Boosting Immunity against Group A Streptococcus

Boosting Immunity against Group A Streptococcus:

Insights into immune response variation across immunization regimens

Shiva Emami

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In the first paper, we conducted subcutaneous immunization on a mouse model to explore the cellular and molecular basis for protective immune responses against GAS-M1. We found that the effectiveness of protection isn't determined solely by the amount of antibody produced, but rather by specific subtypes of protective antibodies. Our research indicates that incorporating the 2W T cell epitope into the M protein brings about crucial qualitative changes in the adaptive immune response against GAS. The insertion of the 2W peptide could potentially enhance the expression of IFN-γ-promoting T-helper cell epitopes within the M protein. This action might disrupt evasion mechanisms employed by the pathogen and boost the effectiveness of attack by complement-fixing antibodies. Our study revealed that a original M1 GAS strain triggers an antibody response that doesn't offer protection. However, when we introduced the immunodominant 2W T helper cell epitope into the M protein of an identical strain, the resulting immune response provided protection against the original non-recombinant M1 GAS strain. Even though both strains induced similar levels of total anti-GAS IgG antibodies, only the strain carrying the 2W epitope increased levels of complement-fixing IgG2c antibodies.

In paper II, the nature of protective immunity against GAS generated with i.p immunization was analyzed. We demonstrate that multiple immunizations are required for the ability to survive a subsequent lethal challenge. our data show that the protection in this model is independent of adaptive immunity and relies on macrophages and the macrophage-activating cytokine IFN-γ.

In the last study, we investigated different adjuvants' effectiveness in enhancing immune responses against GAS infection. We found that adding Poly I:C adjuvant during the immunization significantly enhanced protection against GAS-M1 infection. The study also emphasized the critical role of IFN-γ in protecting against GAS infection. These findings underscore the importance of adjuvant selection and cytokines in developing effective treatments and vaccines.

This thesis offers valuable insights into the development of protective immunity against GAS, paving the way for further research to understand the mechanisms that underlie protection against this bacterium. Understanding these mechanisms is crucial for the development of a functional vaccine with high efficacy.

Key words: Group A Streptococcus (GAS), T cells, B cells, Macrophage, IFN-γ, Antibodies, IgG2c, M protein, Immunization, Protection, lethal infection, survival.

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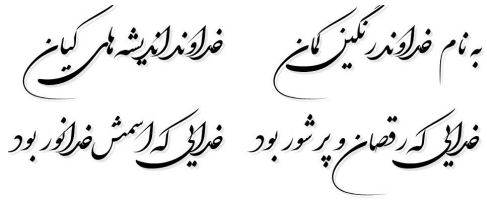

Dedicated to my mother and to my love, Mehrdad♡

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Layman's Summary

Understanding Streptococcus pyogenes: A Tricky Bacterium

What makes Streptococcus pyogenes especially concerning is that there is currently no vaccine available to prevent infections caused by this bacterium.

One of the tricky aspects of this bacterium is its ability to exist harmlessly on the skin surface or in other parts of the body without causing any noticeable symptoms. However, if the immune system is weakened or compromised due to factors such as illness, stress, or certain medications, Streptococcus pyogenes can seize the opportunity to invade and cause infection.

Streptococcus pyogenes is what we call an opportunistic pathogen. This means that it bides its time, waiting for the perfect opportunity to strike its target, which in this case, is the human body. It's like a sneaky intruder waiting in the shadows until it finds a weak point in our defences.

But how does Streptococcus pyogenes (Group A Streptococcus or GAS) manage to evade the immune defence system and cause infections? There are many different strategies that these bacteria use to stay undetected by immune system, one of those tactics is having a protein call M-protein on its surface. This surface-protein has a hyper variable region (HVR) at its end [3] which changes a lot and make more than 200 serotypes of M protein. When immune system detects one serotype of GAS strain, this variation helps bacteria to escape from immune system for the next infection.

In our initial project, we aimed to assist the immune system in detecting M protein more effectively. To achieve this, we introduced a special peptide known as the 2w peptide into the M protein. This peptide has the ability to activate T-cells, which in turn triggers an immune response. Upon completing this modification, we observed a significant improvement in protection against GAS upon subcutaneous (s.c) immunization.

Interestingly, we found that having a high level of antibodies doesn't always guarantee protection. This highlights the distinction between an effective and an ineffective vaccine. Although vaccination may result in a high antibody level, it doesn't necessarily lead to protection. An efficient vaccine is one that stimulates the production of protective antibodies. We found that the insertion of the 2w peptide led to an increase in the levels of IgG2c antibodies that can be connected to the observed protection.

It is known that the immune system can respond differently depending on where the pathogen enters the body. In our second project, we explored how the immune system responds to intraperitoneal (i.p.) immunization and the responsible immune mechanism providing protection against GAS infections. We discovered that the type of immune response is different after i.p immunization, compared to s.c

When antigens were introduced intraperitoneally, antibodies and the adaptive immune response appeared to play no role. Instead, protection against the infection relied heavily on the activity of macrophages and the presence of cytokines such as IFN-γ.

In vaccine production, there are substances called adjuvant, added to vaccines to bolster the body's immune response to a targeted antigen or pathogen. This adjuvant can be made of different substances like sugars or nucleic acids, which can activate certain immune cells.

In our third project, we wanted to see how the immune system responds when we immunize using only the pure M protein along with an adjuvant. We discovered that when we added a Poly I:C adjuvant, it significantly increased the protective immune response and improved protection against GAS infection. This finding highlights the importance of using adjuvants in vaccine development, as they can enhance the effectiveness of the treatment.

In conclusion, GAS is a formidable opponent that challenges our immune system's ability to defend against harmful invaders. Its opportunistic nature and ability to evade detection make it a tricky bacterium to deal with. While there is currently no vaccine for this bacterium, understanding how it infects and how the immune system reacts to it through different routes is crucial. This thesis provides a new perspective on how the immune response can vary in its reaction to GAS depending on the entry route and discover the mechanisms underlying that. Understanding these immune mechanisms helps scientists pave the way for developing effective vaccines that offer strong protection against GAS infections.

Populärvetenskaplig sammanfattning

Förstå Streptococcus pyogenes: En knepig bakterie

Vad som gör Streptococcus pyogenes särskilt oroande är att det för närvarande inte finns något vaccin tillgängligt för att förebygga infektioner orsakade av denna bakterie.

En av de knepiga aspekterna med denna bakterie är dess förmåga att existera harmlöst på hudytan eller i andra delar av kroppen utan att orsaka några märkbara symtom. Men om immunförsvaret försvagas eller kompromitteras på grund av faktorer som sjukdom, stress eller vissa mediciner, kan Streptococcus pyogenes gripa chansen att invadera och orsaka infektion.

Streptococcus pyogenes är vad vi kallar en opportunistisk patogen. Det betyder att den väntar på det perfekta tillfället att slå till mot sitt mål, vilket i detta fall är människokroppen. Den är som en listig inkräktare, lurande i skuggorna tills den hittar en svag punkt i vårt försvar.

Men hur lyckas Streptococcus pyogenes (Group A Streptococcus or GAS) undvika immunförsvaret och orsaka infektioner? Det finns många olika strategier som denna bakterie använder för att förbli oupptäckt av immunförsvaret, en av dessa taktiker är att ha ett protein som kallas M-protein på dess yta. Denna ytantigen har en hypervariabel region (HVR) vid dess ände som ändras mycket och skapar mer än 200 typer av M-protein. När immunförsvaret upptäcker en typ av GAS-stam, hjälper denna variation bakterier att undkomma immunförsvaret för nästa infektion.

I vårt inledande projekt syftade vi till att hjälpa immunförsvaret att upptäcka M-protein mer effektivt. För att uppnå detta introducerade vi en speciell peptid som kallas 2w-peptid i M-proteinet. Denna peptid har förmågan att aktivera T-celler, vilket i sin tur utlöser en immunreaktion. Efter att ha slutfört denna modifiering observerade vi en signifikant förbättring av skyddet mot GAS vid subkutan (s.c) immunisering.

Intressant nog fann vi att en hög nivå av antikroppar inte alltid garanterar skydd. Detta belyser skillnaden mellan en effektiv och en ineffektiv vaccinering. Även om vaccination kan resultera i en hög nivå av antikroppar, leder det inte nödvändigtvis till skydd. Ett effektivt vaccin är ett som stimulerar produktionen av skyddande antikroppar.Vi fann att införandet av 2w-peptiden ledde till en ökning av nivåerna av IgG2c-antikroppar som kan kopplas till det observerade skyddet.

Det är känt att immunförsvaret kan reagera olika beroende på var patogenen kommer in i kroppen. I vårt andra projekt utforskade vi hur immunförsvaret reagerar på intraperitoneal (i.p.) immunisering och det ansvariga immunmekanismen som ger skydd mot GAS-infektioner. Vi upptäckte att typen av immunsvar är annorlunda efter intrauterin immunisering jämfört med s.c.

När antigen introducerades intraperitonealt verkade antikroppar och det adaptiva immunsvaret inte spela någon roll. Istället förlitade sig skyddet mot infektionen i hög grad på aktiviteten hos makrofager och närvaron av cytokiner som IFN-γ.

I vaccinproduktionen tillsätts ämnen till vacciner för att stärka kroppens immunreaktion mot en målinriktad antigen eller patogen. Denna adjuvans kan vara gjord av olika ämnen som socker eller nukleinsyror, vilka kan aktivera vissa immunceller. I vårt tredje projekt ville vi se hur immunförsvaret svarar när vi immuniserar enbart med det rena M-proteinet tillsammans med en adjuvans. Vi upptäckte att när vi tillsatte en PIC-adjuvans ökade den betydligt den skyddande immunreaktionen och förbättrade skyddet mot GAS-infektioner. Denna upptäckt understryker vikten av att använda adjuvanser i vaccinutveckling, eftersom de kan öka behandlingens effektivitet.

Sammanfattningsvis är GAS en formidabel motståndare som utmanar vårt immunförsvar att försvara mot skadliga inkräktare. Dess opportunistiska natur och förmåga att undgå upptäckt gör det till en knepig bakterie att hantera. Även om det för närvarande inte finns något vaccin mot denna bakterie är förståelsen för hur den infekterar och hur immunförsvaret reagerar på den genom olika vägar avgörande. Denna avhandling ger ett nytt perspektiv på hur immunsvaret kan variera i sin reaktion på GAS beroende på inträdesvägen och upptäcker de mekanismer som ligger till grund för det. Förståelsen av dessa immunmekanismer hjälper forskare att banbryta för utvecklingen av effektiva vacciner som erbjuder starkt skydd mot GAS-infektioner.

Abstract

The common pathogen Group A Streptococcus (GAS, Streptococcus pyogenes) is an extracellular bacterium and is linked to various infectious diseases of varying severity. The M protein, which is exposed on the surface, is a significant virulence factor of GAS and is also a target for antibodies that provide protection. Despite its significant impact on society and extensive research efforts, an effective vaccine against GAS has yet to be developed. This highlights the necessity for further understanding of the immunological mechanisms that govern protective immunity. GAS strains can be classified into over 200 different emm (M)-types, and it's believed that protective immunity against GAS relies partly on antibodies that are specific to each type. In our study, we focused on the M1 serotype of GAS (GAS-M1), known to be one of the most aggressive strains of the bacterium.

In the first paper, we conducted subcutaneous immunization on a mouse model to explore the cellular basis for protective immune responses against GAS-M1. We found that the effectiveness of protection isn't determined solely by the amount of antibody produced, but rather by specific subtypes of protective antibodies. Our research indicates that incorporating the 2W epitope into the M protein brings about crucial qualitative changes in the adaptive immune response against GAS. The insertion of the 2W peptide within the M protein could potentially enhance the expression of IFN-γ-promoting T-helper cell. This action might disrupt evasion mechanisms employed by the pathogen and boost the effectiveness of attack by complement-fixing antibodies. Our study revealed that a original M1 GAS strain triggers an antibody response that doesn't offer protection. However, when we introduced the immunodominant 2W T helper cell epitope into the M protein of an identical strain, the resulting immune response provided protection against the original non-recombinant M1 GAS strain. Even though both strains induced similar levels of total anti-GAS IgG antibodies, only the strain carrying the 2W epitope increased levels of complement-fixing IgG2c antibodies.

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This thesis offers valuable insights into the development of protective immunity against GAS, paving the way for further research to understand the mechanisms that underlie protection against this bacterium. Understanding these mechanisms is crucial for the development of a functional vaccine with high efficacy.

Abbreviations

APC	Antigen presenting cell
ARF	Acute rheumatic fever
C4BP	C4b-binding protein
CD	Cluster of differentiation
CR	Complement receptor
DAMP	Danger-associated molecular pattern
DAF	Decay accelerating factor
DCs	Dendritic cells
DT	Diphtheria toxoid
ECM	Extracellular matrix
FH	Factor H
Fn	Fibronectin
GAS	Group A streptococcus
GBS	Group B Streptococcus
HA	Hyaluronic acid
HVR	Hypervariable region
Ig	Immunoglobulin
IL	Interleukin
LTA	Lipoteichoic acid
MAC	Membrane Attack Complex
MASP	MBL-associated serine protease
MCP	Membrane cofactor protein
MHC	Major histocompatibility complex
MMPs	Host matrix metalloproteinases
NF	Necrotizing fasciitis
NK	Natural killer cells

NLRs	NOD-like receptors
OF	Opacity factor
PAMP	Pathogen-associated molecular pattern
PRRs	Pattern-recognition receptors
PSGN	Post-streptococcal glomerulonephritis
RLR	RIG-I like receptors
RLRs	RIG-I like receptors
SAVAC	Streptococcus Vaccine Accelerator Consortium
Ska	Streptokinase
SLO	Streptolysin O
SLS	Streptolysin S
SOF	Serum opacity facto[2]r
Spe	Streptococcal pyrogenic exotoxin
SpeB	Streptococcal pyrogenic exotoxin B
SpyCEP	GAScell-envelope protease
SSA	Streptococcal superantigen
STSS	Streptococcal toxic shock syndrome
Tfh	T follicular helper
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 Streptococcus pyogenes infections

1.1 Epidemiology

Streptococcus pyogenes or Group A Streptococcus (GAS) is a Gram-positive bacterium with facultative anaerobic characteristics, demonstrates chain-like growth patterns and exhibits beta-hemolysis on blood agar plates. It holds the 9th position among the deadliest pathogens worldwide, causing approximately 500,000 deaths each year. This bacterium is a notable human pathogen, capable of inducing a broad range of diseases in immunocompetent individuals. These ailments span from non-invasive superficial infections of the skin and throat, like impetigo and pharyngitis, to severe and life-threatening conditions such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) [4]. Although severe invasive streptococcal infections like NF and STSS are uncommon, affecting roughly three individuals per 100,000, they still pose significant health risks due to their considerable morbidity and mortality rates. Despite receiving intensive care and antibiotic treatment, the overall fatality rate can reach up to 50% [5].

