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Molecular mechanisms of *Streptococcus pyogenes* tissue colonization and invasion

Antonin Weckel

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Université Paris Descartes

Ecole doctorale BioSPC

Institut Cochin INSERM U1016 / Equipe Barrières et Pathogènes

Molecular mechanisms of
***Streptococcus pyogenes* tissue**
colonization and invasion

By Antonin WECKEL

PhD Thesis in Microbiology

Directed by Agnès Fouet

Thesis defense: 30th of October 2018

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“We have to remember that what we observe is not nature herself,
but nature exposed to our method of questioning”

Werner Heisenberg

Physics and Philosophy: The Revolution in Modern Science (1958)



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Abstract

Streptococcus pyogenes (Group A Streptococcus, GAS), is a Gram-positive pathogen responsible for a wide range of diseases, from superficial infections such as pharyngitis to invasive infections such as necrotizing fasciitis and endometritis. Endometritis was a huge social burden in the 19th century, killing one woman out of ten after delivery, and still corresponds to 27% of woman invasive infections in France. GAS strains are genetically diverse and harbor specific repertoires. *emm28* is the third most prevalent genotype in France and is associated with endometritis. By two complementary axes, we analyzed factors and mechanisms involved in GAS endometritis.

Using biochemical and cellular approaches, we characterized the interaction between *emm28*-specific surface protein, R28, and host cells. R28 N-terminal domain, R28_{Nt}, and its two subdomains promote the binding to endometrial, cervical and decidual cells. R28_{Nt} and its two subdomains directly interact with the integrins $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$. R28_{Nt} also promotes adhesion to pulmonary and skin epithelial cells. Our results suggest that R28_{Nt}-integrin interactions contribute not only to *emm28*-elicited endometritis, but also to the overall prevalence of the *emm28* strains.

To further characterize the initial events involved in the establishment of GAS endometritis, we developed a novel approach in which we infected *ex vivo* the human decidua, the mucosal uterine lining during pregnancy. We analyzed the outcome using state-of-the-art imaging setup, image processing and analysis. GAS adheres to the tissue and grows at its surface; secreted host factors triggering this growth. GAS readily forms biofilm at the tissue surface, thread-like and inter-chains filaments ultra-structures composing these biofilms. GAS invades the tissue and this depends on the expression of the cysteine protease SpeB. GAS induces the cell death of 50% of cells within 4 h and this cytotoxicity depends on secreted factors, including the Streptolysin O (SLO). Finally, GAS restrains the tissue immune response at the transcriptional and protein levels, the latter depending on the expression of SLO and SpeB.

Résumé

Streptococcus pyogenes, également appelé Streptocoque du Groupe A (SGA), est un pathogène à l'origine d'une grande diversité d'infections, allant d'infections superficielles comme l'angine aux infections invasives, comme la fasciite nécrosante et les endométrites. Au 19^{ème} siècle, une femme sur dix mourait après l'accouchement de fièvre puerpérale, notamment d'endométrite. En France, les infections gynéco-obstétricales correspondent encore de nos jours à 27 % des infections invasives à SGA chez les femmes. Les souches de SGA présentent une forte diversité génétique et de répertoire de facteurs de virulence. Le génotype *emm28* est le troisième génotype le plus prévalent en France et il est associé aux endométrites. Nous avons analysé par deux axes complémentaires les facteurs et mécanismes impliqués dans les endométrites à SGA.

Par des approches de biochimie et de biologie cellulaire, nous avons caractérisé l'interaction entre les cellules de l'hôte et R28, une protéine de surface spécifique du génotype *emm28*. Le domaine N-terminal de R28 (R28_{Nt}) et ses deux sous-domaines favorisent la fixation des bactéries à des cellules endométriales, cervicales et déciduales. Ils fixent de manière directe les intégrines $\alpha3\beta1$, $\alpha6\beta1$ et $\alpha6\beta4$. Par ailleurs, R28_{Nt} promeut aussi l'adhésion à des cellules épithéliales de la peau et des poumons. Ces résultats suggèrent que la fixation des intégrines par R28_{Nt} concourt, non seulement, aux endométrites dues au génotype *emm28*, mais aussi, et de manière plus générale, à la prévalence de ce génotype.

Afin de mieux caractériser les étapes précoces essentielles au développement des endométrites à SGA, nous avons infecté *ex vivo* la décidue humaine, qui correspond à la membrane utérine durant la grossesse. Nous avons analysé les effets de l'infection de la décidue par des techniques de microscopie et d'analyse d'image de pointes. SGA adhère au tissu et se multiplie au contact de celui-ci grâce à des éléments sécrétés par le tissu. Sur ce tissu, SGA forme des biofilms composés d'ultrastructures ressemblant, pour certains, à des fils reliant deux coques d'une même chaîne et, pour d'autres, à des filaments reliant plusieurs chaînettes ; certains s'organisent en réseau. GAS envahit en profondeur le tissu, ce qui dépend de l'expression de la cystéine protéase SpeB. SGA induit la mort de la moitié des cellules en moins de 4 h à travers la sécrétion de différents facteurs, dont la Streptolysine O (SLO). Enfin, GAS est capable de restreindre la réponse immunitaire du tissu à l'échelle transcriptomique et protéique, le contrôle protéique dépendant de l'expression de SLO et de SpeB.

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List of principal abbreviations

C-ter: C-terminal

ECM: extracellular matrix

EPS: extracellular polymeric substance

Fg: fibrinogen

FH: factor H

FHL-1: factor-H-like protein 1

Fn: fibronectin

GAS: Group A Streptococcus

GBS: Group B Streptococcus

HVR: hypervariable region

LTA: lipoteichoic acid

mRNA: messenger ribonucleic acid

NET: neutrophil extracellular traps

NF: necrotizing fasciitis

N-ter: N-terminal

Pg: plasminogen

Pm: plasmin

QS : quorum sensing

SA regulator : stand-alone regulator

SAGs: superantigens

SEM: scanning electron microscopy

sRNA: small ribonucleic acid

STSS: streptococcal toxic shock syndrome

TCS: two-component system

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Foreword

Streptococcus pyogenes is a strictly human pathogen responsible for a wide range of diseases, from superficial infections, such as pharyngitis, to life-threatening invasive infections, such as necrotizing fasciitis and endometritis. *S. pyogenes* is also involved in post-infectious complications such as rheumatic fever. Strains are genetically highly diverse and classified through the sequencing of the *emm* gene, with more than 200 *emm*-types identified. *emm28* is the third most prevalent *emm*-type in France and is associated with gynecological infections. During the course of infections, numerous ubiquitous or strain-specific virulence factors are involved. However, there are few models that enable an integrative study of *S. pyogenes* early steps of infection which are involved in both superficial and invasive infections. The first part of this dissertation will be an introduction on *S. pyogenes* pathogenesis. The second part will present the main results of my thesis, composed of two complementary aspects addressing *S. pyogenes* and endometritis. The last part of my dissertation will discuss the results and conclude with some perspectives on this work. The introduction will review the most important features of *S. pyogenes* pathogenesis, focusing on the virulence factors, the mechanisms involved and the study models. In the result chapter, we will first present our results on the *emm28*-specific R28 protein involved in promoting adhesion to host cells and its receptors. In the second part, an original model of *ex vivo* infection of a human tissue will be presented, where bacterial colonization, biofilm formation, tissue invasion, induced cytotoxicity and modulation of the immune response are analyzed, with a focus on the role of the Streptolysin O (SLO) and the cysteine protease (SpeB) on several of these phenotypes.

Introduction

Introduction

In this introduction, we will first describe the main bacteriological and clinical characteristics of *S. pyogenes*, then the virulence factors and their regulations, the mechanisms involved in the pathogenesis and finally, the main experimental approaches to understand *S. pyogenes* pathogenesis.

1. *Streptococcus pyogenes* morphology, epidemiology and induced diseases

In this chapter, we briefly introduce the main morphological and microbiological characteristics of *S. pyogenes*, the strain classification, the different *S. pyogenes* induced diseases and their burden, genotypes prevalence and finally we focus on a specific association between *emm28* strains and gynecological-obstetrical diseases.

1.1. *Streptococcus pyogenes* history and morphology

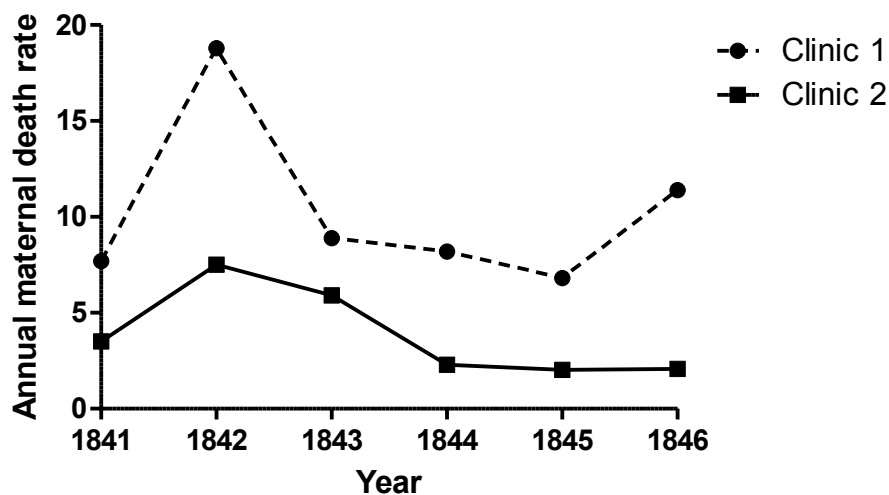
1.1.1. The intimate relation between the history of *S. pyogenes* and puerperal fever

“Everything was in question; everything seemed inexplicable; everything was doubtful. Only the large number of deaths was an unquestionable reality.” Ignaz Semmelweis was a young Hungarian physician in 1847 who worked in a Vienna hospital when he made this statement. The Vienna hospital had two clinics, and as shown in Figure 1, the mortality rate was strikingly higher in one than in the other (1). In the 19th century, up to one woman out of 10 died after delivery and this was not the case before (Figure 2). This had a huge societal impact and was a great society burden. It is during his investigation on the origin of the differences between the mortality rates in the clinics that he discovered that hand washing with specific solution (chlorinated lime) could strongly reduce maternal death rate (Figure 3). Semmelweis’ train of thoughts that lead to his assumption that particles from cadavers were the cause of puerperal fever is summed up in Table 1.

Theodor Billroth is an Austrian surgeon thought to be the first to describe streptococcal infections, cases of erysipelas and wound infections. In 1874, he described “small organisms (Kettenkokken) as found in either isolated or arranged in pairs, sometimes in chains of four to twenty or more links” (2). Louis Pasteur became aware of these results as soon as 1875.

Table 1. Semmelweis' train of thoughts

Observation	Interpretation
<ul style="list-style-type: none"> The maternal death rate in clinic 1 is higher than in clinic 2 Student and professor only visit the clinic 1 Professor Jakob Kolletschka, a forensic, died after a student hit him accidentally with a knife that touched the dead body. Autopsy of the professor revealed similar clinical manifestations as puerperal fever patients Rotten fruit transmit putrefaction to living fruits The death rate was lower in December to April 1847, where the assistant professor went less to the morgue Women with high dilatation were most likely to die than others There are more death in the 19th century than before 	<ul style="list-style-type: none"> Endemic specificities of clinic 1 induce puerperal fever Students and professor could be the vector of the disease Something from the cadaver could explain both the professor and the maternal deaths “Disease” can be transmitted by contact Physician, by coming from the morgue transfer the disease These women were “visited” several times, increasing the risk to get the disease Hospitals and the increase in knowledge in medicine correlates with the increase in maternal death rate

**Figure 1. Maternal death rate in the two clinics**

Clinic 1 was the normal clinic, where students and professors took care of pregnant women until delivery and after. In the Clinic 2, most women already had delivered and were taken care of by midwives. Data from (1)

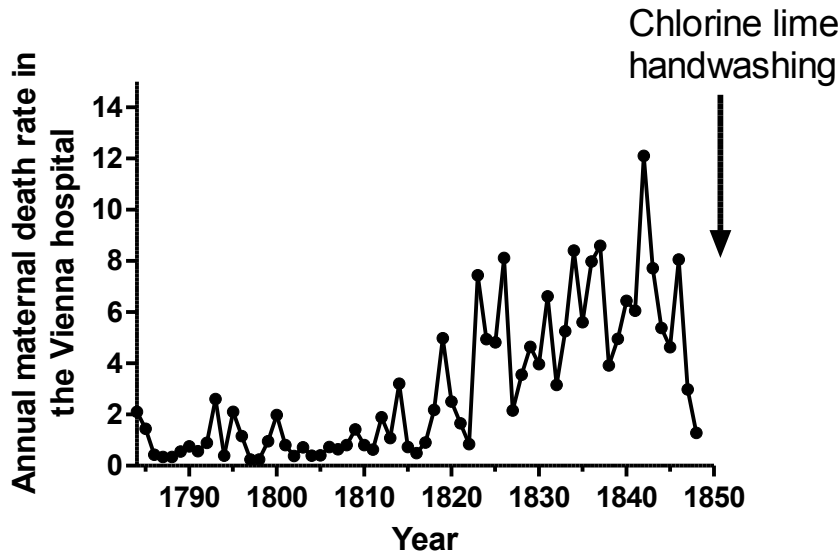


Figure 2. Evolution of the maternal death rate between 1784 and 1848
 Before 1820, maternal death was low in the Vienna hospital (~1%), after 1820 and until handwashing was mandatory, the maternal death rate was around 6%. Data from (1).

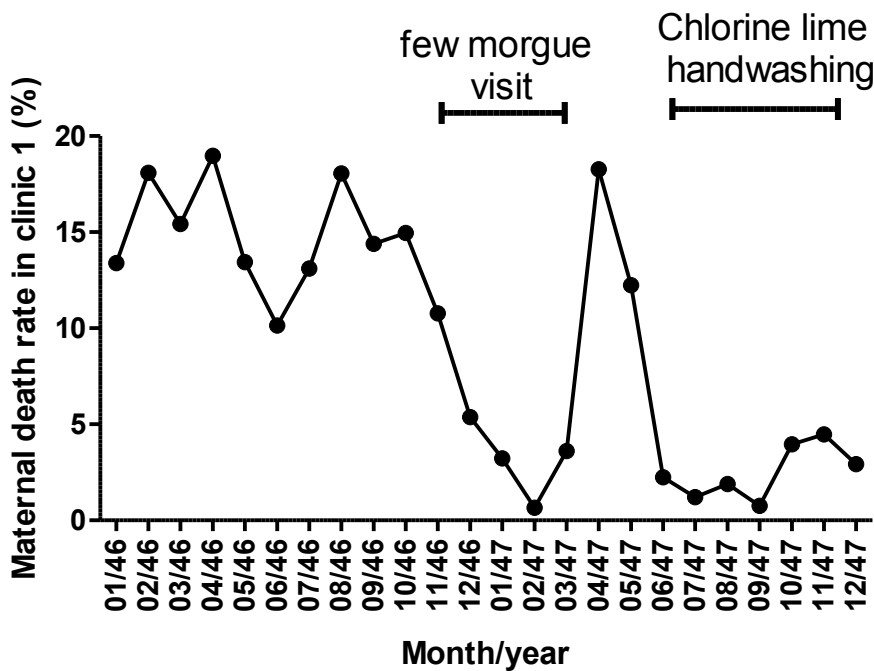


Figure 3. Monthly maternal rate between 1846 and 1847
 There was a change in assistant professor in December 1846 until March 1847. In April 1847, Semmelweis became assistant professor and went every morning to the morgue before taking care of pregnant women. In May 1847, chlorine lime handwashing became mandatory. Data from (1)

On the 21st of January 1879, thirty years after Semmelweis' discovery, Maurice Perrin, a French physician declared: "How can we explain that in the countryside there is not even a case out of a 1000 of puerperal fever, perhaps even one out of 10 000, whereas in hospitals postpartum women suffer dreadful epidemy"¹ (3). In early 1879, Pasteur isolated microbes from a woman with puerperal fever, which looked exactly as the one described by Theodor Billroth. When Dr Hervieux, director of the Port-Royal maternity, claimed that a mysterious "miasma" was the explanation for puerperal fever and not a germ (called vibrion), he ended his speech by declaring: "I have an awful fear [...]: it is to die before we ever find this vibrion"² (4). Pasteur at this point had not validated the Koch postulate for the puerperal fever. However, Pasteur replied on the same day to Hervieux's speech by stating "Let the Academy allow me to draw the dangerous microbe I believe is responsible for puerperal fever"³, and he draw a microbe that Pasteur describes himself as "an organism made of grains in couples or chains"⁴ (4). Later in 1879, Pasteur went to Hervieux's clinic and isolated from multiple women suffering puerperal fever the same organism as that isolated by Theodor Billroth, confirming his initial statement (4). The name *Streptococcus pyogenes* (streptus: chain, pyo: pus, genes: forming) was coined 5 years later in 1884 by Friedrich Julius Rosenbach (5).

Streptococci are classified through one of the very first serotyping methods, developed by Rebecca Lancefield in 1919. Lancefield identified several immunogens to distinguish streptococci, and most of them were carbohydrates. *S. pyogenes* belongs to the Lancefield Group A family of Streptococcus (GAS) (6).

1.1.2. GAS microbiological characteristics

GAS is a Gram positive bacterium, β -hemolytic, with a low G+C % DNA content. Cocci are assembled in chains of minimum two cocci (Figure 4). GAS is grown on blood or Todd Hewitt broth supplemented with 0.2% yeast extract agar plates and its multiplication is favored by CO₂ and anaerobia. GAS is auxotroph for 15 amino acids and its primary source of carbon is glucose [(7, 8), for review: (9)].

¹ «Comment se fait-il qu'à la campagne on ne compte pas un cas de fièvre puerpérale sur mille, peut-être sur dix mille accouchées, tandis que les hôpitaux de femmes en couches sont dévastés par d'effroyables épidémies?» p50

² « Mais, faut-il l'avouer, j'ai une peur terrible, une peur dont je ne puis me défendre, et que l'Académie comprendra : c'est celle de mourir avant qu'on n'ait découvert ce vibrion-là » p 256

³ « Eh bien, que l'Académie me permette de dessiner sous ses yeux le dangereux microbe auquel je suis porté en ce moment à attribuer l'existence de cette fièvre » p 259

⁴[...] organisme formé de couples de grains ou de chapelets de grains". p 272

GAS is a non-motile, extracellular and human specific pathogen. The size of the GAS genome varies from 1.75 to 1.9 megabases, harboring roughly 2000 genes. Eighty-five % of the coding genome is the core genome conserved between all strains. Moreover, 10 % of the metagenome corresponds to prophages, largely contributing to strain diversity (10).

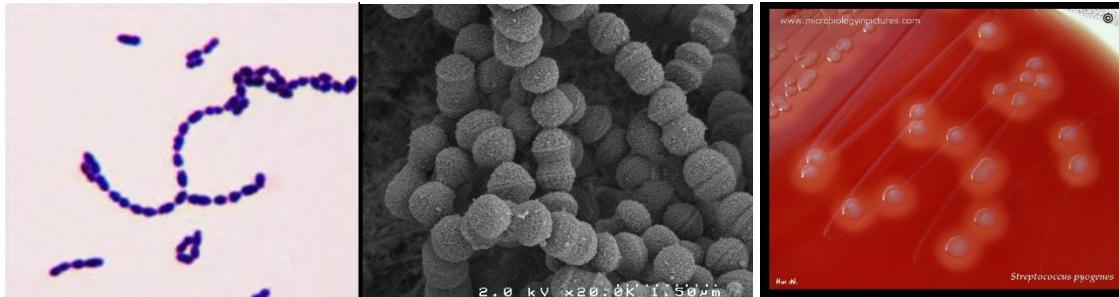


Figure 4. Morphology of GAS

Left: Gram-positive staining of GAS. Middle: Scanning electron microscopy of GAS. Right: GAS β-hemolysis on sheep blood agar plates.

1.2. GAS *emm*-typing

While identifying the carbohydrates involved in the Lancefield Group typing, Lancefield also isolated another major immunogen of GAS which is a protein, and she used this protein as a typing method for GAS strains. After discovering that a non-typable strain was less mucoid, she called this protein the M protein (11). She then set up the first collection and classification of GAS strains depending on the M protein expressed.

The main variations of the M protein are in the N-terminal domain, called the hypervariable region (HVR) (Figure 5) and the serotyping is based on antibodies directed against this region (12). This is an important functional domain, and antibodies against it highly decrease the strain virulence (13). The diversity of M proteins is explained by the immunological pressure and the critical importance of the M protein in virulence: people immunized for one M protein will no longer be affected by the strains presenting this M protein making it a major selective pressure. In addition to this, the more people are infected by a given strain, the smaller the pool of potentially neo-infected individuals is, reducing the strain prevalence. This immunological pressure implies a strong immunization against the M protein encountered, which occur to a lesser extent with antibiotic treatments which stop the infections before a strong immune response is established.

Today the classification no longer stands on serotyping but on genotyping, by sequencing the 5' of the *emm* gene, coding for the 50 first residues of the N-terminal domain of the M protein (14). The sequencing of an array of 1064 invasive strains identified 225 *emm*-types (15).

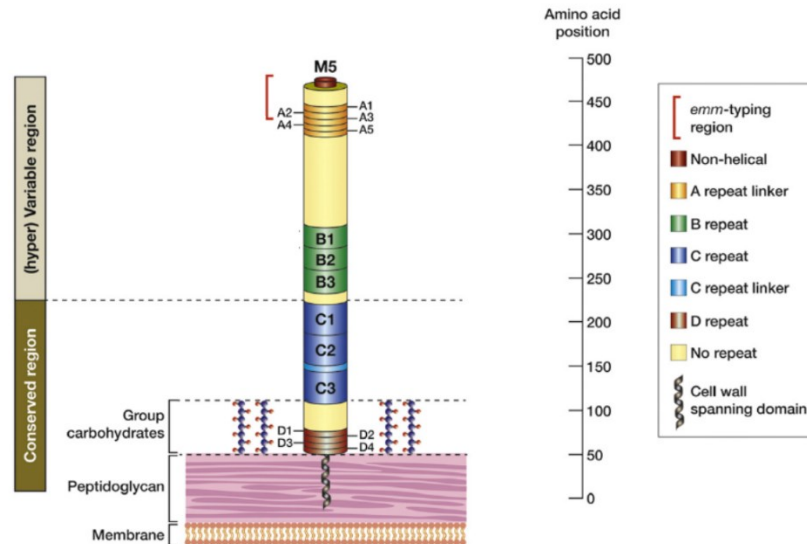


Figure 5. Structure of the M protein

Structure of the M5 protein to highlight the region used for serotyping (hypervariable region), and the 50 residues region now used for the *emm*-typing (in red). Adapted from (12).

1.3. GAS carriage and induced diseases

1.3.1. Asymptomatic colonization

GAS asymptomatic colonization or carriage is frequent in the throat: a meta-analysis showed that around 10% and 6% of children and 2.8% and 2% of adult are colonized in high-income and low-income countries, respectively (16). Some individuals are colonized for long periods of time (several years); the reasons for this persistent state are not well understood, but it appears that it has no role in post-infectious manifestations nor bacterial transmission (17, 18). Moreover, carrier children frequently switch *emm*-types throughout their lives (19).

1.3.2. Superficial infections

Pharyngitis and scarlet fever. Pharyngitis is a superficial infection of the oropharynx and GAS accounts for 4-10% pharyngitis cases in adults (20). GAS is annually involved in 616 million cases of pharyngitis in the world (21). Pharyngitis is more prevalent in OECD (National Organization for Economic Cooperation and Development) than non-OECD countries (16).

The complexity of GAS pharyngitis diagnostic is that most pharyngitis cases are not due to GAS and GAS is found asymptotically in the throat. Consequently, a GAS positive swab does not systematically indicate a GAS-elicited pharyngitis. This could explain the dichotomy between GAS positive pharyngitis and serologically positive pharyngitis, with only around 50% of matches (16).

Impetigo and erysipelas without bacteremia. GAS is also involved in superficial skin infections, such as impetigo and erysipelas. In contrast to pharyngitis, impetigo is predominant in non-OECD countries, and there are an estimated 111 million cases of GAS-elicited impetigo per year (21).

1.3.3. Invasive infections

GAS invasive infections are infections during which GAS is found in normally sterile compartments or with Streptococcal Toxic Shock Sndrome (STSS). GAS invasive infections are responsible for 163 000 deaths per year (21) and, in France, their annual incidence is estimated to be 3.1/100 000, with a case-fatality ratio of 14% (22). Of note, the number of cases of invasive infections has been increasing in the world since 1990s (21), with an increase of 4% per year in France between 2007 and 2014, partly due to an increase in the number of invasive infections of people above 65 years of age (Figure 6).

Necrotizing fasciitis. Necrotizing fasciitis (NF) is the infection of the deeper layers of the skin, below the fascia, and is a rapidly progressing and life-threatening disease, with a mortality around 30% (23). In the United States of America, there are around 700 cases of NF per year and in France, in 2007, there has been 104 cases of GAS-elicited NF, of which 22 % were fatal (22, 24). NF risk factors are varicella, wound, burn and blunt trauma (25).

Bacteremia and septic shock. Bacteremia is the presence of bacteria in the blood and is associated with 60% of French invasive infections (<https://cnr-strep.fr/>). For some bacteremia, the origin of the bacterial translocation is unknown, and is called bacteremia without identified focus; this is the case for around 22% of French GAS invasive infections (22).

STSS. Bacterial toxins in the bloodstream or in a tissue can induce the hyperactivation of the immune system, further inducing a cytokine “storm” and shock followed by organ failures. This is called a septic shock and more specifically the STSS. STSS is the most life-threatening complication of GAS infections, with a mortality of up to 43% (22).

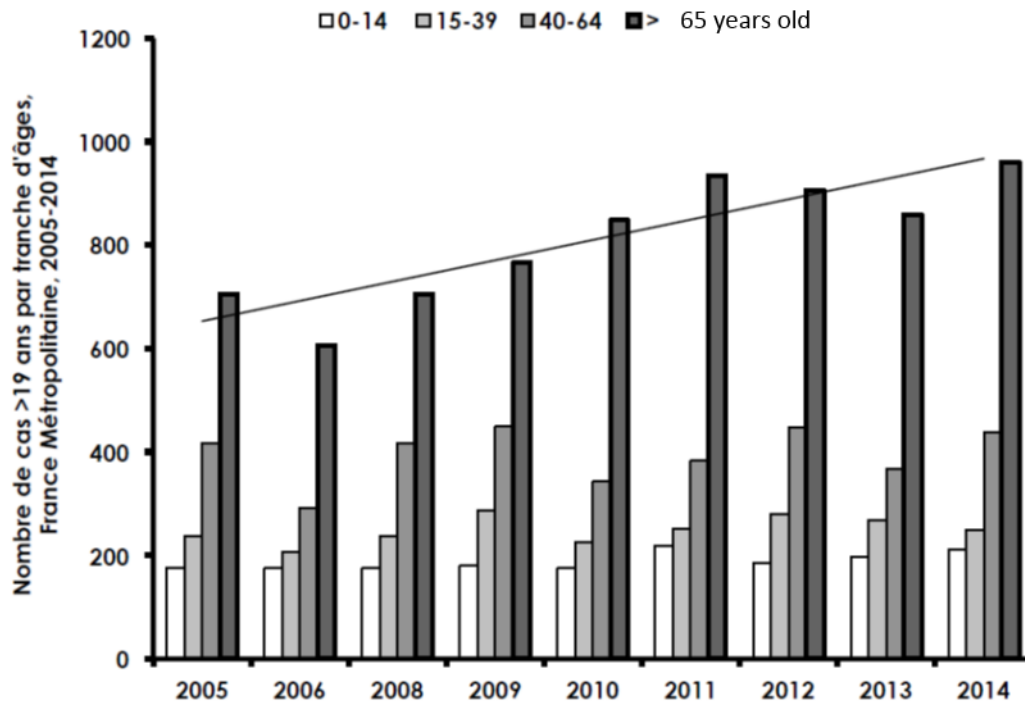


Figure 6. Number of invasive infections in adults in France

Number of cases of invasive infections in France depending on the age. There is an overall increase of the number of invasive infections in France, driven by an increase in the number of invasive infections in people aged above 65 years. From (<https://cnr-strep.fr/>).

Endometritis and Puerperal fever. Puerperal fever, or childbed fever, is the increase of temperature in the 24 h following delivery and is due to an infection, referred to as puerperal infection or post-partum infection. The majority of these infections are in the gynecological tract, including the endometrium. In this thesis, endometritis was studied as a model of GAS invasive infections.

While less frequent than in the 19th century, puerperal fever is still responsible for 75 000 maternal deaths annually in the world, affecting 5-10% of pregnant women (26). A recent analysis in the UK showed an incidence of post-partum endometritis of 109 cases per 100 000 persons/year (27), slightly higher with a US rate of 59 per 100 000 persons/year (28). A population based study in Sweden showed that 2% of women suffered endometritis following delivery (29). The post-partum is a favorable condition for infections with several pathogens: *Escherichia coli*, Group B Streptococcus (GBS), *Staphylococcus aureus*, anaerobic bacteria and *Listeria monocytogenes* (30), but GAS is involved in 50 % of maternal sepsis, making it the pathogen most frequently responsible for maternal infections; furthermore, it is the most aggressive one. In France, infections of the gynecological sphere still correspond to 26.8%

of women, all age groups considered, GAS invasive infections (<https://cnr-strep.fr/>) (Table 2), of which 15.9% are endometritis. Women have a 20-fold increased incidence of GAS invasive diseases compared with non-pregnant women (31). The postpartum, abortion, *in vitro* fertilization and intrauterine devices all are risk factors for endometritis and are involved in more than half of GAS-elicited endometritis France (Table 2).

Table 2. GAS gynecological-obstetrical invasive infections in France 2006-2015, risks factors and prevalence of the main *emm*-genotypes

GAS invasive infections	Women	Gynecological-obstetrical infections	Endometritis	Post-partum and risk factors [*]
All	2489	668/ 26.8%	395/ 15.9 %	201/ 8.1 %
STSS	453/ 18.2%**	72/ 10.8 %	27/ 6.8 %	N.D.
Fatality case	283/ 11.4 %	16/ 2.4 %	5/ 1.3 %	N.D.
<i>emm28</i>		168 /25.1 %	104/ 26.3 %	54/ 26.9 %
<i>emm1</i>		113/ 16.9 %	66/ 16.7 %	27/ 13.4 %
<i>emm89</i>		123/ 18.4 %	75/ 19 %	40/ 19.9%
others (30 genotypes) ^a		240/ 39.5 %	150/ 38 %	80/ 39.8 %

^{*} risk factors: abortion, intra uterine device, *in vitro* fertilization. ^a 30 other genotypes.

**First numbers correspond to the number of cases and strains, the second ones, in italic, to the percentages: first line percentage of all women invasive infections; STSS and fatality case: percentage of the STSS/deaths for each column. The percentage for the strain genotypes corresponds to the percentage of strains for each column. N.D.: not determined. From (<https://cnr-strep.fr/>).

1.3.4. Post-infectious complications

GAS infections are the indirect cause of several pathologies, such as glomerulonephritis and rheumatic arthritis. They all are auto-immune diseases due to the cross-reaction between GAS epitopes and host epitopes (32). Overall, these complications are responsible for 354 000 deaths per year and the incidence is very important in developing countries, with for example 15 to 16 million people suffering from GAS induced rheumatic heart diseases and 282 000 new cases per year (21).

1.4. Epidemiology of the *emm*-types strains

The diversity of strains responsible for superficial and invasive infections is higher in developing countries than in high income countries, which could be explained by climatic differences, genetic susceptibility and social economic reasons (33, 34). The distribution of

strains responsible for invasive infections in France and in Europe is summarized in Figure 7 and Table 3, respectively.

Table 3. Main GAS *emm*-types involved in invasive infections in Europe from 1st January 2000 to 31st May 2017

Country	1 st	2 nd	3 rd	4 th
Czech Republic	<i>emm1</i>	<i>emm81</i>	<u><i>emm28</i></u>	
Denmark	<i>emm1</i>	<u><i>emm28</i></u>	<i>emm89</i>	
England/Wales	<i>emm3</i>	<i>emm1</i>		
Finland	<u><i>emm28</i></u>	<i>emm89</i>	<i>emm1</i>	
France	<i>emm1</i>	<u><i>emm28</i></u>	<i>emm89</i>	
Germany	<i>emm1</i>	<u><i>emm28</i></u>	<i>emm3</i>	
Greece	<i>emm1</i>	<i>emm12</i>		
Hungary	<i>emm1</i>			
Iceland	<i>emm1</i>	<i>emm89</i>	<u><i>emm28</i></u>	
Ireland	<i>emm1</i>	<i>emm12</i>	<u><i>emm28</i></u>	
Italy	<i>emm1</i>	<i>emm12</i>		
Norway	<i>emm89</i>	<u><i>emm28</i></u>	<i>emm3</i>	
Poland	<i>emm1</i>	<i>emm12</i>		
Portugal	<i>emm1</i>	<i>emm89</i>	<i>emm3</i>	
Romania	<i>emm1</i>			
Scotland	<i>emm1</i>			
Spain	<i>emm1</i>	<i>emm3</i>		
Sweden	<i>emm89</i>	<i>emm81</i>	<u><i>emm28</i></u>	<i>emm1</i>

emm-types involved in more than 10% of the invasive infections are represented in the order of prevalence from left to right. The countries are in the alphabetical order. The *emm28* genotype used in this thesis is underlined, the data do not include the most recent French epidemiological data (Figure 4), and *emm28* are no longer the second most prevalent strain in France since 2012. Adapted from (35).

emm1 strains are the most prevalent strains in high income countries, with a clone emerging in the 1980s that is more efficient in colonizing and that increased the *emm1* overall prevalence (36). Since 2008 in Europe, an *emm89* clone has been rapidly emerging (37). While there are more than 200 described *emm*-types, in 2016, 60% of GAS invasive strains in France are from four genotypes: *emm1*, *emm89*, *emm28* and *emm12* (Figure 7) (<https://cnr-strep.fr/>). In Europe, the main genotypes responsible for invasive infections are *emm1*, *emm28*, *emm89*, *emm3*, *emm12* and *emm81* (35).

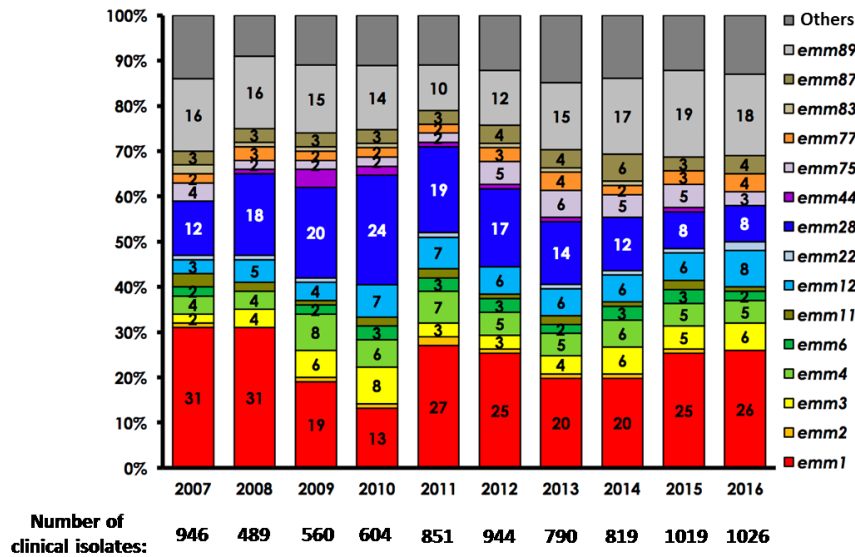


Figure 7. Distribution of GAS *emm*-types involved in invasive infections in France (National Center for Streptococcus activity report, https://cnr-strep.fr/images/CNR-STREP/rapport/rapport_CNR-strep_2016.pdf).

1.4.1. Tissue tropism and *emm* patterns

GAS induces a wide range of diseases and there are few associations between diseases and genotypes (38). GAS strains can be classified with a sequence typing in five gene patterns, A to E, which is based on the analysis of the patterns of the *emm*, *emm*-like genes and *sof/sfbx* genes of numerous clinical strains (Figure 8) (39, 40).

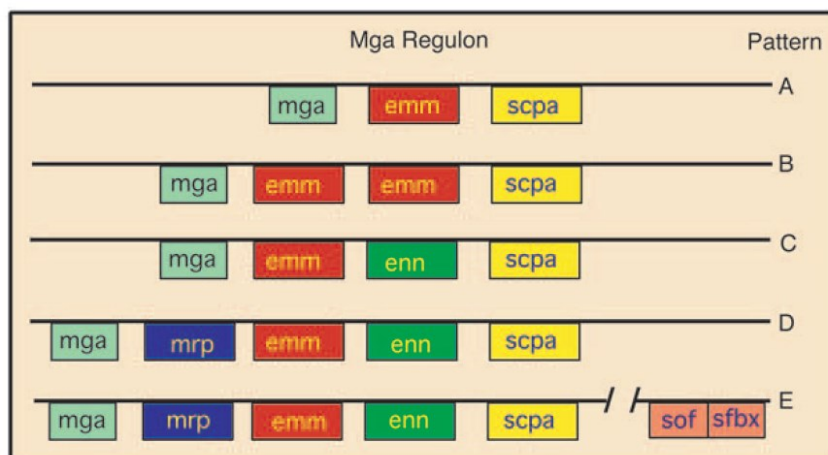


Figure 8. *emm* pattern classification
The organization of the Mga locus and the presence or not of *sof/sfbx* can discriminate GAS strains into 5 *emm* pattern, from A to E. From (41).

There is a clear association between specific *emm* patterns and skin/throat tropisms (40, 42). A-C strains represent ~47% of throat infections, but only 8% of impetigo isolates. In contrast, D

strains represent ~50% of impetigo strains, but only 2% of pharyngitis strain, and E pattern strains are equally represented in both infections, thus called ubiquitous (42).

1.4.2. The *emm28* association with gynec-obstetrical sphere infections

The *emm28* genotype is one of the three main genotypes encountered in Europe (Figure 7, Table 2). It belongs to the E pattern and it is associated with gynec-obstetrical infections, representing around 25% of these infections while being responsible for only 15% of all invasive infections (43). More specifically, the association between puerperal infections and the *emm28* was described in several countries (44–47). *emm28* strains are, together with *emm1*, associated with severe cases of puerperal infection (46). The R28 protein expressed by *emm28* strains has been implicated in the association of *emm28* strains with puerperal infection (48). The first sequence of an *emm28* genome identified two *emm28* specific genomic regions (RD1 and RD2) that could explain this association. RD2 is a 37.4 kb region with genes encoding several cell-wall anchored proteins, including AspA, also known as AgI/II (M28_Spy1325) and R28 (M28_Spy1336) (Figure 9).

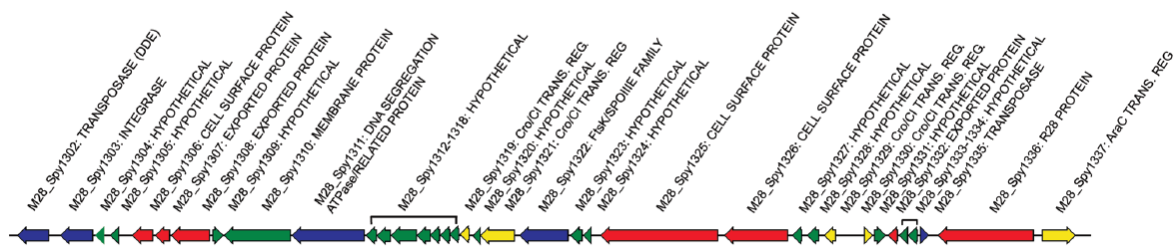


Figure 9. RD2 region of *emm28* strains.

In green are shown inferred hypothetical proteins of unknown function, in red, extracellular proteins, in yellow, putative gene regulators and in blue, putative mobility/transfer ORFs. From (45)

The integrative conjugative element RD2 contains several genes related to GBS genes and was most likely horizontally transferred from GBS (45, 49). Since GBS colonizes 10-30% of healthy women uro-vaginal tract (50), it was suggested that RD2 harbors genes involved in the *emm28* association with gynec-obstetrical diseases.

2. GAS virulence factors and their regulation

2.1. GAS virulence factors

In this chapter, we will describe different factors whose implication in the GAS pathogenesis mechanisms will be discussed in chapter 3 and in the Results section. We will briefly discuss the concept of “virulence” factors in the light of GAS pathogenesis, and we will then describe the different virulence factors and then their regulation.

The virulence concept in the light of GAS pathogenesis

The concept of virulence and virulence factors, coined in the early 20th century aggressins and virulins by Rosenow, was based on the concept that some pathogenic elements could confer the pathogenic property to avirulent organisms (51). This was linked to the concept of intrinsic pathogenicity of a bacterium, in opposition to commensal and avirulent organisms, and to the intrinsic capacity of a factor to yield virulence. However, as described by Casadevall and Pirofski in 1999, virulence emerges from the intersection between the repertoires of factors of an organism and the host environment encountered (52). The pathogenicity of an organism is dependent on how much, where and when it expresses its factors. Moreover, many “virulence factors” expressed by highly pathogenic bacteria are also expressed by commensal bacterium, and the importance of factors in virulence depends on the genetic backgrounds (with the exception of certain toxins). Therefore, virulence properties and specificity of a factor are not intrinsic.

In the following paragraph, we will describe different factors which, in the host environment tested and the bacterial genetic background used, are implicated in the virulence of the tested strains. The virulence property of any factor cannot be extended to all GAS strains or diseases. The biochemical nature and main properties will be analyzed in this section, while the regulation is analyzed in section 2.2, and the role of these virulence factors in GAS infections is analyzed in section 3. Factors are discussed by their importance regarding my PhD work and in alphabetical order. A special focus is made on the R28 protein which is one of the main topics of this dissertation. The order of factors discussed and the focus on some factors/regulation pathways do not pretend to hierarchize them relatively to an importance in virulence.

2.1.1. Secreted factors

2.1.1.1. SLO, NADase, SLS, SpeB and Superantigens

SLO and NADase. Streptolysin O (SLO) is a pore-forming cholesterol-dependent cytotoxin (53), secreted as a 69 kDa protein. SLO forms pores through oligomerization of the protein after cholesterol and galactose binding at the cell surface (54, 55). At low multiplicity of infection, SLO pores induce in keratinocytes an endoplasmic reticulum (ER) stress through a cytoplasmic calcium increase (56). This ER stress induces the unfolded protein response in HeLa cells (cervical cells), which further increases asparagine secretion, used by GAS to multiply (57). Ultimately, these pores induce cell death in different cell types (reviewed in section 3.2). In response to the pores, different pathways were shown to restrain their effect: endocytosis and ectocytosis of the damaged membrane (Figure 10).

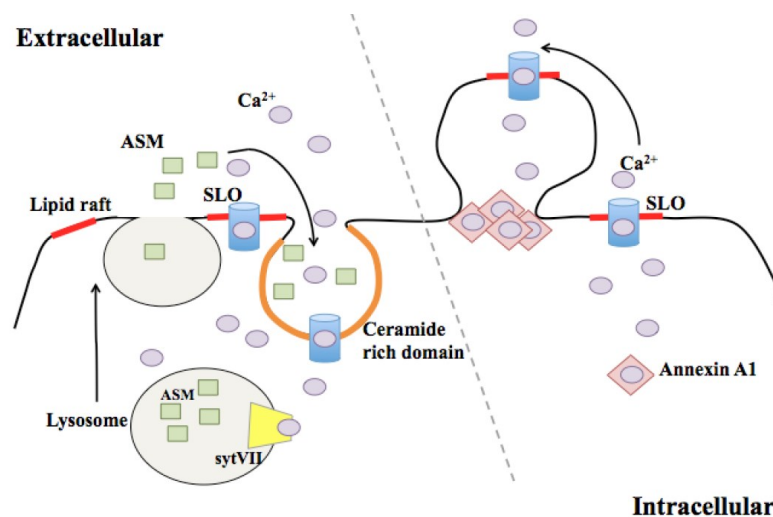


Figure 10. Membrane repair after SLO damages.

Left. Ca^{2+} increase in the cytoplasm is detected by Synaptotagmin VII (sytVII, yellow), which activates lysosome fusion at the membrane. This releases acid sphingomyelinase (ASM, green) in the extracellular compartment, which transforms sphingomyelin in ceramide rich domain, further triggering endocytosis of damaged membrane (58–61). **Right.** Ca^{2+} influx is sensed by annexin A1 which is recruited at the damaged membrane, which triggers ectocytosis of the pore, a mechanism described as “blebbing” (62, 63). From (64)

slo is cotranscribed with *nga*, coding for the streptococcal NAD-glycohydrolase (NADase, Nga or SPN), and *if* coding for NADase inhibitor (IF). NADase is a SLO cofactor and is composed of two domains, one involved in the SLO mediated translocation and the second one is a domain with β -NAD⁺ glycohydrolase activity (65, 66). In the bacterium, the NAD-glycohydrolase activity of NADase is restrained through IF action (67–69). NADase is translocated into cells via a 70 amino acid domain of SLO but the pore formation is not required for NADase translocation

Table 4. SpeB interactions with host components

Protein	Activity	Reference
IgG	C	(94–97)
IgA, IgM, IgD, IgE	D	(97)
C3b	C/D	(98, 99)
Properdin	D	(100)
Chemokines	D	(101)
IL-1 β	A	(102)
H-kininogen	Bradykinin release	(103)
Fibrinogen	D	(104, 105)
Plasminogen	D	(106)
Vitronectin	D	(107)
Fibronectin	D	(107)
Urokinase receptor	C	(108)
MMPs	A	(109, 110)
Decorin	D	(111)
Integrins	B	(93)
Laminin	B	(92)
A1AT	B	(112)
A2M	C	(91)

C= cleavage, A= activation, D= degradation, B= binding. Adapted from (89)

Superantigens. Superantigens (SAGs) are highly potent mitogens of the immune system: they induce hyper-activation and subsequent proliferation of human and some other mammalian T-lymphocytes. In conventional activation, peptides bind to the major histocompatibility class II (MHC) molecules expressed by antigen presenting cells (macrophage, B-lymphocytes and dendritic cells), and the recognition of the peptide/MHC complex by an antigen specific T-cell receptor (TCR) activates the specific T cells. The range of lymphocytes activated is limited by the epitope specificity of the TCR. In contrast, SAGs are able to induce activation of cells by binding non-specifically both the MHC and the TCR molecules (Figure 11).

SAGs are produced by a minority of organisms: some bacteria, among the most common bacterial geniuses, Group A, C and G *Streptococcus* and *Staphylococcus*, and viruses (113). Eleven SAGs have been described in GAS (114) and the overall SAG repertoires are conserved within *emm*-types. However, some strains within an *emm*-type express a specific repertoire of SAGs. The first GAS SAG, later termed SpeA, was identified in 1924 and called the “scarlet fever toxin”. SpeC was discovered in 1960 (115). Because SpeA, SpeB and SpeC are able to induce fever when injected into rabbits (pyrogenicity), Kim and collaborators renamed these toxins streptococcal pyrogenic exotoxin (Spe) A, B and C (116). The SpeB was later shown not to be a SAG but the cysteine protease SpeB (80).

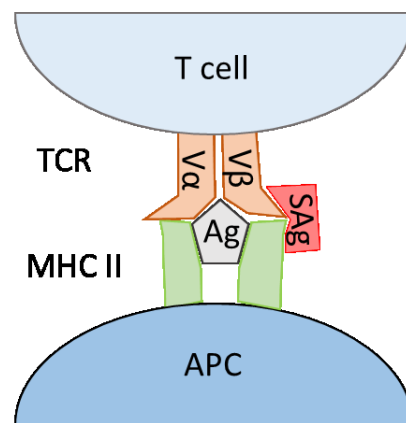


Figure 11. Mechanism of action of superantigens

APC: antigen presenting cell. MHC II: major histocompatibility complex class II. T cell: T lymphocyte. Ag = antigen. SAG = superantigen. TcR = T-cell receptor.

The amino acid sequence of the streptococcal superantigen SSA is very similar to staphylococcal SAGs (117). The streptococcal mitogenic exotoxin Z (SMEZ) is expressed by 94% of invasive strains in France (<https://cnr-strep.fr/>). Genetic analysis of a sequenced strain identified seven other gene coding for SAGs: *speG*, *speH*, *speI* and *speJ*, *speK*, *speL* and *speM* (118–122). The genes are associated with bacteriophages, except for *speG*, *speJ* and *smeZ* which are chromosomally encoded. Genes encoding GAS SAGs are not highly polymorph. Few alleles exist with minor diversity, except for the *smeZ* gene with so far 50 alleles identified, some of them containing nonsense mutations (123, 124). The distribution of SAGs genes in GAS isolates are summed up in Table 5 (125). The strain used in this PhD was sequenced and its genome contains 5 genes coding superantigens: *smeZ*, *speG*, *speJ*, *speC* and *speK* (126).

SAGs bind the variable β chains ($V\beta$) of the TCR and the human T cell $V\beta$ repertoire is composed of 50 different $V\beta$ chains. Each SAG can bind more than one $V\beta$ chain, and one SAG can activate up to 25% of the T cell repertoire. This is to be compared with the ability of a conventional peptide to activate only one subset of naïve T cells from the pool of $10^5 - 10^6$ cells. In addition to $V\beta$ chains and MHC class II molecules, SAGs bind CD28 (127), a co-stimulatory receptor expressed by T cells, and SAGs interact with B7 (CD80/CD86) molecules present on antigen presenting cells. The binding of TCR to MHC molecule is a prerequisite for activation of T cells, but the interaction between B7 molecules of APC and CD28 of T cells is also necessary for T cell activation and the induction of pro-inflammatory cytokine genes (127).

Table 5. Repartition of the SAg_s gene

	<i>smeZ</i>	<i>speG</i>	<i>speJ</i>	<i>speC</i>	<i>ssa</i>	<i>speA</i>	<i>speK</i>	<i>speH</i>	<i>speI</i>	<i>speL/M</i>
% of GAS strains	96	86.9	32.7	51.5	35.4	32.1	24.6	17.1	15.2	9.2

480 nonduplicate GAS isolates from Portugal between 2000-2005 were tested by PCR. Adapted from (125).

Binding of superantigens to T cells induces rapid release of TNF- α , TNF- β followed by IL-2, IL-6, IL-1 and IFN γ . In addition to V β specificities, each SAg induces responses of different intensities, as shown by the ability of SMEZ to induce ten times more cytokines than SpeA (128).

2.1.1.2. Other secreted virulence factors

DNases. DNases are enzymes that cleave DNA and play a potentially crucial role in defense against neutrophil extracellular traps (NETs) (129, 130). Eight types of DNases are expressed by GAS: *sda1*, *sda2*, *spd1*, *spd3*, *spd4* and *sdn*; *spn1* and *sdaB* are the only core genome encoded DNase genes in GAS, and they are present in all GAS strains (131, 132).

Sda1: the strain MIT1 acquired the DNase Sda1 *via* a bacteriophage and is the most important DNase for virulence of this clone (36, 130, 133). The importance of Sda1 in the emergence of the MIT1 strain and in its virulence is questioned (134–136).

Spd1 and Spd3 are bacteriophage encoded DNases (137, 138). Spd1 is reported to have an RNase activity but its role in virulence is unknown (137).

