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Citation for published version:
Wilson, G, Tuffs, S, Wee, B, Seo, KS, Park, N, Connelley, T, Guinane, CM, Morrison, W & Fitzgerald, J 2018, 'Bovine Staphylococcus aureus superantigens stimulate the entire T 2 cell repertoire of cattle.' Infection and Immunity, vol. 86, no. 11. DOI: 10.1128/IAI.00505-18

Digital Object Identifier (DOI):
10.1128/IAI.00505-18

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

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Download date: 05. Apr. 2019

IAI Accepted Manuscript Posted Online 10 September 2018
Infect. Immun. doi:10.1128/IAI.00505-18
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1 **Bovine *Staphylococcus aureus* superantigens stimulate the entire T**

2 **cell repertoire of cattle.**

3

4 Gillian J. Wilson[a,b], Stephen W. Tuffs[a*], Bryan A. Wee[a], Keun Seok Seo[c], Nogi Park[c],

5 Timothy Connelley[a], Caitriona M. Guinane[a‡], W. Ivan Morrison[a] and J. Ross

6 Fitzgerald[a#].

7

8 a. The Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom

9 b. Institute of Infection, Immunity and Inflammation, University of Glasgow,

10 Glasgow, United Kingdom

11 c. Department of Basic Sciences, Mississippi State University, Mississippi State,

12 USA

13

14 Running title: Bovine T cell reactivity with Staphylococcal SAgs

15

16 # Corresponding author: Ross.Fitzgerald@ed.ac.uk

17

18 * Present address: Department of Microbiology and Immunology, Schulich School of

19 Medicine and Dentistry, Western University, London, Canada

20 ‡ Present address: Teagasc/UCC, Food Biosciences, Moorepark Food Research

21 Centre, County Cork, Ireland

22

23

24

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26 **Abstract**

27 Superantigens (SAgs) represent a diverse family of bacterial toxins that induce Vβ-
28 specific T cell proliferation associated with an array of important diseases in humans
29 and animals, including mastitis of dairy cows. However, an understanding of the
30 diversity and distribution of SAg genes among bovine *Staphylococcus aureus* and
31 their role in the pathogenesis of mastitis is lacking. Population genomic analysis of
32 195 bovine *S. aureus* isolates representing 57 unique sequence types revealed that
33 strains encode 2 to 12 distinct SAgs and that the majority of isolates contain 5 or more
34 SAg genes. A genome-scale analysis of bovine reference strain RF122 revealed a
35 complement of 11 predicted SAg genes, which were all expressed *in vitro*. Detection
36 of specific antibodies in convalescent cows suggests expression of 7 of 11 SAgs
37 during natural *S. aureus* infection. We determined the Vβ T cell activation profile for
38 all functional SAgs encoded by RF122 revealing evidence for bovine host-specific
39 activity among recently identified RF122-encoded SAgs SElY and SElZ.
40 Remarkably, we discovered that some strains have evolved the capacity to stimulate
41 the entire T-cell repertoire of cattle through an array of diverse SAgs suggesting a key
42 role in bovine immune evasion.

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51 **Introduction**

52 *Staphylococcus aureus* produces a family of at least 26 distinct superantigens (SAgs)
53 including staphylococcal enterotoxins (SEs), SEA to E, SEG to J, SER to T,
54 staphylococcal enterotoxin-like toxins (SEls), SElK to Q, U, V, X, Y, Z and toxic
55 shock syndrome toxin-1 (TSST-1) (1, 2). SAgs induce the Vβ-specific proliferation of
56 T cells along with the release of pro-inflammatory cytokines including IL-1, IL-2, IL-
57 6, TNF-α, IFN-γ, and chemokines CCL2 and CCL3 (3, 4). The uncontrolled release of
58 pro-inflammatory mediators can lead to rashes, fever, multi-organ damage, coma and
59 death from severe shock (1). The release of pro-inflammatory signals can impede the
60 effectiveness of the immune response by creating a bias towards either the T_h1 or
61 T_h17 response disrupting the appropriate recruitment of effector cells (2). SAgs have
62 been implicated in a wide range of human diseases including staphylococcal food
63 poisoning, endocarditis, necrotizing pneumonia and severe toxic shock (1, 5-7). Taken
64 together the effects induced by SAgs are likely to cause significant deficiency in the
65 ability of the adaptive immune response to contribute effectively to clearance during
66 *S. aureus* infection.

67 *S. aureus* is a common cause of bovine mastitis, an infection of the milk-
68 secreting tissue of the udder, which represents a huge economic problem for the dairy
69 industry worldwide (Miles et al., 1992, Barkema et al., 2006), establishing a typically
70 chronic infection (8). The exact role of SAgs in this disease is currently unknown,
71 however, it has been proposed that superantigenic activity may contribute to the
72 persistence observed (9, 10). Although Vβ specific activation of human T cells in
73 response to staphylococcal SAgs has been well characterised (11-14) relatively little
74 is known for Vβ-specific proliferation of bovine T cells. Previously, SEC and TSST-1
75 have been shown to induce Vβ-specific proliferation of bovine T cells (15-17).

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76 However, these studies were limited by the number of TRBV gene sequences
77 available, with only 5 subfamilies; Vβ1, 2, 4, 13, and 28 included.

