An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections

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The role of host genetic factors in conferring predisposition or protection in infectious diseases has become evident. Infection with group A streptococci causes a wide spectrum of disease ranging from pharyngitis to streptococcal toxic shock syndrome. The release of inflammatory cytokines triggered by streptococcal superantigens has a pivotal role in invasive streptococcal disease. However, individuals infected with the same strain can develop very different manifestations. We report here that the immunogenetics of the host influence the outcome of invasive streptococcal infection, and demonstrate the underlying mechanism for these genetic associations. Specific human leukocyte antigen class II haplotypes conferred strong protection from severe systemic disease, whereas others increased the risk of severe disease. Patients with the DRB1*1501/DQB1*0602 haplotype mounted significantly reduced responses and were less likely to develop severe systemic disease ($P < 0.0001$). We propose that human leukocyte antigen class II allelic variation contributes to differences in severity of invasive streptococcal infections through their ability to regulate cytokine responses triggered by streptococcal superantigens.

Since the early 1980s, a resurgence of severe, invasive, group A streptococcal (GAS; *Streptococcus pyogenes*) infections has occurred. The reemergence of streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) has been reported in several countries including the United States, Canada, Northern Europe, New Zealand and Australia. STSS and NF are rapidly progressive invasive diseases that are associated with high mortality rates, ranging from 30% to 80% despite prompt antibiotic therapy and debridement[1]. The speed with which the disease progresses in some patients, causing severe necrosis and hydrolysis of the flesh, has led some to dub it 'the flesh-eating disease'.

Although distinct strains of GAS have been isolated from invasive infections, a particular subclone of the M1 serotype has globally disseminated, persisting for over 20 years as the most prevalent strain isolated from these infections[2]. We previously reported that this M1T1 clone causes starkly different symptoms ranging from uncomplicated pharyngitis to invasive infection with or without severe systemic disease (SSD; that is, STSS versus bacteremia)[3,4]. The same strain can cause NF, which can also manifest with or without SSD (refs. 3,4). Accordingly, we proposed that host factors must have a role in determining the outcome of the invasive infection.

GAS are armed with many virulence factors that contribute to pathogenesis, but the secreted streptococcal pyrogenic exotoxins (Spes) seem to have a pivotal role in STSS and NF (refs. 5–7). The Spes belong to the family of microbial superantigens (SAgs), which elicit potent inflammatory responses that can lead to organ failure and shock[5,8]. However, the magnitude of inflammatory responses triggered by the same SAgs in different hosts can vary considerably, and there is a direct correlation between intensity of inflammatory cytokine responses and severity of invasive GAS infections[8]. Patients with a stable propensity to produce higher levels of inflammatory cytokines in response to streptococcal SAgs develop significantly more SSD than those who have a propensity to produce lower levels of inflammatory cytokines to the same SAgs (refs. 7,8). These observations led us to propose that variations between individuals in immunogenetic factors that regulate SAg responses may be important determinants of infection severity.

Several candidate genes that map to the human leukocyte antigen (HLA) region have been implicated as determinants of infection outcome[9]. We hypothesized that HLA class II polymorphisms may influence disease outcome because these molecules are receptors for SAgs and their allelic variations can potentiate cytokine responses to these toxins[10].

We determined the distribution of HLA class II haplotypes among patients with invasive GAS infections to test the hypothesis that specific haplotypes will be associated with risk or protection from SSD or from NF. We recruited patients through

a

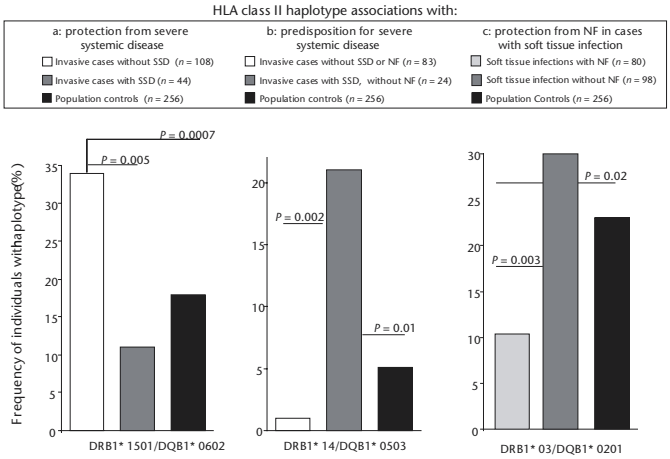

Fig. 1 HLA class II haplotypes associated with outcomes of invasive GAS infections. ***a***, We compared the frequency of the DRB1*1501/DQB1*0602 haplotype in previously healthy invasive infection cases with (n = 44) and without (n = 108) severe systemic disease as well as in healthy Ontario Canadian controls[14] (n = 256). The DRB1*1501/DQB1*0602 haplotype is protective against severe systemic disease. ***b***, We compared the frequency of the DRB1*14/DQB1*0503 haplotype in healthy Ontario Canadian controls[14] (n = 256), in previously healthy invasive infection cases with severe systemic disease without NF (n = 24), and in previously healthy invasive infection cases with neither severe systemic disease nor NF (n = 81). ***c***, We compared the frequency of the DRB1*03/DQB1*0201 haplotype in soft tissue infections with NF (n = 80) with invasive soft tissue infections without NF (n = 98), as well as with healthy Ontario Canadian controls[14] (n = 256).