The EndoS family. Endo-beta-N-acetylglucosaminidase of *S. pyogenes* (EndoS) is a 108 kDa protein that removes carbohydrates from IgG in a specific manner, reducing Fc affinity for its receptors and diminishing the opsonophagocytosis of bacteria (95, 97).

EndoS₂. A variant of EndoS was described in serotype M49, named EndoS2 (139). EndoS2 activity differs from that of EndoS in the hydrolysis of N-linked glycans on native IgG chains and biantennary and sialylated glycans of the alpha1-acid glycoprotein (AGP).

IdeS. The immunoglobulin-degradin enzyme of *S. pyogenes* (IdeS), also known as Mac-1 or MspA is a secreted cysteine protease that specifically cleaves the hinge region of IgG, but not the other immunoglobulins (140, 141). Mac-2 is a variant of IdeS that presents similar functions(142).

Sib35 is another anchorless immunoglobulin-binding protein. In contrast to IdeS or Mac-2, the 35 kDa Sib35 protein can bind IgA and IgM in addition to IgG. Sib35 is present in all GAS strains tested (143). Also, Sib35 binds to mouse B cells, enhances expression of MHC class II and B7-2 (CD86), induces the transition of cells to antibody producing plasma cells and

activates B cells proliferation. However, the overall importance of this phenomenon in GAS pathogenesis remains unexplored.

The Mac-1-like protein is a secreted protein and is a prokaryotic homolog of the human α -subunit of Mac-1, a leukocyte β 2 integrin. This molecular mimicry enables GAS to bind CD16 at the neutrophil surface through the mac-1 like protein (144).

HylA. In GAS, three genes encode a hyaluronidase (HylA), also known as hyaluronate lyase: *hylA*, *hylP*, *hylp2*. *hylP* and *hylp2* are coded by bacteriophages and are thought to facilitate phage penetration in GAS. In contrast, *hylA* is coded in the core genome, but only ~25 % of GAS strains express detectable amount of functional HylA *in vitro* (145, 146). This is due to the presence of a point mutation in *hylA* that abolishes the enzymatic activity of the expressed HylA (147). Strains that express a functional HylA do not have the genes encoding the capsule biosynthetic pathway, the *hasABC* operon (148). There are homologs of the *hylA* gene in *Streptococcus pneumoniae*, GBS and *Staphylococcus aureus* (145), while there are no homologs to the *hasABC* operon (148). It is therefore hypothesized that the acquisition of the *hylA* gene is anterior to the acquisition of the capsule genes: a non-enzymatically active HylA was selected in strains that acquired the production of the capsule.

SIC and CRS. The streptococcal inhibitor of complement (SIC) is a 31 kDa protein whose gene is found in the genome of *emm1* strains and encoded on the same locus as the M protein, the Mga locus. SIC inhibits the formation of the membrane attack complex, avoiding complement mediated lysis of GAS (149). SIC also inhibits α -defensin, LL-37 and lysozyme, β -defensin, secretory leukocyte proteinase inhibitor, the chemokine MIG/CXCL0 (150–153). SIC can also inhibit the contact system; SIC is considered to be a major protein responsible for the increase of virulence of the MIT1 clone (136, 154, 155).

Of note, an *emm57* strain expresses a closely related to SIC protein (CRS), a SIC variant (156) and *emm12* and *emm55* strains express two distantly related SIC variants (157).

SP-SPT, SP-STK, SP-PTP. The eukaryote-type *S. pyogenes* serine/threonine phosphatase (SP-STP) and eukaryote-type *S. pyogenes* serine/threonine kinase (SP-STK) are secreted proteins involved in GAS virulence (158, 159). More specifically, SP-STP crosses cell and nucleus membranes and induces programmed cell death in pharyngeal cells (160). The *S. pyogenes* protein tyrosine phosphatase (SP-PTP) is responsible for Tyr-phosphorylation, even of eukaryotic components after infection. Its absence decreases bacterial growth, cell adhesion and cell internalization (161).

SpyA and SpyB. *S. pyogenes* ADP-ribosyltransferase (SpyA) is a C3 family ADP-ribosyltransferase whose gene is co-transcribed with the gene coding for SpyB (162). SpyA modifies the vimentin cytoskeleton in HeLa cells (162, 163). SpyA also induces the ADP-ribosylation of SpyB (164) which is a small heme binding protein (165). Finally, SpyA is an activator of the macrophage Nlrp3 inflammasome (166).

SpyCEP. *Streptococcus pyogenes* cell envelope protease (SpyCEP) is a serine protease that exists both as a secreted and a cell associated protein. It is secreted as an immature form of 170 kDa composed of two fragments, the N-terminal and C-terminal fragments which non-covalently binds to each other under non-denaturing conditions (167). In contrast to SpeB, it is expressed during the exponential phase. SpyCEP can cleave all chemokines that contain the ELR motif (CXCL-1,-3,-5,-6,-7,-8) (167); notably, SpyCEP is able to degrade IL-8 (168). Expression of SpyCEP decreases GAS adhesion to host cells and biofilm formation (169).

Sse. The role of the *Streptococcal* secreted esterase (Sse) is unknown, but its expression is upregulated in *covRS* mutants (170) and a *sse* null mutant presents a defect in growth in the laboratory rich medium THY (171). The null mutant is less virulent in mice model of necrotizing fasciitis (170) and there is a higher number of neutrophils at the site of infection compared to the wild-type strain (172). SSe hydrolyzes the platelet activation factor (173).

Ska. Plasminogen (Pg) is a single-chain glycoprotein that is part of the plasma and extracellular fluids. Pg is a key component of the fibrinolytic system. Upon binding to the serine proteases urokinase-type and tissue-type plasminogen activators, plasminogen is converted to the enzymatically active plasmin (Pm) that can cleave fibrin clots.

Streptokinase (Ska) is a single chain protein of 414 amino acid residues secreted not only by GAS, but also by group G and C streptococci. While achieving the same process as plasminogen activator, Ska does not harbor in itself a proteolytic activity. By specifically binding human Pg, Ska induces a conformational change in the Pg protein, which reveals an active site in the Ska-Pg complex. The enzymatically active complex can then cleave another Pg protein into Pm (“trigger cycle”). Since Pm has a higher affinity for free Ska than Pg, as soon as plasmin is present, another cycle takes place. Ska-Pm complexes are also enzymatically active and can convert a bound Pg into a bound Pm, creating the “bullet cycle” (Figure 12) (174–176).

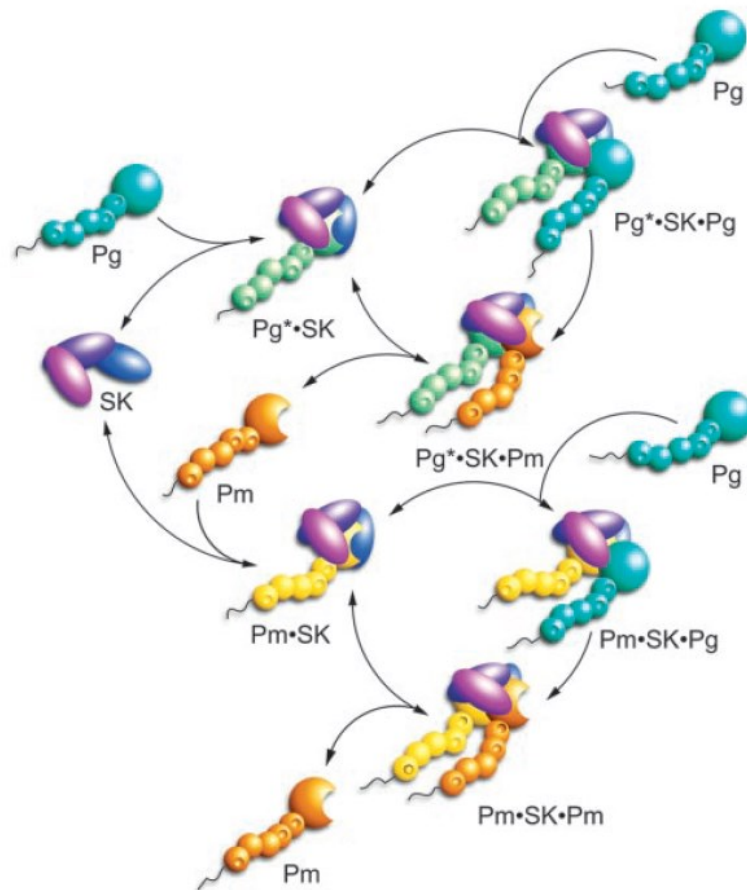


Figure 12. Mechanism of Ska transformation of plasminogen in plasmin. Plasminogen: (Pg), SK: streptokinase, Pm: plasmin. From (177).

2.1.2. Surface-anchored virulence factors

2.1.2.1. Mga locus encoded proteins: M and M-like proteins, ScpA, SIC

M proteins and M-like proteins. The M protein was identified by Lancefield in 1928 (11). It is a major virulence determinant of GAS. It appears in electron microscopy as “tuftlike structures” (178). M proteins are cell-wall anchored protein, with a LPXTG motif close to the C-terminal end (179) and is composed of two chains. In proteins from the A-C genetic pattern (see 1.4.1), the M protein contains 4 repeat domains, A to D, that differ in size and amino acid sequence from one M protein to another. M proteins of strains of the *emm* pattern D do not present the A repeats, and E pattern strains M proteins present neither A nor B repeats (Figure 13). Therefore, E pattern M proteins are shorter than D pattern M proteins, themselves shorter than the A-C pattern M proteins.

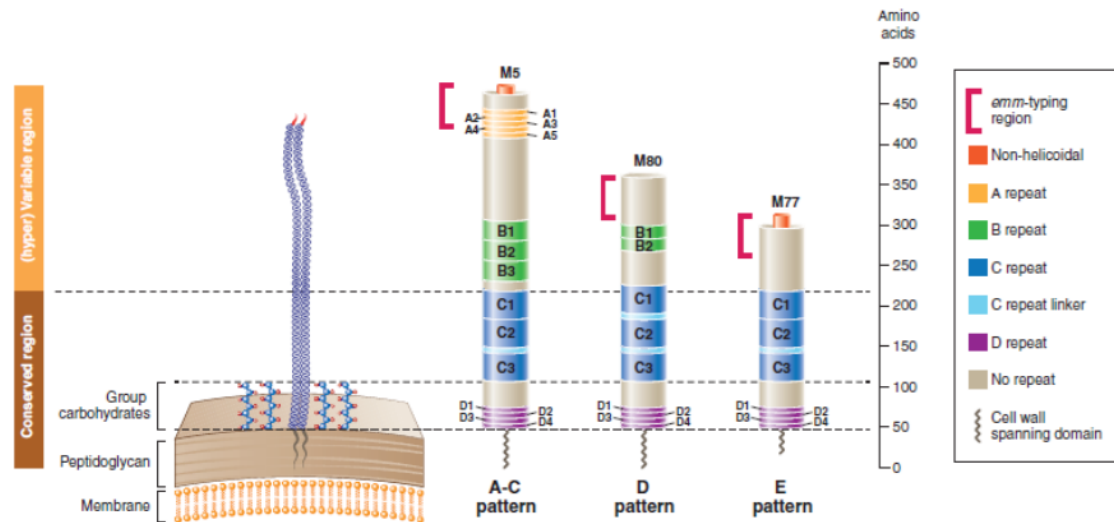


Figure 13. Structure of the M protein from the different *emm* patterns

The number and presence of the repeats A and B differ between the M proteins of the *emm* pattern strains. From (42).

Many strains express, in addition to the M protein, M-like proteins that are thought to come from a duplication of the *emm* gene with subsequent sequence divergence (Figure 8, Figure 14) (180). All strains of the C, D and E *emm* pattern express the Enn protein in addition to the M protein, and D-E strains also express the M-related protein (Mrp). Moreover 30% of M1 strains harbor the protein H, a M-like protein only found in this *emm*-type that belongs to the A-C pattern (181). The diverse host ligands bound by M protein are enumerated in Table 6.

M proteins have a non-ideal coiled-coiled structure (182); at 37°C, M protein dimers do not have stable coiled-coil structures (183). The C repeats, conserved among all M proteins, constitute the stable region of the coiled-coil structure of the M protein, whereas the B repeats contain several amino acid residues destabilizing this coiled-coil structure. Several explanations arose in the past to explain the potential role of this structural non-ideality in the M protein functions. First, these unstable coiled-coil structures could allow anti-parallel arrangement of the M proteins (183); second, the residues that destabilize the coiled-coil structure could increase the affinity for the ligands (184).

Interestingly, the coiled-coil destabilizing residues enable a “capture-and-collapse” model where the binding of fibrinogen brings sufficient energy to stabilize the coiled-coil structure (185, 186). Also the binding of IgG to the protein M dimers stabilizes the coiled-coil structure, enabling a higher C4BP-binding capacity and increasing the virulence (181).

M proteins bind at least one member of the family of inhibitors of the complement: Factor H, Factor H-like, C4BP and the cell surface complement inhibitor CD46 (187–190). M proteins can bind immunoglobulin G by the Fc domain (191). The C and D repeats are highly conserved among the M proteins and are responsible for the binding of factor H and serum albumin (192, 193). The B repeats are considered to be semi-variable and they contain, for most M proteins, the fibrinogen binding domain (194). The functional analysis of the M proteins highlight the clustering of M proteins, with ligand patterns conserved within given *emm* patterns but also M protein specificity within *emm* patterns (195). Strains of the *emm* pattern D cluster 4 represent 18% of the *emm* types and they express M proteins that can also bind plasminogen in the HVR region (194, 195). The M protein HVR is involved in the resistance to antimicrobial peptides and M1 protein inhibits antimicrobial peptides derived from β 2 glycoprotein I (196, 197)

It is only in the beginning of the 1990s that M proteins were described as important adhesins (198, 199). Multiple features of M proteins are not conserved from one to another: for example, some M proteins bind fibronectin, others do not and similarly for the M protein/CD46 interaction (Table 6) (200–203). M proteins bind glycosaminoglycan and this property seems conserved among all M proteins tested (204).

M proteins are involved in bacterial aggregation, which is a critical feature of GAS virulence (205, 206). Antibodies raised against the M proteins are a major source of the autoimmune diseases caused by GAS, since some M protein epitopes resemble host epitopes, and these similarities are called molecular mimicry (32, 207).

Two proteins that share structural similarities to M proteins were also identified. The plasminogen-binding group A streptococcal M-like protein (Prp) isolated from an invasive *emm98* strain is 66% similar to the Plasminogen-binding group A streptococcal M protein (PAM) isolated from a M53 strain. These proteins probably originate from a duplication of *emm* genes, but Prp and PAM coding genes have different phylogenetic history (208). Both PAM and Prp bind plasminogen (208–210).

Table 6. M protein ligands

<i>Emm</i> Pattern	<i>Emm</i> - Type	Fn	Fg	Albumin	Ki	FH	FHL-1	C4BP	IgG	IgA	Pg	CD46	Ref
A-C	M1	■	■	■	■				■				(200, 211–214)
	M3	■	■	■									(200, 215)
	M5		■	■		■	■					■	(188, 190, 216–218)
	M6	■	■			■	■					■	(187, 188, 200, 213, 219–222)
	M12			■					■				(223)
	M24		■										(224)
	M46					■							(213)
	M55		■	■					■				(225)
	M18		■										(226)
	M23		■										(226)
D	M33										■		(194, 227)
	M41										■		(194, 227)
	M52										■		(194, 227)
	M53										■		(194, 210, 227)
	M56										■		(194, 227)
E	M2							■	■				(225, 228)
	M4							■	■	■			(228–233)
	M22							■	■	■		■	(216, 228, 229, 232, 234, 235)
	M28								■	■			(232)
	M49			■					■				(236)
	M50								■				(237)
	M60							■	■	■			(228, 233, 238, 239)

Fg=Fibrinogen, Fn= Fibronectin, Pg= Plasminogen, FHL-1= Factor H-like 1

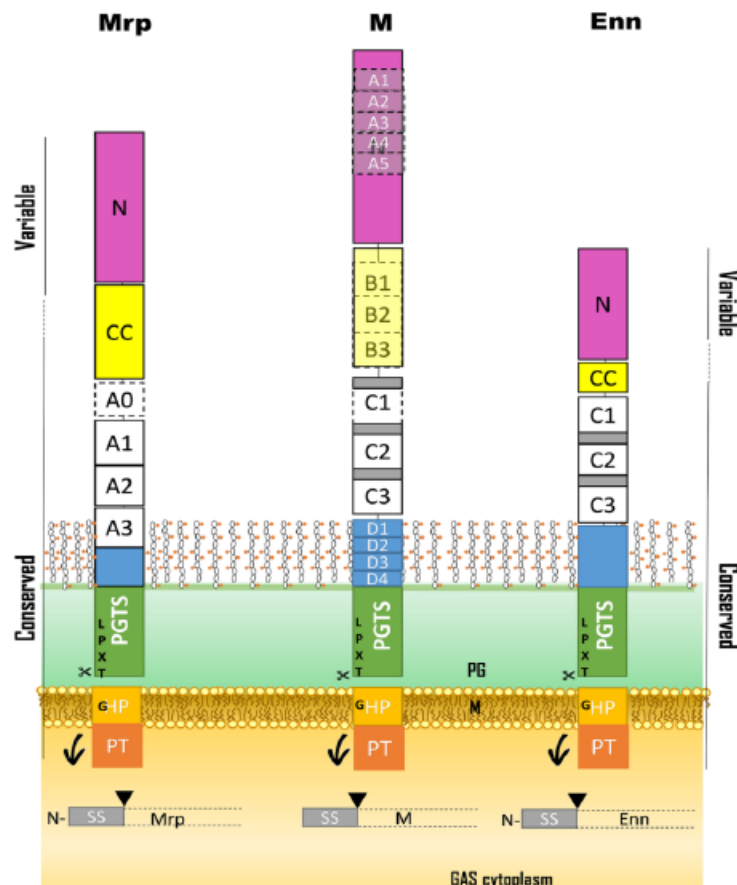


Figure 14. M and M-like proteins structure

Schematic representation of the predicted structure of the M-like proteins, Mrp and Enn, based on known and predicted structural features of the M protein. Following this is the N-terminal, the most variable portion of the proteins of all 3 families although considerably less variable in M-like proteins than in M proteins. The central core (CC) is an EQ rich portion in Mrp and M proteins, and an 18 aa residue motif in Enn proteins. The exposed C-terminal portion of Enn proteins contain C-repeats like those of M proteins, whereas Mrp proteins contain A repeats which differ in both sequence and predicted structure to the C repeats. The cell-wall associated portion is in Mrp proteins half as long as in the Enn proteins. Adapted from (240).

The vast majority of the studies were historically done with the GAS strains of the M1, M3 and M6 serotype. These strains are all of the A pattern that do not express the Mrp or Enn proteins, and they represent only 21 % of the *emm* types (42). Moreover, the M-like proteins, in addition or instead of the M proteins, are responsible for several binding to host proteins [reviewed in (240)] (Table 7). For example, the protein H is responsible for most of the binding of C4BP by M1-expressing strains (241); the Mrp4 is the major fibrinogen binding protein of the *emm4* strains (242); M18 binds Factor H/factor H-like-1 while Enn18 binds C4BP (243). Therefore, while our knowledge of the M protein structure and functions seems extensive, it

does not account for all the M protein patterns nor the M-like proteins. A lot is still to be learned from studies of these M-like proteins.

Table 7. Mrp and Enn host ligands

M-like	Fg	C4BP	IgG	IgA	Ref
Mrp2	■		■		(244)
Mrp4	■		■		(239, 242, 245, 246)
Mrp8	■		■		(247) (242)
Mrp9	■		■		(247)
Mrp13	■		■		(247)
Mrp15	■				(242)
Mrp22	■		■		(239, 242, 248)
Mrp28	■		■		(239)
Mrp49	■		■		(244)
Mrp60	■		■		(239)
Mrp76	■		■		(249–251)
Mrp95	■		■		(244, 252)
Enn2				■	(191, 244)
Enn4				■	(233)
Enn5			■		(253)
Enn18		■	■		(243) (253)
Enn49				■	(244)
Enn95			■		(244) (253)

ScpA. The C5a peptidase, ScpA, is a member of the subtilisin-like serine protease family and ScpA is anchored to the bacterial surface through a LPXTG motif (254). Its gene belongs the Mga locus, together with the genes of the *emm* family (Figure 8). ScpA is produced as a pre-peptide which is processed to the pro-peptide by autocatalytic cleavage (255). ScpA is common to several pathogenic streptococci (256) and is a highly specific endopeptidase which cleaves the C5a protein (255). C5a is an anaphylatoxin (a complement peptide) which plays a key role in the activation and recruitment of neutrophils to the site of infection, and ScpA accentuates GAS colonization in a murine model of infection (257–259). ScpA binds fibronectin (260) and has a role in the adhesion to the pulmonary A549 cells and endothelial HUVEC cells. Recombinant ScpA presents a higher binding to cells in the presence of serum than in its absence, potentially due to the presence of fibronectin (261). In contrast to the ScpA of GBS, ScpA of GAS does not seem to play a role in invasion. Interestingly, the ScpA is also able to cleave the C3a complement peptide, which influences C3 deposition at the bacterial surface. In a murine model of infection, ScpA peptidase activity is not involved in virulence (261).

2.1.2.2. FCT proteins

The fibronectin-binding, collagen-binding, I antigen (FCT) region is an operon including, among others, genes coding for the pilus, *Nra*, *SfBI/PrtfI* and the Protein F2.

Pili. GAS pili are encoded within the FCT region. There are nine FCT types, named FCT 1 to 9 (262). The pilus itself is encoded by three genes: the first one codes for the backbone protein (BP, also known as *FctA*) and the two others for the ancillary proteins *AP1/Cpa* and *AP2/FctB*. The FCT operon also encodes the polymerization machinery composed of the pilus-specific sortase enzymes, *SrtB* and *SrtC2*, and a putative chaperone protein/peptidase (*SipA*). The pilus is composed of multiple copies of the BP protein with one copy of an ancillary protein, and the assembly and export of the proteins is performed by the specific polymerization machinery. GAS strains express different variants of the pilus constituting proteins: 15, 16 and 5 variants of BP, AP1 and AP2 respectively (263). The pili are involved in biofilm formation, adhesion to pharyngeal cells, keratinocytes, primary tonsil cells and primary keratinocytes (264–266). Through its N-terminal domain, the AP1 subunit increases bacterial binding by forming a covalent thioester bond with different cell receptors (267). The AP1 subunit was also found to bind type I collagen and the pilus binds *gp340*, a salivary glycoprotein that induces bacterial aggregation (268, 269). Numerous functions were proposed for the pili, with the possibility that the functions vary from one FCT variant to another.

Sfbi/PrtfI. *SfBI* is coded by a gene in the FCT operon, and is also known as *PrtfI*. It binds fibronectin through five repeats and an upstream binding region (270). It binds indirectly collagen and directly IgG (271, 272); it is involved in adhesion to pulmonary A549 cells and vaginal epithelial Hep2 cells in the presence of fetal calf serum and fibronectin (273). The internalization triggered by *SfBI* is dependent on a mechanism involving caveolae (274). In the absence of the M protein, *SfBI* confers resistance to phagocytosis and reduces complement deposition (275). Finally, *SfBI* covalently cross-links to fibrinogen with a thioester bond, and this interaction increases binding to cells (276).

Protein F2: FbaB and PFBP. *prtF2* is found in 60% of GAS strains and has two mutually exclusive variants: *pfbp* and *fbab* (277), coded in the FCT region. *FbaB* is a fibronectin binding surface protein (143). It is involved in adhesion and invasion of Hep-2 and primary endothelial cells (143, 278).

S. pyogenes fibronectin-binding protein (PFBP) is a 127 kDa protein that binds fibronectin (279).

2.1.2.3. *emm28* specific surface proteins

R28 protein. R28 is a cell-wall anchored protein encoded in RD2, an *emm28* specific region (Figure 9 and see section 1.4.2). It belongs to the Alp family of proteins that all share a common structure with a signal peptide, a similar N-terminal domain, multiple repeats identical at the level of single amino-acid residue within each protein and a LPXTG anchoring motif (280) (Figure 15).

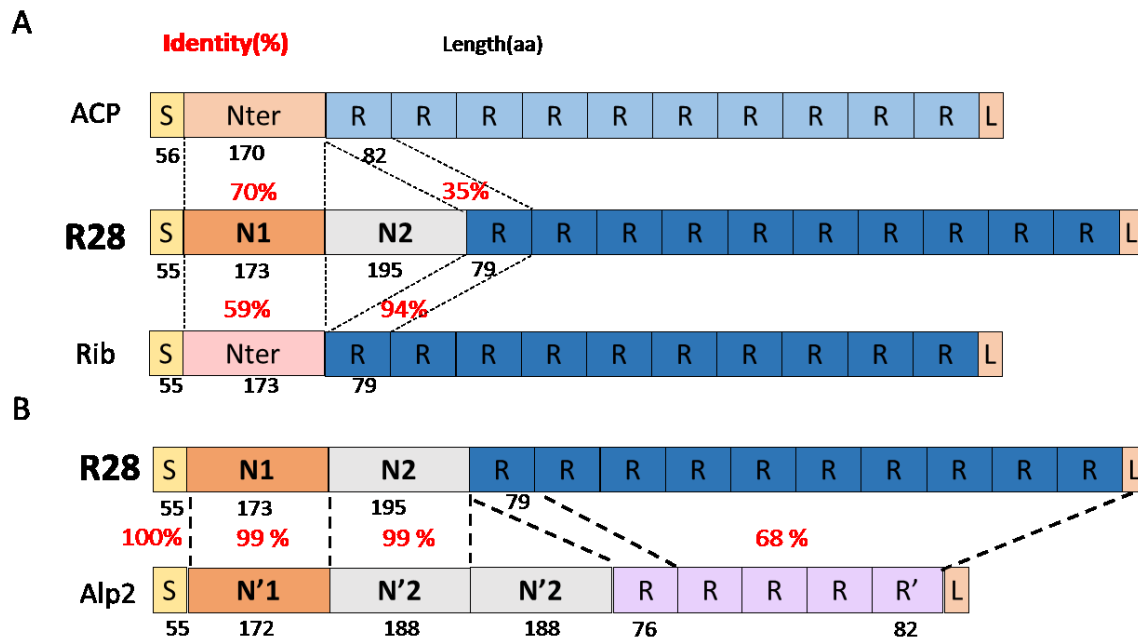


Figure 15. R28 and the structure of the Alp family proteins

S=signal peptide, R=repeats, L=LPXTG. Domain length is indicated in grey below as the number of amino acid residues. Identity between the different proteins and R28 is indicated in red. A. Comparison of the structures and sequences of Rib, R28 and ACP. B. Comparison of R28 and Alp2 proteins.

The Alp family is composed of four proteins expressed in GBS which are evolutionary related: alpha C protein (also known as ACP, or protein α), Rib, R28 (also known as Alp3 in GBS) and Alp2 (48, 280–282). R28 is composed of an N-terminal domain of 41.5 kDa followed by a variable (up to 15) number of repeats depending on the strain (48). R28 N-terminal domain can be divided into two subdomains N1 and N2, with N1 corresponding to the domain similar to all Alp proteins (Figure 15A). The N2 domain is partly similar to the β protein of GBS and presents an IgSF-fold (282). The repeats of R28 are 94% identical to Rib repeats and their structure is related to the Ig-like fold (283); R28 is thought to be a chimera between ACP, Rib and the unrelated β protein. Alp2 N-terminal domain is almost identical to R28 N-terminal domain, with a duplicated N2 domain (Figure 15B).

A R28 deficient *emm28* strain is non-adherent to the cervical cell line ME180, but nothing is known regarding the receptors involved (48). Rib is not involved in adhesion to cells (282) in contrast to ACP. ACP is the most studied member of the Alp family and the crystal structure of the N-terminal domain has been elucidated (284). It binds glycosaminoglycans in a region encompassing the end of the N-terminal domain and the repeats (285, 286), and the $\alpha\beta 1$ integrin through a KTD motif localized in a β -sandwich subdomain at the N-terminal end of the N-terminal domain (287, 288). These interactions increase GBS internalization in ME180 cells (288).

Epf. The extracellular protein factor (Epf) is a 25 kDa surface protein encoded in the pathogenicity ERES island by M1, M4, M12, M28 and M49 strains. It has several C-terminal repeats, a unique N-terminal domain; Epf binds plasminogen (289, 290).

AspA. Antigen I/II (Ag I/II), also called AspA, is encoded in the *emm28* specific RD2 region (45) (Figure 9). The AgI/II is an adhesin that binds to gp340, a component of the human salivary (291). AspA is also involved in biofilm formation (292). AspA is necessary for GAS establishment in the nasopharynx of mice and a null mutant resist to phagocytosis to a lower extent (293).

2.1.2.4. Other surface virulence factors

DNase. SpnA is the only LPXTG bound DNase and it is involved in the escape of NETs (132, 294).

SOF and SfbX. The serum opacity factor is expressed by half of the GAS invasive strains of five states from the United States of America between 1995 and 1999 (295). SOF is both found at the bacterial surface and in the culture supernatants. The name arises from its activity of human serum opacification. The opacification domain avidly interacts with high-density lipoprotein (HDL) in host serum to form neo-HDL and insoluble cholesteryl-ester rich microemulsion (CERM), a property modulated by the interaction with apolipoprotein A-I, the major component of HDL (296). SOF binds to the C-terminal domains of fibronectin and fibrinogen (297–299). Proteins similar to SOF are found in fish and pig pathogenic Streptococci, which suggests an old evolutionary selected bacterial property (300). Apolipoprotein A-I has antimicrobial activity (301) and SOF interaction with it is thought to be a major virulence mechanism.

SfbX is only present in SOF positive strains and its gene is cotranscribed with *sof* (302). It is a fibronectin binding protein (279).

LTA. Lipoteichoic acid (LTA) is a polymer and a component of the cell wall of Gram positive bacteria. It is composed of 1,3-phosphodiester-linked glycerophosphate with a small lipid moiety (a diacylglycerol) which mediates the anchoring of the teichoic acid domains to the bacterial membrane (303). LTA has a dual localization: it is covalently bound to the membrane and it can form complexes with surface proteins of GAS, including M and M-like proteins (41, 304). When bound to surface proteins (and not when membrane bound), LTA readily exposes its lipid moiety, which makes LTA the major factor determining GAS surface hydrophobicity (41, 305) and it induces lymphocytes mitogenesis (306). It is involved in binding to phagocytes and to epithelial cells, and the glycolipid moiety binds to fibronectin (307–310). Since biofilm formation depends on hydrophobicity, the amount of bound LTA to the surface of GAS influences biofilm formation (41). Host apolipoproteins are thought to inhibit LTA effect on host cells, an effect that SOF could counteract.

Hyaluronic acid /Capsule. The capsule is composed of hyaluronic acid, a polymer of N-acetylglucosamine and glucuronic acid, structurally identical to the hyaluronic acid found in the extracellular matrix of humans (311). The capsule biosynthesis proteins are encoded by a 4.2 kb operon composed of the three *has* genes (*hasA*, *hasB*, *hasC*) (312–315). *emm4*, *emm22* and *emm89* strains of the emergent clades do not harbor the *has* operon (37, 148, 316). Moreover, it was reported that *emm87* and *emm28* strains (including the strain used in this study) harbor a frameshift mutation in the *has* operon which induces the production of early truncated HasA protein, suggesting these strains are unlikely to produce a capsule (317). The capsule reduces adhesion to epithelial cells (318). Yet, the hyaluronic acid can act as an adhesin by binding CD44 at the cell surface. Moreover, GAS capsule is an anti-phagocytic factor (11, 319).

GAPDH. Streptococcal surface deshydrogenase SDH or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also independently named streptococcal plasmin receptor (Plr) is an enzyme expressed by numerous organisms, including yeast and mammalian cells. In the cytoplasm, it plays a major role in the glycolysis and GAPDH is also a surface bound protein, although it does not present any known anchoring signal. GAPDH has numerous roles at the bacterial surface and is considered as a major moonlighting virulence factor of GAS. GAPDH binds fibronectin, laminin, myosin, actin, lysozyme, plasmin, uPAR and it induces major

intracellular changes (320–322). It binds C5a which is necessary for C5a cleavage, and this cleavage impairs neutrophil recruitment (323). GAPDH is involved in adhesion to host pharyngeal cells and inhibition of phagocytosis (321). Surface bound GAPDH is involved in the properties and activities of SLO and SpeB among others factors important for virulence (324).

Lmb/Lbp. The laminin binding protein Lbp is a 34.1 kDa membrane bound protein, with a XXGC motif, and is highly conserved among the GAS strains (325, 326). It is 98% similar to the GBS Lmb protein, a laminin- and zinc-binding protein. Lbp binds laminin through a zinc ion (326).

ScII. ScII is a homodimeric protein with a variable domain in the N-terminal globular sequence, a collagen-like domain and a C-terminal anchoring domain (327, 328). ScII interacts with human collagen, binds integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$, promoting GAS internalization (329–331). ScII can also bind fibronectin, laminin, LDL, HDL, factor H and factor H-like I (332–336).

Shp and Shr. The Streptococcal heme receptor is a 145 kDa protein with a N-terminal domain and two heme-binding NEAT domains separated by a leucine-rich segment (337). It is anchored into the membrane and crosses the cell-wall for exposure at the bacterial surface. Shr binds fibronectin and laminin *in vitro* (338). It is expressed by most strains tested and is necessary for blood survival through its capacity to transfer iron to the Streptococcal heme-binding protein (Shp) (339, 340).

CAMP factor. Christie Atkins Munch-Petersen (CAMP) factors are a family of conserved proteins that increase hemolysis of several Gram-positive bacteria, including GAS, with high sequence identity (341, 342). They attenuate the phagocytic activity of murine macrophages RAW 264.7 and promote adhesion and invasion to pharyngeal Detroit cells (342).

SEN. The Streptococcal surface enolase SEN is a 45 kDa plasminogen binding protein; the plasminogen binding site is mapped at the C-terminal domain (343, 344). It is a dimeric to octameric protein (345).

Fba. Fba is a 38 kDa cell wall anchored protein found in the M1, M2, M4, M22, M28 and M49 serotypes. It is highly similar to *S. aureus* FnBPA protein. It binds fibronectin, factor H, factor H-like 1, and is involved in invasion of the cervical Hep-2 cells and the binding to FHL-1

promotes intracellular invasion in pulmonary A549 cells (346–349). It is involved in phagocytosis resistance (348). Fba is shed from the surface by SpeB proteolytic activity (350).

SpyAD. SpyAD is a member of the GAS divisome machinery that binds to keratin and type VI collagen (351).

Beyond the bacterial proteome, the interactome

By binding many host proteins that bind numerous host ligands and by maintaining the host ligand binding capacity or functions, the repertoire of potential functions at the bacterial surface is increased. The binding of a host component can increase the bacterial repertoire of functions as an indirect effect: bound plasmin can degrade host clots, and bound C4BP inactivates the complement cascade, fibronectin can bind host receptors. These different examples highlight the importance of considering that virulence is not only achieved directly by the bacterial repertoire of virulence factors, but also by the whole interactome.

2.2. GAS regulation of virulence factors

Virulence is a means for bacterial multiplication and bacteria differentially express their proteins in response to bacterial resources: protein production has an energy cost, so specific proteins are expressed only when necessary, to optimize the energy used and save the remaining energy for bacterial multiplication (352). Proteins involved in resistance to host responses are also expressed only when necessary. This concept of optimization of protein expression was recently completed by studying *Escherichia coli*, where genes with no detectable benefit account for 31% of protein production *in vitro*, showing that bacteria not only produce essential proteins for their immediate fitness, but also produce at low levels potentially necessary proteins (353). Regulation is therefore the modulation of the intensity of protein production to optimize the fitness while probably maintaining “standby” production of other effectors.

More specifically, the diversity of GAS-induced diseases imply a tight regulation of GAS physiology to face the different hostile environments and nutrient availabilities: difference in the presence and abundance of sugar, proteins/amino acids, oxygen (CO₂), the pH and the osmolarity (9). This is achieved by intrabacterial and external sensing of numerous signals and subsequent physiological changes. Pathways regulating the bacterial metabolism intersect with the pathway regulating virulence: metabolism and virulence are coordinated, as described for other Gram positive bacteria, such as *S. aureus* (354) and as we will describe in the following chapter. Moreover, there are counterproductive effects of different factors during the course of

infections. For example, the capsule is an anti-phagocytic factor, but it also decreases bacterial colonization. SpeB is essential for colonization but is detrimental for blood survival (as explained latter). GAS therefore optimizes its protein production and modulates the expression of its virulence repertoire to adapt to each pathogenesis steps.

As for many pathogens, the “regulatory mesh” is highly complex in GAS, with many interlocked regulatory pathways. In contrast to many Gram positive and negative bacteria, GAS regulation does not depend on sigma factor for the response to stress or growth phase. We will describe in this section successively different levels of regulation in GAS: transcription, post transcription, translation and post translational regulation.

2.2.1. Regulation of gene transcription

We will describe two main systems that enable gene regulation in GAS: two component systems (TCS) and stand-alone activator/repressor.

2.2.1.1. Two-component system: CovR/S

GAS strains express 14 TCS, but not all are expressed by all *emm*-types. Two-component systems are composed of a sensor kinase protein (S) which after detecting a signal autophosphorylates, then phosphorylates a cognate intracellular receptor (R) which transduces the extracellular signal by binding to the regulatory regions of different genes. We will focus on the most studied two-component system, CovR/S.

The control of virulence (CovS/CovR), initially known as capsule synthesis regulator (CsrR/S), is the most characterized GAS TCS. The principal virulence genes and pathway under the control of the oligomerized phosphorylated CovR are listed in Figure 16 (355). CovR/S is involved in the regulation of up to 15% of GAS genes (271 genes), many of which are virulence factors and most of them are downregulated by CovR which therefore acts as a repressor of the expression of virulence genes (355, 356). Host selective pressure selects in the *emm1* and *emm3* genotypes for clones that do not express a functional CovRS system, which is thought to increase the expression of virulence genes involved in the resistance to the immune response, such as the capsule biosynthetic operon *has* (357). Moreover, *speB* is downregulated when CovS is inactive: since SpeB degrades bacterial surface proteins, this down regulation is thought to increase the amount of surface proteins present. However, this overexpression of virulence genes and the subsequent increased presence of the capsule has negative consequences on the colonization: a CovR/S mutant presents decreased biofilm formation, binding to fibronectin and

to keratinocytes (318). This suggests why “hypervirulent” strains are only found after intra-host selection: they present short term increased fitness and are therefore the result of “short sighted” evolution (358), and have long term decreased fitness by colonizing the host and disseminating between individuals with less efficiency.

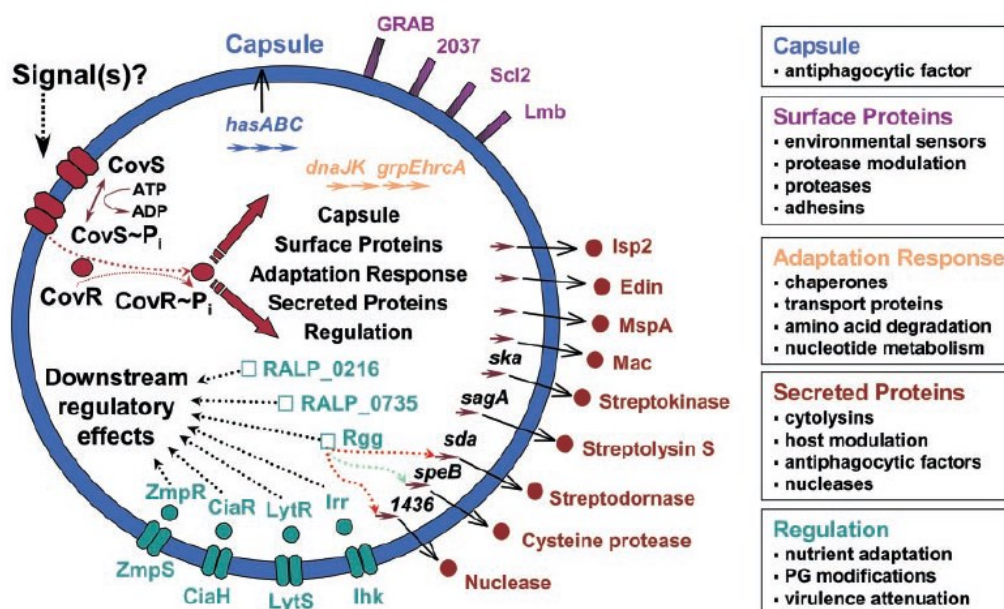


Figure 16. CovR/S system, genes regulated and pathways involved

The different pathways regulated by CovR/S are indicated, in addition to the genes ultimately regulated, and on the right is indicated the different regulated functions. From (355).

CovS phosphorylation depends on host extracellular signals such as Mg^{2+} and the host antimicrobial peptide LL-37: Mg^{2+} increases the repressive effect of CovR/S while LL-37 has an opposite effect, increasing virulence factors expression (359). The operon *cov* is under the repression of CovR itself: autoregulation is functionally explained by the subsequent amplification loop which results in very strong and fast decrease/increase of virulence factors expression. The *cov* operon can be activated by the RocA and Rgg (RopB) regulatory pathways and CovR represses the virulence related regulator RivR (360). Regulatory pathway intertwining results in signal integration from the different bacterial sensors.

2.2.1.2. Stand-Alone regulators: Mga, Rgg/RopB, RofA

GAS strains express 60 “stand-alone” regulators (SA), transcription regulators without determined external sensors. We will present the three most studied regulators: RofA, Mga and Rgg/RopB (Figure 17), and then we will describe briefly regulators important for mechanisms described in section 3.

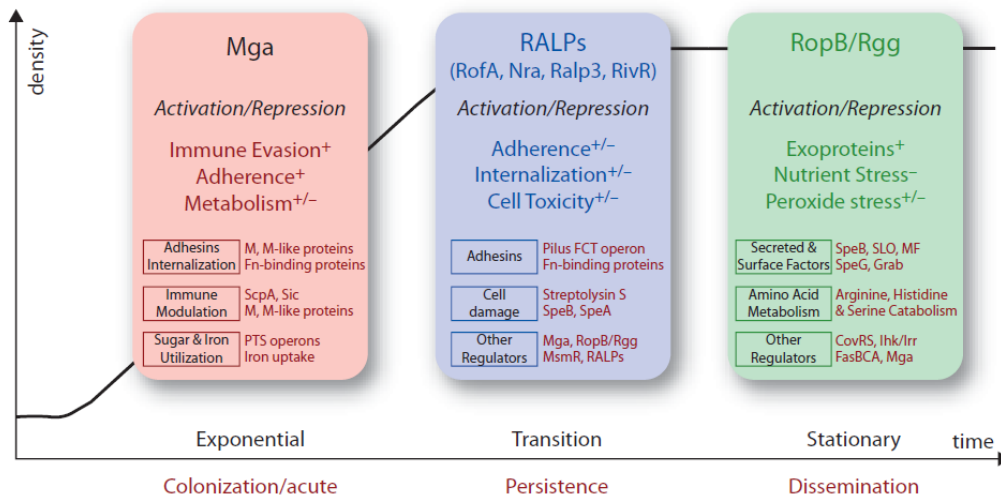


Figure 17. Stand-alone regulators

Growth, metabolism, and stand-alone response regulatory systems of GAS. An *in vitro* growth curve is shown in the background with growth phases indicated. Boxes representing each stand-alone network are placed at the growth phase during which they exhibit maximal expression and the relevant point during infection is given under the x axis. Whether functions are activated (+) or repressed (-) are shown as superscript. Overall processes and specific molecules regulated by each are shown. Adapted from (361).

Mga. Mga stands for multiple gene regulator of Group A Streptococcus (362) and is also known as VirR [for review (363)]. There are two main alleles of *mga*, *mga-1* is mainly found in SOF⁻ strains involved in throat infections, *mga-2* in SOF⁺ strains, found in skin and generalist infections, which suggests an adaptative role for this divergence. *mga* is expressed at its highest level during exponential phase (Figure 17). *mga* is negatively regulated by RALP and Rgg/RopB (364, 365). *mga* expression corresponds to environments that support high bacterial multiplication and *mga* expression itself depends on various environmental cues, and no “sensor” has been identified, but Mga could play the role of sensor and repressor on its own. The phosphotransferase system (PTS) is a multiple protein system that couples the transport of sugars across the cytoplasmic membrane with their phosphorylation (366). Mga presents predicted PTS phosphorylation sites, which establishes a potential link between the carbohydrate metabolism and Mga regulation (Figure 17) (367–369). Either GAS multiplies symptomatically on the pharynx, or remains “silent” on the skin. Mga indirectly regulates many genes, up to 10% of GAS genome (Figure 18) (370): Mga controls the expression of genes coding for ScpA and M/M-like proteins, SIC in certain M1 strains and Fba in others (Figure 18). Mga regulates the synthesis of key virulence factors involved in adhesion and resistance to the immune system. During infection, plasma exudation enables the recruitment of immune

cells, but this also results in a flow of nutrients: favorable conditions are a signal of an hostile environment. Therefore, GAS expression of genes involved in resisting the immune system are increased, allowing to both take advantage of the nutrient flow (due to inflammation) and resist negative consequences of inflammation. Again, this exemplifies the intimate link between metabolism and virulence.

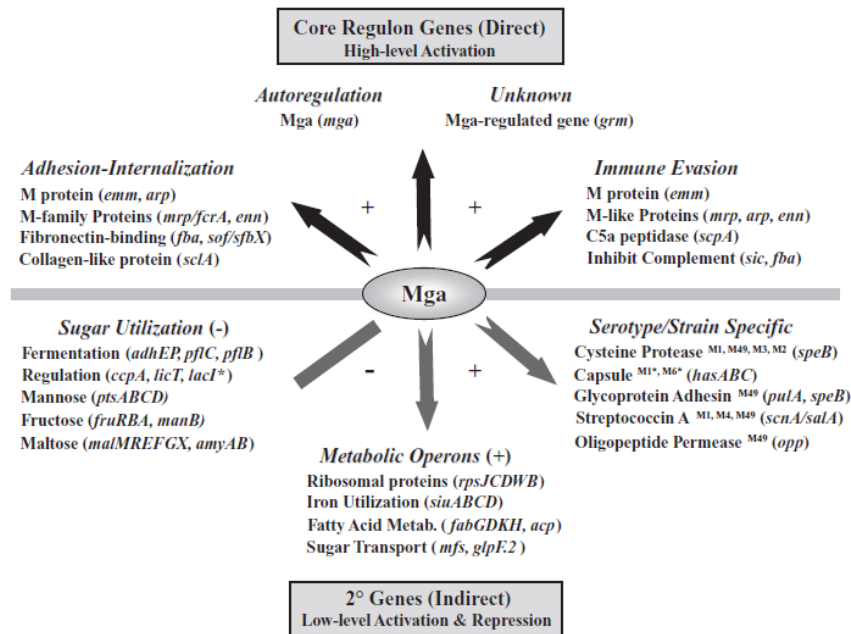


Figure 18. Main genes regulated by Mga

From (363)

RofA and the RofA-like protein type regulators (RALPs). There are four RALPs: RofA, Nra, Ralp3 and RivR (371); strains express either RofA or Nra, and some strains express in addition Ralp3 and/or RivR. The *rofA* gene belongs to the FCT locus and is adjacent to the *sfbI* gene (372); it is autoregulated. RofA upregulates the expression of the FCT region gene and it represses the genes coding SpeB, SpeA and SLS (365, 373).

Nra is expressed by FCT type 3 strains (374). Nra represses the expression of Mga regulated factors (SfbX, ScpA, Lsp, M protein, SpeA, SLO/NADase, AP1, Prtf2) and upregulates several metabolic pathways (375, 376). Nra represses Mga in the exponential phase, and during this phase Mga activates Nra expression. Hence, Nra may be involved in intracellular persistence. Nra also represses Rgg and other RALPs (375, 376).

Ralp3 is encoded in the region coding for the *eno-ralp3-epf-sagA* genes (ERES), and it controls the expression of *epf* and the *sag* operon (375). M1 Ralp3 strongly represses the capsule

biosynthetic operon and *speB* expressions, while the M49 Ralp3 has the opposite effect, which shows *emm*-type specificities (377).

RivR, also known as Ralp4, is a negative regulator of expression of virulence genes, such as the capsule operon and the gene coding for the GRAB protein (378).

The *in vivo* functions of RALPs are difficult to assess since the triggering signals are unknown, and each regulator controls at the same time genes encoding factors implicated in very distinct steps of infection. Moreover, as shown with Ralp3, the role may vary from one strain to another, making RALPs functions even more complex to assess.

Rgg/RopB. Rgg, also known as RopB, is a transcriptional regulator found in many low GC % Gram-positive bacteria. Rgg activates the expression of *speB* which is adjacent to *rgg*, but Rgg is not responsible for the growth-phase regulation of *speB*. Rgg regulates up to 30% of the genome in a given M49 strain but a much smaller set of genes in other M49 strains and other serotypes (379, 380). Rgg also represses the expression of several genes, encoding key virulence factor: SLO and NADase, DNases, GRAB and SpeG [for review see (361)]. Aside from its regulation of virulence genes, Rgg also coordinates amino acid catabolism: the metabolism is switched to the use of amino acids as the primary source of energy. This corresponds to a situation of low nutrients, mimicked by the stationary phase: Rgg/RopB expression is activated in response to stress (Figure 17).

CodY. CodY is a SA regulator found in several low GC gram-positive bacteria; intracellular branched amino acids (valine, isoleucine and leucine) binds to it, which increases CodY affinity for DNA (381). CodY is thus a metabolic sensor involved in the response to amino acid availability. CodY is principally a gene repressor and 17% of genes are differentially expressed in a *codY* deleted strain (382). CodY negatively autoregulates its transcription by directly binding its promoter (382) and an isogenic mutant strain expresses lower levels of genes *grab*, *sagA*, *sdaB/mf-1* and *speB* than the wild-type; and more *nga*, *prtS*, *scl*, *scpA*, *ska*, *slo* and *speH* than the wild-type strain. Moreover, CodY binds *in vitro* to the promoter of the dipeptide and oligopeptide permease genes *dpp* and *opp*.

LacD.1. LacD proteins belong to the family of tagatose bisphosphate aldolase which are metabolic enzymes involved in fructose, lactose and tagatose biphosphate cleavage (383). *lacD.1* originates from a *lacD.2* duplication, with LacD.1 deriving into a regulator and LacD.2 keeping the enzymatic role. There is functional divergence despite 82% similarity (384). After binding carbohydrates in a catabolic domain slightly different from that of LacD.2, LacD.1 is

able to regulate numerous genes: it is consequently a metabolic sensor and regulator. Notably, LacD.1 is a glucose responsive regulator of the expression of *speB* (385).

SRV. SRV is a regulator belonging to the Crp-Fnr transcription regulators family that share structural similarities (386). SRV upregulates *sic* expression and downregulates *speB* expression (382).

2.2.2. Small RNA regulation of virulence

Small regulatory RNAs (sRNAs) are involved in gene regulation through the stabilization or destabilization of mRNA, subsequently increasing or decreasing translation (387).