78 The bovine genome sequencing project and cDNA analyses led to the
79 identification of the full complement of bovine Vβ subfamilies and almost the entire
80 repertoire of bovine TRBV genes (18, 19). This facilitated the development of a
81 quantitative real-time PCR (qRT-PCR) assay to study the bovVβ response to
82 stimulation with the core genome-encoded SAg SElX (14). *S. aureus* strain RF122
83 belongs to the common bovine specific lineage ST151, and was the first animal-
84 associated isolate to be fully sequenced (20). In this study we have carried out a
85 comprehensive, genome-wide analysis of the complement of SAgs encoded by this
86 strain and determined the capacity of each toxin to activate bovVβ specific T cells.
87 We report host-specific functional activity for several SAgs, and reveal the
88 remarkable capacity of bovine *S. aureus* for activation of the full bovine T cell
89 repertoire suggesting a critical role in immune evasion. Importantly, we have also
90 demonstrated that SAgs produced by *S. aureus* may play a role in the development of
91 intramammary infection of dairy cows.
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100 **Results and Discussion**

101 **Population genomic analysis indicates that bovine *S. aureus* strains encode 2 to**
102 **11 intact SAg genes**. We examined 195 bovine *S. aureus* genome sequences
103 representing 57 unique STs for the presence of all 26 known members of the *S. aureus*
104 SAg family (Table 1: Fig. 1). We employed a threshold of 90% sequence identity
105 across the entire CDS to exclude cross matches to other members of the same SAg
106 group (Fig. 2, Supplementary Table 1).

107 Consistent with previous studies, *selw* and *selx*, were found in 100% (195/195)
108 and 79% (150/195) of isolates analysed, respectively. Previous studies identified *selw*
109 to be inactivated in a large number of human *S. aureus* isolates examined, due to the
110 lack of an ATG start codon (21, 22). However, the presence of alternative start codons
111 (TTG), a continuous full-length open reading frame and the high level of sequence
112 conservation across bovine isolates (more than 94% nucleotide sequence identity)
113 suggests a high proportion of isolates have a functional *selw* gene. The *selx* gene was
114 only absent in clonal complex (CC) 30 consistent with previous reports (14). The *egc*
115 cluster was present in 21 of the 57 unique sequence types (ST) analysed and was
116 highly prevalent within CCs 30, 151 and 45. The composition of the *egc* varied with
117 six different gene arrangements characterised but a gene complement of *seg*, *sei*, *selm*,
118 *seln, selo* and *selu/u2* was the most common observed. $SaPI_{bov}$ was less prevalent
119 compared to the *egc* cluster, found in 10 of the 57 STs analysed, primarily in
120 association with CC133 and CC151. Plasmid-encoded SAgs *sed, sej* and *ser* were
121 identified together in 4 strains consistent with the presence of an pIB485-like plasmid
122 as described in human strains (23). The genes encoding *sely* and *selz* are distributed in
123 a lineage-specific manner, (CC151 and CC9 for *sely* and CC151 for *selz),* and SAg
124 genes; *sea*, *seb*, *seh*, *selk*, *selp* and *selq* were randomly distributed across the diversity

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125 of STs examined consistent with horizontal gene transfer. Genes encoding *set* and *ses*
126 were not found in any *S. aureus* genomes examined suggesting they are not important
127 in bovine pathogenesis.

128 All *S. aureus* strains examined contained at least 2 and up to 11 SAg genes.
129 The majority of bovine STs analysed (31/57) encode 5 or more SAgs with CC151
130 isolates such as RF122 generally encoding more SAgs (up to 13) than other bovine *S.*
131 *aureus* strains. Less than half of STs (n=26) contained *selw* and *selx* only. An
132 important example is the bovine reference strain Newbould 305, which has been the
133 focus of a number of studies (24, 25), encodes a functional copy of *selx,* and a
134 pseudogene of *selw* (25). The extensive variation in SAg gene complement between
135 Newbould 305 and RF122 may have a key impact on relative pathogenesis of
136 infections caused by these strains. Newbould 305 is associated with mild and
137 generally subclinical infection as opposed to RF122 and other CC151 isolates which
138 are associated with a more severe and clinical presentation of the disease (25, 26).

139 Analysis of the genome of the bovine *S. aureus* isolate RF122 (Accession:
140 AJ938182, https://www.ncbi.nlm.nih.gov/nuccore/AJ938182.1/) revealed a
141 complement of 11 SAg genes and 2 SAg pseudogenes (Table 1, Supplementary Table
142 1, Fig. 1). Namely, RF122 contains the previously characterized bovine
143 staphylococcal pathogenicity island (SaPIbov) that contains *tstbov*, *sellbov, secbov*,
144 the enterotoxin gene cluster (*egc)* in the genomic island vSaβ containing allelic
145 variants of SAg genes *seg*, *sei*, *selo*, *seln*, *selu* and a pseudogene of *selm.* Spread out
146 across other parts of the genome, RF122 also codes for *selw* (pseudogene -
147 SAB1473c) *selx, sely and selz* (Fig. 1). The SAg family has been previously
148 subdivided into phylogenetic groups I-V (group IV is composed entirely of
149 streptococcal SAgs) (27, 28), and RF122 encodes at least 2 genes from each of the 4

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150 staphylococcal SAg subgroups (Fig. 1). Accordingly, RF122 was selected for
151 genome-scale analysis of the expression and function of bovine *S. aureus* SAgs.
152
153 **Bovine SAg genes are expressed at different levels in a growth phase dependent**
154 **manner *in vitro*.** Relative transcriptional levels of RF122 SAg genes in exponential
155 and stationary phase of growth were determined by qRT-PCR. Transcription was
156 detected for all 11 genes and 2 pseudogenes in both growth phases, with *secbov*
157 exhibiting the highest level of transcription and *selu*, the lowest (Fig. 3). Overall, SAg
158 genes located on SaPIbov were transcribed at higher levels than *selx*, *sely*, *selz*, *egc*-
159 encoded genes and the SAg pseudogenes. The data indicate that SaPIbov SAg genes
160 and *selx* are up-regulated in stationary phase, consistent with regulatory control by
161 *agr*, whereas *sely* and *selz* are transcribed maximally in mid-exponential phase
162 suggesting agr-independent control. Of note, ST151 strains were previously
163 demonstrated to have higher levels of RNAIII transcription in comparison with other
164 ruminant clones and could provide an explanation for the high expression of some of
165 these SAgs (26). In the current study, *egc* genes were transcribed at low levels,
166 independent of growth phase. This finding is consistent with Derzelle *et al* who
167 reported low *egc* transcript levels among 28 human strains (29). However, we cannot
168 rule out the possibility that the *egc* genes are expressed at higher levels *in vivo*, as has
169 been observed for the streptococcal SAgs SPEA and SPEC (30, 31). The differential
170 regulation of SAg transcription *in vitro* suggests that SAgs are expressed at different
171 stages of infection *in vivo*.
172
173 ***S. aureus* SAgs are expressed during bovine infection**. To determine if RF122-
174 encoded SAgs are expressed during bovine infection, we produced recombinant