Various serological and genotyping methods can be utilized to identify and characterize GAS isolates. One of the traditional and widely used techniques is M-typing, which was established by Rebecca Lancefield in the 1920s. This method relies on the M-protein present on the bacterial surface and employs type-specific sera targeting the hyper-variable region [6]. Another serological typing approach targets the T-protein, an alternative surface protein, or the serum opacity factor, a lipoproteinase expressed by approximately half of GAS strains, which leads to increased serum opacity [7].

Currently, the most common method for typing GAS strains involves sequencing the hyper-variable portion of the emm gene, responsible for encoding the M-protein [8]. This approach has identified over 200 different emm types [9]. Antibodies generated against the hyper-variable part of the M-protein are specific to a particular emm type. Consequently, individuals infected with a specific emm type typically develop protective antibodies against that emm type but remain susceptible to other types [10].

Epidemiological studies on GAS strains have demonstrated that the prevalence of different M-types can vary over time and across different geographic areas. Additionally, certain M-types have been associated with specific clinical presentations. For example, M1 and M3 types are frequently observed in severe invasive infections such as NF and STSS [11, 12].

1.2 GAS carriage and induced diseases

1.2.1 Asymptomatic colonization

Throat colonization or carriage of GAS without exhibiting symptoms is a common occurrence. A meta-analysis has revealed that approximately 10% of children and 6% of adults in high-income countries, as well as 2.8% of children and 2% of adults in low-income countries, are colonized by GAS [13]. Some individuals can remain colonized for extended periods, spanning multiple years. The exact reasons behind this persistent state are not yet well understood. However, it is worth noting that this prolonged colonization does not seem to play a role in post-infectious manifestations or bacterial transmission [14, 15]. Furthermore, among carrier, there is a frequent occurrence of switching between different emm-types throughout their lives [16].

1.2.2 Superficial infections

Pharyngitis is associated with GAS infection and refers to a superficial infection of the oropharynx (Figure 1A). GAS is responsible for approximately 4-10% of pharyngitis cases in adults [17]. Worldwide, GAS is involved in an estimated 616 million cases of pharyngitis annually [4]. It is worth noting that the prevalence of pharyngitis tends to be higher in OECD (Organization for Economic Cooperation and Development) countries compared to non-OECD countries [13].

Diagnosing GAS pharyngitis can be complex due to the fact that most cases of pharyngitis are not caused by GAS, and GAS can be present asymptomatically in the throat. As a result, a positive swab for GAS does not necessarily indicate that the pharyngitis is specifically caused by GAS. This discrepancy between a positive GAS swab and serologically confirmed GAS-elicited pharyngitis accounts for only about 50% of cases matching [13].

Scarlet fever: In some cases, GAS pharyngitis coincides with scarlet fever, believed to arise from a pharyngeal infection caused by a GAS strain producing bacteriophage-encoded streptococcal pyrogenic exotoxins, with SpeA being the most notable [18-20]. Also referred to as scarlatina, scarlet fever is characterized by a deep red, finely popular, erythematous rash, often accompanied by a "strawberry tongue" and exudative pharyngitis (Figure 1B) [21]. Scarlet fever can affect people of all ages but is most common in school-age and adolescent children due to the ease of transmission in classrooms and daycares and their lack of immunity to GAS. GAS is not the cause of all pharyngitis cases in children but is responsible for 15-30% of pharyngitis cases in those aged 5-15 years [22]. While scarlet fever posed a considerable threat to childhood health and mortality during the 19th and early 20th centuries, global rates have steadily declined over the past 150 to 200 years, leading to its classification as a relatively rare disease until recently [23]. However, recent outbreaks of scarlet fever in Hong Kong and mainland China highlight that it continues to pose a significant health concern.

Since September 2022, a notable scarlet fever outbreak in children has occurred in Europe and the U.S. This outbreak is notable for its deviation from typical seasonal patterns and has been associated with higher-than-normal mortality rates [22].

While the scarlet fever rash is not harmful, it signals Group A Strep infection, which can lead to serious invasive and fatal diseases like necrotizing fascilitis or toxic shock syndrome if untreated.

Impetigo and erysipelas (Figure 1C and D) are additional skin infections caused by GAS, typically presenting as superficial conditions without bloodstream involvement. Impetigo is a contagious skin infection characterized by pustules that enlarge and rupture, leading to the formation of thick, honey-colored scabs. The infection spreads through direct skin contact and primarily affects children in tropical and subtropical regions with inadequate hygiene and crowded living conditions [24]. In contrast to pharyngitis, impetigo is more prevalent in non-OECD countries, and it is estimated that there are approximately 111 million cases of GAS-elicited impetigo worldwide each year [4].

1.2.3 Invasive infections

GAS invasive infections refer to infections where GAS is detected in normally sterile areas or when accompanied by Streptococcal Toxic Shock Syndrome (STSS). These types of infections are responsible for approximately 163,000 deaths each year [4]. In France, the annual incidence of GAS invasive infections is estimated to be 3.1 per 100,000 population, with a case-fatality ratio of 14% [25]. It is worth noting that the global incidence of invasive infections has been increasing since the 1990s, and in France, there has been a 4% annual increase between 2007 and 2014, primarily driven by a rise in infections among individuals over 65 years of age.

Necrotizing fasciitis (NF) is a severe infection that affects the deeper layers of the skin, below the fascia (Figure 1E). It is a rapidly progressing and life-threatening condition, with a mortality rate of approximately 30% [26]. In the United States, there are approximately 700 cases of NF reported annually, while in France, there were 104 cases of GAS-elicited NF in 2007, with a fatality rate of 22% [25, 27]. Risk factors for NF include varicella (chickenpox), wounds, burns, and blunt trauma [28].

Bacteremia refers to the presence of bacteria in the bloodstream and is associated with 60% of invasive GAS infections in France (https://cnr-strep.fr/). In some cases of bacteremia, the source of bacterial translocation is unknown, leading to a classification of "bacteremia without identified focus." This category accounts for approximately 22% of French GAS invasive infections [25].

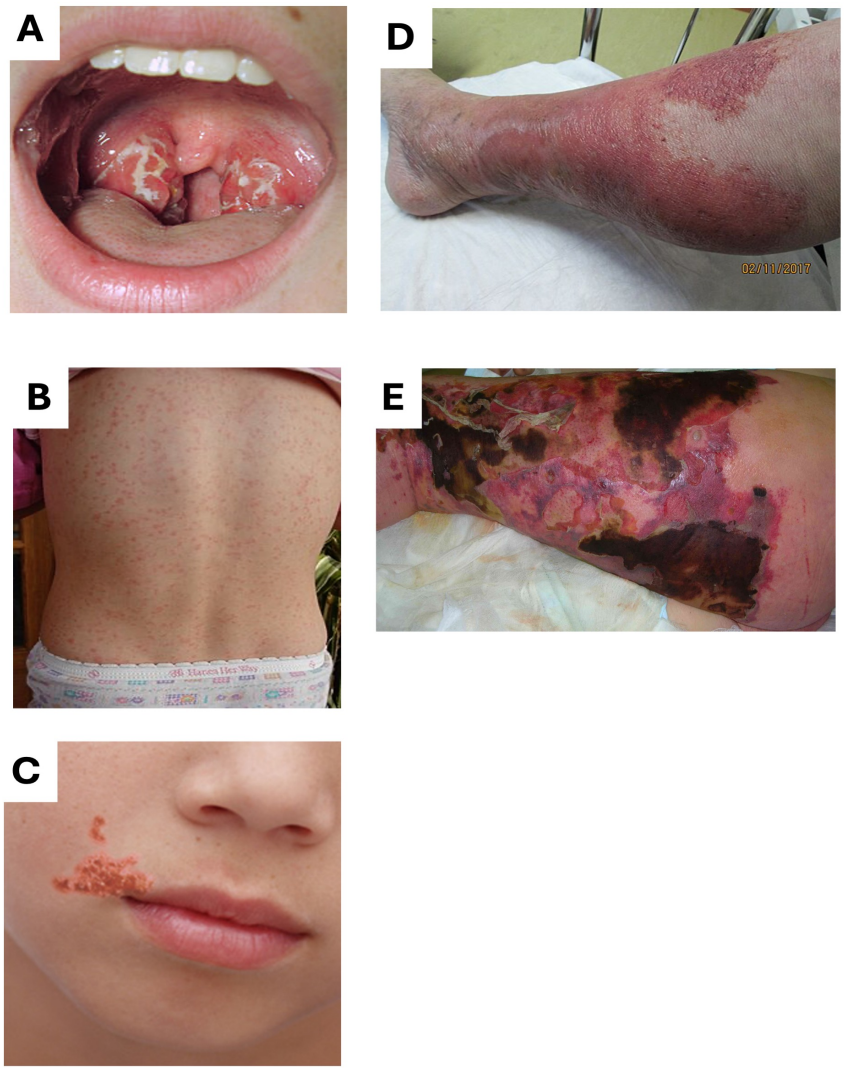

Figure 1. Picture examples of GAS related diseases. (A) Pharyngitis [29].
(B) Scarlet fever [30]. (C) Impetigo [31]. (D) Erysipelas [32]. (E) Necrotizing Fasciitis [33].

Streptococcal Toxic Shock Syndrome (STSS) is the most severe and life-threatening complication that can arise from GAS infections. It occurs when bacterial toxins are present in the bloodstream or tissues, leading to the hyperactivation of the immune system. This immune response triggers a cascade of events, including a cytokine "storm," which can result in shock and subsequent organ failures. Septic shock, specifically STSS, is the term used to describe this condition. STSS carries a high mortality rate, with rates reaching up to 43% [25].

1.2.4 Post-infectious complications

GAS infections can give rise to various pathologies as a result of an indirect process. Conditions such as glomerulonephritis and rheumatic arthritis fall into this category. These complications are considered autoimmune diseases since they occur due to a cross-reaction between epitopes of GAS and the host's own epitopes [34]. Collectively, these post-infectious complications contribute to approximately 354,000 deaths annually. The incidence of these complications is particularly high in developing countries, with an estimated 15 to 16 million individuals suffering from GAS-induced rheumatic heart diseases and 282,000 new cases emerging each year [4].

2 Pathogenesis

GAS demonstrates a remarkable adaptation to the human host, enabling a spectrum of infections ranging from asymptomatic colonization to invasive diseases. Moreover, GAS has the potential to initiate post-infection immune complications [35]. GAS as pathogens, have evolved sophisticated virulence factors to effectively evade the host's immune defences [36].

2.1 Virulence factors

The bacterium exhibits a diverse range of virulence factors, categorized as either surface-associated or secreted factors. It is crucial to note that there exists significant heterogeneity among streptococcal strains, as well as variations in the expressed virulence factors. Moreover, certain factors demonstrate multiple functions and their contribution to pathogenesis may differ across different stages of infection. Below, we discuss several key virulence factors that have proven significant in the development of severe streptococcal infections.

2.1.1 M protein

The M protein, a prominent virulence factor of GAS, is a fibrillar α-helical coiled coil protein that exists in a dimeric form on the surface of the bacterial cell wall. In its mature state, the M protein is covalently linked to the rigid peptidoglycan layer [37]. It can be found in a soluble form that is released during normal bacterial growth or during an infection [38].

All M proteins share a common structure, which includes a conserved signal peptide [39], a hypervariable amino terminus (HVR), a less variable central domain, and a highly conserved C-terminus. Following the hypervariable region, there are a series of repeat sequences known as A, B, C, and D repeats. Figure 2 provides a comparison of nine well-characterized M proteins, revealing that while there is a general conservation in the overall organization, significant differences exist. One notable variation among M proteins is their size, with variations observed in their lengths. The size and number of A and B repeats can vary among different M proteins. In fact, it has become evident that the majority of M proteins (including M1 strain that we used in our study) do not contain any A repeats [37].

There are two methods for typing GAS based on the M protein. One method is emm-typing that sequences a small variable portion (10-15%) of the emm gene, providing limited information about the sequence, structure, or functional domains of the rest of the M protein [40]. Another typing method, known as emm pattern-typing, identifies distinct chromosomal architectures (patterns A-C, D, and E) by examining the presence and arrangement of emm and emm-like genes within the GAS genome [41]. Pattern A-C emm-types have the longest M proteins, featuring a hypervariable region of about 230 residues. In contrast, pattern D and E proteins have shorter hypervariable regions of approximately 150 and 100 residues, respectively. 'A' repeats are generally missing in pattern D and E M proteins, while 'B' repeats are present in most pattern A-C and D emm-types but are usually absent in pattern E emm-types [40] (Figure 2).

The N-terminal portion of the M protein has received particular attention due to two main reasons. Firstly, this region displays hypervariability [36, 42], leading to antigenic variation, which forms the basis for effective serotyping and nucleotide-based emm-typing schemes [36, 43]. By sequencing the HVR, more than 220 distinct types of M protein have been identified. Secondly, the N-terminal portion of the M protein elicits protective antibodies in a type-specific manner, making it a potential candidate for a GAS vaccine [44].

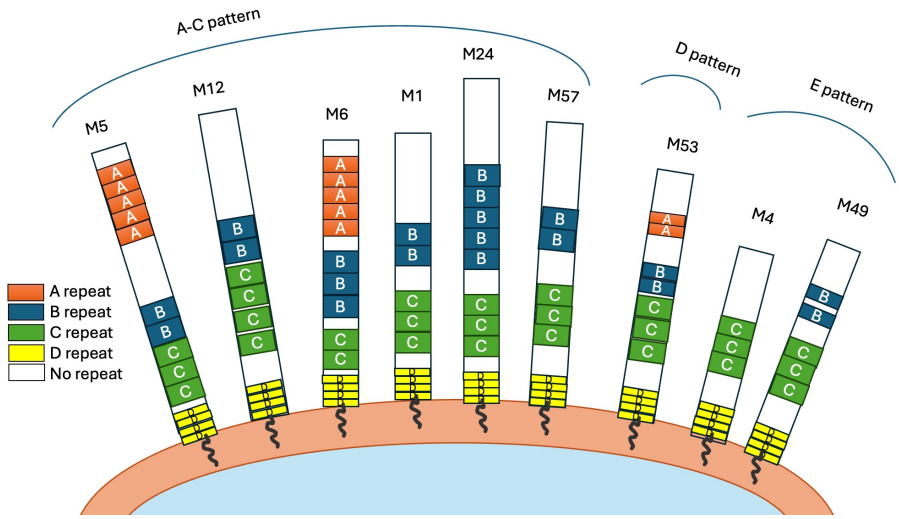

Figure 2. M-protein structure, serotypes and patterns. Drawn by Shiva Emami, taken from a review by Castro & Dorfmueller [1].