In GAS, FasX is a sRNA which stabilizes *ska* transcript, further increasing Ska production. FasX also represses the pilus operon translation, the FCT (388, 389). MarS is another sRNA involved in the stabilization of the *mga* transcript (390). *Pel* transcript is coded in the *sagA* operon, involved in SLS production and processing. *Pel* is involved in the transcriptional regulation of *emm*, *nga* and *sic*, and postranscriptional regulation of SpeB with a *pel* mutant readily producing SpeB, but not in its mature form (391). *rivX* affects the translation of *emm*, *scpA*, *mga*, and *speA* mRNAs (360). Interestingly, CovS negatively regulates *rivR* which controls *rivX* expression. Through a sRNA and different regulators, GAS strains integrate several signals to selectively modulate their virulence repertoire. Apart from these well characterized sRNAs, several bioinformatic analyses with or without Northern blot confirmation identified multiple putative sRNAs. The latest analysis identified 31 sRNAs, from which 15 were confirmed by Northern blot analysis, including 6 regulated by RNases, suggesting a role for these enzymes in mRNA regulation (392). Of note, acting at translational levels, sRNA are thought to act faster than classical regulators of gene transcription, making them critical elements of bacterial plasticity.

2.2.3. GAS quorum sensing

While selection is at the individual level (one bacterium or another is killed) (393) evolution is at the population level [chapter 4 of (394)]. Quorum sensing (QS) is a process of bacteria sensing other bacteria and synchronizing their behaviors: cooperation between genetically linked bacteria and social behavior. One evolutionary advantage of bacterial social behavior is that group production increases the overall effect of an effector: for example, quorum sensing is a mechanism enabling a “All for one, one for all” mechanism (395). The other evolutionary advantage is kinship (also known as kin selection): the behavior of related individuals favors

the overall fitness of all individuals, even at the cost of individual's fitness, a classical example are sterile ants and bees. The fitness of a bacterial clone is linked to the amount of newly infected individuals, not the amount of individuals during one infection. Thus, for pathogens, synchronizing bacteria enable resource management, which optimizes the infectious process. In this light, QS is not only a means to increase effector production (lantibiotic production), but also a means to adjust behavior for the good of all linked genetically individuals (metabolic QS). QS often depends on the density (396): they are activated when there is a sufficient number of inducers, above a specific threshold, corresponding to the proximity of numerous genetically linked bacteria. While QS is often seen as a cooperation to optimize resources when the density is high, quorum sensing is also a way to ensure that secreted effectors will benefit only to bacteria in the vicinity that are genetically closely related: kin bacteria (397).

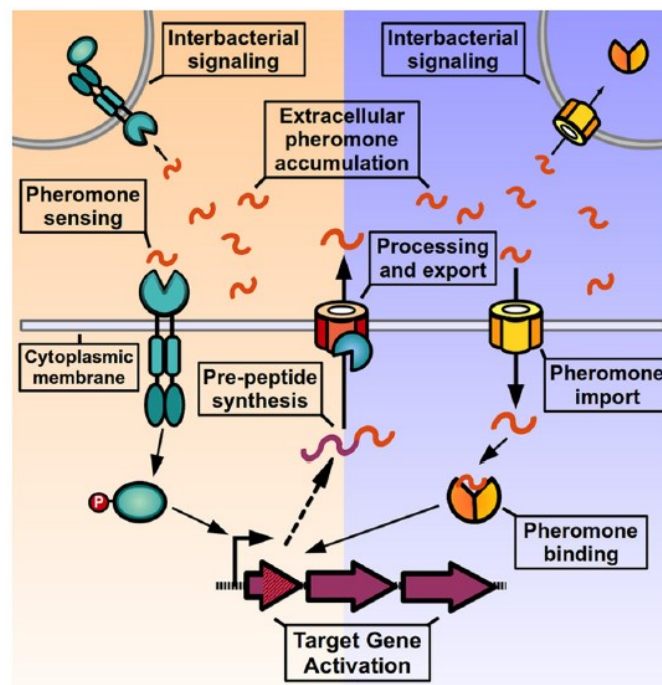


Figure 19. General pathway of quorum sensing

“Pheromone detection can either occur through two-component systems in the bacterial membrane (left side) or by direct binding by transcription factors after peptide import (right side)”. From (398).

The general pathway of QS is as follows: one bacterium senses a signal, it will produce a propeptide which is matured into a peptide (called pheromone), which will trigger in other bacteria a switch of regulation, and the other bacteria will also secrete the propeptide, triggering an amplification loop (Figure 19). There are four main quorum sensing systems in GAS: Rgg, LuxS/AI-2, the lantibiotic system and Sil, the latest being only expressed and functional in 9 % of clinical isolates (399).

As an example, we will only describe the lantibiotic regulatory system. Several bacteria secrete anti-microbial peptides, from which they are protected by immunity proteins, which limits challengers' colonization. Such anti-microbial peptides are called bacteriocins. Class I bacteriocins are called lantibiotics; they are secreted by many Gram-positive bacteria (400). Lantibiotics act as a pheromone that will stimulate the secretion of the lantibiotics in siblings, further increasing the pool of lantibiotics and their effect on challengers.

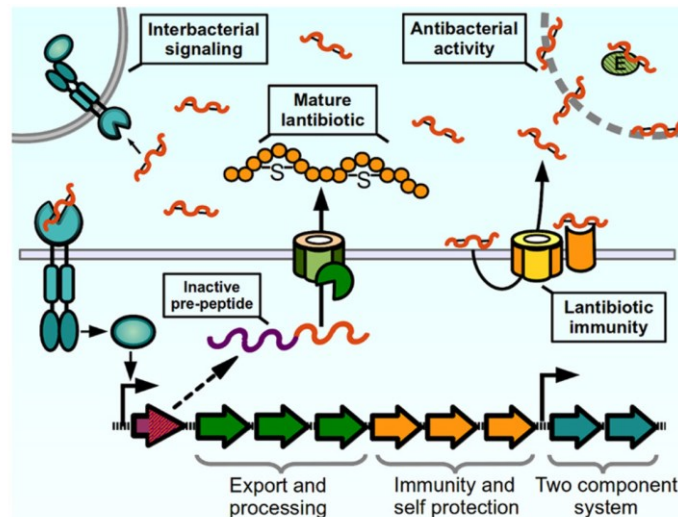


Figure 20. Lantibiotics mechanisms of regulation.

Small orange: pheromone. Left blue: two-component system. On the right, yellow: peptide import machinery. The pheromone either directly interacts with DNA after import, or binds to surface expose two-component systems, inducing a cascade that will ultimately lead to transcription activation. From (398).

2.2.4. Proteome regulation

Extracellular and surface bound GAS proteins are not protected from SpeB proteolytic activity, therefore SpeB produced by GAS influences the amount of GAS surface bound proteins, altering several phenotypes, such as internalization through PrtFI (401). Intra-host *de novo* mutation of CovR/S is thought to decrease the secretion of SpeB, further increasing the amount of surface bound anti-phagocytic proteins such as the M proteins (357). This idea is further supported by the observation that in M1 GAS, there is an inverse correlation between invasiveness and SpeB expression (402). In conclusion, virulence properties of bacteria are altered by the alteration of the surfacome, proteins expressed at the bacterial surface, which depends on the amount of SpeB expressed on top of genetic and transcriptomic regulations.

3. GAS virulence mechanisms

In this chapter, we will describe the different steps involved in GAS pathogenesis of superficial and invasive infections. Since both projects of this thesis focused on the early critical steps of GAS invasive infections, we will emphasize on these critical steps and we will not detail GAS resistance in the blood and dissemination in it, essential features of invasive infections.

3.1. Settling the infection and multiplication

3.1.1. Binding to the ECM

The mammalian extracellular matrix (ECM) is composed of collagen, fibronectin, laminin, proteoglycans and some tissue specific proteins such as keratin. It surrounds cells in conjunctive tissue and the binding of bacteria to ECM is a common critical step for bacterial colonization and invasion of host tissue. GAS strains harbor numerous surface proteins that are able to bind directly or indirectly the ECM components: 10 GAS proteins bind to fibronectin, 4 to laminin, 4 to collagen and one to keratin (Table 8). GAS can also bind to glycosaminoglycans, which could contribute both to adhesion to cells and to ECM (204).

Table 8. ECM components bound by GAS

	Virulence factor	Fibronectin	Laminin	Collagen	Keratin	Ref
All <i>emm</i> types	SpeB					(92)
	C5a peptidase					(260)
	LTA					(403)
	M protein and M-like					(200, 404)
	SfBI/PrtfI*			Indirect		(270, 271)
	Pilus					(268)
	Shr					(338)
	ScII					(333)
	SpyAD					(351)
	Lbp					(325)
<i>emm28</i> strains	Fba					(349)
	Protein F2 (PFBP/FbaB)					(143, 277, 279)
	Epf					(289)
	SOF					(298)
	SfbX					(279)

* : SfBI/PrtfI binds collagen indirectly *via* bound fibronectin.

3.1.2. Biofilm formation

Biofilm are defined as aggregates of bacteria with self-produced extracellular polymeric substances (EPS). It is different from the planktonic stage of the bacteria and bacterial aggregates. Biofilm development is described as a four-step mechanism: stage 1, planktonic transient attachment to a surface; stage 2, formation of aggregates with secretion of EPS which increases the bacterial adherence; stages 3, maturation of the biofilm into a 3D-structure, and step 4, bacterial shedding from the biofilm (Figure 21). The composition of the GAS EPS is poorly described, but carbohydrates, proteins and extracellular DNA are implicated in the biofilm structure (405, 406).

The ability of bacteria to adhere to a surface or a substrate is critical for GAS biofilm formation: it initiates the first step of the biofilm formation (stage 1 and 2). Several matrix binding proteins and adhesins are involved in biofilm formation, such as Scl1, pili, AspA (264, 292, 407). AspA is involved in biofilm formation in the presence of its ligand gp340, but not when bacteria are seeded on polystyrene (292). Indeed, the amount of biofilm is the same in the presence or absence of AspA: on polysterene surface, bacteria are adsorbed to the surface without the need of adhesion. AspA therefore only plays a role in biofilm formation when it is required for adhesion.

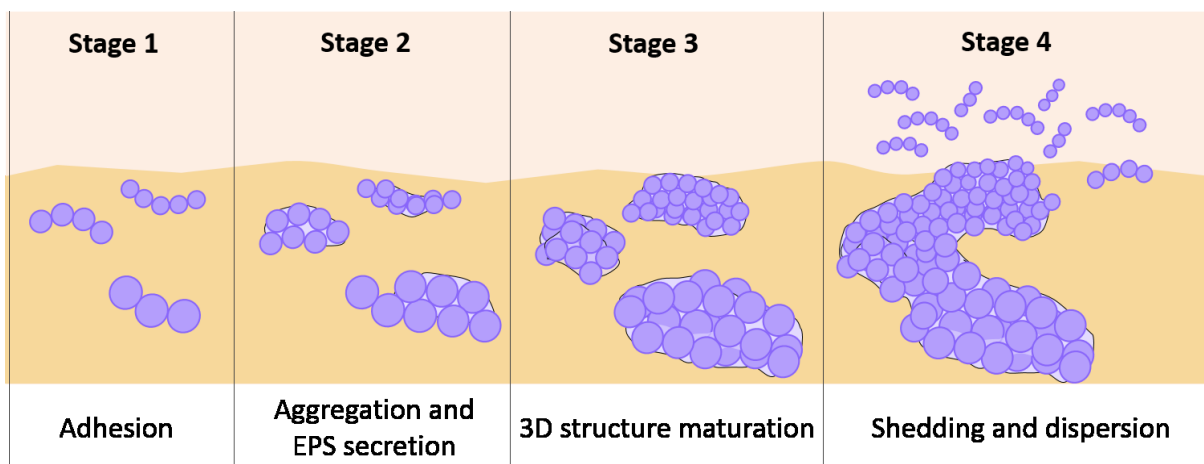


Figure 21. The four stages of GAS biofilm formation

In orange, the surface of a host tissue. In beige, the physiological liquid. In purple, GAS chains. In light purple: EPS.

GAS is a hyper-aggregative bacterium and aggregation in liquid medium is dependent on the homophilic interactions between M and the protein H in a M1 background (205). This aggregation in the absence of M protein could be mediated by other surface factors such as the pili (269). This aggregation step is crucial for GAS biofilm formation, as it initiates spontaneous microcolonies at host surfaces (Figure 21, stage 2). Aggregation contributes to biofilm formation, but does not systematically imply a capacity to form biofilm: a M49 strain readily forming aggregation in liquid culture is not able to form biofilm (408). GAS builds 3D structures without the need of EPS: microcolonies are not necessarily biofilms (408). This complexifies the study of GAS biofilm since EPS are hard to visualize, the only currently used technique is Scanning electron microscopy (SEM), while concanavalin A and wheat-germ agglutinin can be used to visualize lectins.

Multiple GAS surface proteins are involved in biofilm formation. There are striking differences between the different M-types and even between strains of a given M-type in the ability to form biofilms on different substrates (collagen, laminin, etc...) and there are also different biofilm morphologies (408). This indicates that the variation of surface protein repertoires from strain to strain has dramatic consequences on the biofilm formation. Nevertheless, studies point out a correlation between the FCT-type of strains and the capacity of strains to form biofilms (409).

The ability of strains to form biofilms not only depends on the surface protein repertoires and the substrate used, but also on the medium used (409). The presence of glucose stimulates biofilm formation (409); furthermore some strains produce biofilm in a pH-dependent manner (410, 411).

As a pleiotropic phenomenon, any environmental change that would affect the expression of surface proteins involved in the biofilm formation (such as Mga, CodY) and the production of SpeB which degrades surface proteins, will affect biofilm formation. Moreover, some environmental signals probably induce or repress biofilm formation, and it is difficult to distinguish regulators and environmental changes that affect the GAS surfactome, and therefore biofilm formation, from that affecting directly the production of EPS.

Nevertheless, several studies reveal the importance of regulators in biofilm formation. SRV is involved in the control of SpeB production which influences biofilm formation (405). Two other regulators/quorum sensing influence biofilm formation, SilC (408) and the SHP/Rgg pheromone signaling, but there is no clear indication regarding the molecular pathway involved (412).

On patient biopsies of GAS infections, bacteria are present as biofilms and 32% of 31 necrotizing fasciitis biopsies analyzed present biofilms (413). However, the ability of GAS to form biofilm *in vitro* does not reflect their ability to form it during human infection. This highlights that GAS biofilm formation is not an intrinsic properties of GAS: it is a specific program triggered by host signals and/or depending on the host (413).

In conclusion, biofilm formation is a very complex, multi-step process. Most studies were done *in vitro*, even one of the rare studies assessing bacterial formation of GAS on biotic surfaces (keratinocytes) used fixed cells to avoid cell death that would remove cells from plastic glass (414) but this also eliminates potentially host critical secreted factors that could be involved in inducing biofilm formation. While multiple factors are necessary for the first steps of biofilm formation (stage 1 and 2) in GAS, the host and bacterial factors triggering and altering biofilm formation *in vivo* are unknown.

GAS formation of biofilm in human tissue is a critical important virulence mechanism [for review (411)]. Penicillin does not efficiently eradicate GAS infections in up to 30% of pharyngitis despite the absence of penicillin resistance. Two main hypotheses explain antibiotic failure and GAS recurrent infections: bacterial internalization (section 3.1.3) and biofilm formation (415, 416). GAS biofilms are inherently tolerant to immune response and to antibiotic treatments, since they can suffer up to 500 mg/mL of gentamicin (414), by far exceeding clinical use of antibiotics. Moreover, biofilms increase the bacterial load in infected tissues and biofilm-positive biopsies are those with the highest bacterial burden (413).

3.1.3. Adhesion to host cells and internalization

GAS adhesion to host cells

In the battle to attach and remain at the host cell surface, GAS is thought to engage a two-step mechanism: first, the LTA by its lipid moiety overcomes the electrostatic repulsion of host membrane, establishing a labile binding, secondly, surface bacterial adhesins interact with host proteins to induce a high affinity binding (417).

During the initial steps of superficial infections, GAS strains bind to host cell surface. GAS strains express numerous adhesins (Table 9). Of note, not all these factors are expressed by each strain: *emm*-types have been selected to express different repertoires of adhesins during co-evolution. GAS attaches to cells either by directly binding to a host cell receptors, or by binding a host protein which binds to a cell-receptor, mechanism known as indirect binding or bridging. Known direct adhesins are the hyaluronic acid which binds CD44; M6, CD46; Sc11,

integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$ (201, 327, 329, 332). Other proteins mediate adhesion through cell bridging: SfbI/PrtfI, PrtI2, FBP and FbaB, through fibronectin; SEN through plasminogen; protein H through C4BP and SfBI through fibrinogen (143, 241, 276, 349, 418–421). The large majority of these adhesins were tested on Hep-2, initially described as pharyngeal cells and therefore a suitable model for the GAS interactions with host epithelium during pharyngitis, but later recognized as contaminants of HeLa cells, a carcinoma derived cervical cell line (422, 423). Every cell line expresses specific receptors, and the overexpression of certain receptors in Hep-2 cells could overestimate the role of a protein in adhesion. For example, the M protein has a role in adhesion to Hep-2 cells but not to pharyngeal or buccal cells (198). In contrast, several critical major receptors for adhesion to pharyngeal cells might not be expressed by HeLa cells, minimizing the potential importance of several GAS surface proteins.

Plasminogen, FHL-1, fibrinogen, C4BP and fibronectin are plasma proteins. Fibronectin and collagen are two bridging molecules found in the extracellular matrix, however these proteins are mainly found at the cell basal membrane (424). It is unclear if fibronectin is expressed and accessible by cells encountered during the early steps of GAS superficial infections. However, in the presence of a breach, blunt trauma and plasma exudation, where there is inflammation, all indirect interactions involving fibronectin and plasma components probably highly contribute to the bacterial ability to remain attached to cells. Since most invasive infections are subsequent to bacterial induced or already present inflammation, these bridging interactions are critical virulence determinants for GAS invasive infections (349, 425).

GAS internalization in non-phagocytic cells

GAS is an extracellular pathogen, but several reports indicate the presence of viable bacteria within non-phagocytic cells in biopsies and cultured cells (426). Different host and bacterial factors are involved in the invasion process (Table 9). GAS, GBS and *S. aureus* are three of the many Gram-positive extracellular pathogens that can occasionally be found viable inside epithelial cells (285, 427). In the following paragraphs, we will briefly discuss the entry mechanisms of GAS in non-phagocytic cells and we will specifically discuss its role in GAS pathogenesis of superficial and invasive infections.

Table 9. Factors involved in adhesion and invasion

	Virulence factor	Cell-type				Direct Receptor	Indirect receptor	Ref	
		Pulmonary	Endothelial	Pharyngeal	Hep-2				Keratinocytes
<i>All emm</i> -types	M and M-like				A/I(Fn)	A/I(Fn)	GAG, CD46	α 5 β 1 integrin	(203, 204, 212, 428, 429)
	GAPDH			A					(321)
	ScpA	A	A						(261, 430)
	LTA				A	A			(198, 307–309, 431, 432)
	Hyaluronic acid				A		CD44		(432, 433)
	Pilus			A	A/I	A			(265)
	SfbI/PrtfI	A/I (Fn)			A/I(Fn)			α 5 β 1 integrin	(418–420)
	SEN			A (Plg)					(421)
	ScI1		A		A/I		α 2 β 1, α 11 β 1 integrins		(327, 329–331, 434)
	Shr				A				(338)
	Lbp				A				(325)
	CAMP factors			A					(342)
<i>emm</i> 28 strains	Prtf2 (FbaB)	A	A/I		A/I				(143, 278, 435)
	Epf					A/I			(289)
	Fba	I (FHL-1)			A/I (Fn)				(349, 436)
	SOE				A/I				(437, 438)

A(Fn): adhesion (in the presence of Fibronectin), A(Plg): adhesion (in the presence of plasminogen)

I(Fn): invasion (in the presence of Fibronectin)

Internalization could play different roles in the pathogenesis. Internalization induces cell apoptosis (section 3.2) and GAS internalization could enable transcytosis (section 3.2.1). Cytotoxicity and transcytosis through internalization could both contribute to GAS colonization and invasion of tissues (426).

GAS can be found in tonsils of patients with recurrent tonsillitis (439, 440). GAS intracellular bacteria resist to penicillin treatments (441). Strains from asymptomatic carriage and from patients with failure to antibiotic treatment have greater capacity to internalize than other strains (442, 443). This suggests strains with a great capacity to internalize are selected during antibiotic treatments. Strains that resist to erythromycin are also able to invade cells more than strains that are not resistant to erythromycin (444). Since *PrtfI* increases bacterial internalization *in vitro*, the high capacity of erythromycin resistant strains to invade is potentially due to a higher presence of the *prtfl* gene in these strains compared to strains that do not resist to erythromycin (444–447). Either there is a genetic link between *prtfl* and erythromycin resistance genes, or more internalization enable *prtfl* expressing strains to survive β -lactam treatment, leading to a huge selective pressure for erythromycin resistance gene with macrolide treatments. GAS internalization is therefore clinically involved in recurrent pharyngitis, resistance to antibiotic treatments and asymptomatic carriage.

The most described internalization processes involves either SfBI/*PrtfI* or the M1 protein, through fibronectin binding (212, 448). M1 protein-mediated internalization in the presence of fibronectin involves the binding to β 1 integrins and a zipper-like mechanism (212, 428). SfBI/*PrtfI*-mediated internalization in the presence of fibronectin depends on α 5 β 1 integrins and involves caveolae (274, 445, 449). ScII-mediated internalization depends on ScII binding to α 2 β 1 integrins (330). Direct or indirect binding to integrins containing the β 1 subunit appear to be critical in several GAS internalization processes. β 1-integrins and fibronectin interaction induces caveolae endocytosis of fibronectin for its turnover; also, even in the absence of fibronectin, integrins are recycled through caveolae recycling (450). Endocytosis of fibrin bound to α V β 3 enables clearance of fibrin deposition and vitronectin bound to α V β 5 induces endocytosis (451, 452). Therefore, endocytosis of integrin bound elements is a natural physiological pathway involved in many homeostasis processes. Consequently, integrin-mediated internalization of GAS could be seen as a consequence of GAS propensity to bind with high affinity cell surface integrins, activating outside-in signalization pathways involved in homeostatic recycling pathways.

This engulfment may even be an immune mechanism to restrict GAS infection through elimination of internalized GAS. GAS can be eliminated through the autophagy pathway, a phenomenon called xenophagy (453). Strains of serotypes M6, M49 and M3 are efficiently killed by epithelial cells (454–457). However, the highly virulent MIT1 clone can evade xenophagy and replicate in the cytosol of epithelial cells, in a SpeB dependent manner (458). SLO and NADase could also be involved in resistance to xenophagic killing (454), in a SpeB deficient strain. SLO/NADase induce cellular changes that prevent bacterial internalization in keratinocytes, potentially by inhibiting clathrin-dependent uptake of GAS (459, 460), further supporting internalization as a negative effect of GAS colonization.

3.1.4. Bacterial multiplication

Here we will mainly focus on the essential features of bacterial multiplication and its importance in pathogenesis.

Bacterial multiplication is an obvious and critical step in bacterial infection. As already discussed in section 2.2, from a bacterial point of view, infection in itself could be seen as means for the bacteria to acquire nutrients: virulence is a nutrient acquisition process, also termed “nutritional virulence” [for review: (461, 462)]. GAS induces diverse diseases (see section 1.3) and therefore faces various environments with very different nutrient accesses. Moreover, the overall ability of a bacterium to colonize a tissue is the result of bacterial multiplication and host-elicited killing. Many virulence factors have been studied for their involvement in resistance to host killing or increasing bacterial attachment, thus increasing colonization. However, this is assuming nutrient flow is not significantly limiting the colonization, and several reports highlight a direct role of virulence factors in the increase to nutrient access, which we describe in the following section

One central feature of the virulence-metabolic coordination is the SpeB protease. In laboratory medium, SpeB is maximally expressed during the stationary phase, considered as a stage where the amount of nutrients is limiting. Several metabolic regulators affect *speB* expression, such as LacD.1 and CodY, two metabolic sensors (section 2.2.1.2) [see (463) for a complete list of regulators of *speB*]. Opp and Dpp are polypeptide permeases: Opp imports tripeptides and Dpp imports hexapeptides and dipeptides. Inactivation of one or the other polypeptide permease decrease *speB* expression (464). The gene coding for SpeB is under the control of Rgg/RopB, which coordinates amino acid catabolism. It was proposed that the protease SpeB could play a similar role as the *Lactococcus lactis* protease PrtP: the combination

of protein degradation and import by a polypeptide permease is a mechanism of amino acid acquisition (465). This would give a rationale for the overexpression of *speB* in the stationary phase and its activation by Opp, Dpp, Rgg/RopB, LacD.1, CodY which are involved in amino acid catabolism, amino acid import and carbohydrate and branched chain amino acid sensing. By degrading proteins, SpeB would provide a source of amino acids and of energy from amino acid catabolism.

DNA was shown as a potential source of nutrients for different bacteria (466) and GAS strains express several DNAses. While less abundant than polypeptides/proteins, extracellular DNA or DNA released from dead cells could serve as a source of bacterial energy in addition to its role in gene acquisition (467).

Hyaluronidase was mainly studied for its ability to degrade GAS capsule and tissue hyaluronic acid, which increases bacterial invasion. However, hyaluronidase could increase the available source of carbohydrates, which would increase bacterial colonization (468).

GAS induction of cell death (see section 3.2) could also trigger nutrient release since the intracellular reservoir is rich in nutrients [reviewed in (469)]. Moreover, the unfolded protein response (UPR) triggered by SLO and SLS induces secretion of asparagine, which increases bacterial growth in a chemically defined medium devoid of asparagine (57, 470).

3.2. Tissue damage and invasion

Biopsies of GAS infected tissue clearly show that GAS not only colonizes the surface of a tissue, but can also penetrate deeper within the tissue, hereafter called tissue invasion. While breach and trauma are often associated with invasive infections, many invasive infections occur without apparent entry-port, suggesting the capacity of GAS on its own to invade tissues. Without any breach, GAS can cross an epithelium (translocation), and in the presence and absence of breach, GAS invades the extracellular matrix.

3.2.1. GAS crossing the epithelium

The different ways GAS can cross an epithelium are summed up in the Figure 22. To cross the epithelium, GAS can kill the cells that maintain the epithelium integrity (see 3.2). Another way to cross the epithelium is the transcellular route, where bacteria internalize inside cells and are released at the basal side of the cells (Figure 22).

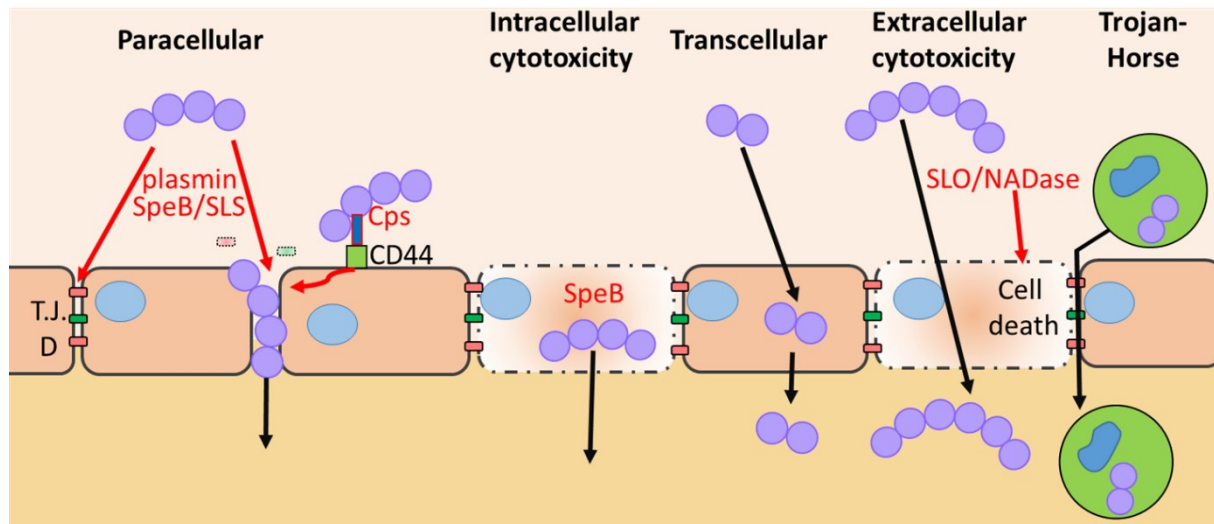


Figure 22. Different routes for GAS translocation across an epithelium

TJ-D: tight junctions- desmosome. Cps: capsule.

Bacteria can also be trapped and released by immune cells, known as the Trojan Horse strategy. This is discussed in the section 3.1.2. Finally, bacteria can cross epithelium in a paracellular mode, by altering intercellular junctions in several ways. First, GAS induces the degradation of the intercellular junctions, which promotes translocation, either through the LTA-CD44 binding or through the action of subcytotoxic dose of SLS (433, 471). GAS also degrades directly, through SpeB proteolytic activity (472), or indirectly through the SEN-plasmin binding (473), the intercellular junctions. Tricellulin is part of tight junctions between three cells and plasmin binding to tricellulin induces GAS to colocalize at these tricellular junctions, which triggers tricellulin degradation and bacterial translocation (421). SpeB can also directly degrade desmogleins, a component of the desmosome which is a type of intercellular junction (474).

3.2.2. Tissue degradation

After a breach or after crossing the epithelial barrier, GAS is in contact with the conjunctive tissue. Two ways to penetrate the conjunctive tissue are the Trojan-horse invasion and cell destruction. Another way consists in degradation of the ECM components (Figure 23). GAS broad spectrum cysteine protease SpeB is able to degrade the ECM components fibronectin and vitronectin (102).

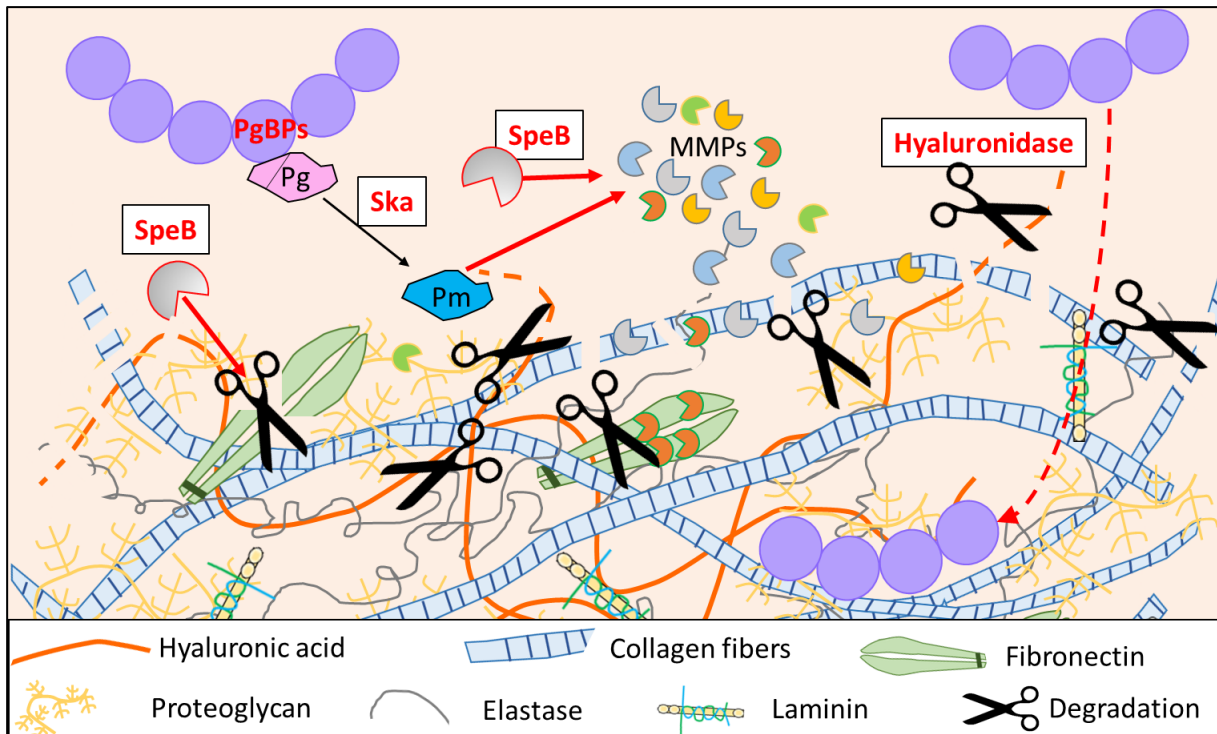


Figure 23 Extracellular matrix and its degradation by GAS

Representation of the ECM components and GAS mechanism of matrix degradation. PgBPs: plasminogen binding proteins. Ska: streptokinase. Pg: plasminogen, Pm: plasmin.

ECM degradation can also occur indirectly through host matrix metalloproteinases (MMPs) activated by SpeB (109, 110). Different GAS factors bind plasminogen (Table 10) which can be activated into plasmin by host effectors (uPA or tPA) or by GAS streptokinase. The bacterial surface-bound plasmin can activate MMPs (475, 476). Activated MMPs can degrade the components of the ECM, enabling bacterial dissemination within the tissue. Bacterial hyaluronidase can also degrade ECM hyaluronic acid, further promoting GAS invasion of the tissue and diffusion of secreted factors such as SpeB and SLO (468) (Figure 23).

3.2.3. Interaction with the fibrinolytic system

Plasma exudation is a source of nutrients for bacterial growth (see section 3.1.4), and the exudation depends on the vascular permeability. The binding of kininogen by the M protein and its subsequent activation in kinin by SpeB or by streptokinase/SpeB activated plasmin may create a local burst of kinin increasing vascular permeability (103, 175, 213). Moreover, as a host defense, the fibrinolytic system is critical to contain bacterial dissemination and is therefore an important defense mechanism against GAS (477). After activation of this system, thrombin cleaves fibrinogen in fibrin, which induces coagulation and bacterial trapping fibrin clots.

Plasmin is a host protease that can degrade these fibrin clots. Consequently, GAS can degrade fibrin clots either directly through SpeB or indirectly through the binding of plasminogen/plasmin at its surface (Table 10) (104–106, 476).

Table 10 Virulence factors interacting with plasma components

	Factor	Albumin	Ig	Fg	Plg/Plm	Complement	Other	Ref
<i>All emm</i> -types	M and M-like	B	B	B	B	C4BP, FH, FHL-1		(191, 193, 194, 478)
	SfbI/PrtfI		B	B				(272, 276)
	SEN				B			(343)
	GAPDH				B			(321)
	Ska				B/A			(174, 175)
	ScpA					D (C5a, C3a)		(255, 261)
	LTA	B						(403)
	ScII					FH, FHL-1	LDL, HDL	(332, 334–336)
	SpeB		D	D	A	D (C3b)		(94, 98, 99, 104, 106)
	EndoS family		D					(95, 139–142)
<i>emm</i> 28	Epf				B			(289)
	SOF			B				(479)
	Fba					FH, FHL-I		(346, 347)
Others	SIC					I (MAC)		(149)
	PAM/Prp				B			(208–210)

B: binds, A: activates, D: degrades

Ig: immunoglobulin, Plg: plasminogen, Fg: fibrinogen, Plg/Plm: plasminogen/plasmin. FHL-1: Factor H like I, FH: Factor H, LDL: low-density lipoprotein, HDL: high-density lipoprotein, MAC: membrane attack complex.

3.2.4. Cytotoxicity

GAS induces cell death in different contexts, to different cell types, and the mechanisms involved (necrosis, oncosis, apoptosis, pyroptosis) depend on the M-type, the virulence factors, the cell type and the experimental procedure. GAS can trigger extracellularly or intracellularly

cell death in lymphocytes, neutrophils, macrophages, keratinocytes, pulmonary cells, pharyngeal cells. This cytotoxicity depends on the independent or concomitant action of SLO, NADase, SLS, SpeB, SpyA, soluble M1 and SP-STP (Table 11). Cytotoxicity can also be induced indirectly: the activation of host matrix metalloproteinases by bacterial bound plasmin or by SpeB can also induce cell death (109, 110).

3.1. Control of the immune response

Host defends itself during the infection: antimicrobial peptides are secreted, pro-inflammatory cytokines are secreted to activate the immune responses, the complement system enable opsonization and bacterial clearance, phagocytic cells kill bacteria and a secondary immune response is induced. To establish infection, GAS expresses many virulence factors involved in countering and resisting the host defenses. In the following paragraphs we will review these different processes, with a special focus on the immunomodulation by GAS, since we analyzed the tissue responses to GAS infection during my thesis.

3.1.1. Resistance to anti-microbial peptides

At a basal level, immune and non-immune cells can secrete numerous anti-microbial peptides that restrain bacterial colonization and multiplication. SpeB releases dermatan sulphate from surface decorin, which inhibits α defensin (111). When SpeB is sequestered by GRAB, it degrades the cathelicidin LL-37 (91). Plasmin bound at the bacterial surface also degrades LL-37 (480). The N-terminal domain of the M1 protein sequesters LL-37, which inhibits the bactericidal effect, and the binding of the M protein to the hCAP-18, a LL-37 precursor, avoids LL-37 proteolytic activation (481). SIC is another critical virulence factor against anti-microbial peptides: SIC interferes with the bactericidal effect of the C3a, inactivates the α -defensin, LL-37 and lysozyme, the β -defensin, and the secretory leukocyte proteinase inhibitor (150–152, 154). The binding of albumin by LTA and M proteins, together with the SIC protein, inhibit chemokines such as CXCL9 that present antibacterial effects (153, 482). Finally, SOF degrades HDL which has an anti-microbial activity (300, 301).

Table 11. GAS-induced cytotoxicity and the factors involved

	Cell Type	M-Type	Cell-Death	Factor	Intra- or Extracellular	Ref
Immune cells	Murine macrophage J774/THP1	M1	Caspase-1 dependent cell death	SpyA	E	(166)
	Murine macrophage J774/THP1	M1	Apoptosis	SLO	E	(483)
	mouse peritoneal leukocytes	M5	unknown	SLS	E	(75)
	THP-1 macrophage	M1	Pyroptosis	Soluble M1	E	(484)
	Monocyte U937	M49	Apoptosis	SpeB	I	(485)
	Mouse neutrophil	M3	Apoptosis	SLS	E	(486)
	Mouse bone marrow derived macrophage	M49	Oncosis	SLO	E	(487)
	B and T lymphocyte	unknown	unknown	SLS	E	(488)
Epithelial cells	Keratinocytes OKP7	M1	Cell death with pyknosis	NADase	E	(489)
	Keratinocyte OKP7	M3	Apoptosis	SLO / NADase	E	(459)
	Keratinocytes HaCaT	M1	Programmed cell death	SLS	E	(76)
	Hep-2	M5	unknown	SLS	E	(75)
	Pharyngeal Detroit 562	M1	Apoptosis	SP-SPT	E	(160)
	Pulmonary A549, Pharyngeal Hep-2	M1, M49	Mostly apoptosis	SpeB	I	(490)
	HeLa	M6	Metabolic and programmed cell death	NADase*	E	(72)

*: NADase induces metabolic cell death, but a NADase natural variant without a NADase activity induces programmed cell death

3.1.2. Resistance to the complement system and phagocytosis

Macrophages recognize bacteria through their PRR (Pattern Recognition Receptor) or their opsonin (immunoglobulins and complement molecules) receptors such as the Fc and C1 receptors.

There are three pathways of complement activation, the classical, the lectin and the alternative pathway (see Figure 24). All lead to the secretion of peptides that recruit inflammatory cells (C5a, C3a, C4a), bind to complement receptor for phagocytosis (C3b) or form the membrane attack complex, MAC (C5b, C6, C7, C8, C9) that lyses bacteria (491). It is critical for bacteria to avoid the complement system activation, and three main strategies are developed to avoid it: 1) avoid recognition by initial peptides (C1q, MBL etc... see Figure 24), 2) degradation of the peptides or inhibition of the different proteases and 3) recruitment of one of the multiple complement inhibitor of the complement pathways: factor H and factor H-like 1 (alternative pathway), C4BP (classical), CD46 etc....

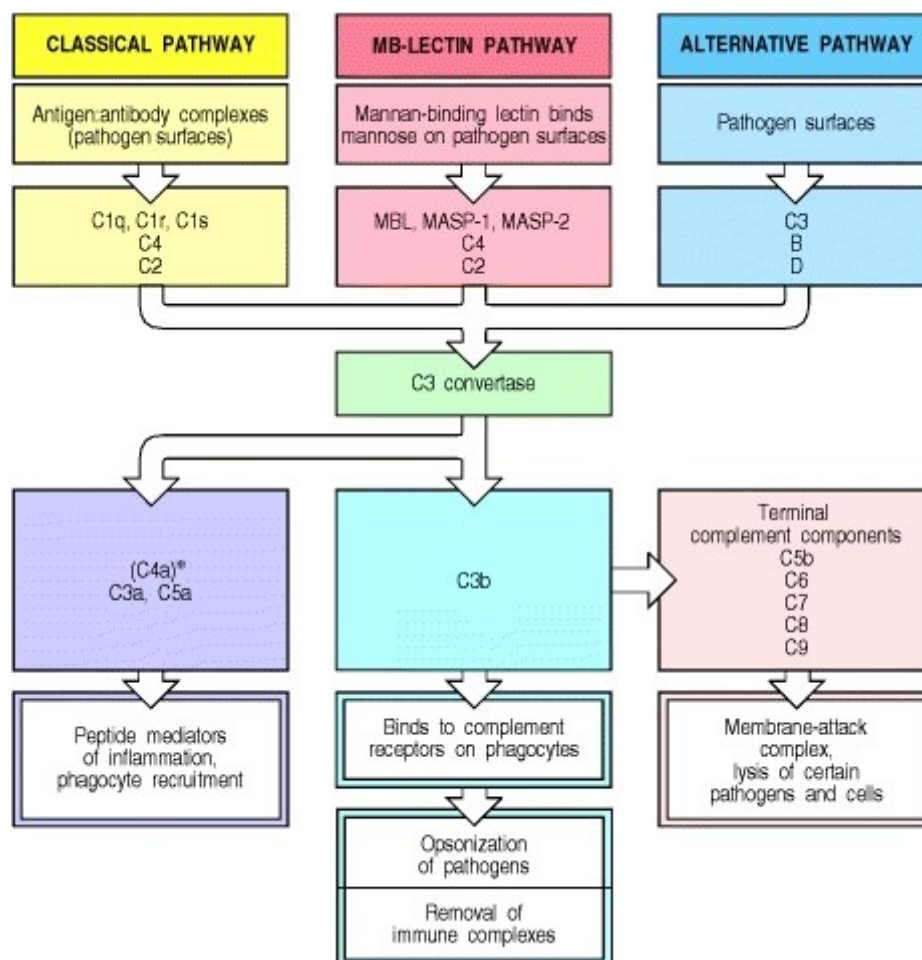


Figure 24. The complement pathway

Description of the main elements involved in the complement cascade. From (491).

3.1.2.1. Avoiding opsonization and inhibiting the complement cascade

There are two types of opsonization, the complement mediated opsonization and the antibody mediated opsonization. The antibody mediated opsonization is based on antibodies recognizing bacterial antigens by the Fab part of immunoglobulins. The available Fc domain can directly

serve as a ligand to trigger phagocytosis through the Fc receptor, or initiate the classical complement cascade through C1q binding to the Fc domain. GAS binds antibodies by their Fc domain through the M and M-like proteins and SfBI/PrtfI, rendering the Fc unavailable for the cellular Fc receptor (492).

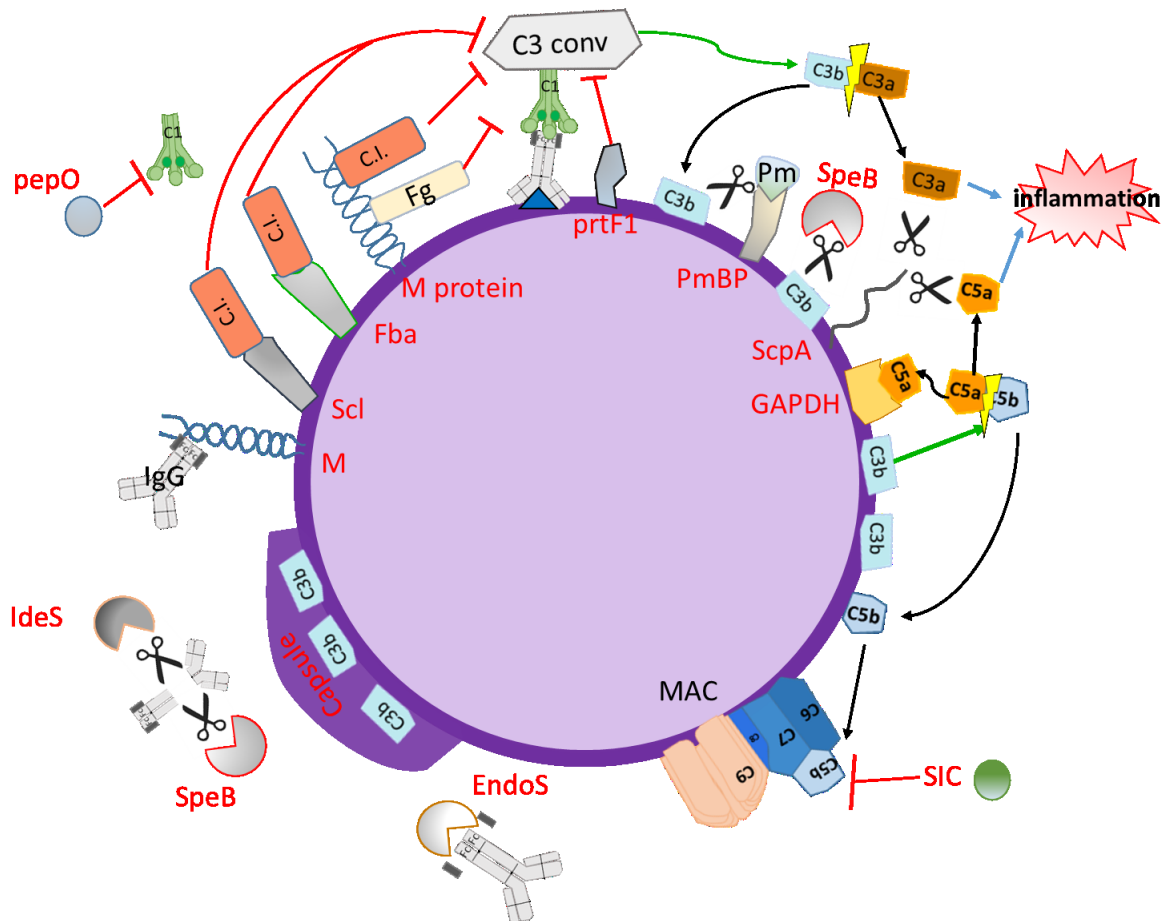


Figure 25. GAS resistance to the complement activation pathway

Schematic representation of the main mechanisms of GAS resistance to the complement cascade. C.I.: complement inhibitors, C3conv: C3 convertase, Fg= fibrinogen, PmBP: plasmin binding proteins, Pm: plasmin. Red arrow: inhibition. Black arrow: step of the pathway. Green arrow: cleavage. Scissors: degradation. Blue arrow: induction.

In addition, to remove the opsonic ability of antibodies, SpeB and IdeS can specifically cleave Fab bound antibodies, freeing the Fc activating domain from the bacterial surface and leaving the bacteria coated by a host-like coat (96, 493). However, SpeB ability to cleave IgG under the physiological, non-reducing, condition was questioned (88). EndoS and the M49 specific EndoS2 remove carbohydrates from IgG, which decreases the affinity of the Fc for the Fc-

receptor by 15-fold (94, 95). M and M-like proteins bind albumin, which plays an important role in determining the location of opsonic and nonopsonic epitopes (193).

Bacterial surface can also directly activate the deposition of C3b (alternative and lectin pathway). C3b can be degraded by SpeB and the plasmin bound at the surface of GAS (98, 99, 494). Moreover, bound plasmin can also degrade C5 (495). C3a is degraded by the C5a peptidase which also degrades C5a, the latter also being inactivated by its capture by GAPDH (261, 323). The alternative pathway is also inhibited by the degradation of properdin by SpeB (100). Recently, PepO, a secreted endopeptidase 68 % similar to *S. pneumoniae* PepO, has been involved in resistance to complement mediated bacteriolysis through the binding to C1q, which also decreases immunoglobulin mediated opsonization (496).

GAS strains can bind fibrinogen through several proteins (Table 10) and this binding inhibits the classical pathway of the complement by inhibiting the formation of C3 convertase through inhibition of the deposition of the C4b molecule (438, 497). In a similar way, Prtfl on its own inhibits C3 deposition at the bacterial surface and SIC inhibits the MAC assembly, in a step prior to the C5b67 binding to the cell membrane (149, 275).

To inhibit complement activation, GAS can recruit the inhibitors of the alternative pathway factor H and factor H-like through the M and M-like proteins, the protein Fba and Sc11 (Table 10) (188, 192, 332, 334, 347, 348). C4BP binds to M and M-like proteins, and the M1 protein binds CD46, but its role in complement inhibition was not shown (201).

3.1.2.2. Inhibiting, limiting and surviving phagocytosis and NETS

GAS has developed several strategies to avoid recognition by immune cells. GAS covers its surface with host proteins, avoiding recognition by phagocytes: LDL and HDL are bound by Sc11, albumin by LTA and by the M protein/protein H (211, 335, 336, 403, 404).

By inhibiting opsonization (3.1.2.1), GAS avoids its recognition by phagocytic cells. Moreover, the capsule prevents the recognition of opsonins and antibodies at the bacterial surface (498, 499).

Some factors can also reduce phagocytosis. Intracellular SIC can interact with ezrin in PMN, which reduces bacterial phagocytosis and Mac-1-like protein can bind CD16 at the surface of neutrophils to reduce their phagocytic capacity (144, 500).

After being phagocytosed, GAS bacteria are able to survive and even replicate within the phagocyte (501, 502). More specifically, viable GAS are found mostly in macrophages, but

also in neutrophils (501). GAS avoids the fusion between the vacuole and the lysosome in a M1 dependent manner (502–504). The ability of M1 strains to inhibit the phagolysosome fusion in a M1 dependent manner was not reproduced with another M1 strain (505). The protein H has also been implicated in the intracellular survival, but the mutant lacking the protein H only was shown to express very low amounts of protein M1 (241, 503). These observations need to be confirmed with a non-M1 strain to see if intra-phagocytic cell survival is a M1-specific characteristic.

SLO and NADase are two factors involved in the prevention of the acidification of the vacuole, increasing bacterial survival in macrophages. At the single cell level, the ability of GAS to replicate in the cytosol of a viable human macrophage depends on SLO (506). Therefore, it is clear that GAS survives within macrophages (501), but the molecular mechanisms involved remain to be elicited.

In addition to the bacterial killing decrease, GAS survival in macrophages could allow for its transport from colonization sites to distant anatomical sites and tissue invasion (507).

Neutrophils not only phagocyte bacteria, they also trap bacteria within neutrophil extracellular traps (NETs) which are mostly composed of extracellular DNA. GAS secretes several DNAses (2.1.1.2) that are involved in the resistance to the NETs by freeing the bacteria from them (130, 133). Finally the M1 protein reduces NETs cytotoxic activity and ScII both reduces NET production and cytotoxicity (502, 508).

3.1.3. Modulation of the inflammation

GAS controls the immune response to increase its survival in tissues and in blood. Paradoxically, different virulence factors can induce anti-inflammatory responses while other, or the same, activate pro-inflammatory responses (Figure 26). SpyCEP is able to degrade IL-8 and other ELR containing cytokine, which decreases neutrophil migration across endothelial barriers (168) and Sse hydrolyzes the platelet activation factor which is a neutrophil chemoattractant (173). SpeB degrades a wide range of anti-inflammatory chemokines: CXCL1-7; CXCL10-14, CXCL16, CCL20 and CX3CL1 (101). The degradation of C5a and C3a by the C5a peptidase both contribute to a reduced inflammation.

