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1 **Vaccine-induced, but not natural immunity, against the Streptococcal**
2 **Inhibitor of Complement protects against invasive disease**

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26 **Abstract**

27 Highly pathogenic *emm1 Streptococcus pyogenes* strains secrete the multidomain
28 Streptococcal inhibitor of complement (SIC) that binds and inactivates
29 components of the innate immune response. We aimed to determine if naturally
30 occurring or vaccine-induced antibodies to SIC are protective against invasive *S.*
31 *pyogenes* infection. Immunisation with full length SIC protected mice against
32 systemic bacterial dissemination following intranasal or intramuscular infection
33 with *emm1 S. pyogenes*. Vaccine-induced rabbit anti-SIC antibodies, but not
34 naturally occurring human anti-SIC antibodies, enhanced bacterial clearance in an
35 ex vivo whole blood assay. SIC vaccination of both mice and rabbits resulted in
36 antibody recognition of all domains of SIC, whereas naturally occurring human
37 anti-SIC antibodies recognised the proline-rich region of SIC only. We therefore
38 propose a model whereby natural infection with *S. pyogenes* generates non-
39 protective antibodies against the proline-rich region of SIC, while vaccination with
40 full length SIC permits development of protective antibodies against all SIC
41 domains.

42

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43 **Introduction**

44 Invasive disease caused by the human specific pathogen, *Streptococcus pyogenes,*
45 also known as group A Streptococcus (GAS), has been increasing since the 1980s
46 and is associated with mortality of approximately 20% [1,2]. Strains expressing the
47 M1 protein, encoded by *emm1*, are overrepresented amongst invasive isolates,
48 and account for over 30% of cases of necrotising fasciitis and streptococcal toxic
49 shock syndrome [3]. The Streptococcal Inhibitor of Complement (SIC) is an
50 extracellular protein, almost uniquely expressed by *emm1 S. pyogenes*, and is one
51 of several virulence factors implicated in the propensity for *emm*1 isolates to
52 cause severe infection[4].

53

54 Three distinct regions of SIC have been described; an N-proximal short repeat
55 region, a central long repeat region, and a C-proximal proline-rich region [4,5]. SIC
56 binds to the C5b67 complex of complement to inhibit the formation of the
57 membrane attack complex [4,6]. SIC also inhibits the function of host antimicrobial
58 factors including lysozyme, alpha and beta defensins, secretory leucocyte
59 protease inhibitor, LL-37 and histones, and additionally has a role in bacterial
60 adherence to epithelial cells [5,7-10]. While transcriptomic and mutagenesis studies
61 have suggested a role for SIC in invasive disease *in vivo* [11,12], expression of SIC has
62 not been directly linked to invasiveness of *S. pyogenes* in the clinical setting.
63 Recently SIC was one of the 15 streptococcal proteins detected in pleural fluid
64 from a child with empyema caused by *emm*1 *S. pyogenes*, indicating that SIC is
65 expressed at high levels during natural infection although it may be degraded [13].

66

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67 Despite a relatively low incidence of anti-M1 antibody, antibody to SIC is found
68 frequently (~40%) among humans from diverse populations, including healthy
69 people and also those with previous streptococcal disease [14-16]. SIC is genetically
70 highly variable with over 300 *sic* alleles described and it is speculated that
71 human antibody at mucosal surfaces may drive SIC variation [17], however the role
72 of anti-SIC antibodies in host immunity remains unclear. We set out to measure
73 SIC production by *S. pyogenes in vitro* and *in vivo*, and then determine whether
74 immunity to SIC can be protective. We found that, despite the prevalence of
75 naturally occurring anti-SIC antibodies in humans, these antibodies do not confer
76 opsonophagocytic protection against *S. pyogenes*. In contrast, vaccine-induced
77 antibodies against full length SIC do confer opsonophagocytic activity against *S.*
78 *pyogenes* and, furthermore, provide protection against experimental invasive
79 streptococcal disease.

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80 **Results**

81

82 **Expression of SIC in vitro among invasive and non-invasive isolates**

83 SIC expression in broth was quantified by western blot and densitometry from
84 101 clinical isolates of *emm*1 *S. pyogenes* to determine whether SIC expression
85 was associated with site of bacterial isolate or original disease phenotype (Figure
86 1). SIC expression varied from 4.14 ng/ml to 434.67 ng/ml (median 83.68 ng/ml,
87 IQR 45.43-126.63) in culture supernatant. Although there was a wide range of
88 expression, there was no significant difference in the detected levels of SIC
89 expression between invasive disease isolates (median 80.58 ng/ml, IQR 43.92-
90 118.4), and non-invasive isolates (median 88.06 ng/ml, IQR 42.69-150.7) (Figure
91 1A). Further categorisation of the 87 strains for which the site of isolation was
92 known did not reveal any association between SIC expression and any specific
93 disease etiology (Figure 1B). Among a subset of 39 isolates for which the
94 sequence of the negative regulatory locus covRS was known, SIC secretion *in*
95 *vitro* was higher in the 6 strains with covRS mutations (median 311.8 ng/ml)
96 than strains without mutations (median 88.06 ng/ml, p=0.0017).

97

98 To quantify SIC expression *in vivo*, FVB/n mice were infected intramuscularly
99 with 5 x 10^7 CFU of *S. pyogenes emm*1 strain AP1 or SIC-negative derivative of
100 AP1 [9]. SIC was detected in infected muscle tissue extracts from AP1-infected
101 mice (n=5) three hours post-infection (Figure 1C); the median quantity of
102 bacteria was 3.6 x 10^7 CFU (0.9 to 6.5 x10^7 CFU) per mg of thigh tissue and the
103 median SIC level detected was 2.49 ng/mg of tissue (0.39 to 3.27 ng/ml of tissue).
104 SIC was not detected in thigh tissue of mice infected with the SIC-negative

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105 derivative (n=3); the median quantity of bacteria was 6.6 x 10^6 CFU (6.5 x 10^6 to
106 1.47 x10^7 CFU) of SIC-negative derivative per mg of thigh tissue (Figure 1C).
107
108 **SIC is immunogenic in mice and immunisation improves outcome following**
109 **intranasal infection**
110 Serum IgG antibodies directed against SIC are common in healthy human
111 populations [14-16], but little is known about the protective role of these antibodies
112 against disease. The potential protective role of anti-SIC antibodies was
113 therefore assessed in a mouse model of infection. To determine whether
114 recombinant SIC protein variant SIC1.300 (rSIC1.300) was immunogenic, mice
115 were immunised with rSIC1.300 or sham vaccine containing PBS and adjuvant.
116 Following immunisation, anti-SIC antibodies could be detected in a 1:64,000
117 dilution of immunised mouse sera (Figure 2A). To assess whether these titres
118 translated into protective immunity, immunised mice were infected with a
119 contemporary *emm*1 *S. pyogenes* strain H584 (a strain that expresses the
120 SIC1.300 variant) via the intranasal route using a volume known to reach the
121 lung and disseminate systemically. *S. pyogenes* lower respiratory tract infection
122 led to noticeable systemic disease. However, mice immunised with recombinant
123 SIC1.300 demonstrated improved outcomes (time to humane endpoints)
124 compared to mice immunised with sham vaccine (Figure 2B), even when the
125 challenge dose was increased (Figure 2C).
126
127 **Immunisation with SIC protects against systemic bacterial dissemination**
128 To determine the mechanism of protection, in a separate experiment
129 SIC-immunised mice were challenged intranasally and bacterial counts at the site

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130 of infection and in distant tissues were quantified 48 hours after infection
131 (Figure 3). Bacterial counts recovered from the nose were similar between both
132 sets of animals, indicating that there was no differences in dose or local bacterial
133 replication between the two groups (Figure 3A). Compared to mice that received
134 sham vaccination, mice immunised with SIC1.300 had significantly reduced
135 bacterial counts in the spleen (Figure 3B) and liver (Figure 3C). Although 3/10
136 SIC-immunised mice were bacteraemic, compared to 7/10 sham immunised
137 mice, there was no significant difference in bacterial counts in the bloodstream
138 (Figure 3D).
139
140 A separate group of SIC-immunised and sham-immunised mice were infected by
141 the intramuscular route and bacterial counts were assessed 24 hours after
142 infection. Again, while no differences in bacterial load were observed at the site
143 of inoculation, in the infected muscle, (Figure 4A) a significant difference in the
144 bacterial counts in the spleen (Figure 4B) and liver (Figure 4C) was measured.
145 Very limited bacterial dissemination to the blood was observed (Figure 4D).
146
147 **Vaccine-induced rabbit anti-SIC antibodies, but not naturally occurring**
148 **human antibodies enhance clearance of *emm*1 *S. pyogenes ex vivo***
149 To assess the protective role of vaccine-induced immunity against SIC *ex vivo*,
150 polyclonal rabbit anti-SIC serum was generated using recombinant SIC 1:300.
151 Cross reactivity with other SIC variants and native SIC from *emm1* GAS culture
152 supernatant was confirmed by ELISA and western blotting (Supplementary
153 Figure S1). To determine if vaccine induced rabbit antibodies and/or naturally
154 occurring human antibodies to SIC could enhance clearance of *emm*1 *S. pyogenes*

