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Tissue tropisms in group A streptococcal infections

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Abstract

Group A *Streptococcus* (GAS) is a human-specific pathogen that is highly prevalent throughout the world. The vast majority of GAS infections lead to a mild disease involving the epithelial surfaces of either the throat or skin. The concept of distinct sets of ‘throat’ and ‘skin’ strains of GAS has long been conceived. From an ecological standpoint, the epithelium of the throat and skin are important because it is where the organism is most successful in reproducing and transmitting to new hosts. This article examines key features of the epidemiology, population biology and molecular pathogenesis that underlie the tissue site preferences for infection exhibited by GAS, with an emphasis on work from our laboratory on skin tropisms. Recombinational replacement with orthologous gene forms, following interspecies transfer, appears to be an important genetic step leading up to the exploitation of new niches by GAS.

Keywords

epithelium; impetigo; pharyngitis; *Streptococcus*; tropisms

Clinical importance of group A streptococcal diseases

Humans are almost exclusively the biological host of *Streptococcus pyogenes*, commonly known as β -hemolytic group A streptococci (GAS). It is estimated that these Gram-positive cocci are responsible for more than 600 million cases of throat infection (pharyngitis and tonsillitis) worldwide per year, and more than 100 million cases of skin infection (primarily nonbullous impetigo) in children living in resource-limited countries [1]. Streptococcal pharyngitis and impetigo are superficial, self-limiting infections that typically cause a mild illness which resolves within 2 weeks, coincident with the rise of specific host immune defenses [2]. Most morbidity and mortality due to GAS arises from invasive and autoimmune disease, each of which is relatively infrequent in occurrence when compared with the highly prevalent infections at the throat and skin epithelium. The throat and skin are the primary reservoirs for

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GAS, and human-to-human transmission is mainly via respiratory droplets or direct skin contact [2].

Carriage at the throat, in which the organism persists in a quiescent commensal-like state and does not cause clinical symptoms, is an important facet of GAS ecology. It is not unusual for throat carriage rates to exceed 20% in populations of school-aged children [3,4]. A subclinical pharyngeal infection can also occur, in which the host mounts a specific immune response, yet lacks obvious symptoms of illness. Antibiotics (β -lactams) are the recommended treatment for GAS pharyngitis and impetigo, and although resistant clones do exist (e.g., resistant to macrolides), treatment failures have not yet emerged as a significant public health problem. Currently, there is no licensed vaccine available to prevent GAS disease.

Genotypic markers of throat & skin strains

The GAS cell surface bears M proteins that form short hair-like fibrils of approximately 60 nm and contain antigenic targets of the major serological typing scheme [5]. Determinants of serotype lie at the distal fibril tips (amino termini). Over the past decade, serological typing has been largely replaced by nucleotide (nt) sequence typing, based on sequence identity within the 5' end of the *emm* gene encoding the type-specific determinants [6]. To date, over 200 *emm* types have been identified [201]. Antibody directed to the M type-specific antigenic epitope mediates opsonophagocytosis of the organism, and provides protective immunity.

Decades of epidemiologic studies have shown that some M protein serotypes of GAS have a strong tendency to cause throat infection (pharyngitis), but not superficial skin infection (impetigo). Similarly, other M types are often recovered from impetigo, but rarely from cases of pharyngitis [2,7]. This observation gave rise to the concept of distinct throat and skin M types. The existence of distinct throat- and skin-tropic strains implies that there must also exist some degree of specialization within this species, and that there are intrinsic biological properties of the organism that confer tissue site preference. Clues may lie in the structure of the *emm* genes that encode the M proteins.

Many strains of GAS have one or two additional *emm*-like gene paralogs lying immediately upstream or downstream from the *emm* locus that encodes the type-specific antigen [8,9]. Together, the *emm* and *emm*-like genes comprise four major lineages based on extensive nt sequence differences within the portion encoding the cell wall-spanning domain [10,11]. Several arrangements of the *emm* chromosomal region are observed, based on the number of *emm* and *emm*-like genes and their lineage. These arrangements are referred to as '*emm* patterns', of which three major groupings are recognized: A–C, D and E. Importantly, for nearly all GAS strains examined, a given *emm* type is restricted to a single *emm* pattern grouping [12]. Thus, based on knowledge of *emm* type, it is reasonable to infer the *emm* pattern of a given strain.

The biological importance of *emm* pattern genotypes is that they are strong markers for preferred tissue site of infection among GAS strains [13,14]. Several population-based collections of GAS isolates recovered from cases of either throat or skin infection [15–25] were assessed for *emm* pattern genotype (Figure 1). The combined surveillance studies yield *emm* typing data on over 4000 isolates collected from the six continents; *emm* pattern was inferred based on the established relationships with *emm* type (Figure 1) [12]. For each study evaluated, the designated skin isolates were obtained from cases of impetigo, but not from other types of skin lesions that can be difficult to discern in non-endemic regions, such as wound infections that are often initiated by throat-derived strains. In addition, the throat isolates were obtained from patients with pharyngitis and/or tonsillitis, and not from subjects with asymptomatic carriage.

Population analysis of the more than 4000 GAS isolates reveals a strong trend in which organisms corresponding to the *emm* pattern A–C group have a strong predilection to cause infection at the throat, and *emm* pattern D strains have a strong tendency to cause impetigo (Figure 1). For the eight pharyngitis and six impetigo studies, differences in the distribution of *emm* pattern A–C versus D strains are highly significant ($t = 0.003$ and $t = 0.002$, respectively; paired t-test). The *emm* pattern A–C strains represent throat specialists, whereas the pattern D strains correspond to skin specialists. The *emm* pattern E strains readily infect both tissues and are considered generalists, accounting for approximately 50% of GAS isolates worldwide. In summary, the population surveillance data provide strong support that *emm* pattern genotype is a highly valid biomarker for preferred tissue site of infection by GAS, although it is not perfect and there are exceptions.