However, SpeB and other virulence factors can induce pro-inflammatory responses. Protein sequestration and protein cleavage are classical pathways in inflammatory response activation. Since SpeB is a broad-spectrum protease, its proteolytic activity can affect multiple

proteins involved in inflammatory pathways, which further stimulates inflammation. SpeB releases the pro-inflammatory bradykinin from the protease inhibitor H-kininogen (103). IL-1 β is a pro-inflammatory cytokine secreted as an inactive form which can be naturally cleaved to its active form by different host proteases during inflammation, or by caspase 1 during a specific cell death activation pathway. *In vitro*, SpeB cleaves the pro- IL-1 β to its mature active form (509): any pathogen's protease which cleaves IL-1 β will trigger a pro-inflammatory response, so IL-1 β can be seen as a sensor of pathogen-associated proteolysis.

Moreover, SpeB, independently from its proteolytic activity, can trap the serine proteinase alpha1-antitrypsin and this could enhance the contact system activation, highly increasing the bactericidal activity of the plasma (112). SpeB production is strongly down-regulated in plasma (106), which could decrease this phenomenon and promote blood bacterial survival.

SpyA induces a pro-inflammatory cell death in murine macrophages (166). In a similar way, SLO induces caspase 1-dependent cell death in murine macrophages, which releases active IL-1 β (510). After infection of murine macrophages, SLO and SLS synergistically induce the production of prostaglandin E₂, a proinflammatory molecule (511). However, in macrophages differentiated from peripheral blood mononuclear cells, a more relevant model, the production of IL-1 β is induced in response to GAS, but independently of SLS or SLO (512). SLO activates human neutrophils by inducing the release of heparin-binding protein, a pro-inflammatory molecule (513).

Similarly to the IL-1 β induction by SLO, data obtained using murine macrophages should be confirmed with human macrophages since it is becoming increasingly clear that murine macrophages differ significantly from human macrophages (514).

GAS strains secrete superantigens (SAGs) that are highly potent mitogens of the immune system (2.1.1.1). A similar effect was reported for LTA (306). SAGs are involved in psoriasis, acute rheumatic fever, Kawasaki disease, STSS and necrotizing fasciitis (119, 515, 516). The systemic hyperactivation of the immune responses are the principal characteristics of toxic shock syndrome, and GAS superantigens are directly involved in STSS [reviewed in (517)]. SAGs are also associated and implicated in different post-infectious complications, while the direct mechanisms involved remain to be established [reviewed in (518)]. The presence in both staphylococcal and streptococcal species of SAGs, the horizontally acquisition through bacteriophages, their dissemination within *emm*-types and the poor variability of the SAGs

genes, all suggest a critical role for these proteins in the setting up of infections. Activating the immune response could increase tissue inflammation which would increase nutrient availability through plasma exudation during the inflammation, however this would also activate a secondary immune response to which GAS cannot survive. The role of SAGs hyper-activation of the immune system within physiological tissue, such as the pharynx and the skin, and their evolutionary advantage remain unclear (519). One clue to the understanding of the role of SAGs in tissue colonization is that SAGs could be involved in anti-inflammatory responses. SAGs hyper-activation could trigger anergy of T cells or avoid a Th2 response which induces cytotoxic T cells proliferation (520). SAGs can induce the transition from CD4 T cells to CD4 T regulatory (Tregs) cells, which results in increased production of anti-inflammatory responses (521). Moreover, SAGs can also trigger CD8⁺ Tregs differentiation in human, decreasing CD4 T cell proliferation (522). Interestingly, the SAG doses required for IL-10 (anti-inflammatory) secretion by induced T cells are much lower than the doses for IFN- γ (pro-inflammatory). These results identify a novel crucial role of SAGs in the pathogenesis of GAS infections.

SAGs have a critical role in GAS colonization in a humanized MHC Class II mice; SpeA is essential in the colonization of mice nasopharynx (523, 524). The same SAG SpeA induces, through its binding of MHC class II molecules, a short term pro-inflammatory response followed by a long term hypo-inflammatory response with secretion of IL-10 and PD-L1, which contributes to T cell anergy by activating Tregs (525). The “hypervirulent” strains of the *emm1* genotypes express a natural variant of SpeA1, called SpeA2, which has a higher affinity for MHC class II molecules (526): this mutation is thought to contribute to the preferential dissemination of this clone, even more suggesting the importance of SAGs in GAS dissemination.

Human skin and pharynx harbor very specific immune resident cells with for example resident memory Tregs in the skin that are very different from systemic circulating cells (527). Resident specific cells probably play a significant role in the establishment of GAS colonization of these tissues and our understanding of SAGs importance for GAS virulence might arise from looking at the effect of SAGs on these different cell subtypes.

By degrading chemokines and pro-inflammatory peptides, GAS decreases the activation and recruitment of immune cells. The question remains: are pro-inflammatory reactions to GAS virulence factor side effects of their evolutionary selected functions (such as SpeB cleavage of IL-1 β) or an evolutionary trait selected to promote the infection? As seen with SAGs, it is clear that bacterial effectors can induce systemic hyper-activation, which results in diseases such as

necrotizing fasciitis and STSS. However, the same systemic pro-inflammatory effectors could have anti-inflammatory effects in tissue. It is therefore important to increase our knowledge of the critical virulence factors responsible for GAS early innate immune response modulation. Targeting the mechanisms of GAS modulation of immunity at the barrier site could be key to the treatments of GAS infection, as shown by the reduction of GAS colonization in mice oropharynx when immunized against SpeA (523).

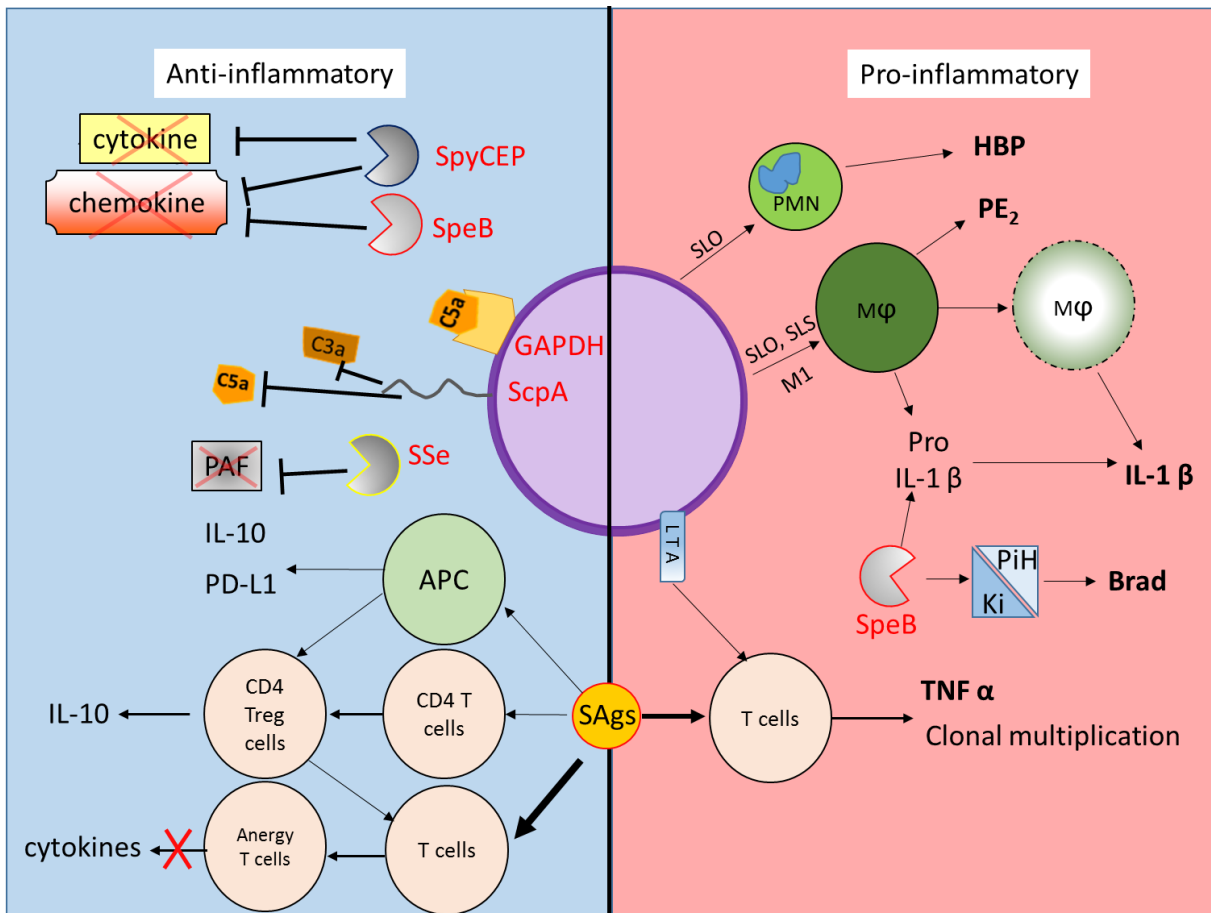


Figure 26. GAS: a "two-faced" modulator of the inflammation

Schematic representation of the different mechanisms of GAS modulation of the immune responses. APC: antigen presenting cells, Mφ: Macrophage, PMN= Polymorphonuclear, HBP= heparin binding protein, PiH= protease inhibitor H-kininogen, Ki= kininogen, Brad= bradykinin, PAF= platelet activation factor, PE₂= prostaglandin E₂.

4. Biopsies and experimental approaches to characterize GAS infections

GAS is responsible for a wide range of diseases that affect different tissues and there is a wide range of disease severity. In addition, GAS is genetically highly diverse and a strictly human pathogen. Finally, GAS ability to induce infections depends on the environmental context (for examples the presence of trauma, including blunt trauma, and the postpartum) and the host genetic background (528). Consequently, many independent different aspects of GAS infections can be studied.

One approach to understand GAS pathogenesis is to analyze disease characteristics in patient infected tissues (biopsies). Then different experimental approaches and models can be built up to mimic the pathogenesis, which enable to analyze the importance of specific factors in the pathogenesis. In the following paragraphs, I will shortly describe several models of infection. First, we will describe the insights from biopsies studies; then the different animal models but we will not discuss the invertebrate model of infection, because I consider it too remote from the questions treated during this thesis. We will also present the 3D organotypic approach and finally the study of human explants. Since in the Manuscript 2 we will present a new model of human explant, the decidua, we will conclude this section by presenting this tissue and its organization.

4.1. Biopsies

Since recurrent pharyngitis require tonsil removal and necrotizing fasciitis also necessitate the removal of infected tissue, researchers have had access to human biopsies since a long time to study the end point of GAS infections. Many parameters of bacterial infections were discovered through this approach, such as biofilm structure, intracellular cells status of GAS in immune and non-immune cells, antibiotic resistance (406, 413, 501, 529). Biopsies analyses are critical to describe phenotypes that models should try to recapitulate, to decrypt the mechanisms involved. One major challenge in biopsy studies is the number of host parameters that are not controlled and could explain the differences of morphologies between one patient and another (413). Biopsies studies unfortunately do not allow addressing all the critical parameters that played during the setup of patient infections.

4.2. Animal models

4.2.1. Rodent models

Mice, rats and chinchillas are three commonly used models for many bacterial infections, including GAS. Not all mouse strains can be used in the different mice models, and significant differences can be seen depending on the mouse genetic background (530). This makes the rodent model versatile, but the study of the genetic differences between mice strain already have unraveled host factors critical for infection (531).

To understand the pathogenesis of skin infections, a common model is injection of 10^6 to 10^8 bacterial in a subcutaneous or air sac model (532). However, this model does not recapitulate all human features of typical skin infections, such as impetigo, pyoderma, erysipelas, cellulitis or necrotizing fasciitis. The architecture of the murine skin might explain the phenotypic differences: murine epidermis is thinner, and the skin contains a lot more hair follicles than does the human skin, hair follicles that contain memory Tregs (533).

GAS is responsible for invasive infections in which bacteria are found in the blood (systemic infections). Several models try to recapitulate these infections: bacteria can be injected intravenously (IV), intraperitoneally (IP), intranasally (IN) or intratracheally (IT). These infection routes bypass all (IV) or most (IP, IN and IT) infectious step. IP is the less relevant, since the barrier to cross is one never encountered by GAS in humans (534). IN and IT systemic infections require the crossing of the lung epithelium (535). Many parameters can be monitored with these models, such as the bacteremia, the number of bacteria in different organs and the lethality. Many of these systemic models suffer from the poor viability of GAS strains in mouse blood compared to human blood. Lethality dose are still used as a marker of bacterial virulence, in spite of the virulence of bacteria in humans not correlating with mice virulence in systemic models (536).

One major feature of GAS is its ability to colonize the human oropharynx, especially the Waldeyer's ring which includes the tonsil: GAS is responsible for 700 million cases of pharyngitis per year (21), the analysis of GAS ability to colonize the oropharynx is therefore a clinical issue. Except for the mouse pathogenic strains B514/33 of the M type 50 (537) which expresses an atypical virulence repertoire (237), most human strains do not colonize on the long term the mouse or rat oropharynx, and to establish short term colonization, huge load of bacteria are instilled, not recapitulating the infection routes. Mice and rats are rodents so their nasopharyngeal organizations significantly differ from human oropharynx, for example there

is no equivalent for the Waldeyer's ring in the rodents. However, this model is informative for the understanding of active immunization: immunization with several virulence factors reduces the bacterial colonization. Another approach to model pharyngitis is the non-lymphoid associated tissue (NALT), which corresponds to the human tonsil. Some GAS strains briefly colonize these tissues but there are the same limitations as for the nasopharyngeal model.

To analyze long term mucosal colonization by GAS, a vaginal model was developed (538). In this model, mice are primed with estradiol and infected with GAS, which results in long term asymptomatic colonization. GAS poorly colonizes human vagina (0.03%) (539) and is responsible for puerperal sepsis in very specific contexts (see section 1.3.3). Mouse vagina recapitulates several human vaginal characteristics and is used to model other vaginal colonizers/ pathogens (GBS and *Candida albicans*). The vagina colonization model allows analysis of long term colonization of a mucosa. However, long term colonization also involves adaptation of bacteria to their environment, and in this case the mouse vagina is an environment very different from most human mucosa.

A rat model was also used to study post-infectious complications resulting from cross-reactivity of antibodies targeting bacterial antigens (autoimmunity). In this model, rats are immunized with GAS antigens and are monitored for their development of post-infectious sequelae such as rheumatic arthritis and endocarditis (540). The developed pathologies resemble those of humans and this model could help understanding the mechanisms involved in post-infectious autoimmune diseases.

GAS is responsible for approximately 3% of ear infections (541). Biofilms are involved in GAS virulence but few models enable the study of biofilm on biotic surfaces. A model of chinchilla ear infections was set up to study biofilm formation and the pathogenesis of ear infections (542). The presence of three-dimensional GAS communities embedded in extracellular polymeric substance indicates that GAS readily forms biofilm in the chinchilla ear. Compared to *in vitro* studies, chinchilla ear infection mimics more closely the biofilm formation that occurs during human infection, and this model can lead to potential critical discoveries that could help fight against *in vivo* GAS biofilms.

Of mice and men: humanization of mice

There are two promising approaches for the study of strictly-human, or not, pathogens such as *Neisseria meningitidis*, *S. aureus* and GAS: humanization of mice and xenograft (543, 544).

Both approaches aim to reduce the inter-species barrier that limits the modeling of bacterial pathogenesis.

Mice transgenic humanization: what you miss, we will give it to you

A limitation of the murine models is the strict specificity of many GAS major virulence factors: GAS does not bind mouse C4BP and factor H, and is therefore intensely targeted by rodent complement cascade and eliminated; GAS binds mouse IgG with a very low affinity compared to human IgG; streptokinase does not target mouse plasminogen and superantigens do not affect murine immune cells (181, 241, 476, 517, 523). This list is not exhaustive and probably many GAS virulence factors are not functional in mice, greatly reducing our ability to understand the role of virulence factors without minimizing the effect of others that are at play in humans.

To solve this issue, mice expressing transgenically human factors in addition or instead of their native factors were generated: hu-plasminogen, hu-Factor H, hu-C4BP and hu-MHC class II (181, 476, 523, 545–548). The contribution to virulence can be assessed compared to the native mice, and these models take advantage of the human factor that functions as the native mouse factor: for example, human C4BP is able to inhibit the mouse complement cascade. With the possibility to generate multiple KO the number of factors tested and the specific contributions, synergy or complementarity, can be assessed (546). These humanized mice do not fully recapitulate the GAS pathogenesis but can yet be useful for specific questions. However, studies of the role of Factor H in virulence in C57BL transgenic mice and BalbC transgenic mice yielded contradictory results (546, 547). As with mouse models, understanding the differences in the genetic background of the mice could contribute to our understanding of critical host parameters in GAS infections.

Xenograft

Another approach is the xenograft: human pieces of skin are grafted onto immunodeficient mice. The tissues come from neonatal foreskin or plastic surgery. Compared to other pathogens, such as *N.meningitidis*, xenografted mice are not commonly used for GAS studies. This approach was used to model impetigo and several features of human impetigo were reproduced (549, 550). Xenograft enables the study of the early steps of bacterial infection of tissues with relevant cells and extracellular matrix components. Immune responses and immune cell recruitment still correspond to the mouse systemic circulation that, furthermore, is immunosuppressed: this model does not allow the study of the humoral response to the infection.

4.2.1. Non-human primate models

Primate models were developed to overcome the species specificity of GAS and to test GAS pathogenesis in the presence of an immune system closer to the human one. GAS readily colonizes the oropharynx of different primates (baboon, rhesus monkey, chimpanzee, and the cynomolgus macaque) and in contrast to rodents, the animals suffer clinical manifestations resembling pharyngitis (551, 552). This colonization is long lasting and enables subsequent humoral immune responses, which could in a near future allow the study of rheumatic fever in this model. Primate is probably the best model to study GAS virulence factors involved in human pharyngitis, and already confirms the importance of the M proteins and the capsule in the colonization (553, 554). As said before, one limitation of mice models is the species-specificity of the superantigens (SAGs), which probably also explains our poor knowledge of SAGs role in GAS virulence. To better understand the pathogenesis of STSS, a model of primate sepsis was set up, which includes the intravenous injection of 10^{10} bacteria in baboons (555). Several human clinical manifestations (hypotension, organ failure) were observed, making primate models a promising model to understand the transition from bacteremia to STSS, and the virulence factors involved.

4.3. 3D organotypic skin model and *ex vivo* infections

4.3.1. 3D organotypic skin model

The 2D resolution, infection of epithelial cells *in vitro* is a good model to analyze GAS-host cells interactions: adhesion, internalization, epithelium crossing, cytotoxicity and cell responses to infection. However, it does not allow long-term study of GAS infections: cytotoxicity induces cell detachment and bacteria are released in the supernatant, not enabling CFU counts or fluorescence microscopy. Moreover, the absence of other cell-types than an epithelial cell line strongly simplifies the interactions that occur during GAS infections: the absence of matrix, immune cells and vertical penetration of a tissue decreases the extent to which epithelial cells can be used as an infection model. Tissue engineering is an old idea that started during world war II (556) and ended up in the setting up of 3D organic tissues with cells from many human organs. 3D skin explant is a tissue that contains epithelial cells seeded in a collagenous matrix. This is the simplest 3D skin explant engineered and it is a very good tool to assess biofilm formation or cytotoxicity of Streptococci strains (413, 557).

Moreover, tissue engineering is being more and more developed, with the generation of fibroblast and epithelial cells 3D tissue (558, 559); bioprinting is now extending the limit of

tissue engineering with 3D tissue incorporating dendritic cells (560–562). While still simpler than normal human skin, these models enable to have only one genetic background, they avoid versatility of human skin and avoid the use of animal models. It is without any doubt that these approaches will be more and more used to study GAS infections.

4.3.2. *Ex vivo* models of infection

To study the very early steps of infection, without grafting tissue to mice, models were developed where human tissue explants are infected *ex vivo* and the infection is monitored on the infected sample (563, 564). Human tonsil were infected *ex vivo* by GAS (265, 565) and human skin explants have been used to study GAS biofilm formation for the first time very recently (566). These models do not allow for long-term observation of the infection compared to the mice, with tissue viable for up to 24 hours. However, recent development of medium enables up to 7 days survival of this tissue (567). The advantage is that the tissue is infected extemporaneously, without the wound healing and possible adaptation that occurs during xenografting. *Ex vivo* models enable to study innate immune response and the limiting elements involved in the early host-pathogen interaction, but does not allow immune cell recruitment. In the second publication of this thesis, we will describe a new model of *ex vivo* infection of a human tissue, the decidua, and how it contributes understanding the critical early steps of GAS infections.

4.3.3. The decidua and maternal-fetal membranes

During the menstrual cycle, the endometrium, the mucosal lining of the uterus, undergoes differentiation in preparation to implantation of an embryo and to facilitate its development. In the second part of the menstrual cycle (after day 18), the differentiation of the endometrium is called decidualization from the Latin *decider*, to fall off or to detach. At the end of the human menstrual cycle, if the oocyte is not fertilized, the decidualized endometrial lining is eliminated during menstruation and the menstrual cycle begins again with a new series of endometrial differentiation.

During decidualization, endometrial stromal fibroblasts transform into specialized secretory cells. This transformation involves both the differentiation of fibroblasts and endometrial stromal cells into large polyploid (epithelioid-like) cells, called decidual stromal cells (DSC), the profound remodeling of the composition and organization of the endometrial extracellular matrix (ECM) and a massive influx of leukocytes, polynuclear cells, macrophages and NK cells.

The decidual extracellular matrix is mainly composed of diffused type I, III and VI collagens. In addition, type IV collagen and laminins are enriched around DSCs (569). The decidua is composed of 40 % of leukocytes and 60 % of DSCs. Circulating monocytes that infiltrate the decidua differentiate into decidual macrophages (CD163⁺) which correspond to 20 % of leukocytes in the decidua (570). Seventy % of leukocytes are decidual NK cells that regulate embryo invasion and the remaining 10 % of leukocytes are T cells involved in the maternal-fetal tolerance (571).

The implantation of the embryo critically depends on the secretion of matrix metalloproteases by the decidua (572). After implantation, the embryo resides within the decidua and during its development, several embryonic cell subtypes associate with maternal cells (Figure 27a). After obliteration of the uterine cavity because of the enlargement of the amniotic cavity, around the fourth month of gestation, the maternal-fetal membranes present their definitive structures with the amnio-chorion membranes surrounding the amniotic cavity (fetal membranes) and the maternal decidua (Figure 27b). These membranes will slough off with the placenta after delivery.

The decidua prevents the embryo from being attacked by maternal immune cells, it supplies nutrition prior to placenta formation and protects both embryo and mother from infections. The large number of immune cells in the decidua is supposed to establish a balance between immune tolerance of the embryo and defense against infections (572). Twenty-four hours of infection with various pathogens (*Escherichia coli*, GBS, *Candida albicans* and *Gardnerella vaginalis*) induce the secretion by maternal-fetal membranes of the pro-inflammatory cytokines IL-6, CXCL8, TNF and the anti-inflammatory cytokine IL-10 (573–576). DSCs could play a role in the modulation of inflammation (572) and they express 4-6 types of TLR (577). Of note, *in vitro*, isolated DSCs produce TNF-alpha, IL-6 and CXCL8 after stimulation with Lipopolysaccharide (LPS) (577, 578). LPS, lipid A and LTA all increase secretion of IL-10 by isolated DSCs (579).

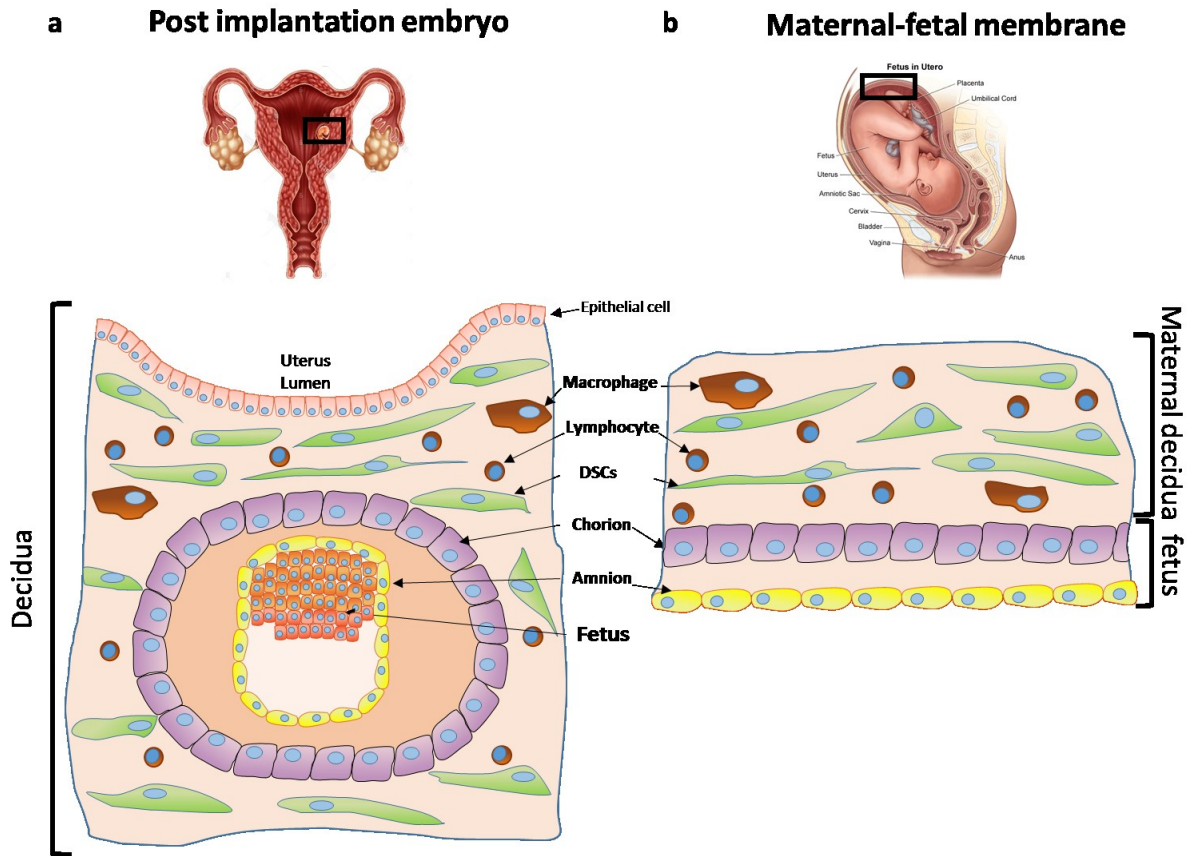


Figure 27. The development of the maternal-fetal membranes

In the top figures are represented the regions of interest (black rectangle) that are described in the schemes below as sections. **a**, the decidua post implantation of the embryo; **b**, a section of a maternal-fetal membrane at the end of pregnancy.

Research context

Research context

The main focus of the team is the deciphering of bacterial mechanisms involved in the crossing of host barriers. The bacteriology department of the Cochin Hospital of Paris hosts the National Reference Center for Streptococci since 2006. The team Barriers and Pathogens mainly focuses on GAS, involved in choc mild to severe infections, pharyngitis to necrotizing fasciitis and septic, and GBS, a pathogen which colonizes the vagina of 10-30 % of women. GAS is responsible for 517 000 deaths in the world, with 163 000 deaths due to invasive infections. These infections are a clinical concern for three reasons. GAS invasive infections are life-threatening and fast evolving diseases with a mortality rate of 25 %, there is a propensity for epidemical dissemination in health-care systems and finally, the number of invasive infections has been increasing in developed countries since the 1980s. The team preferentially analyzes how the mechanisms involved in the onset of GAS invasive infections.

The team is part of the “Département hospital-universitaire” (DHU) Risks in pregnancy, a consortium of obstetrical clinicians and researchers which aims at increasing the knowledge on pregnancy and peripartum diseases. GAS puerperal fevers were, in the nineteenth century, a significant burden, responsible for the death of one out of ten women after delivery. Prophylactic strategies have significantly decreased the number of cases, but endometritis still represents 26 % of women GAS invasive infections in France. In this context, the team studies GAS puerperal fever and endometritis as models of GAS elicited invasive infections. The team confirmed with French epidemiologic data the association between GAS *emm28* strains and gynecological-obstetrical diseases (43), and a representative *emm28* strain was selected on phenotypic and genotypic characteristics from a screening of 50 *emm28* strains involved in endometritis in France. This strain was fully sequenced and called M28PF1 (M28 GAS Puerperal Fever 1) (126). The main goals of this thesis were to characterize mechanisms involved in GAS *emm28*-elicited endometritis, and the role of different factors in these infections. We performed this analysis in two complementary parts: in the first one, we focused on the involvement of the R28 protein in the adhesion to host cells of the gynecological-obstetrical sphere; in the second one, we studied the establishment of GAS invasive infections on an original *ex vivo* model of human tissue infection, using the infection of the decidua as a model of endometritis.

In the first part, we aimed to identify specific *emm28* proteins involved in the association between these strains and gynecological-obstetrical diseases, which prompted us to study R28, an *emm28*-specific protein involved in adhesion to cervical cells. We assayed the role of R28 on

other cell lines potentially involved in gynecological diseases, identified the subdomains involved in promoting the adhesion and their cell receptors (Manuscript 1).

In the second part, we intended to better characterize the pathophysiological scenario of GAS endometritis. The endometrial lining of women after delivery is composed of decidua, a tissue derived from endometrial stromal cells and immune cells. The fetal membrane surrounding the fetus during pregnancy results from the association between maternal decidua and fetal cells. We therefore decided to infect *ex vivo* the decidua from maternal-fetal membranes acquired from healthy caesarians. To analyze the critical parameters involved in the poorly described window of establishment of invasive infections, we focused on the first hours of infection. More specifically, we studied the mechanisms involved in bacterial growth, biofilm formation, tissue penetration and in the modification of the host responses to the infection (Manuscript 2 and additional results).

Results

1. R28 protein and puerperal fever

“The N-terminal domain of the R28 protein promotes *emm28* Group A Streptococcus adhesion to host cells via direct binding to three integrins” (Manuscript 1)

(Manuscript accepted for publication in Journal of Biological Chemistry)

GAS *emm28* strains are associated with gynecological infections, including endometritis (44–47). This *emm*-type is the third most prevalent in France and one of the most prevalent in Europe (35, 43). R28 is a surface protein of GAS expressed nearly exclusively by *emm28* strains and is a member of the Alp family of GBS surface proteins. Noteworthy, GBS is a commensal bacterium of the vagina (50). A GAS *emm28* strain that does not express R28 adheres less to cervical cells than the wild-type strain (48). In this study, we assessed the potential implication of R28 in the association of *emm28* strain and gynecological infections. We dissected the potential role of R28 in the promotion of GAS adhesion on relevant clinical cells potentially involved during the pathogenesis of GAS endometritis.

First, we showed that an isogenic mutant for R28 presents decreased adhesion to primary decidual cells, cells that compose mucosal uterine-lining during pregnancy (Manuscript 1, Fig. 2). Then we show that the purified R28 N-terminal domain (R28_{Nt}) binds specifically to primary decidual cells, to endometrial epithelial cells (HEC-1-A) and to cervical cells (ME180). Beads coated with R28_{Nt} also bind specifically to HEC-1-A. Finally, the exogenous expression of R28_{Nt} increases the adhesion of *Lactococcus lactis* to HEC-1-A cells. Hence R28_{Nt} promotes the adhesion of GAS *emm28* strains to clinical relevant cells possibly involved in endometritis.

We then investigated the subdomains involved in the adhesion to cells (Manuscript 1, Fig. 3). We subdivided the R28_{Nt} in two halves, R28-N1 and R28-N2 domains (Manuscript 1, Fig. 1). N1 is the only domain conserved and similar to all N-terminal domains of proteins of the Alp family. R28-N1 and R28-N2 both contributed to adhesion to HEC-1-A cells as shown by experiments with purified proteins and coated beads. Moreover, R28-N1 and R28-N2 compete one with another suggesting they share common receptor(s).

The analysis of the capacity of R28_{Nt} to bind cells after biochemical modifications of the cell surface showed that the receptors of R28_{Nt} are membrane proteins (Manuscript 1, Fig.

4). A co-immunoprecipitation approach was set up to identify these receptors and a mass-spectrometry analysis identified several surface proteins as putative receptors, including the integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ (Manuscript 1, Table 1). By ELISA, we demonstrated that R28_{Nt}, R28-N1 and R28-N2 all directly interact with $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ but not with $\alpha2\beta1$, an integrin expressed by endometrial cells but that did not co-immunoprecipitate (Manuscript 1, Fig. 5, Fig. S3). We showed that these interactions did not require divalent cations in contrast to natural ligands; yet, these divalent cations altered the binding to $\alpha3\beta1$ and $\alpha6\beta1$ but not to $\alpha6\beta4$.

Finally, since $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$ are expressed by numerous cell-types, we analyzed R28_{Nt} ability to promote adhesion to other than endometrial cell-types (Manuscript 1, Fig. 6). R28_{Nt} increased the binding of beads to pulmonary A549 cells, skin keratinocytes HaCaT and to a lesser extent to intestinal cells TC7, which express apically low amount of $\beta1$ containing integrins.

In conclusion, our work indicates that R28 interacts directly to the three integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ through its subdomains R28-N1 and R28-N2. These interactions contribute not only to adhesion to the endometrium, and consequently potentially to GAS endometritis, but also to the adhesion to other cell types and thus to the overall prevalence of the *emm28* strains.

Manuscript 1

The N-terminal domain of the R28 protein promotes *emm28* Group A Streptococcus adhesion to host cells via direct binding to three integrins

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Running title: GAS R28_{Nt} interacts with integrins to promote adhesion

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Keywords: host-pathogen interaction, *Streptococcus pyogenes* (*S. pyogenes*), infection, adhesion, integrin, protein-protein interaction

ABSTRACT

Group A Streptococcus (GAS) is a human-specific pathogen responsible for a wide range of diseases, ranging from superficial to life-threatening invasive infections, including endometritis and autoimmune sequelae. GAS strains express a vast repertoire of virulence factors that varies depending on the strain genotype, and many adhesin proteins that enable GAS to adhere to host cells are restricted to some genotypes. GAS *emm28* is the third most prevalent genotype in invasive infections in France and is associated with gynecological infections. *emm28* strains harbor R28, a cell wall-anchored surface protein that has previously been reported to promote adhesion to cervical epithelial cells. Here, using cellular and biochemical approaches, we sought to determine whether R28 supports adhesion also to other cells and to characterize its cognate receptor. We show that through its N-terminal domain, R28_{Nt}, R28 promotes bacterial adhesion to both endometrial-epithelial and -stromal cells. R28_{Nt} was further subdivided into two domains, and we found that both are involved in cell binding. R28_{Nt} and both subdomains interacted directly with the laminin-

binding $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins; interestingly, these bindings events did not require divalent cat-ions. R28 is the first GAS adhesin reported to bind directly to integrins that are expressed in most epithelial cells. Finally, R28_{Nt} also promoted binding to keratinocytes and pulmonary epithelial cells, suggesting that it may be involved in supporting the prevalence in invasive infections of the *emm28* genotype

Streptococcus pyogenes, also known as Group A streptococcus (GAS), is a Gram-positive bacterium responsible for a wide range of diseases, from superficial infections such as pharyngitis and dermatitis, to severe invasive infections such as necrotizing fasciitis and endometritis (1–3). GAS infections are also responsible for post-infectious complications such as rheumatic arthritis and glomerulonephritis and, altogether, GAS infections are responsible for 517,000 deaths annually worldwide (4).

GAS strains are genetically diverse and are genotyped through sequencing of the 5' end of the *emm* gene (5) encoding the M protein, a major virulence factor; more than 250 *emm*-types have

been described (6). A link exists between genotype and tissue tropism, with throat- and skin-specialists and ubiquitous genotypes (7); a link between genotype of invasive strains and elicited disease exists for some but not all genotypes (our unpublished data, <https://cnr-strep.fr/>). Approximately 10 % of the GAS genome is composed of exogenous genetic elements encoding virulence factors, with substantial variation between different *emm*-types, which could account for the tropism (8). The first to third most prevalent genotype in Europe, *emm28*, is responsible for 8% of GAS invasive infections in France (9, 10, <https://cnr-strep.fr/>). It is associated to endometritis and for example, in France, 27% of GAS invasive infections in women occur in the gynecobstetrical sphere (10–13). *emm28* strains harbor an integrative conjugative element named RD2 that was likely horizontally transferred from *Streptococcus agalactiae*, also known as Group B Streptococcus (GBS) (13, 14). GBS colonizes 10–30% of healthy women's urogenital-tract (15) and it was suggested that the presence of this integrative conjugative element accounts for the *emm28* GAS gynecobstetrical tropism (13). This remarkable tissue association together with the high prevalence of *emm28* strain invasive infections prompted us to study the role of R28, an RD2 encoded surface protein, in GAS *emm28* infections.

Adhesion to host tissues is the initial step for all GAS infections. It is mediated by different factors, mainly surface proteins, that bind either extracellular matrix components, indirectly the cell surface through plasmatic or extracellular matrix components bridging, or directly to eukaryotic receptors [reviewed in (16) and (17)]. RD2 encodes four surface proteins including R28 and a R28-deficient *emm28* strain is non-adherent to the cervical cell line ME180 (18). R28 is a member of the Alp family composed of GBS proteins that share evolutionary and structural similarity and which includes the alpha C protein (also known as ACP or α), Rib, R28 (also known as Alp3 in GBS) and Alp2 (18–21) (Fig. 1A). These chimeric proteins are composed of a signal peptide, an N-terminal domain, repeats, and an LPXTG anchoring motif. The repeats number varies among clinical isolates and their structure is related to the Ig-like fold (22). The repeats are identical within one protein and the identity percentage between Alp members varies between 35 and 94, the latter being between Rib and R28

repeats. Repeats are considered to properly expose the N-terminal domain at the bacterial surface, potentially the functional domain (21). This domain is composed of one, for ACP and Rib, two, for R28, and three modules, for Alp2. The first modules of R28 and Alp2 share 99% identity and are 70 and 56% identical to the module of ACP and Rib, respectively. The second modules of R28 and Alp2 are 99% identical and is similar to the β protein, unrelated to the Alp family. Alp2 third module is a repeat of the second one (Fig. 1A) (21, 23, 24). Thus, R28 is a chimer of ACP and the β protein for its N-terminal domain and Rib for its repeats and it immunologically cross-reacts with both proteins (19, 25). ACP is the most studied member of the Alp family and the crystal structure of the N-terminal domain has been elucidated (24). It binds glycosaminoglycans in a region encompassing the end of the N-terminal domain and the repeats (26, 27), and the $\alpha 1\beta 1$ integrin through a KTD motif localized in a β -sandwich subdomain at the N-terminal end of its N-terminal domain (24, 28, 29); this motif is absent from R28, Alp2 and Rib (Fig. 1A). These interactions increase GBS internalization in the cervical cell line ME180 (29).

In this study, we analyzed the capacity of R28 to promote adhesion to host cells and characterized the adhesion domain. We demonstrate that the N-terminal domain of R28 ($R28_{Nt}$) is sufficient to promote direct adhesion to different gynecobstetrical cell lines, and further identified two subdomains within $R28_{Nt}$ that are both involved in this adhesion process. We then characterized the chemical nature of the $R28_{Nt}$ receptor and isolated different ligands. We show that $R28_{Nt}$ interacts with the laminin-binding integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$. Finally, we show that $R28_{Nt}$ increases adhesion also to skin and pulmonary cells, further extending the role of R28 as a GAS adhesin involved in GAS *emm28* prevalence.

Results

The N-terminal domain of R28 is sufficient to promote adhesion to human female genital tract cells

Association of GAS *emm28* strains with gynecobstetrical infections could be a consequence of its capacity to colonize the vaginal tract (10). A GAS R28-deleted mutant adheres less than the parental strain to cells from

the cervical ME180 lineage (18). To assay the role of R28 in a more physiological situation, we tested whether the phenotype could also be observed on human decidual stromal cells (hDSC) isolated from decidual biopsy of specimens obtained after caesarian delivery (Fig. 2A). The decidua is the lining of the uterine during pregnancy and consists of differentiated endometrial stromal fibroblasts and recruited leukocytes. It may be a direct target of GAS infection, a major cause of severe puerperal sepsis. The R28-deleted strain adhered significantly less (18%) to hDSC than the wild-type strain (Fig. 2A), confirming the role of R28 as an adhesin on physiologically relevant cells. We then sought to characterize the adhesion domain of the R28 protein using different cells from the female genital tract, hDSCs as well as two cell lineages, cervical cells (ME180), often used to study female genital tract infections, and endometrial epithelial cells (HEC-1-A). The six to 17 highly conserved 79-residue long repeats of R28 are considered to expose the 368 residue-long N-terminal domain at the bacterial surface, potentially the functional domain (Fig. 1A) (18, 29). Consequently, to analyze the ability of R28 to promote adhesion to various cells, we focused on the N-terminal domain of R28, R28_{Nt}, using biotinylated R28_{Nt} (Fig. 2B-D). R28_{Nt} displays higher binding capacity to hDSC, ME180 and HEC-1-A cells than soluble BSA (200, 10 and 10 times more binding at 10 μ M, respectively). To confirm the capacity of R28_{Nt} to promote adhesion, we tested whether R28_{Nt}-coated beads or a heterologous bacterium expressing R28_{Nt} could bind more to HEC-1-A cells than the controls (Fig. 2E-I). R28_{Nt}-coated beads adhered three times more than the BSA-coated control beads ($p < 0.05$) (Fig. 2E-G) and this binding was blocked by the addition of purified R28_{Nt} antibodies ($p < 0.05$) (Fig. 2H). Also, the *Lactococcus lactis* strain expressing R28_{Nt} (Fig. S1A) adhered significantly more than the control strain harboring the empty vector (+35%, $p = 0.0066$) (Fig. 2H). The increased adherence may seem weak. However, in the absence of the repeats, R28_{Nt} may be poorly exposed at the *L. lactis* cell surface or hidden by the protective polysaccharide pellicle (30) compared to the exposition of the complete R28 protein in GAS *emm28* strains; this could lead to an underestimation of R28_{Nt} capacity to promote adhesion. Thus, the N-terminal domain of R28 is sufficient to increase the adherence of a Gram-positive bacterium to endometrial cells.

Altogether these results demonstrate that R28_{Nt} promotes bacterial adhesion to female genital tract cells.

Both N-terminal subdomains R28-N1 and R28-N2 are sufficient to promote adhesion to HEC-1-A cells.

The R28_{Nt} domain is composed of two halves, from amino-acid residue 56 to 229 and from residue 230 to 424, which we termed R28-N1 and R28-N2, respectively (Fig. 1A and Fig. S1B (21)). A BLAST alignment indicated that R28-N1 and R28-N2 share no similarity (E-value, 0.27). To test which sub-domain mediates adhesion, we produced and purified the corresponding peptides (Fig. S1B, S1C) and incubated them after biotinylation with ME180 and HEC-1-A cells (Fig. 3A-C). Both the R28-N1 and R28-N2 subdomains showed significant binding with both cell types compared to BSA ($p < 0.001$ for both cell-types), with R28-N2 displaying an affinity two-to three-fold higher than that of R28-N1 (Fig. 3C). The R28-N1 and R28-N2 binding values indicate an additive contribution of R28-N1 and R28-N2 to the R28_{Nt} binding on HEC-1-A cells and a synergetic one on ME180 cells (Fig. 3C).

To assess the binding of these subdomains in more physiological conditions, we tested whether R28-N1- and R28-N2-coated beads bound HEC-1-A cells (Fig. 3D). Twenty-seven % and 19% of R28-N1- and R28-N2-coated beads bound to HEC-1-A cells, respectively, a percentage significantly higher than BSA-coated beads (6.3%, $p < 0.001$ and $p < 0.05$ for R28-N1 and R28-N2 with HEC-1-A cells, respectively). We checked the binding specificity of the coated beads by incubating cells with an excess amount of soluble peptides (20 μ M) and assessing the percentage of beads still bound to cells (Fig. 3D). Purified R28-N1 and R28-N2 peptides competed with their respective coated beads (6.3% and 4.5 % vs 27 % and 19%, for R28-N1 and R28-N2, respectively) confirming the binding specificity. Altogether, these data clearly indicate that both the R28-N1 and R28-N2 subdomains are sufficient to promote adhesion to HEC-1-A cells.

We then sought to determine if R28-N1 and R28-N2 have independent receptors. We performed competition assays in which we assessed the percentages of R28-N1- and R28-N2-coated beads bound to cells pre-incubated with excess amounts (20 μ M) of soluble R28-N2 and R28-N1, respectively (Fig. 3D). These subdomains compete with one another ($p < 0.005$),

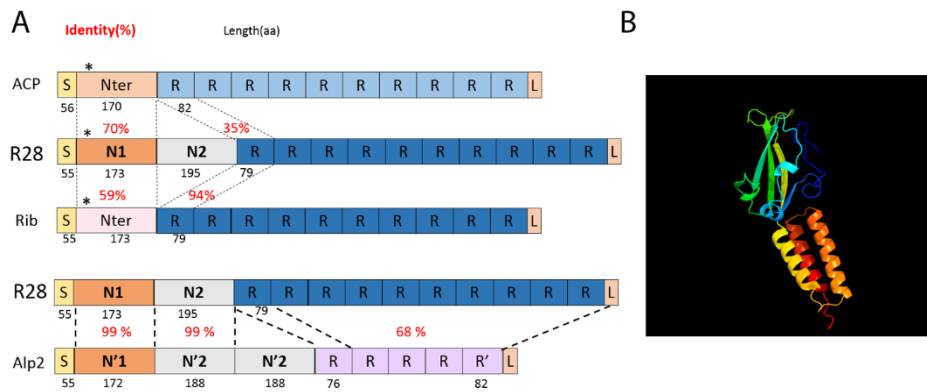


Figure 1. Schematic representation of R28, Alp family members and R28-N1 predicted 3D structure. *A*, Schematic representation of the Alp family members structure and similarity. Numbers in dark below the scheme indicate the length in amino-acid residues and in red the percentage of identify between two domains. S=Signal peptide, R= repeats, L= LPXTG. *= KTD in ACP, KAD in R28 and KPD in Rib. Modified with our sequence comparisons from (1–3). *B*, R28-N1 structure as predicted by Phyre. Green and yellow, the β -sandwich; yellow to red, the three-helix bundle

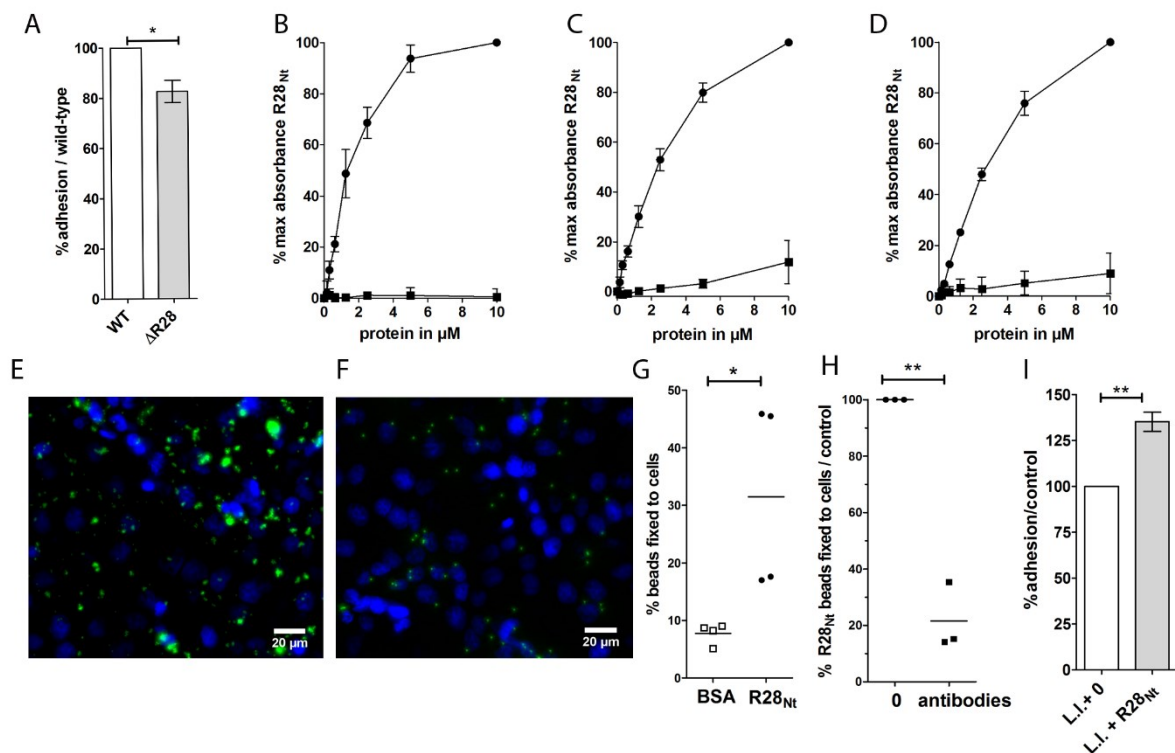


Figure 2. R28_{Nt} is sufficient to promote adhesion to female genital tract cells. *A*, Binding of the Δ R28 strain to human decidual stromal cells (DSCs) is expressed as a percentage of that of the wild-type strain; Two-tailed t-test was performed on 6 independent experiments performed in triplicates. *B-D*, ELISA-based protein-cell interaction assay with biotinylated proteins, expressed as percentage of the maximum binding, on: *B*, secondary decidual cells (DSCs); *C*, ME180 cells and *D*, HEC-1-A cells. Circle, biotinylated R28_{Nt}; square, biotinylated BSA. Error bars correspond to SEM of four independent experiments. *E-F* representative immunofluorescence of coated fluorescent beads with *E*, R28_{Nt} or *F*, BSA, on HEC-1-A cells. Coated beads in green and DAPI staining in blue. *G*, Binding of R28_{Nt} or BSA coated fluorescent beads to HEC-1-A cells. One-tailed t-test. *H*, Binding of R28_{Nt}-coated fluorescent beads to HEC-1-A cells in the presence of purified anti-R28_{Nt} antibodies, expressed as the percentage of that in the absence of anti-R28_{Nt} antibodies. *I*, Adhesion of R28_{Nt} expressing *L. lactis* (L.l. + R28_{Nt}) to HEC-1-A cells, expressed as the percentage of that of the *L. lactis* empty vector strain (L.l. + 0). *H-I*, two-tailed t-test. *, $p < 0.05$; **, $p < 0.01$

suggesting that these subdomains share common receptor(s). Moreover, both displaced R28_{Nt}-coated beads lowering its binding level to unspecific binding level (BSA-coated beads, 6%), further supporting that R28-N1 and R28-N2 share a common receptor.