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155 in the *ex vivo* whole blood assay, rabbit and human anti-SIC antibodies were
156 affinity purified from rabbit polyclonal serum and commercially-available pooled
157 human immunoglobulin (ivIg) respectively. ELISA and western blot analysis
158 confirmed that anti-SIC antibody from both humans and rabbits, that was affinity
159 purified using SIC1.300, was able to detect full length recombinant (r) SIC of
160 three different variants and also native SIC from *emm1 S. pyogenes* culture
161 supernatant, suggesting that immunogenicity was not restricted to a single SIC
162 variant (Supplementary Figure S2).
163
164 Purified rabbit and human anti-SIC antibodies were added to human whole
165 blood from healthy individuals and growth of *emm1 S. pyogenes* was assessed
166 over 3h using a modified Lancefield assay. Rabbit anti-SIC antibody reduced
167 growth of the *emm1* isolates H584 and AP1 compared to rabbit IgG isotype
168 control antibody (Figure 5A). In contrast, human anti-SIC IgG did not inhibit
169 growth of the *emm1* isolate H584 in whole human blood, compared to a control
170 antibody (Figure 5B). We further evaluated naturally occurring SIC antibodies
171 using a panel of human sera previously determined to have high anti-SIC titres [16].
172 There was no correlation between anti-SIC titre and bacterial growth inhibition
173 when heat-inactivated human serum from antenatal donors [16] was co-incubated
174 with *emm1 S. pyogenes* growing in human whole blood (Supplementary Figure
175 S3), further indicating that natural anti-SIC antibodies in human serum do not
176 promote opsonophagocytic killing of *S. pyogenes*. Together with the *in vivo* data
177 from mice, the findings indicated that immunisation of mice or rabbits with SIC
178 results in antibodies that have the potential to protect against *emm1 S. pyogenes*

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179 infection. In contrast, naturally occurring human anti-SIC antibodies lacked the
180 protective activity that was observed for vaccine-induced antibody.
181

182 **Human, rabbit and mouse anti-SIC antibodies detect different fragments of**
183 **SIC**

184 To determine the basis for the apparent difference between natural human anti-
185 SIC antibodies, and vaccine-induced rabbit or mouse anti-SIC antibodies, the
186 regions of SIC recognised by each of the anti-SIC antibodies were studied using
187 polypeptide fragments of SIC (fragments 1, 2 and 3), which are based on SIC from
188 the *emm*1 *S. pyogenes* strain AP1 (Supplementary Figure S4). Whilst purified
189 rabbit anti-SIC antibodies were able to recognise all three SIC fragments by an
190 ELISA-based assay, purified natural human anti-SIC was able to detect only
191 fragment 3, with limited detection of fragment 1 or fragment 2 (Figure 6A).
192 Serum from mice that had been immunised with full length rSIC1.300 for the
193 earlier infection challenge experiments, also detected all three SIC fragments
194 similar to findings in the rSIC1.300-immunised rabbit serum (Figure 6B). These
195 findings were confirmed by Western blot; rabbit and mouse anti SIC detected all
196 three fragments while human anti-SIC detected only fragment 3 (Figure 6C).
197 Furthermore, when the anti-SIC response was quantified from individual donor
198 human antenatal sera, the predominant response was against SIC fragment 3
199 (Figure 6D).
200

201 **Immunisation with SIC fragments does not protect against invasive disease**

202 To ascertain whether immunity to any single SIC fragment would be sufficient to
203 induce protective immunity, mice were immunised with recombinant SIC

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204 fragment 1, fragment 2, fragment 3 or a sham vaccine containing PBS and
205 adjuvant. Fragment 3 immunization was complicated by an unexpected
206 hypersensitivity reaction in 5/8 mice. Following immunisation, strong reactivity
207 was obtained against SIC fragment 1 and fragment 3 respectively with serum
208 from mice immunised with the homologous SIC fragment when analysed by
209 ELISA (Figure 7A) and western blotting (Figure 7B). No antibodies against SIC
210 fragment 2 were detected by either method.

211

212 Mice immunised with individual SIC fragments were challenged with 2 x 10^8 CFU
213 of *emm*1 *S. pyogenes* AP1 by the intramuscular route and the bacterial counts in
214 the organs and at the site of infection were quantified 24 hours after infection.
215 There was no significant difference in dissemination to the bloodstream, liver,
216 spleen or at the site of infection between any of the groups (Figure 7C). Thus, the
217 protection conferred against *S. pyogenes* by immunisation with full length rSIC
218 could not be recapitulated by any single SIC domain.

219

220 **Discussion**

221 Antibodies against SIC are widespread in populations worldwide [14-16], although
222 the role that these antibodies play in protection against infection with *S.*
223 *pyogenes* remains unclear. In this present study, we have demonstrated that SIC
224 is expressed ubiquitously by invasive and non-invasive *emm1* bacterial strains *in*
225 *vitro* and we have quantified SIC production *in vivo*. Immunisation of animals
226 with full length SIC provided protection against systemic bacterial dissemination.
227 In contrast to vaccine-induced immunity, natural human immunity to SIC is

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228 directed against only one domain of SIC, and this is insufficient to confer
229 immunity.
230
231 Multiple functions have been attributed to SIC which all appear to aid bacterial
232 evasion of host innate immunity [6,8,9]. The upregulation of *sic* by invasive *emm1*
233 isolates that had undergone a mutation in the bacterial two-component regulator
234 *covRS* [11] suggested that SIC may contribute to *S. pyogenes* invasiveness as part of
235 the *covRS* regulon. Whilst previous studies have examined the role of the
236 variation in sequence and size of SIC [17-21], we instead hypothesised that variation
237 in SIC expression levels would reflect the invasive phenotype of a strain. SIC
238 expression by single bacterial isolates has been quantified in two separate
239 reports [8,9], however, to our knowledge this is the first report to examine SIC
240 expression in a wider collection of invasive and non-invasive *emm*1 GAS clinical
241 isolates. Although SIC expression levels varied widely, levels were not overall
242 significantly higher amongst invasive isolates. Of note, invasive isolates
243 represented almost two thirds of the strains investigated and it is likely that only
244 some have mutations in covRS. Among a subset of isolates for which covRS
245 sequencing was undertaken we did however observe significantly heightened
246 expression of SIC. We have, for the first time, also demonstrated that SIC is
247 detectable in infected murine thigh tissue, which was the site of infection, in
248 infected animals. We attempted to detect SIC in muscle from a patient with
249 necrotising myositis caused by *emm*1 *S. pyogenes*, however samples were
250 obtained after treatment with intravenous immunoglobulin, and showed too
251 much cross reactivity with reagents used, precluding detection of SIC (data not
252 shown). Recently SIC was one of just 15 *S. pyogenes* proteins detected in pleural

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253 fluid from a child with empyema caused by *emm*1 *S. pyogenes*, underlining the
254 potential for virulence factors such as SIC to be upregulated during *in vivo*
255 infection.
256
257 Antibodies against SIC are widespread and persistent amongst human
258 populations in geographically distinct regions [14-16]. Intriguingly, anti-SIC
259 antibodies are identified more frequently than antibodies to M1 protein (14). We
260 set out to assess whether immunisation with one SIC variant (SIC1.300) would
261 be protective *in vivo* in two mouse models of infection. rSIC immunised mice had
262 increased survival compared to sham-immunised mice following respiratory
263 tract infection. Whilst there was no difference in bacterial counts in the nasal
264 tissue and lungs, immunity to SIC appeared to impact bacterial dissemination
265 beyond the site of infection; the observation that rSIC immunisation also reduced
266 bacterial dissemination from intramuscular infection provided further evidence
267 to support this.
268
269 The ability of antibodies raised against full length SIC to enhance clearance of *S.*
270 *pyogenes* in whole blood and in the mouse models is likely to reflect inhibition of
271 SIC function rather than any opsonic activity, since we found little convincing
272 evidence of surface-localised SIC. The data are consistent with previous work
273 demonstrating the role of SIC *in vivo*: SIC enhanced virulence in both
274 intraperitoneal [12] and subcutaneous mouse models of GAS infection [9,22]. By
275 immunising mice with SIC and demonstrating reduced bacterial dissemination
276 following infectious challenge, our data suggest an important role for SIC in

