocervical epithelial cells from patients undergoing hysterectomy, and to compare their susceptibility and inflammatory response with that of HeLa cells upon *C. trachomatis* infection. We showed that primary epithelial cells were less permissive to *Chlamydia* development than HeLa cells. As discussed on our paper, part of the difference could be due to culture medium. However, intrinsic features of the cells could also be implicated, and it could be that ecto-cervix derived epithelial cells are less permissive than cells from columnar epithelia that distributed on the surface of upper genital tract (endo-cervix, uterus and oviduct). Indeed, Moorman et al. reported lower infection of cervical squamous epithelial cells than endometrial columnar epithelial cells (Moorman et al., 1986). In addition, we also demonstrated that these primary epithelial cells have a stronger capacity to mount an inflammatory response to *Chlamydia* infection than HeLa cell line. The difference of the basal level of synthesis of cytokines between primary cells and HeLa cells is interesting and suggest that HeLa cells make a poor model of the situation faced by invading bacteria. Therefore, when looking at the innate response of epithelial cells to infection, primary ecto-cervical epithelial cells make a better model, particularly of what happen in the lower FGT.

In our study the primary epithelial cells were immersed in the cell culture medium, a lay-out known as ‘submerged culture’. However, under normal physiological conditions, mucosal epithelial cells in the lower FGT are directly exposed to external environment with a very thin layer of mucus on their surface, a lay-out referred to as air-liquid interface. Such air-liquid interface approach [Figure 49] (Chen and Schoen, 2019), could be obtained by growing the primary epithelial cells on trans-well filters. This model would better mimic the FGT epithelium *in vitro* and allow to investigate the tropism of the inflammatory cytokine secretion (apical and/or basal), the integrity of epithelial layer, and the migration of inflammatory cells such as neutrophils upon *Chlamydia* infection. Moreover, the submerged culture of primary epithelial cells formed monolayers, while the lower genital tract of women has stratified epithelial cells. Using liquid-air interface approach, we might be able to reproduce the stratification of the primary ectocervix epithelium. Indeed, HaCaT cells, a spontaneously transformed aneuploid immortal keratinocyte cell line derived from adult skin (Wilson, 2013), exposed to a liquid-air interface can form organized 3D cultures with differentiations in different layers, similar to the non-keratinizing squamous epithelium of the mucosa (Stark et al., 2004). Upon *Chlamydia* infection, different layers owns distinct infectivity in this cell model (Nogueira et al., 2017).



**Figure 49. Schematic cross-sectional representation of an air-liquid interface culture with epithelial cells** (mucociliary phenotype). The culture system consists of an apical and a basal compartment, which are separated by a porous filter support and the confluent epithelial tissue. Upon differentiation, epithelial cells of mucociliary phenotype secrete mucus fluid into the apical compartment. (From Chen and Schoen, 2019)

Organoid 3D models have also been established by culturing epithelial progenitor cells isolated from different compartments of the FGT of patients or mice in Matrigel matrix (Heremans et al., 2021). As an example, endometrium-derived organoids have a lumen structure surrounded by epithelia, similar to the physiological environment of the upper genital tract local in women (Boretto et al., 2019). One advantage of this model, compared to our primary epithelial cell model which could be maintained in culture for only 4-5 passages, is that these organoids can be maintained in culture for up to 1 year. Therefore, it will be interesting to develop human ecto-cervix organoids. Co-culture of organoid with neutrophils has recently been shown to reconstitute recruitment of these immune cells to *Chlamydia* infection (Dolat and Valdivia, 2021). One limitation of the organoid model is however its cost, and the extensive manipulation required. Finally, animal models represent another tool to investigate *Chlamydia* infection in a complex system. Mice have been largely used in that respect, although they present limitations

discussed in the introduction of this dissertation. The use of HLA humanized transgenic mice infected with *C. trachomatis* infection in upper genital tract, might improve our ability to investigate the chronic tissue damage and immune response to *Chlamydia* infection in mice.