b

Table 1 Clinical manifestations of invasive GAS infection cases[a]

Clinical manifestation	Total cases	Previously healthy cases[b]
Invasive cases without severe systemic disease	194	108
Invasive cases with severe systemic disease	75	44
Invasive cases with neither severe systemic disease nor NF	151	83
NF cases without severe systemic disease	42	24
NF cases with severe systemic disease	35	20

Analysis of HLA effects on severe systemic disease was done on only the previously healthy cases to avoid misclassified cases who developed severe systemic disease due to a preexisting chronic condition. [a]Clinical classification of cases is as detailed in methods. [b]Previously healthy cases are patients without chronic underlying illness who were <85 y old.

c

Table 2 Haplotypes associated with different manifestations of invasive GAS infections

Manifestation of invasive GAS infection	Haplotypes with protective effect	Haplotypes with predispositional effect
Severe systemic disease	DRB1*1501/DQB1*0602	DRB1*14/DQB1*0503
NF	DRB1*03/DQB1*0201	DRB1*11/DQB1*0301*
Severe systemic disease in the presence of NF	DRB1*1501/DQB1*0602	DRB1*07/DQB1*0201*

*The DRB1*11/DQB1*0301 haplotypes showed only a trend towards an association with risk for NF. The trend for an association of the DRB1*07DQB1*0201 haplotype with risk for SSD in the presence of NF was functionally validated.

ongoing active surveillance of all invasive GAS infections in Ontario[2]. Invasive disease is defined as illness associated with isolation of GAS from a normally sterile site. Invasive cases presented with or without SSD depending on the presence or absence of hypotension and multiple organ failure (MOF). NF cases were defined as previously described[11], and these cases presented with or without SSD. Confounding factors, such as age and underlying disease, that may lead to misclassification of severe invasive cases were clearly defined and recorded. We flagged patients who may have had hypotension and MOF due in part to old age (greater than 85 years) and/or chronic underlying diseases, and conducted the analysis of HLA association with SSD in previously healthy patients aged 85 years or lower (Fig. 1).

We carried out molecular typing of the DRB1, DQA1 and DQB1 loci[12,13] and assigned the determined HLA class II DRB1, DQA1 and DQB1 haplotypes based on the known linkage disequilibrium in healthy Canadian controls[14]. We found 11 haplotypes in frequencies of greater than 3% in our entire patient population; of these only 2 showed uneven distribution between severe and non-severe cases or between cases and controls, and 2 showed uneven distribution among NF cases compared with non-severe cases without NF or with healthy controls.

HLA class II haplotype association with SSD

We determined class II haplotype frequencies in previously healthy invasive infection cases with and without SSD (that is, with and without MOF and hypotension), and compared them with healthy Canadian controls (n = 256) from southern Ontario[14]. Previously healthy invasive cases with and without SSD showed significant differences in DRB1*1501/DQB1*0602 and DRB1*14/DQB1*0503 haplotype frequencies. The frequency of the DRB1*1501/DQB1*0602 haplotype in cases with SSD was significantly lower than in the cases without SSD (11% versus 34%; odds ratio (OR) = 0.25; 95% confidence interval (CI) = 0.09, 0.68; P = 0.005; Pc = 0.05). Moreover, this haplotype was significantly higher in the non-severe cases compared with healthy controls (34% versus 18%; OR = 2.5; 95% CI = 1.4, 4.3; P = 0.0007; Pc = 0.008; Fig. 1*a*). Thus, the DRB1*1501/DQB1*0602 haplotype confers strong protection from the severe systemic manifestations of invasive GAS infection regardless of the infecting serotype. In analysis adjusted for potential confounders (age, the serotype of infecting strains and presence of NF), the DRB1*1501/DQB1*0602 haplotype remained associated with significant protection from SSD (OR = 0.24; 95% CI = 0.08; 0.70; P = 0.008; see Supplementary Note online).

The frequency of the DRB1*14/DQB1*0503 haplotype was higher in previously healthy cases with SSD than in those without (14% versus 3.7%; OR = 4.1; 95% CI = 0.9, 2.1; P = 0.03; Pc = 0.39). However, multivariable analysis revealed an interaction between SSD, NF and this haplotype. Risk for SSD was significantly conferred by this haplotype in the absence of NF (21% versus 1%; OR = 21; 95% CI = 2.4, 196; P = 0.002; Pc = 0.04), but not in the presence of NF (P = 0.61; Fig. 1*b*). These data are suggestive that individuals with the DRB1*14/DQB1*0503 haplotype are at risk of SSD complicating their invasive infection.