Population genetics of throat & skin specialists

Not only are the throat and skin specialists physically separated by their distinct ecological niches, but there is also geographic separation as evidenced by the prevalence of throat versus skin infection in different parts of the world. Upper respiratory tract infections predominate in temperate regions, whereas impetigo is mostly observed in communities having a warm and tropical climate and is often endemic (Figure 1) [2,26]. There are also seasonal differences in some locales, with pharyngitis peaking in winter months and impetigo emerging during the summer [2,26]. It appears that climate-related risk factors (e.g., warm humid climate; time spent indoors) for GAS infection are a driving force in promoting adaptation to narrow ecological niches among the specialist strains. Spatial separation, due to both geographic partitioning and innate tissue site preference for infection, might impose a physical barrier to lateral gene exchange between throat and skin specialist strains.

This raises the question of whether *emm* pattern A–C and D strains are at the beginning stages of forming new species due to reproductive isolation. Several analyses on core housekeeping genes provide strong support for extensive genetic recombination across the three *emm* pattern groupings and within the GAS population as a whole [14,27–31]. The extensive recombination follows frequent horizontal gene transfer events that occur between different strains. Consequently, ancestor–descendent relationships among GAS strains are not obvious, and genetic signatures for early stages of speciation within the GAS population are lacking. Based on housekeeping genes, there is no evidence that pattern E strains are intermediate to pattern A–C and D strains.

Genes linked to markers for preferred tissue site of infection

Against a background of random associations among core housekeeping genes, other genes that exhibit linkage disequilibrium with *emm* pattern are strong candidates for having a direct role in causing infection of either the throat or skin. For example, if there is a particular set of accessory genes that is essential for causing superficial throat or skin infection, then the corresponding group of specialist strains should have the full complement of adaptive genes.

By screening a genetically diverse collection of approximately 100 GAS isolates, several GAS genes that display strong linkage with the *emm* pattern groupings have been identified [32–34]. Included are genes having a differential presence or absence among strains (*cpa*, *prtF1*, *sof*), and loci having alleles that form discrete phylogenetic lineages (*mga*, *rofA/nra*, *ska*). Furthermore, most of the linked genes identified to date are clustered within two genomic regions: the *emm* region itself or the FCT region [35], positioned approximately 300 kB from *emm* and containing pilus biosynthesis genes (Figure 2). The pili contain the immunodeterminants of T type, which provides the basis for another serotyping scheme of GAS [36]. Additional *emm* pattern-linked genes located distal to the *emm* and FCT regions

have been uncovered by comparative genomic hybridization studies [Bessen DE *et al.*, Unpublished Data].

The *mga* and *rofA/nra* loci encode transcription regulators of gene expression [37,38]. Alleles at the *mga* locus comprise two discrete phylogenetic lineages having approximately 28% nt sequence divergence, and are referred to as the *mga-1* and *mga-2* lineages [32]. The *nra* and *rofA* lineages of alleles occupy the same locus within the FCT region, and display approximately 35% nt sequence divergence. All GAS isolates have either an *mga-1* or *mga-2* lineage allele, but not both. Likewise, all isolates also have either a *rofA* or *nra* allele. The products of each allelic lineage also display a high level of divergence in amino acid sequence.

The *mga-1* alleles are present in nearly all *emm* pattern A–C isolates, whereas *mga-2* alleles are present in 100% of the *emm* pattern D and E strains examined [32]. Several other loci display strong linkage with *emm* pattern and lie within the surrounding region of approximately 55 kB (Figure 2). The *sof* gene encodes serum opacity factor (SOF), a multifunctional extracellular protein that modulates high-density lipoprotein [39], and binds fibrinogen and fibronectin (Fn) [40,41], and is largely restricted to *emm* pattern E strains, present in 100% of them [34]. In addition, SOF provides the basis for a third GAS serological typing scheme. The *pam* gene encodes a specialized plasminogen (Plg)-binding M protein (PAM) that is restricted to *emm* pattern D strains [42]. The *ska* gene encodes the Plg activator streptokinase, and has three main lineages of alleles exhibiting approximately 40% overall sequence divergence [33]. The cluster 2b *ska* alleles are largely restricted to *pam*-positive pattern D strains [33].

Given its genome map position is immediately adjacent to the *emm* gene, it is perhaps not surprising that *mga* lineage alleles display linkage disequilibrium with *emm* pattern group (Figure 2). Strong linkage of *emm* pattern to *mga*, *sof*, *pam* and *ska* might be explained by physical proximity, yet there is also evidence for genetic recombination within the region of the genome surrounding the *emm* locus [13,43,44]. Even though *emm* and *sof* loci lie only approximately 16-kB apart, a given M type is found in association with a wide array of SOF types, and *vice versa*, indicative of a history of genetic recombination [45]. Since physical linkage is broken by recombination, the strong genetic linkage that is observed between *emm* pattern group and neighboring genes might otherwise be preserved via a functional necessity arising from a phenotype that enhances fitness.

Mga functions as a positive regulator of *emm* gene expression in numerous strains examined [46–48], representing all three *emm* pattern groups. Several genes that lie outside of the *emm* region are also under direct transcriptional control of Mga. Comparison of the Mga regulons of *mga-1*- versus *mga-2*-positive strains fails to reveal differences among genes likely to be under the direct control of Mga [38,48], except for Mga-regulated genes that are *emm* pattern restricted (e.g., *sof*) in their distribution among strains.

