Review

Molecular and genomic characterization of pathogenic traits of group A *Streptococcus pyogenes*

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Abstract: Group A streptococcus (GAS) or *Streptococcus pyogenes* causes various diseases ranging from self-limiting sore throat to deadly invasive diseases. The genome size of GAS is 1.85–1.9 Mb, and genomic rearrangement has been demonstrated. GAS possesses various surface-associated substances such as hyaluronic capsule, M proteins, and fibronectin/laminin/immunoglobulin-binding proteins. These are related to the virulence and play multifaceted and mutually reflected roles in the pathogenesis of GAS infections. Invasion of GAS into epithelial cells and deeper tissues provokes immune and non-immune defense or inflammatory responses including the recruitment of neutrophils, macrophages, and dendritic cells in hosts. GAS frequently evades host defense mechanisms by using its virulence factors. Extracellular products of GAS may perturb cellular and subcellular functions and degrade tissues enzymatically, which leads to the aggravation of local and/or systemic disorders in the host. In this review, we summarize some important cellular and extracellular substances that may affect pathogenic processes during GAS infections, and the host responses to these.

Keywords: *Streptococcus pyogenes*, GAS, infection, virulence factor

Introduction

Streptococcus pyogenes are gram-positive, non-motile, facultatively anaerobic cocci. Clinical isolates of β-hemolytic streptococci have been classified into serological groups A, B, C, etc., based on the immunochemical specificity of their cell wall polysaccharides. Group A streptococcus (GAS) includes a single species, *S. pyogenes*. The genus *Streptococcus* contains *ca.* 130 species and subspecies, most of which have their natural habitat in humans and/or animals. Based on 16S rRNA and multilocus sequence type analysis (MLSA), streptococcal species have been separated into distinct groups such as pyogenic, mitis, mutans, and bovis. Among these, the pyogenic group comprises multiple human and animal pathogens such as *Streptococcus agalactiae* (Lancefield group B), *Streptococcus equi* (group C), *Streptococcus dysgalactiae* (group C), as well as GAS. Thus, the pyogenic streptococcal species are of medical and/or veterinary importance.[1),2)]

GAS usually colonizes the throat or skin epithelial surfaces and causes a wide variety of clinical manifestations such as noninvasive pharyngitis,

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Abbreviations: C5aBP: C5a-binding protein; CRISPR: clustered regularly interspaced short palindromic repeats; ECM: extracellular matrix; FBP: fibronectin-binding protein; FCT: fibronectin- and collagen-binding T-antigen; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GAS: group A streptococcus; GcAV: GAS-containing autophagosome-like vacuole; HA: hyaluronic acid; HLA: human leukocyte antigen; IAV: influenza A virus; Ig: immunoglobulin; Lbp: laminin-binding protein; LPXTG: leucine-proline-any amino acid-threonine-glycine; Mrp: M-related protein; NCBI: National Center for Biotechnology Information; NETs: neutrophil extracellular traps; NF: necrotizing fasciitis; NO: nitric oxide; PRP: proline-rich protein; ROS: reactive oxygen species; Scl: streptococcal collagen-like surface protein; Sda: streptodornase; Ska: streptokinase; SLO: streptolysin O; SLS: streptolysin S; Spe: streptococcal pyrogenic exotoxin; Srt: sortase; STSS: streptococcal toxic shock syndrome; SWAN: streptococcal wall-anchored nuclease; uPAR: urokinase plasminogen activator receptor.

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dermatitis, and scarlet fever, as well as invasive systemic infections such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) in humans. Additionally, glomerulonephritis and rheumatic fever are post-streptococcal non-suppurative immune sequelae. In humans, noninvasive GAS infections occur most frequently in various age groups, while cases of deep-seated soft-tissue infections are occasionally encountered. While treatment with high doses of β-lactam antibiotics is effective against noninvasive GAS infections, it is not effective in the case of invasive infections. The incidence of invasive GAS infections has been increasing globally since the mid-1980s and is associated with high morbidity and mortality.[3),4)] The incidence and severity of the infections are highest in winter.[5)]

A systematic review of the Medline and WHO databases in 2005 estimated that 18.1 million existing cases of severe GAS diseases, with 1.78 million new cases occurring globally each year, led to 500,000 deaths yearly due to severe acute rheumatic fever, rheumatic heart disease, post-streptococcal glomerulonephritis, and invasive infections. The global burden of invasive GAS infections deserves greater attention because of 663,000 new cases with 163,000 deaths each year. In addition, 616 million new cases of pharyngitis and 111 million existing cases of pyoderma have been noted. These estimates indicate that the importance of GAS infections is undervalued in many countries worldwide.[6)]

GAS possesses various cell-surface components such as hyaluronic acid, M and T proteins, and proteins binding to host components such as fibronectin (FN), laminin, immunoglobulins (Igs), lipoteichoic acid, and peptidoglycan, which may contribute to pathogenesis. Additionally, GAS produces extracellular enzymes including streptokinase (Ska), proteinases, hyaluronidase, nucleases, and neuroaminidase, and toxins such as streptolysins, pyrogenic exotoxins (Spe), and streptococcal superantigens, some of which induce fever and shock. Following adherence of GAS to human host-cell surfaces, these factors may function in invading host tissues/organs, resulting in exacerbation of the disease manifestations.[7),8)] Some of these extracellular products induce the production of specific antibodies in hosts, which protect them from further infection by the same GAS strain. Here, we reviewed the current state of GAS research with special emphasis on the molecular pathogenesis and prevention of GAS infections.

Genomic features of GAS

Since the first genome sequence of an M1 strain of *S. pyogenes* has been published by Ferretti *et al.*[9)] complete genome sequences of 23 GAS strains and 201 permanent draft genomes have been reported. Some genomic features of 19 strains deposited in the National Center for Biotechnology Information (NCBI) database are shown in Table 1. The GAS genome is a single circular chromosome with a sequence length of 1.8–1.9 Mb. The average GC content is 38–39%, indicating that GAS belongs to the low-GC% gram-positive bacterial species. The genome possesses 8 to 10 prophages and nonfunctional phage remnants. We previously performed whole-genome sequencing of an M3 isolate from a Japanese STSS patient and found large-scale genomic rearrangements between the homologous *rrn-comX1* regions and between two prophage-coding regions across the replication axis (Fig. 1). As a result, 1 Mb of genomic DNA is inverted across the axis in this strain, and new phages are reconstructed according to this large genomic rearrangement. Notably, the genomic rearrangement occurred in 64 out of 94 clinical isolates collected during 1990–2002, while we observed it in only 25% of isolates obtained before 1985. Thus, prominent genomic rearrangements and integration of phages into the GAS chromosome may cause genomic diversity and unbalanced genomic architecture, which may result in the shuffling of virulence-related genes, thus generating new clones with modified gene cassettes.[10)] In fact, extensive rearrangement of multiple genetic factors and phage integration gave rise to a hypervirulent serotype M23 strain of GAS.[11)]

Bacteriophages play an important role in bacterial evolution by mediating DNA transfer and controlling the bacterial life cycle.[12)] There are two major outcomes of bacteriophage behavior in an infected bacterial cell: lysogenization and bacteriolysis. Bacterial cells lyse when a temperate bacteriophage infects a bacterial cell, whereas in lysogenization, bacteriophage DNA is integrated into the bacterial genome, resulting in a prophage region in the bacterial genome. Prophage regions encode virulence factors such as exotoxins,[13)] and the diversity of prophages might affect the variety in GAS pathogenicity. In fact, various prophages have been found in GAS strains isolated at different disease stages (Table 1).