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277 *emm*1 *S. pyogenes* pathogenesis and, based on observations in the whole blood
278 model, a potential role in resistance to opsonophagocytic killing.
279
280 Whilst human anti-SIC antibodies are abundant in populations, our data suggests
281 that these antibodies are not protective. One possible explanation for this is that
282 following immunisation with full length SIC, protective antibodies are raised to
283 epitopes throughout the mature SIC protein, however, following natural infection,
284 antibodies are only generated against epitopes within fragment 3 of SIC. A
285 previous study using sera from 29 individuals, identified ten linear epitopes in
286 SIC1.01 that were identified by ≥ 50% of the human anti-SIC sera. These
287 epitopes were at sites in SIC in which polymorphisms commonly occur, and were
288 evenly distributed within the equivalent of SIC fragments 1, 2 and 3 [14]. When
289 this was further analysed by phage display using two sera to detect natively
290 folded SIC peptides, 7/8 peptides recognised by one serum and 11/12 peptides
291 recognised by the other serum spanned regions present in the equivalent of SIC
292 fragment 3 (from residue 168 onwards). Our data using ELISA and western blot
293 confirm that epitopes within fragment 3 are the most readily recognised by
294 human anti-SIC that had been purified from ivIg pooled from over 1,000 donors
295 [23]
296
297 The reasons that human anti-SIC antibody responses are directed to a single
298 domain only are unclear. One possibility is that anti-SIC responses represent
299 cross-reactive responses to another, unrelated antigen, that has structural
300 similarity to fragment 3. Notably, SIC readily undergoes proteolysis by human
301 proteases such as human neutrophil elastase and bacterial proteases such as

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302 SpeB [14,24]. The digestion of SIC by unknown bacterial or host factors within
303 human saliva [25] provides an alternative mechanism by which SIC fragments,
304 rather than full length SIC, might be presented to the immune system.
305
306 The lack of protection following immunisation with separate SIC fragments was
307 surprising, especially considering that several immune inhibitory functions of
308 SIC have been localised to the SRR and LRR contained within SIC fragments 1 and
309 2 respectively [5]. Indeed, immunisation with SIC fragment 2 elicited minimal
310 antibody response, despite epitopes in this fragment being detected with
311 antibodies raised against full length SIC, and also human anti-SIC serum in a
312 previous study [14], suggesting that this fragment may form part of a discontinuous
313 epitope. Data generated using various biophysical methods indicate that SIC
314 contains low levels of regular secondary and tertiary structures (unpublished
315 data); this could mean that discontinous epitopes form long range contacts
316 resulting in either stable or dynamic tertiary structure assemblies. Resolution of
317 the complete folded structure of SIC would provide a better understanding of the
318 nature of these epitopes. Interestingly, few immune inhibitory functions of SIC
319 have been localised to the PRR of SIC contained within fragment 3. Thus, an
320 alternative explanation to the varying immunogenicity of SIC domains is that, in
321 humans, SIC fragments 1 and 2 bind to host ligands concealing these regions
322 from the host, whilst epitopes in SIC fragment 3 are abundantly available. Whilst
323 SIC binds to both human LL-37 and mouse cathelicidin [12], previous studies
324 assessing other ligands of SIC have used only human proteins [5,8,9,22,24]. It
325 remains unclear whether SIC binds to other mouse proteins and hence the lack of

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326 immunogenicity of fragment 2 compared to fragment 1 may be due to
327 differential binding to mouse proteins.
328
329 At first glance, a vaccine based on an antigen found in only one serotype, does
330 not seem an attractive proposition. However, in light of difficulties in developing
331 an effective GAS vaccine over the last 70 years, and the dominance of the *emm*1
332 lineage, alternative approaches must be considered. The successful introduction
333 of multi-component vaccines for other bacterial infections indicates that the
334 inclusion of multiple novel antigens in a vaccine may be important. Additionally,
335 antibodies directed against single virulence factors are being developed as
336 adjunctive therapy for a number of serious diseases caused by other bacteria.
337 Our data suggest that the inclusion of SIC in a multi-component vaccine has
338 potential to reduce the burden of disease caused by highly invasive *emm*1 *S.*
339 *pyogenes* and goes someway to explain the anomaly of widespread anti-SIC
340 immunoreactivity in an otherwise susceptible population.
341
342

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343 **Materials and Methods**

344

345 **Bacterial strains**

346 *S. pyogenes emm*1 strain H584 was isolated from a case of lethal postpartum
347 sepsis [16]. An additional 100 *emm*1 clinical *S. pyogenes* isolates from the 1930s
348 through to 2013 were referred to Imperial College from diagnostic laboratories,
349 linked to available clinical data, and anonymised as approved by the local
350 research ethics committee (06/Q0406/20). 68 isolates were from invasive
351 disease and 32 were from non-invasive disease. Specific disease etiologies were
352 not known for 13 isolates. *S. pyogenes* isolates were identified as *emm1* via
353 sequencing of the *emm* gene, which encodes the M protein
354 (https://www2a.cdc.gov/ncidod/biotech/strepblast.asp). Invasive disease-
355 associated isolates were defined as *S. pyogenes* isolated from a normally sterile
356 site or a non-sterile site with clinical diagnosis of necrotising fasciitis or septic
357 shock. Non-invasive disease-associated isolates were defined as *S. pyogenes*
358 isolated from non-sterile sites with no clinical signs indicating severe/invasive
359 disease. *S. pyogenes emm*1 strain AP1 and SIC-, a mutant derivative not
360 expressing SIC, were used in experiments where a SIC negative *emm1* isolate was
361 required and have been described previously [9].

362

363 All streptococcal strains were cultured on Columbia horse blood agar (CBA)
364 (E&O Laboratories Ltd, Bonnybridge, Scotland) or in Todd-Hewitt broth (THB)
365 (Oxoid, Basingstoke, UK) at 37 °C, 5% CO_2 without shaking. *Escherichia coli* were
366 cultured in Luria-Bertani broth (Oxoid) with 100 µg/ml ampicillin (Sigma–
367 Aldrich, Dorset, UK).

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368

369 **Recombinant SIC proteins**

370 Recombinant full length SIC proteins were purified using affinity
371 chromatography with a His-bind column as per manufacturer's instructions
372 (Novagen, Merck, Darmstadt, Germany). *sic*1.300, from *emm*1 strain H584,
373 *sic*1.02 and *sic*1.301, used in initial validation experiments were expressed in *E.*
374 *coli* using the pET19b expression vector (Novagen, Nottingham, UK), as
375 previously described [16]. Recombinant SIC fragment 1 (amino acids 1-69),
376 fragment 2 (amino acids 70-167) and fragment 3 (amino acids 168-278) were
377 based on the originally published *sic* sequence of the *emm*1 strain AP1 [4] and
378 were expressed in *E. coli*, purified by nickel affinity chromatography, processed
379 with TEV protease to remove the His-tag, purified by reversed phase
380 chromatography and lyophilized.

381

382 **Murine immunisation and infection challenge**

383 Female six to eight-week-old FVB/n mice (Charles River, Margate, UK) were
384 immunised intramuscularly with 30 µg of recombinant SIC protein or
385 recombinant SIC fragments 1, 2 and 3 emulsified 1:1 in Freund's incomplete
386 adjuvant (Sigma–Aldrich) on days 0, 21 and 35; sera were collected by tail bleed
387 on day 39-41. A control group of mice were immunised with phosphate buffered
388 saline (PBS) and adjuvant (sham vaccination). On day 42-45, mice were infected
389 with *emm*1 *S. pyogenes* strain H584 after full length SIC immunisation, or *S.*
390 *pyogenes* strain AP1 following SIC fragment immunisation, (as SIC fragments
391 were derived from SIC AP1). For respiratory tract infection, two invasive disease
392 isolates were used. Mice were briefly anaesthetised with isoflurane and 2 x 10^7

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393 CFU or 2 x 10^8 CFU of *S. pyogenes* were administered by inhalation (5 µl of
394 bacterial suspension per nostril). Mice were monitored for seven days and any
395 mice reaching defined humane endpoints were euthanized. For intramuscular
396 infection, mice received 5 x 10^7 CFU of GAS directly into thigh muscle. In some
397 experiments mice were euthanized (after 48 hours for intranasal infection and
398 24 hours for intramuscular infection) and blood and tissue (homogenised in
399 PBS) were taken for culture.