The *rofA/nra* locus of the FCT region encodes a transcriptional regulator that controls the expression of pilus structural genes [37,49–51]. Nearly all *emm* pattern A–C and E strains have *rofA*, whereas most *emm* pattern D strains harbor *nra* [32]. The association of *rofA* versus *nra* with *emm* pattern group differs from the distribution of *mga* alleles, in that the pattern E generalists tend to share *rofA* with pattern A–C strains, but share *mga-2* with pattern D strains. The effect of RofA and Nra on gene transcription varies from strain to strain [50,51]. For example, Nra can function as either an activator or repressor of pilus gene transcription. Similar to Mga, RofA/Nra is a global regulator of GAS gene transcription, affecting the expression of numerous genes lying outside of the FCT region [52].

T type is found in association with numerous M- and SOF-type combinations [53], suggestive of past recombination events involving genetic crossover somewhere between the FCT and

emm chromosomal regions. Yet, several loci within the FCT region display strong linkage disequilibrium with *emm* pattern. FCT region-encoded colonization factors (e.g., *cpa* and *prtF1*) may facilitate tissue-specific infection, as evidenced by their strong linkage with *emm* pattern genotypes [34]. *Cpa* is a collagen-binding protein, and is also an accessory protein of the pilus [36,54]. The *cpa* gene is detected by PCR in more than 90% of *emm* pattern D strains, but in only 25% of the *emm* pattern A–C strains examined [34]. *PrtF1* is a Fn-binding protein that has a strain distribution opposite to that of *Cpa*, being present in 75% of pattern A–C strains, but in only 20% of pattern D strains [34]. The differential distribution of *Cpa* and *PrtF1* among throat versus skin specialists is supportive of a role for each adhesin in mediating tissue-specific adherence. A high proportion (93%) of pattern E strains harbor *prtF1*; however, only 55% have *cpa* [34]. Therefore, if *Cpa* is a determinant for infection of the skin, then many pattern E strains appear to utilize an alternative molecular strategy.

A striking feature of the FCT region is that its structure is highly homogenous among the *emm* pattern D strains but, by contrast, the *emm* pattern A–C throat specialists exhibit a wide array of gene combinations sharing little in common across the group (Figure 2) [34]. The *emm* pattern E generalists are intermediate in this regard. The highly heterogeneous structure of the FCT regions of throat specialist strains is consistent with the idea that there may be multiple genotypes giving rise to a similar phenotype.

GAS adherence in cell & organ cultures

Adhesins can potentially contribute to bacterial tropisms for host species, tissue and/or cells. The body of knowledge on GAS adherence to epithelial cells is very extensive and only a few of the many important published studies are mentioned below. A key characteristic expected for a tissue-specific GAS adhesin is differential binding to throat- versus skin-derived cells or tissue of human origin. Furthermore, a tissue-specific adhesin is expected to be distributed among the GAS population in accordance with GAS strain tropisms. Human host-derived cells and tissues used to study GAS adherence include tonsils, respiratory tract-derived cell lines, human transgenic cell lines, skin tissue sections, primary keratinocytes and keratinocytes induced to undergo differentiation in culture. However, solid proof for an adhesin conferring tissue tropisms for superficial throat versus skin infection has not yet clearly emerged.

The pilus accessory protein *Cpa*, derived from an M-type 49 (M49) GAS strain (*emm* pattern E), selectively binds type I collagen [54], which is abundant in the dermal layer of skin and present in other tissues as well. Localization of *Cpa* to the pilus tip in an M-type 3/T-type 3 (M3T3) strain may allow it to extend beyond the polysaccharide capsule and become accessible for mediating adherence [55]. A critical role for pili in adherence to human palatine tonsil tissue is demonstrated for the *emm* pattern A–C strain SF370 (M1T1) [56], whose pili genes display extensive sequence divergence with most other GAS strains [34]. Pili-mediated adherence to tonsillar tissue is not blocked following pre-incubation of GAS with either fibronectin or collagen. The pili of the M1T1 strain also promote adherence to primary skin-derived keratinocytes, yet have no effect on GAS adherence to cell lines originating from the respiratory tract (HEp-2, A549); in another study using an M49 strain, a *cpa* mutant also failed to show differences in adherence to HEp-2 cells [57]. However, the M1 protein of the M1T1 strain enhances adherence to tonsils but decreases GAS binding to keratinocytes, suggestive of a complex set of competing interactions among adhesins [56]. M1 strains are usually found in association with pharyngitis, although in a surveillance study in Nepal, M1 isolates were recovered from nearly 20% of the impetigo cases [17]. Using the same M1T1 strain and a pharyngeal-derived cell line (Detroit-562), the role of pili in GAS adherence was confirmed by another group of investigators, and a role for GAS pili in biofilm formation was also demonstrated [58].

The GAS population displays numerous combinations of genes encoding the structurally distinct Fn-binding proteins PrtF1, PrtF2, SOF, SfbX, FbaA and FBP54 [59–63]. The Fn-binding proteins appear to exhibit differences in cell surface expression and binding properties. Using an *in situ* adherence assay with histological sections of human skin, PrtF1 directs GAS adherence to Langerhans cells, in contrast to GAS bound to keratinocytes via M6 protein, suggestive of cell-specific tropisms [64]. Other factors, such as integrin expression and the multimeric tissue form of Fn, can influence GAS adherence to mammalian cells [65,66]. The degree to which the individual Fn-binding proteins of GAS differ in their binding affinities for specific cell types remains to be fully explored.

In addition to adherence to human tissue, GAS must acquire nutrients in order to reproduce within the host and, at the same time, circumvent host defenses. Conceivably, any process that contributes to pathogenesis might be specific for either the throat or skin.