Bacteria have evolved protection mechanisms against bacteriophage infection such as the restric-

Table 1. Genomic features of complete *Streptococcus pyogenes* genomes

Strain	*emm* type	Genome size (bp)	GC content (%)	Number of CDS	Number of tRNAs	Number of rRNA operons	Number of phages	NCBI accession no.
A20	1	1,837,281	38.5	1,808	67	6	3	NC_018936
Alab49	53	1,827,308	38.6	1,791	67	6	3	NC_017596
HSC5	14	1,818,351	38.5	1,785	67	6	3	NC_021807
M1-476	1	1,831,128	38.5	1,808	57	5	3	NC_020540
Manfredo	5	1,841,271	38.6	1,822	66	6	4	NC_009332
MGAS315	3	1,900,521	38.6	1,912	67	6	6	NC_004070
MGAS1882	59	1,781,029	38.5	1,703	57	5	1	NC_017053
MGAS2096	12	1,860,355	38.7	1,774	67	6	2	NC_008023
MGAS5005	1	1,838,554	38.5	1,811	67	6	3	NC_007297
MGAS6180	28	1,897,573	38.4	1,864	67	6	4	NC_007296
MGAS8232	18	1,895,017	38.5	1,908	67	6	5	NC_003485
MGAS9429	12	1,836,467	38.5	1,782	67	6	3	NC_008021
MGAS10270	2	1,928,252	38.4	1,913	67	6	5	NC_008022
MGAS10394	6	1,899,877	38.7	1,868	67	6	3	NC_006086
MGAS10750	4	1,937,111	38.3	1,908	67	6	4	NC_008024
MGAS15252	59	1,750,832	38.5	1,667	57	5	2	NC_017040
NZ131	49	1,815,785	38.6	1,769	66	6	3	NC_011375
SF370	1	1,852,441	38.5	1,811	60	6	4	NC_002737
SSI-1	3	1,894,275	38.6	1,917	57	5	6	NC_004606
Mean ± SD		1,854,917 ± 48,786	38.5 ± 0.2	1,822 ± 71	64 ± 4	6 ± 0.4	4 ± 1.3	

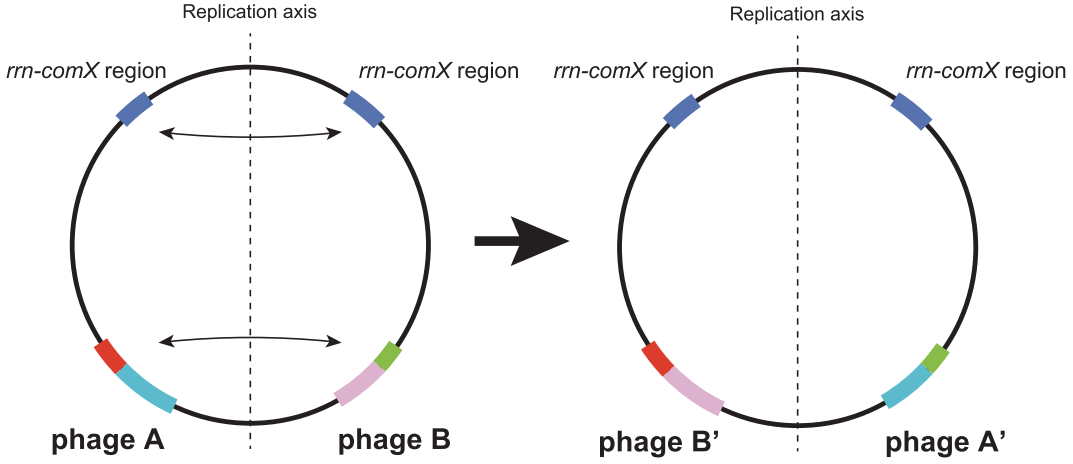

Fig. 1. Phage-related genomic rearrangements result in the diversity of the GAS chromosome. The virulence genes in the phage regions A and B are exchangeable, forming phages A′ and B′.

tion-modification system and clustered regularly interspaced short palindromic repeats (CRISPR).[14)–16)] CRISPR regions are responsible for acquired immunity in bacteria; they consist of repeat sequences separated by spacers of invading DNA that are essential for recognition and degradation of the target upon secondary infection.[17),18)] We previously reported that GAS has at most two distinct CRISPR loci, which contain fewer spacers than several other *Streptococcus* species.[19),20)] Since a negative correlation has been observed between the number of CRISPR spacers and the number of prophages, the CRISPR system in GAS likely regulates the acquisition of new prophages.[20)]

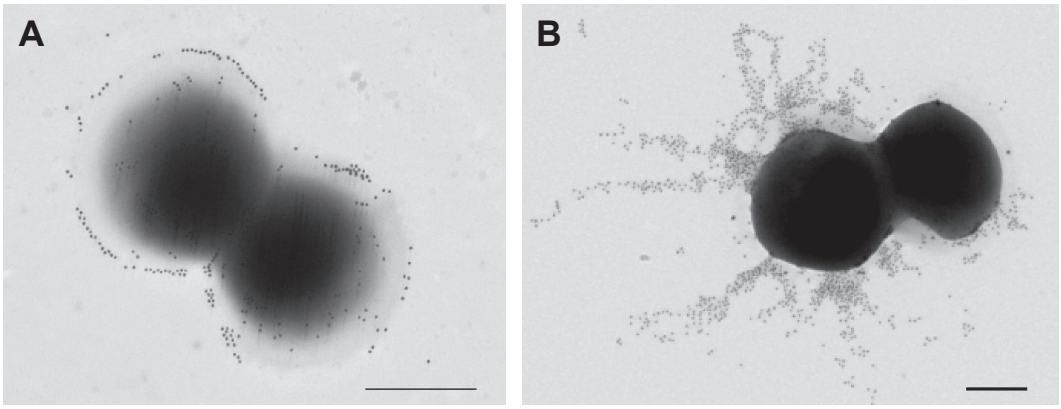

Fig. 2. Immunoelectron microscopy of the fibrillar structures on the surface of GAS. M proteins (A) and pili (B) are visualized by incubation with specific antibodies, followed by incubation with gold-conjugated secondary antibodies. Bar, 0.5 μm.

Virulence factors that may contribute to the pathogenesis of GAS

M protein. M protein is a fibrillar surface protein that exhibits an α-helical coiled-coil configuration with a hypervariable, non-helical N-terminal region and a conserved C-terminus with an LPXTG motif for anchoring of the proteins to the cell wall peptidoglycan (Fig. 2A). It plays an important role in pathogenesis due to its anti-phagocytic properties against neutrophils and promotes the adhesion/invasion of GAS into host cells. M protein is strongly antigenic, and serotype-specific antibodies against M protein protect the host from attack by GAS with the particular M protein serotype. Thus, M protein is a pivotal virulence factor of GAS.[21] M protein interacts with various host serum components including FN, Igs, fibrinogen, and plasminogen. The interaction of M1 protein with fibrinogen may trigger the activation of neutrophils, resulting in the release of inflammatory mediators inducing vascular leakage, which is a key phenomenon of STSS.[22] Additionally, M protein shows resistance to neutrophil extracellular traps, DNA-based innate immunity structures, through inhibition of the antimicrobial peptide cathelicidin (LL-37).[23),24]

M protein has been used for the serologic classification of GAS isolated from patients or healthy subjects for epidemiologic surveillance of GAS. Diversity in M serotypes has been reported in terms of both geographical distribution and disease profiles. Recently, serologic M-typing has been replaced with sequence typing of the *emm* gene encoding the M protein. Genotyping is based on sequencing of the 5′ end of the *emm* gene, and GAS strains have been separated into more than 250 *emm* genotypes.[25] Epidemiological studies have indicated that restricted *emm* genotypes generally predominate in developed, high-income countries, while in developing countries, a higher diversity of *emm* types has been reported.[26] For example, 25 *emm* types accounted for >90% of all tested isolates in high-income countries, whereas 26 *emm* types accounted for 61–62% of the isolates from the Pacific region or Africa.[27] In Japan, *emm* types 12 and 28 were the most prevalent among 1994–1999 isolates, followed by types 1, 4, and 13,[28] whereas type 1 represented the majority of the 2001–2005 isolates from severe invasive GAS infections.[29] In Thailand, the most prevalent types were, in descending order, *emm* types 44, 1, 81, 104, 75, 22, and 25.[30]