To summarize, the submerged primary epithelial cell model described in my thesis, and the air-liquid interface culture of primary epithelial cells that could also be implemented, are suitable models to examine *Chlamydia*-epithelia interaction in the lower genital tract of women. 3D organoid and HLA-humanized mouse model constitute models even closer to the physiological conditions, and could be implemented, depending on the aims and read-outs of the study. Finally, it is important to note that while the use of primary cells should be preferred, these cells are difficult to obtain, and not always amenable to mechanistic approaches. Therefore, in the second part of my PhD I combined experiments using primary cells and HeLa cells to investigate the innate response of epithelial cells to infection.

### **III. Does *Chlamydia* restrain inflammation during infection?**

Upon *Chlamydia* infection, host epithelial cells sense the bacteria and activate the inflammatory response to eliminate the invading pathogen. I showed in the 2nd part of my work that the transcription of *IL-6* and *IL-8*, genes coding for two major inflammatory cytokines associated with *Chlamydia* infection, were increased by the infection in both primary and HeLa epithelial cells. Interestingly, when analysis at the single cell level by flow cytometry showed that only less than 10% of infected cells were above detection threshold for IL-6 or IL-8 at 30 h post infection, even though over 85% cells had been infected by *C. trachomatis*. This reflects heterogeneity, both in the cells and in the bacteria. We tried to examine whether the same was observed in primary epithelial cells, unfortunately most of the cells were lost during the staining procedure, precluding analysis. If most primary epithelial cells also show a low or undetectable level of IL-6 and IL-8 upon *Chlamydia* infection, this would suggest that the host inflammatory response is dampened by *Chlamydia*. A proteomic study showed that *Chlamydia* infection globally reduced host cell protein synthesis, a phenomenon that might contribute to the relatively low cytokine production (Ohmer et al., 2019). Effector proteins secreted by the type III machinery might also specifically target the host immune response. One such effector is a deubiquitinating enzyme that decreases the sensitivity of *C. trachomatis* to IFN $\gamma$ . Absence of this enzyme reduced bacterial burden in infected mice (Fischer et al., 2017). TepP, an effector of *C. trachomatis* secreted at early stage of infection, can also manipulate the host signals associated with innate immunity of host cells (Carpenter et al., 2017; Chen et al., 2014). But whether these effectors or others dampen the expression of IL-6 and IL-8 needs to be further investigated. Our observation that increasing TLR3 expression is sufficient to increase the synthesis of IL-6 and IL-8 indicates that, if such a brake exists, it might play, at least in part, on the capacity of the cells to detect the pathogen. Also, it is very surprising that *C. trachomatis* infection fails to induce the nuclear translocation of NF- $\kappa$ B, a major player of the innate immune response. The data we reported here were consistent with previous work by the Caldwell lab, who attributed the absence of nuclear translocation of NF- $\kappa$ B to the action of the chlamydial protease CPAF based on the phenotype of a CPAF-KO strain (Patton et al., 2016).

In fact, the same study showed that the CPAF-KO displayed defects in the maturation of several effectors, indicating that other effectors might be implicated in preventing NF- $\kappa$ B activation during *C. trachomatis* infection.

#### IV. Effect of IFN-I on *C. trachomatis* infection and physiological relevance

IFNs belong to a superfamily of molecules, among which IFN $\alpha$  and  $\beta$  are the well-known members of type I subfamily and IFN $\gamma$  for type II. IFN $\gamma$  has been shown to inhibit *Chlamydia* growth in epithelial cells (De la Maza et al., 1987), which is accounted for by its ability to induce the expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan (Beatty et al., 1994; Yasui et al., 1986). Since tryptophan is required for the growth of *C. trachomatis*, IDO expression prevents bacterial growth (Allan and Pearce, 1983). In addition, IFN $\gamma$  plays a role in the cellular immune response of the host to infection. The role of IFN-I during *Chlamydia* infection is less clear. The present study showed that IFN-I (both IFN $\alpha$  and  $\beta$ ) significantly enhanced the expression of inflammatory cytokines IL-6 and IL-8 induced by *C. trachomatis* infection, while IFN-I alone had no effect. It will be necessary to compare the size of inclusions and bacterial progenies in the presence or absence of IFN-I, to know whether IFN-I, like IFN $\gamma$ , influences bacterial development per se.