HLA class II haplotype association with NF

Using the cases with soft tissue infections, we compared class II haplotype frequencies in patients with and without NF. The

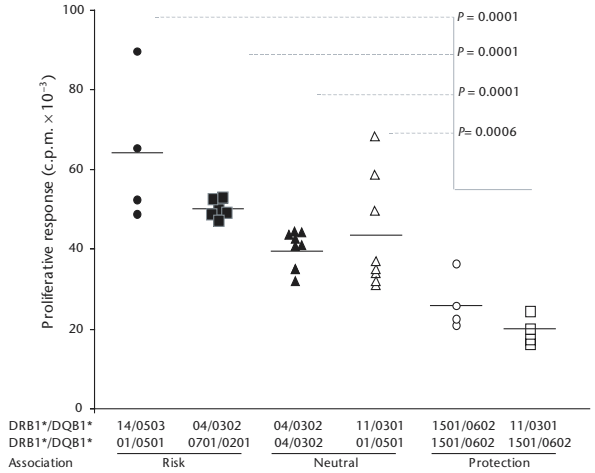

Fig. 2 Responses of healthy individuals with risk, neutral or protective haplotypes to M1T1 SAgs. We isolated PBMCs from the blood of healthy individuals who carried class II haplotypes associated with risk or protection from severe systemic GAS disease, stimulated them with the mixture of SAgs produced by different clonal M1T1 isolates[8,21], and then measured the proliferative response.

DRB1*03/DQB1*0201 haplotype was associated with protection from NF. The frequency of this haplotype was significantly lower in the NF cases compared with cases without NF (11% versus 30%; O*R* = 0.31; 95% CI = 0.13, 0.68; *P* = 0.003; *Pc* = 0.07; Fig. 1*c*). There seemed to be an interaction between NF and SSD with the DRB1*03/DQB1*0201 haplotype—cases with this haplotype were much less likely to have NF in the absence of MOF (4.8% versus 30%; O*R* = 0.12; 95% CI = 0.03, 0.53; *P* = 0.001; *Pc* = 0.03). When the independent contribution of the class II haplotype was assessed in a multivariate analysis model, which included age and the serotype of infecting strains, the DRB1*03/DQB1*0201 haplotype remained associated with significant protection from SSD (O*R* = 0.28; 95% CI = 0.11, 0.72; *P* = 0.006).

The DRB1*11/DQB1*0301 haplotype showed a trend toward an association with risk for NF in the absence of SSD. The frequency of this haplotype was 29% in cases with NF compared with 12% in cases without NF (*P* = 0.045), or compared with 13% in healthy controls (*P* = 0.017). The trend toward an association between this haplotype and NF was also apparent in multivariate analysis adjusted for confounders (O*R* = 2.3; 95% CI = 0.93, 5.8; *P* = 0.06).

HLA class II haplotype association with SSD plus NF

NF combined with SSD is one of the deadliest forms of invasive GAS infections. Although the DRB1*1501/DQB1*0602 haplotype conferred no protection against NF, patients with NF who have this haplotype seemed to be protected from concurrent SSD and toxic shock in addition to their NF disease. Previously healthy patients who had this deadly form of invasive GAS infection (*n* = 20) had a significantly lower frequency of the DRB1*1501/DQB1*0602 haplotype compared with cases with neither NF nor SSD (5% versus 35%; O*R* = 0.09; 95% CI = 0.004, 0.761; *P* = 0.006). A trend toward an association between the DRB1*07/DQA1*0201/DQB1*0201 haplotype and risk for the combined NF and severe disease was also observed. The frequency of this haplotype was higher in cases of NF and SSD compared with invasive cases who had neither of these syndromes (35% versus 11%; O*R* = 4.4; 95% CI = 1.2, 16.2; *P* = 0.014).

Molecular basis for HLA class II association with SSD

Having identified specific class II haplotype associations in different forms of the invasive infection (Fig. 1), we next conducted functional studies to investigate the molecular basis for these associations. Inasmuch as our previous studies have shown that the development of severe disease is associated with significantly higher levels of production of inflammatory cytokines during the acute infection[7,8,15] and that the propensity of severe cases to respond vigorously to GAS SAgs is reproducible during convalescence[8], we hypothesized that individuals who carry the SSD-protective haplotype will mount lower responses to GAS SAgs compared with individuals with risk haplotypes. We assessed responses of healthy individuals with protective, risk or neutral haplotypes (that is, those with no association to disease outcomes) to the SAgs produced by the clonal M1T1 strain, and found that individuals with the