Attempts have been made to develop safe vaccines to prevent GAS infections. Recently, a vaccine containing 30 different M-protein peptides has been found to induce bactericidal antibodies against the particular serotypes. Furthermore, the antibodies exhibited bactericidal activity against 24 out of 40 non-vaccine M-protein serotypes, suggesting that at least some protective epitopes are shared between non-vaccine and vaccine serotypes of GAS.[31]

A significant percentage of GAS strains releases M-related protein (Mrp), which is structurally similar to M protein. Mrp, like M protein, has anti-phagocytic activity.[32] It preferentially binds human IgG (IgG1 > IgG4 > IgG2 > IgG3), but not IgM or IgA. The non-immune binding of Mrp enhances the ability of GAS to resist phagocytosis, which may explain the restriction of GAS infections to the human host. In this context, Mrp functions as a virulence factor of GAS.[33]

Pilus protein. Pili or fimbriae extending from the cell surface have been observed in various gram-

negative bacterial species, whereas little was known regarding the existence of pili in GAS until recently. Mora *et al.*[34] reported the presence of highly diverse filamentous pili on the cell surface of several M types of GAS (Fig. 2B). Since then, nine variants of the pilus-gene island located within the fibronectin- and collagen-binding T-antigen (FCT) region have been reported.[35),36] Serotype M6 FCT type 1 pili are assembled from two precursor proteins, *i.e.*, pilus backbone T6, which constitutes the shaft, and ancillary protein FctX. Anchoring of the pilus to the cell wall requires both a membrane-bound housekeeping sortase (SrtA) and pilus-associated sortase (SrtB). Pilus subunits are assembled through the LPXTG or a similar motif, which is cleaved between the threonine and glycine residues by SrtA.[37]

Trypsin treatment of GAS substantially removed M protein, while pili were not affected. The pili are used for Lancefield T-typing of GAS. FCT type 2 pili from serotype M1 strain have been shown to mediate adhesion to pharyngeal cell surfaces, which promotes biofilm formation and autoaggregation under static conditions. Pilus-deficient knockout mutants show impaired ability to adhere to the same cell line and are markedly less efficient in forming cell aggregates in standing liquid culture. These mutants produce no typical three-dimensional cell layer as indicated by confocal laser microscopic analysis.[38] In contrast, while FCT type 1 pili participate in biofilm formation, they inhibit bacterial aggregation, indicating that GAS pili may play variable roles during infection of hosts.[39] Purified recombinant pilus protein has been demonstrated to be a protective antigen in mice immunized with the protein along with adjuvant.[34]

In addition to GAS, other pathogenic streptococci possess LPXTG-anchored pili that are assembled by sortase and share homology among various streptococcal species including GAS, group B streptococcus, and *Streptococcus pneumoniae*. However, each of these species is expected to exhibit different modes of interaction with the host-cell surface or deep penetration into the cells or tissues.[40),41]

FN-binding protein. FN is an extracellular matrix (ECM) glycoprotein that is secreted by a range of host cells. It is a major soluble constituent of plasma and other body fluids. The insoluble form of FN is present in the ECM. FN is known to bind a wide variety of proteins and integrins on the surface of host cells (Fig. 3A). Various bacterial species including staphylococci and streptococci have been reported to bind FN in the initial adhesive stage before proliferation on the host cell surface and invasion into host tissues.[42),43] GAS possesses several cell surface proteins that are able to bind FN.[44)–46]

We have identified two novel surface-located FN-binding proteins (FBPs), FbaA and FbaB, both of which possess the LPXTG cell wall-anchor domain in the C-terminal region (Fig. 3B and C). FbaA is a 37.8-kDa protein, and the *fbaA* gene was found in 40 out of the 59 GAS strains examined. The *fbaA*-positive GAS strains comprised 18 of the 26 different M types. No test strains of groups B, C, D, and oral streptococci possessed the gene. Biotinylated recombinant (r) FbaA bound FN, but not IgG, IgA, and IgM. Mutants lacking FbaA exhibited decreased adhesion to and invasion of HEp-2 human epithelial carcinoma cells.[47] Functional analysis of FbaA revealed that a central proline-rich repeat domain, but not the N- and C-terminal domains, bound FN, and that antibodies specific for both repeat domain and the N-terminal portion of FbaA showed strong bactericidal activity that resulted in protection from GAS infection.[48] In contrast, FbaB (calculated molecular mass, 79 kDa) was present only in the M3 and M18 strains. All the M3 isolates (13 STSS and 14 pharyngitis isolates) possessed the *fbaB* gene; however, only 8 of 13 STSS isolates expressed FbaB protein on their cell surface. The FbaB-deficient mutant TR-47 of strain SSI-1 (M3), obtained from an STSS patient, exhibited 6-fold lower adhesion to and invasion of HEp-2 cells than the wild-type strain. Moreover, TR-47 caused 40% mortality, while SSI-1 yielded 90% mortality in mice on day 4 after infection with 4×10^6 CFU. These results suggested that FbaB plays an important role in the pathogenesis of GAS that expresses the protein on the cell surface.[49]

Some FBPs induce protective antibodies and are vaccine candidates for the prevention of GAS infection.[43] In this context, among the GAS FBPs reported, FBP54[50] is of special interest; sera of patients with acute glomerulonephritis and rheumatic fever contained antibodies specific for this protein. We found the *fbp54* gene in all clinical isolates of various M types examined, including types 1, 3, 11, 12, 18, 19, and 80. Mice subcutaneously or orally immunized with rFBP54 produced specific IgG and IgA antibodies and survived significantly longer than non-immunized control mice. Thus, FBP54 is a good vaccine candidate because of its wide distribution among different GAS serotypes and its strong antigenicity both in mice and humans.[51]