Animal models for GAS infection of the upper respiratory tract

A major challenge to studying GAS tissue tropisms and tissue-specific aspects of molecular pathogenesis at the epithelium lies in the shortcomings of experimental animal models to accurately represent diseases caused by this human-specific pathogen. Also, the extensive genetic diversity among strains often give rise to strain-dependent phenotypes in the various models.

The molecular pathogenesis of GAS pharyngitis is summarized in a recent review [67]. The GAS disease model in cynomolgus macaques probably provides the best demonstration of tonsillitis, following intranasal (IN) inoculation of live GAS [68,69]. Numerous studies have used nonhuman primate models for pharyngeal colonization and/or infection by GAS; however, high inoculating doses of bacteria are consistently employed, ranging from more than 3×10^5 to 10^8 CFU [68,70–73]. Nonetheless, transcription of several GAS genes that are upregulated in organisms recovered from pharyngitis patients are also expressed at elevated levels during infection in the macaque model [71].

Whether or not the high nonphysiological doses of GAS employed in nonhuman primate models for upper respiratory tract infection are essential to cause disease remains unclear. High doses might be necessary if innate host defenses need to be overcome and/or there is a lack of high-affinity binding sites for GAS adhesins in nonhuman species. Transgenic mice expressing human-derived genes are valuable tools for studying GAS infections [74–78], but none have yet to be applied to superficial infections at the throat or skin epithelium.

Delivery of GAS to mice or rats by an IN route has been widely used to study colonization at the mucosal epithelium [79–82], but evidence for progression to pharyngitis in the mouse has been difficult to obtain. However, GAS are observed in association with nasal-associated lymphoid tissue, a human tonsil homologue [83]; there is also evidence that nasal-associated lymphoid tissue may be an entry point for GAS into deeper tissue. In fact, mice receiving GAS via the IN route often succumb to a disseminated systemic infection. The doses of GAS used for IN inoculation of mice are typically quite high, often exceeding 10^6 or 10^7 CFU. Somewhat lower inoculum doses of GAS delivered IN to immunodeficient mice (BALB/c *nu*⁺) lead to a high rate of mortality [84], consistent with the idea that naturally acquired cross-protective immunity may limit GAS infection in mice.

Animal models for superficial GAS infection of the skin

There are numerous models for GAS infection that involve the skin; however, when bacteria are delivered by injection, the epithelial barrier is bypassed and infection is no longer considered to be superficial. GAS causes not only impetigo – a superficial skin infection often

limited to the epidermis – but can also cause wound infections that penetrate into deeper tissue and sometimes lead to abscess formation. Impetigo and wound infections represent two distinct pathological processes.

Several animal models for GAS infection leading to skin involvement deliver high doses of bacteria by injection under the skin. It has been reported that macroscopic, impetigo-like lesions are induced in hamsters via intradermal injection of GAS [85]. However, these injections are better described as subcutaneous; subdermal abscesses form that gradually penetrate through the epidermis and rupture on the skin surface, releasing a purulent exudate. Injection of GAS into the hind flanks of hairless mice is used to study skin ulcers and tissue necrosis that originates in deeper tissue and involves the epidermis after the infection has progressed to a later stage [86–88]. Coinjection of bacteria with a foreign substance (e.g., Cytodex™ beads) is often employed to further enhance the inflammatory response. In the mouse skin air sac model, GAS are injected subcutaneously; this model can be used to study bacterial dissemination from a focal infection site to distal organs [89]. In another mouse skin infection model, GAS are applied topically following the complete removal of the epidermis by tape stripping; unusually high nonphysiologic inoculum doses (10^7 CFU) of GAS are employed here as well [90].

Mouse skin is structurally distinct from human skin. Mouse epidermis is typically three cell layers thick, and the dermis is loaded with mast cells; except in hairless mice, it has a very high density of hair follicles. In sharp contrast, most anatomic areas of human skin epidermis have three morphologically distinct viable layers (basal, spinous and granulosum), each several cell layers thick. Since humans are the only natural host for GAS, a model that accurately reflects the key events during human infection is highly desirable.

A humanized mouse model for superficial skin infection (hu-skin SCID mouse) was developed in which SCID mice are engrafted with human neonatal foreskin [47,51,91–95]. SCID mice fail to reject xenotransplants because they lack functional B and T lymphocytes. For infection, freshly cultured GAS is topically applied to grafts slightly damaged by gentle scratching of the skin surface; inoculation is followed by occlusion of the area with a bandage. These treatments are part of an attempt to mimic the risk factors for impetigo in humans, which include both a nick in the skin and a warm, humid environment.

The humanized mouse model for GAS impetigo has high sensitivity, whereby inoculum doses as low as approximately 300 CFU of a virulent GAS strain can give rise to a purulent exudate at 7 days. This low inoculum dose probably approaches physiologic levels for naturally occurring infection in the human host. The hu-skin SCID mouse model also displays high specificity and can discriminate the GAS strain tropisms observed in human populations, in that *emm* pattern D skin specialist strains are significantly more virulent than *emm* pattern A–C throat specialist strains, based on blinded gross pathology scores. The primary outcome measure in the humanized mouse model is the net change in the number of CFU between inoculation and tissue biopsy. This measure is evolutionarily relevant because successful transmission to a new host depends on the ability to generate new progeny. Virulent strains of GAS undergo reproduction and net growth at the skin, resulting in an increase in CFU that can exceed 10,000-fold, a hallmark of evolutionary success.