400

401 **Purification of antibodies from serum or human intravenous**
402 **immunoglobulin**

403 Rabbit anti-SIC polyclonal serum was raised against recombinant SIC1.300. To
404 purify anti-SIC antibodies from rabbit anti-SIC serum or human pooled
405 intravenous immunoglobulin ((ivIg), Privigen immune globulin, CSL Behring, PA,
406 USA)) recombinant SIC1.300 (1 mg/ml) in coupling buffer (0.1M sodium
407 bicarbonate, 0.5M sodium chloride, pH8.3) was applied to a chromatography
408 column containing CnBr activated agarose (Sigma-Aldrich) at room temperature
409 for 2 hours. Following washing with coupling buffer, 0.2M glycine was applied at
410 room temperature for 2 hours. The SIC-CnBr resin was then washed extensively
411 by alternating between coupling buffer and 0.1M acetate buffer containing 0.5M
412 sodium chloride, pH4. The resin was equlibrated in Tris buffered saline 1
413 [(TBS1), 50mM Tris, 150mM NaCl, pH 7.5)], then either serum from a rabbit
414 immunised with SIC1.300 or human pooled ivIg was applied to the SIC-CnBr
415 resin. The resin was then washed extensively with TBS1 and Tris buffered saline
416 2 [(TBS2), 20mM Tris, 2M NaCl, pH7.5). Purified antibodies against SIC were
417 eluted in fractions from the resin using 0.2M glycine-HCl, pH2.2 and neutralised

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418 with 1M Tris-HCl, pH 8.0. Fractions containing purified anti-SIC antibodies were
419 pooled and dialysed into PBS overnight at 4°C. Antibody concentrations were
420 determined using the Coomassie-Bradford assay. From 12 ml of rabbit anti-SIC
421 serum, approximately 2 ml of purified rabbit anti-SIC antibody (0.5mg/ml) was
422 obtained, and from 20 ml of human pooled ivIg, approximately 0.5 ml of purified
423 human anti-SIC antibody (0.1mg/ml) was obtained.
424
425 **Human whole blood phagocytosis assay**
426 Lancefield whole blood assays were performed to assess the protective effect of
427 rabbit and human anti-SIC antibodies *ex vivo*. Approximately 50 CFU of *emm*1 *S.*
428 *pyogenes* strain H584 or AP1 were inoculated into heparinized human whole
429 blood obtained from healthy donors as described [26]. Mixtures of whole blood,
430 bacteria and anti-SIC were incubated for 3 hours at 37°C with end-over-end
431 rotation. Bacterial survival was quantified as the multiplication factor of number
432 of surviving colonies relative to the starting inoculum and tested in triplicate. To
433 assess the effect of rabbit anti-SIC, purified rabbit anti-SIC antibodies were added
434 to the whole blood. Rabbit IgG isotype control antibody (ab176094, Abcam,
435 Cambridge, UK) was used as a control. For separate studies with H584 using
436 human anti-SIC, either serum from 79 healthy antenatal patients or purified
437 human anti-SIC from human pooled ivIg were added to the whole blood.
438 Antenatal sera were previously used and tested for anti-SIC1.300 titres [16]. A
439 human IgG isotype control (Novus biological, CO, USA) was used as a control.
440
441 **ELISA-based assay**

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442 To assess the antibody response of immunised mice, 96-well polystyrene plates
443 (Nunc, ThermoScientific, MA, USA) were coated with 100 ng of full length
444 SIC1.300 or SIC fragments 1, 2 or 3 overnight at 4°C, washed, blocked for one
445 hour with 3% normal goat serum (Sigma-Aldrich) diluted in PBS-0.1% Tween20
446 (PBST). Test sera were then added at a range of dilutions. Binding was detected
447 using HRP-conjugated goat anti-mouse IgG (Abcam) and incubated for one hour
448 at room temperature. The substrate (ONPG, Sigma-Aldrich) was added to wells,
449 the reaction was stopped with 3N HCl, and the OD A_{492} read with a µQuant
450 spectrophotometer (Biotek, VT, USA). To compare cross detection of
451 recombinant SIC variants or SIC fragments by rabbit polyclonal anti-SIC1.300
452 serum or purified SIC antibody, plates were coated with SIC1.02, SIC1.301 and
453 SIC1.300 or SIC fragments 1,2 and 3 and binding was detected using 1 in 25,000
454 dilution of HRP-conjugated goat anti-rabbit IgG (Life Technologies, Paisley, UK).
455 To determine detection of SIC fragments by human purified anti-SIC antibody or
456 antenatal serum, plates were coated with SIC1.300 or SIC fragments 1, 2 and 3, and
457 binding detected using 1 in 25,000 dilution of HRP-conjugated goat anti-human
458 IgG (Sigma-Aldrich).
459
460 **SDS-PAGE and Western blot**
461 Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel
462 electrophoresis (SDS-PAGE) using NUPAGE 10% Bis-Tris Gels (Life Technologies),
463 run in NUPAGE MES Running Buffer (Life Technologies). Following SDS-PAGE,
464 proteins were transferred onto PVDF membranes (Amersham Hybond-LFP
465 membranes [GE Healthcare, Buckinghamshire, UK]), or nitrocellulose membranes
466 (Amersham Protran, GE Healthcare) using cold tris-glycine transfer buffer (0.025M

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467 Tris, 0.2M Glycine). For quantification of SIC and cross detection of SIC variants,
468 following blocking in PBST with 5% skimmed milk powder, SIC was detected by
469 probing with 1 in 10,000 dilution of rabbit polyclonal anti-SIC1.300 for two hours at
470 room temperature. Proteins were visualized by incubation in 1 in 50,000 dilution of
471 HRP-conjugated goat anti-rabbit IgG (Life Technologies) for one hour followed by
472 Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) exposed
473 to Amersham Hyperfilm ECL (GE Healthcare). For cross detection of SIC variants
474 using purified human anti-SIC, membranes were probed with 1 in 10,000 dilution of
475 human anti-SIC followed by 1 in 50,000 dilution of HRP-conjugated goat anti-human
476 IgG (Sigma-Aldrich). To directly compare detection of SIC fragments by purified
477 human and rabbit anti-SIC antibodies, purified antibodies were first diluted to the
478 same concentration (0.1mg/ml in PBS), then used at 1 in 1,000 dilution to probe
479 membranes, followed by secondary antibodies as outlined. Finally, for detection of
480 SIC fragments by mouse anti-SIC fragment 1, 2 and 3, serum from groups of mice
481 immunised with each fragment was pooled and used to probe membranes at 1 in 100
482 dilution, followed by 1 in 50,000 dilution of HRP-conjugated goat anti-mouse IgG
483 (Abcam). Full, unmodified western blots for Figures1C, 6C and 7B are displayed in
484 separate Supplementary materials.
485

486 **Quantification of SIC expression in culture supernatant and *in vivo***

487 GAS strains were grown overnight in THB at 37°C with 5% CO2. Cultures were
488 then centrifuged at 2,500 x g for 10 min at 4°C and proteins from the supernatant
489 were precipitated using 10% tricholoroacetic acid (TCA) with acetone (Sigma-
490 Aldrich). 1,700 µl of TCA-acetone was added to 300 µl of culture supernatant and
491 incubated at -20°C for one hour. Samples were washed twice with ice-cold

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492 acetone and allowed to dry before being re-suspended in 30 µl of sample
493 treatment buffer [Lithium Dodecyl Sulphate Sample Buffer (Life Technologies);
494 100mM DL-Dithiothreitol, (Sigma-Aldrich)], heated at 70°C for 10 minutes, and
495 used for Western blot analysis.
496
497 For *in vivo* determination of SIC, female six to eight-week old FVB/n mice
498 (Charles River, Margate, UK) were infected intramuscularly with either 5 x 10^7
499 CFU of *emm*1 *S. pyogenes* strains AP1 or SIC- and culled 3 hours after infection.
500 Infected thigh muscle was homogenised in PBS and centrifuged at 16,000 xg for
501 five minutes. The supernatant was added to sample treatment buffer, heated at
502 70°C for 10 minutes, and used for Western blot analysis. Serial dilutions of
503 known quantities of SIC1.300 were included on each gel to generate the standard
504 curve, and densitometry was undertaken with Image J software (National
505 Institutes of Health, MD, USA) enabling pixel values to be converted to
506 concentrations.
507
508 **Statistical Analyses**
509 Non-parametric tests were used for comparisons and were performed with
510 GraphPad Prism 6.0 software (GraphPad Software, CA, USA). Samples sizes were
511 selected based on pilot studies or previous studies [27]. No randomisation or
512 blinding was performed. All animal procedures were approved by the local
513 ethical review process and conducted in accordance with the relevant, UK Home
514 Office approved, project license (PPL70/7379).
515
516

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517 **Study approval**

518 The analysis of anonymised samples and bacteria from patients with suspected
519 infection was approved by an NHS Research Ethics Committee (REC reference
520 06/Q0406/20). Human blood cells from normal donors was obtained following
521 informed consent from a sub-collection of the Imperial College Healthcare NHS
522 Trust Tissue Bank. All animal procedures were conducted in accordance with UK
523 Home Office guidance and approval.