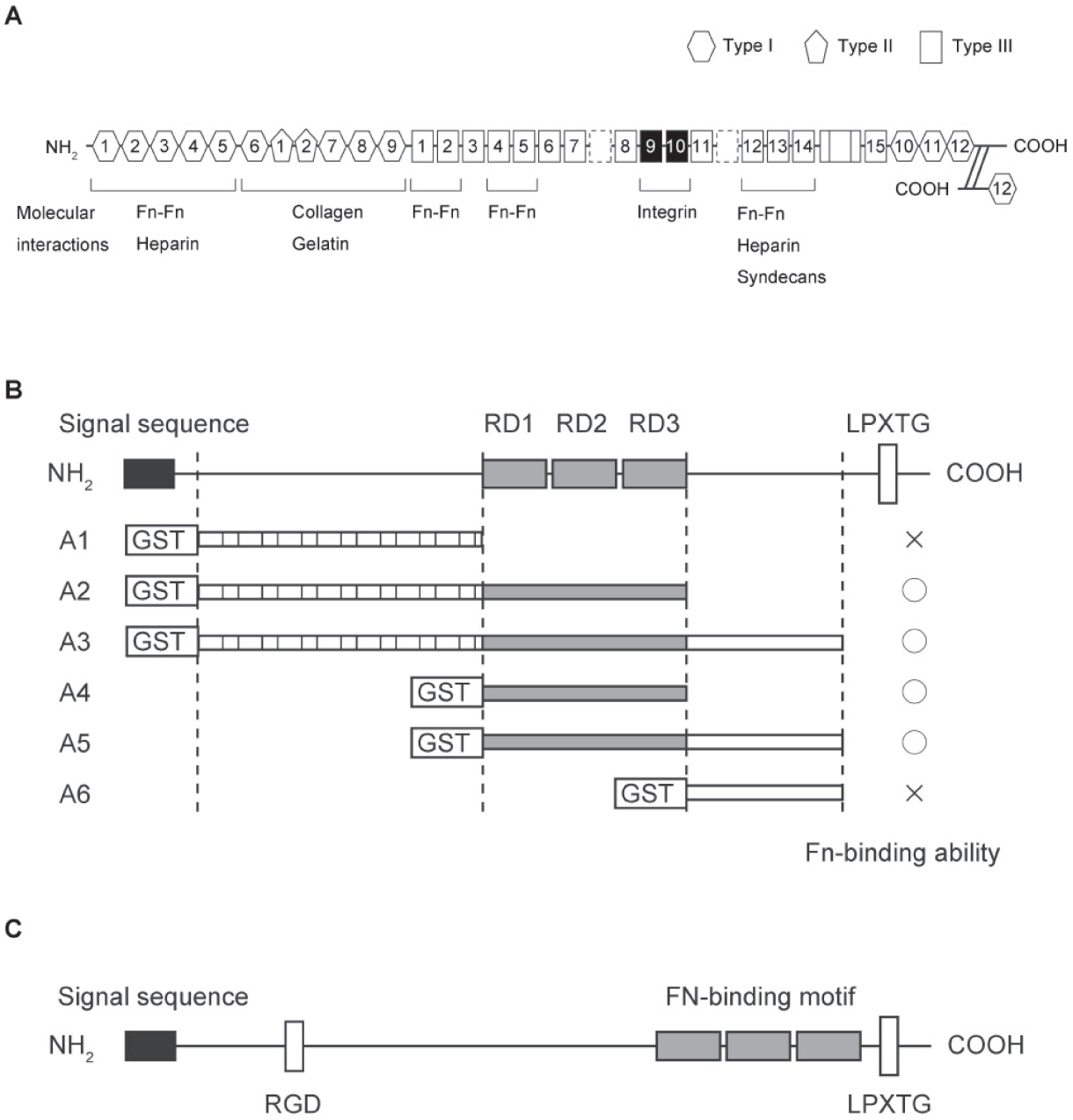

Fig. 3. Schematic illustration of FN, FbaA, and FbaB. (A) FN is constituted by subunits I, II, and III that are represented as hexagons, pentagons, and squares, respectively. FN can interact with various proteins. (B) FbaA possesses 3 repeat domains (RDs). Among 6 genetically modified recombinant FbaA proteins, those possessing RDs bind FN. (C) FbaB contains an RGD motif in addition to an FN-binding motif.

FBPs may directly or indirectly function as modifiers of GAS-epithelial cell adhesion and invasion by inducing various signal pathways that affect the outcome of infection. In addition, FBP contributes to GAS evasion of phagocytosis by inhibiting complement activation.[43)]

Other surface proteins. In addition to various FBPs, we previously characterized a 34-kDa laminin-binding protein (Lbp) in an M12 strain.[52)] Laminin is a 900-kDa glycoprotein found in the basement membrane. Lbp harbors an XXGC motif in the N-terminus and was well conserved among all GAS strains tested, belonging to 10 different M types. It is

noteworthy that *S. agalactiae* has been reported to possess a laminin-binding adhesive protein, Lmb,[53] which shows 98% similarity to Lbp. Patients with streptococcal infections produced antibodies specific for rLbpA of M1 GAS.[54] A mutant strain SSI-9 (M1) deficient in Lbp exhibited less adhesion to and invasion of the cells.[52]

Sib35 a 35-kDa surface protein that does not possess the cell wall-anchoring motif LPXTG.[55] All tested GAS strains, but not those of other streptococcal species, possess this protein. The IgG-binding proteins enhance resistance of GAS to opsonophagocytosis, resulting in increased survival of GAS-infected mice. rSib35 stimulated mouse B-cell proliferation and differentiation into Ig-producing plasma cells. Thus, Sib35 is a B-cell mitogen and provides protective immunity against GAS to hosts.[56),57]

Lzp is a 66-kDa surface protein with 24 leucine-zipper motifs consisting of a sequence of leucine residues spaced seven amino acids apart (LXXXXXXLXXXXXXL).[58] This protein binds human IgG, IgM, and IgA, whereas no other Ig-binding proteins so far reported bound either one or two Ig classes.[58),59]

HtpA is a 92.5-kDa protein possessing five histidine triad motifs. The *htpA* gene is located downstream of the *fbaA* and *lbp* genes. All tested strains of group A and B streptococci, and some of groups C and G, possess HtpA, as revealed by western blotting using rabbit rHtpA-specific antiserum. An *htpA* mutant strain exhibited complete loss of pathogenicity in mice. Conversely, mice immunized by intracutaneous injection of wild-type rHtpA produced specific IgG antibodies that protected them from subsequent GAS infections. Therefore, HtpA may be an important GAS virulence factor.[60] Recently, it has been shown that acidic environment dramatically stimulated GAS to invade into deeper tissue and promoted the secretion of 33 extracellular proteins including HtpA.[61]

Two novel streptococcal collagen-like surface proteins (Scls) in GAS, SclA and SclB, have been reported.[62),63] Both proteins are widely distributed among GAS strains and possess various collagen-specific GXX motifs that are involved in the formation of a triple helix structure in the absence of hydroxyproline.[64),65] Scls participate in bacterial adhesion to ECM protein in the host cell surface and promote biofilm formation.[66]

GAS is frequently isolated from the pharynx and tonsils where salivary flow bathes. Human salivary secretions contain various substances including proline-rich protein (PRP), amylase, statherin, lysozyme, lactoferrin, and secretary IgA (sIgA). PRP plays an important role in the promotion of GAS invasion into cultured epithelial cells, and anti-PRP inhibited this phenomenon. GAS strain NY-5, lacking both M protein and FBP, adheres to HEp-2 cells via a 28-kDa surface protein. The 28-kDa protein has been identified as the chaperone protein GrpE, of which the N-terminal region has been demonstrated to bind to PRP. GrpE appears on the bacterial surface during septum formation in bacterial binary division.[67]

GAS possesses surface-associated C5a-binding protein (C5aBP) that could be extracted with 8 M urea and purified to a 34-kDa protein. Analysis of the protein showed that it is identical to streptococcal plasmin receptor/streptococcal surface dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The open reading frame of the *plr/sdh/gapdh* gene consists of 1008 nucleotides encoding a GAPDH protein composed of 336 amino acid residues with a calculated molecular mass of 36 kDa.[68)–71] GAPDH is present in a cell-associated form, but is ultimately released from the cells. Retention of this protein in the cytoplasm was found to attenuate the virulence of GAS, while secretion of the protein was associated with significant regulatory function and exploitation of the GAS virulence properties.[72] GAPDH is a multifunctional protein that shows various activities seemingly unrelated to its classical role in metabolism;[73] it allows evasion of the complement system via cleavage and trapping of C5a, thereby inhibiting chemotaxis and H_2O_2 production of neutrophils.[71] Furthermore, human pharyngeal cells express urokinase plasminogen activator receptor (uPAR) or CD87 on their surface that serves as a receptor for GAPDH. Thus, adhesion of GAS to pharyngeal epithelial cells for the establishment of infection might occur through GAPDH and uPAR/CD87.[74]

Capsule molecules. A number of clinical isolates from patients with invasive GAS infection exhibit mucoid colonies on blood agar plates. The bacterial cells in these colonies are highly encapsulated, which is indicative of pathogenic capability. The capsule is made of high molecular weight hyaluronic acid (HA) or hyaluronan. In GAS, HA is synthesized via the *hasABC* operon present in all serotypes except M4 and M22, and the *hasA* gene is necessary for the production of HA in GAS. The *hasABC* operon is under the control of the P1

promoter and the upstream P2 putative promoter, both of which are completely repressed by the *CovR* response regulator of the CovR/S two-component signal transduction system.[75] Capsular HA and the HA ubiquitously expressed in the host ECM are structurally identical; therefore, GAS can undermine host immune surveillance and phagocytosis. However, most GAS isolates from uncomplicated cases form nonmucoid colonies, which do not synthesize HA. These findings suggested that the HA capsule is essential for colonization and infection by GAS, and encapsulation of GAS provides a strong advantage over the nonencapsulated one.[76]