R28_{Nt} adhesive properties are not conserved among all Alp family members

Among members of the Alp family, alpha C protein (ACP) binds, through its N-terminal domain, the $\alpha 1\beta 1$ integrin (28) and, through a region encompassing the end of the N-terminal domain and the repeats, glycosaminoglycans (26, 27). In contrast, attempts to demonstrate that Rib is an adhesin have been unsuccessful (31). We focused on the shared N-terminal domains of these proteins, leaving out from ACP_{Nt} the region binding glycosaminoglycans. A BLAST alignment indicated that R28-N1 is 70% and 56% identical to the N-terminal domains of ACP and Rib respectively (Fig. 1A). Furthermore, a Phyre analysis predicted, with a confidence of 100%, that R28-N1 has the same structure as ACP, that is two domains, an N-terminal β -sandwich, sharing structural elements with the type III fibronectin fold, and a C-terminal three-helix bundle (Fig. 1B) (24). We thus wondered whether the adhesive properties of R28-N1 to cervical and endometrial cells are conserved among the ACP or Rib N-terminal domains. We tested the binding capacity of ACP_{Nt} and Rib_{Nt+2R} which contains, in addition to the N-terminal domain, two repeats which are 94% identity to the R28 repeats (Fig. 1A). We expressed and purified these peptides and analyzed their direct binding to ME180 and HEC-1-A cells (Fig. S1C, S1D; Fig. 3A, 3B). ACP_{Nt} binds to both cell-types, yet less than R28-N1 and consequently even less than R28-N2 or R28_{Nt}. Since ACP_{Nt} binds HEC-1-A, despite the absence of $\alpha 1\beta 1$ integrin on its surface (32), we tested whether it shared a receptor with R28-N1 or R28-N2 by assaying, as for the R28-N1 and R28-N2 competitions, whether it can compete with R28-N1 and R28-N2 (Fig. 3D, 3E). ACP_{Nt} competes with itself and R28-N1 ($p < 0.005$), suggesting that these subdomains share a common receptor, but not with R28-N2 (Fig. 3B). Moreover, we did not detect a competition between R28-N1 and ACP-bound beads (Fig. 3E). In contrast to ACP, Rib_{Nt+2R} does not bind to either ME180 or HEC-1-A cells, which indicates that this binding property is restricted to some Alp family members (Fig. 3 A, 3B). Moreover, because Rib_{Nt+2R} contains two R28-like repeats, its

inability to bind ME180 and HEC-1-A cells suggests that R28 repeats do not mediate the adhesion on their own.

R28_{Nt} receptor is a membrane protein

Our experiments were carried out in the absence of added extracellular or plasmatic protein; we consequently hypothesized that R28_{Nt} binds directly to a cell surface receptor(s). To identify it, we first defined its chemical nature. As previously described (33), we applied to HEC-1-A cells different treatments that affect potential receptors depending on their chemical nature before incubating them with soluble R28_{Nt} and quantifying bound R28_{Nt} by flow cytometry (Fig. 4). Pronase and trypsin treatments significantly reduced, in a dose dependent manner, the percentage of positively labelled cells down to 10% and 29%, respectively. Also, neither heparinase I nor sodium periodate treatments affected R28_{Nt} binding to cells (data not shown), excluding an interaction between R28_{Nt} and glycosaminoglycans or carbohydrates which harbor vicinal hydroxyl groups. These results suggest that the R28_{Nt} receptor is a cell-surface exposed protein. Similar results were obtained with R28-N1 and R28-N2 (Fig. S2A-S2B). Altogether, our data suggest that the R28_{Nt}, R28-N1 and R28-N2 receptor(s) is (are) a cell-surface exposed protein(s).

Identification of several putative receptors of R28_{Nt}

To identify R28 receptor(s), a co-immunoprecipitation experiment using HEC-1-A cells was set up followed by mass spectrometry analysis. Cross-linked R28_{Nt} - cellular proteins complexes were resolved on acrylamide gels and Western blotting highlighted multiple high molecular, above 170 kDa, complexes (data not shown) absent in the co-immunoprecipitation without R28_{Nt} incubation (control). The zones above 170 kDa of R28_{Nt} and control immunoprecipitations were excised from gels and the protein contents were analyzed by mass spectrometry (Supplementary file). Hits enriched in the co-immunoprecipitation in the presence of the bait R28_{Nt} in all three independent experiments were initially selected. From this list, and since the R28_{Nt} receptors are cell-surface exposed proteins (Fig. 4), only such proteins were further selected (Table 1). Hence, through co-immunoprecipitation of R28_{Nt} we identified several potential R28_{Nt} receptors.

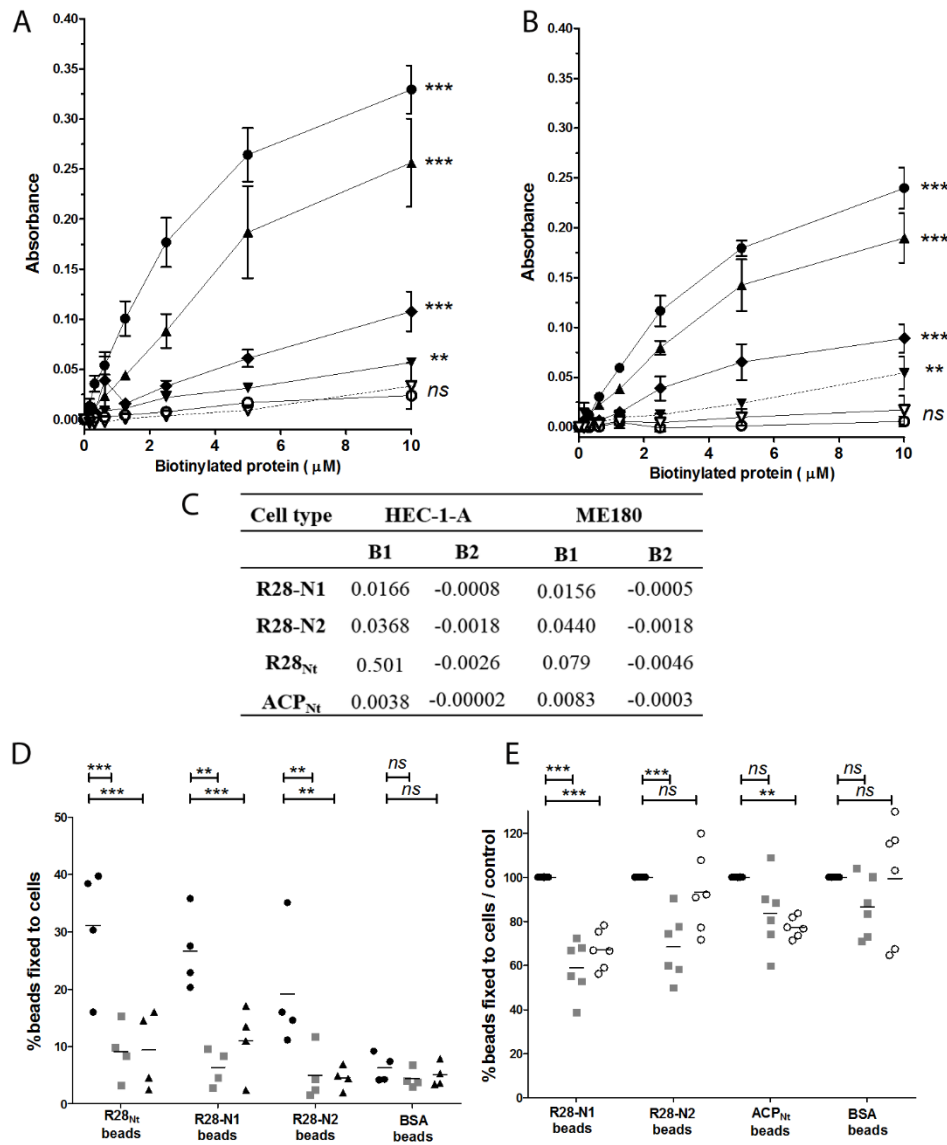


Figure 3. R28-N1, R28-N2 subdomains and ACP_{Nt} are sufficient to promote adhesion to HEC-1-A cells and compete differentially with each other binding. *A-B*, ELISA-based cell interaction assay with purified biotinylated R28_{Nt} (●), the subdomains R28-N1 (◆) and R28-N2 (▲); ACP_{Nt} (▼), Rib_{Nt+2R} (○) and BSA (▽). Peptide solutions were incubated with *A*, ME180 cells or *B*, HEC-1-A cell, washed, fixed with paraformaldehyde and bound biotinylated proteins were detected as in an ELISA. The experimental data for biotinylated R28_{Nt} and BSA are those used for Fig. 2C and 2D. The calculation for the absorbance is described in Experimental procedures. Error bars correspond to SEM of four independent experiments performed in duplicates. *C*, parameters of the non-linear fitting of figures *A* and *B*. *D-E*, Fluorescent (*D*) R28_{Nt}-, (*D, E*) R28-N1-, R28-N2-, (*E*) ACP_{Nt}- or (*D, E*) BSA-coated beads were incubated with HEC-1-A cells pre-incubated with 20 µM of recombinant R28-N1 (■); R28-N2, (▲), ACP_{Nt} (○) or not (●). **, $p < 0.005$; ***, $p < 0.001$; *ns*, not significant.

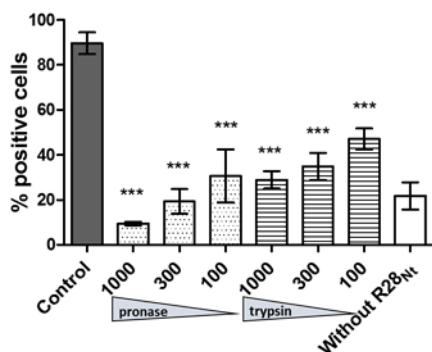


Figure 4. The R28_{Nt} receptor is a cell surface protein. Analysis of R28_{Nt} receptor chemical nature. Treatments were applied to HEC-1-A cells in suspension prior to incubation with purified R28_{Nt}; bound R28_{Nt} is immunostained and cells were analyzed by flow cytometry. Dark columns: untreated cells; clear column: unspecific labeling of cells without incubation of peptide. Statistical analysis was performed against the untreated condition. Error bars correspond to SEM of three independent experiments. Two-Way ANOVA: ***, $p < 0.001$.

Table 1. Main hits from mass spectrometry analysis of specifically co-immunoprecipitated R28_{Nt} receptors

Common Name	Gene name	Relative raw intensity		
		Exp1	Exp2	Exp3
Basal cell adhesion molecule	<i>BCAM</i>	A	3.50	5.00
Cadherin-1;	<i>CDH1</i>	A	A	A
CD166 antigen	<i>ALCAM</i>	A	3.70	8.30
Desmoglein-2	<i>DSG2</i>	A	A	6.01
Ephrin type-A receptor 2	<i>EPHA2</i>	A	4.03	3.20
Integrin alpha-3	<i>ITGA3</i>	A	8.32	65.32
Integrin alpha-6	<i>ITGA6</i>	A	18.37	A
Integrin alpha-V	<i>ITGAV</i>	A	1.74	7.20
Integrin beta-1	<i>ITGB1</i>	A	3.50	6.55
Integrin beta-4	<i>ITGB4</i>	A	5.84	5.62
Prostaglandin F2 receptor negative regulator	<i>PTGFRN</i>	A	A	A
Transferrin receptor protein 1	<i>TFRC</i>	A	3.54	5.10

A, Absent from control condition.

R28_{Nt}, R28-N1 and R28-N2 interact directly with integrins

Of all the proteins identified as present in the complexes co-immunoprecipitated with R28_{Nt}, we decided to focus on integrins as possible R28_{Nt} receptors. To test this, we assayed by immunofluorescence whether soluble R28_{Nt} and highlighted integrin monomers are in close proximity at the surface of HEC-1-A cells (Fig. 5A-5C). At the cell surface, R28_{Nt} indeed binds into clusters in close proximity to integrin $\alpha 3$, $\alpha 6$, and $\beta 1$ clusters.

To confirm a direct interaction between R28_{Nt} and integrins, we analyzed the binding of the extracellular domains of integrins to R28_{Nt}, R28-N1 and R28-N2 by ELISA (Fig. 5D-5F). Integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ all interact significantly more with R28_{Nt}, R28-N1 and R28-N2 than with BSA ($p < 0.001$). To assess the binding specificity further, the same experiment was carried out with an integrin containing a subunit which, although it is expressed by HEC-1-A cells, had not been found in the mass spectroscopy analysis, namely $\alpha 2\beta 1$ (Fig. S3) (32). In contrast to a natural ligand, type I collagen, neither R28_{Nt} nor its two subdomains, R28-N1 and R28-N2, displayed interaction with $\alpha 2\beta 1$, indicating that the integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are true receptors of R28_{Nt}. The influence of divalent cations on the binding efficiency was assessed. As expected, binding of R28_{Nt} to the different integrins in the presence of EDTA reflects that in PBS (Fig. 5G). Addition of the divalent ions Ca^{2+} significantly lowers binding of R28_{Nt} to $\alpha 3\beta 1$ and $\alpha 6\beta 1$ (51% and 72% compared to in the presence of EDTA, respectively) and that of Mn^{2+} to $\alpha 3\beta 1$ (45%). In contrast, binding to $\alpha 6\beta 4$ was unchanged by the addition of divalent cations. Altogether, these data demonstrate the direct interaction between R28_{Nt}, R28-N1 and R28-N2 and the three integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$.

R28_{Nt} is sufficient to promote adhesion to different cell lines

So far, our study focused on the capacity of R28 to promote adhesion to endometritis-related cells; yet, *emm28* is the first to third most prevalent genotype for GAS invasive infections in Europe (9, 10, <https://cnr-strep.fr/>). Moreover, the R28-interacting integrins are expressed by numerous cell types, so we assayed whether R28 contributes to adhesion in other GAS-elicited invasive infections. We tested R28_{Nt} ability to promote adhesion to other cell lines by testing the

binding capacity of R28_{Nt}-coated beads to skin keratinocytes, HaCaT, pulmonary epithelial cell, A549, as well as the gut intestinal cells TC7, that do not display the integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ apically (Fig. 6) (35). R28_{Nt}-coated beads bind significantly and to a similar level (approximately 30%) A549, HaCaT and HEC-1-A cells (Fig. 2G and 6). In contrast, only 13% of R28_{Nt}-coated beads bind TC7 cells, not significantly different from the BSA-coated control beads. In conclusion, R28_{Nt} is sufficient to promote adhesion to cell lines relevant not only for GAS endometritis but also for other GAS invasive infections.

Discussion

R28, a surface protein specifically expressed by *emm28* strains, which are associated with endometritis, promotes adhesion to cervical cells; its eukaryotic ligand was unknown (18). Currently in France, 50 % of GAS elicited endometritis are associated with puerperal fever, abortion, *in vitro* fertilization or spontaneous abortion (our unpublished data, <https://cnr-strep.fr/>), all situations where stromal decidual cells line the endometrium. Thus, GAS elicited endometritis may be favored by GAS adhering to decidual cells, in addition to epithelial cervical or endometrial cells. The R28 deleted strain was hampered in its capacity to adhere to human decidual stromal cells (hDSCs) (Fig. 2). That the mutant strain still adheres can be explained by the number of adhesins displayed by GAS strains (36–38), the deletion of a single one being insufficient for a total loss of adhesion capacity, as shown for example for Epf (39) and C5a peptidase (40). R28_{Nt} binds to hDSCs, endometrial epithelial cells and cervical epithelial cells; it also promotes the adhesion of beads or heterologous bacteria to endometrial epithelial cells (Fig. 2). These data altogether confirm the role of R28 as an adhesin and extends its role to other cells potentially involved during endometritis.

R28 belongs to the Alp family of proteins, shared with GBS, which includes ACP, Rib and Alp2. The ACP N-terminal domain, but not that of Rib, also bound endometrial and cervical epithelial cells indicating that this property is not limited to R28 but not shared by all the members of the Alp family either (Fig. 3). Noteworthy, the ACP N-terminal domain shows a higher sequence identity to R28 N-terminal sequence than does Rib N-terminal domain, as well as a structural

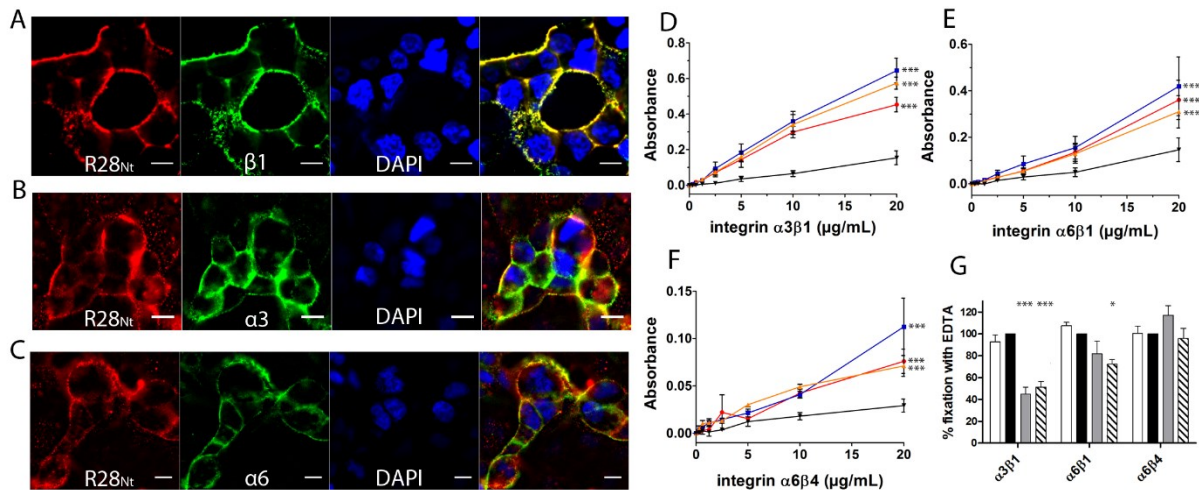


Figure 5. R28_{Nt} and its subdomains interact with integrins α3β1, α6β1 and α6β4. *A-C* Immunostaining of R28_{Nt} and different integrin monomers on HEC-1-A cells: *A*, β1; *B*, α3; *C*, α6. R28_{Nt}, red; the specified integrin monomer, green; DAPI, blue; the right-hand side image, merge. Scale bar corresponds to 5 μm. *D-F* Assessment of integrin binding: *D*, α3β1; *E*, α6β1; *F*, α6β4 binding to R28_{Nt}, red; R28-N1, blue; R28-N2, orange; BSA, black, by ELISA. Error bars correspond to SEM of three to five independent experiments. Two-way-ANOVA at 20 μg/mL with Bonferroni post-tests against BSA. *G*, role of divalent cations in the binding of integrins to R28_{Nt}, PBS, clear column; PBS + EDTA, dark column; PBS + Mn²⁺, grey column; PBS + Ca²⁺, hatched column. One-Way ANOVA of four independent experiments. *, p<0.05; ***, p<0.001.

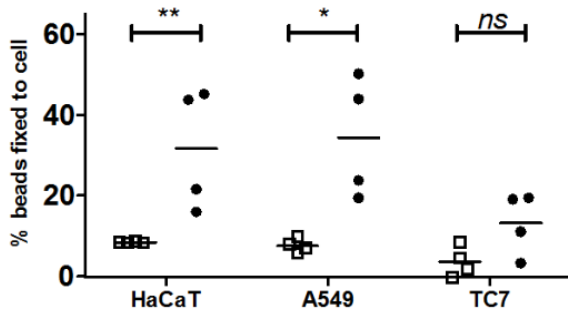


Figure 6. R28_{Nt} increases binding to different cell lines. Binding of R28_{Nt}, black circles; BSA-coated, empty squares, fluorescent beads to different epithelial cell types: HaCaT (keratinocytes), A549 (pulmonary) and TC7 (intestine). Four independent experiments performed in duplicates. Two-Way ANOVA. *, p<0.05; **, p<0.005; ns, not significant.

identity (18, 24); this could account for ACP but not Rib sharing the binding property. Furthermore, R28_{Nt} and Alp2 N-terminal domain are near identical (20, 21), consequently this binding property could be extended to Alp2. Fifteen to 30% of women vaginal tract is colonized by GBS and all GBS strains present the gene encoding for one member of the Alp family (15, 41); most of these proteins are likely involved in GBS colonization.

The GBS proteins ACP and Alp3 (*i.e.* R28) bind glycosaminoglycan (26). Analysis using a set of constructions with different ACP domains suggested that a site beginning in the N-terminal region of ACP and extending into the repeat region is responsible for this binding (26, 27). Here, the R28_{Nt} and ACP_{Nt} peptides we used were devoid of repeats region sequences, suggesting that the binding we observed was not that to glycosaminoglycan; this was confirmed by the absence of heparinase treatment effect on the R28_{Nt} – HEC-1-A binding. This suggested that other receptors than glycosaminoglycan are involved in the R28-endometrial or -cervical cell binding.

In this study, pull-down experiments on whole cells indicate that R28_{Nt} binds other integrins namely $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ (Fig. 5). Furthermore, we demonstrate by protein-protein binding experiments that the binding is direct. Among already described GAS direct interactions, Sc11 interacts with integrins $\alpha 11\beta 1$ and $\alpha 2\beta 1$ (42, 43), SDH with urokinase plasminogen activator receptor (44), M6 and M1 with CD46 (45, 46) and hyaluronic acid capsule with CD44 (47). The direct binding of GAS to $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ had never been described thus far. Cervical cells, endometrial epithelial cells and decidual cells all express, among others, the integrin subunits $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$ (32, 48–50). The R28_{Nt} interaction with integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ could therefore account for the binding of R28_{Nt} to all these endometritis-related cells (Fig 2).

The $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ may not be the sole R28_{Nt} ligands. Indeed, we did not assay the direct interaction of R28_{Nt} with all pulled-down proteins and the other candidates may interact indirectly, *via* the pulled-down integrins, or directly with R28_{Nt}. Other cell surface proteins could also interact with R28_{Nt} and be missed in our screen since our co-immunoprecipitation experiments were carried out with HEC-1A cells; different cell types express different proteins,

including different integrins. Further analyses may thus highlight other eukaryotic R28 receptors. Similarly, ACP binds $\alpha 1\beta 1$ but may also bind other integrins (28) and among them $\alpha 3\beta 1$, $\alpha 6\beta 1$ or $\alpha 6\beta 4$.

Our data suggesting that both R28-N1 and R28-N2 shared a receptor, we tested whether they both interacted directly with the three integrins, which they did (Fig. 5), supporting our competitive assay conclusion. To further characterize the interactions between R28_{Nt} and integrins we assayed the influence of Mn^{2+} and Ca^{2+} on them. Our data suggested that at the molecular level the interactions between R28_{Nt} and the various integrins are different and that cations induced conformational changes to $\alpha 3\beta 1$ and $\alpha 6\beta 1$, but not of $\alpha 6\beta 4$, which interferes with R28_{Nt} binding. Most integrin ligands require cations for their interactions, but, although not often described, cation independent interactions of integrins with proteins from pathogens have already been reported, for example between the $\alpha V\beta 5$ integrin and the HIV Tat protein (51). Defining the R28 amino-acid residues involved in the interaction would provide clues regarding the molecular mechanism taking place. Establishing the structure of R28-N2 would shed light on the sequences potentially interacting with the various receptors and provide a framework for mutational analysis. ACP binds $\alpha 1\beta 1$ through a KTD sequence located in a β -sandwich subdomain (24, 28); however, R28 harbors no KTD or related sequence and interacts with other integrins, it will be interesting to determine whether the same β -sandwich subdomain is nevertheless involved (Fig. 1A).

The absence of significant competition between ACP and R28-N2 could be related to the strong difference of affinity between R28-N2 and ACP for HEC-1-A binding. Also, the absence of competition between R28-N1 and ACP-bound beads could be a consequence of ACP and R28-N1 presenting distinct affinity for some receptors or ACP recognizing additional receptors.

R28 promotes direct binding to the cells. However, this is not the sole binding involved in the adhesion of GAS with hDSC as indicated by the fact that the mutant strain still adheres to the cells. GAS adhesion is a two-step process, a first more labile adhesion involving lipoteichoic acid followed by a second more specific and stronger adhesion involving adhesins (52). Many adhesins have been described and the interactions with epithelial cell surface receptors thoroughly

studied (reviewed 32, 47, 48). Most interactions are indirect, proteins of the extracellular matrix, such as fibronectin, form bridges with the host cells. GAS possesses numerous fibronectin-binding proteins, including the M protein that is variable among the *emm*-types and may not play always the exact same role. Also, M1 binds directly CD46 (46) and indirectly $\alpha 5\beta 1$, *via* fibronectin. The dual interaction is required for efficient epithelial cell invasion (46). The interaction between R28 and integrins could potentiate other association(s) mediated by independent adhesins. More experimental support is needed to draw conclusions.

The involvement of R28 in the association between *emm28* and endometritis has been suggested (18) and our data support this suggestion. However, other factors may be involved in this association. They may be adhesins restricted to few genotypes, including *emm28*, such as Mrp28, M28, Enn28, FCT type 4 pilus proteins (Sfb1, Cpa) (53), Epf (39), and some RD2, a *emm28*-specific genomic region, encoded surface proteins (13). The unique conjunction of these in *emm28* strains may confer an advantage for binding to the endometrium. RD2 also encodes other proteins of unknown function as well as a regulator and they may be involved in diverse colonization or invasion steps of the endometrial or cervical tissue. The adhesion, colonization and invasion capacities of single or multiple mutant strains should be assessed.

R28 promotes adhesion to female genital tract cells including epithelial cells and it was suggested that it may contribute to GAS adhesion to different types of epithelial cells (18). We have shown that, indeed, it also promotes adhesion to endometrial epithelial cells and decidual cells, but also to pulmonary epithelial cells and to keratinocytes, natural targets for GAS invasive infections (Fig. 6). HaCaT and A549 both express $\alpha 3$, $\alpha 6$, $\beta 4$ and $\beta 1$ at their surface (54, 55) and that R28_{Nt} interacts with the different corresponding dimers probably accounts for the increase of binding of R28_{Nt} beads to these cell lines (Fig. 6). TC7 cells also express these integrins, but their localization is mainly basolateral (35); this could explain the lower binding of R28_{Nt} to the apical side of TC7 cells compared to the other cell lines. This ability of R28_{Nt} to promote adhesion to several cell lines, at least partially through integrins, suggests that expressing R28 is an advantage for *emm28* strains that is not limited to endometritis. This may represent a physiological

explanation for the fact that *emm28* genotype is a prevalent genotype, being the first to third most encountered *emm*-type in invasive infections, in most European countries (9, 10, <https://cnr-strep.fr/>). Other *emm28* specific virulence factors could also participate in the prevalence and warrant further study.

In conclusion, through direct interaction with integrins, R28 supports *emm28* strains elicited endometritis and also contributes to the prevalence of this genotype.

Experimental procedure:

Bacterial strains and growth conditions

The strains used in this study are described in Table S1. The M28PF1 strain is a clinical isolate responsible for an endometritis (French National Reference Center (CNR) for Streptococci, <https://cnr-strep.fr/>) that was selected on phenotypic and genotypic bases from a collection of 50 *emm28* independent clinical isolates (56). GAS strains were grown under static condition at 37°C in Todd Hewitt medium supplemented with 0.2% Yeast Extract (THY) or on THY agar (THYA) plates. *L. lactis* strains were grown in Todd Hewitt (TH) medium, supplemented with 10 µg/mL erythromycin when necessary, at 30 °C without agitation or on TH agar (THA) plates. *Escherichia coli* strains were cultured in Tryptic Soy medium at 37 °C with agitation with, when necessary, added antibiotics at the following concentrations: erythromycin 150 µg/ml, ticarcillin, 100 µg/ml.

Genetic constructions and generation of the AR28 mutant

The DNA fragments encoding the proteins without signal peptide of R28_{Nt}, R28-N1, R28-N2, ACP and Rib_{Nt+2R} (nt 169-1272, nt 169-688, nt 688-1272, nt 154-681 and nt 171-1188, respectively) were amplified and cloned into pET2818 as described in (57). All fragments were amplified by PCR using genomic DNA from M28PF1, except for the fragments encoding ACP and Rib_{Nt+2R}, which were amplified from GBS strains A909 and BM110, respectively; the primers used are described in Table S2. The plasmid pOri_R28_{Nt}, contains the promoter of *hvgA* deleted of the *covR* consensus boxes, the signal peptide of the GBS BM110 surface protein HvgA, and the LPXTG anchor signal of HvgA subcloned from pAT28-covSP +SPA (57), with the addition of the sequence encoding R28_{Nt} subcloned from pET2818_R28_{Nt}. The different

plasmids constructed and used during this study are described in Table S1.

The Δ R28 strain corresponds to an in-frame deletion mutant of the gene *Spy1336* encoding the R28 protein and was obtained by homologous recombination of the plasmid pG1-R28 following the same protocol as previously (57). The strain was entirely sequenced as described previously (56). No other significant mutation was found compared to the parent strain M28PF1 except for the gene deletion (56). The primers used for the generation of the plasmid pG1-R28 are described in Table S2. *L. lactis* strain was transformed by electroporation (58) with the empty vector (CCH2022) or pOri_R28_{Nt} (CCH2023).

Protein production and purification

Peptide expressions were performed with the corresponding derived pET2818 plasmids (Table S1) and purification as described in (57), except for the gel filtration step which we did not perform. Peptides purity were confirmed using a 12% SDS-PAGE acrylamide gel and Coomassie Blue staining (Fig. S1C).

Antibodies

All animal experiments described in this study were conducted in accordance with guidelines of Paris Descartes University, in compliance with the European animal welfare regulation (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.html) and were approved by the Institut Cochin University Paris Descartes animal care and use committee (n° 12–145). Mouse anti-R28_{Nt} anti-serum was produced as follows. BalbC 6 weeks mice (Janvier laboratory) were subcutaneously injected with 100 μ L of 50 μ g/mL R28_{Nt} solution in PBS+aluminium adjuvant. Two and four weeks after initial injection, mice were injected with 100 μ L of a 25 μ g/mL solution. The antibody purity and titer were determined a week after the last injection and assessed by western blot experiments. The purified R28-N1 or R28-N2 were injected into rabbits to produce polyclonal antibodies (Covalab). The following antibodies were used throughout the study: anti α 3 integrin (P1B5; DSHB), rabbit anti α 6 integrin (Novus) for ELISA, mouse anti α 6 integrin (P5G10; DSHB) for immunofluorescence, mouse anti β 1 integrin (P5D2; DSHB) and rat anti β 1 integrin (AIIB2, DSHB).

Bacterial cell wall extracts

Overnight cultures of *L. lactis* were diluted 1/100 in 50 mL of TH broth and cultivated to DO = 0.5. Bacteria were centrifuged 10 min at 5000 rpm at 4°C and the pellet was washed once in wash buffer (Cold PBS 1X + EDTA 10 mM + PMSF 1 mM). Bacteria were centrifuged and the pellet was resuspended in 250 μ L of mutanolysin buffer (Tri-HCL 20 mM pH 7.5 + Sucrose 1 M + EDTA 10 mM + PMSF 1 mM + protease inhibitor cocktail 1X (Roche) + mutanolysin 200 u/mL (Sigma)) and incubated for 90 minutes at 37°C. The protoplast suspension is then centrifuged 15 minutes at 10 000 rpm and 30 μ L of the supernatant containing the cell wall extract is loaded on an acrylamide gel.

Cell culture

HEC-1-A (ATCC® HTB-112™) and ME-180 (ATCC® HTB-33™) cells were cultured as recommended, in McCoy's 5A medium (GibCo) supplemented with 10% fetal bovine serum (FBS) at 37 °C, 5% CO₂. A549 (ATCC® CCL-185™), HaCaT (AddexBio T0020001) and Caco/TC7 cells (CVCL_0233), were cultivated as recommended, in RPMI + 10 % FBS.

Isolation and culture of human decidual stromal cells (hDSC)

Decidual stromal cells were isolated from decidua parietalis, obtained from fetal membranes of non laboring women after a normal term (> 37 weeks of gestation) singleton-pregnancy delivered by elective caesarean section. The study of the human fetal membranes was approved by the local ethics committee (Comité de Protection des Personnes Ile de France XI, n° 11018, 03/03/2011) and informed consent was obtained from all donors. Furthermore, the study abides to the Declaration of Helsinki principles. Briefly, fetal membranes were dissected from placenta under sterile conditions and decidua attached to chorion leaf was peeled off amnion and placed in PBS. After the removal of blood clots, choriodecidua was cut in small pieces and digested with 0.2% collagenase B (Roche Diagnostics, Mannheim, Germany) in DMEM-F12 (Invitrogen, Cergy-Pontoise, France) at 37°C for 1h30. After the addition of DMEM-F12 containing 5% FCS and 100 μ M EDTA, the cell suspension was filtered through a 100 μ m nylon gauze and centrifuged at 400 g for 10 min. The cell pellet, resuspended in complete medium (DMEM-F12 containing 5% FCS, 100 IU/ml penicillin (Invitrogen), 100 μ g/ml streptomycin

(Invitrogen)), were plated at a density of 10^5 cells/cm² and cultured in complete medium at 37°C in 5% CO₂ and 95% air in 75cm² flasks overnight. Medium was then renewed after several PBS washes. Cells were harvested with trypsin/EDTA when the cells were 90% confluent. The hDSCs were expanded further to passage 2 to 4 and thereafter, used in the experiments. hDSCs from at least two different women were used.

Adhesion of bacteria to cells

GAS strains M28PF1 (WT) and ΔR28 were diluted from an overnight culture, grown to the exponential phase at an OD = 0.5 and diluted in RPMI without glutamine to obtain a multiplicity of infection (MOI) of one. Confluent hDSCs in 24 well-plates were washed three times, bacterial solution was added and plates were centrifuged 5 min at 1000 rpm to synchronize bacterial adhesion. After 1 h of incubation at 4 °C, cells were washed three times with PBS and lysed with distilled water. Serial dilutions of cellular lysates were plated on THYA plates and the number of CFUs was determined after 24-48 hours growth at 37 °C. Six independent experiments were performed in triplicates and ΔR28 values were normalized to WT adhesion for each independent experiment. Statistical analysis was performed using One-Sample T-test with Graph-Pad Prism 5.0.

For adhesion assay of *Lactococcus lactis* on HEC-1-A, cells were seeded 72 h prior to infection to reach confluence at $5-10 \times 10^6$ cells/well in 24-well plates. The protocols for the *L. lactis* CCH2022 and CCH2023 bacterial preparation and adhesion to cells was the same as above. For all experiments, three independent assays in triplicate were carried out for each infection. Paired T-test were performed with Graph-Pad Prism 5.0.

ELISA-based protein-cell interaction assay

The cell-protein binding assay protocol derives from Bolduc *et al.* 2007 (28). Briefly, cells are incubated with biotinylated proteins in PBS + BSA 1% for 1 hour at 4°C, then washed and bound proteins are revealed by streptavidin-HRP (GE Healthcare) and subsequent O-phenyldiamine (Sigma) revelation. Number of moles of biotin per mole of protein was evaluated using an HABA kit (Lifetechnologies): the ratio of biotin molecule per peptide molecule was around 5, except for Rib_{N1+2R} for which it reached 9. Therefore, the fluorescence values obtained for Rib_{N1+2R} were divided by 2 for comparison sake.

Experiments were performed four times independently, except for Rib_{N1+2R} performed three times. Statistical analysis was performed after non-linear fitting using a second order polynomial quadratic model and comparison of the extra sum of squares F-test of the best fit values using GraphPad Prism 5.0. Curves are fitted with an equation: Absorbance = B1 * concentration + B2* (concentration)². Since this equation did not fit the BSA curve, statistical analysis to BSA were performed with Two-Way ANOVA with Bonferroni post-tests at maximum binding.

Beads fixation to cells

Fluosphere (Invitrogen, yellow green, 1 μm) were coupled with the different peptides following the manufacturer's recommendations. Correct coupling was confirmed by cytometric analysis with appropriate antisera. Indicated confluent cells in dark 96 well plates (Nunc, Lifetechnologies) were washed three times with cold PBS supplemented with 1 mM Ca²⁺ and 1 mM Mg²⁺ (PBS⁺⁺). Beads were diluted to a concentration of 10⁸ beads/mL in cold PBS⁺⁺ and 150 μL of beads solution was added to cells and incubated 1 h at 4 °C. Cells were washed three times in PBS⁺⁺, fixed 15 min at room temperature in paraformaldehyde 1%, quenched with 50 mM NH₄Cl. Fluorescence of the inoculum solutions, cells and cells incubated with beads were measured with a TECAN fluorescent plate reader with a GFP filter. Fluorescence of adherent beads corresponds to the fluorescence of the total well subtracted by the autofluorescence of a well containing cells only.

For competition assays, prior to beads adhesion, cells were incubated with 20 μM of specified peptide in PBS⁺⁺ for 1 h at 4 °C; beads were then added and the experiments performed as described above. For antibody competition, beads were incubated with 10 μg/mL of purified anti-R28-N1 and anti-R28-N2 rabbit antibodies for 1 h at 4°C prior to incubation with cells. Experiments were performed at least three times in duplicates. Statistical analyses were performed with Two-Way ANOVA.

Flow cytometry analysis of protein binding to eukaryotic cells after different treatments

Confluent 72 h HEC-1-A cells were dissociated with cell dissociation buffer (Lifetechnologies) and treated with NaIO₄ (Sigma), pronase (Lifetechnologies), trypsin (Worthington Biochemical Corporation),

phospholipase A₂ (Sigma) or heparinase I (NEB) at the specified concentrations and as described by Gallotta *et al.* 2014 (33). Cells were then incubated with 20 μ M soluble R28_{Nt}, R28-N1 or R28-N2 in PBS- BSA 0,5% and stained with mouse antiserum, and secondary anti-mouse PE coupled antibody. Fluorescence of cells was measured in an Accuri C6 BD cytometer. 100% staining was considered with cells untreated (Control). Unspecific labelling of cells by mouse antiserum and secondary antibodies was measured on untreated cells that were not incubated with the peptides, but underwent the same labeling protocol (without peptide condition). Experiments were performed at least three times independently. Two-Way ANOVA analysis was performed with GraphPad Prism 5.

Protein sequence comparison

These comparisons were carried out using the BlastP algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&DATABASE=n/a&QUERY=&SUBJECTS=)

In silico protein structure prediction

Peptide structure was made using the Phyre software (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>).

Co-immunoprecipitation of cross-linked R28_{Nt} receptors

HEC-1-A cells grown to confluence in T25 flask were washed three times with cold PBS++. A 6 mL solution of cold PBS++, 1% BSA, with or without (control) 20 μ M R28_{Nt} was added and incubation pursued for 1 h on ice. Cells were washed three times in cold PBS++ and 10 mL of 1 mM DTSSP (ThermoFisher) was added for 2 h on ice. After one wash with PBS++, cross-linking was blocked with 10 mL of 50 mM Tris pH 7.5 for 10 min on ice. An equimolar mix of rabbit polyclonal anti R28-N1 and R28-N2 antibodies (1 mg/mL total, 1.7 mL per flask) was added to two freshly washed HEC-1-A T25 flask for 10 min to adsorb unspecific cell binding antibodies. Pre-adsorbed antibodies (1.5 mL) were added to each flask and incubated for 1 h at 4 °C. Unbound antibodies were removed by three washes with PBS++ and cells were lysed for 1 h at 4 °C with 1.5 mL of lysis buffer (PBS, protease inhibitor cocktail 1X (ThermoFisher),

phenylmethylsulfonyl fluoride 1 mM (Sigma), 1% SurfactAmp Triton X100 (ThermoFisher)). Lysates were centrifuged at 15 000 g for 15 min at 4 °C and supernatants were incubated with 100 μ L magnetic protein A beads (Pierce) for 1 h at 20 °C. Beads were then washed six times with 1.5 mL PBS, 500 mM NaCl, Triton 1% and complexes were eluted in Laemmli Buffer in disulfide-bonds preservation conditions (60mM Tris pH6.8, 1% SDS) at 95°C for 5 minutes. Gels were run in duplicate; one gel was used for western blotting with 15 % of the eluates, revealed with mouse anti-R28_{Nt} antiserum to reveal cross-linked complexes and the other one for their identification by mass spectrometry. The western blotting indicated that R28_{Nt} complexes were found above 170 kDa.

Short migration SDS-PAGE, protein trapping and peptide extraction

Eighty-five % of the eluate were loaded in the same condition as above for a two centimeters-migration, then stained with colloidal Coomassie blue (Quick Coomassie Stain from Clinisciences). Stained gels allowed visualization of protein abundance and molecular weight distribution. Protein-containing gel lanes from the two conditions (with the bait R28 and in its absence, control condition) were excised above the 170 kDa MW marker (Lifetechnologies Pageruler prestained protein ladder). The polyacrylamide gel constitutes a matrix where successive protein treatments were performed: salt, buffer and detergent removal by successive washes of 100 mM NH₄HCO₃ (or ABC)/Acetonitrile 50%, disulfide bonds removal by cystein reduction in ABC + 10 mM DTT at 56 °C for 30 minutes; free thiols protection by alkylation in ABC + 55 mM chloroacetamide for 30 minutes at room temperature and overnight digestion with trypsin. Peptides were then extracted using washes of 5% formic acid intercalated with gel shrinkages in 50% acetonitrile (ACN). All washes were pooled and evaporated before analysis.

C18 liquid nanochromatography and mass spectrometry

Mass spectrometry analyses were performed at the 3P5 proteomics facility of the University Paris Descartes using an U3000 RSLC nano-LC-system hyphenated to an Orbitrap-fusion mass spectrometer, all from Thermo Fisher Scientific. Peptides were solubilized in 7 μ L of 0.1% TFA containing 10% ACN. They were loaded, concentrated and washed for 3 min on a

C18 reverse-phase precolumn (3 μm particle size, 100 \AA pore size, 75 μm inner diameter, 2 cm length, Thermo Fischer Scientific). Peptides were separated on a C18 reverse-phase resin (2 μm particle size, 100 \AA pore size, 75 μm inner diameter, 25 cm length from Thermofisher scientific) with a 35 minutes binary gradient starting from 99% of solvent A containing 0.1% formic acid in H_2O and ending in 40% of solvent B containing 80% acetonitrile, 0.085% formic acid in H_2O . The mass spectrometer acquired data throughout the elution process and operated in a data-dependent scheme with full MS scans acquired with the Orbitrap, followed by as many MS/MS ion trap CID spectra 5 seconds can fit (data-dependent acquisition with top speed mode: 5 seconds cycle) using the following settings: full MS automatic gain control (AGC): 2.10×10^5 , maximum ion injection time (MIIT): 60 ms, resolution: 6.10×10^4 , m/z range 350-1500 and for MS/MS; isolation width: 1.6 Th, minimum signal threshold: 5000, AGC 2.10×10^4 , MIIT: 100 ms, peptides with undefined charge state or charge state of 1 were excluded from fragmentation, dynamic exclusion time: 30 s.

Identifications (protein hits) and quantifications were performed by comparison of experimental peak lists with a database of theoretical sequences using MaxQuant version 1.6.1.0. (59). The databases used were the human sequences from the curated Uniprot database (release June 2018) and a list of in-house contaminant sequences. Carbamidomethylation of cysteines was set as constant modification while acetylation of protein N-terminus and oxidation of methionines were set as variable modifications. False discovery rate (FDR) was kept below 1% on both peptides and proteins. The “match between runs” option was not allowed. For analysis, results from MaxQuant were imported into the Perseus software version 1.6.1.1. (60). Reverse and contaminant proteins (human keratins and non-human proteins) were excluded from analysis. Proteins of interest were selected based on reported intensity in 3 over 3 replicates for each group. All hits are presented in the supplementary file.

Selection of positive hits

The enrichment is defined as the ratio of raw intensity of the co-immunoprecipitation identified proteins in the R28_{Nt} bait conditions compared to the control condition. Hits with a ratio above 1 and/or no peptide found in the control condition for all three independent experiments performed were considered as

specifically enriched in the presence of R28. From this list, only surface exposed proteins are shown in Table 1.

Immunolabelling of bound R28_{Nt} and coated fluospheres on HEC-1-A

Seventy-two hours after seeding on glass coverslips in 24 well plates, confluent HEC-1-A cells were washed three times in cold PBS++. A 10 μM solution of R28_{Nt} in PBS++, 1% BSA was added to the cells for 1 h at 4 $^\circ\text{C}$. Cells were washed three times in cold PBS++ and fixed 15 min at 20 $^\circ\text{C}$ with paraformaldehyde 1%. Cells were quenched with ammonium chloride 50 mM and blocked for 30 min with PBS-BSA 3%. Cells were incubated with primary antibodies for 1 h at 20 $^\circ\text{C}$ in PBS-BSA 1%. Cells were washed and incubated with secondary antibodies and DAPI at 1/5 000 for 1 h at 20 $^\circ\text{C}$. Coverslips were mounted on slides with Mowiol, imaged on a Leica DMI6000 and images were analyzed with ImageJ. For the observation of bound coated fluospheres, 10^8 coated beads in PBS++ were added to the cells and treated as above. Beads did not require labelling since they are intrinsically fluorescent.

ELISA

To evaluate integrin binding to immobilized R28_{Nt} or its sub-domains R28-N1 and R28-N2, ELISA were performed essentially as described in Six *et al.* 2015 (57). Proteins were coated at 5 $\mu\text{g}/\text{mL}$ overnight. Fifty μL of integrins (human recombinant extracellular domain, tested for their ability to interact with known ligands, R&D) were incubated 2 h at 37 $^\circ\text{C}$ at the specified concentrations diluted in PBS-BSA 1%. Rat anti- $\beta 1$ (AIIB2, DSHB) and rabbit anti- $\alpha 6$ (R&D) antibodies were used at a 1/200 dilution. Experiments were performed at least three times. Statistical analyses were performed as described above for the ELISA based protein-cell interaction assay.

The importance of divalent cations for the interaction between R28_{Nt} and integrins was tested by ELISA with the same coating protocol as above with fifty μL at 10 $\mu\text{g}/\text{mL}$ of integrins added to the wells. All solutions for the assay were made in PBS, PBS + 10 mM EDTA, PBS + 1 mM Mn^{2+} and PBS + 1 mM Ca^{2+} , as specified. Detection and revelation were performed as above. Values were normalized on the binding to the integrins in the presence of EDTA. One-Way ANOVA was performed to compare the four independent experiments.

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FOOTNOTES

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The abbreviations used are: GAS, Group A Streptococcus; GBS, Group B Streptococcus.

Supporting information

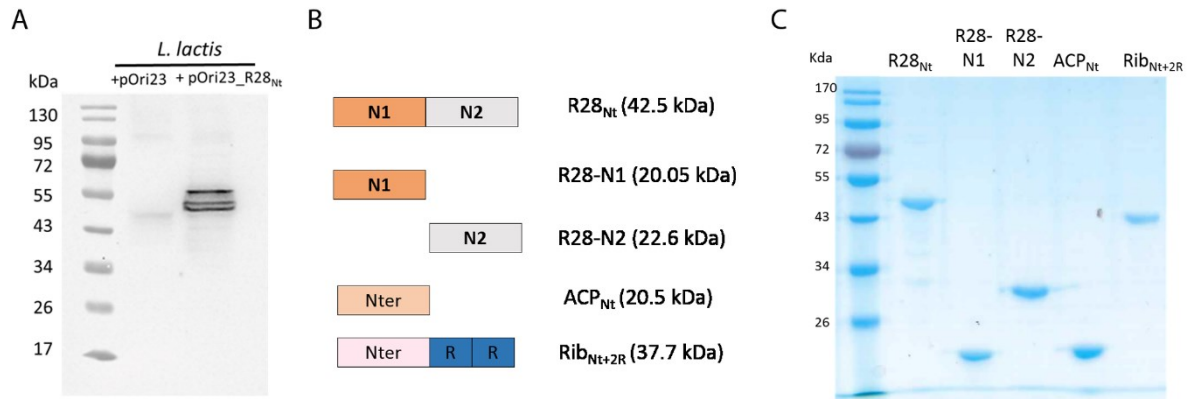


Figure S1. Schematic representation of constructed peptides used in this study, and their expression in *L. lactis* or *E. coli*. *A*, Western blot with anti-R28_{Nt} antiserum of cell wall extracts of *L. lactis* expressing R28_{Nt}, + pOri_R28_{Nt}; or not, +pOri. *B*, schematic representation of the different peptides used in the study. Color code corresponds to Fig. 1A. *C*, Coomassie blue staining of an acrylamide gel with 1 μ g of each peptide loaded.

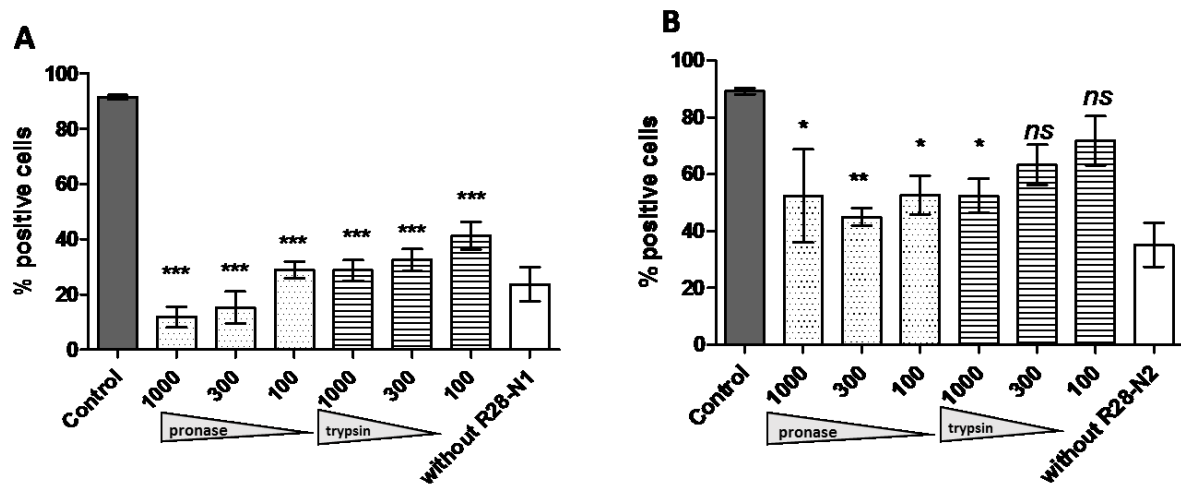


Figure S2. Effect of different treatments on the binding of *A*, R28-N1 and *B*, R28-N2 to HEC-1-A cells. Treatments were applied to HEC-1-A cells in suspension prior to incubation with purified R28-N1 and R28-N2; bound peptides were immunolabelled and cells were analyzed by flow cytometry. Statistical analysis was performed against the untreated condition. Error bars correspond to SEM of three independent experiments. Two-Way ANOVA: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; ns, not significant.

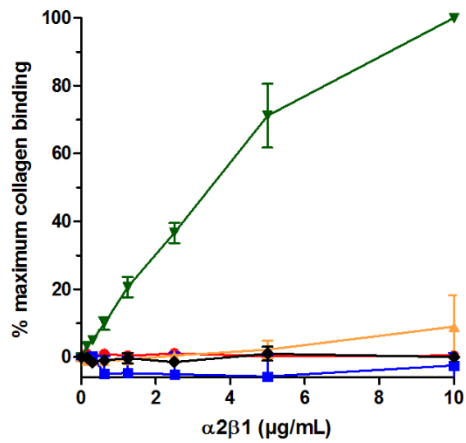


Figure S3. R28Nt, R28-N1 and R28-N2 do not bind the $\alpha 2\beta 1$ integrin. Assessment of integrin $\alpha 2\beta 1$ binding to R28_{Nt}, red; R28-N1, blue; R28-N2, orange; Type I collagen, green; BSA, black. The binding is expressed as percentage of that of the collagen at 10 $\mu\text{g/mL}$ integrin.