2.1.2 Virulence mechanisms of the M protein

Adherence to host cells: The recognition of M proteins as significant adhesins emerged in the early 1990s [45, 46] and now, the adhesive characteristics of the M protein have been well recognized. M protein has the ability to bind and adhere to host cells through interactions with extracellular matrix proteins such as collagen and fibronectin [37]. All

tested M proteins have shown a consistent capability to bind glycosaminoglycan [47]. Most M protein isoforms (75%) include a CD46-binding region within their C repeat domain which facilitates the adherence of GAS to human keratinocytes and promotes its internalization into epithelial tissues [48]. Moreover, M proteins play a role in bacterial aggregation, a crucial characteristic associated with the virulence of GAS [49, 50].

Phagocytosis inhibition by binding to host proteins: The interactions between M proteins and host ligands are well-documented, with numerous host proteins known to bind to M proteins. Certain M proteins exhibit binding capabilities for plasminogen and collagen, and the specific interactive motifs within these M proteins have been extensively studied [51, 52]. One of the well-known characteristics of the M protein is its ability to inhibit phagocytosis by interfering with the opsonization process and the deposition of complement. This effect is achieved by the M protein's ability to bind to host plasma proteins such as albumin and fibrinogen. By coating the bacterial surface with these proteins, the M protein hinders the deposition of complement and subsequent activation of the complement cascade. This inhibition of complement deposition contributes to the evasion of phagocytosis by the bacterium [37].

Preventing complement deposition: M protein can also bind to the human complement inhibitors like Factor H [53], CD46 [48] and C4b-binding protein (C4BP) [54]. Binding to factor H leads to C3-convertase destruction and preventing the opsonization by C3b consequently [55, 56]. Studies have indicated that C4BP retains its inhibitory function even after binding to the M protein and can effectively prevent complement deposition on the bacterial surface [54, 57]. Binding of C4BP to the HVR of M22, a specific M protein variant, has been shown to play a significant role in conferring resistance to phagocytosis [54].

Residing in neutrophils and macrophage: Recent studies have shown that the M1 serotype of GAS, a human pathogen, can survive and multiply inside human neutrophils after being engulfed [58, 59]. GAS strains with M or M-like proteins block the fusion of azurophilic granules with phagosomes. This prevents the delivery of antimicrobial substances to phagosomes containing these bacteria. However, bacteria without M or M-like proteins allow the efficient delivery of granule content to phagosomes [58]. It has also been found that the ability of GAS to survive inside macrophages is linked to the M1 protein, which interrupts the phagosomal-lysosomal pathway, allowing the bacteria to persist [60].

Acting as super antigen: It has been shown that the soluble form of the M1 protein effectively stimulates the proliferation of T cells and triggers the release of cytokines associated with the Th1 type immune response. The researchers proposed that this soluble M1 protein represents a new type of streptococcal superantigen, potentially contributing to the heightened activation of T cells and the hyperinflammatory reaction observed in severe invasive streptococcal infections [61].

2.1.3 Other virulence factors

Hyaluronic acid (HA) capsule: Notably, GAS expresses an anti-phagocytic HA capsule that closely resembles the HA found in human connective tissue. This HA capsule serves a dual purpose: firstly, it allows the bacteria to disguise itself as part of the host's tissues, and secondly, it facilitates adherence to epithelial cells by interacting with CD44 receptors [36, 62].

Fibronectin-binding proteins: In order to gain entry into host tissues, GAS utilizes its own fibronectin-binding proteins to recognize and bind to fibronectin (Fn). This interaction enables GAS to access epithelial and endothelial cells. Following colonization, GAS faces the challenge of evading the host's innate immune system to disseminate into blood vessels and deeper organs. Some of the Fn-binding proteins expressed by GAS play a role in evading the host's innate immune response, specifically targeting the complement system and phagocytosis. Furthermore, certain Fn-binding proteins have demonstrated their ability to inhibit complement opsonization of the bacteria, thereby acting as antiphagocytic factors [63].

SpyCEP: On the surface of streptococci, there is an additional enzyme called GAS cell-envelope protease (SpyCEP). This enzyme possesses the ability to cleave and deactivate chemoattractants for human immune cells [24]. Specifically, SpyCEP targets and cleaves interleukin-8 (IL-8), an important chemoattractant for human neutrophils. This action is thought to impair the recruitment of neutrophils to the site of infection. In murine models, it has been observed that SpyCEP activity leads to a decrease in IL-8-dependent neutrophil recruitment and bacterial clearance, playing a significant role in bacterial dissemination [64, 65].

C5a peptidase: C5a, which is generated through the cleavage of C5, functions as a potent inflammatory peptide. It promotes complement activation, facilitates the formation of the MAC (Membrane Attack Complex), attracts innate immune cells, and induces histamine release, which is implicated in allergic responses [66]. C5a peptidase is an enzyme found on the surface of streptococci, which possesses the ability to cleave and deactivate human C5a. [67].

2.2 GAS virulence mechanisms

Here, I will outline the steps involved in GAS pathogenesis. This will cover the mechanisms behind superficial infections, focusing on adherence, colonization, and tissue damage. Additionally, I will address the development of invasive infections, emphasizing the invasion of deeper tissues and the resulting tissue damage. By understanding these steps, we can gain insights into the mechanisms driving GAS infections and explore potential strategies for prevention.

2.2.1 Adhesion to host tissue

GAS is primarily spread from person to person through contaminated air droplets. Once on the skin or inside the host, GAS can survive for several hours to days [1] . GAS utilizes a variety of surface proteins to attach to the skin, allowing it to establish and spread the infection. These proteins play a crucial role in the initial attachment and colonization of GAS on the skin, facilitating the transmission of the bacteria [1].

Cell attachment, although still not fully understood, is currently described as a two-step process in the case of GAS. The initial attachment is facilitated by the GAS surface carbohydrate called lipoteichoic acid, which interacts weakly but sufficiently with the pharyngeal or dermal epithelial cells of the host through hydrophobic interactions [68]. During the initial stages of infection, GAS settles and multiplies within the host. One critical step in this process is the binding of GAS to the mammalian extracellular matrix (ECM) (Figure 3). The ECM is a complex structure consisting of various components such as collagen, fibronectin, laminin, proteoglycans, and tissue-specific proteins like keratin [69]. Bacterial colonization and invasion of host tissue often rely on the attachment of bacteria to the ECM. GAS produces adhesins that interact with fibronectin, collagen and laminin allowing attachment to the ECM, which is a critical step in establishing infection [70].

In the later stages of cell attachment, GAS utilizes high-affinity binding events facilitated by pili, as well as interactions between lectins and carbohydrates, and proteins. These interactions are mediated by GAS adhesion proteins and result in a strong attachment to specific tissue sites within the human host (Figure 3) [68]. It is important to note that bacterial adherence is a dynamic process. GAS has the ability to detach from tightly adhered surfaces and transfer to more favourable environments where it can survive and multiply. This flexibility allows the pathogen to adapt to different conditions and optimize its chances of survival [68, 71]. After GAS attaches to the surface of the host's skin or pharynx, it initiates the formation of microcolonies. These microcolonies are visible macroscopic structures that undergo multiplication, ultimately leading to the development of streptococcal infections. The upper respiratory tract provides a favourable environment for the growth of various pathogens. In the case of GAS, it competes with the epithelial lining of the respiratory tract to establish colonization and invade the host's epithelial cells [72]. This competitive interaction is crucial for the pathogen's ability to persist and cause infections in the upper respiratory tract.

Furthermore, GAS is characterized as a hyper-aggregative bacterium. This aggregation process plays a critical role in GAS biofilm formation as it serves as the initial step in the development of spontaneous microcolonies at host surfaces [73].GAS aggregation in liquid medium relies on the homophilic interactions between the M protein and the protein H, particularly in a M1 background [49]. However, in the absence of M protein, other surface factors such as pili may potentially mediate this aggregation [74].

2.2.2 GAS Internalization into host cells

Although GAS is primarily considered an extracellular pathogen, there have been reports indicating the presence of viable bacteria within non-phagocytic cells in both biopsies and cultured cells [75]. This phenomenon has also been observed with other Gram-positive extracellular pathogens such as Group B Streptococcus and S. aureus [76, 77].

The impact of high-frequency intracellular invasion on systemic streptococcal disease remains uncertain. However, the intracellular state of the bacterium may enhance its dissemination among humans. Strains from carriers show the highest internalization frequency, indicating a potential role in spreading [78]. After receiving treatment with penicillin, a range of 10% to 40% of children still continue to carry and release streptococci bacteria [79]. This indicates that during antibiotic treatments, strains of GAS with a higher ability to internalize are favored and selected. Moreover, GAS strains that exhibit resistance to erythromycin also tend to have a greater capability to invade cells compared to non-resistant ones [80]. This allows certain strains to thrive and potentially cause more severe infections in the presence of antibiotic pressure.

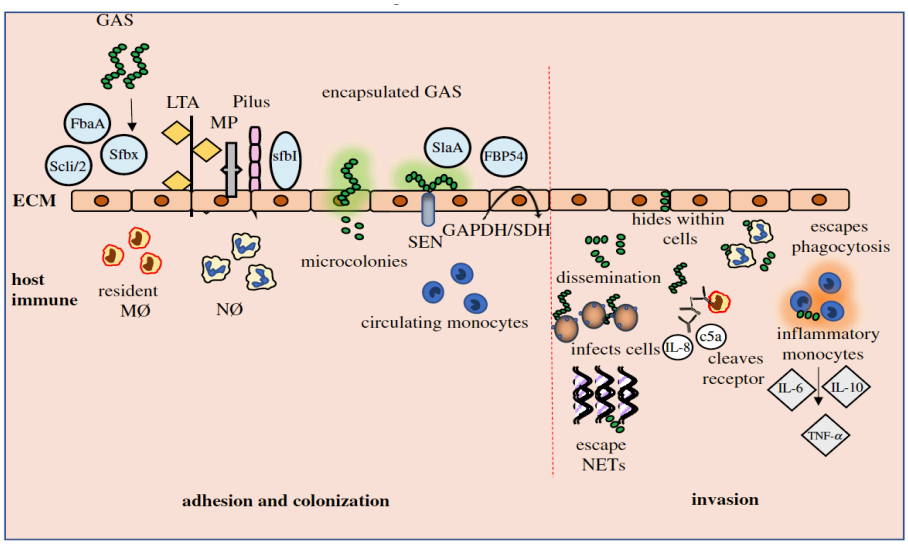

Figure 3. Steps of GAS adhesion and invasion of the host tissue [1].Initially, bacterial protein adhesins facilitate the attachment and colonization of GAS to the extracellular matrix (ECM) of host tissues. After the initial attachment, GAS forms microcolonies with the help of cell wall-anchored adhesins and anchorless enzymes. Once colonized, GAS disseminates within the host, surviving and multiplying through various mechanisms. These mechanisms include hiding within epithelial cells, inhibiting phagocytosis, and degrading neutrophil extracellular traps (NETs) using DNase. The infection triggers a strong inflammatory response in the host, leading to a cytokine storm. ECM: Extracellular matrix, LTA: Lipoteichoic acid, MP: M-protein, Protein adhesins: FbaA, Scli/2, sfbX, sfbI, SlaA, FBP54, NØ: Neutrophils, MØ: Macrophages, SEN: Streptococcal surface enolase, GAPDH/SDH: Streptococcal surface dehydrogenase, NETs: Neutrophil extracellular traps.The figure is Published by the Royal Society under the terms of the Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0/,

The process of engulfment, where host cells internalize GAS, can serve as an immune mechanism to restrict GAS infection by eliminating the internalized bacteria. This elimination of internalized GAS can occur through the autophagy pathway, known as xenophagy [81]. Certain strains, such as those belonging to serotypes M6, M49, and M3, are effectively killed by epithelial cells [82-85]. However, the highly virulent M1T1 clone has the ability to evade xenophagy and replicate within the cytosol of epithelial cells, specifically in a SpeB-dependent manner [86]. This further supports the idea that internalization is a negative consequence of GAS colonization.

2.2.3 GAS crossing the epithelium

There are various mechanisms by which GAS can cross the epithelium. One way is by causing cell death of the epithelial cells that maintain the integrity of the epithelium. Another method involves the transcellular route, where the bacteria are internalized by cells and subsequently released at the basal side of those cells.

GAS can also utilize a Trojan Horse strategy, wherein they are trapped and released by immune cells. Additionally, GAS can cross the epithelium through the paracellular mode by altering intercellular junctions in several ways. Firstly, GAS induces the degradation of intercellular junctions, facilitating translocation. This can occur through the binding of lipoteichoic acid (LTA) to CD44 or through the action of sub-cytotoxic doses of streptolysin S (SLS) [62, 87]. GAS can directly degrade intercellular junctions through the activity of the SpeB protease [88] or indirectly through the SEN-plasmin binding [89]. Tricellulin, a component of tight junctions between three cells, is involved in GAS colocalization at tricellular junctions. Plasmin binding to tricellulin triggers tricellulin degradation, facilitating bacterial translocation [90]. SpeB can also directly degrade desmogleins, which are components of desmosomes, a type of intercellular junction [91].

2.2.4. Tissue degradation

After breaching or crossing the epithelial barrier, GAS encounters the connective tissue. GAS produces a broad-spectrum cysteine protease called SpeB, which can degrade ECM components like fibronectin and vitronectin [92].

Host matrix metalloproteinases (MMPs) can also be activated by SpeB, leading to indirect degradation of ECM components [90, 93]. These processes enable GAS to infiltrate the connective tissue and establish infection.

GAS possesses factors that can bind plasminogen. Plasminogen can be converted into active plasmin by host effectors or by GAS streptokinase [94, 95]. Activation of plasmin on the bacterial surface can subsequently activate MMPs, which degrade ECM components and assist in GAS dissemination within the tissue. Additionally, GAS produces a bacterial hyaluronidase that degrades ECM hyaluronic acid. This

degradation promotes GAS tissue invasion and facilitates the diffusion of secreted factors such as SpeB and SLO [96].