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175 proteins for each of the encoded SAgs, and carried out western immunoblot analysis
176 with convalescent sera from cows (Table 2). A serum sample obtained from a cow
177 without a history of *S. aureus* mastitis, did not contain antibody reactive for any of the
178 SAgs tested and was used as a negative control (Table 2). IgG antibodies specific for
179 8 of the 11 SAgs were detected in at least 1 of the 4 bovine sera samples tested
180 whereas $rSEI_{bov}$, $rSEG_{bov}$ and $rSElO_{bov}$ were not reactive with any of the samples
181 tested (Table 2). In a previous study by Wilson et al, $rSElX_{bov}$ was demonstrated to be
182 reactive with all bovine sera samples tested. Most human adults have antibodies
183 specific for an array of *S. aureus* SAgs including SEA, SEB, SEC, SED, SEE, SElX
184 and TSST-1, as a result of exposure during colonisation or infection (14, 32, 33). The
185 current study corroborates previous observations, which found, that despite the
186 relatively high prevalence of the *egc* cluster in clinical isolates of *S. aureus*,
187 neutralising antibodies are rare (34).

188 This suggests that either the *egc* SAgs are poorly expressed during infection or
189 that the host is unable to generate antibodies due to low T or B-cell reactivity.
190 Importantly in this study we have shown that SElY, SElZ, and to a lesser extent SElU
191 and SEI are expressed by *S. aureus in vivo.* Antibodies against SElY and SElZ have
192 been detected in at least one serum sample of bovine and human origin, consistent
193 with a role in pathogenesis of both host-species.

194 Although our data suggest low levels of expression of some SAgs, it is
195 feasible that they can contribute to *S. aureus* immune modulation. For example, we
196 recently demonstrated that suboptimal stimulation of human T cells with a low
197 concentration of SAg (1 ng/ml) induced CD8+CD25+FOXP3+ regulatory T cells that
198 strongly suppress activation of effector T cells (35). A similar phenomenon can be

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199 observed in the bovine system as immunosuppressive CD4+CD25+FOXP3+ are
200 activated with equivocally low concentrations of SAg (1 ng/ml) (36).
201
202 **RF122-encoded SAgs are mitogenic for bovine T cells.** In order to examine the
203 mitogenicity of each of the 11 identified SAg, we constructed a SAg-deficient mutant
204 of *S. aureus* strain RF122 to facilitate plasmid-mediated expression of each SAg in
205 isolation by its native *S. aureus* strain. *S. aureus* RF122-1, a TSST-1-deficient
206 derivative of RF122 had been constructed previously by allele replacement of the *tst*
207 gene with a tetracycline resistance cassette (17). In turn, we sequentially deleted the
208 *sec*, *sel*, egc genes, *selx, sely and selz* by allele replacement (Fig S1) resulting in the
209 sequential mutants RF122-2 to RF122-8 and the final SAg-deficient derivative
210 RF122-8 (Table S3 and Fig S1). Finally, to limit the Hla-mediated toxicity for T cells,
211 we constructed *hla* mutants in the parent RF122 and SAg-deficent derivatives
212 resulting in strains RF122t-α and RF122-8α, respectively (Supplementary Table 3).
213 The mutants were validated to rule out spurious mutations accrued during *in vitro*
214 passage that impact on secreted virulence proteins (Fig S2). Analysis of the
215 mitogenicity of stationary and mid-exponential phase culture supernatants of RF122
216 and RF122-8 confirmed loss of all detectable mitogenic activity (Fig S2).
217 Previously, superantigenic activity of RF122-encoded SEC_{bov}, TSST-1_{bov} and
218 $SElX_{bov}$ has been described (14-17). In order to examine the mitogenic potential of all
219 SAg encoded by RF122 expressed in a native *S. aureus* background, SAg genes were
220 cloned into the inducible expression plasmid pALC2073. This allowed controlled
221 expression in the SAg-deficient RF122-8α, facilitating analysis of the effect of
222 individual SAgs produced in their native strain context on bovine T cells *in vitro*.
223 Proteins of the predicted molecular weight were detected in supernatants of induced

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224 RF122-8α cultures for each SAg plasmid construct with the exceptions of $SEG_{bov,}$
225 $SElN_{bov}$, SEI_{bov} and $SElO_{bov.}$ (Fig. S3). To examine the mitogenicity of RF122-
226 encoded SAgs for bovine T cells, culture supernatants of RF122-8α containing
227 pALC2073::SAg constructs and recombinant SAg proteins were used to stimulate
228 bovine PBMC, and proliferation was measured using a thymidine incorporation assay
229 (Fig. 4). Mitogenic activity for bovine T cells was detected for 7 of the 11 SAgs
230 expressed in the SAg-free RF122-8α including TSST-1, SECbov, SEL_{bov} SEI_{bov},
231 $SElN_{bov,,}$ SElX, and $SElZ_{bov}$ at total protein concentrations ranging from 10 pg/µl to 10
232 ng/ml, but there was no detectable mitogenic activity for $SElO_{bov}$, SEG_{bov} ,
233 $SElU_{bov..}$and SElY (Fig 3a). However, recombinant proteins $rSEG_{bov}$ and $rSElU_{bov}$ and
234 rSElY expressed in *E. coli* could stimulate T cell proliferation at higher
235 concentrations (Fig. 3b). Accordingly, of the 11 SAgs encoded by RF122, only
236 $SElO_{bov}$ did not exhibit any capacity for stimulation of bovine T cells. Taken together,
237 these data indicate that RF122 encodes an array of SAgs that are potent bovine T cell
238 mitogens.
239
240 **RF122-encoded SAgs have the capacity to stimulate the entire bovine Vβ**
241 **repertoire.** Most previous studies of the bovine Vβ-dependent T cell activation
242 capacity of staphylococcal SAgs have been limited by the number of identified bovine
243 Vβ subfamilies (15, 17). Recently, we developed a novel qRT-PCR assay which is
244 representative of the full complement of bovine Vβ subfamilies (14). Supernatants
245 from tetracycline induced cultures of RF122-8α containing pALC2073::SAg
246 constructs were used to stimulate bovine T cells. If the supernatant was unable to
247 induce proliferation at a total protein concentration of 0.01µg/ml then purified
248 recombinant protein was used as an alternative to determine the bovVβ profile (Fig.