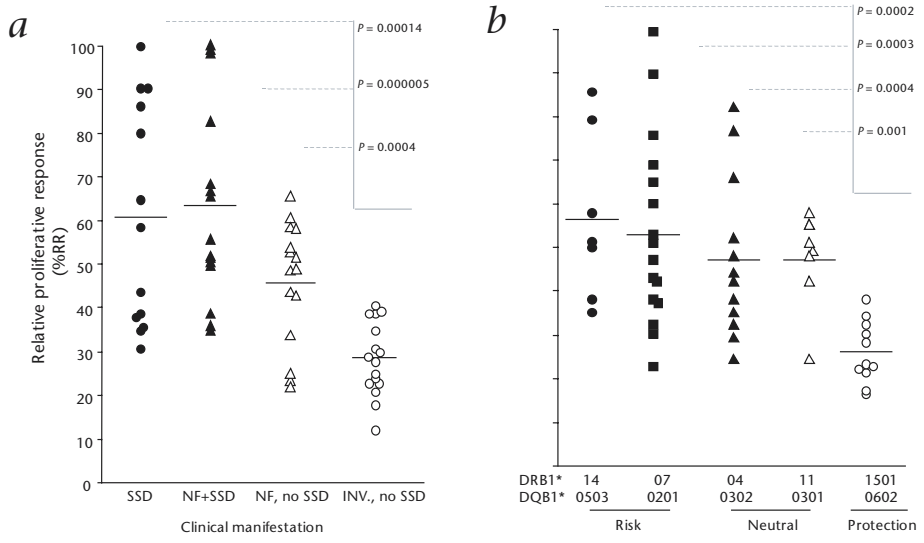

Fig. 3 Responses of convalescent patients to the SAgs produced by their isolate. We isolated PBMCs from blood of patients at least 1 month after discharge (convalescent samples) and stimulated them with the mixture of SAgs produced by their own respective isolate as described[8,21]. We then compared the relative proliferative responses, which are presented as percentage of maximum PHA response[8], in patients with and without severe systemic disease SSD in the presence or absence of NF (***a***), or patients with class II haplotypes associated with risk or protection from severe systemic GAS disease (***b***). INV., invasive infection; NF, necrotizing fasciitis; SSD, severe systemic disease. Statistical differences were calculated by the two-tailed Student's *t*-test GraphPad software.

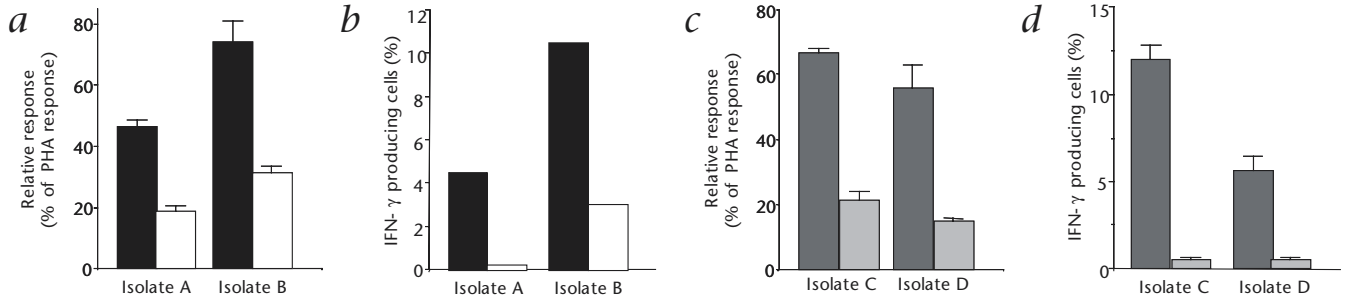

Fig. 4 Proliferative and cytokine responses of matched pairs of convalescent patients. We drew blood from pairs of age, gender and underlying disease-matched NF cases with SSD (cases A (■) and C (■)) and without SSD (cases B (□) and case D (■)) who were also infected with the same clonal M1T1 strain of GAS[4]. Cases B and C carried the protective DRB1*1501/DQB1*0602 haplotype; cases A and C lacked it. We tested the PBMCs from the paired patients case A versus case B (***a*** and ***b***), and case C versus case D (***c*** and ***d***) in the same assay for responsiveness to the SAgs produced by their isolate as well as each other's isolate. We determined the proliferative response (***a*** and ***c***) and the percentage of IFN-γ producing cells in 72-h cultures (***b*** and ***d***) as detailed[8,21], and calculated statistical differences by the two-tailed Student's *t*-test using GraphPad software. Case A (■) had NF + SSD and carried the DRB1*04/DQB1*0302;DRB1*11/DQB1*0301 haplotypes; case B (□) had NF, no SSD and carried the DRB1*15/DQB1*0602;DRB1*15/DQB1*0602 haplotypes. Case C (■) had NF + SSD and carried the DRB1*04/DQB1*0302;DRB1*14/DQB1*0503 haplotypes; case D (■) was an invasive nonsevere case (Inv., no SSD) and carried the DRB1*03/DQB1*0201;DRB1*15/DQB1*0602 haplotypes.