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530

531

532 **Competing Interest Statement.** The authors have declared that no conflict of
533 interest exists

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534 **Authors' contributions**

535 LKKT, MR, LB and SS conceived the study. LKKT and MR analysed the data and
536 wrote the manuscript. LKKT, MR, DT, NR, VN, LEML, CT, IMF and MW performed
537 the experiments. LKKT and MR prepared the Figures. All authors reviewed and
538 approved the final version of the manuscript.
539

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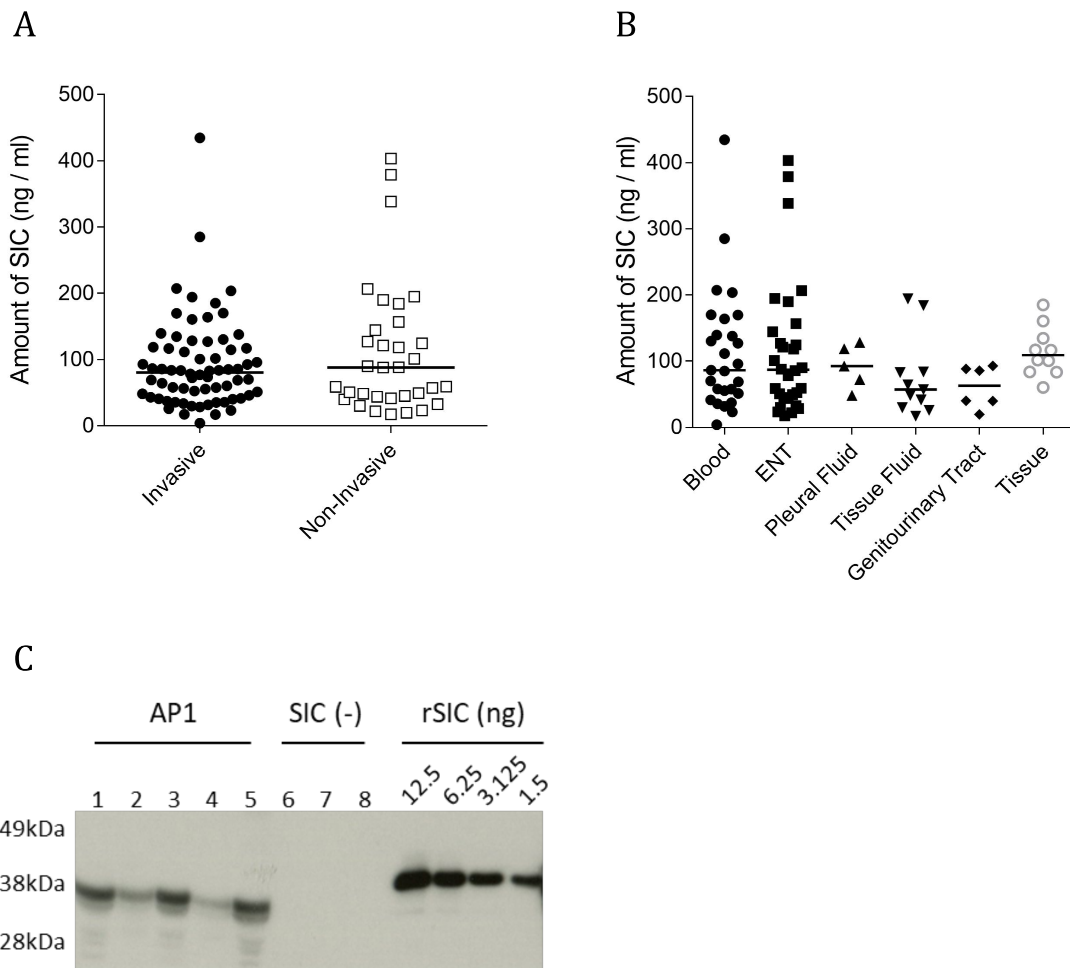

637
638

639 **Figure 1. Quantification of *in vitro* and *in vivo* SIC production**

640 A-B) The concentration of SIC in overnight culture supernatants from 101 *emm1*
641 *S. pyogenes* clinical isolates grouped by A) invasive vs non-invasive disease
642 phenotype or B) site of isolation was quantified. (C) SIC was quantified in the
643 thigh tissue of mice following a 3 h intramuscular infection with the *emm1* GAS
644 isolate AP1 (5 mice, lanes 1-5) or a SIC-negative AP1 derivative (3 mice, lanes 6-
645 8). Quantifications were performed by Western blotting and densitometry using
646 a recombinant SIC (rSIC) standard ranging from 12.5 ng to 1.56 ng per well .
647
648

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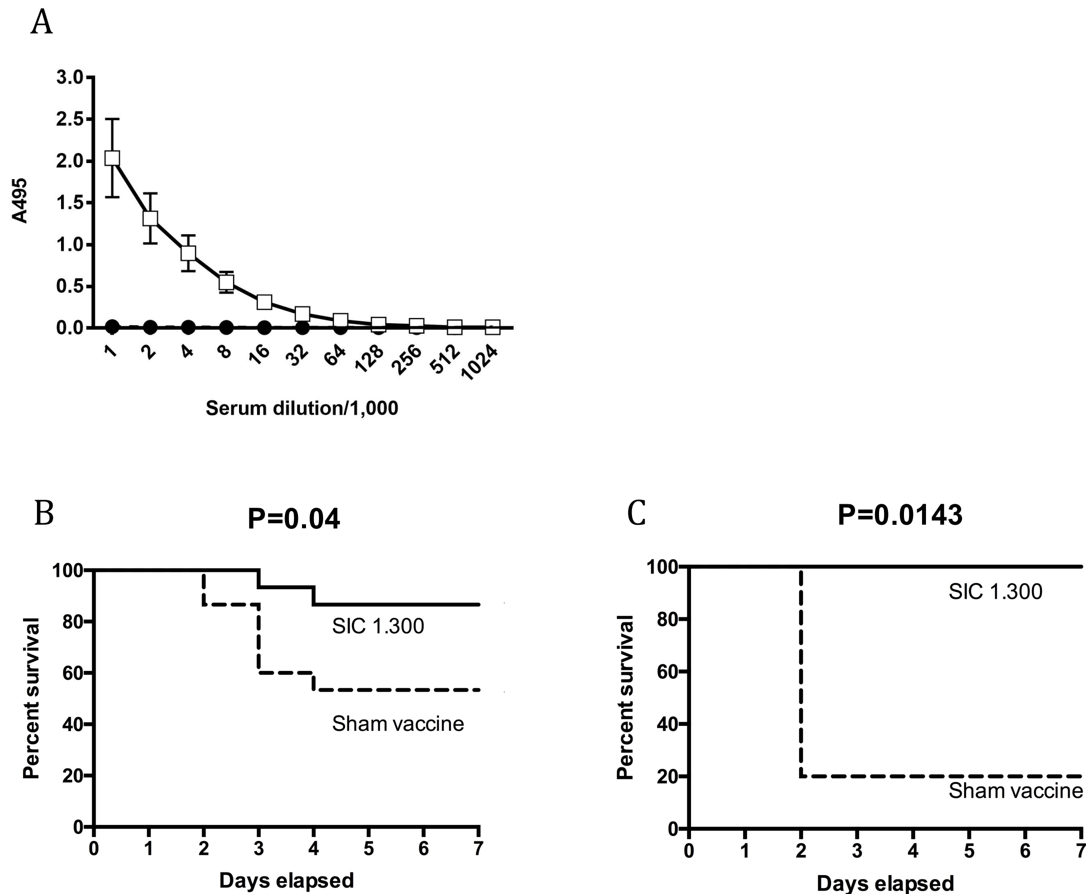

649
650
651 **Figure 2. SIC 1.300 vaccination is immunogenic and induces protective**
652 **response against lower respiratory tract infection**
653 A) Serum was obtained from mice on day 39 post-immunization with rSIC1.300
654 (open squares) or PBS (closed circles) and SIC1.300-specific IgG was measured
655 by ELISA. Data were obtained from 10 mice per group, over three vaccination
656 experiments. (B-C) FvB/n mice immunized with SIC 1.300 (solid line) or sham
657 vaccinated (dashed line) were infected intranasally with B) $2x10^7$ CFU (n=15,
658 pooled data from two independent experiments) or (C) $2x10^8$ CFU (n=5) of the
659 *emm1 S. pyogenes* isolate H584 and culled when experimental endpoints were
660 reached. Survival was compared using the log-rank test.

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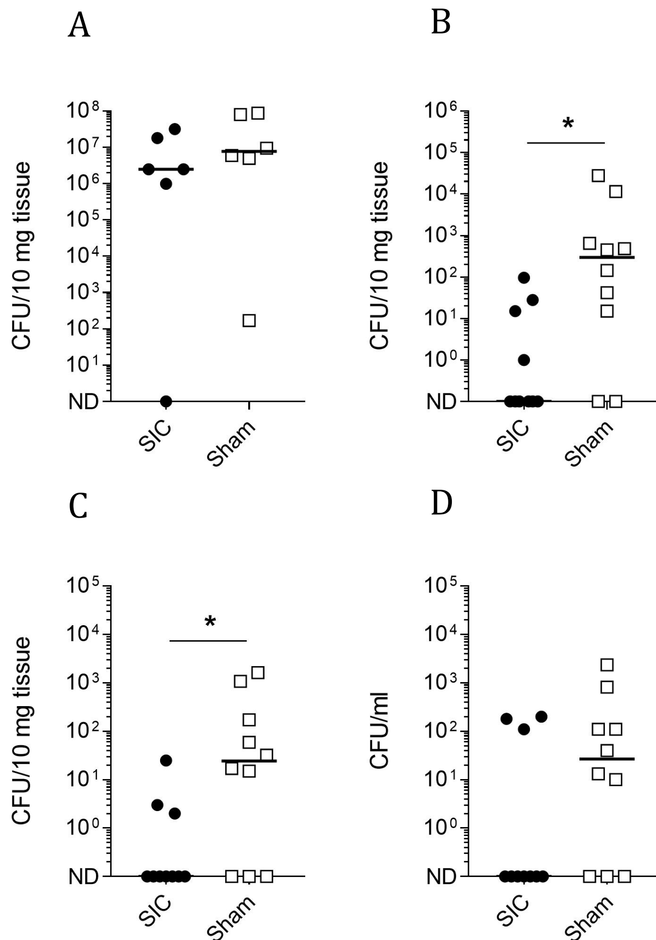

661

662 **Figure 3. SIC 1:300 vaccination prevents *S. pyogenes* dissemination from an**
663 **respiratory tract focus of infection.**

664 FvB/n mice immunised with SIC 1.300 (n=10) or sham vaccine (n=10) were
665 infected i.n. with $5x10^7$ CFU of the *emm1 S. pyogenes* isolate H584. Mice were
666 culled 48 h post-infection and bacterial loads within the nose (A), spleen (B),
667 liver (C) and blood (D) were enumerated. For the nasal tissue, bacterial
668 enumeration was only performed on 6 mice per group. Solid lines indicate the
669 median CFU recovered from each organ. *p < 0.05 one-tailed Mann–Whitney U.
670 ND: Not Detected.