Table S1. Strains and plasmids used in this study

Strains or plasmid	Relevant properties	Source or reference
<u><i>Escherichia coli</i></u>		
BL21 λ DE3	F ⁻ <i>ompT gal (dcm) (lon) hsdSB (rB⁻ mB⁻) endA1 hsdR17(rK⁻mK⁺)</i>	(1)
<u><i>Streptococcus pyogenes</i></u>		
M28PF1	<i>emm28</i> source of DNA for R28 _{Nt} , R28-N1, R28-N2 amplifications	(2)
Δ R28	M28PF1 lacking the <i>Spy1336</i> coding for R28	This study
<u><i>Streptococcus agalactiae</i></u>		
A909	serotype Ia, sequence type 7 , used for amplification of the gene of ACP	(3)
BM110	serotype III, sequence type 17, used for the amplification of the gene encoding Rib	(4)
<u><i>Lactococcus lactis</i></u>		
MG1363	Lac ⁻ Prt ⁻ ; NCDO 712 derivative	(5)
CCH2022	MG1363+ pOri23	This study
CCH2023	MG1363+ pOri23_R28 _{Nt}	This study
<u>Plasmids</u>		
pG+host5	Erm; ColE1 replicon, thermosensitive derivative of pGK12; MCS pBluescript	(6)
pG1_R28	500 pb upstream (R28F1-R28R1) and 500 bp downstream (R28F2-R28R2) sequence of <i>Spy1336</i> cloned in pG+host5	This study
pET2818	Amp, oriR pBR322, T7 promoter, His-Tag coding sequence, pET28/16 derivative.	S. Mesnage (Pers. com.)
pET2818_R28 _{Nt}	pET2818 with the sequence encoding the R28 _{Nt} , without signal peptide. For the production of the peptide in <i>E. coli</i> with a His Tag	This study
pET2818_R28.N1	Ibidem with the sequence encoding R28-N1	This study
pET2818_R28.N2	Ibidem with the sequence encoding R28-N2	This study
pET2818_ACP	Ibidem with the sequence encoding ACP	This study
pOri23	Erm; <i>ermB</i> , ori ColE1, thermosensitive derivative of pIL253, P23 promoter of <i>L. lactis</i> MG1363	(7)
pOri23_R28 _{Nt}	High level of expression of R28 _{Nt} at the cell wall of Gram-positive bacteria	This study

Table S2. Primers used in this study for cloning and protein purifications

Primer Name	Sequence*
R28-F1	CGACTCTAGAGGATCCGCCTGTGAGAGACGATCATA
R28-R1	ATCATTCTTATCGGCCCGTTGTTTCGTCTGTGAAG
R28-F2	GCCGATAAGAATGATCCAGC
R28-R2	CCATGATTACGAATTCGCCTTAACTCGTATTCCTTT
R28_F	CCATGGCTACAATTCCAGGGAGTGC
R28_R	GGATCCACCTTTTGGTTCGTTGCTATC
R28_N1_F	CCATGGGGTCTACAATTCC
R28_N1_R	GGATCCTGGTAGCGATAACAATAA
R28_N2_F	CCATGGATAAAAATTAAGTATTCGCC
R28_N2_R	GGATCCTTTTGGTTCGTTG
ACP_F	CCATGGGGTCAATAGTTGCTGCATCTACAAT
ACP_R	GGATCCTACTGACAATACTAACAATTTCTC

* restriction enzyme sites used for cloning are highlighted in bold

Supporting information file

The raw data of hits found by mass-spectrometry analysis of the co-immunoprecipitations are available in the supporting information file. In green and yellow are highlighted hits that are significantly enriched in the presence of R28_{Nt} in all three independent experiments. In yellow are the surface exposed membrane proteins that correspond to Table 1 hits. In pink are highlighted the samples in which the given protein was not detected. CT-1, CT-2, CT-3 and R28-1, R28-2, R28-3, samples eluted from the control zones and R28_{Nt} co-immunoprecipitated zones of experiments 1, 2, 3, respectively

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2. GAS interaction with the decidua

“Group A Streptococcus efficiently colonizes and invades a human tissue and curtails its immune response during the early steps of infection” (Manuscript 2 and Additional results)

(Manuscript to be submitted to Nature Microbiology as a Letter)

To better understand the critical early steps of GAS endometritis, as a model of GAS invasive infections, we developed a model of *ex vivo* infection of a human tissue, the decidua. Maternal-fetal membrane surrounds the fetus during pregnancy and the maternal side, the decidua, derives from and is similar to the uterine lining in the post-partum.

These membranes obtained from healthy women with cesarean section were infected with a GFP-producing wild-type *emm28* GAS strain and the pathogenic mechanisms were analyzed and quantified by the use of customized imaging set-up and high-throughput image analysis. The different codes I wrote and used for this study are explained and available in the additional results p 98. The tissue surface is composed of fibronectin, and type IV collagen embedding decidual stromal cells and immune cells (Manuscript 2, Fig. 1). GAS efficiently colonizes the tissue and multiplies until it covers the whole surface; this multiplication is triggered by a tissue secreted element. Furthermore, element(s) secreted by decidual stromal cells are sufficient to trigger bacterial growth (Additional results, Fig. 1).

GAS forms microcolonies at the tissue surface and the bacterial layer is up to 16 μm thick (Manuscript 2, Fig. 1). We showed by scanning electron microscopy that this layer presents several extracellular polymeric substances, and thus corresponds to biofilms. These biofilms were characterized by distinct ultra-structures: thread-like structures, micrometric filaments and webs (Manuscript 2, Fig. 1 and S1). The kinetic of the formation of these structures depended on the donor and the presence of a flow accelerated the kinetic of the biofilm formation.

GAS bacteria, despite their absence of motility, are not restricted to the tissue surface during clinical infections. We therefore investigated the capacity of GAS to penetrate the decidua (Manuscript 2, Fig. 2 and S2). GAS invades actively the tissue, with twice more bacteria after 8 h than at 4 h of infection; furthermore, the invasion depends on the expression

of the cysteine protease SpeB. We showed in real-time *in situ* GAS internalization in phagocytic cells, and our results suggested that some GAS bacteria found deeper in the tissue correspond to end point of phagocytosis events. We further characterized bacteria within immune cells (Additional results, Fig. 2) and showed that GAS bacteria were not only phagocytosed by macrophage-like cells but also by neutrophil-like cells reaching the surface. We see at late time-points both phagocytic cells filled with GAS in CD45+ vesicles, with signs of immune cell death (Additional results, Fig. 2).

Then, we assessed the consequences of GAS infection on the tissue integrity (Manuscript 2, Fig. 3). GAS induced dramatic damages to cells, with abnormal nuclear morphology and cellular blebbing. We showed by TUNEL staining that more than 50 % of the cells are killed within 4 h and that all types of cell are affected. This cell-death is induced by secreted effectors, including the Streptolysin O (SLO).

Finally, we assessed the decidua immune responses to GAS infection (Manuscript 2, Fig. 4, S3 and S4). In the early time-points of infection, GAS induced the expression of 10 out of the 133 immunity-related genes tested, implying that GAS restrains the immune response at the transcription level. At the protein level, we confirmed a significant but limited accumulation of pro-inflammatory cytokines after infection with several cytokines that do not accumulate although their genes are overexpressed. SpeB and SLO mutants did not play a significant role in the expression of the pro-inflammatory cytokines genes. Yet, the SLO mutant induced lower accumulation of several tested cytokines at 4 h, and SpeB induced higher accumulation of some cytokines at 8 h. This indicates that SLO and SpeB are involved in the modulation of the immune response at the protein level. Altogether, these results demonstrate that GAS controls the inflammatory response by acting on the expression and the protein levels of inflammatory molecules.

Manuscript 2

Group A Streptococcus efficiently colonizes and invades a human tissue and limits its immune response during the early steps of infection

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Before the introduction of prophylactic measures, up to 10% childbirths were followed by the mother's death due to puerperal fever¹. The vast majority of the cases were due to invasive infections elicited by *Streptococcus pyogenes*, also known as Group A Streptococcus (GAS). GAS is a Gram-positive human-specific pathogen also responsible for other invasive infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome, causing 163,000 deaths per year in the world². Little is known regarding the establishment of GAS invasive infections. The decidua is the port of entry of post-partum endometritis, leading to puerperal fever. To identify critical features of GAS early steps of infection, including the potential manipulation of the host response, we analyzed, after infecting *ex vivo* human decidua, the establishment of the infection with state-of-the-art customized tools. Here we show that GAS benefits from tissue secreted products to multiply at the tissue surface and forms biofilms. GAS invades the tissue; the cysteine protease SpeB^{3,4} is involved in the invasion and the streptolysin O^{5,6} in the cytotoxicity leading to the death of half the cells within the first two hours. Infection induces limited tissue immune response, with overexpression of less than 1/10th of the tested immunity-related genes. We demonstrate the remarkable capacity of GAS to infect a human tissue and restrain the immune response during the onset of invasive infections. Furthermore, mutant-infected tissues display weakened phenotypes paving the way to a prophylactic treatment against these life-threatening infections.

Most GAS studies focus on the acute phase of invasive infections to understand and improve the clinical treatments of these diseases. However, it is fundamental to unravel the mechanisms involved in the establishment of these infections which include multiple steps with, among others, adhesion to host tissue, nutrients acquisition and growth, invasion of the tissue and resistance to immune responses. The decidua is the mucosal uterine-lining during pregnancy and is made up of endometrial stromal cells and of 40% of leucocytes⁷. To characterize the steps involved in the first hours of GAS puerperal fever, we infected *ex vivo* the upper portion of the decidua that is sloughed off with the placenta⁸. GAS is genetically diverse; the *emm28* GAS strains are associated with obstetrical and gynecological infections⁹⁻¹¹. We therefore used a wild-type (WT) *emm28* clinical strain isolated from a puerperal fever and its GFP and mutant derivatives¹².

Human decidual explants were infected with a GFP expressing wild-type strain (GFP-WT) derivative. GAS efficiently adheres to the tissue surface that is composed of a fibronectin layer covering cells embedded in an extracellular matrix containing type IV collagen (Fig. 1a). After 24 h of infection, GAS formed microcolonies of up to 16 μm thick (Fig. 1b) indicating the successful tissue surface colonization by GAS. We analyzed GAS multiplication at the tissue surface during the first hours of infection. In contact with the tissue, GAS multiplies until it covers the whole tissue surface (Fig. 1c,d, Supp video 1). Noteworthy, the kinetic was nearly identical on the tissues from the five subjects, out of six, on which growth occurred (Fig. 1d). We measured the growth parameters of initially scattered bacteria (Fig. 1e,f, Supp video 2). GAS grows exponentially at the tissue surface with a doubling time of 80 minutes, twice more than in THY, an optimized laboratory growth medium (Fig. 1f). We then quantified bacterial thickness increase (Fig. 1g,h). The mean thickness of the bacterial doubled in 4 hours reaching a mean value of 4.2 μm (Fig. 1h). For any given subject, the increase in thickness was locally highly heterogeneous with, after 6 h, hot-spots of more than 10 μm thick (Fig 1g). Yet, and similarly to the 2D-multiplication, the overall increase of the thickness was alike on all subjects considered (Fig. 1d,h). To analyze if diffusible host factors promote GAS growth *ex vivo*, we compared GAS growth in infection medium, RPMI, and in tissue-conditioned supernatants, which contain tissue-secreted products (Fig. 1i). GAS was able to multiply in conditioned supernatants from all subjects tested, whereas, in contrast to *Staphylococcus aureus* and Group B Streptococcus (GBS), other Gram-positive species responsible for endometritis^{13,14}, it is not able to multiply in RPMI (Fig. 1i and data not shown). This demonstrates that GAS benefits from tissue secreted products to multiply.

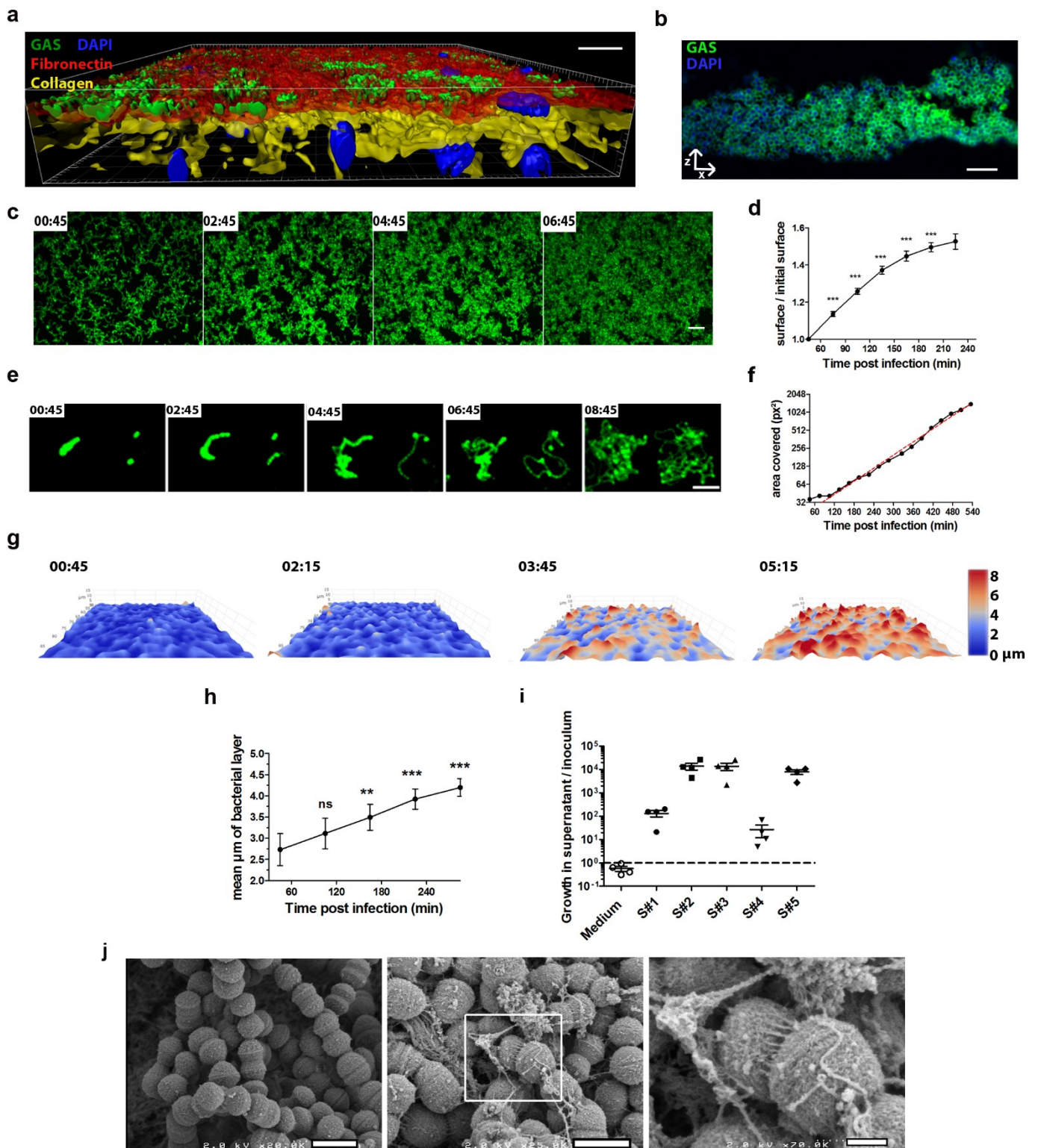


Fig. 1. Elements secreted by the tissue trigger GAS multiplication and formation of biofilm. **a**, Imaris 3D representation of a tissue infected under static conditions for 16 h. Fibronectin, red; type IV collagen, yellow; GFP-GAS, green; DAPI, blue. Scale bar: 20 μm . Magnification: 40X. **b**, Immuno-histofluorescence of a paraffin embedded tissue slice after 24 h of infection under static conditions. Anti-GAS, green; DAPI, blue. Scale bar: 10 μm . Magnification: 100X. **c** and **e**, z-max intensity projections of GFP signals from live acquisition images of GFP-WT at the tissue surface at the indicated time points, h:min. Scale bar: 10 μm (**c**) and 20 μm (**e**) Magnification: 25X. **d**, GFP signal area quantification of figure (**c**) and of the tissue of four other subjects: overtime ratio of the area covered by GFP-WT GAS compared to initial covered surface. The mean area of 3 to 11 fields for each time point for each tissue was used and the standard-error of the mean corresponds to the values of the 5 subjects. Two-Way ANOVA to the first time point. ***, $p < 0.001$. **f**, Quantification of the surface covered by GFP GAS from image (**e**). An exponential curve was fitted (red dotted line) starting at time point 105 minutes. $R^2 = 0.9948$, $T_{1/2} = 82$ min. **g**, 3D surface heat-map of the thickness of the bacterial layer at different time points, observed by live imaging h:min. x, y and z are scaled. The color code is indicated on the right of the figure, in μm . **h**, Quantification of the mean thickness of the bacterial layer, the mean of 2 to 13 fields analyzed per time point per tissue from 5 independent subject was calculated and the standard-error of the mean corresponds to the values of these 5 independent tissues. **i**, Multiplication factor after incubation of GAS for 8 h in tissue-conditioned medium or RPMI. The ratio corresponds to the CFU after 8 h of growth compared to the inoculum. **j**, Left panel. Scanning electron microscopy (SEM) of S#6 tissue 1 h after infection, with no observable extracellular polymeric substance. Middle panel, SEM of tissue infected 1 h, washed and incubated for another 7 h under flow conditions. Right, higher magnification of the middle panel image. “Thread-like” structures ~ 20 nm thick can be observed connecting the cocci and ~ 200 nm long and filaments ~ 30 nm thick and up to several μm long are observed connecting streptococcal chains. Scale bars (in white), magnification: left (1 μm , 20 000 X), middle (1 μm , 25 000 X), right (250 nm, 70 000 X).

Biofilms are critical features of GAS invasive infections, involved in resistance to the immune system, to antibiotic and persistence at a tissue surface¹⁵. We analyzed whether GAS readily formed biofilm at the early time points of infection. At 4 and 8 h after decidua infection, different ultrastructures, not found one hour after infection, are present: intra-chains “thread-like” structures and inter-chains filaments (Fig. 1j, S1). The formation was time dependent, donor dependent and was accelerated by the presence of flow (Fig. S1). This shows that GAS already forms biofilms in the very first hours of infections. GAS therefore adheres to the tissue, uses secreted tissue factors to grow and forms biofilms.

On biopsies of GAS necrotizing fasciitis, GAS is observed deep within the tissue¹⁶; yet, the mechanisms involved in GAS tissue penetration are poorly characterized. While GAS is non-motile, bacteria were readily present at 4-5 micrometers below the tissue surface after 16 h of infection (Fig 2a, S2a). We then quantified at earlier time points the tissue invasion. An average of 600 invasion events per mm^2 were counted after 4 hours of static infection (Fig 2b); this corresponds to approximately two chains invading per 10^3 surface chains. Furthermore, there

are three times more intra-tissular bacteria at 8 h than at 4 h (Fig 2c); we hypothesized that it is partly due to tissue degradation, especially fibronectin degradation. We tested whether SpeB, a GAS major protease⁴, is involved in tissue penetration. At 4 h the Δ SpeB strain invaded the tissue 2-fold less efficiently than the WT strain ($p=0.0428$) demonstrating that SpeB contributes to tissue invasion and supporting the involvement of matrix protein degradation (Fig 2c). Another potential source of bacterial invasion is internalization into host cells. We observed phagocytosis of GAS by immune cells 20 min after infections and bacteria remained inside immune cells 6 μ m under the tissue surface up to at least 4 h after infection (Fig. 2d,e, S2b, Supp video 3). Our results suggest that GAS can actively invade host tissues during the onset of invasive infections, the invasion depending on SpeB and possibly relying partly on GAS survival in host phagocytic cells.

We then analyzed the consequences of infection on the state of the cells. As soon as 4 h after infection, we observed blebbing and dying immune cells (Fig. 3a, Supp video 4). After 16 h, we observed nuclei with altered morphology and DNA damages (Fig 3b). All types of cells, immune (CD45+) and non-immune (CD45-) cells, were damaged (TUNEL+) after 16 h of infection (Fig. 3c). Already at earlier time points, infection significantly induced cytotoxicity (Fig. 3d), with 27% and 57% TUNEL positive cells at 2 h and 8 h, respectively, compared to 7% and 11% without infection (Fig. 3e). To determine if cell death was induced by a GAS secreted factor, we measured cytotoxicity when bacteria were physically isolated from the tissue (Fig. 3f). In these conditions, GAS was still able to induce 21% and 37% of cell death at 2 h and 8 h, respectively, which demonstrates that GAS secreted factors induce cell death (Fig. 3e,f). SLO^{5,6} being a pore-forming toxin described to induce cell death, we tested to which extent this protein was involved in GAS cytotoxicity on the whole tissue at early time points. At 2 and 4 h, the Δ SLO strain is twice less cytotoxic than the WT strain (25 and 33% versus 43 and 60 %, respectively) (Fig. 3g). However, even in the absence of SLO, GAS induces a significant cytotoxicity (Fig. 3g). In conclusion, GAS dramatically damages the cells within a few hours and SLO and other secreted factors are involved in the cytotoxicity.

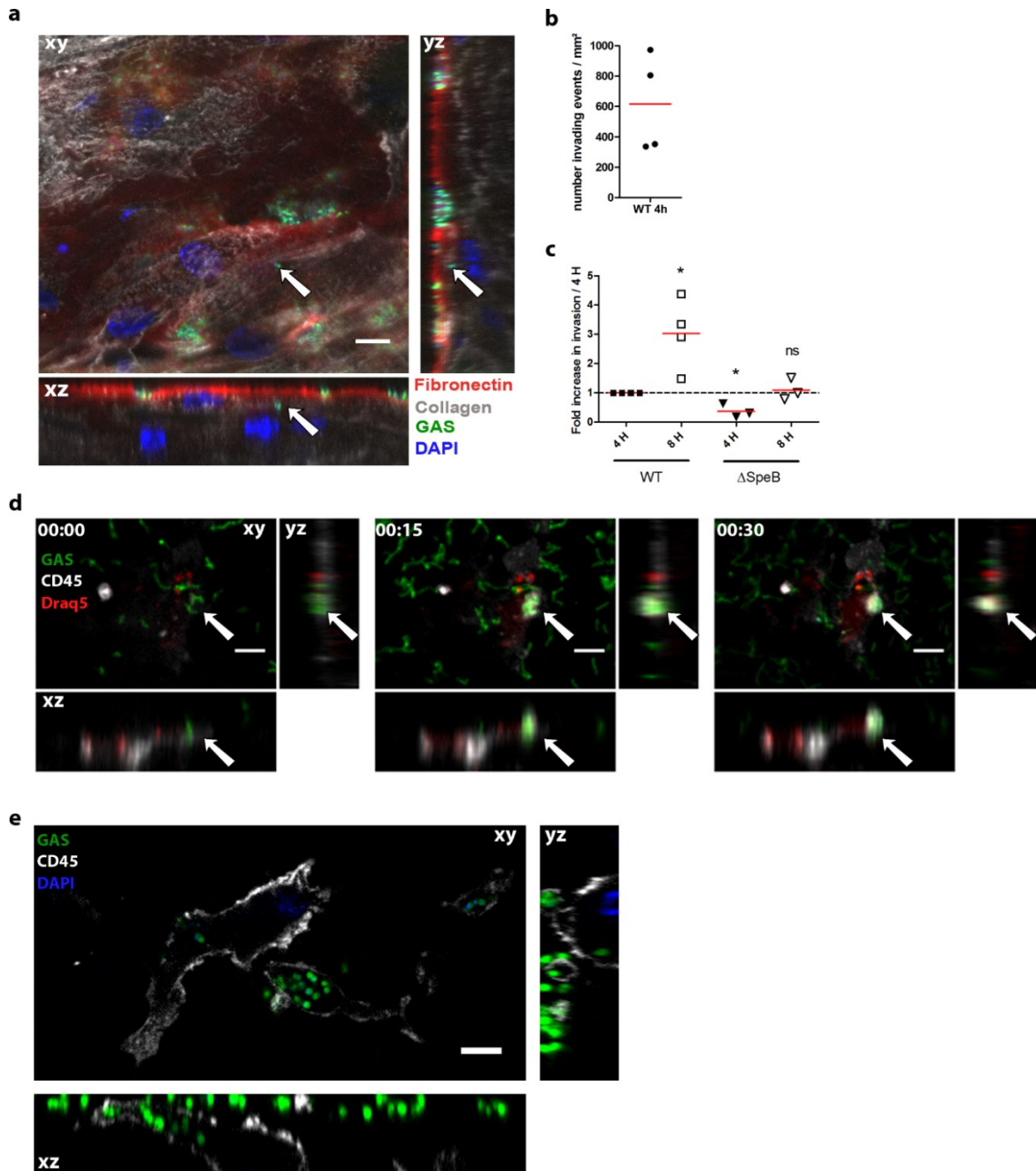


Figure 2. GAS invades the tissue using non-exclusively a protease activity and a Trojan horse mechanism. **a**, Orthogonal view of a tissue infected for 16 h. Arrows indicate an invading particle. Fibronectin, red; type IV collagen, grey; GAS, green; DAPI, blue. Scale bar: 10 μ m. Magnification: 40X. **b**, Mean number of invading events per mm^2 at 4 h with the GFP-WT strain calculated using high throughput image analysis. Four subjects are shown. **c**, Ratio of GFP-WT and GFP- Δ SpeB invading events compared to WT at 4 h with the indicated strain at the indicated time. One-sample t-test to the WT at 4 h. N=4 GFP-WT, N=3 for GFP- Δ SpeB. *, $p < 0.05$. **d**, Time lapse of an immune cell phagocytosing GAS *in situ*, with orthogonal view. Anti-CD45 (immune cell), grey; GAS, green; Draq5 (DNA), red. White arrow indicates the localization of the phagocytosis event. Scale bar: 10 μ m. Magnification: 25X. **e**, GFP-WT inside the tissue and within an immune cell. Tissue infected under flow condition for 3 h. Anti-CD45, grey; bacteria, green; DAPI, blue. This image is taken 6 μ m under the tissue surface. Scale bar: 5 μ m. Magnification: 100 X.

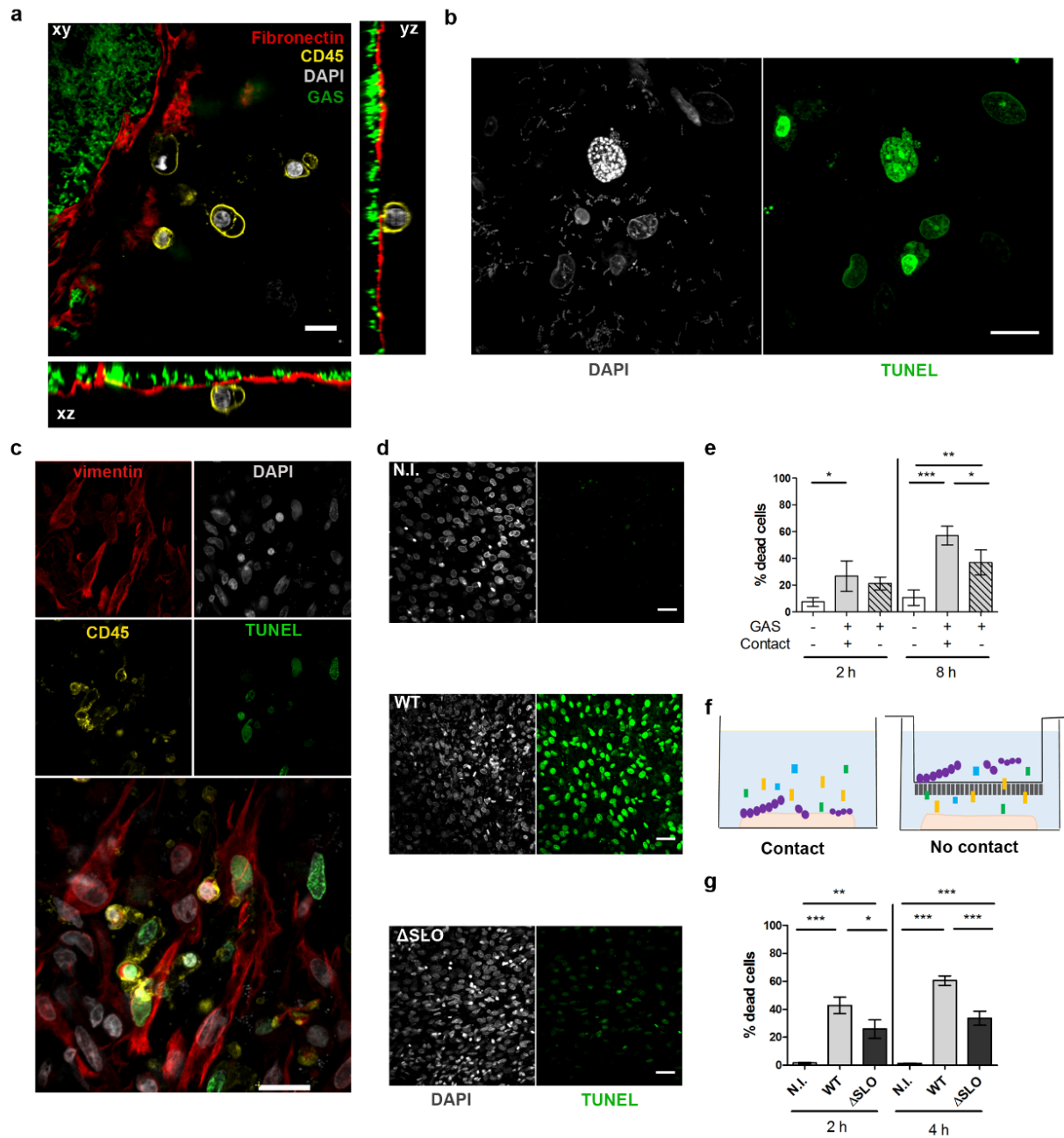
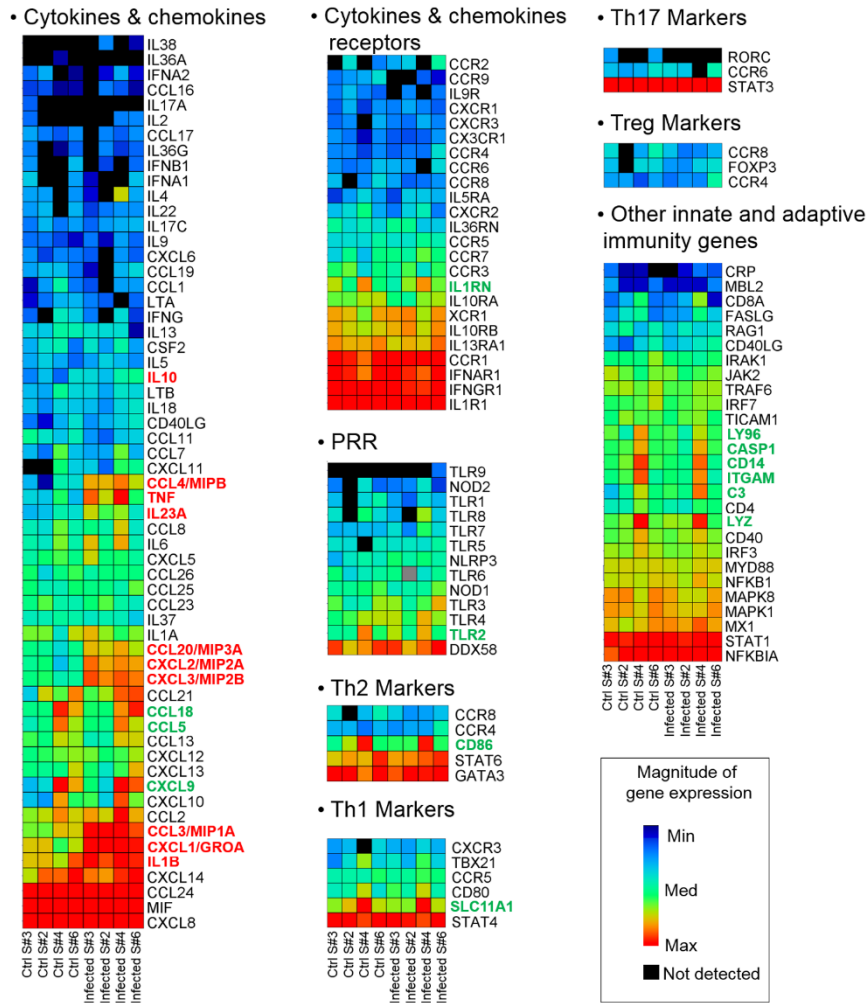


Figure 3. GAS induces stromal and immune cells death via processes involving SLO and other secreted factors. **a**, Immune cells are affected by infection. Orthogonal view of a tissue prestained with anti-fibronectin (red) and anti-CD45 antibodies (yellow) and infected under static conditions 5 h with WT-GFP bacteria (green), and stained with DAPI (grey). CD45 positive cells present blebs and altered nuclear morphology. Scale bar: 10 μ m, Magnification 40X. **b**, Immunofluorescence approach highlighting the state of the nuclei in a tissue after 16 h of static infection DNA, grey; TUNEL, green. Nuclei present altered morphology and DNA damages, as shown by the TUNEL staining. Scale bar: 10 μ m. Magnification: 63X. **c**, All cells can be damaged. Immunofluorescence of tissue after 16 h of static infection. Decidual cells (vimentin⁺, CD45⁻) and immune cells (CD45⁺) present damaged nuclei (TUNEL⁺). Vimentin, red; DAPI, grey; CD45, yellow; TUNEL, green. Scale bar: 10 μ m. Magnification: 40X. **d**, Immunofluorescence of tissue infected 4 h with the indicated strains. N.I., non-infected. DNA, grey (left panel), TUNEL, green (right panel). Scale bar: 40 μ m. Magnification: 20X. **e,g** Percentage of dead cells. **e**, quantification of cytotoxicity, with 3 subjects; contact, + or -, in the absence or the presence of a Transwell® insert, respectively. **f**, Schematic representation of the experimental set-up with a Transwell® used in figure (e). Bacteria, mauve; tissue, beige; rectangles, secreted factors. **g**, Quantification of cytotoxicity, 4 subjects. Statistical analysis (**e** and **g**): Two-Way ANOVA with a Bonferroni post-test, *, p<0.05; **, p<0.005; ***, p<0.001

When growth occurred at the tissue surface, it was with similar kinetics (Fig. 1f,h); however, one tissue did not elicit bacterial growth at all (S#8, data not shown). Also, *in vitro* the conditioned media promoted either a 10,000-fold (S#2, S#3 and S#5) or a 10- to 100-fold CFU increase (S#1 and S#4) (Fig. 1i) suggesting that subject/tissue-peculiarities influence GAS capacity to establish infection. This correlates with the fact that host genetic factors favor GAS invasive infections¹⁷ and suggests that the immunological state of the various tissues differs. We therefore analyzed the expression of 133 unique immune genes related to “Inflammatory cytokines and receptors” and “Human innate & adaptive response” pathways in four tissues. In the absence of infection, there is low to moderate expression of the majority of the genes tested (Fig. 4a). The 10% most highly expressed genes are related to “Macrophage” and “cytokine-mediated signaling pathway”, reflecting the innate immune response capacity of the tissue. Interestingly, the basal expression levels of these genes are subject dependent (Fig. 4a, S3a) and those of S#4 tissue that supports low bacterial growth (Fig. 1i) differs from the others, its basal expression level of ten genes, including CD14, ITGAM, and SLC11A1, signing “macrophages” being markedly higher than those of the three other subjects (Fig. 4a,S3a). This suggests that initial presence of a high proportion of macrophage/phagocytic cells in the tissue impairs GAS capacity to establish infection.

a



b

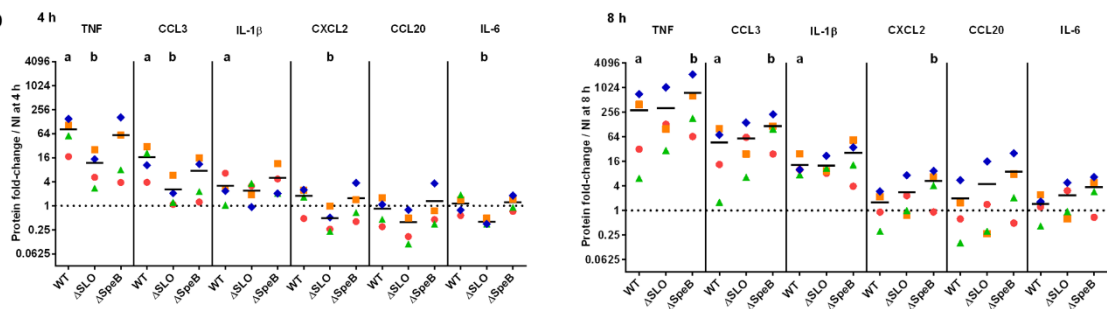


Fig. 4. Host responses to GAS infection a, Heat-map of the results from the qRT-PCR screening using RT²qPCR “Inflammatory cytokines and receptors” and “Human innate & adaptive response” arrays on 4 subjects, control (Ctrl) or infected 8 h with the WT strain (Infected). Genes are regrouped according to their ontogeny, and classified top to bottom depending on the intensity of expression, the 10% most highly expressed genes are related to the ontology pathways “Macrophage” (ARCHS4 database¹, p values: 0.001) and “cytokine-mediated signaling pathway” (Gene Ontology Biology process GO:0019221, p value: $3.48 \cdot 10^{-16}$). Genes that are significantly overexpressed after infection are in red and are related to “acute inflammatory response” and “macrophages” (GO:0002526, p value: $1.41 \cdot 10^{-17}$; ARCHS4, p value: $4.26 \cdot 10^{-10}$, respectively). Genes that are expressed differently at the basal level on subject S#4 are indicated in green and are related to a signature of macrophage (ARCHS4 database¹, p value: $4.26 \cdot 10^{-10}$). Statistical analysis: Student’s two tailed t -distributed for paired samples; *, $p < 0.05$. **b**, Fold-change increase in the concentration of the indicated cytokines compared to the non-infected condition (NI) at; left 4 h and right, 8 h post-infection on tissues from 4 different subjects. Mean is indicated with a black line. S#7, red circle; S#8, orange square; S#9, green. Statistical analysis: Friedman and post hoc pairwise comparison tests, a, $p < 0.05$ as compared to NI; b, $p < 0.05$ as compared to WT

We next investigated the host immune response during the early steps of GAS tissue infection by testing the change in expression of these 133 genes after 8 h of infection. Ten genes among the 133 (7.5%); they all encode cytokines and chemokines related to the ontology pathways, “acute inflammatory response” and “macrophages”, showed a significantly overexpression (Fig. 4a, Table S1). To assess the breadth of this immune response, we compared our results to the already published response of human decidua to a GBS infection¹⁸. After 8 h of infection, GBS induced the expression of 32 genes that include 9 of the 10 genes overexpressed after a GAS infection (Table S1). The principal molecules activated specifically during the GBS infection are signaling molecules related to “cellular response to cytokine stimulus”. This demonstrates that GAS impairs the tissue robust immune response normally elicited by a Streptococcus pathogen also involved in endometritis. Strikingly, the only gene specifically overexpressed by a GAS infection is IL10, an anti-inflammatory cytokine. Altogether, these results suggest that GAS specifically induces a low and limited inflammation during the initiation of infection.

To further analyze the host immune response and because GAS is able *in vitro* to modulate cytokine expression and accumulation¹⁹⁻²¹, we quantified at 4 and 8 h the expression of seven of these ten genes and that of IL6, the classical pro-inflammatory cytokine, in five tissues and also the protein concentration of six cytokines in the explant supernatants of four subjects (Fig. S3b-c, 4b, S4, Table S2, S3). Overexpression of three genes, TNF, CCL20 and CCL3, occurs as soon as 4 h after infection; yet fewer genes are upregulated than at 8 h where the expression of CXCL2, CXCL1, and CCL4 is also increased (Fig. S3b). After GAS infection, the classical

pro-inflammatory cytokines IL6 and IL1b are not overexpressed in contrast to the observed overexpression with other pathogens including GBS at 8 h (Table S1)²². The basal level of accumulation of the six cytokines differ between subjects (Fig. S4). GAS infection increases significantly the accumulation of TNF, CCL3 and IL-1 β as soon as at 4 h, and with a higher fold at 8 h (Fig. 4c). Interestingly, the overexpression of the CXCL2 gene at 4 h and of the CXCL2 and CCL20 genes at 8 h (Fig. S3) are not paralleled by an increase in the concentrations of these two proteins, suggesting that GAS also controls the immune response at the protein level (Fig. 4c).

GAS limiting the immune response at the transcriptional and post-transcriptional levels, we analyzed the potential roles of SLO and SpeB on that control. The Δ SLO strain yielded at 4 h a similar activation of the gene expressions than the WT strain (Fig. S3c). In contrast, it generated a lower accumulation of all cytokines except IL-1 β and CCL20, indicating that the presence of SLO increases their secretion at 4 h but not at 8 h (Fig. 4c). In contrast, at 4 h the SpeB mutant has no effect on the accumulation of cytokines. Yet, at 8 h, it induced a higher accumulation of TNF, CCL3, and CXCL2 than the wild-type strain, indicating that SpeB tampers with cytokine accumulation. Overall, GAS controls the immune response at the RNA and protein levels as soon as 4 h post-infection and SLO and SpeB are involved in the control of the immune response at the protein level.

GAS was, until the 20th century, the major cause of infectious maternal death¹ while not being a colonizer of the vaginal flora²³, suggesting a particular propensity for GAS to elicit puerperal fever. A comprehension of the crucial initial steps of GAS invasive infections and in particular puerperal fever is lacking. GAS possesses a large collection of virulence factors mainly described for their implication in later stages of invasive infections, but their role in the initiation of these infections is discussed²⁴. Furthermore, GAS human species specificity hinders the *in vivo* analysis of GAS infections. We therefore used the conjunction of *ex vivo* infection of a human decidua, bacterial genetic manipulation, state-of-the-art image and immune response analyses to dissect at the molecular level GAS capacity to establish puerperal fever. We demonstrate the remarkable capacity of GAS to colonize, invade and damage the decidua within the first hours of infections. Also, GAS simultaneously induces pro- and anti-inflammatory responses during the onset of infection. Moreover, an overall limited inflammation, including the production of an anti-inflammatory cytokine, is elicited; this tampering of the inflammation is supported by the comparison with GBS elicited response. This is in accordance with previous conclusions that the genuine role of several GAS effectors is to impair the immune system, favoring GAS colonization²⁵. This constrained inflammation

may favor GAS infection initiation by delaying or decreasing the recruitment of immune cells. Altogether, our data account for the GAS – endometrium puerperal fever association.

We further show the involvement of two major virulence factors, SLO and SpeB, in the early steps of infection, both for the bacterial invasion process *per se* and the host response. The weaker efficiency of the mutant strains, for the tested phenotypes, together with the potential critical influence of the host basal immune stage on the GAS capacity to establish infection, point to the necessary equilibrium between pathogen and host factors for GAS infection to be successful.

Our holistic approach supported by the toolbox we developed can be applied to the study of the initial steps of GAS and other pathogens human infections on many tissues, and as such be a template for future *ex vivo* infections. GAS capacity to divert the host response during the early stages of decidua infection and to establish very early in the infection niche to resist antibiotics, such as intracellular bacteria and biofilms, confirms the necessity for prophylaxis. GAS remarkable efficiency to initiate invasive infections support the necessity for vaccination and the use of models such as ours to assess GAS potential targets.

Methods

Strains and culture conditions. The strains used in this study are described in Table S4. The M28PF1 strain is a clinical isolate responsible for an endometritis (French National Reference Center (CNR) for Streptococci, <https://cnr-strep.fr/>) that was selected on phenotypic and genotypic bases from a collection of 50 *emm28* independent clinical isolates¹². GAS strains were grown under static condition at 37°C in Todd Hewitt broth supplemented with 0.2% Yeast Extract (THY) or on THY agar (THYA) plates. For GFP strains, the medium was supplemented with 10 µg/mL erythromycin.

Genetic constructions and generation of GFP-producing and SLO and SpeB deleted mutant strains. The strains harboring an integrated inducible *gfp* gene were constructed as follows. We sequentially cloned into the pG1 vector²⁶, the Perm - *gfp* transcriptional fusion surrounded by the KpnI and HindIII sites from pATΩgfp²⁷ giving rise to pG1-Perm-gfp. The *lacA-lacR* intergenic sequence, enabling a single cross-over in M28PF1 and derivatives, was amplified using the primers F_lacA and R_lacA and cloned by In-fusion© into pG1-Perm-gfp digested with EcoRI, giving rise to pG1-lacA-Perm-gfp. The *erm* promoter was replaced by the tetO tetR Pxyl promoter region by amplifying it from pTCV_TetO²⁸ using the primers F_tetO and R_tetO and cloning it into pG1-lacA-Perm-gfp previously digested with EcoRI-EcoRV, giving rise to pG1-lacA-PTetO-gfp. The plasmid was checked by sequencing and transferred into M28PF1 or derivatives by electroporation as previously described²⁹. The correct localization of the construct was confirmed by sequencing the region using a primer outside the construct (R_extlacA) and one hybridizing to the vector, RP48. The uninduced GFP-producing strain, M28-GFP, grew like M28PF1 in laboratory THY. The construct was stable even in the absence of erythromycin for at least eight generations. The minimum anhydrotetracycline concentration required for full expression of the GFP is 20 ng/mL, the fluorescence is stable for at least 6 h when the bacteria are in the stationary growth phase. GFP fluorescence is observable when GAS is intra-cellular.

The ΔSLO and ΔSpeB strains correspond to in-frame deletion mutants of the genes *slo* and *speB* genes and were obtained by homologous recombination of the plasmid pG1-SLO and pG1-SpeB following the same protocol as previously³⁰. The regions surrounding the construct were sequenced as described previously. The primers used for the generation of the plasmids pG1-SLO and pG1-SpeB are described in Table S5.

Antibodies. The following antibodies were used during this study: AlexaFluor 594 mouse anti-CD45 (Biolegend, clone M5E2), rabbit anti-Fibronectin (Sigma, #F3648), rabbit anti-vimentin (Abcam, ab92547), mouse anti-Type IV collagen (DSHB, M3F7), rabbit anti whole GAS, gift from I. Julkunen.

Human tissue collection. Human placenta with attached maternal-fetal membranes were collected from healthy women with an uncomplicated singleton pregnancy, undergoing a planned cesarean delivery prior the onset of active labor at term (between 38 and 40 weeks of pregnancy). The study of the human maternal-fetal membranes was approved by the local ethics committee (Comité de Protection des Personnes Ile de France III, n°Am5724-1-COL2991, 05/02/2013). All participants provided written informed consent prior to inclusion in the study at the Department of Obstetrics, Port Royal Maternity, Cochin University Hospital, Paris, France. Except for per-operatively administered antibiotics, women were excluded if prescription antibiotics were used during the two weeks preceding delivery. Table S6 indicates in which experiments each sample tissue was used.

Materno-fetal membrane processing. Within 15 min of collection, biological samples were processed in the laboratory. Maternal-fetal membranes were detached from placenta under sterile conditions, rinsed extensively in phosphate-buffered saline (PBS) and carefully examined. Pieces of membranes in RPMI, at distance of placenta and of the remodeling zone overlying the cervix⁷, free of surface blood clots, were cut and glued to Petri dishes with a veterinary glue (Vetbond glue 3M, St Paul, MN), the fetal side sticking to the plastic.

Infection procedure. For all experiments, GAS strains were prepared as follows. GAS strains were diluted from an overnight culture, grown to the exponential phase at an OD = 0.5 in THY and diluted in RPMI. For GFP strains, exponential phase bacteria were diluted to an OD = 0.1 in THY supplemented with 10 µg/mL erythromycin and 20 ng/mL anhydrotetracyclin (Sigma), grown for 1 h 30 at 37°C and diluted in the specified medium.

Static: 1.5 cm² or 8 cm² of maternal-fetal membranes were glued (Vetbond 3M) by the fetal side in a 35mm Petri dish or on a glass coverslip respectively. When specified, tissues were pre-stained with 500 µL RPMI with 5 µL of anti-CD45 Alexafluor-594 coupled (Biolegend) for 30 minutes at 37°C, 5% CO₂. Tissues were infected with 0.2 mL/cm² of a solution of 1.7 10⁸ bacteria/mL in RPMI, supplemented by 10 µg/mL erythromycin and 50 ng/mL anhydrotetracycline when GFP strains were used. After infection, tissues were washed and fixed in formalin for 24 h, and then stored in 70% ethanol at 4°C.

For cytotoxicity assay, 500 μL of RPMI were added to 1.5 cm^2 tissue glued to a coverslip in a 24-well plate. 100 μL of M28PF1 at 5.10^8 bacteria/mL was added directly or in the upper chamber of a 6.5 mm Transwell® insert (polycarbonate, 0.4 μm membrane, Costar). For the non-infected condition, 100 μL of RPMI was added directly. After the specified time of infection, tissues were washed with PBS and fixed in formalin for 24 h, and then stored in 70% ethanol at 4°C.

Flow infection condition: 8 cm^2 maternal-fetal explant were glued by the fetal side to 35 mm Petri dish and stained with Draq5 (1/2000, Biolegend) and anti-CD45 Alexafluor-594 coupled (Biolegend) in infection medium (RPMI without phenol, 10 $\mu\text{g}/\text{mL}$ erythromycin, 50 ng/mL anhydrotetracycline (Sigma)) for 30 min at 37°C, 5% CO_2 . Tissue was extensively washed with infection medium and 3 mL of GFP-producing bacteria (1.10^8 CFU/mL) in infection medium were added to the tissues in a 37°C microscopy chamber for 45 minutes until 20-30 % of tissue surface was covered by bacteria. Supernatant was then discarded, tissue was washed with infection medium to remove unattached bacteria, a peristaltic pump with circulating infection medium bubbled with 95% O_2 and 5% CO_2 was activated in an open circuit at 0.3 mL/min.

Immunofluorescence on fixed tissue. Fixed tissues were cut into 0.5 cm^2 pieces and glued to coverslips, permeabilized with 0.2% triton X100 (Sigma) for 30 minutes at 20°C. 500 μL of blocking solution (PBS+BSA 3%) was added for one hour at 20°C, and tissue were stained for 4 h at 20°C with 500 μL of blocking solution containing primary antibodies: 2 μL anti-fibronectin, 10 μL anti-type IV collagen, 2 μL of rabbit anti-Vimentin. Tissues were washed with PBS and secondary antibodies with DAPI (1/1000) in blocking solution were added for 1 hour at 20°C.

TUNEL staining protocol is based on a modified manufacturer's protocol (DeadEnd™ Fluorometric TUNEL System, Promega). Tissues fixed and glued to coverslips were permeabilized with 500 μL PBS with 0.2 % Triton X100 for 30 min at 20°C. 100 μL of equilibration buffer was added on the tissue in a wet chamber for 15 min, liquid was removed and 50 μL of labelling solution was added for 3-4 h at 37°C. Tissues were washed twice with saline-sodium citrate (SSC) 2X buffer (from the manufacturer's kit) and stained with DAPI (1/2000) for 15 min. For double vimentin and TUNEL staining, TUNEL staining was performed first.

Image acquisition of fluorescently labeled tissues. Live microscopy: infected tissue images were acquired with an upright confocal Leica Yokogawa CSU-X1 Spinning Disk (Yokogawa,

Tokyo, Japan) coupled with a DMI6000FS Leica microscope (Leica microsystems Gmbh, Wetzlar, Germany), with a 25X objective (NA 0.95, Olympus). Acquisitions were made with MetaMorph 7 software and an image was taken every 30 min for 3 h with a z-step of 2 μm . For long-term acquisition, axial drift was manually compensated after 3 h and acquisition was continued for another 3 h.