The humanized mouse model provides a striking similarity to the histopathology observed during natural GAS impetigo in humans [96–101]. Infection of the wounded graft with an *emm* pattern D skin specialist strain typically leads to loss of the stratum corneum over large sections of skin by 48 h postinoculation (Figure 3). At 48 h, polymorphonuclear leukocytes (PMNs) of mouse origin are observed in the dermal vessels, within the epidermis and on the external surface of the skin. Single cocci and short chains are adherent to the outer epidermal cell layer, with nearby PMNs often present. As the GAS infection progresses further, there are

dramatic changes. By 4 days postinoculation, there can be a thick coat of PMNs over some sections of skin. As the PMNs move from the vasculature, a small amount of plasma extravasates and gradually accumulates. Together, the PMNs and plasma constitute the purulent exudate, or pus, which can be seen on gross examination. Under high magnification, one can observe massive aggregates of cocci concentrated at the outer rim of the pus layer. Presumably, M protein prevents phagocytosis of the organism, but steric effects due to the aggregates might play a role as well. Importantly, the bacteria are in an environment rich in at least some key nutrients that may facilitate reproductive growth. The GAS at the outer rim of the pus layer also seem well positioned for transmission to a new host. In addition, sometimes GAS can survive within PMNs, which may provide a vehicle for dissemination [102]. From the standpoint of evolutionary success, this is what matters the most to a microbial pathogen: reproduction and transmission to the next host.

The dynamics of reproductive growth of GAS in the humanized mouse has been studied using a constant inoculum dose of approximately 2000 CFU of the virulent *emm* pattern D strain Alab49 [Bessen DE *et al.*, Unpublished Data]. A growth curve at the skin can be constructed using biopsy times postinoculation of 2, 3 and 6–7 days (Figure 3). The average number of bacterial population doublings at these time points is 2.2, 9.1 and 13.5, respectively. The saturation value for GAS on the human skin graft, irrespective of inoculum dose, is approximately 2×10^7 CFU, equivalent to approximately 13.3 population doublings assuming an inoculation dosage of approximately 2000 CFU. The occluding bandage keeps the skin lesion moist, preventing crust formation and healing.

Most exponential phase growth of GAS at the skin occurs between 2 and 3 days postinoculation. The average population doubling time between the 48 and 72 h time points is 3.45 h. In sharp contrast, the doubling time of GAS grown in enriched broth (e.g., Todd-Hewitt with yeast extract) is approximately 40 min during log phase. Using the estimated doubling time of 3.45 h during exponential growth at the skin, saturation of the GAS population is expected to be attained by approximately 86 h postinoculation, after which time the population enters stationary phase. During log phase at the skin, the bacterial growth rate may be slowed by a lack of certain key nutrients, or replication may be countered by a loss of new progeny via innate host defenses.

Role of skin-specific gene candidates in pathogenesis

A set of criteria can be used to provide evidence that a particular gene is a determinant of tissue-specific infection. The first requirement is a strong association between the tissue-specific gene and *emm* pattern genotype, which, in turn, correlates with the host tissue site of isolation as established by population-based surveys of GAS infection (Figure 1). Several genes that are strongly linked to the *emm* pattern markers for preferred tissue site of infection have been identified (Figure 2). The second requirement for a tissue-specific determinant of infection is that inactivation of the candidate gene leads to an alteration in virulence using a relevant animal model of disease.

The humanized mouse model for superficial GAS skin infection was used to measure the effect of mutations in genes displaying linkage disequilibrium with the *emm* pattern D genotype. The virulent *emm* pattern D strain Alab49 (M53) was the target of mutagenesis. Alab49 harbors *mga-2* and *nra* lineage alleles, as well as the *pam* form of *emm*, the *ska-2b* lineage of the streptokinase gene and the FCT region genes *cpa* and *prtF2*. The *sof* gene that is present in all pattern E strains is missing from Alab49; also absent from Alab49 is the *prtF1* gene found in most pattern A–C strains and in many pattern E strains. Differences in virulence at the skin having statistical significance for mutants versus the parental wild-type (wt) Alab49 strain are summarized in Table 1. To prepare the inoculum, GAS is grown to either log or stationary

phase in enriched broth; the physiological state of GAS during human-to-human transmission is unknown, but presumably consists of a heterologous mix.

Data show that inactivation of genes encoding the transcriptional regulator Mga leads to a significant and near-complete loss in virulence of strain Alab49 in the humanized mouse (Table 1) [47]. Mga functions as a positive regulator of several Alab49 genes, including *pam/emm53* (Plg-binding M53-type protein), *speB* (secreted cysteine proteinase), *sclA* (collagen-like surface protein), *scpA* (C5a peptidase) and *fbaA* (Fn-binding protein). Mga-regulated gene products are potential determinants of tissue tropisms.

Inactivation of either the *pam/emm53* or *ska* genes in Alab49 leads to a statistically significant but partial attenuation of virulence in the humanized mouse (Table 1) [93]. In addition, both mutants display a complete loss in bacterial-bound plasmin activity. PAM is a specialized multifunctional M protein containing a high affinity binding site for Plg [103], and is restricted to *emm* pattern D strains [42]. PAM and streptokinase work synergistically; Plg binds to the bacterial cell surface via PAM and, in combination with streptokinase, the bound Plg is activated to form plasmin, a broad-spectrum protease. The plasmin may act to degrade fibrin, which would normally contribute to scab formation. Thus, plasmin may prevent the impetiginous skin lesion from drying out and healing by allowing inflammatory cells and plasma to accumulate over a longer time frame and, in turn, expand the window of opportunity for GAS transmission to new hosts. Like most other *emm* pattern D strains, Alab49 harbors a *ska-2b* lineage allele [33]. The product of Ska-2b differs from the other streptokinase forms in that fluid-phase plasmin activity is essentially absent [104; Bessen DE *et al.*, Unpublished Data]