DRB1*1501/DQB1*0602 haplotype responded at a significantly lower level compared with individuals with risk or even neutral haplotypes (P = 0.001–0.0001; Fig. 2). We saw the same results when we analyzed responses of recovered patients in convalescence, who had had invasive GAS infection. We obtained blood from representative invasive cases with various degrees of severity at least 1 month post-recovery, and stimulated their peripheral-blood mononuclear cells (PBMCs) with the SAgs produced by their respective isolate[8]. The response of cells of patients who had SSD was significantly higher than that of patients who had no SSD (P = 0.0001–0.000005; Fig. 3*a*). Cells from patients with the DRB1*1501/DQB1*0602 haplotype mounted consistently lower responses than patients' cells with risk or neutral haplotypes (Fig. 3*b*). The response of cells from patients with the DRB1*14/DQB1*0503 or DRB1*07/DQB1*0201 haplotypes, which are respectively associated with risk for SSD in the absence and in the presence of NF, was significantly higher than that of cells from patients' PBMCs with the DRB1*1501/DQB1*0602 haplotype (P = 0.0003–0.0002).

Although convalescent patients' PBMCs were stimulated with their own isolate, low responders elicited low responses and high responders elicited high responses when stimulated with each other's isolates. To better illustrate this point and to minimize assay variability, fully recovered pairs of age, gender and underlying disease-matched cases with and without SSD, who were also infected with the same clonal strain of GAS[4], were bled and tested in the same assay for responsiveness to the SAgs produced by their isolate as well as by each other's isolate. We matched two cases of NF with SSD (cases A and C) with two invasive cases without SSD, but with NF (case B) and without SSD or NF (case D; Fig. 4). Case B was homozygous for the protective DRB1*1501/DQB1*0602 haplotype and case D was heterozygous with the protective haplotype and the DRB1*03/DQB1*0201 haplotype. In this type of response experiment, both DRB1*1501/DQB1*0602 cases without SSD mounted a significantly lower proliferative and interferon-γ (IFN-γ) response than their paired counterparts who had combined NF and SSD, and lacked this protective haplotype (P < 0.003; Fig. 4).

To further confirm results obtained from patient responses, we took advantage of the HLA-unrestricted SAg presentation and used HLA-homozygous lymphoblastoid B-cell lines to look at the differential response to GAS SAgs when presented by different class II haplotypes. SAgs produced by the M1T1 clone were presented to pure, antigen-presenting cell (APC)-depleted T cells from healthy responders by these homozygous APCs. Responses to the M1T1-derived SAgs were significantly lower when presented by APCs homozygous for the DRB1*1501/DQB1*0602 protective haplotype than by the DRB1*14/DQB1*0503 APCs homozygous for the haplotype that confers risk for severe invasive disease (P < 0.001; Fig. 5*a*), or even by APCs expressing the neutral DRB1*04/DQBB1*0302 haplotype (P < 0.01; Fig. 5*b*). Similarly, SAg proliferative and cytokine responses in the presence of the DRB1*1501/DQB1*0602 haplotype were significantly lower than those in the presence of the DRB1*07/DQB1*0201 haplotype (P < 0.0001; Fig. 6*a*), which showed a trend toward an association with risk for the combined NF and SSD. The number of cells producing tumor necrosis factor-β (TNF-β) and IFN-γ, which are typical SAg-induced TH1 cytokines, were respectively 47% and 70% lower in the presence of the DRB1*1501/DQB1*0602 haplotype than in the presence of the DRB1*07/DQB1*0201 haplotype (Fig. 6*b*). In contrast, production of the TH2 cytokine, interleukin-4 (IL4), was 50% higher in the presence of the DRB1*1501/DQB1*0602 haplotype than in the presence of the DRB1*07/DQB1*0201 haplotype.

The above functional data reflect the results seen with the patient's PBMCs and provided validation for the genetic association data. The data provide a clear molecular basis for the association of the DRB1*1501/DQB1*0602 haplotype with protection from SSD in invasive GAS infections.

Discussion

We have shown that individuals with low levels of protective anti-streptococcal antibodies in their plasma are at risk of developing invasive GAS infection; however, once the bacteria invaded a normally sterile site, the severity of the invasive infection was unrelated to the levels of these antibodies inasmuch as invasive cases with or without SSD had significantly low levels of anti-M serotype antibodies and low levels of neutralizing anti-SAg antibodies compared with age and geographically matched controls[3]. Instead, we provided evidence that the propensity to mount a high or low proliferative and cy-

tokine response to SAgs produced by the invasive GAS isolates was directly and significantly correlated to the disease severity[8]. Based on these findings we proposed that host immunogenetic factors involved in regulating SAg responses may influence the severity of invasive GAS infections. We focused on the HLA class II haplotypes because these molecules serve as SAg receptors and variation in these molecules is known to influence SAg responses.