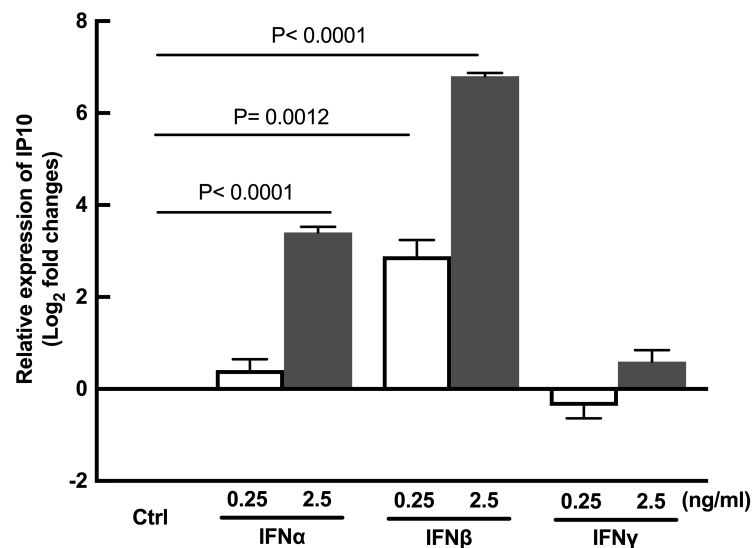
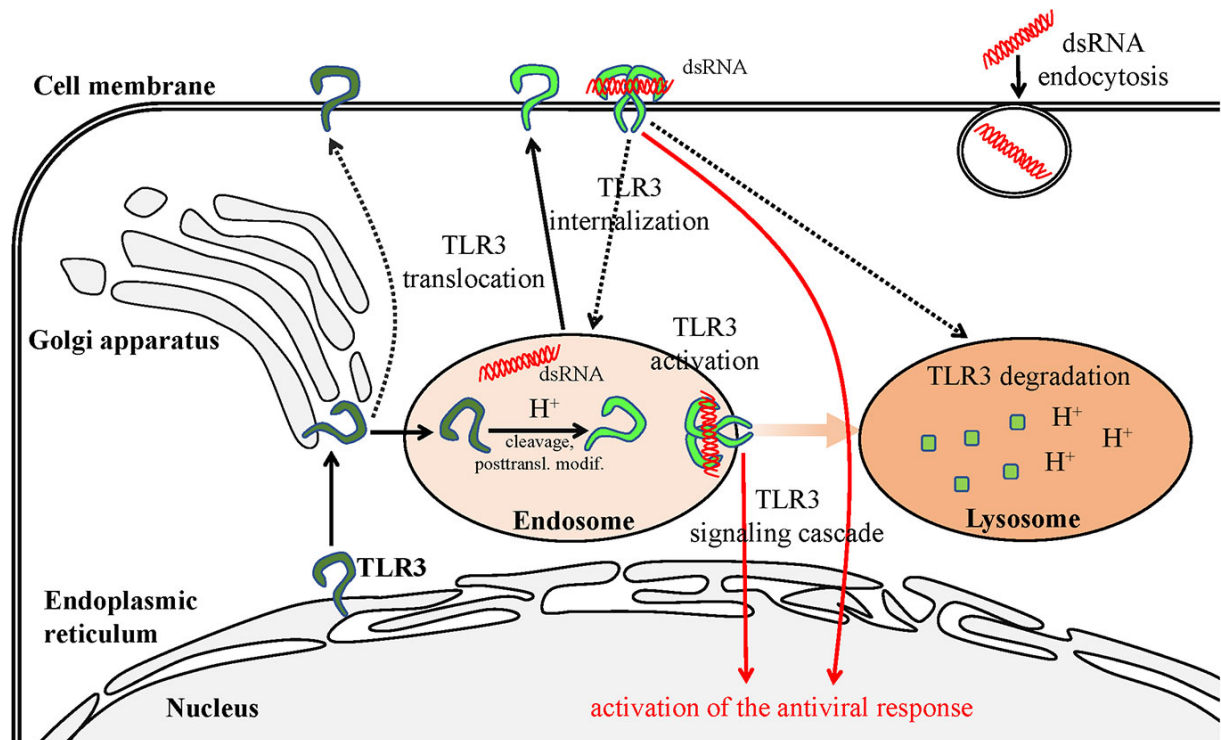