2.2.5 Escape from the immune system

Complement C3b-degradation: C3b was demonstrated to be absent in sera of patients with severe invasive GAS infections, whereas it was clearly detected in the sera of healthy volunteers [97]. C3b, a complement fragment, plays a crucial role in the complement system by promoting opsonization [97]. The secreted SpeB protease has been found to function as an inactivator of the complement system by degrading C3b in the bloodstream [98]. SpeB also degrades properdin, a positive regulator of the alternative pathway. Properdin stabilizes the alternative C3 convertase and enables C3b amplification on the bacterial surface [99]. Disruption of properdin hinders efficient phagocytosis and phagocyte recruitment, facilitating bacterial dissemination [98].

Complement C5a-inhibition: In most cases of invasive GAS infection, there is no neutrophil infiltration around the site of infection. It is known that complement C5a acts as a chemotaxin, attracting and recruiting neutrophils to the site of infection [97]. It has been found that GAS has a C5a peptidase enzyme that removes a specific fragment from human C5a, leading to its inactivation and thus allowing the bacteria to evade neutrophil recruitment and survive in the host [100, 101].

IgG-degrading enzymes: IgG molecules, the most prevalent antibodies in human serum, play a vital role in combating infections. They activate the complement system by binding to C1q and facilitate effective opsonophagocytosis by interacting with Fcγ receptors on phagocytes [98]. To overcome this host immune response, GAS secretes three major immunoglobulin-degrading enzymes, namely IdeS/Mac-1, Mac-2 and EndoS.

The interaction between IdeS/Mac-1 and IgG results in the cleavage of the lower region of the IgG heavy chain. This cleavage efficiently inhibits complement binding and recognition of the Fc region [102]. This hydrolysis generates circulating F(ab')2 fragments that bind to the bacterial surface without activating complement or immune cell signalling, thus providing a shelter against the immune response [103].

EndoS cleaves a specific region in the heavy chain of human IgG [104]. This cleavage is important for the interaction between IgG and Fcγ receptors on phagocytic cells, as well as for IgG-mediated complement activation [105].

3 Immune responses against GAS

The primary role of the immune system is to detect and defend the host against infectious agents and foreign substances. Comprising various cell types, tissues, and organs, the immune system is commonly categorized into innate and adaptive immunity. Innate immunity is characterized by its rapid response to conserved microbial patterns, involving numerous cells. Conversely, adaptive immunity is composed of a smaller population of cells with the ability to recognize specific pathogens. Due to this limited number, these cells need to undergo proliferation and expansion to achieve adequate quantities for an effective response, a process that takes several days. Notably, the adaptive immune response can generate long-lasting cells that remain dormant until reactivated upon encountering the same pathogen, resulting in immunologic memory. This memory allows for a more robust response against the particular pathogen upon subsequent encounters [106]. Although innate and adaptive immunity are often considered separate components, they usually work together in harmony, with their synergy being vital for an effective immune response.

3.1 Innate immunity

The innate immune response is often referred to as the "first line of defence" against pathogens and is phylogenetically well-conserved, existing in a similar form from primitive life-forms to humans [107]. Before encountering innate effector cells, invading pathogens must breach anatomic and physical barriers surrounding the body, such as the skin, respiratory epithelium, and gastrointestinal tract epithelium. These barriers possess various antimicrobial mechanisms, including mucus secretion, cilia, peristalsis, resident microbial flora, and antimicrobial peptides [108].

Once outer barriers are breached, the innate immune system's next line of defence comprises various effector cells, including professional phagocytes like macrophages, dendritic cells (DCs), and neutrophils, as well as lymphoid cells such as innate lymphoid cells, natural killer cells, gamma delta T cells, and mucosal-associated invariant T-cells [108].

The cells of the innate immune system possess the ability to recognize a wide spectrum of pathogens through evolutionarily conserved patterns known as pathogen-associated molecular patterns (PAMPs). Receptors responsible for detecting PAMPs are grouped together as pattern-recognition receptors (PRRs). Different types of PRRs can recognize

various structures, such as RNA and DNA from replicating intracellular pathogens, lipopolysaccharide (LPS) from Gram-negative bacteria, and lipoteichoic acid from Gram-positive bacteria [109].

Among the well-characterized PRRs are the Toll-like receptors (TLRs), with 10 distinct TLRs identified in humans. Approximately half of these are expressed on the cell surface, while the rest are located inside intracellular vesicles [110]. Two other crucial forms of PRRs are the NOD-like receptors (NLRs) and RIG-I like receptors (RLRs), present in the cytoplasm of the cell. RLRs mainly recognize viral RNA, whereas NLRs recognize a wide range of ligands, including bacterial RNA [109].

In addition to PRRs, Fc- and complement receptors are also important in the host defence against pathogens. These receptors indirectly interact with invading pathogens through antibodies or complement deposited on the bacterial surface, promoting phagocytosis by innate immune cells.

3.1.1 Innate immune cells in GAS infection

Macrophages: Macrophages are pivotal players in our body's innate immune defence mechanisms, crucial for combating invading pathogens [111]. Their multifaceted capabilities include the engulfment and subsequent elimination of microorganisms through phagocytosis, as well as serving as adept antigen-presenting cells. Moreover, macrophages release a plethora of signalling molecules such as cytokines and growth factors, which not only initiate but also modulate local inflammatory responses [112-114].

However, despite the undeniable importance of macrophages in immune function, their potential role in the pathogenesis of streptococcal infections has received little attention.

In a noteworthy study, researchers found compelling data that align with our findings from Paper II. They discovered that resident macrophages possess the remarkable ability to efficiently phagocytose and eliminate GAS even in the absence of opsonic antibodies [115]. The importance of macrophages in host defence becomes particularly evident in the early stages of the host-pathogen encounter, before specific immunity has fully developed. It's been demonstrated that macrophages play a critical role during this phase. They possess the ability to recognize surface domains expressed by various pathogens, even in the absence of specific antibodies. This innate recognition mechanism underscores the versatility and efficiency of macrophages as key players in the initial defence against invading pathogens [116].

Research findings have unveiled that in patients with severe GAS infections, the affected tissues exhibit pronounced inflammation, marked by the infiltration of a significant number of both macrophages and neutrophils [117, 118]. Furthermore, these reports suggest that GAS possesses the capability to infect and persist within macrophages at the site of infection [117]. This dual observation highlights the complex interplay between the pathogen and immune cells.

In mice studies, it has been demonstrated that macrophages play a critical role in protecting mice from GAS infections and preventing bacterial spread from the primary subcutaneous inoculation site [115, 119]. It was shown that depleting macrophages in mice increases their susceptibility to the infection, resulting in higher mortality compared to non-depleted control animals. Bacterial loads in the blood and organs also significantly increased in macrophage-depleted mice. It was also found that blocking macrophage phagocytic function leads to resistance to GAS. This highlights the essential contribution of macrophages in protecting against GAS infection [115].

In response to GAS infection, both macrophages and conventional DCs (cDCs) exhibit a strong production of TNF and IL-6, which relies on the MyD88 pathway Interestingly, TNF-deficient mice did not accumulate macrophages at the infection site, but neutrophil infiltration remained unaffected. These findings suggest that tissue-resident macrophages require support from newly recruited macrophages to establish protective defences. The source of this support, possibly macrophage-derived TNF, likely acts in autocrine and paracrine ways to facilitate the chemotactic relocation of macrophages to the site of infection. Alternatively, tissue-resident DCs, either alone or in conjunction with macrophages, might be the critical source of this cytokine [120, 121].

Dendric cells: DCs are among the first cells to encounter GAS in the respiratory mucosa or skin. These cells are highly specialized antigen-presenters, serving as a bridge between the innate and adaptive immune systems [122, 123]. When DCs interact with a pathogen, they undergo a series of activation events that include phagocytosis, antigen processing, and migration to lymphoid tissues where they present antigens to T cells [124, 125]. Furthermore, activated DCs send danger signals that alert the immune system and influence the activation and differentiation of lymphocytes [126]. In vitro studies have shown that GAS rapidly induces the maturation and activation of both human [127, 128] and murine [129] DCs upon exposure. DCs are the primary source of interleukin-12 (IL-12) *in vivo* during infection, which is essential for developing protective immune responses against GAS [129]. A study has demonstrated the protective role of DCs during infection with GAS. It was found that the ablation of DCs exacerbates the infection, highlighting their importance. Additionally, the depletion of DCs completely eliminated the production of IL-12 [129].

DCs play a significant role in producing IL-12 in response to GAS [119, 121], which in turn stimulates NK cells to generate IFN-γ. While both IL-12 and IFN-γ are known to contribute to immune responses against GAS, their specific functions are not yet fully understood [130, 131]. IFN-γ is a major cytokine that activates macrophages [132], suggesting that the DC/NK cell circuit involving IFN-γ might primarily enhance macrophage antimicrobial functions. Interestingly, NK cells have been implicated in streptococcal toxic shock syndrome, indicating that uncontrolled stimulatory activity of NK cells can be harmful to the host [133].

NK cells: NK cells, along with macrophages and polymorphonuclear neutrophils, play a crucial role in the body's innate immune defense. They are guided to infection site by signals from cytokines and chemokines [134] and are capable of destroying infected

cells from the same organism without prior sensitization [135-137]. NK cells play a role in immune defense against pathogens by producing IFN-γ [138-140]. During septic shock, their high secretion of IFN-γ can activate NK cells and contribute to the development of this severe reaction [18].

In mice, NK cells have been identified as significant sources of IFN-γ during the initiation of a generalized Shwartzman reaction. This reaction is a fatal cytokine-induced shock response triggered by sequential exposure to bacterial elements [141, 142].

In a summary, it has been shown that NK cells are crucial elements of the innate immune system, playing a key role in the early defense against pathogens by releasing IFN-γ before the adaptive immune response is activated [135-138, 140]. However, if this defense mechanism is overactivated, the excessive production of proinflammatory cytokines can trigger various harmful reactions, potentially resulting in septic shock and death [141-143].

Neutrophils: Neutrophils are derived from the bone marrow and make up around 70% of our circulating leukocytes. Every day, up to $0.5\text{-}1x10^{11}$ neutrophils are produced in the bone marrow [144]. It has been shown that the neutrophil response to various streptococcal supernatants differs depending on the strain tested. The M3 strain, for example, elicits a significantly stronger neutrophil response compared to the M1 strain. The presence or absence of SpeB also plays a crucial role in determining the impact of the bacteria on neutrophils [145].

3.2 Adaptive immunity

Lymphocytes serve as the primary effector cells in adaptive immunity and are produced in the bone marrow, differentiating into B-lymphocytes and T-lymphocytes.

3.2.1 B-cells

B-cells express B-cell receptors, enabling them to produce antigen-specific antibodies upon activation, which can recognize and neutralize pathogens and toxins. Antibodies are also crucial for opsonization of bacteria, making them susceptible to phagocytosis and destruction by effector cells like neutrophils and macrophages [146].

3.2.2 Antibodies

(IgG is a highly abundant protein in human serum, constituting approximately 10–20% of plasma protein. It is the predominant class among the five classes of immunoglobulins found in humans, which also include IgM, IgD, IgA, and IgE. These glycoproteins, with 82–96% protein and 4–18% carbohydrate composition, have

distinct heavy chain structures and different effector functions. IgG can be further categorized into four subclasses: in humans IgG1, IgG2, IgG3, and IgG4, with varying levels of abundance [147]. The discovery of these IgG subclasses occurred in the 1960s through extensive studies using specific rabbit antisera against human IgG myeloma proteins [147]. While the IgG subclasses share over 90% similarity in their amino acid sequences, each subclass exhibits a unique profile concerning antigen binding, immune complex formation, complement activation, stimulation of effector cells, half-life, and placental transport [147], as summarized in table 1.

IgG1: Soluble protein antigens and membrane proteins primarily induce IgG1 antibody responses, along with lower levels of other subclasses like IgG3 and IgG4 [148]. IgG1 deficiency can lead to reduced total IgG levels (hypogammaglobulinemia) and is associated with recurrent infections [149].

IgG2: Antibody responses to bacterial polysaccharide antigens are mostly restricted to IgG2 [148, 150, 151]. Deficiency in IgG2 may lead to a lack of IgG anti-carbohydrate antibodies [152], compensated by increased IgG1 and IgG3 levels [153]. IgG2 plays a role in defending against certain bacterial infections [154].

IgG3: IgG3 antibodies are highly effective in inducing effector functions and possess potent pro-inflammatory properties. Their shorter half-life helps to control excessive inflammatory responses [153]. During viral infections, IgG1 and IgG3 subclasses of IgG antibodies are commonly produced, with IgG3 antibodies appearing early in the infection [148].

IgG4: Allergens can stimulate the production of IgG1 and IgG4 antibodies, alongside IgE. In non-infectious situations, IgG4 antibodies are often generated after repeated or prolonged exposure to antigens, and they may become the dominant subclass [155]. Additionally, infections caused by helminths or filarial parasites can trigger the formation of IgG4 antibodies [156, 157], and elevated IgG4 levels might be linked to asymptomatic infections [158].

Table 1. functional properties of subclasses of human IgG [2]. Available via license: CC BY 4.0

Properties	IgG1	IgG2	IgG3	IgG4
Approximate molecular weight (kDa)	146	146	165	146
Hinge length (number of amino acids)	15	12	62	12
Antibody-dependent cell-mediated cytotoxicity	+++	+/−−	++	+/−−
Antibody-dependent cell-mediated phagocytosis	+	+	+	+/−−
C1q binding	+	+/−	+++	−
Complement-mediated cytotoxicity	++	+/−	++	−
FcRn binding	+	+	+/−	+
Plasma half-life (days)	21	21	5–7.5	21
Approximate average plasma concentration (mg ml−1)	9	3	1	0.5

Antibody responses against GAS: Protective immunity against GAS is generally weak, and recurrent infections, particularly in children, are common [159]. This occurs despite the fact that most individuals do mount an adaptive immune response and produce high levels of IgG antibodies against various GAS antigens [160-163]. The reason for this lack of protection is not fully understood but can be partly attributed to the extensive variety of GAS serotypes and the associated variability in surface antigens [40]. Additionally, GAS can undermine adaptive immunity by impairing IgG function. This can occur through nonimmune binding of IgG to Fc-binding proteins on the streptococcal surface, such as the M and M-related proteins [164, 165], or through direct degradation of IgGs. For instance, GAS secretes the IgG-degrading enzyme of GAS (IdeS), an IgG-specific protease that cleaves antibodies at the hinge region, separating the antigen-binding Fab fragments from the Fc region responsible for effector functions [102]. Additionally, GAS secretes the endoglycosidase of GAS (EndoS), which specifically cleaves the conserved Fc N-glycan from IgGs [104].