The pharynx serves as a reservoir for GAS that causes pharyngitis or sore throat, as well as invasive infections with STSS. The HA-binding cell-surface molecule CD44 functions as a receptor for the adhesion of GAS to the pharyngeal epithelial cells. This was demonstrated by the fact that adhesion of GAS to CD44-deficient keratinocytes was clearly lower than adhesion to wild-type keratinocytes.[77] Similarly, we have previously demonstrated that encapsulated GAS strains (M3, M18, and M6) carrying the wild-type *hasA* gene showed higher adhesion and invasion capability to HEp-2 cells than nonencapsulated isogenic mutants.[78] However, the invasive M1T1 clone that recently emerged in the western world exhibits enhanced HA production, but decreased adhesion to epithelial cells.[79]

Native capsular HA inhibited internalization by macrophages and increased GAS survival in the blood of mice. Partial degradation of the HA by GAS hyaluronidase was found to enhance the uptake of GAS by macrophages *in vitro* and to limit the infectivity and disease severity in a mouse model system. Digestion of tissue HA lowered the extent of GAS infection in mice.[80] Interestingly, two M4 isolates from Australia lacking antiphagocytic HA have recently been reported. Whole-genome sequencing of the two isolates revealed that the *hasABC* operon was absent; however, these isolates produced hyaluronidase that is rendered nonfunctional in other GAS because of a point mutation. They were highly virulent and caused severe invasive infections in children. Thus, M4 isolates may possess unknown antiphagocytic factor(s) enhancing adhesion and invasion.[81]

SpeB. GAS secretes several pyrogenic exotoxins including SpeA, SpeB, SpeC, and SpeD. These are responsible for pyrogenic activity, superantigenic activation of T cells of specific repertoire, and augmentation of endotoxic shock.[82),83] However, SpeB was found not to be a toxin, but a cysteine proteinase.[84] Most clinical GAS isolates release cysteine proteinase when grown to stationary phase in broth medium. The enzyme is synthesized as a 40-kDa surface-associated zymogen and is released as a 28-kDa mature enzyme by self-truncation.[85]

SpeB degrades a wide variety of host and bacterial cellular and extracellular components including matrix glycoproteins (FN, vitronectin, laminin, and integrin), IL-1, chemokines, Igs, and complement components. Further, it regulates some essential subcellular activities related to apoptosis and autophagy in the host cells. Therefore, SpeB is involved in bacterial evasion from the host defense system and in systemic dissemination. It is known that neutrophil infiltration to the GAS infection site is absent in the early stage of STSS. We have recently shown that wild-type GAS and rSpeB degraded C3b very efficiently, and that wild-type GAS was not mobilized to the infection site, whereas SpeB$^-$ GAS mutant was phagocytized. These findings suggested that SpeB functions in escape of GAS from phagocytosis at the infection site, which allows invasion of GAS in the host.[86] In addition, SpeB degrades C1-esterase inhibitor, a component of the complement system that controls the complement cascade, as well as complement factors C2 to C9. When the wild-type GAS and an isogenic SpeB$^-$ mutant were incubated with fresh human serum, the surface integrity of the mutant, but not that of the wild-type strain, was clearly affected; the cell surface showed pore-like structures. These findings indicated that loss of SpeB activity permits bacteriolysis due to activation of the complement cascade. Thus, SpeB participates in the inhibition of complement factors, which allows GAS to escape from elimination.[87]

More direct evidence indicates that SpeB cleaves epithelial cell-cell junction complexes, including occludin and E-cadherin, thereby allowing bacterial penetration across the epithelial barrier. SpeB-induced destabilization of the paracellular barrier may permit bacterial virulence factors, including pyrogenic exotoxins exhibiting superantigenic activities, to enter, which eventually leads to exacerbation of clinical manifestations.[88] In addition, SpeB digests the cell wall-anchored FBP F1, which results in reduced internalization of GAS.[89] In conclusion, SpeB plays an important role in the internalization and penetration of GAS into human cells and tissues. In addition, SpeB contributes to various stages in the interaction between the host and GAS as discussed below.

Streptolysins. GAS produces two distinct hemolysins, streptolysin O (SLO) and streptolysin S (SLS). SLO is a well-characterized oxygen-sensitive prototype of a cholesterol-dependent bacterial exotoxin that oligomerizes to form large pores in host cell membranes. SLO has been shown to disrupt membrane integrity in multiple cell types such as epithelial cells, neutrophils, and macrophages. The cytotoxic activity of SLO is associated with GAS pathogenesis, including modulation of bacterial internalization and intracellular killing by epithelial cells. Our reports provided evidence that SLO is critical for bacterial escape from endosomes into the cytosol, followed by autophagic clearance of internalized GAS organisms.[90),91)] Furthermore, SLO-mediated epithelial damage allowed SpeA penetration in an *ex vivo* model of the mucosal barrier.[92)] In addition to its direct membrane-damaging activity, SLO functions as a vehicle for translocation of the enzymatically active co-toxin NAD-glycohydrase (NADase) into the cytoplasm of host epithelial cells. The coordinated action of SLO and NADase leads to intracellular signaling responsible for bacterial survival in oropharyngeal keratinocytes and macrophages.[93),94)]

SLS is an oxygen-stable and non-immunogenic peptide primarily responsible for the formation of a β-hemolytic zone surrounding colonies grown on blood agar. It is a 2.7-kDa peptide with broad cytolytic spectrum against lymphocytes, neutrophils, platelets, and epithelial cells. An interesting feature of SLS is that its cytolytic activity is rapidly lost in the absence of bacterial cells or carrier molecules such as albumin and the RNase-resistant fraction of yeast RNA (RNA core). Therefore, SLS exists primarily in a cell-bound form and is delivered most effectively to target cells by direct contact with bacterial cells. A contiguous nine-gene locus (*sagA* to *sagI*) has been shown to be involved in SLS production. The structural *sagA* gene product is enzymatically processed, modified, and exported by downstream gene products. Although recent studies have provided insights into the structure–activity relationship of SLS, the exact chemical structure of mature SLS is not yet fully elucidated.[95)]

The mechanism by which SLS contributes to the pathogenesis of GAS infection has been the subject of numerous investigations over the years. *In vitro* studies have suggested that cell-associated SLS actively destroys neutrophils and allows GAS to evade the innate immune response.[96)] Further, SLS has been shown to be involved in cellular injury, phagocytic resistance, and virulence in a murine model of necrotizing soft-tissue infection.[97),98)] Notably, a recent study of ours indicated a novel biological aspect of SLS associated with the mediation of bacterial translocation via a paracellular route (Fig. 4). SLS recruits the host cysteine protease calpain to the plasma membrane during the early stage of infection, and GAS utilizes its proteolytic activity to invade into deeper tissues.[99)]

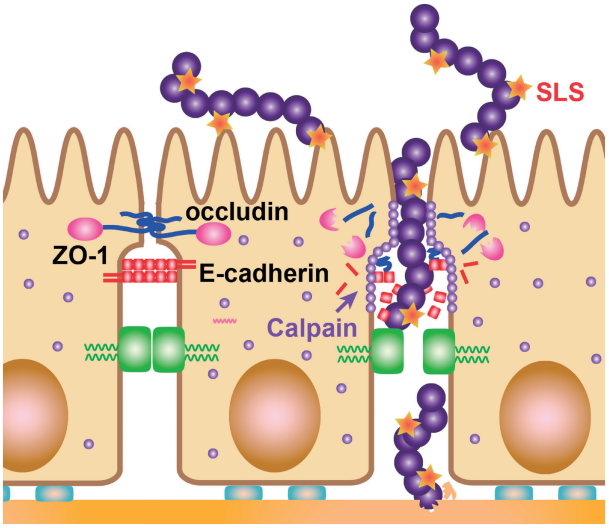

Fig. 4. SLS promotes translocation of GAS via a paracellular route. SLS signals the activation of host calpains and augments the penetration of GAS across the epithelial barrier via degradation of epithelial intercellular junctions.