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249 5). Accordingly, in the current study we were able to comprehensively evaluate the
250 response of 18 bovVβ subfamilies to stimulation with all RF122-encoded SAgs by
251 qRT-PCR (Table 3 and Fig. 5). In order to examine the host-specificity of bovine
252 SAgs, we also examined the capacity of recently characterized SAgs SElY and SElZ
253 to stimulate Vβ-dependent activation of human T cells (Fig. 6 and Table 3). We found
254 that all SAgs encoded by RF122 with the exception of $SElO_{bov}$ induced Vβ-specific
255 stimulation of bovine T cells (Fig. 5) with a unique bovVβ activation profile similar to
256 humVβ activation profiles T cell (37). Of note, the data indicate that each of the 18
257 bovVβ subfamilies tested are activated by at least one RF122-encoded SAg, such that
258 RF122 has the potential to stimulate the entire bovVβ repertoire (Fig. 5). Remarkably,
259 the 3 SAgs encoded by SaPIbov alone activate 13 of 18 bovVβ subfamilies
260 highlighting the potential importance of SaPIbov in bovine immune evasion. In
261 comparison, despite being twice in number, the *egc* SAgs activate only 11 of 18
262 subfamilies. Extensive duplication within the bovVβ repertoire has resulted in 9
263 multimember subgroups, the largest of which, bovVβ 1, 10 and 13, contain 23, 9 and
264 20 functional TRBV genes, respectively (18, 19). Each of the SaPIbov encoded SAgs,
265 SEC_{bov}, $SElL_{bov}$ and TSST-1_{bov} and *egc* encoded SEI_{bov} can activate at least one of
266 these large subfamilies each (Fig. 5). $SElL_{bov}$ activates both bovVβ 1 and 10 which is
267 consistent with the large proportion of T cells which are induced in response to
268 stimulation with this SAg (Fig. 4 and 5). It has been shown previously that all humVβ
269 subfamilies (with the exception of humVβ 4 and 11) are activated by at least one SAg
270 (2). Our data also indicates that some bovVβ subfamilies can be activated by multiple
271 SAgs, for example, Vβ 16 and X are activated by 6 RF122-encoded SAgs, and Vβ 24
272 and 17 are activated by 5 of them. This apparent functional redundancy implies that
273 activation of these Vβ subfamilies is of critical importance in *S. aureus* infection. A

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274 similar redundancy has been observed in the humVβ response to SAgs, with Vβ1, 3,
275 5, 6, 9, 12, 18 and 21 targeted by at least 5 or more different SAgs (2).
276
277 **Evidence for host-adaptation by bovine *S. aureus* SAgs.** For the recently
278 characterized SAgs SElY and SElZ, we examined the Vβ-dependent activation of
279 human and bovine T cells. We utilised protein variants for both SElY and SElZ
280 derived from human and bovine isolates to investigate the possibility of host-
281 adaptation. Both human and bovine variants of SElY and SElZ induced similar levels
282 of expansion of human T cells (Fig. 6a). SElY induced expansion of a broad number
283 of human Vβ subfamilies (Fig. 6b), while SElZ induced expansion of a single human
284 Vβ subfamily (13.2). In contrast, to the human Vβ expansion profile, both human and
285 bovine variants of SElY and SElZ activated different bov Vβ subfamilies. $SElZ_{bov}$
286 activated bov Vβ subfamilies 1, 3, 7, 11, 16, 17, 24, 28, and X, while the $SElZ_{hum}$
287 activated bov Vβ subfamilies 24, 28, and X (Fig. 6c). It is also noteworthy that
288 $SElZ_{bov}$ exhibited a 10-fold greater potency than $SElZ_{hum}$ for stimulating bovine T cell
289 proliferation (Fig. 6a). This could be explained by activation of a broader number of
290 bov Vβ subfamilies by $SElZ_{bov}$ compared to the human variant (Fig. 6c). Strikingly,
291 $SElY_{bov}$ induced expansion of a broad array of bov Vβ subfamilies, while $SElY_{hum}$
292 was unable to induce activation of bovine T-cells (Fig. 6a, c). Combined, these results
293 suggest adaptive evolution of SElY and SElZ to the bovine host.
294 Analysis of the protein variants SElY and SElZ revealed a number of unique
295 residues which may be responsible for the difference in phenotype observed between
296 the human and bovine variants (Fig. S4). For SElY, three positions varied between the
297 bovine allele from RF122 (ST151) and the human allele from MSA2020 (ST121)
298 (E19G, T67A and I183V). In particular the glutamic acid residue at position 19 was

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299 identified in SElY allele of ST151 and other cattle isolates (STs 3140, 504, 706 and
300 3099), but not in any of the SElY variants of human origin. For SElZ, four positions
301 varied between the bovine allele from RF122 (ST151) and the human allele from
302 MSA1695 (P6L, N55S, D75N and G106A). Of note, the glycine residue at position
303 106 of RF122 SElZ was found in all but one of the bovine SElZ variants analysed and
304 was absent among the majority of human variants (6/8).