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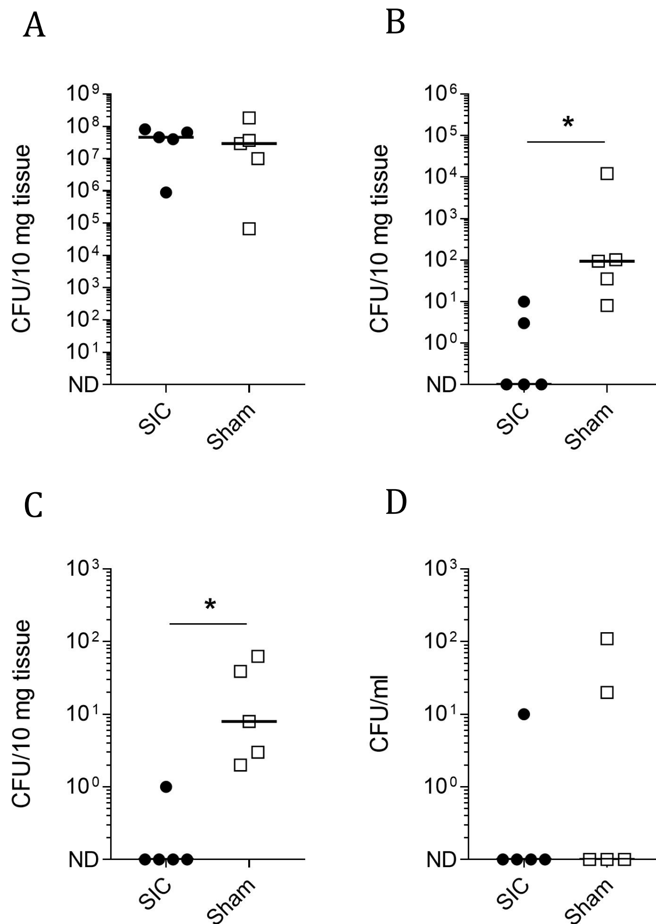

671

672 **Figure 4. SIC 1:300 vaccination prevents *S. pyogenes* dissemination from an**
673 **intramuscular focus of infection.**

674 FvB/n mice immunised with SIC 1.300 (n=5) or sham vaccine (n=5) were
675 infected I.M. with $5x10^7$ CFU of the *emm1 S. pyogenes* isolate H584. Mice were
676 culled 24 h post-infection and bacterial loads within the thigh muscle (A), spleen
677 (B), liver (C) and blood (D) were enumerated. Solid lines indicate the median
678 CFU recovered from each organ. *$p < 0.05$ one-tailed Mann–Whitney U. ND: Not
679 Detected.

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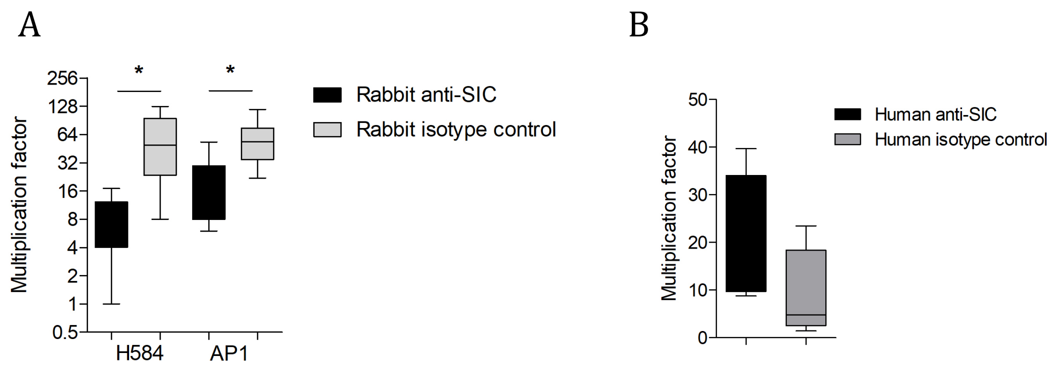

680
681
682 **Figure 5. Rabbit but not human anti-SIC antibodies are protective in *ex-vivo***
683 **whole blood assay.**
684 A) The *emm1 S. pyogenes* isolates H584 and AP1 were grown in human whole
685 blood with the addition of affinity purified rabbit anti-SIC 1.300 polyclonal
686 antibodies (black bars) or rabbit IgG isotype control antibodies (grey bars).
687 Bacterial growth (multiplication factor) was determined after rotation at 37°C
688 for 3 hours. Median and range shown for two separate experiments with three
689 different donors. *$p < 0.05$ Wilcoxon matched-pairs signed rank test. B) The
690 *emm1 S. pyogenes* isolate H584 was grown in human whole blood with the
691 addition of purified human anti-SIC antibodies (black bars) or human isotype
692 control antibodies (grey bars). Bacterial growth (multiplication factor) was
693 determined after rotation at 37°C for 3 hours. Mean and standard deviation
694 shown for experiment repeated in triplicate from one donor.

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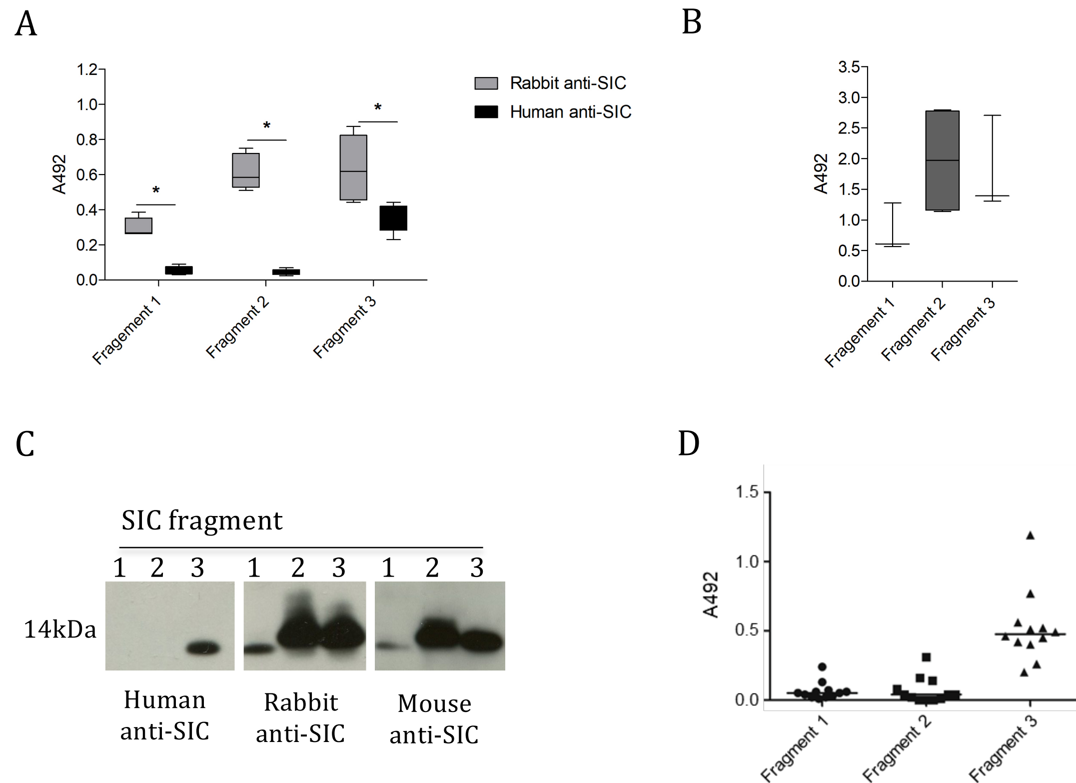

695

696

697 **Figure. 6. Rabbit and mouse but not human anti-SIC antibodies recognise**
698 **all three SIC fragments**
699 A-B) Immobilised recombinant SIC fragments 1, 2 and 3 were incubated with A)
700 0.1mg / ml of affinity purified human anti-SIC or rabbit anti-SIC1.300 antibodies,
701 or B) a 1:100 dilution of pooled serum from mice immunised with full length
702 SIC1.300. Mean and standard deviation shown of ELISAs repeated at least twice.
703 C) Equal quantities of recombinant SIC fragments 1, 2 and 3 were visualized by
704 western blotting using 0.1mg/ml of human anti-SIC or rabbit anti-SIC antibodies,
705 or a 1:250 dilution pooled serum from mice immunised with full length SIC1.300.
706 (D) Immobilised recombinant SIC fragments 1, 2 and 3 were incubated with a
707 1:100 dilution of heat inactivated sera from individual antenatal donors in which
708 anti-SIC 1.300 titers had been determined previously. Data points represent
709 mean A492 readings from two independent experiments and solid lines indicate
710 the median.