Indeed, the protective role of antibodies against GAS has been a subject of controversy. While some studies suggest that anti-M protein antibodies provide protection against GAS infection, others highlight potential negative effects, including the development of autoimmune diseases [34, 166].

In a recent study, the role of strain-specific antibodies in immunity against GAS has been investigated using intravenous immunoglobulin (IVIG) from healthy donors. The authors investigated the role of strain-specific antibodies, particularly those targeting the M-protein, in immunity against various GAS strains using functional killing assays. It found that for GAS strains in the E pattern group, M-type specific antibodies do not contribute to killing the bacteria, unlike strains in pattern A–C and D groups [167]. This challenges the traditional view that the M-protein is the main protective antigen for all GAS strains.

There is substantial evidence that antibodies develop following exposure to GAS or GAS-related antigens and that these antibodies possess protective capabilities. This protective response has been observed in various experimental settings [168-172]. For example, intranasal delivery of an adjuvanted multivalent M protein vaccine can induce protective antibody responses [169]. Furthermore, low antibody levels against the M1 antigen were found in Swedish patients with bacteremia, particularly in fatal cases [173]. This suggests that low levels of antibodies specific to the M antigen of the infecting strain might contribute to the severity of the infection and its associated complications. However, not all antibodies against the M protein appear to be protective and only those targeting the HVR of the M protein were found to efficiently protect against infection in passive immunization studies [174]. This highlights the complexity of the humoral response against GAS. A recent study adds to this debate by demonstrating that specific IgG antibodies against the M1 protein can actually worsen the outcome of GAS infection. These antibodies form complexes with M protein and fibrinogen, leading to increased release of Heparin-Binding Protein (HBP) and contributing to the development of STSS [175]. This challenges the conventional belief that antibodies always confer protection. Instead, it suggests that under certain

circumstances, antibodies can exacerbate inflammatory responses, potentially leading to more severe disease. This highlights the complexity of the immune response to GAS and emphasizes the need for further research to fully understand its mechanisms and implications for disease management.

3.2.3 T-cells

T-cells express T-cell receptors and can be classified into $CD4^+$ T-helper (Th) cells and $CD8^+$ cytotoxic T-cells, based on surface markers and functions. $CD8^+$ cytotoxic cells play a significant role in eliminating infected cells and tumour cells, while $CD4^+$ Th cells are essential for activating and regulating the immune response [146]. Upon activation, $CD4^+$ Th cells can differentiate into various subsets depending on signals and cytokines present at the activation site. These subsets, including Th1, Th2, Th17, and regulatory T-cells (T-regs), have distinct roles in controlling the immune response and maintaining homeostasis by either activating or suppressing inflammatory pathways [106].

The initiation of adaptive immunity occurs when lymphocytes recognize foreign antigens through their respective antigen-specific receptors. While B-cells can recognize antigens directly, T-cells require antigen presentation by major histocompatibility complex (MHC) class I or II molecules on antigen-presenting cells [176].

T follicular helper (Tfh) cells represent a unique subset of $CD4^+$ T cells and are critical for the formation of germinal centers within secondary lymphoid organs during immune responses (Figure 4). Within the germinal centers, they support rapid B cell proliferation, antibody diversification, and the production of antibodies with higher affinity for the target antigen. Tfh cells provide co-stimulation to B cells through CD40-CD40L interaction and produce the cytokine IL-21, driving B cell proliferation. In the absence of Tfh cells, germinal centers s do not form, resulting in antibody defects [177]. B cell lymphoma 6 (Bcl6) is a critical transcription factor in $CD4^+$ T cells, essential for Tfh cell differentiation and the development of germinal centers [178-180].

Vaccine development has predominantly concentrated on antibodies and to a lesser extent, on cellular T cell-based immunity. However, T cell responses are crucial for several reasons: they help induce high-affinity antibodies, ensure the longevity of antibody-producing B cells by providing necessary survival and differentiation signals [181, 182] and support the induction and maintenance of memory B cells [183]. Additionally, cytokines secreted by T cells, such as IFN-γ and IL-4, play a pivotal role in class-switch recombination, leading to the production of specific immunoglobulin classes and subclasses [184, 185].

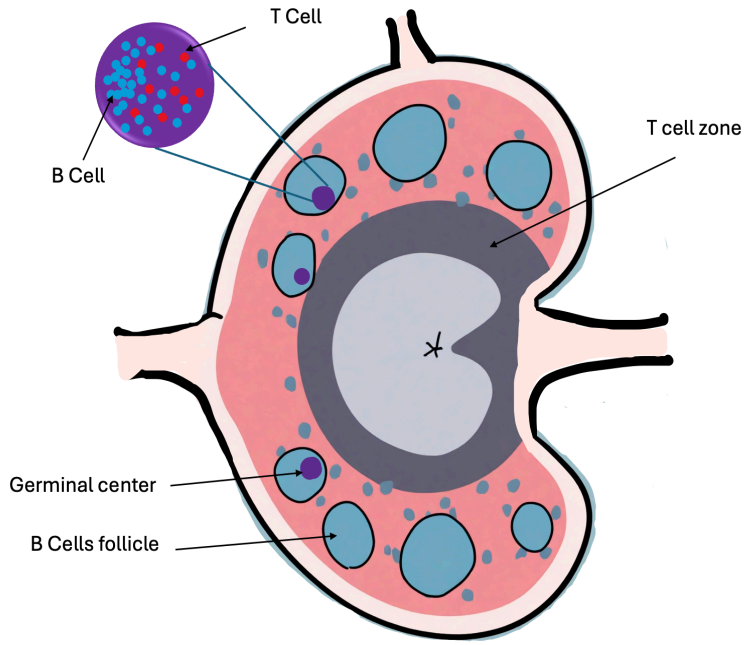

Figure 4. Lymphnode structure. Drawn by Shiva Emami

Moreover, Th17 cells are known to offer protection against bacterial infections like Streptococcus pneumoniae [186]. Recent studies in animal models of GAS infections have suggested that Th17 cells may also contribute to defense against GAS infections [187, 188]. It has been found that after intranasal inoculation with GAS, antigen-experienced CD4+ T cells in the nasal-associated lymphoid tissue (NALT) produced IL-17A, or both IL-17A and IFN-γ if the infection was recurrent. This dominant Th17 response was specific to the intranasal route, while intravenous or subcutaneous inoculations mainly resulted in IFN-γ-producing CD4+ T cells [189].

In the same study, it has been revealed that the cellular immune response to GAS is Th1 polarized. When T cells from adults were stimulated with two non-M protein antigens of GAS, ScpA (streptococcal C5a peptidase) and Isp (immunogenic secreted protein), the highest cytokine levels were observed for IFN-γ, followed by TNF-α and IL-17. This demonstrates that human cellular immunity against GAS involves Th1 and Th17-associated cytokines, with minimal involvement of Th2 cells [189].

CD4 T cells targeting M protein have been identified in the tonsils of patients suffering from recurrent tonsillitis and tonsillar hypertrophy. These CD4 T cells produce a range of cytokines, including IFN-γ, IL-2, IL-4, IL-5, and IL-6 [190].

3.2.4 Immunodominance and tracking of specific T cell responses

T cell responses are significantly influenced by the peptide:MHC (p:MHC) ligands presented by antigen-presenting cells (APCs) in secondary lymphoid organs. Key factors include the amount and duration of peptide presentation, in turn affected by how abundant the corresponding proteins are, how efficiently they are processed, and how strongly and stably the ensuing peptides bind to MHC molecules [191, 192]. Immunodominant peptides are likely to be displayed in greater amounts and for longer periods on APCs, leading to more intense TCR signaling in the cognate T cells. Therefore, the immunodominant peptides promote greater clonal expansion, stronger effector functions and/or the formation of larger memory T cell pools. Additionally, the size of the naive T cell population specific to certain p:MHC ligands can also affect the strength of the immune response [193, 194].

Studies of endogenous in vivo polyclonal antigen-specific T helper (Th) cell responses have been advanced through p:MHCII tetramer technology. This method involves the multimerization of soluble and biotinylated p:MHCII complexes by fluorescently labeled streptavidin, enabling the detection of rare endogenous p:MHCII-specific CD4+ T cells via flow cytometry [195]. The technology relies on identifying pathogen-derived peptides that form p:MHCII epitopes with the MHCII alleles expressed by the host. While the tetramer technology has been used successfully to identify and enumerate even naive CD4+ T cells specific for different foreign p:MHCII ligands in C57BL/6 (B6) mice [196], the generation of statistically meaningful results on phenotype and effector functions of p:MHCII specific T cells usually requires larger populations of T cells specific for a given p:MHCII complex. Therefore immunodominant p:MHCII complexes are preferentially used to target larger subsets of antigen specific Th cells. The use of immunodominant p:MHCII tetramer technology has enhanced the ability to study Th cell responses in vivo, providing valuable insights into the dynamics of T cell immunity.

As no immunodominant p:MHCII complexes have been identified for GAS in B6 mice, a recombinant M1 strain was created, containing the 2W variant of peptide 52-68 from the MHCII I-E α-chain, inserted into the N-terminal of the M1 protein (GAS-2W.M1) [188, 197]. In B6 mice, which lack I-E molecules, the precursor frequency of naive CD4+ T cells recognizing 2W:I-Ab is very high. This high precursor frequency is associated with one of the most robust p:MHCII-specific CD4+ T cell response magnitudes observed in this mouse strain [196, 198]. The The 2W:I-Ab tetramer was accordingly used in the aforementioned studies to track GAS-specific Th cell responses in mice immunized with the GAS-2W.M1 strain. In paper I, we however hypothesized that difficulties to establish protective immunity in B6 mice following s.c. GAS immunization could be related to suboptimal Th cell responses. To test this hypothesis, we utilized the GAS-2W.M1 strain for immunization and compared the ensuing response to the response induced by the wild type GAS-M1 strain. As described in paper I, our results demonstrate enhance protective immunity against the wild type GAS-M1 strain when the recombinant strain is used for immunization, indicating that the insertion

of the immunodominant 2W peptide into the M1 protein confers an altered and more protective Th cell response.

3.3 Adjuvant

An ideal protein vaccine formulation includes several key components: immunostimulants to activate the innate immune system and provide the co-stimulatory signals that guide adaptive immunity, the antigen that prompts the adaptive immune response, and a delivery system that ensures these components are combined and presented at the right place and time for optimal immune stimulation.

In the late 1990s, researchers found that activating receptors like Toll-like receptors (TLRs) on dendritic cells triggers immune responses by recognizing microbial patterns [199, 200]. This supports Charlie Janeway's idea that the innate immune system senses microbes through pattern recognition receptors (PRRs), like TLRs [201]. Over the next decade, other PRRs such as RIG-I, DNA sensors like STING, and NLRs were discovered, all demonstrated to influence adaptive immune responses. These receptors are now being explored for their potential in vaccine development [202].

Adjuvants can be classified based on their component sources, physicochemical properties, or mechanisms of action. Modern vaccines typically feature two main classes of adjuvants:

1. Immunostimulants: These adjuvants directly act on immune cells to enhance responses to antigens. Examples include:

- TLR ligands
- Cytokines
- Saponins
- Bacterial exotoxins

2. Vehicles: These adjuvants present vaccine antigens to the immune system in an optimal manner, often utilizing controlled release and depot delivery systems to amplify the specific immune response to the antigen. Examples include:

- Mineral salts
- Emulsions
- Liposomes
- Virosomes (nanoparticles made of viral proteins such as influenza hemagglutinin and phospholipids)
- Biodegradable polymer microspheres
- Immune-stimulating complexes (ISCOMs, ISCOMATRIX)

Vehicles can also serve the dual purpose of delivering immunostimulants, thereby enhancing the overall immune response by combining antigen presentation with immune cell activation [203].

Adjuvants are categorized into two generations. The first generation includes insoluble aluminum salts, commonly referred to as Alum. Alum was first identified in the 1920s [204] and is now a component of licensed vaccines worldwide [205]. Emulsion adjuvants, introduced by Freund in the 1930s [206], are also part of the first generation as well as polymeric particles, and liposomes [207]. These early adjuvants have played a crucial role in enhancing vaccine efficacy and remain foundational in many vaccines used today.

First-generation adjuvants are designed as particulate-based structures, making them ideal for uptake by phagocytic cells such as macrophages and dendritic cells because their size and shape closely mimic those of pathogens. These adjuvants enhance the immune response by adsorbing or encapsulating vaccine antigens, which improves their persistence and delivery [203, 207, 208]. For instance, when antigens are adsorbed onto alum, they become more stable and remain longer at the injection site [207]. Alum and MF59 (an adjuvant that is added to influenza vaccines [209]) are particularly effective at inducing antibody and TH2 responses [210, 211]. However, like all first-generation particulate adjuvants, they have weak efficacy at stimulating antibody production to some protein subunit antigens and are poor at inducing cell-mediated immunity [212].

Second-generation adjuvants were developed 40–50 years after the introduction of Alum and MF59. These adjuvants are composed of multiple components, typically combining first-generation adjuvants with immunostimulants [213]. The most potent immunostimulants are often pathogen-associated molecular patterns, which include bacterial products such as lipopolysaccharide (LPS), toxins like cholera toxin (CT), and bacterial nucleic acids such as unmethylated bacterial CpG DNA. These specific immunostimulants promote the maturation of DCs by binding to PRRs on the DCs, leading to the expression of co-stimulatory molecules necessary for shaping the adaptive immune response [214, 215]. Overalls, these natural pathogen-associated signals can cause systemic inflammation and shock, making them too toxic for use in human clinical applications [216]. However, monophosphoryl-lipid A (MPLA) which is derived from LPS, is designed to retain the immune-stimulating properties of LPS while reducing its toxicity, making it a safer and more effective option for vaccine formulations. MPLA is a widely recognized TLR4 agonist and can induce a strong CD4+ Th1 immune response, which is crucial for antibody affinity maturation. Recently, MPLA has been licensed as an adjuvant for the human papillomavirus (HPV) vaccine in Europe and the USA [217, 218].