Host–GAS interactions: bacterial invasion or protection from infection

Neutrophil extracellular traps. Neutrophils, as part of the innate immune machinery, work as a first line of host defense. They are recruited to the infection site to fight against microbes entering via the skin surface or the mucosal membrane. Neutrophils recognize and ingest invading microbes to eventually kill them in the vacuoles. Phagocytosis is promoted by opsonization of the microbe with IgG and/or C3b. During phagocytosis, neutrophils produce reactive oxygen species (ROS) and antibacterial peptides that are essential for their bactericidal activity.[100),101)] Neutrophils discharge extracellular fibrous traps termed "neutrophil extracellular traps" ("NETs"), composed of DNA, histones, LL-37, defensins, myeloperoxidase, and elastase.[102)] NET formation results in the binding and killing of invading microbes, which is an active response of stimulated neutrophils against infection.[103)]

Serotype M1 strains produce DNase named streptodornase (Sda) 1, which degrades the DNA in the NET, eventually allowing the GAS to escape from NET-mediated killing. A mutant lacking Sda did not decompose the DNA and was trapped in the NET, resulting in very few bacteria surviving in humans and mice. Wild-type M1 GAS lost resistance to neutrophils in the presence of G-actin, a DNase I inhibitor. Within the NETs, antibacterial peptides and proteases are highly concentrated to exert efficacious bacterial killing.[104),105)] Furthermore, SclA, a ubiquitous surface protein of GAS, supports its survival in NETs and protects it from cathelicidin antimicrobial peptides (*e.g.*, LL-37) found in the NETs. In addition, SclA inhibits myeloperoxidase release, resulting in hampered NET formation.[106)] *Streptococcus sanguinis* possesses a cell wall-anchored nuclease (termed "SWAN") that digests various forms of DNA including DNA in the NETs and human RNA in the presence of divalent cations such as Mg^{2+} and Ca^{2+}. NET killing of a $SWAN^-$ mutant was significantly enhanced as compared to that in the wild-type *S. sanguinis*, indicating that SWAN plays an important role in the survival of *S. sanguinis* in the NETs during infection.[107)]

Apoptosis of host cells. Apoptosis is a regulated, multistep cell death process that can be activated by extracellular as well as intracellular triggers. Adhesion to and invasion of epithelial cells by GAS induces apoptosis.[108)] HEp-2 cells underwent apoptotic cell death with DNA fragmentation upon invasion of protein $F1^+$ GAS strains, but not $F1^-$ isogenic mutants, indicating that F1 plays a crucial role in efficient bacterial entry into epithelial cells.[46),109)] Interestingly, SpeB decomposes the surface-exposed F1, resulting in reduced internalization of GAS.[89)] Further, caspase inhibitors reduce apoptotic cell death; however, $SpeB^-$ GAS mutants did not cause significant apoptosis, unlike wild-type GAS. These findings clearly indicated that SpeB acts as an inhibitor or a trigger for the induction of apoptosis in GAS-infected epithelial cells. SpeB has been found to activate matrix metalloproteinases and to induce the production of proapoptotic molecules such as TNF-α and soluble Fas ligand in a murine model of severe invasive GAS infection.[110)] Furthermore, serine/threonine phosphatase secreted by GAS causes apoptosis in human pharyngeal respiratory cells. Both extracellular and intracellular serine/threonine phosphatases induce apoptosis, but not necrosis.[111)]

Apoptosis of epithelial cells induced by GAS internalization is associated with cytochrome c release and Bax translocation to the mitochondria and with reduced levels of Bcl-2 and Bcl-X_L at 4 h after infection. The caspase-9 inhibitor Ac-LEDH-CHO inhibits the release of nuclear histone from infected cells.[109)] Serial analysis of gene expression following GAS invasion showed downregulation of voltage-dependent anion-channel genes 1 and 2 and upregulation of the genes encoding cytochrome c oxidase and calcium-binding protein. Macroarray and RT-PCR analyses revealed that the genes encoding IL-1β, IL-12, p35, p40, and GM-CSF, as well as caspases 1, 9, and 14, are induced upon GAS infection. These findings indicated that GAS-induced apoptosis is caused by mitochondrial dysfunction and calcium regulation.[112)] It has been found that ROS triggers apoptosis in HeLa cells; however, HBD98-2-4 cells overexpressing Bcl-2 suppress apoptosis induction. In the latter cells, ROS production and mitochondrial dysfunction were clearly reduced upon GAS infection.[113)] Several GAS strains (M1, M3, M6, and M18) have been reported to express multiple cell death pathways. Strains of types M3 and M18 preferentially induced host cell apoptosis, while M6 rather promoted necrotic or lytic cell death.[114)]

An encapsulated M3 strain induced apoptosis in human primary keratinocytes triggered by SLO-mediated calcium influx and associated with vacuolation of the endoplasmic reticulum and mitochondrial damage. In this process, binding of CD44 to capsular HA might play a role in the initiation of apoptosis.[115)]

Autophagy. Autophagy is an intracellular process by which eukaryotic cells degrade damaged organelles, macromolecular components, and even invading microbes through the formation of a double-membrane autophagosome that fuses with the lysosome for digestion of its content. Thus, autophagy plays an important role in the innate immune system.[116)–118)]

In relation to GAS infection, Nakagawa *et al.*[90)] were the first to report that autophagic processing effectively eliminated the serotype M6 strain JSR4 upon invasion in HeLa cells; the GAS colocalized with LC3, a marker for GAS-containing autophagosome-like vacuoles (GcAVs) (Fig. 5). LC3 and LAMP-1, a lysosomal membrane protein, also colocalized. The number of cells bearing GcAVs increased over time and reached maximum at 3 h after infection. At 4 h after infection, the GcAVs

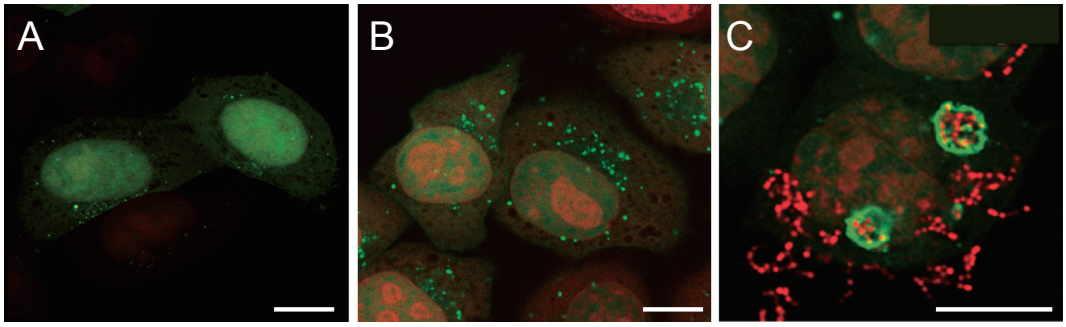

Fig. 5. Autophagosome formation in GAS-infected HeLa cells. In the control cell culture (A), a limited number of autophagosomes is seen in the cytoplasm, while multiple autophagosomes (diameter = 0.5 μm) exist in the cytoplasm under starvation (B). In GAS-infected cells, large autophagosome-like vacuoles (diameter > 5 μm) containing GAS are found in the cytoplasm (C). Green; EGFP-LC3 (autophagosome marker), Red; PI staining. Scale bar = 10 μm.

fused with lysosomes forming single-membrane-bound compartments that contained degraded cytosol as revealed by electron microscopy. Intracellular GAS was killed over time. However, in autophagy-deficient Atg5$^{-/-}$ cells that cannot form autophagosome, the GAS could survive and proliferate, and was ultimately released from the cells.

Although the autophagic process is an essential part of cellular membrane dynamics, genetic screening for autophagy-defective mutants in yeast has led to the identification of a distinct set of core autophagic components that differ from other components of membrane transport such as Rab and soluble N-ethylmaleimide-sensitive factor activating protein receptor.[119] It is still unclear whether proteins functioning in vesicular membrane trafficking are important for autophagy completion,[120] especially during bacterial infection.