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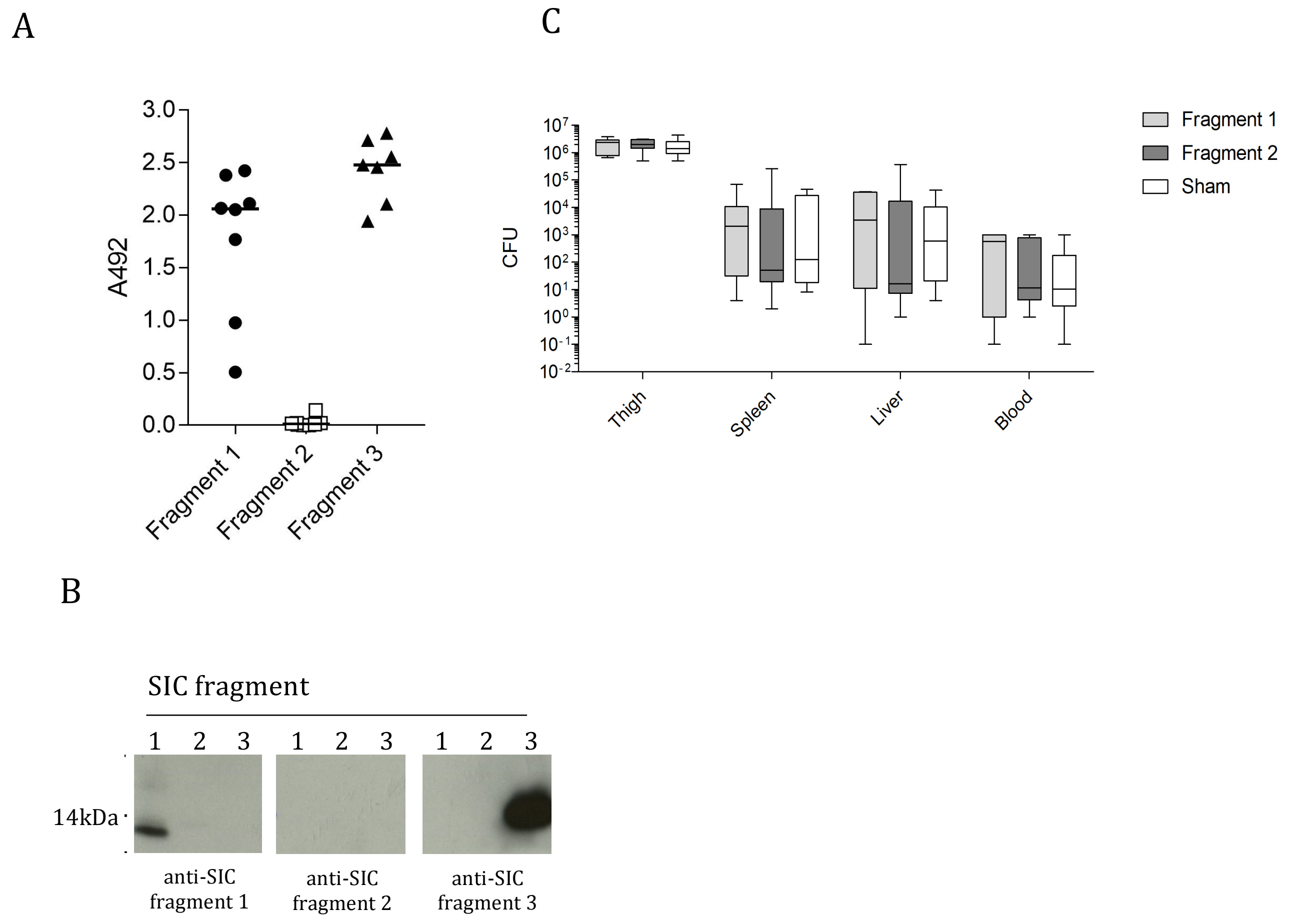

713 B
714
715 SIC fragment
716 1 2 3 1 2 3 1 2 3
717 14kDa
718 anti-SIC fragment 1 anti-SIC fragment 2 anti-SIC fragment 3
719

720 **Figure 7. SIC fragments are variably immunogenic and non-protective**

721 A) Sera were obtained from individual mice (n=8) immunized with recombinant
722 SIC fragments 1, 2 or 3 and SIC fragment specific IgG was measured by ELISA.
723 Data points represent mean A492 readings from individual mice obtained in two
724 independent experiments and solid lines indicate the median. B) Equal quantities
725 of recombinant SIC fragments 1, 2 and 3 were transferred to a membrane and
726 incubated in in 1:100 dilution of mouse anti-SIC fragment 1, 2 or 3 antiserum. C)
727 FvB/n mice immunised with SIC fragment 1, fragment 2 or sham vaccine (n=8)
728 were infected intramuscularly with 2 x 10^8 CFU of the *emm1 S. pyogenes* isolate.
729 Mice were culled 24 h post-infection and bacterial loads within the thigh muscle
730 spleen, liver and blood were enumerated. Data are displayed as CFU/10 mg
731 tissue (thigh, spleen and liver) or CFU/ml (blood) (median and range).
732
733

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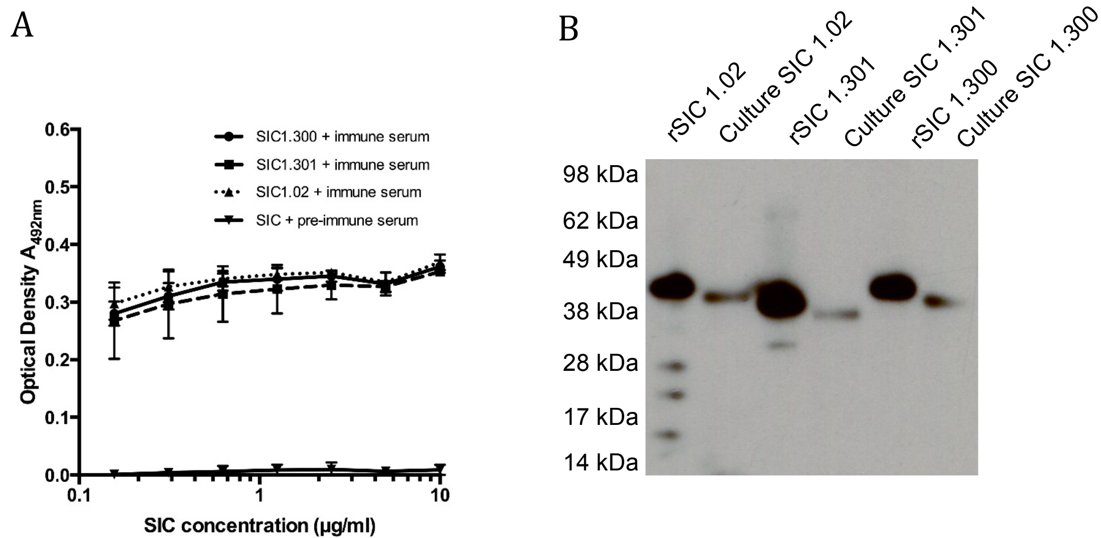
734 **Supplementary figures**

735



736

737 **Supplementary figure S1. Rabbit anti-SIC1.300 cross detects other SIC**

738 **variants**

739 (A) Increasing concentrations of different recombinant (r)SIC variants (rSIC1.02

740 dotted line, triangles; rSIC1.300 solid line, circles; and rSIC1.301 dashed line,

741 squares) were bound to ELISA wells and incubated with 1:10,000 dilution rabbit

742 anti-SIC1.300 serum. Each recombinant SIC variant bound to ELISA wells was

743 also separately incubated in pre-immune rabbit serum (triangles). Mean and

744 standard deviation shown of experimental triplicates. (B) Recombinant SIC

745 variants rSIC1.02, 1.300,1.301 and proteins from concentrated culture

746 supernatant of *S. pyogenes* isolates naturally expressing the same SIC variants

747 were transferred to a membrane and incubated with 1:10,000 dilution of rabbit

748 anti-SIC1.300. SIC1.301 differs from SIC1.300 by a 29 amino acid deletion in

749 SIC1.301, within the long repeat region. SIC1.02 is the common SIC allele in the

750 UK, and differs from both SIC1.300 and SIC1.301 by a 5 amino acid insertion and

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751 an amino acid substitution of glutamine (Q) to lysine (K) in the short repeat

752 region [16].