Plasminogen-binding M protein has a measurable function during skin infection in the humanized mouse, despite the finding that binding of mouse Plg is approximately 20-fold lower than that of human Plg [42,93]. Furthermore, activation of fluid-phase Plg by at least some forms of streptokinase is specific for the mammalian species from which the bacterium was isolated [105]. The modest partial attenuation observed for the Δ *pam* and Δ *ska* mutants in the hu-skin SCID mouse may reflect a human species restriction that severely limits the amount of mouse-derived plasmin that can be formed. Plg is primarily synthesized by the liver; however, Plg mRNA is detectable in numerous extrahepatic tissues [106–108]. It remains to be established whether the human-derived cells of the skin graft synthesize Plg during GAS infection. In addition to its role in bacterial-bound plasmin activity, PAM/M53 has an antiphagocytic function that may prevent GAS uptake by infiltrating PMNs. However, as observed for other strains having multiple *emm*- and *emm*-like genes, selective loss of the type-specific *emm* gene does not necessarily result in complete killing via opsonophagocytosis [93,109,110].

Transcription of *pam/emm53* is positively regulated by Mga. Transcript levels for *pam/emm53* are decreased more than 100-fold in the Δ *mga* mutant relative to Alab49 wt; by contrast, *ska* transcript levels are largely unaffected by Mga [47]. The magnitude of the loss of virulence observed for the Δ *mga* mutant appears to be far greater than that experienced by the Δ *pam/emm53* mutant, which is only slightly attenuated [93]. Thus, additional Mga-regulated genes probably contribute to skin infection by Alab49.

Expression of the cysteine protease SpeB is regulated by Mga, most likely through an indirect circuit since *speB* transcript levels are decreased by only approximately threefold in the Alab49 Δ *mga* mutant [47]. All GAS strains harbor *speB*, but there is wide variation in the level of SpeB activity. With the exception of numerous M1 strains [111], SpeB activity is generally quite low for the *emm* pattern A–C group of throat specialists [92]. By contrast, SpeB activity is high for most strains of the *emm* pattern D group of skin specialists. Inactivation of *speB* leads to a

complete loss in Alab49 virulence in the humanized mouse model for impetigo (Table 1) [92,94]. The combination of a strong association between the SpeB phenotype and *emm* pattern genotype, and the absolute requirement of SpeB for skin infection, makes SpeB another compelling candidate for being a key determinant of tissue tropism. Addition of exogenous SpeB to an aviru-lent *emm* pattern A–C strain leads to a relative increase in the number of organisms recovered from skin grafts, although the magnitude of the enhancing effect of added SpeB on the throat specialist strain is less than that observed for a Δ *speB* mutant of a pattern D strain. This latter finding is consistent with the idea that additional *emm* pattern D-specific factors are required for virulence [92].

Molecular interactions between the extracellular virulence factors reveal even greater complexity, as evidenced by the post-transcriptional control of Ska activity via SpeB. The digestive activity of SpeB peaks during the stationary phase of bacterial growth, and Ska is among its many degradation targets [93]. The broad-spectrum SpeB proteinase also interacts with the human host to release biologically active kinins from kininogen [112], degrade chemokines [113], cleave Fn [114] and activate IL-1 β [115], in addition to several other activities.

Inactivation of *nra* results in a significant loss in virulence of strain Alab49 in the humanized mouse model (Table 1) [51]. In Alab49, *nra* encodes a positive regulator of pilus gene transcription and is localized to the FCT region of the genome (Figure 2). Several of the *nra*-regulated genes display strong linkage with *emm* pattern, most notably *cpa* and *fctA*, which are present in nearly all pattern D strains, but in only approximately half of pattern A–C strains [34]. Inoculation of the Δ *cpa* mutant, following growth to mid-log phase in nutrient-rich broth, leads to a significant decrease in virulence relative to Alab49 wt; however, attenuation of the Δ *cpa* mutant is not observed following its growth to stationary phase (Table 1) [94]. The lack of Cpa dependence on GAS virulence following stationary phase growth in broth might be explained by a redundant function conferred by an unidentified virulence factor that is highly expressed during stationary phase only. One can reasonably speculate that the growth-phase dependency of the GAS inoculum on virulence signifies a critical role of Cpa during the initial stages of infection.

Cpa is an accessory protein of the GAS pilus, and immunoblots show that pilus polymers are produced in the Δ *cpa* mutant, albeit at a reduced level when compared with Alab49 wt [94]. By contrast, inactivation of *fctA*, encoding the major pilus backbone subunit, results in a (near) complete loss of pilus polymers. Yet, the Δ *fctA* mutant retains full virulence in the humanized mouse following growth to either log or stationary phase (Table 1) [94,95]. Conceivably, the lack of an FctA virulence effect may be due to the topical mode of delivery of the GAS inoculum, circumventing a pilus-dependent step normally encountered during person-to-person transmission. The *prtF2* gene also resides within the FCT region but is not under *Nra* control. The Δ *prtF2* mutant of Alab49 behaves similarly to the Δ *cpa* mutant in the humanized mouse, showing attenuation following growth in culture broth to log phase, but no change in virulence following growth to stationary phase (Table 1). An explanation may lie in the finding that PrtF2, like Ska, is degraded by SpeB, whose activity peaks during stationary growth. Similar to *fctA*, the *prtF2* gene is present in over 90% of *emm* pattern D strains but in only approximately half of pattern A–C strains [34].

Orthologous gene replacements & altered virulence

A third criterion set forth for establishing that a gene is a determinant of tissue-specific infection requires that substitution of an impetigo-associated gene for a pharyngitis-associated gene leads to altered virulence, and *vice versa*. Three of the *emm* pattern D-linked genes that are essential

for maximum virulence of strain Alab49 in the humanized mouse model – *mga*, *nra* and *ska-2b* – are present in the GAS population as discrete lineages of alleles.