Rab GTPases constitute the largest family of small GTPases, and more than 60 members have been identified in humans. Rab proteins localize to distinct intracellular membranes and vesicles and function as molecular switches that alternate between two conformational states: the GTP-bound "on" form and the GDP-bound "off" form.[121] Rab proteins are key molecules for the regulation of membrane-fusion processes. Because autophagy involves membrane traffic and fusion, it is important to identify Rab proteins that regulate autophagosome formation and maturation to understand the underlying molecular mechanisms. Two signaling GTPases, Rab5 and Rab7, have been found to be associated with autophagosome formation, bacterial invasion, endosome fusion and maturation, and the fate of intracellular GAS. In addition, the cytotoxin SLO promoted escape of GAS from endosomes into the cytoplasm. Interestingly, an SLO-deficient mutant remained viable longer than the wild-type strain although it failed to escape the endosomes.[91] Furthermore, Rab9a, Rab23, and Rab17 have been identified as novel autophagy regulators. Rab9a is recruited to GcAVs after autophagosome maturation to assist GcAV enlargement and eventual fusion with lysosomes, while Rab23 is essential for GcAV formation and acts in GAS targeting of autophagic vacuoles. Knockdown of these G proteins resulted in diminished degradation of intracellular GAS.[122] Rab17 mediates the supply of membrane from recycling endosomes to GcAVs. In other words, recycling endosomes contribute to antibacterial autophagy (Fig. 6).[123]

Serotype M1T1 SpeB^{-} mutant strain 5448 escapes from autophagy for intracellular replication, in contrast to serotype M6 strain JSR4. The clear difference has been ascribed to avoidance by strain 5448 of ubiquitination and recognition by the autophagy marker LC3 and ubiquitin-LC3 adaptor proteins NDP52, p62, and NBR1, which are degraded by SpeB. In fact, this strain was targeted to autophagy, resulting in diminished intracellular proliferation.[124),125]

Nitric oxide (NO) is another pivotal factor in autophagy of GAS as has been demonstrated using cultures of GAS-infected macrophage-like cell line RAW264.7 and mouse peritoneal macrophages. NO induced its downstream mediator 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), leading to Lys63-linked polyubiquitination and exclusion of GAS from the cells. Intracellular GAS was modified by protein *S*-guanylation in autophagosome-like vacuoles, suggesting that *S*-guanylation is a marker for selective autophagy.[126]

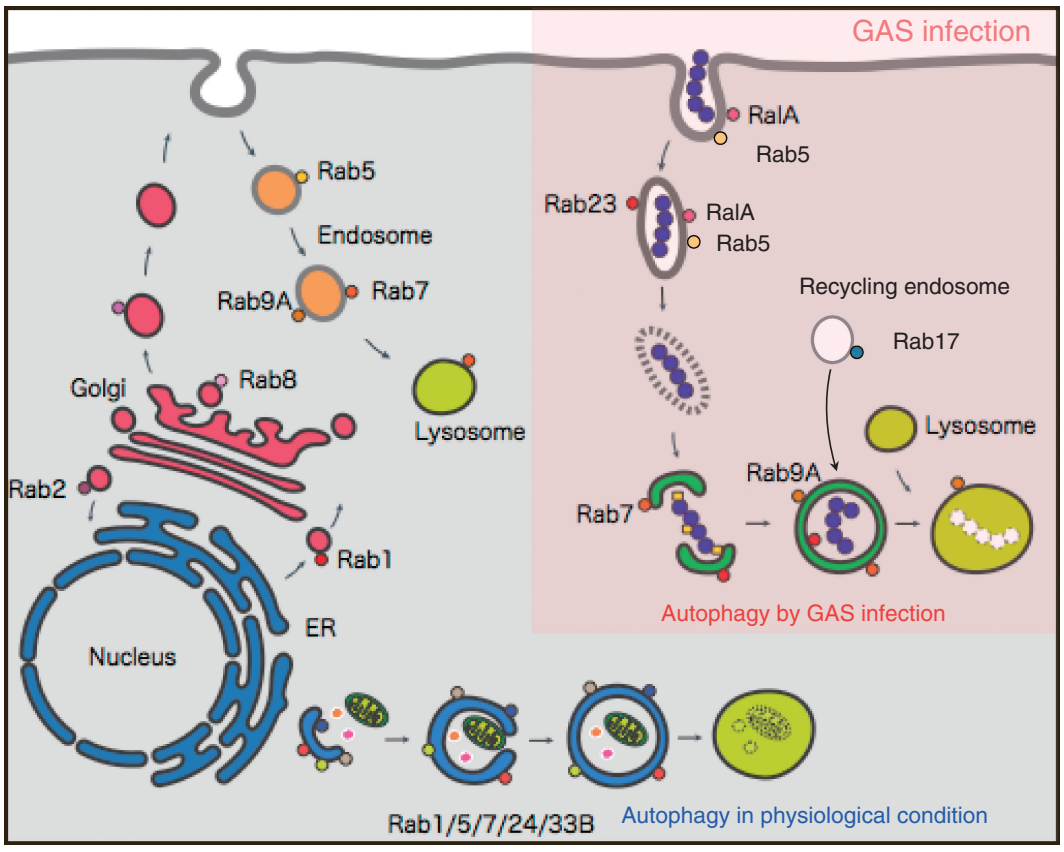

Fig. 6. Membrane trafficking of autophagosome-like vacuoles in GAS-infected cells. The endocytic pathway is tightly regulated by several Rab GTPases in non-infected conditions (bottom panel). In case of GAS infection, infection-specific Rab GTPases (Rab23, Rab9a, and Rab17) are required for maintaining the large autophagic vacuoles to degrade the intracellular GAS (right panel).

Enhanced pathogenesis of GAS due to co-infection with influenza virus

GAS infections occur most frequently in the winter season, and are more likely to be severe during January and April. The infection risk is highest among the elderly and lower in young children.[5),127)] Since the late 1980s, resurgence of invasive GAS infections has been documented in many countries including those in Europe, the US, and Japan. Clinical manifestations are usually severe, resulting in STSS that may lead to a fatal outcome. However, the mechanisms underlying aggravation of the invasive GAS infections remain to be elucidated.

Recent reports have indicated that co-infection with GAS and influenza virus causes severe and occasionally fatal syndromes in humans. For example, during a seasonal influenza epidemic in 2007–2008 in Basel, 3 female patients were hospitalized and were found to have dual severe infections of influenza B and GAS in 2 cases (1 died later) and *S. pneumoniae* in the other case.[128)] An outbreak of invasive GAS infections occurred in South East England between December 2010 and January 2011, in which 10 out of 19 patients died. A prodrome of influenza-like illness was reported in 14 cases, and co-infection of influenza B was confirmed in 4 cases, three of which eventually died.[129)] In Hong Kong, 4 cases of severe pneumonia due to influenza B virus were reported in 2013. Two of them were found to be co-infected with *S. pneumoniae* and 1 with GAS.[130)] Data from 683 critically ill patients in 35 intensive care units in the US during the 2009 influenza A (IAV; H1N1) pandemic disclosed that bacterial co-infection frequently occurs, accompanying increased fatal cases. *Staphylococcus aureus*, *S. pneumoniae*, and GAS were the most common bacteria isolated from respiratory cultures.[131)]

We have demonstrated that intranasal superinfection of BALB/c mice with GAS 2 days after preinfection with IAV caused pneumonia, fasciitis, and sepsis, occasionally with fatal outcome. Mice

monoinfected with GAS or IAV did not die during the 12-day observation period. Prior nonlethal IAV infection is critically important for GAS to invade into the lower respiratory tract, because GAS infection prior to or simultaneously with IAV infection did not enhance mortality. HA was expressed on the alveolar cells within 24 h after IAV infection, followed by internalization of GAS. These processes were inhibited by administration of monoclonal antibody specific for HA.[132] Targeted disruption of *hasA*, *slo*, *speB*, or *sagA* in the clinical M1-type GAS strain SSI-9 resulted in mutants with altered virulence as compared to the wild-type SSI-9 when co-infected with IAV in mice. The number of capsule-depleted $hasA^-$ mutant cells adhering to IAV-infected alveolar epithelial cells decreased sharply, while other mutants did not show such differences. Thus, capsular HA plays an important role in the invasion of GAS into host tissues following co-infection with IAV.[133]

A recent study in C57BL/6 mice has revealed that neuraminidase from IAV enhances the expression of host adhesins such as FN and α5 integrin through activation of TGF-β, leading to increased bacterial loading in the lungs.[134] This indicates that TGF-β and host adhesins may be attractive targets for prevention of IAV and bacterial co-infections.