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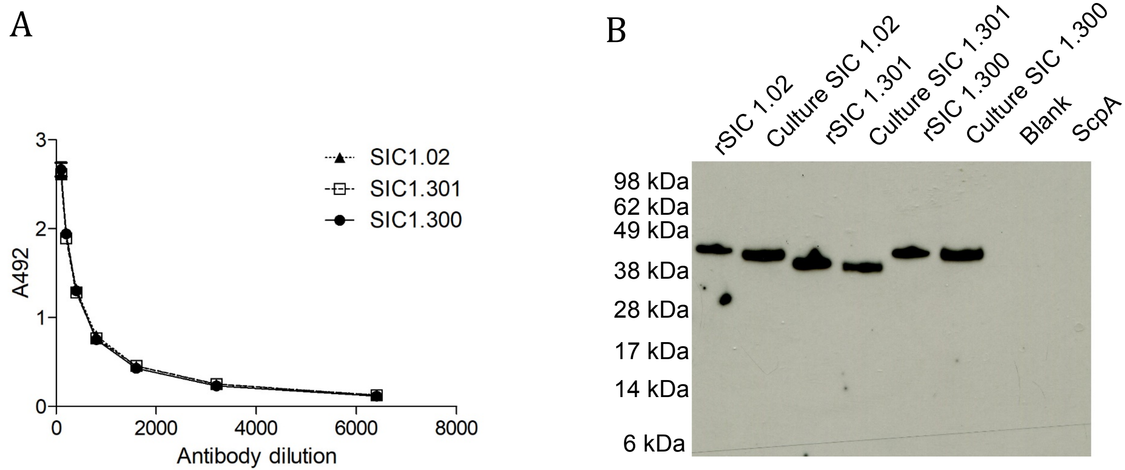

753

754 **Supplementary figure S2. Purified human anti-SIC cross-detects SIC**
755 **variants**

756 (A) Affinity purified human anti-SIC antibodies were obtained from pooled
757 human intravenous immunoglobulin and SIC-specific IgG against rSIC1.02
758 (dotted line, triangles), rSIC1.300 (solid line, circles), rSIC1.301 (dashed line,
759 squares) was measured by ELISA. Data show the mean and standard deviation
760 from experimental triplicate.

761 (B) Recombinant SIC variants (25 ng) rSIC1.02, rSIC 1.300, rSIC 1.301, and
762 proteins from concentrated culture supernatant (10 µl) of *S. pyogenes* isolates
763 naturally expressing the same SIC variants were transferred to a membrane and
764 incubated in 1:1,000 dilution of purified human anti-SIC antibodies.
765 Recombinant streptococcal protein ScpA acted as a negative control.

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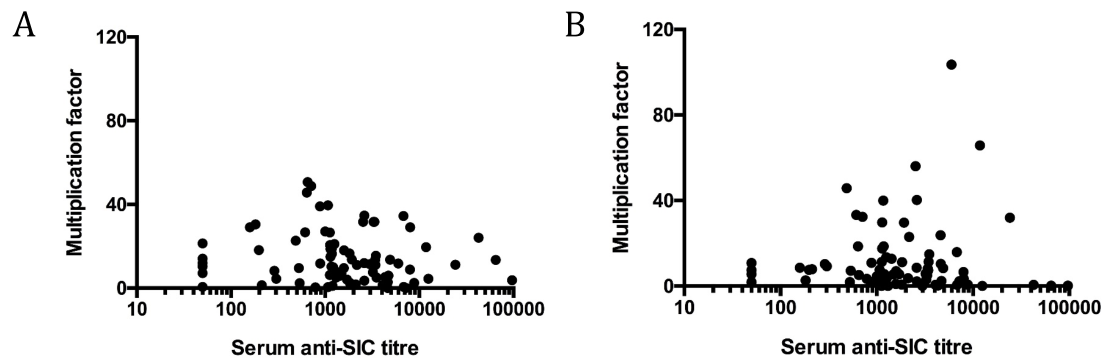

766

767 **Supplementary figure S3. Lack of correlation between human serum anti-**
768 **SIC levels and bacterial killing**

769 *S. pyogenes emm*1 strain H584 was grown in human whole blood from two
770 different donors (A) and (B) co-incubated with individual heat inactivated sera
771 from antenatal donors (n=79) in which anti-SIC 1.300 titres had been previously
772 determined. Bacterial growth (multiplication factor) was analysed after rotation
773 at 37°C for 3 hours and correlated with anti-SIC serum titres. There was no
774 correlation between anti-SIC titres and bacterial growth (Spearman rank
775 coefficient) for donor A (p=0.120) or donor B (p=0.2487).

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```
CLUSTAL O(1.2.4) multiple sequence alignment

                Ss                                      SRR    Fragment 1
Fragments       ------------------------------GMETYTSRNFDWSGDDWPEDDWSGDGLSKY  30
AP1             MNIRNKIENSKTLLFTSLVAVALLGATQPVSAETYTSRNFDWSGDDWPEDDWSGDGLSKY  60
SIC1.300        MNIRNKIENSKTLLFTSLVAVALLGATQPVSAETYTSRNFDWSGDDWPEDDWSGDGLSKY  60
                              . ****************************

                         Fragment 1                             LRR Fragment 2
Fragments       DRSGVGLSQYGWSQYGWSSDKEEWPEDWPEDDWSSDKKDETEDSMEDKTRPPYGEALGTG  90
AP1             DRSGVGLSQYGWSQYGWSSDKEEWPEDWPEDDWSSDKKDET----EDKTRPPYGEALGTG  116
SIC1.300        DRSGVGLSQYGWSQYGWSSDKEEWPEDWPEDDWSSDKKDET----EDKTRPPYGEALGTG  116
                *****************************************    ***************

                                 Fragment 2
Fragments       YEKRDDWGGPGTVATDPYTPPYGGALGTGYEKRDDWGGPGTVAIDPYTPPYGEALGTGYE  150
AP1             YEKRDDWGGPGTVATDPYTPPYGGALGTGYEKRDDWGGPGTVAIDPYTPPYGEALGTGYE  176
SIC1.300        YEKRDDWGGPGTVATDPYTPPYGGALGTGYEKRDDWGGPGTVATDPYTPPYGGALGTGYE  176
                ******************************************* ******** *******

                      PRR Fragment 2            Fragment 3
Fragments       KRDDWRGPGHIPKPENEQSPNPSMSHIPEPPQIEWPQWNGFDELSFGPSDWGQSEDAPRF  210
AP1             KRDDWRGPGHIPKPENEQSPNP--SHIPEPPQIEWPQWNGFDELSFGPSDWGQSEDAPRF  234
SIC1.300        KRDDWRGPGHIPKPENEQSPNP--SHIPEPPQIEWPQWNGFDGLSSGPSDWGQSEDTPRF  234
                **********************  ****************** ** *********:***

                                      Fragment 3
Fragments       PSEPRVPEKPQHTPQKNPQESDFDRGFSAGLKAKNSGRGIDFEGFQYGGWSDEYKKGYMQ  270
AP1             PSEPRVPEKPQHTPQKNPQESDFDRGFSAGLKAKNSGRGIDFEGFQYGGWSDEYKKGYMQ  294
SIC1.300        PSEPRVTEKPQHTPQKNPQESDFDRGFSAGLKAKNSGRGIDFEGFQYGGWSDEYKKGYMQ  294
                ****** *****************************************************

                 Fragment 3
Fragments       AFGTPYTPSAT         281
AP1             AFGTPYTPSAT         305
SIC1.300        AFGTPYTPSAT         305
                ***********
```

776 **Supplementary figure S4. Alignment of amino acid sequences for SIC**
777 **fragments 1, 2, and 3, SIC 1.300 and SIC from AP1 *emm*1 GAS**
778 Recombinant SIC fragment 1 (amino acids 1-69), fragment 2 (amino acids 70-
779 167) and fragment 3 (amino acids 168-278) were based on published *sic*
780 sequence of *emm*1 strain AP1 [4]. Arrows mark the start of key regions of SIC: the
781 Signal Sequence (Ss); NH2-terminal short repeat region (SRR); Long repeat
782 region (LRR); Proline rich region (PRR). SIC fragment 1 corresponds to the SRR,
783 fragment 2 corresponds to the LRR and the first 13 amino acids of the PRR, and
784 fragment 3 corresponds to the remainder of the PRR. The start of a SIC fragment
785 is delineated by ⊢ and the end of a fragment is delineated by ⊣. Regions of
786 differences between SIC fragments 1, 2 and 3, SIC1.300 and AP1 SIC are indicated
787 with spaces, dashes (-) indicating absent amino acids, stars (*) indicate identical
788 amino acids between variants and colons (:) indicate equivalent but not the same
789 amino acids. Alignment made using Clustal Omega software and sequences
790 accessed from GenBank.