A *rofA* lineage allele derived from an *emm* pattern A–C throat strain, plus its upstream region containing *cis*-binding sites for autoregulation, was introduced into the Alab49 skin strain in place of the *nra* gene [95]. The *rofA* chimeric construct displays a significant increase in the levels of pilus gene transcription and pilus protein production. Thus, despite its extensive sequence divergence, RofA restores Nra function in regulating pilus gene transcription, a finding that underscores the evolutionary flexibility of transcriptional regulatory networks [116]. The *rofA* chimera also displays an increased growth rate in the humanized mouse model for impetigo, as evidenced by the number of bacterial population doublings on a time course that is approximately 24 h ahead of the Alab49 wt strain. Perhaps throat strains are more heavily pilated than skin strains, to help counter the flow of mucus over the oropharyngeal epithelium.

The hypervirulence observed for the *rofA* chimeric construct points to an increased fitness of the organism during infection of an individual host. Short-term fitness is readily captured in the humanized mouse model for impetigo. However, properties underlying short-term fitness do not necessarily coincide with long-term fitness attributes. At the level of the host population, transmission success is paramount. If an organism is hypervirulent, infection may run its course more quickly and, thereby, shorten the time period for infectiousness and transmission to new hosts. The relationship between hypervirulence and longer-term fitness is not always clear cut, and survival of the pathogen in the human host population may depend on properly balanced pathogenicity [117]. Precisely how the RofA-associated hypervirulence might relate to long-term fitness, and explain tissue site preferences for GAS infection, will require further exploration.

Hypervirulence in the humanized mouse model for infection is also evident for a chimeric construct involving the *ska* locus, wherein the *ska-2b* lineage allele of Alab49 is replaced with the divergent *ska-1* allele [Bessen DE *et al.*, Unpublished Data]. Bacterial-bound plasmin activity is equivalent for the chimera and wt Alab49 strain, indicating that, like Ska-2b, the Ska-1 form can activate bacterial-bound Plg. However, fluid-phase plasmin activity, which is lacking in the Ska-2b-containing parent strain, is a newly acquired phenotype of the *ska-1* chimera, and may underlie the hypervirulence phenotype observed for the Alab49-based construct.

The existence of discrete lineages of alleles at the *mga*, *rofA/nra* and *ska* loci within the GAS population is highly suggestive of a genetic origin in another bacterial species. A possible donor species for both the *rofA* and *ska-2a* lineage alleles is the close genetic relative of GAS, *Streptococcus dysgalactiae* subspecies *equisimilis*, also known as group C and G streptococci (GCS/GGS) [32,33]. GCS/GGS typically act as commensals colonizing the oropharynx of the human host and, on occasion, they are recovered in association with disease. All GCS/GGS strains examined harbor both *rofA* and *ska-2a* genes. Neither the *mga-1* nor *mga-2* gene is detected in GCS/GGS, or in the numerous related streptococcal species screened.

The presence of *rofA* and *ska-2a* in 100% of GCS/GGS strains is consistent with the idea that interspecies gene transfer from a GCS/GGS donor to a GAS recipient resulted in recombinational replacement of the GAS gene with an orthologous form [32,33]. Experimental orthologous gene replacement of *nra* with *rofA*, and *ska-2b* with *ska-1*, preserves key functions associated with the original GAS phenotype, yet also introduces novel phenotypes. Thus, orthologous gene replacement may constitute an important step in bacterial evolution, by providing the raw material for new adaptations, such as exploitation of an ecological niche.

Conclusion

The history of horizontal movement of GAS core genes provides a background of random associations against which one can measure functionally driven linkage between adaptive genes and disease phenotypes. Tissue site preference for infection strongly correlates with *emm* pattern group. Accessory genes that display linkage disequilibrium with the *emm* pattern marker for throat specialist, skin specialist and generalist strains are strong candidates for conferring tissue-specific tropisms. Several of the tissue-specific candidate genes are required for virulence in a humanized mouse for skin infection. Exchange of impetigo-associated genes with pharyngitis-associated genes can lead to hypervirulence at the skin. The throat specialist-linked gene encoding the transcriptional regulator RofA was likely acquired by GAS from a commensal donor species. The finding of hypervirulence in a skin strain construct harboring RofA, combined with preservation of the transcriptional regulatory network, provides evidence that orthologous gene replacements can lead to new adaptations while maintaining critical functions.

Future perspective

The strong association of certain GAS strains with particular diseases points to an underlying genetic organization of GAS that might explain the molecular basis for disease-specific pathogenic mechanisms, such as throat and skin tropisms. A major challenge faced in trying to relate genotype to phenotype is the enormous range of genetic diversity among GAS strains. Complicating this further is the prospect that some phenotypes may arise via any one of multiple genetic pathways that converge to yield similar biological properties.

To reach the next level of understanding will likely require a high-throughput systems biology approach that simultaneously incorporates a diverse population of GAS strains, so that biologically relevant groups of strains can be compared and contrasted. The complete set of microbial factors that give rise to the tissue tropism phenotype, and their well-orchestrated interactions, need to be identified and fully characterized. Further improvements in experimental models for GAS disease are also essential. Transgenic animals harboring a human-derived gene are valuable in that the impact of singular molecular interactions can be assessed. Human-derived organs are more complex, but have the potential to capture many more of the key interactions between pathogen and host.

Executive summary

Clinical importance of group A streptococcal diseases

- Group A *Streptococcus* (GAS) is a human-specific pathogen having high disease prevalence worldwide.
- The vast majority of GAS infections involve the epithelium of the throat or skin.
- The throat and skin are the primary ecological reservoirs for GAS.