305 Some of the differences between human and bovine Vβ activation profiles are
306 due to absence of an orthologous subgroup, such as the activation of humVβ 12, 14,
307 20, 22, and 23 (absent in bovine), and bovVβ 10, 28 and X (absent in human) (18).
308 However, there are cases where Vβ subfamilies from one host are activated, but the
309 orthologous subgroup from the other is not (Table 3). For example, $SElL_{bov}$ activates
310 bovVβ 6 and 24 but not humVβ 6 and 24, TSST-1_{bov} activates only bovine Vβ 4, and
311 24, SEI_{bov} activates bovVβ 16 and humVβ 5 and 6, but not the equivocal variants in
312 the opposite species, and $SElN_{bov}$ activates bovVβ 3, 16 and 24 and humVβ 7 and 8,
313 but not the equivalent human or bovine subgroups. It is important to note that with the
314 exception of SEC_{bov} and $SElX_{bov}$, the human Vβ profiles described here were
315 determined in previous reports in response to stimulation with SAgs derived from
316 human *S. aureus* strains (11, 12). It is feasible that distinct human Vβ profiles could
317 be stimulated by bovine SAg variants. Our analysis of SElY and SElZ, and previous
318 analysis of SElX (14) support the notion that allelic variants of SAgs made by *S.*
319 *aureus* from different host species have evolved to preferentially activate the Vβ
320 repertoire of the strains target host. Together, these data indicate that some SAgs
321 encoded by bovine *S. aureus* have undergone host-adaptation associated with broader
322 stimulation of Vβ–subfamilies, and increased potency of bovine T cell activation.
323 Furthermore, we report that SElY and SElZ are classical SAgs in that they have a

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324 unique Vβ activation profiles with capacity to mediate immune modulation in both
325 humans and cattle.
326
327 **Preliminary examination of the role of SAgs in the pathogenesis of bovine**
328 **mastitis**. The functional analysis of bovine SAgs made by a single strain in the
329 current study suggests a profound role in host-pathogen interactions and pathogenesis.
330 In order to examine the role of SAgs in *S. aureus* bovine mastitis, preliminary
331 experimental infections of bovine mammary glands were carried out using RF122 and
332 RF122-8 over a course of 21 days. Seven healthy dairy cows in their 1st to 4th
333 lactation were enrolled in two groups of 4 and 3 cows, challenged with wild type
334 RF122t or the SAg-deficient RF122-8, respectively. There were no differences
335 observed between the groups in terms of somatic cell count, milk yields and core
336 body temperatures (Fig. S5). *S. aureus* was isolated from the mammary gland of all
337 animals during the trial, taken together with the milk quality and somatic cell counts,
338 these data indicate that SAg are not required to establish sub-clinical mastitis. The
339 group infected with wild type RF122 exhibited clinical mastitis at least once, in three
340 out of the four animals infected during the course of the study (Fig. 7). In contrast,
341 clinical mastitis was not observed in the animals infected with the SAg-deficient
342 mutant. Although the study was not powered for statistical significance, the data are
343 suggestive of a role for bovine SAgs in the development of staphylococcal clinical
344 mastitis. Further experimentation would be required to confirm this preliminary
345 observation.
346 We speculate that SAgs may contribute to pathogenesis through expression of
347 some SAgs such as SEC1 and TSST-1 at high concentrations to promote the release
348 of proinflammatory cytokines which in turn induce tissue damage, inflammation, and

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349 clinical pathology Furthermore, some SAgs such as *egc* SAgs expressed at low
350 concentrations may induce immunosuppressive regulatory T cells to promote
351 colonisation of the host.
352
353 **Concluding comments.** In conclusion, the role of SAgs during pathogenesis is very
354 complex. The array of identifiable staphylococcal SAgs is expanding and has been
355 expedited with advances in genomic analyses. The extensive diversity is potentially
356 driven by the need to activate a large number of T cells, and bind to MHC class II
357 molecules in multiple ways, contributing to immune evasion. Our findings contribute
358 to the understanding of staphylococcal SAg diversity and provide a comprehensive
359 analysis of the bovine T cell response to SAgs. In addition, we report examples of
360 toxins that contribute to the capacity of *S. aureus* to adapt to different host species.
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382 **Methods**

383 *Ethics Statement*

384 All *in vivo* work was done after local ethical review and under the oversight of the
385 Kalamazoo IACUC, and in accordance with local, state, and federal animal welfare
386 regulations. Bovine venous blood was taken under the authority of a UK Home Office
387 Project License (PPL 604394) within the terms and conditions of the regulations of
388 the UK Home Office Animals (Scientific Procedures) Act 1986 and the code of
389 practice for the housing and care of animals bred, supplied, or used for scientific
390 purposes. Human venous blood was taken from healthy donors in accordance with a
391 human subject protocol approved by the national research ethics service (NRES)
392 committee South East Scotland under the research ethics committee reference
393 11/AL/0168. Volunteers were recruited by a passive advertising campaign within the
394 Roslin Institute (University of Edinburgh) and written consent was given by each
395 volunteer before each sample was taken.

396

397 *Bacterial culture conditions*

398 *S. aureus* strains were grown in tryptone soya broth (TSB) or brain heart infusion
399 broth (BHI) (Oxoid, UK) shaken at 200 rpm, or on tryptone soya agar (TSA) (Oxoid,
400 UK) at 37 °C for 16 h unless otherwise stated. *E. coli* strains were grown in Luria-