Associations of HLA with various infectious diseases have been reported. This study, however, is the first to document an HLA association with different outcomes of a Gram-positive infection and to provide a molecular basis for an association with an infectious disease. The findings presented here support our hypothesis that variation in class II alleles/haplotypes can influence the outcome of a SAg-mediated disease, including invasive GAS infection. Our data also suggest that this effect is mediated through differential presentation of streptococcal SAgs by distinct class II alleles/haplotypes, resulting in significant differences in the magnitudes of inflammatory responses.

Although the incidence of invasive GAS infections has been on the rise since the 1980s, these remain relatively rare diseases. The annual incidence of invasive GAS infections found in a prospective study carried out in Canada was 1.5 cases per 100,000 persons, with STSS and NF occurring in 13% and 6% of invasive infections, respectively[1]. The fact that we detected specific HLA associations with different outcomes of the invasive infection (Fig. 1 and Table 2) was remarkable given that other host factors also contribute to disease outcome. We designed the study to control for some of these variables by flagging elderly patients with possible confounding preexisting health conditions that can lead to case misclassification and by correcting for potential confounders in multivariable analyses. As would be expected in genetic associations with an infectious disease, the strongest association was with protection from the severe systemic form of the invasive infection. The DRB1*1501/DQB1*0602 haplotype was highly represented in invasive cases without SSD compared with severe invasive cases or with healthy population controls. The protective effect of this haplotype against SSD was even evident in the presence of NF. Functional studies supported the protective effect of the DRB1*1501/DQB1*0602 haplotype by showing that the proliferative and cytokine responses to streptococcal SAgs were considerably lower in the presence of the protective DRB1*1501/DQB1*0602 haplotype than in the presence of a predisposing or even a neutral haplotype. Although individuals with the DRB1*14/DQB1*0503 or the DRB1*07/DQB1*0201 risk haplotypes were the highest responders to the streptococcal SAgs, responders with neutral haplotypes also mounted significantly more potent responses than individuals with the DRB1*1501/DQB1*0602 haplotype. We propose that the presence of the DRB1*1501/DQB1*0602 haplotype somehow attenuates inflammatory responses to the streptococcal SAgs and we are further investigating the underlying mechanism of this phenomenon.

The effects of class II allelic variation on SAg responses or disease outcome may be potentiated by polymorphisms in other host immunogenetic factors, including variations in class II–linked genes and cytokine genes[9]. In fact, we have preliminary data to suggest a significant protective effect conferred by particular TNF microsatellite haplotypes, which we found to be in strong linkage disequilibrium with the DRB1*1501 allele[16]. Ongoing studies with carefully selected genotyped APCs will help us discern the individual and/or interactive effects of class II and cytokine gene polymorphisms on streptococcal SAg responses and on the severity of invasive GAS infections.

Another important finding in this study is that HLA associations with predisposition and protection for STSS and NF were different. This substantiates a long-standing opinion among many infectious disease physicians that these are different diseases that may require different intervention strategies. European studies, which reported an increase in severe GAS infection, did not report the concomitant presence of NF (refs. 17,18). Other researchers have reported an increased incidence and severity of GAS, with only a small number of NF cases[19]. A retrospective survey of the medical records of all ten hospitals in Pima County, Arizona, designed to identify sterile site isolates of GAS between 1985 and 1990, found significant changes in the clinical spectrum of invasive infections, with an increase in patients with clinical features of STSS during the last 3 years of the study[20]. NF was not associated with any of the other clinical features of STSS. Another study found that only 36 of 77 cases of GAS NF were associated with the presence of STSS[11]. Therefore, the genetic associations reported here underscore the difference between two major manifestations of invasive