For fixed tissue, the maternal side of stained tissue was placed facing a 35 mm high glass bottom μ -dish (IBIDI) and directly imaged using a Yokogawa CSU-X1 Spinning Disk (Yokogawa, Tokyo, Japan) coupled with a DMI6000B Leica microscope (Leica microsystems Gmbh, Wetzlar, Germany). Acquisitions were made with MetaMorph 7 software.

For cytotoxicity quantification, images were acquired with a 20X objective (NA=0.7), 1 μm z-step and approximately 70 slices per stack were acquired. For quantification of bacterial invasion, images were acquired with a 40X objective (NA=1.25), 0.3 μm z-step and approximately 90 slices per stack were acquired.

Image treatments and data analysis. All images were processed with ImageJ. 3D drifts of live images were corrected with homemade routines. For surface area measurements, bacteria were segmented based on a threshold on the maximum intensity z projection images of the GFP signal at the different time points, with the same threshold for each time point. For local thickness measurement of the GFP signals corresponding to bacteria, whole field was subdivided in region of interest and a home-made ImageJ macro measured the full width at half maximum (FWHM) of the z-axis GFP signal. For thickness and surface measurements of bacteria, 3 to 12 fields per tissue were analyzed. For cytotoxicity measurements, TUNEL and DAPI positive nuclei of images were segmented and automatically counted. At least 5 fields per condition were analyzed. To measure bacterial invasion, another ImageJ macro was used to segment bacterial particles on z-max projection image of GFP signal. For each bacterial particle, the axial localization was determined and compared to fibronectin and type IV collagen localization. Any bacterial particle whose signal was below 1.2 μm to fibronectin or collagen signals was considered as an invasion event. 5 to 8 fields (153 x 169 x 30 μm) per conditions were analyzed. A GAS chain was considered to be in mean 4 cocci, corresponding to the mean size of invading particles. Taking into account surface and thickness of bacteria (mean thickness, 4 μm , bacterial coverage of a field: 90 %), we estimated the amount of invading chains.

Biofilm thickness 3D surface heat map was generated by a custom R code based on the plot-ly library.

A 3D image representation of the tissue was performed using Imaris 7.4 (Bitplane AG).

Immunohistofluorescence (IHC). After static infection, tissues were fixed in formol and embedded in paraffin; IHC was performed as described previously⁸. 10 µm-thick slices were cut and stained with home-made whole GAS rabbit antiserum and with DAPI.

Scanning electron microscopy. Tissues were infected 1 h as described above for static infections, washed once with RPMI, fresh RPMI was added for 3 h (1+3 h condition) or 7 h (1+7 h condition) and then the samples were fixed. Samples were immersed in a fixative solution (2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4) and stored 1 h at 20°C and overnight at 4°C. The fixative was removed, and samples were rinsed three times for 10 min in a sodium cacodylate solution (0.1M, pH 7.4). The samples underwent progressive dehydration by soaking in a graded series of ethanol (50 to 100%) before critical-point drying under CO₂. Samples were mounted on aluminum stubs (10 mm diameter) with carbon adhesive discs (Agar Scientific, Oxford Instruments SAS, GOMETZ-LA-VILLE, France) and sputter coated with gold-palladium (Polaron SC7640, Milexia, Verrières-le-buisson, France) for 200 s at 10 mA. Samples were visualized by field emission gun scanning electron microscopy. They were viewed as secondary electron images (2 kV) with a Hitachi S4500 instrument (Milexia, Verrières-le-buisson, France). Scanning Electron Microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRA, Jouy-en-Josas, France).

RNA extraction. After static infections, tissues were washed once and stored in TriReagents (Sigma) at -80°C until used. Total RNA was isolated using a Qiagen RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity and concentration of total RNA were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), by measuring absorbance at 260 nm.

RT-qPCR analysis. RNA was treated with deoxyribonuclease (Invitrogen, Life Technologies, St Aubin, France) to remove any contaminating DNA. Four µg of total RNA were reverse transcribed using random primers and M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative PCR was carried out on a Light Cycler 480, 96 well apparatus (Roche Diagnostics, Mannheim, Germany), with 160 ng of cDNA as a template, using the amplification kit SensiFAST SYBR No-Rox kit (Bioline, London, UK), according to the manufacturer's instructions. The RT² Profiler "Inflammatory cytokines and receptors" and

“Human innate and adaptive response” arrays PCR Arrays (Qiagen) were performed according to the manufacturer's instructions. Gene expression was normalized using a panel of 5 housekeeping genes: ACTB, B2M, GAPDH, HPRT1, RPLP0. Δ Ct ranging from 0 and below to 6.0 were considered as maximum magnitude of gene expression, from 6.1 to 9.9 as moderate and from 10 to 15 as low. Primers for additional RT-PCR analysis were chosen using PRIMER3 software, based on published sequences (available upon request). Primers were obtained from Eurogentec (Angers, France) and used at 10 nM in the PCR reaction. In this series of experiment, the set of internal controls included the geometric mean of three different reference genes: SDHA, PPIA, and GAPDH.

Genes with a nominal p value ≤ 0.05 were considered to be differentially expressed. Genes showing > 2 -fold variation were further considered in the analysis. Heatmaps were created using the MeV Package. Enrichr was used for pathways enrichment analysis, restricted to Gene Ontology (GO) terms and ARCHS4 databases.

GBS infection of maternal-fetal membranes microarray subanalysis. Data (Control 8 h: GSM2535518, GSM2535524, GSM2535529, GSM2535535 and GBS infection 8 h: GSM2535517, GSM2535526, GSM2535530, GSM2535536) from GSE96557 were extracted using GEO2R. After conversion of the Gene annotation using DAVID gene Id Conversion tool, fold-changes and p values for the 133 immune-related genes we previously analyzed with the RT²Profiler Arrays were retrieved from these 8 samples.

Protein analysis. After static infections, supernatants of explants were stored at -80°C until use. The levels of IL6, TNF, CCL20, CXCL2, CCL3, and IL-1 β were measured in a multiplex assay (Bio-Plex BIORAD). The concentrations are reported as pg/mL medium. The samples were quantified by duplicate according to the manufacturer's instruction.

In vitro bacterial growth measurement. RPMI was added to materno-fetal tissues for 8 h at 37°C , 5% CO_2 and the supernatants (conditioned medium) were filtered and diluted 5 times with RPMI. A thousand bacteria/mL was added to these diluted tissue supernatants or to RPMI alone. After 8 h of incubation at 37°C , 5% CO_2 , solutions were serially diluted and plated. After 24 h at 37°C , CFU were counted.

Computer code availability. Computer codes used for this work will be transferred upon request.

Statistical analyses. Data were analyzed by Prism 6 software (GraphPad Software, Inc. San Diego, CA) or Xlstat version 2018.1 (Addinsoft, Paris, France). When indicated, we used Two-

Way ANOVA. We used Student's t-test or non-parametric tests for quantitative variables as indicated in the text and Pearson's chi-square for qualitative variables, as appropriate. A p -value <0.05 was considered to be significant.

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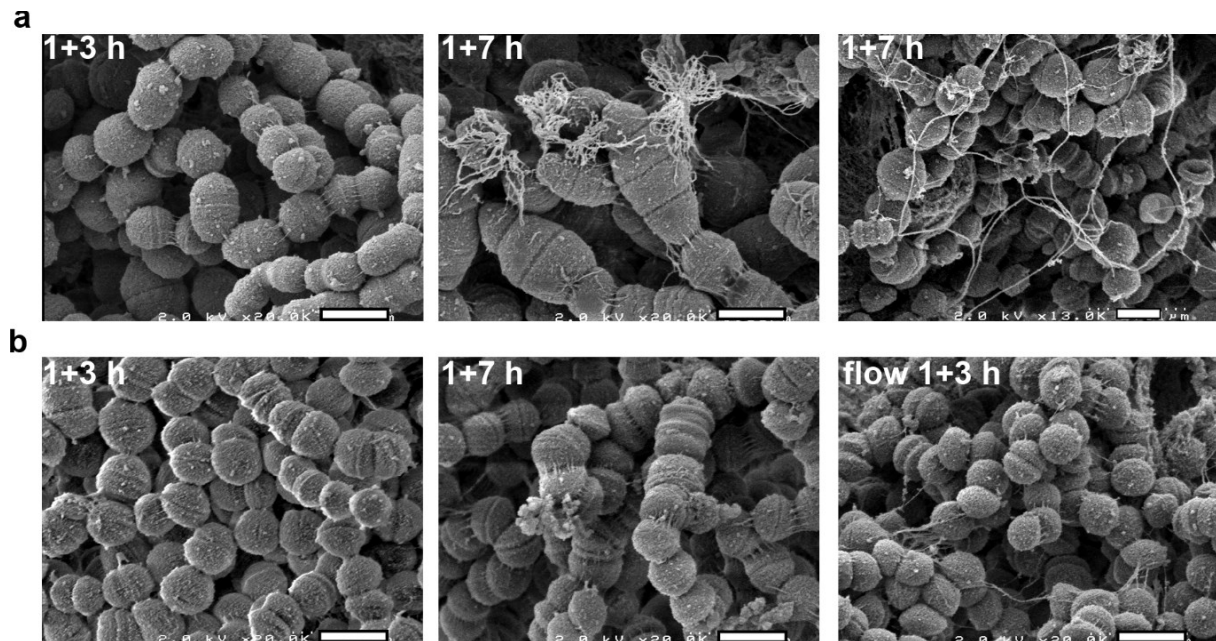
Supplementary figures

Figure S1. Kinetic of GAS biofilm formation at the surface of the tissue analyzed by scanning electron microscopy (SEM) with two subjects and different experimental conditions. After 1 h of infection, the tissues were washed and the experiment continued during indicated times **a**, Subject S#10. Morphological details of biofilm obtained under static conditions: left, after 3 h, thread-like structures, ~20 nm thick and ~200 nm long, but no filaments; middle: after 7 h, thread-like structures and micrometric webs; right: after 7 h, abundant filaments, ~30 nm thick and up to several μm . **b**, Subject S#5, displaying a different kinetic of biofilm formation. Under static condition: left, after 3 h, no thread-like structures nor filaments in contrast to S#10 (panel a); middle, after 7 h, thread-like structures, but no micrometric in contrast to S#10 in (panel a). Under flow condition, after 3 h, (right), thread-like structures and filaments absent in static conditions (S1b), showing that flow accelerates biofilm formation. Scale bar: 1 μm . Magnification: 20 000 X, except for figure (a) right panel, 13 000 X.

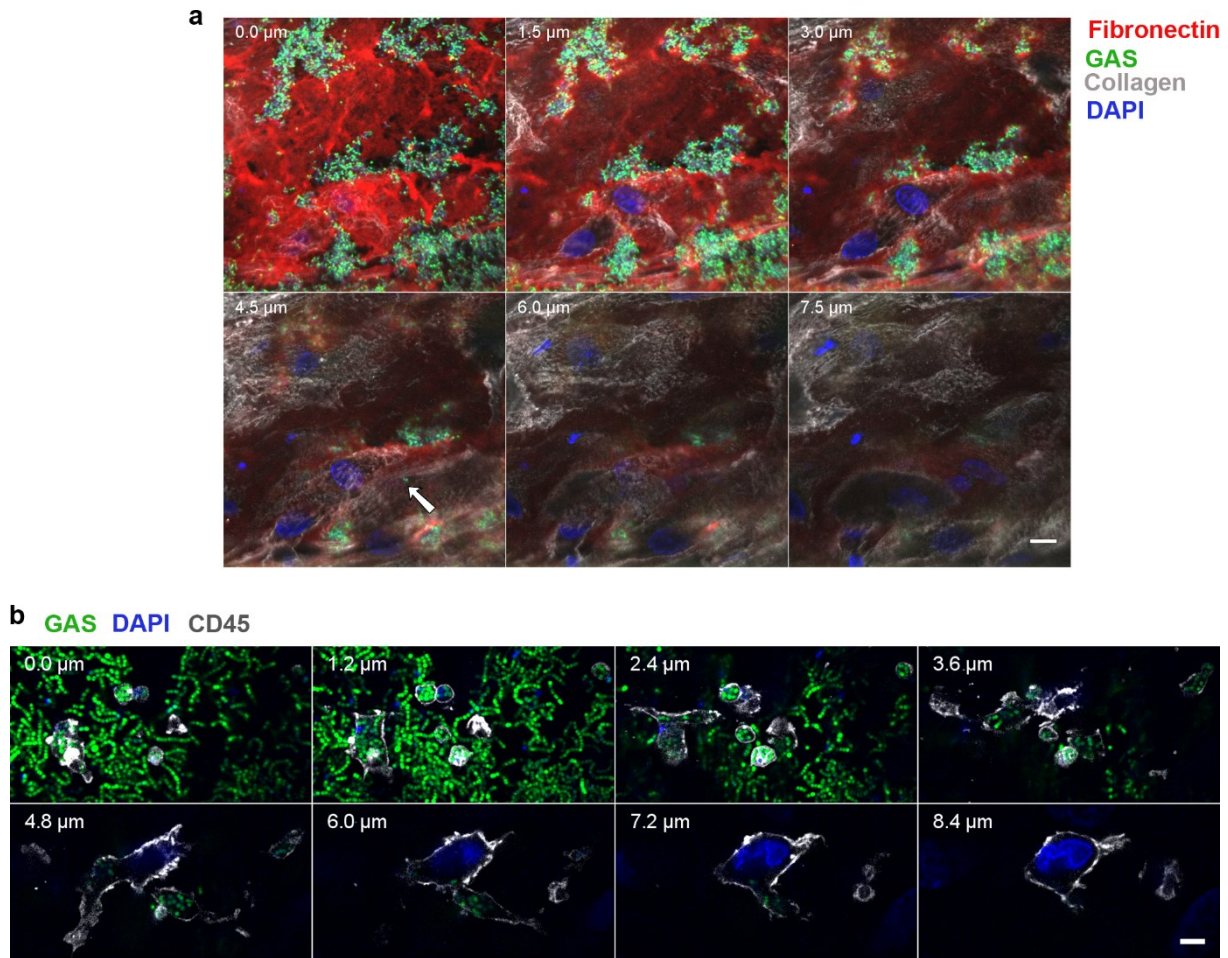


Figure S2. GAS invades the tissue. Montages of the same field at multiple depths. On both panels, the depth is indicated at the top left. 0 corresponds to the beginning of the GAS layer and is the first slice shown. **a**, ImageJ montage showing an example of GAS in the tissue. Tissue infected for 16 h under static conditions. Fibronectin, red; type IV collagen, grey; GFP-WT, green; DAPI, blue. A white arrow indicates the position of a GFP-WT coccus in the tissue. Scale bar: 10 μ m. Magnification: 40X. Same sample as in Fig. 1a and 2a. **b**, ImageJ montage of GFP-WT inside the tissue and within an immune cell. Tissue infected under flow condition for 3 h. Anti-CD45, grey; GFP-WT, green; DAPI, blue. Scale bar: 5 μ m. Magnification: 100 X. Same sample as in Fig. 2e.

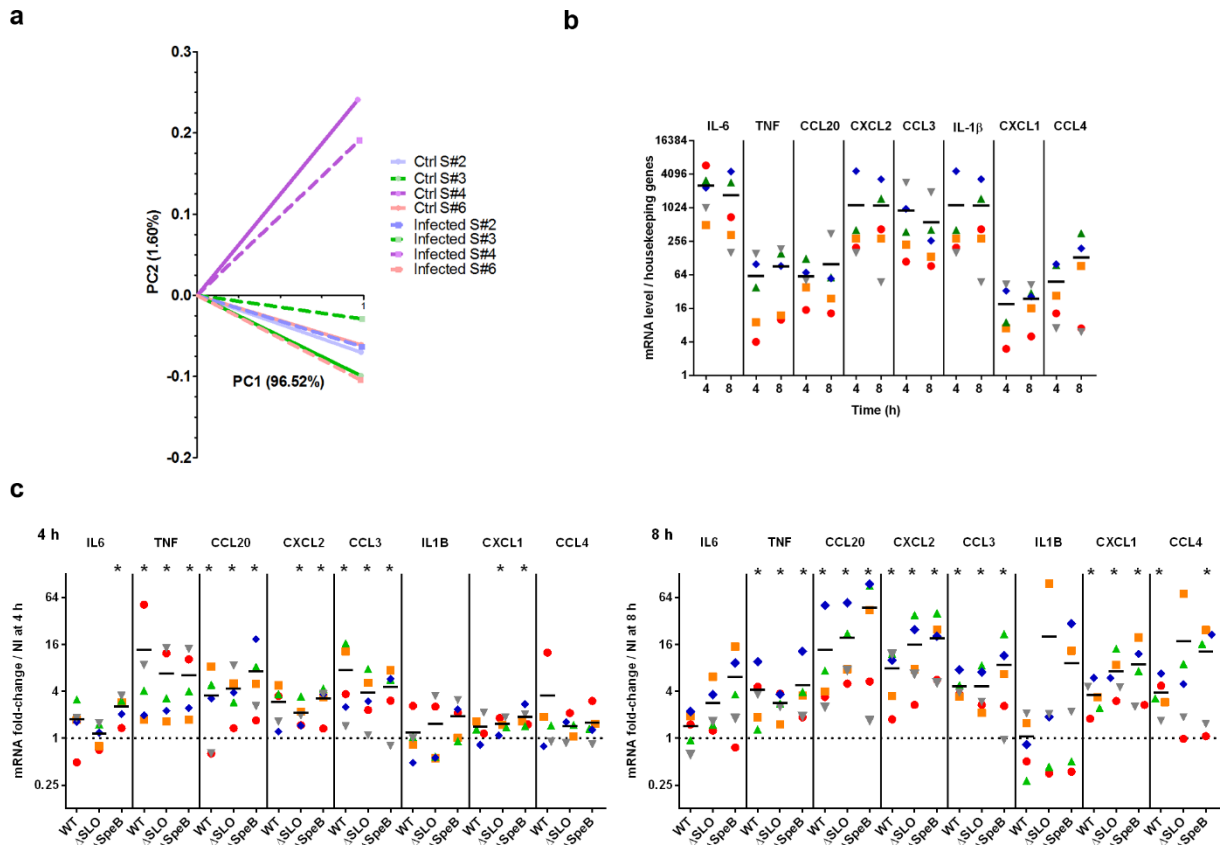


Figure S3. Basal levels and variation in expression of cytokine genes. **a**, principal component analysis of the basal expression of the 133 immune related genes across all samples showing that that of tissue #S4 differs from those of the other tissues. **b**, Basal level of expression of the indicated immune-related gene in the non-infected conditions compared to the housekeeping genes at 4 and 8 h. **c**, Fold-change variation of the expression of the immune-related genes after infection with the indicated strains for 4 h (left panel) and 8 h (right panel) compared to the non-infected expression. Axis is in log2. S#7, red circle; S#8, orange square; S#9, green triangle; S#10, blue diamond; S#11, grey triangle. Dotted lines correspond to the value of 1. Statistical analysis: Mann-Whitney U test; *, $p < 0.05$ as compared to 1, ratio Strain-infected / non-infected conditions.

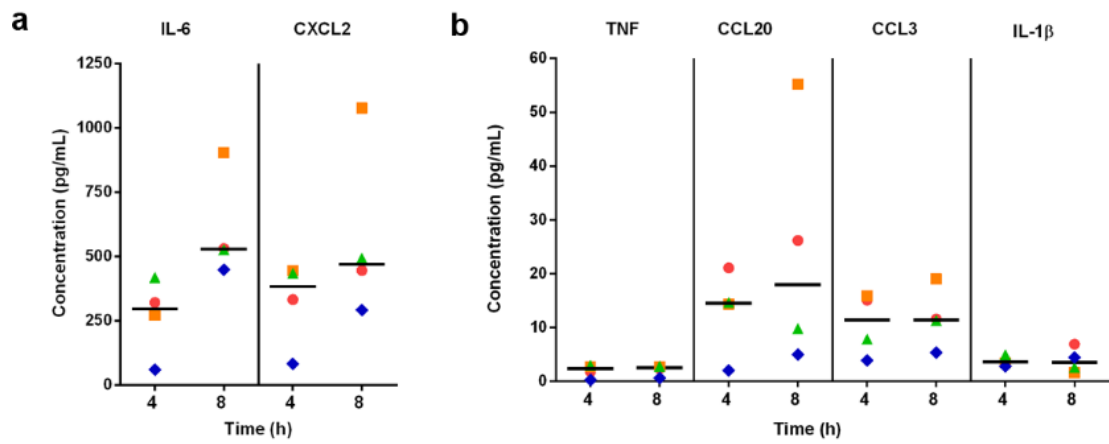


Figure S4. Basal levels of cytokine secretion. **a-b**, Basal levels of secretion of the indicated cytokine at the indicated time points for supernatant of non-infected decidual tissues. The scales of **(a)** and **(b)** are different. S#7, red circle; S#8, orange square; S#9, green triangle; S#10, blue diamond; S#11, grey triangle.

Table S1 Comparison of the overexpression of genes involved in the immune response

	GAS		GBS	
	mRNA Fold-change	<i>p</i> -value	mRNA Fold-change	<i>p</i> -value
IL6	3.44	0.2443517	20.08	0.0000001
IL1B	6.06	0.0101306	7.79	0.0000004
TNF	40.37	0.0106377	32.25	0.0000016
CXCL2	29.34	0.0005470	10.46	0.0000044
CCL20	12.77	0.0005246	33.91	0.0000144
CXCL8	1.90	0.0911197	4.80	0.0000319
IL1RN	0.94	0.9482826	3.96	0.0000747
CCL4	111.43	0.0032225	12.69	0.0000815
IL1A	1.83	0.4538192	6.85	0.0001290
NFKB1	1.11	0.6764782	2.25	0.0001450
ICAM1	2.08	0.4791674	5.01	0.0001730
NFKBIA	2.75	0.0500612	5.51	0.0001780
CXCL3	28.34	0.0000184	9.92	0.0001850
TLR2	1.26	0.7816158	4.52	0.0002380
TLR6	1.15	0.7529346	0.43	0.0017900
CCL13	0.76	0.7698270	0.47	0.0018400
NLRP3	2.36	0.0521823	4.39	0.0022200
CCR7	2.62	0.1155378	3.36	0.0064500
CCR1	2.00	0.0623347	0.36	0.0065000
CCL3	14.52	0.0009604	4.88	0.0072300
CD80	1.61	0.5656690	3.08	0.0081700
CD14	0.53	0.5807491	0.64	0.0084000
IFNA2	0.84	0.8758766	0.71	0.0125000
CCL2	2.58	0.2099790	2.38	0.0137000
CCL8	1.71	0.5478146	3.10	0.0146000
IL10RA	0.56	0.2238125	1.55	0.0186000
CXCL1	14.52	0.0007640	3.20	0.0200000
TICAM1	0.87	0.7283693	1.69	0.0226000
IL23A	9.47	0.0229023	13.52	0.0229000
CXCL5	1.48	0.5061415	2.98	0.0233000
IL37	0.64	0.1534900	0.68	0.0356000
CD40LG	2.38	0.3139426	0.74	0.0411000
IL18	1.19	0.7368512	2.15	0.0510000
LTA	0.35	0.2617627	1.42	0.0511000
TLR8	0.94	0.9689146	1.72	0.0609000
CXCR2	0.51	0.3713690	0.54	0.0629000
NOD1	0.78	0.5487833	0.60	0.0639000
CXCR3	1.58	0.4743766	0.77	0.0803000
IL9R	0.23	0.1263553	1.27	0.0815000
IRAK1	0.69	0.3244791	0.74	0.0861000
TLR4	1.40	0.4818540	0.63	0.0879000
TBX21	1.09	0.9344079	0.75	0.0966000
RAG1	0.74	0.5642718	0.80	0.1000000
CSF2	1.18	0.8418437	1.33	0.1030000
IFNGR1	0.74	0.3574953	1.25	0.1120000
STAT3	1.21	0.4820490	1.27	0.1330000
CXCL10	3.57	0.4609962	5.21	0.1360000
TLR1	0.93	0.9379901	0.70	0.1380000
CASP1	0.76	0.7458079	1.42	0.1440000
CD86	0.94	0.9553241	0.64	0.1730000
IL10	4.73	0.0490744	1.43	0.2190000

In green and bold the genes significantly upregulated after infection with GAS and GBS compared to tissue non infected; in orange the genes significantly only upregulated after infection with GBS¹⁸. In red and bold genes only upregulated after GAS infection. Several genes only overexpressed by GBS, such as NFKB1, ICAM, NFKBIA, TLR2, NLRP3, CD80, are related to the ontogeny pathways “cellular response to cytokine stimulus” (GO:0071345, *p* value= 2.4 10⁻³¹). Hits were classified in the decreasing order of *p*-value for GBS, until no gene was significantly differentially regulated for GAS and GBS. n=4 for GAS and for GBS.

Table S2 Concentration of inflammatory molecules in the supernatants at 4 h

	4H			
	CT	WT	Δ Slo	Δ SpeB
CXCL2	323 (81-444 \pm 84)	544 (158-1091 \pm 222)	167 (42-441 \pm 92)	340 (132-629 \pm 104)
IL1b	3.7 (2.8-4.9 \pm 0.44)	11 (5.2-25 \pm 4.5)	9.7 (2.6-18 \pm 3.3)	18 (5.7-39 \pm 7.4)
IL-6	267 (60-417 \pm 75)	346 (46-778 \pm 159)	106 (21-145 \pm 29)	279 (107-393 \pm 68)
CCL3	11 (3.9-16 \pm 2.9)	186 (40-481 \pm 102)	31 (8-92 \pm 20)	82 (18-250 \pm 56)
CCL20	13 (2-21 \pm 4)	9.6 (2.2-23 \pm 4.6)	3.5 (1.6-7.1 \pm 1.3)	8.2 (5.1-11 \pm 1.2)
TNF	1.9 (0.25-21 \pm 2.9)	130 (31-23 \pm 287)	23 (3.7-7.1 \pm 70)	58 (6.9-11 \pm 161)

Concentration in pg/mL. Results are expressed as: Mean (minimum-maximum \pm standard error). 5 subjects.

Table S3 Concentration of inflammatory molecules in the supernatants at 8 h

	8H			
	CT	WT	Δ Slo	Δ SpeB
CXCL2	576 (291-1077 \pm 172)	932 (153-2311 \pm 482)	1115 (487-2115 \pm 351)	3078 (410-7190 \pm 1453)
IL1b	3.9 (1.6-6.9 \pm 1.2)	43 (19-69 \pm 10)	49 (15-97 \pm 18)	75 (27-154 \pm 29)
IL-6	602 (448-904 \pm 102)	951 (222-2203 \pm 432)	1202 (495-2127 \pm 401)	2274 (359-4283 \pm 853)
CCL3	12 (5.4-19 \pm 2.8)	616 (18-1908 \pm 437)	503 (73-761 \pm 158)	1202 (279-2192 \pm 391)
CCL20	24 (5-55 \pm 11)	33 (1.6-85 \pm 18)	33 (3.1-79 \pm 17)	147 (13-429 \pm 98)
TNF	2.1 (0.6-55 \pm 2.8)	401 (17-85 \pm 1090)	320 (83-79 \pm 622)	938 (152-429 \pm 1798)

Concentration in pg/mL. Results are expressed as: Mean (minimum-maximum \pm standard error). 5 subjects.

Table S4 Strains and plasmids used in this study

Strains or plasmid	Relevant properties	Source or reference
<i>Streptococcus pyogenes</i>		
M28PF1	Wild-type representative <i>emm28</i> clinical isolate	31
ΔSpeB	M28PF1 lacking the <i>speB</i> gene coding for SpeB	This study
ΔSLO	M28PF1 lacking the <i>slo</i> gene coding for SLO	This study
M28PF1-GFP	M28PF1 with the integrated pG1-lacA-PTetO-gfp	This study
ΔSpeB-GFP	ΔSpeB with the integrated pG1-lacA-PTetO-gfp	This study
Plasmids		
pG+host5	Erm; ColE1 replicon, thermosensitive derivative of pGK12; MCS pBluescript	26
pATΩgfp	pAT28 derivative containing the <i>gfp</i> gene	27
pTCV_TetO	Plasmid containing the tetO tetR Pxyl promoter, inducible with anhydrotetracycline	28
pG1-Perm-gfp	pG+host5 containing the <i>gfp</i> gene from pATΩgfp	This study
pG1-lacA-Perm-gfp	pG1-Perm-gfp with the lacA intergenic region to allow stable integration in GAS genome	This study
pG1-lacA-PTetO-gfp	pG1-lacA-Perm-gfp with the Erm promoter replaced by the tetO tetR Pxyl promoter of pTCV_TetO. Can be integrated in GAS genome for anhydrotetracycline inducible <i>gfp</i> expression	This study

Table S5. Primers used in this study for cloning and checking cloning

Primer Name	Sequence*
F_tetO	GTGGAATTGTGAGCGGATAAC
R_tetO	GGTACCTTTT CACTCGTTAAAAAGTTTTGAGAATATTTTATATTTTGTTCATGTAATCACTCCTTCTTAATCTGTTAACGCTACGATCTAGCT
F_lacA	ACATGATTACGAATT TCAACGACTTCGTATTTACCTT
R_lacA	ACACTCTTAAGAATTC GCGGTCATATCTGAGATGTT
R_extlacA	CCACCATGGGTCCTGATA
RP48	AGCGGATAACAATTTCACACAGGA
SLO-F1	GACTCTAGAGGATCC GGTGCCAAAGGGTTTAGAA
SLO-R1	CTCAGGGG GATAAGAGCTGCCGTTAGTAG
SLO-F2	TCTTATC CCCTGAGCCCATATGGTTCGAT
SLO-R2	CATGATTACGAATTC GGGACAGTTGGGGTCAAATC
SpeB-F1	GACTCTAGAGGATCC GAGCATCTACTAGCCACAATA
SpeB-R1	GGGTTAGCAAGAACAAATCC
SpeB-F2	TGTTCTTGCTAACCC TTCAACGGTTACCAAAGTGC
SpeB-R2	CATGATTACGAATTC ATTAGTAGGCGTTGATGACC

* restriction enzyme sites are highlighted in bold and sequences used for the In-fusion© cloning are shown in red.

Table S6 List of experiments in which each tissue sample was used

Subject	Figure
S#1	Fig. 1i
S#2	Fig. 1i, Fig. 2b-c, Fig. 4a, Fig. S3a
S#3	Fig. 1i, Fig. 4a, Fig. S3a
S#4	Fig. 1i, Fig. 4a, Fig. S3a
S#5	Fig. 1i, Fig. 2e, Fig. 3e, Fig. S1, Fig. S2b
S#6	Fig. 4a, Fig. S3a
S#7	Fig. 1h, Fig. 3c, Fig. 4c, Fig. S3b-c, Fig. S4, Table S3-4
S#8	Fig. 2b-c, Fig. 4c, Fig. S3b-c, Fig. S4, Table S3-4
S#9	Fig. 1c-d, Fig. 1g-h, Fig. 2b-d, Fig. 4c, Fig. S3b-c, Fig. S4, Table S3-4, Supp video 1
S#10	Fig. 1d, Fig. 1h, Fig. 1k, Fig. 4c, Fig. S1, Fig. S3b-c, Fig. S4, Table S3-4, Supp video 3
S#11	Fig. S3b-c
S#12	Fig. 3g
S#13	Fig. 3e
S#14	Fig. 3e
S#15	Fig. 3g
S#16	Fig. 1a, Fig. 1h, Fig. 2a-c, Fig. 3a, Fig. S2a
S#17	Fig. 1h, Fig. 3d, Fig. 3g
S#18	Fig. S1d-f, Supp video 2
S#19	Fig. 1f, Fig. 3b, Supp video 4
S#20	Fig. 1b
S#21	Fig. 3g

The numbering does not correspond to the order in which subjects were included in the study

Supplementary video:**Supp video 1. GAS multiplication at the tissue surface**

Live confocal microscopy. GAS is in green. Magnification: 25X. Scale bar: 20 μm . Time step= 30 minutes. The images are the same as in Fig. 1c.

Supp video 2. Isolated GAS multiplication at the tissue surface

Live confocal microscopy GAS is in green. Magnification: 25X. Scale bar: 5 μm . Time step= 30 minutes. The images are the same as in Fig. 1e.

Supp video 3. Real-time *in situ* phagocytosis of GAS

Live confocal microscopy. GAS in green, CD45 in grey and DNA in blue. Magnification: 25X. Scale bar = 5 μm . Time step= 30 minutes. The images are the same as in Fig. 2d.

Supp video 4. Immune cell blebbing and death after infection

Live confocal microscopy of tissue infected under flow conditions. Intact nucleus, red; permeabilized nucleus (dead cell), blue; CD45, yellow. Magnification: 25X. Scale bar: 10 μm . Time step= 30 minutes.

Additional results and experimental approaches related to manuscript 2

Additional results to Manuscript 2

Involvement of decidual cells in the *ex vivo* bacterial growth

Decidual explants trigger bacterial growth *ex vivo* (Manuscript 2, Fig. 1). While the overall kinetic is conserved between donors (Manuscript 2, Fig. 1d), we observed discrepancies over the 9 fields acquired on one donor tissue (Fig. 1a). Two groups of fields can be distinguished, one that induced high bacterial growth and the other which induced low bacterial growth. We wondered if the immune cell types in the different fields could suggest the origin of such differences. There was no correlation between the number of CD45 positive cells within a field and the growth of GAS (data not shown). However, there was a direct correlation between the number of decidual cells per field and the capacity of bacteria to multiply (Fig. 1b). We therefore investigated if elements secreted by decidual stromal cells were sufficient to trigger bacterial growth. We used primary decidual cells isolated from two donors, and we observed that decidual stromal cells conditioned media were sufficient to trigger GAS growth (Fig. 1c). In conclusion, our results suggest that secreted elements by tissue decidual cells are involved in GAS growth.

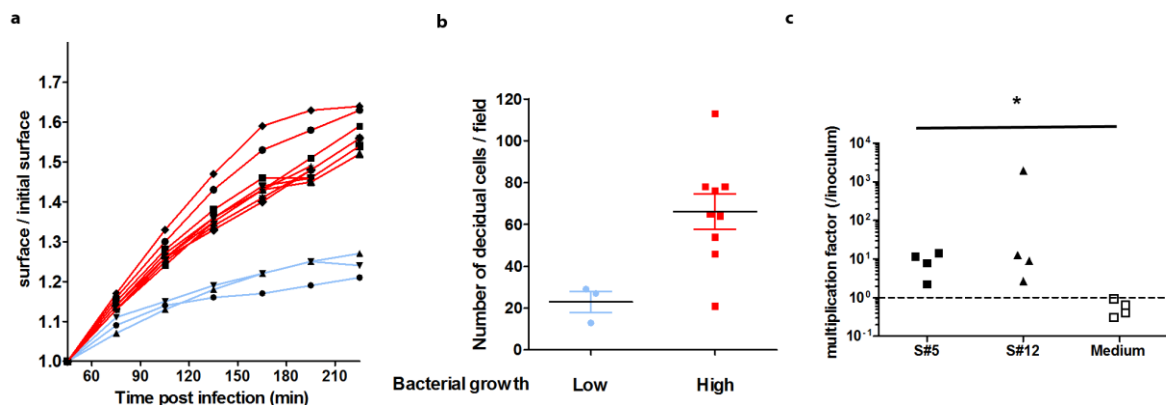


Figure 1 Secreted elements by decidual stromal cells are sufficient to trigger bacterial cell growth a, The increase in the area covered by GFP-producing bacteria on 9 fields of the same acquisition of the same tissue (S#9) are represented. The color code is meant to highlight fields inducing high (red) and low (blue) growth. b, number of decidual stromal cells (vimentin⁺, CD45⁺) in each field of (a). c, *in vitro* growth of GAS in primary decidual cells conditioned medium, from two donors (S#5 and S#12). Each experiment was performed 4 times independently in duplicates. Medium corresponds to RPMI. Friedman test with Dunn's multiple comparison test. *, p<0.05.

In situ phagocytosis

We observed that two types of cells phagocytosed GAS in infected decidua: large CD45 cells (Manuscript 2, Fig. 2d-e) and neutrophil-like cells (Additional results Fig. 2a). Using live-confocal imaging, we witnessed in real-time in situ phagocytosis of GAS (Manuscript 2, Fig. 2a and supplementary video 3). This suggests that PMN-like cells reach the surface within 15 minutes and phagocytose GAS. We observed neutrophil-like cells (Fig. 2b) filled with GAS in CD45+ vacuoles. The nuclei are condensed and there is a rupture of the plasma membrane in one of the two cells (Fig. 2b).

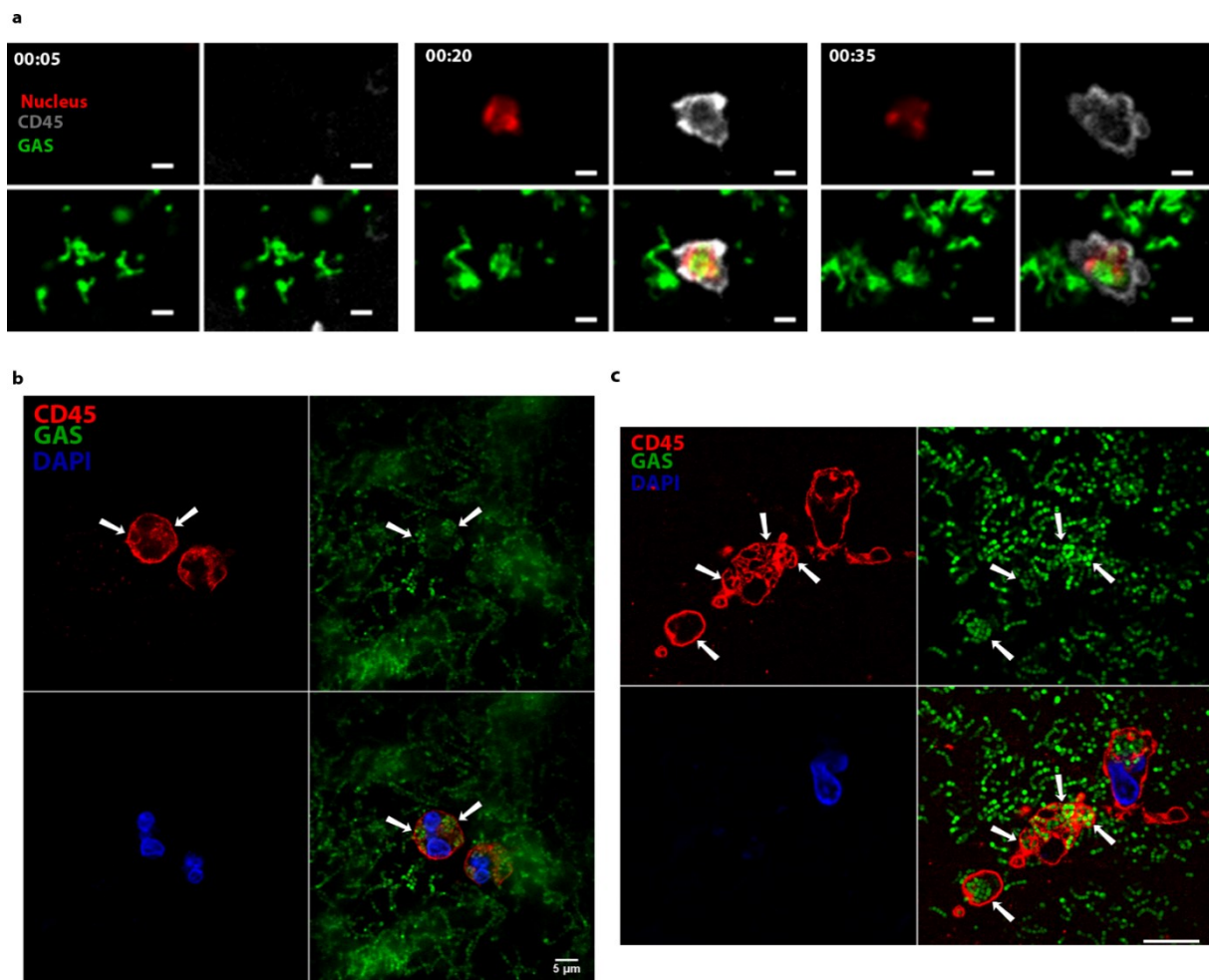


Figure 2 Real-time phagocytosis of GAS and intracellular bacteria surrounded by vacuoles. a, PMN-like CD45+ cells with intracellular GAS at the tissue surface in live confocal microscopy. Live acquisition of an immune cell phagocytosing bacteria on tissue of subject S#7. CD45 in grey; Draq5 in red and GAS in green. Scale bar: 5 μ m. Magnification: 25X. b, Tissue pre-stained with anti-CD45 antibodies, infected 4 h under static conditions (S#19) showing a neutrophil-like cells with GAS in vacuoles. The arrow shows the membrane rupture. Scale bar: 10 μ m; Magnification: 100X. c, Pre-stained tissue (S#5) was infected with GFP-producing bacteria for 1h under static conditions, then 3 h under flow conditions. Macrophage-like cell with GAS in vacuoles, as indicated by arrows. Scale bar: 5 μ m; magnification: 100X. GAS in green; CD45 in red; DAPI in blue for b and c.

Large CD45+ cells with large extension present multiple GAS within CD45+ membranes. Since we incubated the sample with anti-CD45 antibodies before the infection, the CD45+ vacuole

corresponds to plasma membrane engulfed with bacteria, suggesting phagocytosis. Cells are filled with bacteria and present an altered nucleus morphology with high condensation, suggesting the phagocytic cell died (Fig. 2c).

We therefore observed GFP-producing bacteria within vacuoles in macrophages and neutrophils *in situ*, suggesting that phagocytic cells efficiently phagocytose GAS. These bacteria appear to escape from these cells after cell lysis through mechanisms we did not assess.

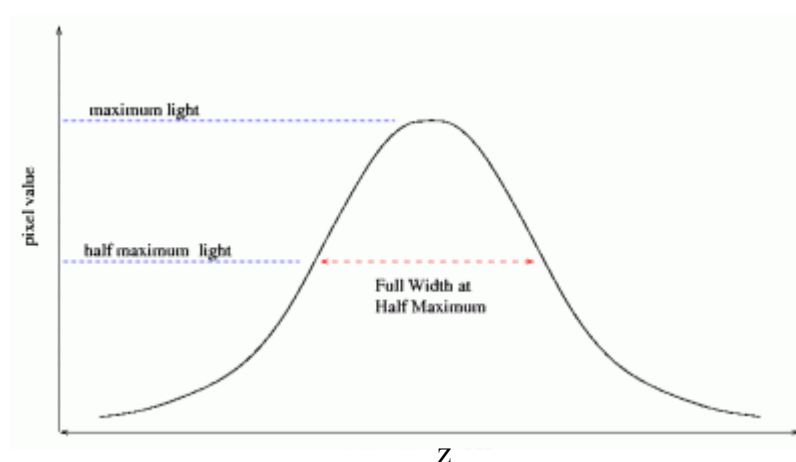
Experimental procedure

***In vitro* growth in decidual cell supernatant.** Decidual cells were isolated and cultured as described in the experimental procedure of Manuscript 1. Decidual cells from two different donors were starved to eliminate residual FBS by removing culture medium, washing once with PBS and adding RPMI 8 h. RPMI was then removed and fresh RPMI was added overnight. Supernatant was collected, and 10^3 bacteria in exponential growth phase were added to 1 mL of decidual cell supernatant or fresh RPMI and incubated 8 h at 37°C, 5% CO₂. GAS was plated and cfus were numbered as described in the experimental procedure of Manuscript 2.

Computer codes used in Manuscript 2

Thickness measurement

This macro takes advantage of the fact that the thickness of an object is proportional to the full width at half maximum (FWHM) of the Gaussian curve of the intensity plot in the axial (depth) direction.



We can therefore estimate the thickness of a bacterial layer by:

- fitting a Gaussian curve
- calculating the FWHM
- transforming this value in μm : for this we calculated the FWHM of isolated bacteria and considered their diameter to be $1 \mu\text{m}$

Single bacteria display an intensity curve that is a perfect Gaussian curve in the axial direction (z-axis). However, aggregates of bacteria present a Gaussian curve in the z axis only if the distance between single objects is inferior to the axial resolution, 500 nm. This is locally correct on dense aggregates. Moreover, the bacterial layer is not perfectly flat and homogenous. We therefore need to locally fit a Gaussian curve, on small regions to have accurate measurements of the thickness.

The macro basically subdivides the field in 2500 regions of interest, and fit a Gaussian curve to the z-axis plot of intensity of the GFP signal (bacteria), from which the FWHM is extracted. The macro filters results for Gaussian curves that are incorrectly fitted (R^2 too low etc...). Finally, we have for each of the 2500 regions a value in pixel that is transformed in μm . We then extract the mean value, and repeat the analysis for each time-point of each stage for each donor tissue.

```

1 //ScanslideThickness.
2 //The purpose of this macro is to measure the Local thickness of a bacterial layer.
3 //This macro will fit a gaussian to a plot signal of a subrectangle of an image and will measure the FWHM of the gaussian, and the complete image will be scanned by this rectangle.
4 //Written by Antonin Weckel, Institut Cochin, Barriers and Pathogens. February 2018.
5
6 start = getTime();
7 Image_Name = getTitle();
8 getDimensions(image_width, image_height, image_channels, image_slices, image_frames);
9 getVoxelSize(voxel_width, voxel_height, voxel_depth, voxel_unit);
10 //image_slices=image_slices*2;
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21 dir = getDirectory("Choose directory");
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```


R code for 3D surface heat map.

This surface is generated from the 2500 values of local thickness (from FWHM). To smooth out the surface, every single region was attributed the mean of the values of 4 adjacent tiles.

```
library(plotly)

pathPrep <- function(path = "clipboard") {
  y <- if (path == "clipboard") {
    readClipboard()
  } else {
    cat("Please enter the path:\n\n")
    readline()
  }
  x <- chartr("\\", "/", y)
  writeClipboard(x)
  return(x)
}

pathPrep()
#Replace "Downloads" with the directory where your input file is
folder = pathPrep()

x <- seq_len(nrow(volcano)) + 50
y <- seq_len(ncol(volcano)) + 50
col3 <- c(`0` = "blue", `5`="white", `10` = "orange", `20` = "red", `30` = "black")
axx <- list(
  nticks = 10,
  range = c(50,100)
)

axy <- list(
  nticks = 10,
  range = c(50,92),
  title=""
)

axz <- list(
  title="m",
  nticks = 5,
  range = c(0,15)
)

m <- list(
  l = 0,
  r = 0,
  b = 0,
  t = 0,
  pad = 0)

input.file = "FWHM value T10.csv"
lieu<-paste(folder,"/",input.file,sep="")
mat2 <- read.csv(lieu,header=FALSE,sep=";")
test<-as.matrix(mat2)
p=plot_ly(x = ~x, y = ~y, z =test,text= "T10", width = 900, height = 300,
type="surface",showscale=T, cmin=0, cmax=9, cauto = F, autocolorscale=F,colorscale=col3

  )%>%layout(autosize=F, margin = m, scene = list(aspectmode = "manual", aspectratio =
list(x=1.5, y=1.05, z=0.225),xaxis=axx,yaxis=axy,zaxis=axz, camera=list(eye = list(x = 0.005, y
= 1, z = 0.5))))
p
```

Measurement of cytotoxicity

This macro is a 3D segmentation of nuclei based on intensity and morphology (Plug-in 3D object counter) to count TUNEL positive (dead cells) and DAPI positive nuclei (all cells). To avoid false positive in the DAPI channel that also contains the signals from bacteria that could be interpreted as nuclei, there is a small step where we exclude the first stacks of the image containing only bacterial signal, and an “open” treatment of the binary signal (which “erases” sharp structures such as bacteria).

```
1 // macro tunnel counting made for 20X pictures, bin=2
2 //CHANGE are places where the user needs to change in the text the value for each image analysis. Not user friendly, sorry. Lines 41,45,81
3 //Copyright Antonin Weckel, Institut Cochin
4
5 //unspecific setting up
6 run("Close All");
7 setForegroundColor(0,0,0);
8 setBackgroundColor(255,255,255);
9 setOption("BlackBackground", true);
10
11 if (isOpen("Log")) {
12     selectWindow("Log");
13     run("Close");}
14
15 // specific set up
16 dir = getDirectory("Choix du repertoire contenant les images");
17
18 Dialog.create("nom de la manip");
19 Dialog.addMessage("attention aux caracteres utilises,pas nom du canal");
20 Dialog.addString("nom manip","a remplacer");
21 Dialog.show();
22 filename=Dialog.getString();
23
24 Dialog.create("nombre de fichier à mesurer");
25 Dialog.addString("nombre","a remplacer");
26 Dialog.show();
27 nb=Dialog.getString();
28
29 // beginning of the macro
30 //the channel with TUNEL staining is analyzed with 3D object counter
31 for (h=1;h<=nb;h++){
32     numero=h;
33     filenamenumber=filename+numero;
34     name488=filenamenumber+"_w2491".TIF";
35     direct488=dir+name488;
36     open(direct488);
```

```

36 open(direct488);
37 selectWindow(name488);
38 run("Subtract Background...", "rolling=30 stack");
39 // security check: 3D object counter induces bugs if you have thresholded above the maximum value presented in the image. So the macro can only be run if one px>threshold
40 run("Z Project...", "projection=[Max Intensity]");
41 getRawStatistics(nPixels, mean, min, max);
42 if (max>=220){ //the maximum value of the zprojection is the maximum px present in all the image. CHANGE
43     close();
44     selectWindow(name488);
45     run("3D Objects Counter", "threshold=300 slice=33 min.=700 max.=16233360 objects summary"); //CHANGE the threshold
46 } else { //if no px above threshold, no positive nuclei
47     print(name488+"no object");
48 }
49
50 selectWindow("Log");
51
52 savefilename=filename+". "+number TUNEL positive nuclei+".csv";
53 saveAs("text",dir+savefilename);
54 close();
55
56 if (isOpen("Log")) {
57     selectWindow("Log");
58     run("Close");
59 }
60 // beginning of the analysis of the DAPI image: all the nuclei
61 count405=newArray(0);
62 h=getNumber("if bacteria, say 1, else 0",1);
63 for (i=1;i<=nb;i++){
64     filenamenumber=filename+i;
65     name405=filenamenumber+"_w3405".TIF";
66     direct405=dir+name405;
67     open(direct405);
68     selectWindow(name405);
69     z=nSlices;
70     slicestart=1;
71
72     slicestart=1;
73
74     if (h==1){ //image with bacteria which are not nuclei but DAPI+
75         waitForUser("choose the number of the first slice to start analysis");
76         slicestart=getNumber("slice start",1);
77     }
78     run("Duplicate...", "title=subs duplicate range="+ slicestart + "-" + z); // we analyze the image starting at the top of nuclei, not top of bacteria
79     rename("subs"+name405);
80     run("Subtract Background...", "rolling=30 stack");
81     setAutoThreshold("Default dark");
82 //run("Threshold...");
83 setThreshold(60, 4800); // CHANGE
84 setOption("BlackBackground", true);
85 run("Convert to Mask", "method=Default background=Default black");
86 run("Options...", "iterations=2 count=1 black pad edm=8-bit do=Open stack"); // small trick to get rid of non ellipsoid small patches of bacteria
87 run("3D Objects Counter", "threshold=128 slice=28 min.=700 max.=13773760 objects summary");
88 }
89 selectWindow("Log");
90 savefilename=filename+". "+nombre noyau+".csv";
91 saveAs("text",dir+savefilename);
92 run("Close All");
93

```

Analysis of the 3D position of GAS

This macro segments bacterial particles from the maximum z-projection of the GFP signal. Each particle is a Region of Interest (ROI).

For each particle, the z-position is calculated from the maximum of the z-axis intensity plot (Find Peak plug-in on the z-axis profile). The position of collagen and fibronectin is analyzed at the same ROI in a similar way. Z-position of the different elements are compared: if the z-position of the bacterial particle is lower than collagen or fibronectin (the most superficial element), with a distance superior to 1.2 μm , the bacteria is considered as “invading particle”. Though we did not use it in the manuscript, this macro also exports the distances between invading particles and the topical signal, and the size of the particles.

Since cellular granules have sometime the same intensity as GFP-producing bacteria (due to autofluorescence, autoF), particles are filtered to check they are bacteria by analyzing the presence of a DAPI (DNA) signal in the vicinity of the GFP signal (they should overlap).