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401 Bertani Broth (LB) (Melford Laboratories, UK) shaken at 200rpm, or on LB-agar
402 (Melford Laboratories, UK) at 37 °C for 16 h unless otherwise stated. Media were
403 supplemented where appropriate with 150 µg/ml X-gal, 50 µg/ml ampicillin, 10 µg/ml
404 erythromycin or chloramphenicol (Sigma-Aldrich, Dorset, UK). For growth curve
405 analysis of *S. aureus*, strains were cultured overnight in 5 ml Brain–Heart Infusion
406 (BHI) broth (Oxoid Ltd., Basingstoke, UK) in triplicate. After 12 h strains were sub-
407 cultured at a dilution of 1 in 100 into 30 ml fresh BHI broth in 250 ml Erlenmeyer
408 flasks and placed in a shaking incubator at 37 °C and 200 rpm. Absorbance readings
409 were measured at 600 nm (OD_{600}) using a spectrophotometer (Cecil Aurius CE2021,
410 Thistle Scientific Ltd., Glasgow, UK) over a period of 12 h and a growth curve was
411 determined.
412
413 *Sequence analysis of staphylococcal SAg genes*
414 The sequences of characterised staphylococcal SAg genes were obtained from the
415 NCBI Genbank database (Supplementary Table 1). SAg homologs were identified in
416 publicly available whole genome sequences of bovine and representative human *S.*
417 *aureus* genomes using BLASTn with a minimum alignment of 90% nucleotide
418 identity averaged across the entire gene sequence using the Blastable script
419 (github.com/bawee/blastable). Representative genomes with unique sequence types
420 and SAg content were selected and a core genome alignment was built using Parsnp
421 (38). The association between SAg content and phylogeny was visualised using iTol
422 (39). Nucleotide sequences corresponding to each reference SAg were aligned at the
423 codon level using translatorx and mafft (40, 41). A Maximum-Likelihood tree was
424 constructed from the translated amino acid alignment using RAxML (v8.2.10) with
425 the following settings: -m PROTCATAUTO -f a -N 1000 -x 123 -p 123 (42). BRIG

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426 (43) was used to construct the circular genome representation and GC content plot
427 with *S. aureus* RF122 genome sequence (Accession number: AJ938182
428 https://www.ncbi.nlm.nih.gov/nuccore/AJ938182.1/) as a reference.
429
430 *Transcriptional analysis of SAg genes*
431 Total RNA was extracted from *S. aureus* strains RF122 exponential (OD_{600}=0.6) and
432 stationary phase (12 h) cultures using the RNeasy miniprep kit (QIAgen, UK) as
433 described in the manufacturer's instructions except for an added lysis step with re-
434 suspension of the bacterial pellet in TE buffer with 100 μg/ml Lysostaphin and
435 incubation at 37°C for 20 min. RNA was treated with Turbo DNase (Thermo Fisher,
436 UK). 0.5 μg mRNA was analysed for gene transcription using the same protocol
437 outlined in Wilson *et al* (2011), SAg primers are listed in Supplementary Table 2.
438
439 *Allelic replacement of SAg genes*
440 Gene deletion constructs of SAg genes in RF122 were performed using constructs
441 prepared in the plasmid pMAD (44) (Supplementary Table 3). Plasmid construction
442 and allelic replacement were performed as described elsewhere (14, 44). The resulting
443 mutant strain which had lost the gene of interest (GOI) was analysed by PCR for no
444 amplification with primers within the deleted region or with pMAD MCS primers
445 (Supplementary table 2). The mutant strains were also sequenced using primers
446 upstream (E) and downstream (Z) of the GOI to confirm the predicted deletion event.
447 Sequencing reactions were carried out by Edinburgh Genomics (King's Buildings,
448 University of Edinburgh, UK). To investigate the possibility that deletion of the genes
449 could have pleiotropic effects, the phenotype of WT and mutant strains was
450 compared. Firstly, a growth curve was determined for RF122, RF122t and RF122-8,

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451 grown in BHI liquid culture for 10 h at 37 °C, which revealed growth rates and yields
452 were similar for each strain (Fig S2). In addition, the haemolysis of rabbit
453 erythrocytes incubated with culture supernatants of RF122 and SAg-deficient
454 derivative strains was investigated. In each case the haemolytic titre of RF122 and
455 SAg-deficient derivatives was 1022, indicating that the deletion of SAg genes had no
456 effect on haemolytic activity and that the *agr* locus was functional (Fig S2). Deletion
457 of the *hla* gene in RF122 resulted in a reduction in haemolytic titre indicating these
458 strains are less toxic than the wild type. Analysis of the profile of secreted and CWA
459 proteins of WT and mutant strains revealed no unexpected differences (Fig S2).
460
461 *Analysis S. aureus secreted and CWA proteins*
462 Secreted and cell-wall associated (CWA) proteins were extracted from *S. aureus* mid-
463 exponential (OD_{600}=0.6) and stationary phase (12 h) cultures grown in BHI. Cells
464 were centrifuged at 4000 x g and supernatant fractions containing secreted proteins
465 were removed and concentrated with Amicon Ultra-15 Centrifugal Filter units with a
466 10 kDa MWCO as described in the manufacturer's instructions (Merk Millipore, UK).
467 To extract CWA proteins, pelleted cells were washed with 1 ml PBS (Oxoid,
468 Cambridge, UK), re-suspended in 1 ml lysis buffer (50 mM TrisHCl, 20 mM $MgCl_2$,
469 30% Raffinose (Fluka, UK), adjusted to pH 7.5) containing 200 µg/ml Lysostaphin
470 (AMBI products LLC, NY, USA) and protease inhibitors (Roche, UK) and incubated
471 at 37 °C for 20 min. Samples were centrifuged at 6000 x g for 20 min and CWA
472 proteins were recovered from the supernatant fraction. Protein preparations were
473 separated on 10% SDS-PAGE gels, stained overnight at room temperature with
474 Coomassie Blue (Severn Biotech), or transferred to Nitrocellulose membranes
475 (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western blot