3.3.1 ALUM

The clinically approved alum adjuvants are made up of aluminum phosphate or aluminum hydroxide precipitates, which adsorb antigens [219, 220]. For over seventy

years since its first approval in the 1920s, alum has been the sole adjuvant used in licensed products like vaccines for hepatitis B, diphtheria, tetanus, pertussis, and human papillomavirus [205]. Despite numerous other adjuvants showing promising effectiveness in preclinical studies during this time, the majority have not received licensure for use in humans, largely due to safety or tolerance issues.

While it was initially believed that alum primarily creates a long-lasting depot for antigens and promotes their uptake by APCs, it is now understood that alum's primary adjuvant activity involves innate immune stimulation [219, 220]. Alum mainly enhances antibody production and does not rely on TLR for its function in vivo [221]. In humans, responses to proteins adsorbed to alum involve a mix of Th2 and Th1 cells, However, in mice, alum induces a strongly polarized Th2 cell response, leading to Th2 cell-dependent antibody isotypes for nearly all protein antigens [222, 223].

3.3.2 Poly I:C

Polyinosinic-polycytidylic acid (poly I:C) is a synthetic double-stranded (ds) RNA that mimics viral infection by activating various components of the host defence system. When combined with an antigen, poly I:C acts as a PAMP adjuvant, enhancing and optimizing the antigen-specific immune response [224]. Poly I:C can induce several receptors like TLR3, RIG-I, MDA-5 and NLRP. Binding of these Receptors to poly I:C leads to the release of inflammatory cytokines and type I interferons (IFNs) which can activate both innate and adaptive immune responses [225]. It has been shown that poly I:C can activate TLR3 expressed in NK cells, leading to production of pro-inflammatory cytokines like IL-6 and IL-8, as well as IFN-γ [226].

Previous studies have demonstrated the antibacterial effects of poly I:C. For instance, lipoproteins from Staphylococcus aureus cause excessive inflammatory responses when poly I:C is present [227]. Golshani et al. found that poly I:C enhances immunity and protection provided by outer membrane vesicles (OMVs) against Brucella challenge in mice [228]. Additionally, it was shown that poly I:C boosts humoral and cellular immune responses to multi-epitope antigens, acting as a strong Th1-inducing adjuvant in vaccine formulations against brucellosis [229]. These findings suggest that poly I:C has various immune-enhancing effects, some of which are still not fully understood.

3.4 Cytokines and chemokines

Upon activation, the immune system produces and releases a diverse array of cytokines and chemokines that induce various effector functions, including growth, differentiation, migration, and activation of immune cells.

Innate immune cell activation triggers the activation of nuclear factor-κB (NF-κB), leading to the transcription and translation of cytokines and chemokines like TNF-α, IL-1β, IL-6, and IL-8. These early released molecules play a crucial role in recruiting

leukocytes to the site of infection. TNF-α and IL-1β affect the vasculature by promoting vasodilation and increased capillary permeability. IL-6 contributes to the acute phase response, IL-12 is essential for T-cell activation and adaptive immunity, and IL-8 acts as a significant chemoattractant for neutrophils [230]. Concurrently, the immune system produces anti-inflammatory mediators, including IL-10, transforming growth factor (TGF)-β, and various soluble cytokine receptors, to counterbalance inflammation and maintain controlled immune responses [230].

As previously mentioned, different cytokines are produced by T cells based on the route of infection. The Th17 response, producing IL-17, was dominant in the intranasal route, whereas intravenous or subcutaneous inoculations of GAS mainly resulted in IFN-γ-producing CD4+ T cells. The ability of these T cells to produce IL-17A and the survival of mice after infection depended on the cytokine IL-6. IL-6-deficient mice that survived the infection became long-term carriers despite having many IFN-γ-producing CD4+ T cells. These findings suggest that an imbalance between IL-17- and IFN-γ-producing CD4+ T cells might contribute to GAS carriage in humans [188].

It has been proposed that all individuals gradually develop immune responses to GAS. Alongside antibody reactions, exposure to GAS triggers the development of specific cellular responses. These cellular reactions, including cytokine patterns like IFN-γ, appear similar in adults and children initially. However, upon closer examinations, variations in the intensity of these responses emerge. Specifically, IFN-γ levels were notably lower in children compared to adults [189].

IFN-γ

There is a prevalent belief that myeloid cells, including polymorphonuclear leukocytes (PMNs), play a central role in the survival of individuals from GAS infections. Additionally, IFN-γ is deemed essential for the full activation and proper function of PMNs. Interestingly, while IFN-γ at the infection site is considered critical for protection, elevated systemic levels appear to have detrimental effects on survival following GAS infections. This dichotomy underscores the delicate balance required for effective immune responses against GAS infections and suggests that fine-tuning the regulation of IFN-γ levels could be crucial for improving outcomes in such cases. [231].

Previous studies have suggested that T cells and NK cells may contribute to the production of IFN-γ during GAS infections [118, 231-234]. However, in another study, it was demonstrated that γIMCs (gamma interferon-producing innate myeloid cells) in various anatomical sites such as the peritoneal cavity, skin, spleen, kidney, peripheral blood, and bone marrow—rather than T cells or NK cells—produce IFN-γ in vivo during the early stages of severe invasive GAS infections. This highlights the diverse cellular sources of IFN-γ during GAS infections and emphasizes the pivotal role of innate immune cells in orchestrating the initial immune response against the pathogen [235].

Administration of IFN-γ reduces the bacterial burden in the blood. However, intriguingly, when γIMCs are transferred, but not IFN-γ alone, the survival rate of mice following GAS infection improvs. This indicates that while IFN-γ is necessary for protection, it is not sufficient on its own to confer full protection against severe invasive GAS infections. This underscores the complexity of the immune response to GAS infections and highlights the noticeable role of γIMCs in mediating the protective effects observed [235].

Two recent studies on infections with group B streptococci have revealed that IL-12 confers significant protection against neonatal infection in mice. Moreover, both in vivo and in vitro investigations demonstrated that this protection is mediated by IFN-γ [236, 237].

Notably, treatment of animals with IL-12 prior to infection resulted in enhanced transcription of IFN-γ, both locally at the infection site and systemically in the spleen. This observation suggests that, akin to other bacterial infection models, IFN-γ serves as a potential mediator of the protection induced by IL-12.

4 Development of GAS vaccines

4.1 The elements of a vaccine

A vaccine comprises two primary components: antigens and adjuvants. Antigens, typically proteins or carbohydrates derived from the pathogen, instigate an immune response. Adjuvants, on the other hand, are additives incorporated into vaccines to stimulate and prolong this immune response. The term "adjuvant," stemming from the Latin word 'adjuvare' meaning 'to help,' was coined by French veterinarian Gaston Ramon in 1920. Ramon observed that horses vaccinated against diphtheria exhibited stronger immune responses when they developed inflammatory abscesses at the injection site [238]. His experimentation revealed that the addition of substances such as breadcrumbs or starch to the vaccine boosted antibody production. [239].

4.1.1 Vaccine adjuvant design

Adding adjuvants to a vaccine improves and directs the immune cell response to the antigens, thereby reducing the required amount of antigen or the number of immunizations and enhancing the vaccine's efficacy [203].

Traditional "live vaccines," which are based on attenuated pathogen cells, do not require the addition of adjuvants. Similarly, vaccines based on inactivated viruses or bacteria are often sufficiently immunogenic without adjuvants, although some include adjuvants to further enhance the immune response. In contrast, current generation vaccines often consist of highly purified recombinant proteins or protein subunits, synthetic peptides, or plasmid DNA (pDNA). These vaccines have well-defined compositions and are less reactogenic, making them safer and less toxic, but they are also less immunogenic compared to traditional vaccines [208, 210]. Therefore, these new vaccines increasingly require adjuvants to enhance the quality and magnitude of the adaptive immune response elicited by the specific antigen alone.

4.1.2 Obstacles in Developing Vaccine Adjuvants: Balancing Humoral and Cellular Immunity

Present-day vaccines predominantly offer protection via humoral immunity [240]. These responses are induced by various vaccine platforms, such as live attenuated, recombinant proteins, toxoids, or polysaccharide-protein conjugates. The antibody

responses to many current vaccines are durable, often requiring little to no additional boosting to maintain their protective effects [241]. Despite the success of these vaccines, there are significant groups for whom current vaccines, including those with alum adjuvant, fail to achieve adequate seroconversion rates or protective antibody levels. Furthermore, vaccine efficacy tends to decline in healthy adults after the age of 40–50 years [242] and can be compromised by health conditions like chronic kidney disease [243]. Enhancing vaccines by adding an adjuvant, as seen with influenza vaccines [244], or replacing alum with a more potent adjuvant, as done for the hepatitis B virus (HBV) [243, 245], can provide significant advantages for these populations.

Different adjuvants distinctly influence the polarization of helper T cells. Adjuvants like MF59, ISCOMs, and ligands for TLR2 and TLR5 boost T cell and antibody responses without changing the Th1/Th2 balance. Conversely, adjuvants incorporating TLR3, TLR4, TLR7-TLR8, and TLR9 agonists induce more polarized Th1 responses. Complete Freund's adjuvant (CFA) and CAF01 promote mixed Th1 and Th17 responses. Therefore, choosing the right adjuvant depends on the desired $CD4^+$ T cell response required for effective protection [199].

The challenge of generating strong, lasting T cell immunity with current vaccines and adjuvants has significant clinical implications. There are still no fully effective vaccines for many common infectious diseases, including HIV-AIDS, malaria, and tuberculosis. While humoral immunity helps preventing HIV infection and affects malaria stages [246, 247], Th1 and $CD8^+$ T cells play a crucial role in controlling these infections. Developing adjuvants for cancer and chronic viral infections is even more challenging, requiring potent, multifunctional T cell responses in patients with immune regulation issues. Effective adjuvants must stimulate $CD8^+$ T cells and bypass regulatory mechanisms limiting host responses [248, 249]. These challenges highlight the urgent need for vaccines that induce strong, lasting T cell immunity.

4.2 History of GAS vaccine

GAS vaccine development and clinical studies have a history spanning over 100 years, encompassing various antigen approaches such as inactivated whole cell, scarlet fever toxin, M protein, and other non-M protein antigens [250]. The M protein, a significant virulence factor of GAS, has been a major focus of development since 1940. This endeavour evolved from using whole M protein to refining approaches with N-terminal polypeptides and C-repeat peptides [251].

Before the 1960s, M protein vaccines, including those based on whole M protein, were administered to tens of thousands of participants, including children. In the 1970s, human challenge studies involving purified M protein for GAS pharyngitis demonstrated vaccine efficacy of up to 89%, and no severe adverse events were detected among the vaccinated individuals [252-254].

Despite promising progress, vaccine development against GAS faced a setback in 1979. The US FDA introduced a regulation (21 CFR 610.19) of using GAS organisms or their derivatives" in vaccines due to concerns about potential harmful tissue reactions from GAS antigens in vaccines [251]. This was influenced by an uncontrolled study involving a type 3 M protein vaccine. In this study, higher rate of rheumatic fever was observed in vaccinated patients with M3 protein compared to non-vaccinated patients [255]. However, the regulation inadvertently hindered further vaccine progress. In 2006, the FDA revoked this regulation, opening the door for future development [256]. Currently, the FDA does not impose specific requirements for a GAS vaccine, allowing for potential advancements in the field.

Between 2006 and 2016, progress in GAS vaccine development was slow due to the enduring impact of the 1979 FDA regulation. Despite this, advancements occurred, primarily focusing on M protein-based vaccines, with notable pre-clinical developments in non-M protein vaccines like group A streptococcal C5a peptidase [257]. Clinical efforts included early-phase trials in healthy adult volunteers involving four vaccine candidates: a 6-valent N-terminal M protein vaccine, a 26-valent N-terminal M protein vaccine, a 30-valent N-terminal M protein vaccine, and a conserved C-repeat region M protein vaccine [44, 258-260]. These trials reported no significant safety concerns and demonstrated promising immunogenicity data across all vaccine candidates.

4.3 M-protein based vaccines

4.3.1 Conserved region M-protein based vaccines

An alternative approach to N-terminal M peptide vaccines involves utilizing the conserved C-terminal region, particularly the C-repeat region, from the extracellular domain of the M-protein. This C-repeat region, highly conserved across various GAS strains, holds potential as a vaccine candidate capable of offering protection against multiple strains of the bacteria [10]. Several research groups have delved into the conserved region vaccine strategy. Currently, three main approaches have been explored:

1. Utilizing the complete C-terminal region of the M6 strain as a recombinant protein [261]. Bessen and Fischetti demonstrated that peptides representing the conserved region of the M-protein from an M6 GAS strain could induce IgA antibodies. These antibodies offered passive protection to mice when combined with GAS and administered intranasally [262]. It is known that the mucosal epithelium of the pharynx serves as a primary site for GAS infection in humans and IgA plays a crucial role in defending against bacterial infection in this context. Expanding this approach, the peptides were linked to the cholera toxin B subunit (CTB). Mice vaccinated with these peptide-CTB conjugates showed significantly reduced pharyngeal colonization upon intranasal GAS challenges, compared to control mice [263].

2. Utilizing a 12 amino-acid minimal B-cell epitope from the C-repeat region (J8) [168]. An epitope named peptide 145, situated within the conserved C-terminus of the M protein, has been identified as the target of opsonic antibodies in humans and mice, detected in serum of adults in streptococcal-exposed areas [264]. However, concerns about potential cross-reactivity with host cardiac myosin due to T cell activation arose [265]. To mitigate this, a shorter sequence called J8 was developed, preserving the beneficial immune features of the M protein epitope while excluding a potentially harmful T cell epitope [266]. The synthetic peptide J8 from GAS' C-repeat region initially showed poor immune response in genetically diverse mouse populations. To enhance its immunogenicity, it was conjugated to diphtheria toxoid (DT) [168]. The resulting J8-DT conjugate, when administered with adjuvants like CFA or alhydrogel, demonstrated high immunogenicity in both inbred and outbred mice [168]. Vaccination with J8-DT led to the development of memory B cells and long-lasting antibody responses, providing protection against systemic infection in mice [267]. Additionally, similar promising results were observed for the related peptide J14 as an intranasal vaccine [268].

3. Utilizing the B and T cell epitopes as a synthetic peptide from the C-repeat region. Brazilian researchers have developed a potential GAS vaccine called "StreptInCor." This vaccine is based on amino acid sequences from the conserved region (C2 and C3) of the M5 protein. By analyzing a broad panel of around 900 sera and peripheral blood mononuclear cells (PBMC), they identified crucial B and T immunodominant epitopes. These findings led to the creation of StreptInCor, comprising 55 amino acid residues [269]. This construction offers the advantage of potentially inducing both memory T and B cells, likely resulting in a more robust protective immune response. The safety of this vaccine was validated due to not having any autoimmune reactions [270, 271].