Genotypic markers of throat & skin strains

- M protein provides the basis for a serotyping scheme; there are over 200 *emm* types.
- The concept of distinct throat and skin strains arose from the strong association of certain M types with one disease or the other (pharyngitis versus impetigo).
- *emm* types can be classified by *emm* pattern genotype, based on the chromosomal arrangement of *emm* and *emm*-like genes.

- The phenotype of tissue site preference for infection correlates strongly with *emm* pattern genotype, which in turn is used to define groups of throat specialist, skin specialist and generalist strains.

Population genetics of throat & skin specialists

- The GAS population genetic structure is characterized by extensive recombination.
- There is no evidence for early stages of speciation among throat and skin specialist strains.

Genes linked to markers for preferred tissue site of infection

- Against a background of random associations between core housekeeping genes, noncore accessory genes that display strong linkage to *emm* pattern are good candidates for being determinants of tissue tropisms.
- Tissue tropism candidates include transcriptional regulators, and secreted and surface proteins, such as pili.

GAS adherence in cell & organ cultures

- Many GAS adhesins are encoded by accessory genes having a differential distribution among strains.
- Pili mediate GAS adherence to human tonsil tissue and primary keratinocytes.
- Solid proof for an adhesin conferring cell-specific tropisms representing throat versus skin infection has not yet clearly emerged.

Animal models for GAS infection of the upper respiratory tract

- Since GAS is a human-specific pathogen, animal models tend to fall short in providing a complete and accurate representation of GAS disease.
- A nonhuman primate model provides an excellent demonstration of GAS tonsillitis.
- Animal models for throat colonization and/or infection employ high nonphysiological doses of GAS for inoculation.

Animal models for superficial GAS infection of the skin

- Many models deliver GAS by injection, and do not reflect superficial infection.
- Human skin engrafted on SCID mice provides a model for superficial skin infection by GAS that has high specificity and high sensitivity, and shows pathological changes similar to those observed in patients with impetigo.

Role of skin-specific gene candidates in pathogenesis

- Inactivation of the major gene candidates for skin-specific infection results in a decrease in virulence in the humanized mouse model for impetigo.

Orthologous gene replacements & altered virulence

- Substitution of impetigo-associated genes with pharyngitis-associated genes leads to hypervirulence in the humanized mouse model for impetigo.
- Several candidate genes for tissue tropism were acquired by GAS via lateral transfer from a donor commensal species.

- Acquisition of a divergent orthologous gene from a related species results in the preservation of many key functions.
- Orthologous gene replacement may provide the raw material for important new adaptations, such as exploitation of a new ecological niche.

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Figure 1. Population-based surveys of group A *Streptococcus* infection

Group A streptococci isolated from patients with either superficial throat or skin infections, in several locales throughout the world by numerous investigators, had been characterized for *emm* type [15–25]; based on the *emm* type, the *emm* pattern group is inferred [12]. The percentage of isolates in each *emm* pattern group is depicted for the eight pharyngitis and six impetigo collections of group A streptococci.

[†]Aboriginal populations of tropical Australia.

Figure 2. Map of the *emm* and FCT regions of the group A *Streptococcus* genome

The most common combinations of genes present within the *emm* and FCT regions of over 100 group A *Streptococcus* strains are depicted in accordance to *emm* pattern grouping, based on findings reported in [32–34,42]. Shown are *emm* pattern A–C (blue), D (red) and E (purple). For pattern D and E strains, the upstream *emm*-like genes is also known as *mrp* and the downstream *emm*-like gene is also known as *enn*.

Figure 3. Growth dynamics of group A *Streptococcus* during skin infection in the humanized mouse Pathological alterations throughout the time course of GAS infection of skin grafts in the humanized mouse are described. The time scale (top) designates the day postinoculation of skin with GAS. The growth stage of infecting GAS, determined based on the number of colony-forming units recovered from the skin at different time points, is also described. Panels on the right represent hematoxylin- and eosin-stained tissue sections obtained from GAS-infected skin biopsies.

Black arrow: GAS aggregates; green arrowhead: dermal capillary containing PMNs.

GAS: Group A *Streptococcus*; PMN: Polymorphonuclear leukocyte.

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Alab49 mutants having a statistically significant decrease in virulence relative to wild-type, when tested in the humanized mouse model for superficial skin infection.

Table 1

Mutant	Function of gene product	Control of gene transcription	Cell association	Virulence at skin (log inoculum) [†]	Virulence at skin (stat inoculum) [†]
Δmga	Transcriptional regulator	Autoregulated (activated) [‡]	Cytoplasm	Decreased	Decreased
Δnra	Transcriptional regulator	Autoregulated (activated) [‡]	Cytoplasm	Decreased	Decreased
Δpam ($\Delta emm53$)	Plg-binding M protein	Activated by <i>mga</i>	Surface	N.D.	Decreased (partially)
Δska	Plg activator (streptokinase)	No effect by <i>mga</i>	Secreted	N.D.	Decreased (partially)
$\Delta speB$	Cysteine protease	Activated by <i>mga</i> (indirect)	Secreted	Decreased	Decreased
$\Delta fcrA$	Pilus backbone subunit	Activated by <i>nra</i>	Surface	No effect	No effect
Δcpa	Collagen-binding protein (pilus)	Activated by <i>nra</i>	Surface	Decreased	No effect
$\Delta prf2$	Fn-binding protein	Activated by <i>msmR</i>	Surface	Decreased	No effect

[†] Virulence at the skin was measured for group A Streptococcus inoculum cultures grown to either log or stationary phase in enriched broth.

[‡] Assumed but not completely proven for strain Alab49; Mga and Nra function as autoregulators in other strains [37].

Fn: Fibronectin; N.D.: Not determined.