We previously examined whether influenza vaccination protects hosts from severe attack following co-infection with IAV and GAS. Subcutaneous immunization with formalin-inactivated IAV induced production of IAV-specific serum IgG antibodies and suppressed mortality of mice with effective clearance of IAV and GAS. A similar protective effect was found in mice when intranasal immunization was carried out with influenza vaccine with adjuvant cholera toxin.[135] Immunization with these vaccines limited the induction of pro-inflammatory cytokines including IFN-γ in the lungs, which restricted the recruitment of macrophages and neutrophils to the pulmonary surface and decreased the mortality of mice.[136] The lethal synergism between influenza virus and bacteria such as GAS, *S. pneumoniae*, or *S. aureus* demonstrated in experimental mice may represent an impending danger for humans in their daily life.[137]

Mechanisms of development of invasive GAS infections

Invasive GAS diseases are characterized by extensive tissue damage, vascular dissemination, and systemic disease manifestations (Fig. 7). A highly complex molecular transition in GAS has been implicated in the progression from localized to invasive infection. Microarray analysis has revealed that multiple GAS isolates from pharyngeal and invasive infections have distinct transcriptome profiles.[138] When GAS isolates associated with pharyngitis were passaged through mice, they acquired an invasive-type expression profile and enhanced virulence. In addition, transition from local to systemic infection was related to spontaneous mutations within the genes encoding the CovR/S two-component signal transduction system.[139] GAS generally utilizes the CovR/S system for survival under stress conditions such as acidified pH, presence of LL-37, high salt concentration, and iron starvation. Mutations in *covR/S* result in strong transcriptional upregulation of multiple virulence-associated genes such as the operon for synthesis of the HA capsule and the genes encoding Ska, SLO, SpyCEP and Sda1. Additionally, loss of *SpeB* expression is one of the most important changes in *covR/S* mutants.[140]

Although SpeB plays a crucial role in the establishment of localized GAS infection, its potent proteolytic activity degrades Ska, M protein, and host plasminogen. Ska-mediated activation of surface-acquired plasminogen to plasmin has been contributed to destruction of the host tissue barrier and transition of the bacteria from the site of localized infection to the bloodstream. Thus, CovR/S signal-induced preservation of these proteins enables GAS to enhance cell surface plasmin activity, promoting systemic dissemination.[7]

Streptococcal superantigens belong to a family of exotoxins that interact with host MHC class II and T-cell receptors, provoking massive production of proinflammatory cytokines. Furthermore, superantigens are thought to interfere with the development of the host adaptive immune response by inhibition of specific antibody production and disruption of antigen-specific T-cell responses.[82),83] Structural characterization using several superantigen-MHC complexes has demonstrated that the structure of the superantigen-binding domain is highly variable among MHC class II isoforms.[141] Indeed, human leukocyte antigen (HLA) class II haplotypes have been shown to influence the outcome of invasive GAS infection. For example, the DRB1*1501/DQB1*0602 haplotype was associated with strong protection against severe systemic disease through the elicitation of anti-inflammatory cytokines. In contrast, patients infected with the DRB1*14/DQB1*0503 haplotype showed increased risk of severe diseases. These studies suggested that HLA class II allelic variation leads to differences in severity of invasive GAS infections

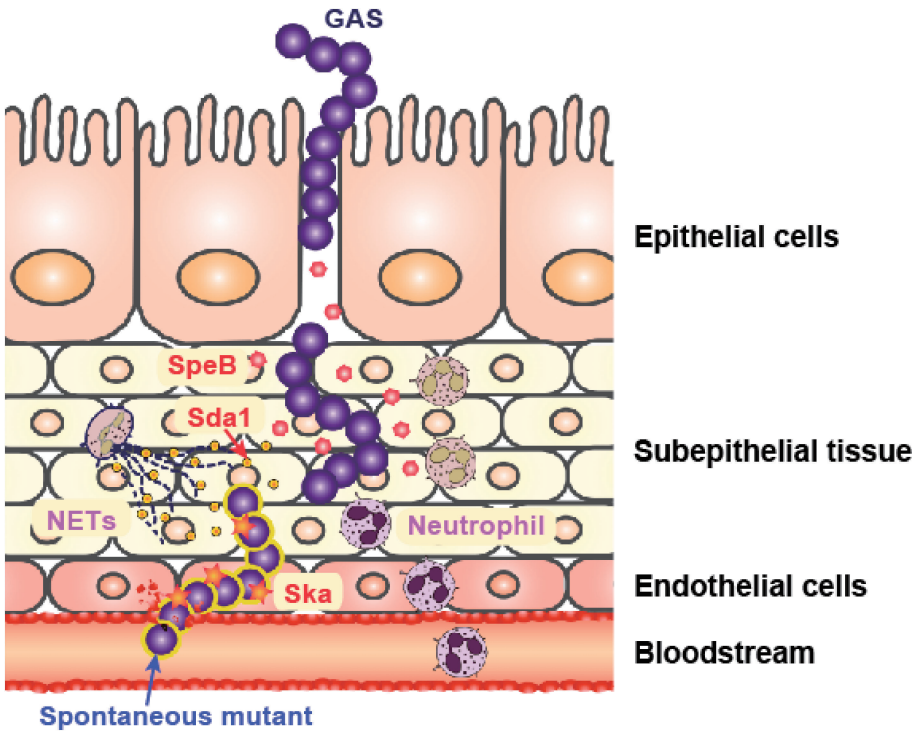

Fig. 7. Systemic dissemination of GAS during infection. Following bacterial entry into the subepithelial tissue, GAS expresses SpeB for the establishment of localized infection. Spontaneous mutations in the *covR/S* operon trigger loss of SpeB expression and strong transcriptional upregulation of several virulence factors including Sda1 responsible for bacterial escape from NETs. Loss of SpeB allows the accumulation of surface plasmin through Ska, which ultimately results in tissue destruction and systemic GAS infection.

through their ability to regulate cytokine responses triggered by streptococcal superantigens.[142)]

Concluding remarks

GAS is a globally spread human pathogen that occasionally generates highly virulent clones due to flexible genomic rearrangements accompanied by phenotypic changes that may be linked with altered pathogenicity. Reflecting its variable nature, GAS induces a variety of clinical manifestations ranging from moderate skin and pharyngeal infections to severe and frequently lethal STSS and fasciitis. Notably, invasive GAS infections have resurged since the mid-1980s. The annual death toll from these infections has been estimated at more than 0.5 million patients worldwide, which is a considerable number, and remains a concern to many countries. To prevent severe GAS infections, numerous studies have been carried out in an attempt to elucidate virulence factors that facilitate disease progression, and various cellular and extracellular molecules of GAS have been reported to exhibit prominent pathobiological activities *in vitro* and/or *in vivo*. Furthermore, host immune and non-immune responses are important to protect the body from invading GAS. To date, no GAS component(s) that could serve as candidate vaccine(s) to prevent GAS infection or to mitigate severe symptoms following the GAS infection have been identified. Furthermore, no animal models that allow faithful reproduction of the disease processes are available. A better understanding of the genomic/molecular mechanisms underlying the pathogenesis of GAS infections should aid the development of preventive measures of this important infectious disease in future.

Acknowledgments

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Profile

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