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476 analysis. The membrane was incubated with primary antibody for 1 h with 1:2500
477 dilution of anti-SEC (Santa Cruz Biotechnology, Heidelberg, Germany), or 2 h with a
478 1:2000 dilution of rat antisera specific for rTSST-1, rSElL, or rSElX$_{bov.}$ Membrane
479 was incubated with secondary antibody for 1 h at dilutions; 1:2500 (Rabbit anti-
480 mouse IgG, Zymed, Invitrogen, UK), or 1:1500 Goat polyclonal antibody to rat
481 IgG/HRP, (Abcam, Cambridge, UK), and visualised by ECL.
482
483 *Cloning of SAg genes into pALC2073*
484 5´ oligos to amplify RF122-encoded SAg genes for cloning into the expression
485 plasmid pALC2073 were designed to prime upstream of the predicted ribosome
486 binding site (RBS) with a *Kpn*I site incorporated to facilitate cloning (Supplementary
487 table 2). The 3´ primer was designed to include the stop codon of the gene with a *Sac*I
488 site incorporated (Supplementary table 2). PCR reactions were carried out with 10 ng
489 RF122 gDNA, 100 nmol forward and reverse primers, as listed in Supplementary
490 Table 2, using 1 U Vent polymerase (New England Biolabs, Herts, UK) as described
491 in the manufacturer's instructions. PCR products were cloned into the Strataclone
492 pSC-B plasmid (Agilent, Cheshire, UK), inserts were released by digestion with *Sac*I
493 and *Kpn*I for 3 h at 37 °C, purified by gel extraction, ligated with digested pALC2073
494 plasmid DNA using T4 DNA ligase, and transformed into *E.coli* DH5α. The resulting
495 pALC2073::SAg plasmids were isolated from DH5α and transformed by
496 electroporation into an intermediate electro-competent strain of *S. aureus,* RN4220.
497 Subsequently the plasmids were re-isolated and transformed into the SAg deficient
498 strain RF122-8. *S. aureus* strains were made competent as described previously (14).
499 RF122-8 strains containing each of the pALC2073::SAg constructs were induced with
500 a sub-inhibitory concentration of tetracycline (50 ng/ml) (Sigma-Aldrich, Dorset, UK)

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501 when cultures reached mid-exponential phase, and grown for a further 4 h (Bateman
502 B.T *et al*, 2001).
503
504 *Recombinant expression of SAg genes*
505 5′ primers for cloning into the pET15b (Merk Millipore, UK) or pQE30-Xa (QIAgen,
506 UK) plasmids, were designed to anneal immediately after the signal peptide coding
507 region, as predicted by Signal P 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/),
508 and 3′ primers were designed to include the stop codon of the gene (Supplementary
509 Table 2). The cloning procedure was performed as outlined for pALC2073 and ligated
510 constructs were transformed into *E. coli* DH5α or XL-1 blue (for pQE30-Xa
511 constructs) cells. pET constructs were isolated from DH5α using the QIAprep Spin
512 Miniprep kit (QIAgen, UK), and transformed into *E. coli* BL21 (DE3). BL21 or XL-1
513 blues cells containing expression constructs were cultured in Luria-broth containing
514 50 µg/ml ampicillin (Sigma-Aldrich, Dorset, UK) and induced in mid-exponential
515 phase of growth (OD_{600}=0.6), with 1 mM isopropyl β-D-1-thiogalactopyranoside
516 (IPTG) (ForMedium Ltd., Norfolk, UK) for 4 h. Cells were recovered by
517 centrifugation at 8000 xg, disrupted using a French Press, and His-tagged
518 recombinant proteins were purified by affinity chromatography on a Ni-NTA nickel
519 affinity column (GE healthcare, UK). Proteins were dialysed using Spectra/Por Float-
520 A-Lyzer tubing with a 8000 to 10000 molecular weight cut off (MWCO) (Spectrum
521 Laboratories, California, USA).
522
523 *Immunoblot analysis of convalescent bovine serum*
524 SDS-PAGE and western blotting was carried out on SAgs overexpressed in *E. coli*.
525 The nitrocellulose membrane (Amersham Hybond™ ECL™, GE Healthcare, Slough,

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526 UK) was incubated with 10ml of blocking buffer containing 5% (w/v) skimmed milk
527 powder (Sigma Aldrich, UK) in PBST (PBS with 0.05% Tween 20 (Sigma Aldrich,
528 UK)) overnight at 4°C. The membrane was then incubated for 2 hours with a 1:1000
529 dilution of pooled bovine convalescent serum in PBST with 1% (w/v) skimmed milk
530 and washed three times with PBST. Secondary antibody (goat anti-bovine IgG-HRP
531 (Santa Cruz Biotechnology, Heidelberg, Germany)) was added at a concentration of
532 … for 1 h at room temperature. The blot was washed again as. Immunoreactivity was
533 visualised by chemiluminescence from ECL.
534

535 *T cell proliferation assays*

536 Blood was obtained from Holstein-Friesian cattle aged 18 to 36 months via jugular
537 vein puncture. Animals were reared indoors and maintained on a ration of hay and
538 concentrates. PBMC were isolated by density gradient centrifugation using Ficoll
539 Paque PLUS (GE Healthcare, UK) as described previously (45). Human PBMC were
540 isolated from venous blood, drawn from healthy human volunteers and mixed with
541 acid-citrate-dextran (ACD) (25 g D-glucose (Sigma-Aldrich, UK) and 20.5 g
542 trisodium citrate (Sigma-Aldrich, UK) added to 1 L of ddH2O). The buffy coat was
543 isolated by spinning the blood at 1500 x g for 15 min with no break and then PBMC
544 were isolated using Ficoll Paque PLUS (GE Healthcare, UK) according to the
545 manufactures specification. PBMCs were adjusted to a concentration of 1×10^6
546 cells/ml in complete cell culture medium (RPMI 1640 (Sigma Aldrich, UK)
547 supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Gibco, UK), 100
548 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine. (PSG) (Gibco,
549 UK)), were stimulated in triplicate at least with concentrated total protein *S. aureus*
550 supernatant fraction or recombinant protein. Culture media and 50 μg/ml