4.3.2 Multivalent M protein-based vaccines

Over 200 emm types are identified based on the 5' emm gene sequence encoding the N-terminus or the HVR of the mature protein [272]. The HVR has been found to evoke antibodies with potent bactericidal activity and lower potential for tissue cross-reactivity. This has led to the development of recombinant multivalent subunit vaccines containing 4–30 different HVR M peptides [273-276].

The most recent 30-valent vaccine, StreptAnova™, encompasses prevalent M types in the US, Canada, and Europe, potentially providing immunity against around 85% of pharyngitis and invasive infections in these regions [276]. Phase 1 evaluation in adults demonstrated StreptAnova™ to be safe, well-tolerated, and immunogenic without triggering autoimmunity [259]. Additionally, an innovative approach involved expressing N-terminal M peptides through Lactobacillus lactis, serving as mucosal immunogens that protected mice from mucosal challenge infections. This approach could offer a cost-effective vaccine production method for low- and middle-income countries [277, 278].

4.3.3 Advantage and disadvantages of M-protein based vaccines

As a vaccine candidate, C-repeat region has the potential to induce host protection against all GAS strains; however, concerns about the effectiveness of conserved region epitopes and their immunogenicity have been raised. A study by Jones and Fischetti, involving 19 monoclonal antibodies, showed that only one of them could opsonize the M6 streptococci strain by targeting the amino-terminal region of the M-protein. Notably, antibodies targeting the C-repeat region did not opsonize but were able to fix complement in the study [279].

The multivalent M protein vaccine approach is questioned due to its potential limitations in providing vaccine coverage for low- and middle-income countries, as well as disadvantaged populations in high-income countries. These concerns arise from the higher diversity of M types in these regions, where children and young adults face the greatest risk of acute rheumatic fever (ARF) and rheumatic heart disease (RHD) [280, 281]. However, recent investigations indicate that the development of broadly protective vaccines containing N-terminal HVR peptides might not be as challenging as initially thought, as not all M types are immunologically distinct [9]. This suggests the possibility of achieving broader protection against streptococcal infections than previously anticipated.

4.4 Non-M protein antigens used in GAS vaccine candidates

Several vaccine candidates against GAS target non-M protein antigens as well. These antigens exhibit high conservation levels, being present in 95-99% of all known GAS isolates worldwide [1, 282, 283]. In these types of vaccines, crucial virulence factors of GAS have been carefully chosen and incorporated.

Fibronectin-binding proteins: GAS expresses a diverse range of at least 11 different Fibronectin (Fn)-binding proteins, each contributing to its virulence. Many of these proteins have multifunctional roles. The Fn-binding proteins of GAS play essential roles in virulence through binding to host fibronectin, and their potential as vaccine targets is of significant interest [24, 63].

Streptococcal C5a protease: The streptococcal C5a protease (SCP) is expressed on the surface of various GAS serotypes and many human isolates of groups B, C, and G streptococci, functioning to specifically degrade C5a. This enzyme also binds fibronectin and acts as a low-level 51nvasion for GAS [284]. Intranasal and subcutaneous immunization using recombinant SCP along with adjuvants, elicits strong anti-SCP IgG and IgA responses. These responses facilitate the rapid clearance of streptococci from pharyngeal- and nasal-associated lymphoid tissue after challenge with various serotypes [257, 285-287]. The antibodies against SCP inhibit C5a cleavage and exhibit opsonic activity against both GAS and group B streptococci [287]. As

anticipated, intranasal administration of anti-SCP antibodies in mice prevented colonization of NALT, which corresponds to human tonsils. This research highlights SCP's potential as a target for immunization against streptococcal infections [286].

Carbohydrate capsule antigens: Carbohydrate capsule antigens have been integrated into various vaccines targeting streptococcal species. However, the capsule of GAS is made of hyaluronic acid, which is a molecule also present in human tissues and recognized as a self-antigen. Consequently, using this molecule as a vaccine component is not feasible due to its self-antigen nature [288, 289].

Another significant component in GAS is the group A carbohydrate (GAC), making up around half of the cell wall [290]. Initially, the vaccine potential of purified GAC was explored by linking it to tetanus toxoid. Mice immunized with this combination were safeguarded against systemic and intranasal challenges [291]. However, the N-acetylglucosamine side chain of GAC has been implicated as a trigger for post-infection complications caused by GAS [292].

Pili: GAS infections are dependent on the pathogen's ability to adhere to host tissues and form cell aggregates. Mutants lacking GAS pili demonstrated reduced attachment to pharyngeal cell lines. Additionally, in vivo evidence of pilus expression was shown as sera from patients with GAS-mediated pharyngitis reacted to recombinant pili proteins. These findings underscore the significance of pili in GAS infections by facilitating attachment and aggregation processes [293].

Streptolysin O is a toxin secreted by GAS that forms pores in host cell membranes, contributing to the pathogen's virulence. This toxin is upregulated in more aggressive strains of GAS[1].

SpyCEP, is a protease produced by the bacterium. As previously mentioned, tt plays a role in evading the host immune response by cleaving IL-8. By breaking down IL-8, SpyCEP helps the bacterium avoid detection by the immune system [294].

SpyAD is an adhesin found on the surface of S. pyogenes. This protein enables the bacterium to interact with and attach to host cells. This interaction is a crucial step in the process of infection, allowing the bacterium to establish itself within the host's tissues [295].

Trigger factor (TF) is an essential protein that assists in the proper secretion and maturation of another protein, the cysteine protease, in S. pyogenes. This process is important for the bacterium to effectively deploy its virulence factors [296].

Arginine deiminase (ADI) is an enzyme produced by GAS that plays a role in colonization and manipulating the host immune response. ADI converts arginine, an amino acid, into citrulline and ammonia. This metabolic conversion helps the bacterium adapt to the host environment and influence the immune response to its advantage [297].

4.5 Future of vaccine development

The development of GAS vaccines lags behind other high-burden infectious diseases, prompting the establishment of the GAS Vaccine Accelerator Consortium (SAVAC) in 2019 [3]. SAVAC brings together experts to expedite vaccine development and highlights the cost-effectiveness of a GAS vaccine. Key knowledge gaps include limited data from low- and middle-income countries, incomplete understanding of protection measures, lack of immune correlates and functional assays, absence of standardized safety surveillance, and the need for advocacy. SAVAC aims to address these gaps, advance vaccine development, and raise awareness about the burden of GAS disease [298]. SAVAC's next phase focuses on research coordination to address gaps, improve disease estimates, establish safety standards, enhance advocacy, and engage stakeholders. They've developed an innovative framework to guide vaccine development based on accurate burden of disease data [299].

The experience of developing vaccines for SARS-CoV-2 offers valuable lessons, including the utilization of advanced vaccine technologies like mRNA and streamlined clinical trial approaches. These insights can be applied to GAS vaccine development [300]. As potential lead GAS vaccine candidates advance towards clinical trials, SAVAC is planning to support the field over the next five years. This involves several key steps:

1. Gathering epidemiological, economic, and societal data for vaccine efficacy trials in low- and middle-income countries. Strengthening surveillance, laboratory activities, and clinical trial capacity through a network of sentinel sites in these countries.
2. Engaging with vaccine developers and manufacturers to emphasize the need and commercial viability of a GAS vaccine. Addressing barriers to accelerate the GAS vaccine pipeline.
3. Collaborating with non-industry stakeholders such as WHO, global funders, national policy makers, and experts in laboratory and safety surveillance. This aims to address barriers and enhance implementation efforts for a future GAS vaccine.

These steps collectively aim to pave the way for successful GAS vaccine development and deployment [251].

5 General methods and methodology

5.1 Immunizations and Infections

Immunization and infection protocols are critical for studying the immune response and the effectiveness of vaccines or treatments. In my experiments, antigens, often in the form of inactivated or killed bacteria, are administered to mice to stimulate an immune response. Subsequent exposure to live bacteria allows to assess the protective effects of the immunization.

Application and Rationale: In my study, I aimed to investigate the immune response and protective efficacy of immunization with heat-killed (HK) and formalin-fixed (FF) bacteria. Specifically, I used a GAS M1strain (GAS-M1) to immunize mice and later challenged them with a live, lethal dose of the same bacteria. This approach helps to determine the effectiveness of the immunization in providing protection against the infection.

Experimental Procedure

1. Preparation of Bacteria:

Heat-Killed (HK) Bacteria: Log-phase cultures of GAS-M1 were washed with PBS and incubated at 60°C for two hours to achieve complete killing. This process ensures that the bacteria are no longer viable while maintaining their antigenic properties.

Formalin-Fixed (FF) Bacteria: Log-phase cultures of GAS-M1 were fixed with 1% formalin to kill the bacteria. This method preserves the bacterial structure and antigens, ensuring they can still elicit an immune response.

Confirmation of Killing: The killing of bacteria was confirmed by plating on blood agar and checking for the absence of bacterial growth.

2. Storage:

HK Bacteria: The suspensions were diluted to the appropriate concentration and stored at -20°C.

FF Bacteria: The suspensions were stored at 4°C and used in max one month.

3. Immunization of Mice:

Mice were injected intraperitoneally (i.p.) or subcutaneously (s.c.) with 10^8 CFU of HK or FF GAS-M1. This dose was administered one to three times at three-week intervals.

Blood samples were collected 19 days after each injection to monitor the immune response.

4. Challenge with Live Bacteria:

For protection experiments, a lethal dose of 10^8 CFU live GAS-M1 was administered i.p. three weeks after the last immunization.

The cages were blinded to prevent any bias in determining the morbidity and mortality of the mice after the challenge.

Rationale for Method Selection

1. Effective Antigen Presentation: Using HK and FF bacteria ensures that the antigens necessary to stimulate the immune system are preserved while eliminating the risk of infection. This method allows the immune system to recognize and respond to the bacterial antigens effectively [301].

2. Mimicking Natural Infection: Injecting mice with a lethal dose of live bacteria after immunization simulates a scenario where an individual is exposed to a pathogen after vaccination. This approach is essential for evaluating the protective efficacy of the immunization.

3. Blinding to Prevent Bias: Blinding the cages during the challenge phase ensures objective assessment of the outcomes, reducing the potential for bias in determining the effectiveness of the immunization.

5.2 Flow Cytometry

Flow cytometry is a powerful analytical technique used to measure the physical and chemical properties of cells or particles suspended in a fluid. By passing cells through a laser beam, this method enables the detection and quantification of multiple parameters simultaneously, such as cell size, granularity, and the presence of specific markers identified by fluorescent antibodies. It is widely used in immunology, cell biology, and clinical diagnostics for its ability to analyze complex cell populations at the single cell level with high precision.

Application and Rationale: In my experiments, I employed flow cytometry to detect and analyze various cell types in the lymph nodes and spleen, including germinal center B cells, Tfh cells, IFN-γ production by T cells, 2W-specific Th cells, macrophages, monocytes, neutrophils, and eosinophils, etc**.**

The rationale for using flow cytometry in this context includes several critical factors:

Sample Preparation: After preparing single-cell suspensions form the lymph nodes or spleen, the cells were stained with specific antibodies that target antigens on the cell surface or intracellular antigens. These antibodies are conjugated with fluorochromes, which emit light of different wavelengths when excited by the laser in the flow cytometer. This preparation step is crucial for ensuring that each cell type of interest can be accurately identified based on its unique antigen profile.

Multiparametric Analysis: Flow cytometry allows for the simultaneous measurement of multiple cell surface and intracellular markers. This capability is essential for identifying and differentiating between the diverse cell types of interest in my study, such as Tfh cells and IFN-γ producing T cells, which all require the detection of specific combinations of markers.

Quantitative Data: Flow cytometry provides quantitative data on cell populations, allowing for a detailed analysis of the frequency and number of each cell type within the lymph nodes and spleen. This quantitative aspect is crucial for understanding the dynamics and regulation of immune responses.

Detection and Analysis: After staining, the cells are washed to remove excess antibodies and then analyzed by the flow cytometer. The machine detects the emitted light from the fluorochrome-labeled antibodies bound to the target cells, allowing for the identification and quantification of each cell type based on the specific light emitted. The data collected by the flow cytometer is subsequently analyzed using the FlowJo software, which provides a comprehensive analysis of the cell populations, including gating strategies and statistical analysis.

Speed and Efficiency: The method allows for the rapid analysis of thousands to millions of cells in a relatively short period. This efficiency is beneficial when processing multiple samples or large volumes, ensuring timely and consistent data collection.

5.3 ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used technique for detecting and quantifying soluble substances, such as peptides, proteins, antibodies, and hormones. The method involves the binding of an antigen to a surface (usually a

microtiter plate) and the subsequent binding of a specific antibody to the antigen. This interaction is then detected using an enzyme-linked secondary antibody, which produces a measurable color change upon the addition of a substrate.

Application and Rationale: In my experiments, I utilized the ELISA method to measure the antibody levels in mouse sera. The procedure involved coating a 96-well plate with the desired antigens (intact GAS, M1 protein or HVR) and subsequently detecting specific antibodies against these antigens in the serum samples.

Experimental Procedure

1. Coating the Plate: The 96-well plate was coated with the desired antigens. These antigens were adsorbed onto the surface of the wells, providing a target for the antibodies in the serum samples.

2. Blocking: The wells were then blocked with a blocking buffer to prevent nonspecific binding. This step is critical to reduce background noise and improve the specificity of the assay.

3. Sample Addition: Serially diluted serum samples from mice were added to the wells. Any antibodies present in the serum that are specific to the coated antigens will bind to them.

4. Detection: After washing away unbound antibodies, a secondary antibody conjugated with an enzyme (such as horseradish peroxidase, HRP) was added. This secondary antibody binds to the primary antibodies that are attached to the antigens.

5. Substrate Addition: A substrate for the enzyme was then added to the wells. The enzyme catalyzes a reaction that produces a color change, the intensity of which is proportional to the amount of bound antibody.

6. Measurement: The colour change was measured using a spectrophotometer, providing quantitative data on the antibody levels in the serum samples. A pooled serum from animals previously immunized with HK GAS M1 was used as a standard serum. A standard curve was employed to calculate other values based on this standard. The values presented in the figures are expressed in arbitrary units.

By using the ELISA method, I aimed to accurately measure the levels of specific antibodies in the mouse sera. This detailed analysis is essential for understanding the systematic immune response and evaluating the effectiveness of immunization or other interventions in my research.