Figure 50. The induction of IP10 by IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$  in HeLa cells. (24 h incubation)

Another open question is the identification of the IFN-I, i.e. IFN $\alpha$  or IFN $\beta$ , that is relevant in patients. Previous studies have shown that *Chlamydia* infection induced IFN $\beta$  synthesis in oviduct epithelial cells and in the FGT of mice (Derbigny et al., 2007; Prantner et al., 2011). Treatment of mice with neutralizing antibodies against murine IFN $\beta$  decreased *C. muridarum* dissemination and alleviated the tissue damage (Prantner et al., 2011). A retrospective study in *C. trachomatis* infected women concluded that IFN $\alpha$  induction did not differ in patients with and without ascending *Chlamydia* infection, while IP10, a IFN responder, did (no data is reported on IFN $\beta$ ) (Poston et al., 2019). Our preliminary data demonstrated a higher ability of IFN $\beta$  in inducing IP10 synthesis than IFN $\alpha$  and IFN $\gamma$  in HeLa cells, a finding that we plan to

test in primary cells [Figure 50]. These data indicate that in patients IFN $\beta$ , rather than IFN $\alpha$ , might enhance the innate response to *Chlamydia* infection. Finally, regarding the IFN-I producing cells, it is reasonable to speculate that at the onset of infection, the infected cells produce IFN-I that can act in an autocrine manner to amplify bacterial detection, with a positive feedback loop between TLR3 and IFN-I expression. Later in infection, other cells, including non-epithelial cells, might contribute to the local increase in IFN-I concentration and the exacerbation of infection.

## V. The black box of TLR3 stimulation during *C. trachomatis* infection



**Figure 51. Scheme of TLR3 transportation in the cell.** The transportation route is initiated in the ER and terminates in the lysosome-site of degradation. TLR3 present on the cell surface may recognize the dsRNA, which is a viral replication intermediate for some viruses, derived from necrotic infected cells. The dsRNA can also be endocytosed and recognized by TLR3 in the endosomes. Pathways requiring further examination are marked with dashed lines. (From Mielcarska et al., 2021)

By screening the expression of PRRs we observed that IFN-I increased expression of TLR2, TLR3, TLR4 and NOD1, which have been shown previously to sense *Chlamydia* infection. Only silencing or knocking-out TLR3 attenuated the synergy between IFN-I and *Chlamydia*, pointing to TLR3 as a key player in this process. To know how TLR3 senses the infection it will be interesting to know the location of TLR3 in *Chlamydia*-infected cells. TLR3 is usually located in the endosome, but distribution of TLR3 on the cell surface has also been reported [Figure 51] (Matsumoto et al., 2003; Mielcarska et al., 2021; Ueta et al., 2005). Neutralizing surface TLR3 on human fibroblast with anti-TLR3 antibodies inhibits TLR3 responses in fibroblasts, suggesting a functional role of cell surface TLR3 (Matsumoto et al., 2002, 2003). We tried several commercial anti-human TLR3 antibodies to detect endogenous TLR3 either by immunoblot or by immunofluorescence, with no success, even upon TLR3 induction by IFN-I. Overexpression of human TLR3 with an epitope tag at the N-terminal of TLR3

(Addgene, #13084) can be used. It may be that a fraction of TLR3 is localized at the inclusion membrane, in contact with the bacteria or PAMPs present in this compartment.