```

1 //macro3Dbacteriaposition. Developed by Antonin Mechel, INSERM u1016, Institut Cochin, Paris, FRANCE. Last modified: March 2018
2 //Objectives: Identify the number of bacterial particles found within the tissue
3 //Pipeline: 1) bacteria are thresholded in a z-max projection of the 491 channel, particles are segmented and ROI list is saved.
4 // 2) For each ROI, the z position of the bacteria (z bact) is determined through the "find peaks" plugin applied to z-axis profile. If two bacteria are one on another (two peaks), the z bact corresponds to the deepest peak.
5 // 3) ROI are checked for behind real bacterial signal and not autofluorescence. For each ROI, DNA peaks are analyzed and the absence of a DNA peaks in the vicinity of the bacterial peaks exclude the ROI of the macro. The number of excluded events is indicated.
6 //ROI number of excluded events can be retrieved with a specified Line.
7 // 4) Z position of objects of interest in the channel 635 and 591 are determined (z591 and z635) with "Find Peaks". This time, the closest object to the surface (smaller z) is kept.
8 // 5) Z position of filtered ROI is compared to z635 and z591, in this order. Bacteria are considered below z635 and z591 if they are 3 stacks below (1.2 µm).
9
10 setForegroundColor(0,0,0);
11 setBackgroundColor(255,255,255);
12 setOption("BlackBackground", true);
13 dir = getDirectory("Folder containing images"); // folder where images are, and where the output files will be saved
14 list = getFileList(dir);
15 firstimage=0;
16 for (fileposition=0;fileposition<list.length;fileposition++){ //this a line which gives me automatically the name of all the files in a folder
17     if (endsWith(list[fileposition],".tif")){
18         filename=substr(list[fileposition],0,9); //check this line, depends on the length of the name
19         name488=filename+"_u3491*.tif";
20         name561=filename+"_u2561*.tif";
21         name635=filename+"_u1635*.tif";
22         name405=filename+"_u4005*.tif";
23
24         direct488=dir+name488; //image directory
25         direct635=dir+name635;
26         direct561=dir+name561;
27         direct405=dir+name405;
28
29         print(name488); // the value to segment images is only determined on the firstimage
30         // This is the different file Main loop.
31
32         //Initiation of the macro
33         if (isOpen("Results")) {
34             selectWindow("Results");
35             run("Close");
36         }
37         if (isOpen("Log")) {
38             selectWindow("Log");
39             run("Close");
40         }
41
42         if (isOpen("Plot Values")) {
43             selectWindow("Plot Values");
44             run("Close");
45         }
46         if (isOpen("Num@ro des particules en invasion")) {
47             selectWindow("Num@ro des particules en invasion");
48
49             selectWindow("Num@ro des particules en invasion");
50             run("Close");
51         }
52         if (isOpen("ROI Manager")) {
53             selectWindow("ROI Manager");
54             run("Close");
55         }
56         if (isOpen("Numerobacterielnu")) {
57             selectWindow("Numerobacterielnu");
58             run("Close");
59         }
60         //Initiation of the variables
61         nbpartunder=0;
62         nbbacterunder=0;
63         nbbacterleunder=0;
64         nbpartcol=0;
65         nbbactcol=0;
66         nbmatrice=0;
67         nbbactmatrice=0;
68         Nbpars=0;
69         m=0;
70
71         //to be checked for every acquisition
72         amplitude488=0;
73         amplitude405=0;
74         amplitude635=0;
75         amplitude561=0;
76
77         filename=filename+h; //incrementation of the filename
78         name488=filename+"_u3491*.STK";
79         name561=filename+"_u2561*.STK";
80         name635=filename+"_u1635*.STK";
81         name405=filename+"_u4005*.STK";
82
83         direct488=dir+name488; //image directory
84         direct635=dir+name635;
85         direct561=dir+name561;
86         direct405=dir+name405;
87         if (File.exists(direct488)) {
88             open(direct488);
89
90             run("Duplicate...", "duplicate"); // I work on a duplicated image to apply a threshold on it
91             rename("488analyse");
92
93             // the deeper we get, the bigger chance we have to find autof granules. Since no bacteria can be found very deep, we only look for bacteria on the first 30µm
94             ...

```



```

93 // the deeper we get, the bigger chance we have to find autof granules. Since no bacteria can be found very deep, we only look for bacteria on the first 30um
94 if (nSlices>90)
95   run("Z Project...", "steps=90 projections[Max Intensity]");
96
97 else {run("Z Project...", "projections[Max Intensity]");}
98
99
100 run("Mean...", "radius=1");
101 setAutoThreshold("Default dark");
102
103 //If it is the first image, the user is asked to give a threshold value (minthreshold)
104 if (firstimage==0)
105   loop;
106   waitForUser("Check threshold ");
107   Dialog.create("min threshold bacteria");
108   Dialog.addMessage("it must be a number");
109   Dialog.addString("min threshold value", "200");
110   Dialog.show();
111   minthreshold=Dialog.getString();
112   firstimage=1; //at the next image h, the thresh will not be asked
113 }
114
115 //run("Threshold...");
116 setThreshold(minthreshold, 4200);
117 setOption("BlackBackground", true);
118 run("Convert to Mask");
119 run("Watershed"); // I add a Watershed to decrease the size of the particles analyzed
120 run("Analyze Particles...", "size=30-Infinity showNothing display clear include summarize add");
121 roiManager("Show All with labels");
122
123 n = roiManager("count"); // n is the number of particles
124 Arrayunder = newArray(n); //array containing the particles invoting
125
126
127 open(direct635);
128 open(direct561);
129 open(direct485);
130
131 //on the microscope I use, the 485 channel is not at the same z position as the other channels so it is essential to correct it through a translation of 4 voxels. Note induces false peaks, always better with a subtract background
132 selectWindow(name485);
133 run("Subtract Background...", "rolling=50 stack");
134 run("Transform> Translate", "x-distance=0.0 y-distance=0.0 z-distance=4 voxel interpolation=linear background=0.0");
135 rename("485translated");
136 selectWindow(0, 1306);
137
138 selectWindow(name485);
139 run("Close");

```

```

139 run("Close");
140
141
142 // Step 2. Loop on every single ROI
143 for (i=0; i<n; i++) {
144   tooClose=0;
145   z335=0;
146   nopeak535=0;
147   nopeak561=0;
148   z561=0;
149   zbact=0;
150   Zint=0;
151   Zint561=0;
152   tempzbact=0;
153   a1=newArray(1);
154   selectWindow(name485);
155   roiManager("Select", i);
156   getStatistics(area);
157   m=area;
158   run("Plot Z-axis Profile");
159   rename("Z-axis profile-"+name485);
160   run("Find Peaks", "min_peak_amplitude=amplitude485 min_peak_distance=0 min_values[] exclude max_values=10 list"); //The amplitude depends on every acquisition. Change it before the run
161   selectWindow("Z-axis profile-"+name485);
162   run("Close");
163   selectWindow("Plot Values");
164   listbacteria=FindPeakList(a1); //listbacteria contains the value of the peaks of bacteria. See function description for details
165
166   if (listbacteria.length>1) { //if more than one peak, we check if the peaks are close or not. when too close, DNA peaks can't be resolved
167     for (u=1; u<listbacteria.length; u++) {
168       tempu=u;
169       delta=abs(parseInt(listbacteria[u])-parseInt(listbacteria[tempu]));
170       if ((parseInt(delta)<9) {
171         tooClose=1; //this is a security. Too close peaks in 481 sometimes induce DNA peaks that merge.
172       }
173     }
174   }
175   if (listbacteria.length==1 && listbacteria[0]!=0) {
176     zbact=parseInt(listbacteria[0]); //when there is one peak, zbact equal to the only peak position
177   }
178   if (listbacteria.length>1) {
179     array.sortIn(listbacteria, min, max, mean, stdDev);
180     zbact=parseInt(max); // when several peaks, the deepest is taken (max value of listbacteria)
181   }
182   close("Peaks");
183 }

```

```

185 close("Peaks");
186 //concl DNA 485. Step 3 Here the main idea is that cells granules are highly autof, and the peak of a granule can be mistaken as bacteria. There is no DNA in a granule, in contrast to bacteria
187 bact=0; // when 0, bacteria is not a bacteria. when 1, we have confirmed it is a bacterium.
188 selectWindow("485translated");
189 roiManager("Select", i);
190 run("Plot Z-axis Profile");
191 rename("Z-axis profile-"+name485);
192 run("Find Peaks", "min_peak_amplitude=amplitude485 min_peak_distance=0 min_values[] exclude max_values=10 list");
193 selectWindow("Z-axis profile-"+name485);
194 run("Close");
195 selectWindow("Plot Values");
196 a1=newArray(1);
197 listdna=FindPeakList(a1);
198
199 if (tooClose==0) {
200   if (listdna.length==1 && listdna[0]!=-1) { //there is only one DNA peak
201     zDNA=parseInt(listdna[0]);
202     if ((zbact-8)<=zDNA && (zbact+8)>=zDNA) { //the DNA peak is close to the bacteria peak
203       bact=1; // when bact=1, we confirm the ROI is a bacterium
204     }
205   } else if (listbacteria.length>1) { //there is more than a bacteriopeak
206     for (t=0; t<listbacteria.length; t++) { // we go back to the list of bacteria peak to check if one correspond to the DNA peak
207       tempzbact=parseInt(listbacteria[t]);
208       if ((tempzbact-8)<=zDNA && (tempzbact+8)>=zDNA) {
209         bact=1;
210         zbact=tempzbact; // the "correct" bact peak is saved. if it exists
211       }
212     }
213   }
214 }
215
216
217 if (listdna.length>1 && listbacteria[0]!=-1) { // there are several DNA peaks. Some can come from nuclei.
218   for (t=0; t<listbacteria.length; t++) { // we check if any bacterial peak correspond
219     tempzbact=parseInt(listbacteria[t]);
220     for (g=0; g<listdna.length; g++) { //we check all the dna peaks correspondence to a bacterial peak.
221       if ((tempzbact-8)<=listdna[g] && (tempzbact+8)>=listdna[g]) {
222         zbact=tempzbact;
223         bact=1;
224       }
225     }
226   }
227 }
228
229 if (bact==0) // this is to check the particle that were disqualified as no DNA
230   print("Not a bacterium"+i+1);
231

```

```

232 }
233 }
234 //channel 561 (beginning of step 4)
235 selectWindow(name561);
236 roiManager("Select", 4);
237 run("Plot Z-axis Profile");
238 rename("Z-axis profile" name561);
239 run("Find Peaks", "min_peak_amplitude=Amplitude561 min_peak_distance=0 min_values[] exclude_max_values=10 list"); //this is the line to change at ever acquisition. Amplitude varies
240 selectWindow("Z-axis profile" name561);
241 run("Close");
242 selectWindow("Plot Values");
243 setNameArray(4);
244 list561=FindPeaksList(a1);
245
246 if(list561.length==1){ // either there is no peak, or one peak
247     if(list561[0]==-1){ // no peak
248         npeaks561=1;
249     }
250     else { // one peak, so z561 gets this unique value
251         z561=parseInt(list561[0]);
252     }
253 }
254
255 if (list561.length>1){ //several 561 peaks
256     Array_getStatistics(list561, min, max, mean, stdDev);
257     z561=parseInt(min); //the peak the closest to the surface is taken into account
258 }
259
260 close("Peaks");
261
262 //canal fibronectine 635
263 selectWindow(name635);
264 roiManager("Select", 4);
265 run("Plot Z-axis Profile");
266 rename("Z-axis profile" name635);
267 run("Find Peaks", "min_peak_amplitude=Amplitude635 min_peak_distance=0 min_values[] exclude_max_values=10 list");
268 selectWindow("Z-axis profile" name635);
269 run("Close");
270 selectWindow("Plot Values");
271 setNameArray(1);
272 list635=FindPeaksList(a1);
273
274 if(list635.length==1){ //symetric to 561 analysis
275     if(list635[0]==-1){
276         npeaks635=1;
277     }
278     else {
279         z635=parseInt(list635[0]);
280     }
281 }
282
283 if (list635.length>1){
284     Array_getStatistics(list635, min, max, mean, stdDev);
285     z635=parseInt(min);
286 }
287
288
289 //Step 5. the zbaceria position is compared to the other elements of the tissue
290 if(bactcol){ //it is a bacterium with a 492 peak and a DNA peak
291     if(npeaks635==1){ // no 635 element
292         if(npeaks561==0) { // but a 561 element
293             if (zbact-3>z561){
294                 nbpartunder=nbpartunder+1;
295                 nbactunder=nbactunder+(m/37);
296                 Zint561=((zbact-z561)*0.3);
297                 print("ROI under 561 signal" +i+" &" distance to 561 peaks " +Zint561);
298             }
299             else if (zbact-3<=z561){
300                 nbpartcol=nbpartcol+1;
301                 nbactcol=nbactcol+(m/37);
302             }
303         }
304         if(npeaks561==1){ // no 561 element
305             nbpartcol=nbpartcol+1;
306             nbactcol=nbactcol+(m/37);
307         }
308     }
309     if(npeaks635==0){ //a 635 peak
310         if(npeaks561==0) { // and a 561 peak
311             if(zbact-3>z561){ //bacteria below 561
312                 nbpartunder=nbpartunder+1;
313                 nbactunder=nbactunder+(m/37);
314                 if (z561<=z635){ //when 635 peak is above 561 peak
315                     Zint561=((zbact-z561)*0.3);
316                     print("ROI under 561 signal is:" +i+" &" distance to 561 = " +Zint561); // distance bacteria to surface = distance bacteria 561
317                 }
318                 if (z561>=z635){ // distance bacteria to surface distance to 635
319                     Zint561=((zbact-z635)*0.3);
320                     print("ROI under 561 signal is:" +i+" &" distance to 635= " +Zint561);
321                 }
322             }
323         }
324     }
325 }
326 }
327 }
328 }
329 }
330 }
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Discussion

Discussion and perspectives

GAS is responsible for a wide range of diseases and is able to infect multiple and diverse tissues (skin, pharynx, endometrium). It infects people of all age groups, ethnicities and sex. More than 200 GAS *emm*-types have been described (15), they present diverse and distinct repertoires of virulence factors. There is no single hypervirulent strain of GAS and the proportion of the different *emm*-types involved in invasive infections is dynamic, with several clones emerging in the past decades, after acquisition or loss of different factors (capsule, Sda1 etc...). These emerging clones are involved in the increase of the number of GAS invasive infections in developed countries (580, 581) and they indicate that GAS strains are still evolving and adapting to the human host. GAS harbors a very complex network of virulence factors acting with one another. These factors present functional redundancy, as shown with the number of fibronectin binding proteins. Moreover, GAS virulence factors are tightly regulated (Introduction 2.2). Consequently, GAS is fully equipped to face various environments. Host genetic background (528) and local parameters (inflammation, trauma, breach, post-partum) are already-known risk factors for superficial and/or invasive infections. Yet why certain individuals suffer from invasive infections is still not understood.

The main focus of our research team is the establishment of GAS invasive infections, with the endometritis as a model. Interestingly, GAS barely colonizes the human vagina in contrast to other bacteria (539). A recent report showed that women with previous GAS genital infections had a higher carrier stage than a control group, suggesting that particularities in some women favor their colonization with GAS, increasing the risk for GAS endometritis (582). GAS appears to take particularly advantage of the postpartum compared to other bacteria colonizing the woman gynecological sphere and the number of cases dropped due to current prophylactic measures (31). To understand the origin of GAS infections in the postpartum, it is important to consider the specificity of this context, and how it favors GAS infections (583). While in our studies we considered the specific cells from the tissues involved in the onset of GAS endometritis (cervical, endometrial, decidual stromal and immune cells), we did not consider other factors that are very specific of the postpartum, such as the pH, that could be critical for GAS pathogenesis (583).

To explore the mechanisms involved in GAS endometritis, we developed during this PhD two complementary research axes analyzing diverse aspects of GAS pathogenesis. Binding to host epithelium (endometrium, skin or pharynx), through R28 among other direct

and indirect adhesin-mediated bindings, is critical to infection. In the presence of a breach, after epithelium disruption or in any situation where the stroma is accessible to GAS, the multiple steps we characterized are critical.

Direct interactions with integrins and their implications in pathogenesis

The N-terminal domain of R28 binds to three integrins, $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$. Integrins are plasma membrane dimers with one β and one α subunit; there are 9 types of β subunits and 24 types of α subunits and altogether 24 different integrins (584). Integrins promote cell binding to the extracellular matrix, and their ligands are fibronectin, vitronectin and laminins (585). $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins are laminin binding integrins (586). We could investigate if R28 competes with laminins (and *vice-versa*) to bind integrins.

Integrins exist under two conformations, active or inactive and natural ligands often bind a defined biochemical region overlapping the α and β subunits, accessible only when the integrin is in the active form (584). Divalent cations change the conformation of integrins to the active form and the binding of ligands to integrins is partly mediated by these divalent cations. R28_{Nt} does not require divalent ions to bind integrins indicating it can bind the inactive form of integrins. Since R28_{Nt} binds to a lesser extent integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$ in the presence of divalent cations, this suggests that the affinity of R28_{Nt} for integrins is lower in the active form than in the inactive form. All this indicates that R28 does not bind to the exact same site as laminins.

Intracellular domains of integrins bind to the cytoskeleton and are involved in the transduction of outside-in signals: $\alpha 3\beta 1$ and $\alpha 6\beta 1$ bind to actin, and $\alpha 6\beta 4$ to intermediate filaments (585). Binding of natural ligands or inside-out signals induce integrins to change from the inactive to the active form. In our experiments, R28_{Nt} did not induced intracellular transduction of signals as evaluated by assaying the phosphorylation of several proteins of classical downstream pathways of integrin activation (such as FAK), but our results were not conclusive and will require more sensitive approaches. Ligand binding to integrins can induce the internalization of bound elements (see Introduction 3.1.3). We looked for the effect of R28_{Nt} on the internalization of coated beads and found no difference between R28_{Nt}-coated beads and the controls. In contrast to natural ligands such as laminins, R28_{Nt} may bind to a domain on integrins that does not change the activation state of the integrins, explaining absence of effects on the internalization and intracellular transduction.

The integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are receptors of R28_{Nt} and are most probably not its sole receptors. Silencing simultaneously the $\beta 1$ and $\beta 4$ integrins did not significantly reduce the binding of R28_{Nt} to HEC-1-A cells (data not shown). R28_{Nt} significantly binds to K562 hematopoietic cells, which express only the $\alpha 5\beta 1$ integrin (data not shown). This confirms that on HEC-1-A cells and other cell lines, $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ might not be the sole receptors of R28_{Nt}.

Few GAS proteins bind directly to integrins: so far, only one variant of SpeB and the ScII proteins have been shown to bind to integrins (93, 330, 331); GAS interaction with $\alpha 3\beta 1$, $\alpha 6\beta 1$ or $\alpha 6\beta 4$ was not described. Therefore R28 completes the vast repertoire of adhesins of *emm28* strains by binding to integrins not bound by already described adhesins, promoting adhesion to several epitheliums.

To our knowledge few bacterial adhesins bind directly $\alpha 3\beta 1$, $\alpha 6\beta 1$ or $\alpha 6\beta 4$ integrins. Only *Yersinia pestis* invasin directly binds to both the integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$ (587) and the BBB07 and BB172 proteins of *Borrelia burgdorferi* directly bind to $\alpha 3\beta 1$ (588, 589). Among viral proteins, the glycoprotein B (gB) of *Herpesviridae* binds directly to $\alpha 3\beta 1$ and $\alpha 6\beta 1$, the gB of cytomegalovirus directly binds to $\alpha 6\beta 1$ (590, 591) and Adenovirus Serotype 5 penton base protein directly binds to $\alpha 3\beta 1$ (592). No direct interaction between a viral, fungal or bacterial protein with $\alpha 6\beta 4$ has been described so far. No bacterial interaction with cells was described to involve $\alpha 6\beta 4$, but several viruses might interact with this integrin (593). A protein binding any of these three integrins is not only unique among GAS, but also rare among bacterial and viral pathogens. The mechanism of R28_{Nt} binding to these integrins has therefore no equivalent in other organisms and this interaction warrants further structural, biochemical and mechanistic studies.

***Ex vivo* infection of the decidua: strength and limits of an original model**

We developed a novel model of *ex vivo* infection of a human tissue, the decidua. The tissue comes from healthy subjects, it is a normally sterile tissue. The fibronectin layer provides an early abundant source of adhesion, which allows the infectious bacteria to settle. Decidualization is the differentiation of stromal endometrial cells in specialized cells, which support embryo growth and maintain early pregnancy (594). As described in the introduction, half of the endometritis in France are in the context of postabortum, postpartum, *in vitro* fertilization, and intra-uterine device: in all these contexts the endometrium is decidualized. Moreover, decidualization starts at the 18th day of the menstrual cycle. The decidua is therefore

a good model to study endometritis. It is immunotolerant to the fetus until labor, through the presence, among other factors, of fetal specific T regulatory lymphocytes (595, 596). However, it contains multiple cell types thought to react/control the infection of materno-fetal membrane: the decidua is an “immune-competent” tissue (583, 597, 598). These membranes constitute an immune barrier protecting mother from fetal infection, and the fetus from maternal infection. It is therefore a good model to study immune response in a tissue barrier. We infected the tissues less than three hours following the caesarians to avoid stress due to changes from *in vivo* conditions. We used a high inoculum and only 10 % of the bacteria adhere to the tissue within the first hour in static conditions. Moreover, static infection implies accumulation of host and bacterial elements, enhancing their effects on the host tissue and the bacteria. During the *in vivo* routes of infection, the initial inoculum and the concentration of effectors are probably lower than the one we used in our model. However, such a high inoculum allows the appearance of reproducible phenotypes despite inherent differences between the tissues we study. In contrast, in the experimental set-up with flow conditions, we washed the tissue after 45 minutes to let a ~20% initial colonization; the wash and the flow removed bacteria in the supernatant. In these conditions, there is dilution of host and bacterial elements. *In vivo* conditions are probably reflected in part by the static and in part by the flow conditions, and both are complementary to study GAS pathogenesis.

GAS 3D colonization and multiplication at the tissue surface

In contrast to all other Gram-positive bacteria tested, GAS does not multiply in RPMI. Metabolic needs and pathways differ between these Gram-positive bacteria. Tissue secreted elements promote bacterial growth; furthermore, the supernatants of primary decidual stromal cells of HEC-1-A cells (data not shown) are sufficient to sustain it. Our study is different from Baruch and collaborators' (57), since they used a chemically defined medium devoid of the essential amino acid asparagine; the endoplasmic reticulum stress due to SLO and SLS releases asparagine, enabling bacterial growth. Our current hypothesis is that amino acid residues are not lacking in RPMI: all 20 amino acids are present, we inoculated a thousand bacteria per mL and there is no multiplication. Adding asparagine to RPMI did not trigger GAS growth (laboratory unpublished results). The only way we were able to restore bacterial growth in RPMI was by adding HEC-1-A and decidual stromal cells supernatant, the laboratory medium THY or BSA. We hypothesized that peptides, most probably di/tri/hexa peptides released by cells and imported in GAS (through the Dpp or Opp permeases) could be the missing elements for bacterial growth in RPMI. We are currently investigating whether small peptides enable

bacterial growth in RPMI. Either GAS exploits small peptides in conditioned medium, or it degrades proteins into small peptides. We will perform dialysis to remove small peptides from RPMI and test *in vitro* GAS multiplication in these depleted media. How GAS exploits host molecules for bacterial growth *in vivo* in the early steps of infections is scarcely investigated. However, multiplication is a fundamental for virulence and there is a relation between Dpp/Opp and the activation of several virulence pathways, notably the production of SpeB [(464), Introduction 3.1.4]. Therefore, acting on GAS metabolism or sensing of the host could unravel new opportunities for the development of treatments against GAS infections.

We showed that GAS is able to form biofilms at the surface of the decidua; we analyzed the dynamics of this formation and the EPS structures involved in it. Several reports describe the involvement of extracellular DNA and carbohydrates in these structures (405, 406). In our model, we did not detect extracellular DNA by DAPI staining in contrary to Siemens and collaborators (413), nor inter-bacterial concanavalin A (ConA) positive structures and we did not look at lipids. However, most already-published descriptions of GAS biofilms were made after 24-48 h after inoculation: late structures may contain extracellular DNA that 8 h biofilms do not possess. We observed ConA labelling of bacteria after infection of the decidua, but the ConA also labels several host elements. Furthermore, GAS aggregates were stained by ConA after one hour of infection, though we did not observe any EPS under SEM analysis at one hour, suggesting that ConA labels GAS surface. Considering the size of the biofilm that is present after 8 h, the structures we observe under SEM might be carbohydrates; that was not confirmed during immunofluorescence experiments, but the amount of material produced or the immunofluorescence resolution may not be sufficient to observe them. To study GAS biofilm, Lembke and collaborators (408) used multiple M-types on different *in vitro* surfaces and under static or flow conditions and assessed the biofilm structures after 24-72 h. While their conditions are unrelated to ours, they also observed at low resolution (3500X) “thread-like” structures. In their experimental approach, they only see these structures with a M2 strain seeded on a fibronectin-coated surface and under flow conditions. We also found that biofilm formation is different under flow and static conditions, with more EPS structures under flow than in its absence. Several hypotheses could explain this difference. First, some bacterial and host effectors could accumulate in static but not in flow conditions and degrade or repress the synthesis of these structures. Of note, it has been suggested that quorum sensing might be involved in biofilm formation (413). If the quorum sensing inhibits biofilm formation, diluting the supernatant with flow would decrease the amount of quorum sensing pheromones,

enhancing biofilm formation. However, this would be the opposite to what was demonstrated *in vitro*, where quorum sensing is described as an activator of biofilm formation (408). We did not observe significant changes in the dynamic of appearance of biofilm structures with the Δ SpeB mutant, compared to the wild-type strain, under SEM (data not shown), confirming that SpeB is not critical for biofilm formation as shown on 3D organotypic skin tissue (413). The flow imposed in our chamber set-up (0.3 mL/min) was only to compensate medium evaporation for confocal live imaging which probably does not provide a significant shear stress. We therefore think that the shear stress from the flow does not explain the difference in biofilm presence. Our main hypothesis is that supply in nutrients in flow conditions might lead to bacteria with differentially regulated metabolisms and different levels of production of EPS.

We identified three structures: thread-like structures, filaments and micrometric webs. Thread-like structures and filaments do not present the same diameter (20 nm vs 30 nm), localization (intra-chain vs inter-chains) and length (~200 nm vs > 1 μ m). We hypothesize that thread-like structures and filaments correspond to structures of different origins and perhaps of nature. Webs may be a late stage of filaments structures intertwined. We still distinguish these two structures because we observed filaments in several conditions, but webs only at 7 h on the subject (S#10). To further study these structures, we could compare early formation of biofilm *in vitro* in a conditioned tissue/cell medium on a fibronectin coated, or not, surface, and compare it to formation in a laboratory culture medium, BHI (409). Though GAS multiplies in both media, there could be striking differences in the amount of biofilm and structures produced due to specific signals in the conditioned media. Using only fibronectin would also avoid other host elements that are secreted by the tissue and that cover the bacteria, such as small amount of plasma derived components, enabling more precise identification of prokaryotic structures. Finally, we used SEM on ethanol fixed samples, which alters the appearance of the small structures, especially the hydrated structures. Recent advances in SEM allow acquiring samples without ethanol fixation and this would give insights on the native structures of the biofilm. Finally, we could investigate the biochemical nature of the EPS structures we observe by treating infected decidua with protease, DNase or metaperiodate (405).

We observed different kinetics of appearance of structures depending on the subjects. This could be due to different growth kinetics, higher secretion of host elements degrading the structures or promoting their synthesis, or less signals triggering biofilm formation. Host factors appear critical in the establishment of GAS biofilms in NF biopsies (413). To identify key

elements governing the formation of biofilm structures, and correlation with other phenotypes such as bacterial growth at the tissue surface, will require studying tissues from more subjects.

Invasion of the tissue and the intracellular state of GAS

Clinical samples of GAS invasive infections show high infiltration of GAS, indicating GAS can penetrate host tissue. However, studying GAS invasion of stromal tissue requires a 3D matrix, which is provided by explants. There are more invading events at 8 h than at 4 h. From all the “invading particles”, a basal level corresponds to artefacts due to the imaging and the quantification, and small breaches may enable a basal level of unspecific invading particles in the early time-points. The fact that GAS wild-type and Δ SpeB invade more at 8 h than at 4 h could have two origins. Either the tissue is more degraded at 8 h than 4 h due directly to GAS effectors, or the tissue is degraded by host metalloproteases, activated by host inflammation or by bacterial effectors. To analyze if host elements of the tissue increase the permeability of the tissue overtime, we could compare the invasion on fresh tissue and on tissue preincubated for 8 h, in which host factors could have accumulated and affected the tissue integrity. In static conditions of infection and when analyzing the invasion process, we used an excess amount of bacteria to minimize differences in colonization between the wild-type and Δ SpeB strains that could explain differences in invasion. The Δ SpeB invades less at 4 h than the wild-type, and there is a similar trend at 8 h. Therefore the level of invasion of the WT strain at 4 h is not only due to passive penetration and before 4 h, bacterial effectors, including SpeB, already exert their effects. SpeB degrades fibronectin, which we hypothesize is the major explanation for the decreased of invasion of the SpeB mutant. However, since SpeB is a broad-spectrum protease, its absence induces multiple distinct changes, and discriminating which change(s) is involved in the decreased invasion of the Δ SpeB strain is difficult to assess. Of note, SpeB regulates the GAS proteome [(401) and Introduction 2.2.4]. It would be interesting to analyze if SpeB readily degrades *ex vivo* the surface fibronectin of decidual tissue or *in vitro* a fibronectin layer. Treatment of the tissue with purified SpeB prior to infection with the Δ SpeB strain or the wild-type strain with the cysteine protease inhibitor E64 might confirm the importance of GAS degradation of the tissue for invasion. Finally, we could analyze the invasion of a SpeB expressing *Lactococcus lactis* strain compared to a control strain. The invasion events are rare at the 8 h time point, but they could be critical for GAS invasive infections. Our results confirm that during infection, GAS produces factors favoring invasion of the tissue. Clinically, this might be important events for invasive infections, where GAS penetrates the tissue, favoring the access to new sources of nutrients and increasing bacterial loads.

We were able to follow in real-time and at high resolution phagocytosis by two different types of phagocytic cells. Cytometric analysis of uninfected tissue so far did not seek neutrophils since they represent a low fraction of all cells. Our image analysis showed that these cells are highly mobile in the tissue and sometimes recruited to the surface to phagocytose bacteria. We also showed large CD45⁺ phagocytic cells with surface protrusion capture surface bacteria. We are currently investigating which type(s) of immune cells, CD11b/c⁺ and CD14⁺ performs this phagocytosis. Moreover, different reports highlight GAS capacity to survive in phagocytic cells (501), but the molecular mechanisms are still under investigation, with the M1 protein and SLO incriminated (502–506). Also, some GAS bacteria are found trapped in CD45⁺ vacuoles at very late time-points, which shows that not all bacteria escape these vacuoles. GFP-GAS trapped in these vacuoles maintained their fluorescence over several hours, which suggests the vacuole is not fully acidified, providing an explanation for their survival. Survival in phagocytic cells could also originate from a more global dysfunction of the cells, due to the stress induced by GAS pore-forming toxins and other effectors, highly suggested by our observation that several macrophage-like cells produce blebs after phagocytosis. We will track phagocytosis events at higher time/spatial resolution *in situ* and for longer periods (using 40X objective instead of 25X, and time steps of 5 minutes instead of 15 minutes), to track the bacterial future and analyze if they survive to phagocytosis and multiply inside the phagocytic cells.

GAS consequences on host cells and cytotoxicity

GAS induces dramatic cell-death through secreted molecules, including SLO. The Transwell® system avoids the contact between bacteria and the tissue. However, it also increases the distance between GAS and the tissue, potentially decreasing the diffusion of GAS effectors and explaining the slightly lower cytotoxicity when infection was carried out in the Transwell® system instead of a direct contact with the tissue.

More than half the cells are killed during a 4 h GAS infection. Of note, there is a gradient of cytotoxicity: we observed that the cells the closest to the surface are the first to die. The most abundant cells in the decidua are from top to bottom: stromal cells, then stromal and immune cells (mainly lymphocytes) and finally lymphocytes with macrophage-like cells. This might be important regarding the immune response: macrophages are the latest to suffer from the infection, and at 4 h a significant proportion of macrophages might not be affected. To confirm this hypothesis, we will specifically label macrophages (CD14), other immune cells (CD3 and

CD4) and decidual cells (Vimentin) in addition to TUNEL staining, and we will quantify the proportion of each cell type that is killed at different time-points and depending on their depth.

We observed blebbing of cells during infection with GAS. These dramatic morphological changes could arise from two mechanisms: ectocytosis, as a resistance mechanism to pore formation (Introduction, Figure 10), or cell death (599). These blebs are rare on non-infected compared to infected tissue under static conditions. However, we observed also blebbing on non-infected tissue during live acquisition, but to a lesser extent than in the presence of GAS. Since UVs from the live acquisitions (for DAPI imaging) induce apoptosis, we will compare blebbing formation between infected and non-infected tissue in the absence of UV excitation. Several events of blebbing are concomitant with DAPI incorporation in flow conditions, and for lymphocytes, nuclear shrinkage appears before blebbing: this suggests that the blebbing is linked to cell death pathways. Some of the events observed during live microscopy may be apoptotic events: we observed the formation of beaded apoptopodia on some infected tissues (data not shown), a feature of apoptosis (600). We could analyze the Caspase 3 and 7 activation on the whole tissue as an indication of apoptosis/pyroptosis, and compare the activation after infection with several mutants and in uninfected tissue. In live microscopy, we could use Nucview to follow in real-time the activation of caspases. However, we observed large blebs originating from CD45⁺ cells, which resemble macrophages, and these blebs are formed before cell death. These structures could correspond to ectocytosis and blebs as previously described (62, 63). We tried to label the A1 annexin recruitment in blebs as a marker of ectocytosis, but this was not conclusive and needs further development. We showed that both immune and non-immune cells are killed. Our approach did not allow following in real-time stromal cells death nor blebbing because there is no specific cell surface marker to label stromal cells and because DAPI is weakly excluded from these cells under flow conditions. We tried several unspecific labelling of cells: lipid dyes do not penetrate homogeneously in the tissue and molecules such as Celltracker require metabolic activity and did not stain efficiently decidual stromal cells. We could use cytometric analyses for quantifying cell death, but these analyses are highly biased, since dead cells are more sensitive to the mechanical disruption of the tissue used for the generation of the cell suspension. Such cytometric analysis would thus probably underestimate the amount of dead cells. We already performed multiple tests on the decidua, which appears to be very sensitive to all tested disruption protocols, and this was even more complicated on infected tissue with many cells lysed during the disruption of the tissue. As for phagocytosis of bacteria, our imaging set up

was not optimized to follow in real-time membrane modifications. The concomitant use of DAPI incorporation, membrane labelling (CD14, CD3 and CD4), real-time marker of apoptosis and high time and spatial resolution might indicate different changes in membranes depending on the immune cell type, and if blebbing is associated to cell death, or a form of ectocytosis.

Our Transwell® system suggests that the elements involved in cytotoxicity are secreted. SLO is a well described pore-forming toxin, but as shown in our study, though its deletion reduces significantly the cytotoxic effect of GAS, it is not the only source of cytotoxicity. Three candidates are SLS, NADase and SP-SPT (75, 76, 160, 488). We could perform the same analysis we did with strains deleted for one or many of these secreted molecules. Moreover, we could compare blebbing in live acquisition between a wild-type strain and a Δ SLO, or other mutants, which might highlight a direct involvement of bacterial effectors in the triggering of cell blebbing.

GAS pathogenesis and the host response: an intimate relation depending on the context

GAS is well-known for inducing strong inflammatory responses during invasive diseases, such as necrotizing fasciitis, potentially leading to STSS (517). Regarding GAS and puerperal fever, there are very few reports that analyzed the immune response to this infection. In a murine model of vaginal colonization, IL-17A is important for GAS colonization (601). In our model and during decidua infection with GBS, IL-17A was not upregulated, and is probably not critical for the early response to these infections (602).

GAS has a strong capacity to control, delay or destabilize the immune responses, increasing GAS ability to colonize and infect (523). In our model, GAS induces an overexpression of very few immune-related genes, less than GBS for instance (602). We infected the decidua with a higher amount of bacteria than Park *et al.*, however they used FBS in the medium which might promote bacterial growth yielding a number of bacteria equal or higher to that we used. We will analyze the overexpression of several genes after infection with GBS in the same condition as GAS to refine our comparison between GAS and GBS infections. The latter activates the expression of 32 genes out of 133, 9 in common with GAS infection. Notably, GBS specifically activates the expression of IL-6, TLR2, TLR6, NLRP3 and NFKPB1, CD80. The fact IL-6 is not activated ($p=0.24$ by GAS compared to $p=0.0000001$ for GBS) is the most striking result: TNF is highly overexpressed and together with IL-6 they correspond to the early acute response to infection. CD80 is a marker of activation of antigen presenting cells, required for the activation of effector T cells (603). Since recruitment of

circulating immune cells is impossible in explants, an increase in whole tissue of CD80⁺ cells indicates a differentiation of some of immune cells, consequent to inflammation and/or microbial recognition. Leucocytes represent 40 % of the tissue cells, and 70 % of these leucocytes are lymphocytes but no lymphocyte is naïve (personal communication). The absence of significant overexpression of TLR, NLRP and NFκB shows that lymphocyte did not respond to GAS infection in contrast to GBS. Either these cells are more sensitive to GBS than to GAS antigens, or GAS is able to inhibit their activation. After infection with GAS, there is only the primary tissue response (cytokines, including many chemokines of the same family, the MIP), but no development of the long-term immune response. Finally, the only cytokine overexpressed in GAS but not in GBS is IL-10, an anti-inflammatory cytokine. It is striking that an infection triggers the production of an anti-inflammatory molecule early during the infection. However, superantigens are known to induce the secretion of IL-10 in immune cells (521), which could contribute to the overall tempering of the immune response. We will test the ability of GAS to temper the immune response by co-infecting the tissue with GAS and GBS, and compare the activation of several cytokine genes (genes coding for IL-6, IL-1β, TNF, Nlrp3 etc..) to uninfected tissue, GAS or GBS infections at 8 h. GAS ability to silence lymphocyte (re)activation could occur by multiple mechanisms, either by killing these cells before they can react, or by the secretion of superantigens that induces anergy to lymphocytes (520). Moreover, GAS infection results in a significant increase in the concentration of several pro-inflammatory molecules: TNF, CCL3 and IL-1β. While higher than in the absence of infection, the levels of these cytokines could still be lowered by GAS. The difference we observed when the infections were carried out with the SLO and SpeB mutant strains are very minor compared to the overall control of the immune response. We should therefore test the importance of other GAS factors, such as superantigens, to understand how GAS controls the tissue response.

One major limitation of our study is that we used whole RNA analysis, which limits the sensitivity, and we could miss important changes in expression in small cell subsets. As shown with the infection of the decidua with GBS, this approach still allows to identify many genes upregulated after infection with a pathogen (602). RNA analysis after cell sorting could enrich for immune cells. However, this approach involves enzymatic and mechanical disruption of the tissue, which eliminates fragile or damaged cells. We could use *in situ* hybridization to identify which cells express/overexpress specific genes on the whole tissue after infections (604).

In contrast to infection with the wild-type strain and the SpeB mutant, infection with the SLO mutant yielded lower concentration of cytokines in the supernatant than in the absence of infection. This indicates that the absence of accumulation of cytokines with the wild-type strain is the concomitant result of mechanisms that increase and decrease the amounts of cytokines, and that SLO is involved in a pro-inflammatory response. Stressed and dead cells release pro-inflammatory signals, and by having a delayed kinetic and lower intensity of cytotoxicity, the infection in the absence of SLO could result in lower induction of inflammation (605). In apparent contrast, at 8 h, in experiments on three subjects out of four, the amounts of cytokines accumulated after infection is higher in the absence of SLO than in its presence. We hypothesize that SLO mediated cell-death affects the 8 h time-point immune population involved in the secretion of these cytokines. By reducing the pool of secreting cells, SLO reduces the mounting of the inflammatory response.

The SpeB mutant presents higher accumulation of cytokine at 8 h but not 4 h. SpeB degrades several pro-inflammatory cytokines, including the overexpressed CXCL1, CXCL2 and CCL20 (101). However, by acting on some but not all cytokines, SpeB might reduce the activation of immune cells, further decreasing the production of cytokines. One reason for SpeB absence of effect at 4 h might result from its lower gene expression since *speB* is highly expressed during the stationary phase. Lower concentrations of SpeB and cytokines at 4 h than at 8 h may not be sufficient to observe an effect of SpeB on cytokine accumulation at 4 h. SpeB is not the only protease involved in the degradation of cytokines; we could test the importance of SpyCEP on cytokine accumulation (167).

Finally, we only tested the effect of mutant strains on genes that were overexpressed during infections with the wild-type strain. However, some genes not overexpressed in these conditions may be overexpressed during infection with the SLO and SpeB mutants, and similarly for the accumulation of different cytokines. RT-profiling with the mutants at 8 h could be performed to analyze these possibilities.

The implication of differences in host and bacterial genetic backgrounds in the pathogenesis

GAS efficiently multiplies at the tissue surface except with one subject (S#7). *In vitro* growth in the supernatant of uninfected tissue depends on the subject and one of them that elicited lower growth, compared to other subjects, presented a higher amount of cells with a “macrophage” signature. The levels of expression and production of cytokines in the absence

of infection differ between the subjects, demonstrating difference in the immune status of the subjects. We analyzed the available clinical information on patients, and nothing was significantly different from the other subjects: it is crucial to analyze the specificities of each subject at the tissue level to understand the differences of bacterial phenotypes. We could better characterize the tissue in the absence of infection, by flow cytometry to enumerate the different cell subsets, by analyzing the levels of expression of key genes by qPCR, by analyzing the tissue secretion of anti-microbial peptides, which might enlighten our understanding of host properties favoring or limiting GAS pathogenesis. In a longer time, we could also compare the differences between the genetic backgrounds of the subjects (528).

We focused our study on the *emm28* genotypes, which is an E pattern genotype highly prevalent in Europe but poorly studied in comparison to the *emm1*, *emm6*, *emm3* (A pattern) or *emm89* strains (E pattern). All *emm*-genotypes harbor specific repertoires of virulence factors and the *emm28* might be specifically equipped for infection of the gynecological sphere. The high efficiency of infection we observed on the decidua might be specific to this genotype. For example, the capacity of formation of biofilm might be *emm28*-specific. *In vitro* studies on GAS formation of biofilm point out that some strains are, or not, biofilm producers (408, 409). Moreover, the *in vitro* capacity of GAS strains to form biofilm does not correlate with their ability to form biofilms in biopsies (413). While R28 is specific to *emm28* strains, the study we performed on the tissue did not focus on *emm28*-specific factors: SpeB and SLO are expressed by all clinical strains. However, the factors influencing the regulation of expression of *slo* and *speB* depend on the genotype considered (see Introduction 2.2). Although being expressed by all strains, their importance in the infection may be different in other *emm*-types. We could perform infection of the decidua with strains from other *emm*-types to study the specificity of the *emm28* wild-type phenotypes. In addition, we used a representative strain of a collection of *emm28* strains, and we could compare the phenotypes of different strains of this collection.

We explored the possibility that R28 contributes to GAS *emm28* strains association with endometritis. We showed that R28 might be involved in the *emm28* association with endometritis by increasing adhesion to cells of the gynecological sphere. Studying factors independently may hide that a combination of factors promotes infections in the specific environment of the gynecological sphere. Surface proteins like M28, Mrp28, Enn28, Epf or RD2-encoded proteins such as, in addition to R28, Ag I/II and other yet uncharacterized putative surface proteins, might contribute, alone or together, to increasing adhesion to cells of gynecological sphere. However, our understanding of GAS *emm28* association with

endometritis might not rely uniquely on its capacity to adhere, and other specificities of this environment might specifically favor this *emm*-type, such as the acidity, osmolality of the endometrium.

General perspectives

This study was performed on human explants. As specified in the introduction, only few studies of GAS were done on human explants, such as skin and tonsil. However, our study developed numerous tools that could be applied to these other tissues and might improve our understanding of the early steps of skin/throat infections. We can apply similar approaches on other explants to determine the specificity of the decidua in the phenomena described and we could extend our discoveries to other superficial and invasive infections.

Explant studies provide the ability to analyze the tissue-specific response without being “polluted” by recruitment of circulating cells. Yet, this also implies that we see differential secretion of cytokines without analyzing if it actually significantly delays the immune response, for example by decreasing the immune cells recruitment. The only highly motile cells in the tissue are cells with several nucleus lobes, probably neutrophils. However, these cells are rare and the few movements in the tissue we analyzed were hectic and complex since the tissue is not flat and the time steps we used were too long (30 minutes) to track cells. Apart from isolated events, we did not witness significant immune cell recruitment, and to analyze it, we could use xenografting of explants on mice, as already done with skin samples (See introduction 4.2.1).

This study mainly focused on the endometritis. However, several phenomena we describe may not rely on properties specific to the decidua and may consequently reflect what occurs during GAS invasive infections. What struck us when performing this study on the decidua was, despite inherent genetic differences between subjects, the robustness of all the phenotypes we analyzed: this reminds the fact that GAS is a remarkable and well-equipped bacterium able to face various situations with high efficiency. GAS is a rare example of a widely spread human-specific pathogen which is not a commensal and has a parasitic mode of dissemination: its main dissemination route is superficial infections of the skin and the throat. Its rare lifestyle makes this pathogen an interesting source of fundamental questions on co-evolution and virulence strategies, while making highly complex the battle to reduce this pathogen burden on health and disease.

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Résumé

Streptococcus pyogenes, également appelé Streptocoque du Groupe A (SGA), est un pathogène à l'origine d'une grande diversité d'infections, allant d'infections superficielles comme l'angine aux infections invasives, comme la fasciite nécrosante et les endométrites. Au 19^{ème} siècle, une femme sur dix mourait après l'accouchement de fièvre puerpérale, notamment d'endométrite. En France, les infections gynéco-obstétricales correspondent encore de nos jours à 27 % des infections invasives à SGA chez les femmes. Les souches de SGA présentent une forte diversité génétique et de répertoire de facteurs de virulence. Le génotype *emm28* est le troisième génotype le plus prévalent en France et il est associé aux endométrites. Nous avons analysé par deux axes complémentaires les facteurs et mécanismes impliqués dans les endométrites à SGA.

Par des approches de biochimie et de biologie cellulaire, nous avons caractérisé l'interaction entre les cellules de l'hôte et R28, une protéine de surface spécifique du génotype *emm28*. Le domaine N-terminal de R28 (R28_{Nt}) et ses deux sous-domaines favorisent la fixation des bactéries à des cellules endométriales, cervicales et déciduales. Ils fixent de manière directe les intégrines $\alpha3\beta1$, $\alpha6\beta1$ et $\alpha6\beta4$. Par ailleurs, R28_{Nt} promeut aussi l'adhésion à des cellules épithéliales de la peau et des poumons. Ces résultats suggèrent que la fixation des intégrines par R28_{Nt} concourt, non seulement, aux endométrites dues au génotype *emm28*, mais aussi, et de manière plus générale, à la prévalence de ce génotype.

Afin de mieux caractériser les étapes précoces essentielles au développement des endométrites à SGA, nous avons développé un modèle original d'infection : nous infectons *ex vivo* la décidue humaine, qui correspond à la membrane utérine durant la grossesse. Nous avons analysé les effets de l'infection de la décidue par des techniques de microscopie et d'analyse d'image de pointes. SGA adhère au tissu et se multiplie au contact de celui-ci grâce à des éléments sécrétés par le tissu. Sur ce tissu, SGA forme des biofilms composés d'ultrastructures ressemblant, pour certains, à des fils reliant deux coques d'une même chaîne et, pour d'autres, à des filaments reliant plusieurs chaînettes ; certains s'organisent en réseau. GAS envahit en profondeur le tissu, ce qui dépend de l'expression de la cystéine protéase SpeB. SGA induit la mort de la moitié des cellules en moins de 4 h à travers la sécrétion de différents facteurs, dont la Streptolysine O (SLO). Enfin, GAS est capable de restreindre la réponse immunitaire du tissu à l'échelle transcriptomique et protéique, le contrôle protéique dépendant de l'expression de SLO et de SpeB.

Abstract

Streptococcus pyogenes (Group A Streptococcus, GAS), is a Gram-positive pathogen responsible for a wide range of diseases, from superficial infections such as pharyngitis to invasive infections such as necrotizing fasciitis and puerperal fever that includes endometritis. Puerperal fever was a huge social burden in the 19th century, killing one woman out of ten after delivery, and gyneco-obstetrical infections still correspond to 27% of woman GAS invasive infections in France. GAS strains are genetically diverse and harbor specific virulence factors repertoires. *emm28* is the third most prevalent genotype in France and it is associated with endometritis. By two complementary axes, we analyzed factors and mechanisms involved in GAS endometritis.

Using biochemical and cellular approaches, we characterized the interaction between *emm28*-specific surface protein, R28, and host cells. R28 N-terminal domain, R28_{Nt}, and its two subdomains promote the binding to endometrial, cervical and decidual cells. They directly interact with the integrins $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$. R28_{Nt} also promotes adhesion to pulmonary and skin epithelial cells. Our results suggest that R28_{Nt}-integrin interactions contribute not only to *emm28*-elicited endometritis, but also to the overall prevalence of the *emm28* strains.

To further characterize the initial events involved in the establishment of GAS endometritis, we developed a novel infection model: we infected *ex vivo* the human decidua, the mucosal uterine lining during pregnancy. We analyzed the outcome of the infection using state-of-the-art imaging set-up, image processing and analysis. GAS adheres to the tissue and grows at its surface; secreted host factors promote this growth. GAS readily forms biofilm at the tissue surface; thread-like and inter-chains filaments ultra-structures compose these biofilms. GAS invades the tissue and this depends on the expression of the cysteine protease SpeB. GAS induces the cell death of half of the cells within 4 h and this cytotoxicity depends on secreted factors, including the Streptolysin O (SLO). Finally, GAS restrains the tissue immune response at the transcriptional and protein levels, the latter depending on the expression of SLO and SpeB.

PhD dissertation by Mr Antonin WECKEL

“Molecular mechanisms of *Streptococcus pyogenes* tissue colonization and invasion”

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