5.4 CBA Assay

The Cytometric Bead Array (CBA) assay is a multiplex flow cytometry technique used to measure multiple soluble analytes, such as cytokines, chemokines, and growth factors, in a single sample. It involves the use of beads coated with specific capture antibodies that bind to target analytes. These beads are then detected using a flow cytometer, allowing for the simultaneous quantification of multiple analytes from small sample volumes.

Application and Rationale: In my study, I utilized the CBA assay to measure cytokine levels in blood, spleen, and i.p. wash samples from mice. These measurements were performed on both immunized and non-immunized mice to assess the differences in cytokine production before and after infection. This approach provided crucial insights into the immune response elicited by the immunization and subsequent infection.

Experimental Procedure

1. Sample Collection:

Blood: blood was collected from mice and processed to obtain serum.
Spleen: spleens were harvested, homogenized, and processed to obtain suspensions containing cytokines and chemokines.
i.p. Wash: Intraperitoneal washes were performed by injecting and aspirating PBS to collect peritoneal exudate cells and fluid.

2. Preparation of CBA Assay:

Bead Preparation: CBA beads coated with capture antibodies specific to various cytokines were prepared according to the manufacturer's instructions.
Sample Incubation: Serum, spleen suspensions, and i.p. wash samples were incubated with the prepared beads. Cytokines in the samples bind to the corresponding capture antibodies on the beads.

Detection: Detection antibodies conjugated with fluorescent markers were added to the bead-sample mixtures. These antibodies bind to the captured cytokines, allowing for fluorescence detection.

3. Flow Cytometry Analysis:

The bead-sample mixtures were run through a flow cytometer. The flow cytometer detects and quantifies the fluorescence emitted by each bead, corresponding to the concentration of specific cytokines.

Data Analysis: The collected data were analyzed using flow cytometry analysis software to determine the levels of various cytokines in the samples. There was a standard with a defined concentration for each cytokine. The concentration of cytokines in each sample can be calculated based on these standards.

Rationale for Method Selection

1. Multiplexing Capability: The CBA assay's ability to measure multiple cytokines simultaneously from a single sample is highly advantageous. This feature is crucial for obtaining a comprehensive cytokine profile, especially when working with limited sample volumes.

2. Sensitivity and Specificity: The CBA assay provides high sensitivity and specificity for cytokine detection. This is essential for accurately measuring low-abundance cytokines and detecting subtle changes in cytokine levels between immunized and non-immunized mice.

3. Quantitative Data: The CBA assay yields quantitative data on cytokine concentrations, which is critical for assessing the magnitude of the immune response. This quantitative analysis allows for a detailed comparison of cytokine production before and after infection.

4. Comparative Analysis: By measuring cytokine levels in blood, spleen, and i.p. wash samples from both immunized and non-immunized mice, the CBA assay enables a comprehensive assessment of the systemic and local immune responses. This comparative analysis is importnt for understanding the impact of immunization on cytokine production, with possible impact on immune protection.

6 Ethical statement

This study was conducted with the approval of the Lund/Malmö Animal Ethical Committee, permit numbers: 7342/2017 and 07178/2020 in accordance with local legislation and institutional requirements. Both my supervisor and co-supervisor possess the necessary ethical permits for conducting in-vivo experiments with mice. Additionally, we have obtained ethical permits for working with GAS.

The ethical permits for this project encompass various experimental procedures involving mice, including immunization, euthanization, and infection. Given the sensitive nature of the experiments, which involve the injection of mice with lethal doses of bacteria at specific intervals, strict measures are taken to minimize the suffering of the animals. Mice are monitored every four hours for signs of distress such as shaking, bending, slow movement, or closed eyes. If any of these symptoms are observed, indicating discomfort, the mice are euthanized promptly to alleviate suffering. Furthermore, if any signs of sickness, shaking, or lesions resulting from the injections are noted, the mice are sacrificed immediately to prevent further suffering.

Working with infectious bacteria like GAS entails significant risks to laboratory personnel, necessitating adherence to rigorous biosafety protocols to prevent accidental exposure or release. All personnel involved in the experiments are trained in these protocols to ensure a safe working environment.

By adhering to these ethical guidelines and protocols, we aim to conduct our research responsibly and humanely, prioritizing the welfare of the animals and the safety of the research team.

7 Present investigations

7.1 Aim of the thesis

- Boosting the protective immune response against GAS in a subcutaneous immunization model and to investigate the underlying protective mechanisms.
- To establish an intraperitoneal immunization protocol to provide protective immunity in mice against GAS infection and to assess the immune response responsible for this protection.
- To assess the effectiveness of various adjuvants in enhancing immune responses against GAS, with a particular focus on the IFN-γ-inducing molecule poly I:C.

7.2 List of papers

Paper I:

Insertion of an immunodominant T helper cell epitope within the Group A Streptococcus M protein promotes an IFN-γ dependent shift from a nonprotective to a protective immune response.

Shiva Emami, Thiago Rojas Converso, Jenny J. Persson and Bengt Johansson-Lindbom.
Front. Immunol. 2023-August-15, 14:1241485.

Paper II

Protection acquired upon intraperitoneal Group A Streptococcus immunization is independent of concurrent adaptive immune responses but relies on macrophages and IFN-γ.

Shiva Emami, Elsa Westerlund, Thiago Rojas Converso, Bengt Johansson-Lindbom and Jenny J Persson

Paper III

A whole-cell immunization regimen with Group A Streptococcus and the adjuvant poly I:C conferring IFN-γ -dependent protective immunity.

Shiva Emami, Jenny J Persson, and Bengt Johansson-Lindbom

Paper I: Insertion of an immunodominant T helper cell epitope within the Group A Streptococcus M protein promotes an IFN-γ dependent shift from a nonprotective to a protective immune response.

GAS represents a prevalent human pathogen causing around 700 million infections and 500 000 deaths annually worldwide and GAS infections are also linked to aberrant immune responses underlying onset of severe autoimmune diseases, including rheumatic fever and heart disease.

Despite decades of attempts to develop a vaccine against GAS, no such vaccine exists. Some vaccine candidates are being tested in early human trials, with those based on the bacterial surface M protein showing promise. However, there's a problem with the M protein's variability, especially in the HVR at the N-terminal. This makes it difficult to create a vaccine that can protect against all the different types of GAS.

In the current study, we investigate the efficacy of heat-killed GAS of the M1 serotype (HK GAS-M1) in inducing protective immunity in B6 mice. While administration of heat-killed GAS elicits significant IgG responses, it fails to confer protective immunity. However, insertion of the immunodominant T helper cell epitope 2W into the N-terminal part of the M1 protein leads to the development of a recombinant GAS strain (GAS-2W.M1) that induces protective immunity in B6 mice.

The generation of non-protective antibodies underscores challenges in vaccine development, emphasizing the importance of antibody subclass, epitope targeting, and immune response quality. Comparing antibody subclasses in our study's mouse model, revealed that mice immunized with GAS-2W.M1 had higher levels of IgG2c compared to those immunized with the original strain. Additional experiments with mice lacking Tfh cells and IFN-γ deficient mice showed that protective immunity depends on a T cell-dependent antibody response and IFN-γ, possibly involving the production of IFN-γ-dependent antibodies of the complement-fixing IgG2c subclass.

In summary, this study suggests that the M protein of GAS has evolved mechanisms to limit IFN-γ production by Th cells, thereby evading efficient adaptive immune responses. Notably, mice immunized with the recombinant GAS strain display similar levels of total IgG against GAS-M1 as those immunized with the non-recombinant strain but exhibit a selective increase in antibodies of the IgG2c subclass.

Conclusion: Insertion of the immunodominant CD4 T cell epitope 2W into the N-terminal of the M1 protein results in a recombinant GAS strain with an enhanced ability to induce protective immunity against the parental GAS-M1 strain, which depends on IFN-γ and associated with increased and IFN-γ-dependent IgG2c responses.

Key findings:

- Inserting the immunodominant CD4 Th cell epitope 2W into the N-terminal of the M1 protein overcomes the lack of protective immunity observed after GAS-M1 immunization.
- Immunization with GAS-2W.M1 does not provide protection against GAS-M1 infection in the absence of T cell-dependent B cell responses.
- The 2W Th cell epitope enhances a robust CD4 T cell IFN-γ response in GAS-2W.M1-immunized mice.
- The inability of GAS-2W.M1 to induce protective immunity in IFN-γ deficient mice is linked to a specific reduction in IgG2c responses.
- The absence of IFN-γ does not hinder germinal center B cell or Tfh cell responses after GAS-2W.M1 immunization.

Paper II: Protection acquired upon intraperitoneal Group A Streptococcus immunization is independent of concurrent adaptive immune responses but relies on macrophages and IFN-γ.

In Paper I, we explored the immune mechanisms responsible for the protection induced by s.c. immunization. In Paper II, which actually preceded Paper I in terms of experiment initiation, we conducted i.p. immunization and investigated the immune mechanisms underlying protection. Interestingly, we discovered two distinct pathways contributing to protection depending on whether the immunization was administered subcutaneously or intraperitoneally.

Recent research has expanded our understanding of immune protection beyond traditional adaptive B and T cell memory, revealing innate immune mechanisms such as trained immunity, which confer non-specific protection against both homologous and heterologous infections. While type-specific immunity against GAS has been extensively studied, broader protection observed in adults and the potential role of trained immunity in GAS infections remain unexplored.

Research Aim and Conclusion:

This study aims to investigate protective immunity induced by i.p. of mice with intact HK GAS. Results demonstrate that repeating injections with HK GAS-M1 generate protective immunity, seemingly independent of canonical adaptive immune responses. Although GAS-specific IgG production is induced, the protective mechanisms do not

involve germinal center development, CD4 T cells, or antibodies. The production of non-protective antibodies has always been a challenge in vaccine development. Differences between s.c. and i.p. immunization models underscore tissue-specific immune responses. Insights from the concept of trained immunity and previous studies on Gram-positive bacteria suggest potential innate immune mechanisms that mediate protection.

Our data indicate that the protection conferred by immunization in this model is associated with changes in the acute cytokine profile during subsequent infection. Additionally, the survival of immunized mice following a lethal infection relies on macrophages and the cytokine IFN-γ, which activates these macrophages. These findings represent the first evidence suggesting that GAS may trigger forms of trained innate immunity.

C**onclusion**: our findings shed light on a potentially new mechanism in protective immunity against GAS, not relying on adaptive memory responses and targetable through immunization. We observed that immunized animals displayed changes in their cytokine profile and an increase in the recruitment of monocytes, which differentiated into macrophages, during subsequent infection. Further research is needed to understand how innate immune cells are trained and the specific role of different mediators in immunized mice. This will help us to better understand the importance of innate responses in GAS infections and vaccine development strategies.

Key findings:

- Repeated intraperitoneal immunization with heat-killed GAS-M1 confers protection
- Intraperitoneal immunization-induced protective immunity does not depend on antibodies produced by germinal center B cells and T cell assistance
- Immunized mice exhibit a selective increase in monocytes/macrophages and an altered cytokine profile after lethal challenge with GAS-M1.
- Protection against GAS infection in immunized mice relies on macrophages and IFN-γ.

Paper III: A whole-cell immunization regimen with Group A Streptococcus and the adjuvant poly I:C conferring IFN-γ–dependent protective immunity.

In both Paper I and Paper II, we utilized heat-killed GAS alone for immunizing mice. However, we were curious about the protective efficacy if we immunized mice with pure protein and adjuvant. In the current study, our objective was to assess the

effectiveness of two different adjuvants in boosting immune responses and providing protection against GAS infection. Our results after s.c immunization with pure M1 protein indicate that immunization with the Poly I:C adjuvant resulted in significantly better survival outcomes compared to Alum adjuvant, emphasizing the importance of adjuvant selection in vaccine formulations. Moreover, we observed that adding the Poly I:C adjuvant to heat-killed GAS M1 (HK-GAS M1) improved protection against GAS-M1 infection, highlighting the potential of adjuvant-mediated immune modulation in vaccine strategies against GAS.

Furthermore, our findings indicate a crucial role of IFN-γ in conferring protection against GAS infection. IFN-γ, a key cytokine produced by activated T cells and natural killer cells and possesses potent antimicrobial and immunomodulatory effects. In several murine models of GAS infection, IFN-γ has emerged as a critical mediator of protective immunity [235, 302] . Our study suggests that the failure of the whole-cell GAS-M1 immunization regimen to confer protection at least partially can be explained by too weak IFN-γ responses and that including poly I:C to the formulation enhances IFN-γ, leading to protection during subsequent GAS infection. Our study provides insights into the mechanisms underlying host defence against this pathogen.

Conclusion: In summary, our research indicates that selecting the right adjuvant can enhance the immune response and improve protection against GAS infection. We also found that IFN-γ plays a critical role in protecting against GAS following immunization with intact bacteria and poly I:C. These discoveries help us better understand how immune system fights off this infection and provide clues for developing new treatments and vaccines.

Key findings:

- Immunization of mice with recombinant M1 protein with Poly I:C as adjuvant leads to increased survival compared to immunization using Alum as adjuvant.
- Adding Poly I:C as adjuvant to the HK GAS-M1 immunization increases protection against subsequent GAS-M1 infection.
- Following this immunization regimen, IFN-γ is crucial for protecting against GAS infection.

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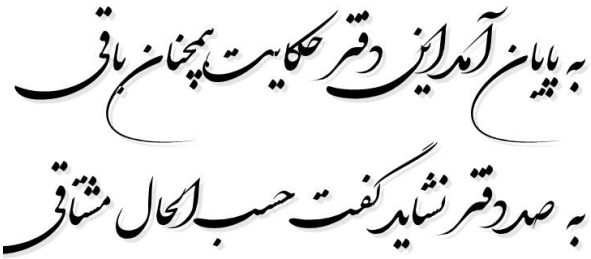
برای مادرم که دلیل بودنم در اینجاست. کلمات قادر به بیان نیستند که چقدر دوستت دارم و چقدر عمیقاً مدیون تو هستم، مامانی. تو همیشه دوست داشتی من و خواهرم شاد و موفق باشیم و هر کاری انجام دادی تا این امکان را فراهم کنی. از همه حمایت ها و فداکاری هایت برای بهتر شدن زندگی ما ممنونم. دوستت دارم تا دنیا دنیاست ❤



سعدی

This book has come to an end, but the story still remains…
In hundred books it can't be expressed how happy I am now…

Saadi

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