Beside the question of the location of TLR3 activation, one major question raised by our data is the nature of TLR3 ligand in *Chlamydia*-infected cells. dsRNA, the known inducer of TLR3 activation, are usually observed in viruses. In bacteria a variety of small non-coding RNAs are known to be involved in post-transcriptional gene regulation, often in response to stress, and might adopt dsRNA structure. For instance, limitation of iron in *E. coli* causes the synthesis of a small RNA (RyhB) that binds to the target mRNA to form dsRNA (Massé and Gottesman, 2002). A high amount of dsRNA is also found in lactic acid bacteria, which triggers IFN $\beta$  induction by infected cells through activating TLR3 signaling pathway (Kawashima et al., 2013). Whether such dsRNA-like structures are formed during *Chlamydia* infection, in the inclusion or in the infected epithelial cells, need to be investigated. Beside dsRNA, other TLR3 activators might remain to be discovered. For instance, the microtubule regulator stathmin has been identified as an endogenous agonist of TLR3 (Bsibsi et al., 2010; Werkman et al., 2020). Identification of TLR3 trigger in the context of *C. trachomatis* infection thus represents a challenging question for the future.

## VI. Beyond TLR3 activation

Having shown the central role of TLR3 in sensing the infection, we initiated the study of the intermediates between TLR3 activation and IL-6/IL-8 transcription. We showed that MAPK ERK is activated by *Chlamydia* infection, consistent with other reports (Su et al., 2004; Tang et al., 2021b; Zadora et al., 2019b). Silencing TLR3 abolished ERK activation triggered by *Chlamydia*. In addition, ERK inhibitor U0126 attenuated ERK activation and inflammation upon *Chlamydia* infection as well as the synergy between IFN-I and bacteria. These clues suggested that ERK lies downstream of TLR3 for *Chlamydia*-triggered inflammatory response. It will be interesting to identify the downstream transcription factor(s) responsible for the increase in *IL-6* and *IL-8* transcription. The transcription factors AP1 has been implicated in *Chlamydia*-induced *IL-8* expression in HeLa cells (Jolly et al., 2019). MAPK-activated AP1 is also involved in TLR3 signaling pathway (Muccioli et al., 2012). Thus, we will test whether AP1 participates in *Chlamydia*-induced inflammation in our cell model, and in the synergy between *Chlamydia* and IFN-I.



## CONCLUSION

During my PhD, I combined experiments in primary cells and HeLa cells to understand the innate response of epithelial cells to *C. trachomatis* infection, and more specifically the synergetic effect of infection and IFN-I on the expression of some key inflammatory cytokines. This synergy between IFN-I and the bacteria on the inflammatory response of the tissue may contribute to the hyperinflammation observed in the FGT in some women infected with *C. trachomatis*. The pathological consequences of *C. trachomatis* infection are precisely the irreversible tissue damages provoked by the infection, presumably due to local hyperinflammation and fibrosis during scarring. Thus, anti-IFN treatment or TLR3 antagonist might represent interesting therapeutic strategies for symptomatic women undergoing acute *C. trachomatis* infection, to limit the inflammation and improve prognosis.

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## RÉSUMÉ

*Chlamydia trachomatis* est une bactérie intracellulaire obligatoire, qui se développe principalement dans cellules épithéliales des muqueuses du tractus génital. Asymptomatiques dans la plupart des cas, les infections par ce pathogène peuvent entraîner des inflammations pelviennes. L'inflammation peut elle-même engendrer des fibroses et mener à une infertilité tubaire chez les femmes. Il est important d'étudier la réponse des cellules épithéliales, qui constituent la première ligne de défense contre l'infection par *C. trachomatis*, pour mieux comprendre ses manifestations pathologiques dans le tractus génital féminin (FGT).