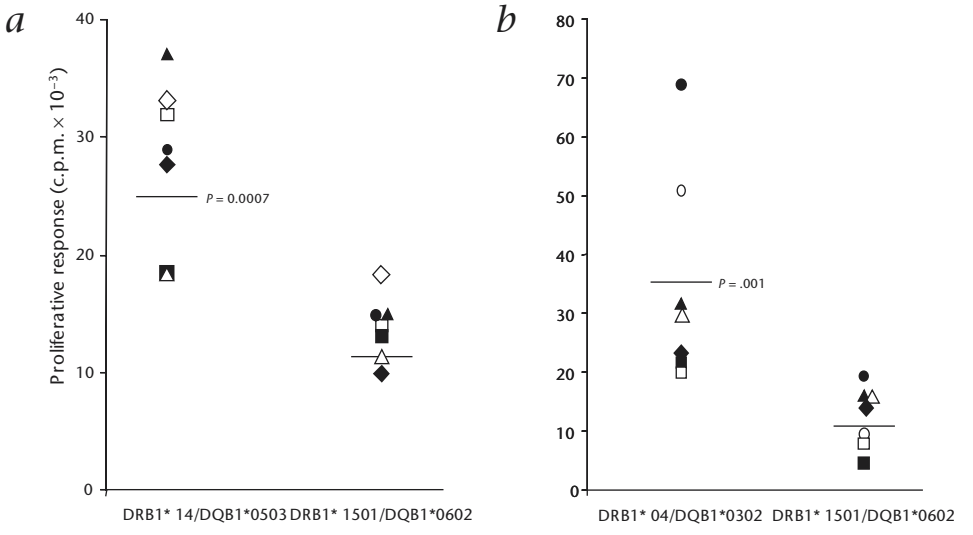

Fig. 5 Presentation of GAS SAgs by cells carrying SSD protective/predispositional haplotypes. APC-depleted T cells from healthy individuals[21] were stimulated with the SAgs derived from representative clonal M1T1 isolates[4,8] in the presence of mitomycin C–treated homozygous lymphoblastoid cell lines[21] carrying the protective DRB1*1501/DQB1*0602 haplotype (***a***) or the risk DRB1*14/DQB1*0503 haplotype (***b***). Each symbol represents the response cells from the same T-cell donor to the same isolate, with the only variable being the HLA class II-expressing APCs. Statistical differences were calculated by the two-tailed Student's *t*-test GraphPad software.

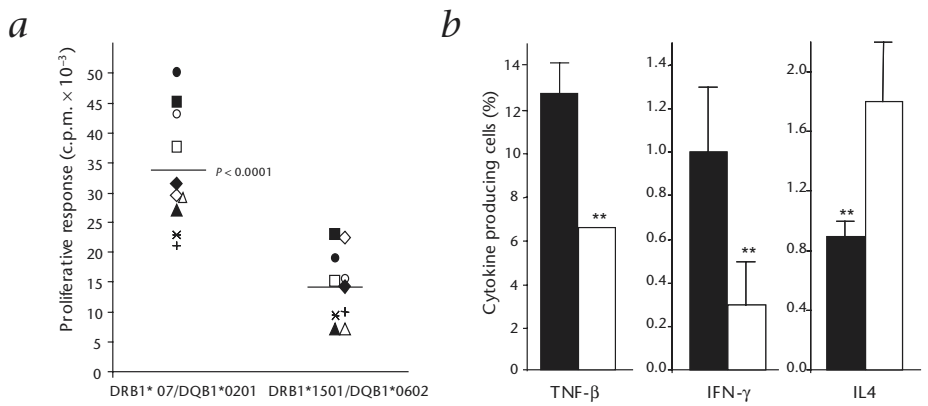

Fig. 6 Presentation of GAS SAgs by cells carrying NF + SSD protective/predispositional haplotypes. APC-depleted T cells from healthy individuals[21] were stimulated with the SAgs derived from representative clonal M1T1 isolates[4,8] in the presence of mitomycin C–treated homozygous lymphoblastoid cell lines carrying the indicated haplotypes[21]. ***a***, Proliferative responses. Each symbol represents the response of cells from the same T-cell donor to the same isolate, with the only variable being the HLA class II-expressing APCs. ***b***, Cytokine responses to superantigens presented by the DRB1*07/DQB1*0201 haplotype (black column) or by the DRB1*1501/DQB1*0602 haplotype (white column): The percentage of TNF-β, IFN-γ and IL4 producing cells was determined at the optimal times in the 48-h, 72-h and 24-h cultures, respectively, by immunohistochemical staining and by counting the Golgi-stained cells as described[8,21]. Statistical differences were calculated by the two-tailed Student's *t*-test GraphPad software.

GAS infection, STSS and NF, supporting clinical and epidemiological observations. Although the HLA association with NF and SSD were distinct, multivariate analyses revealed an interesting interaction between these two conditions such that a significant association with risk for either disease was only evident in the absence of the other. Together, the clinical and genetic data are suggestive that NF and STSS have different underlying mechanisms. A deeper understanding of these differences should lead to a better understanding of the disease mechanism and may lead to more effective and specific therapeutic modalities.

As new pathogens emerge and old diseases recur with increased virulence, it has become evident that host genetic factors have an important role in modulating the infection outcome. The wide scope of GAS diseases provides an intriguing model for investigating the role of host–pathogen interactions in determining the outcome of an infectious disease and for understanding the underlying mechanism of these genetic associations.