À cette fin, nous avons mis au point un protocole simplifié pour isoler une fraction très pure de cellules épithéliales primaires, à partir du FGT de patientes subissant une hystérectomie. Nous avons observé que ces cellules épithéliales primaires étaient moins permissives à l'infection par *C. trachomatis* que la lignée cellulaire classiquement utilisée en laboratoire, *i.e.* la lignée HeLa. Nous avons montré que la différence de milieu de culture et l'ajout de sérum dans les cultures de cellules HeLa, expliquent une grande partie de ces différences. Cependant, testées dans des milieux identiques, les cellules épithéliales primaires issues de l'ectocol se sont révélées moins permissives que les cellules HeLa vis-à-vis du développement de *C. trachomatis*. Enfin, les cellules épithéliales primaires exprimaient globalement davantage de cytokines pro-inflammatoires, au niveau basal et après infection à *C. trachomatis*, que les cellules HeLa, suggérant une forte capacité des cellules épithéliales primaires à monter une réponse inflammatoire.

Nous nous sommes ensuite attachés à comprendre pourquoi l'interféron de type I (IFN-I) agissait-t-il en synergie avec l'infection par *C. trachomatis* vis-à-vis de la réponse proinflammatoire des cellules. Nous avons démontré que l'IFN-I, mais pas *C. trachomatis*, augmentait l'expression de plusieurs récepteurs de reconnaissance de motifs bactériens. L'extinction de l'expression du récepteur TLR3, ou la délétion de ce gène, empêche la synergie entre l'IFN-I et *C. trachomatis*. Nous avons identifié la voie de signalisation intermédiaire entre l'activation du récepteur de l'IFN-I et l'expression du TLR3, ainsi que la signalisation en aval de l'activation de TLR3, qui aboutit à l'expression des cytokines pro-inflammatoires interleukines-6 et 8. Ainsi, l'IFN-I exacerbe la réponse inflammatoire de l'hôte déclenchée par l'infection à *C. trachomatis* via une augmentation de l'expression du TLR3. La synergie entre l'IFN-I et les bactéries à stimuler la signalisation pro-inflammatoire des cellules épithéliales pourrait jouer un rôle dans les lésions tissulaires qui résultent de l'infection chez certaines patientes.

## ABSTRACT

*Chlamydia trachomatis* is an obligate intracellular bacterium, which grows mainly in epithelial cells of the mucous membranes of the genital tract. Asymptomatic in most cases, infections with this pathogen can lead to pelvic inflammations. The inflammation itself can lead to fibrosis and tubal infertility in women. To better understand the pathological manifestations in the female genital tract (FGT), it is important to study the response of epithelial cells, which constitute the first line of host defense against *C. trachomatis* infection.

To this end, we developed a simplified protocol to isolate a very pure fraction of primary epithelial cells from the FGT of patients undergoing hysterectomy. We observed that these primary epithelial cells were less permissive to *C. trachomatis* infection than the cell line classically used in laboratories, i.e. HeLa cells. We have shown that the difference in culture medium and the addition of serum in HeLa cell cultures explain a large part of these differences. However, when tested in an identical culture medium, primary ectocervical epithelial cells were found to be less permissive than HeLa cells towards *C. trachomatis* infection. Finally, primary epithelial cells expressed overall more pro-inflammatory cytokines, both basally and after *C. trachomatis* infection, than HeLa cells, suggesting a strong capacity of primary epithelial cells to mount an inflammatory response.

We then focused on understanding why type I interferon (IFN-I) acts synergistically with *C. trachomatis* infection towards the proinflammatory response of epithelial cells. We demonstrated that IFN-I, but not *C. trachomatis*, increased the expression of several bacterial pattern recognition receptors (PRRs). Expression silencing of TLR3 receptor, or deletion of this gene, prevented synergetic effect between IFN-I and *C. trachomatis*. We also identified the intermediate signaling pathway between IFN-I receptor activation and TLR3 expression, as well as the signaling downstream of TLR3 activation, which results in the expression of the pro-inflammatory cytokines interleukin-6 and 8. Based on these data, we conclude that IFN-I exacerbates the host inflammatory response triggered by *C. trachomatis* infection via increased TLR3 expression, and that this synergetic effect between IFN-I and bacteria on pro-inflammatory signaling in epithelial cells may play a role in the tissue damage that results from infection in some of the patients.