Methods

Patient material and clinical classifications. We identified the patients that were included in this study (mean age 47.3 y; male-to-female ratio 1.17) through active surveillance for all invasive GAS infections in Ontario, Canada, from 1994 to 1999. Informed consent was obtained from all patients and healthy controls; the consent form and study design were approved by the Human Subjects Review Committee of the University of Toronto, by the University of Tennessee Health Science Center and by the Veteran's Affairs Medical Center, Memphis, Tennessee. Invasive disease is defined by isolation of GAS from a normally sterile site. SSD (for example, STSS) is invasive disease with hypotension (systolic BP ≤ 90 mmHg, or less than the fifth percentile for patient's age) plus at least 2 of the following: acute renal failure (serum creatinine concentration more than 176 μmol/ml or twice baseline in the absence of chronic renal failure); coagulation abnormalities (platelets greater than 100,000 × 10^9/l or evidence of disseminated intravascular coagulation); hepatic dysfunction (AST or ALT at least twice the upper limit of normal or twice baseline in the absence of chronic liver disease); and adult respiratory distress syndrome. NF was defined as described[11]. The NF cases presented either with or without SSD (Table 1).

Confounding factors, such as age and underlying disease, that may lead to misclassification of severe invasive cases were documented. Attending physicians and infection control practitioners collected demographic data and information regarding underlying chronic illnesses and severity of illness. We flagged patients who may have had hypotension and MOF due in part to age and underlying diseases, and removed them from the analysis of HLA association with SSD. Thus, we conducted primary analyses of HLA association with SSD in patients without chronic underlying illness who were <85 y old (designated 'previously healthy cases'; Table 1). We conducted primary analyses of HLA association with NF in cases with a soft tissue site of infection.

HLA molecular typing and statistical analyses. Staff not aware of the clinical data carried out the HLA class II genotyping. We determined DRB1, DQA1 and DQB1 alleles by molecular typing using described methods[12,13] and assigned haplotypes based on known linkage disequilibrium between the three loci in healthy controls from Ontario, Canada[14]. We carried out DQA1 typing on all patients to further verify haplotype assignments[13].

We used the GraphPad Software (San Diego, CA) to calculate OR and 95% CI for marker association, and evaluated significant associations by the two-tailed Fisher's exact test, with *P* values corrected for multiple comparisons. We used as covariates, in a multivariate regression analysis, other potential risk factors for severe disease or NF (including age and the serotype of infecting strains) and these data are included in the Supplementary Note online. We experimentally validated the statistically significant disease associations detected in this study using functional assays, described below.

Assessment of the response of convalescent patients to SAgs produced by their GAS isolate. We identified the GAS isolate from each patient with the same number as the patient to allow further individualized testing of specific immunological parameters. We prepared the partially purified mixture of secreted M1T1 GAS SAgs as described[3,8].

We isolated PBMCs from blood of patients at 1 month after full recovery and cultured them at 10^6 cells/ml in RPMI medium with 10% heat-inactivated endotoxin-free FBS, treated with polymyxin B–agarose to adsorb any residual endotoxin[8,21]. We stimulated the PBMCs with predetermined optimal concentrations of GAS culture supernatants prepared from the patient's isolate or with PHA (1 μg/ml). After 72 h of culture, we pulsed the cells for 6 h with 1 μCi [^{3}H]thymidine, and counted them in a β-scintillation counter. We assayed all samples in triplicate and present the data as mean counts per minute ± s.d., or as percentage relative responses (%RR) to PHA response. We used the two-tailed *t*-test to assess differences.

Presentation of GAS SAgs by homozygous B cell lines expressing protective and predisposing class II haplotypes. To test the effect of HLA class II haplotypes on the presentation of SAgs, we purified pure T cells by depleting autologous APCs using class II magnetic beads[22,23], checked their purity by flow cytometry as well as by their lack of response to SAg and PHA, and then stimulated them with the mixture of SAgs produced by isolates representing the clonal M1T1 strain that is responsible for approximately 40% of our cases. We used, as APCs, mitomycin C–treated, immortalized, homozygous B-cell lines expressing the haplotypes DRB1*1501/DQB1*0602 (line 9081), DRB1*14/DQB1*0503 (line 9061) or DRB1*04/DQB1*0302 (line 9033). These lines were purchased from the European Cell Culture Collection (UK). We then stimulated the APC-depleted T cells with or without the mitomycin C–treated APC with the

partially purified mixture of secreted M1T1 GAS SAgs or with PHA. We assessed the differences in proliferative responses as detailed above.

Assessment of cytokine responses. We analyzed cytokine production by enumerating the number of cytokine-producing cells using Golgi-confined stain as a marker[8,21]. We harvested the cultures at specified times and stained them for intracellular cytokines, after permeabilization with 0.1% saponin (Sigma, St. Louis, MO), using cytokine-specific antibodies as detailed[8]. We counted at least 1,000 cells in duplicate slots, and present the data as percentage cytokine-producing cells ± s.d. We evaluated the differences by the two-tailed *t*-test.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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