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551 Concanavalin A were used as negative and positive controls respectively.
552 Proliferation of bovine and human PBMCs were assessed by [^{3}H]-thymidine
553 incorporation assay as described previously (14). Total RNA was extracted from
554 bovine PBMC (4× 10^6 cells) using Tri-reagent (Sigma–Aldrich, Dorset, UK) as
555 described in the suppliers instructions or using the RNeasy plus kit (Qiagen, UK)
556 according to the manufactures instructions. First-strand cDNA was generated from 0.5
557 μg of RNA using Power SYBR® Green RNA-to-CT 2-Step Kit or High Capacity
558 RNA-to-cDNA Kit and Power SYBR® Green PCR Master Mix (Thermo Fisher,
559 UK). The reverse transcription reaction was performed in a 20 μl volume according to
560 the manufacturer's specifications. Bovine Vβ subfamily specific qRT-PCR reactions
561 were carried out as described previously (14). Human Vβ activation analysis was
562 performed as described previously (12, 46).
563
564 *Experimental infection of dairy cattle*
565 Adult cows (Holstein) in their 1st to 4th lactation at 92 to 174 Days in Milk (DIM)
566 were used in this study. Overnight cultures of *S. aureus* were inoculated 1:50 into
567 fresh TSB and grown until an OD_{600} of 1.1 was reached. Staphylococci were diluted
568 in TSB to obtain an inoculum of $5x10^7$ CFU/ml. Inocula were determined by CFU
569 enumeration following serial dilution, plating on TSA, and growth at 37°C. Animals
570 were challenged via teat dip immersion twice daily (22mm immersion) until a score
571 of 1 or greater for milk appearance or udder evaluation was observed and the animal
572 developed an intra-mammary infection twice within a five day period. Following
573 infection animals were observed for a total of three weeks. Somatic cell counts (SCC)
574 and cultures were taken twice a week. Udder and milk clinical scores, milk yield and

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575 milk conductivity data were collected at each milking which was performed twice
576 daily.

577

578 *Statistical analysis*

579 All statistical analysis was performed in Graphpad Prism 7. Fold change enrichment
580 data was analysed using student t-test with Welches correction if required. Tests were
581 unpaired and two-tailed and significant differences were considered when the p-value
582 was <0.05.

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584

585 **Acknowledgements**

586 The authors are grateful to Dr Gregory Bohach for sharing his expertise and
587 contributing to this project. The authors would like to thank Dr Sara Clohisey and Dr
588 Kenneth Baillie for their assistance with organising the blood donation study and the
589 volunteers from the Roslin Institute who provided human blood samples. We would
590 like to extend our thanks to Sarah Salmon and Dennis Peterson of Zoetis Animal
591 health for their assistance with the animal infection study. J.R.F. was supported by
592 grants BB/K00638X/1, BB/I013873/1 and institute strategic grant funding
593 (BBS/E/D/20002173 from the Biotechnology and Biological Sciences Research
594 Council (UK), a Medical Research Council (UK) doctoral training grant, and Zoetis
595 Animal Health. This work was also partially supported by grants from Center for
596 Biomedical Research Excellence in Pathogen-Host interactions, National Institute of
597 General Medical Sciences, NIH (1P20GM103646-01A1) and Animal and Plant
598 Quarantine Agency, South Korea (I-1543081-2015-17-01) to K.S.S.

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611 **Tables**

612 **Table 1: SAgs encoded by *S. aureus* strain RF122**

Gene	Toxin	Size (kDa)[a]	Locus tag	Homology with characterised SAg gene (%)
tstbov	Toxic shock syndrome toxin-1 (TSST-1_{bov})	22	SAB_RS01910	98% (*tst*)
secbov	Staphylococcal enterotoxin C-bovine (SEC_{bov})	27.6	SAB_RS01930	99% (*sec1*)
sellbov	Staphylococcal enterotoxin like toxin L-bovine ($SElL_{bov}$)	24.7	SAB_RS01935	99% (*sel1*)
segbov	Staphylococcal enterotoxin G-bovine (SEG_{bov})	20.6	SAB1696c	77% (*seg1*)
seibov	Staphylococcal enterotoxin I-bovine (SEI_{bov})	24.9	SAB_RS09045	97% (*sei1*)
selnbov	Staphylococcal enterotoxin like toxin N-bovine ($SElN_{bov}$)	26.1	SAB_RS09035	95% (*sen1*)

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selubov	Staphylococcal enterotoxin like toxin U-bovine ($SElU_{bov}$)	27.2	SAB_RS09040	97% (*selu1*)
selmbov	Staphylococcal enterotoxin like toxin M-bovine ($SElM_{bov}$)	N/A	SAB1700c	87% (*selm1*)
selobov	Staphylococcal enterotoxin like toxin O-bovine ($SElO_{bov}$)	27.1	SAB_RS09055	98% (*selo2*)
selw	Staphylococcal enterotoxin like toxin W (SE26)	N/A	SAB1473c	54% (*sea1*)
selxbov	Staphylococcal enterotoxin like toxin X-bovine ($SElX_{bov}$)	19.5	SAB_RS01710	45% (*tst*)
selybov	Staphylococcal enterotoxin like toxin Y-bovine (SElY)	22.5	SAB_RS13070	58% (*set*)
selzbov	Staphylococcal enterotoxin like toxin Z-bovine (SElZ)	27.1	SAB_RS00140	57% (*seg1*)

613 [a] Predicted size of the mature protein based on amino acid sequence. Pseudogenes not included.

614 **Table 2: Immunogenicity of recombinant SAg proteins from RF122 with sera**

615 **from bovine and human *S. aureus* infections.**

Serum sample [a]	SElZ	SElY	SEG	SEI	SElO	SElU	SElN	SEC	SElL	TSST-1	SElX[b]
Human											
IE19	+	+	-	-	-	-	-	+	-	+	+
IE37	+	+	-	+	-	-	-	+	+	+	+
IE41	-	-	-	-	-	-	-	+	-	+	+
IE51	-	-	-	-	-	-	-	+	-	+	+
IE54	-	-	-	-	-	-	-	-	-	+	+
Bovine											
2480	+	-	-	-	-	-	-	-	-	-	+
2487	+	+	-	-	-	-	-	-	-	+	+
2521	+	+	-	-	-	+	-	+	+	+	+

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4227	+	-	-	-	-	+	-	+	+	+	+
2211	-	-	-	-	-	-	-	-	-	-	-

616 + or – indicate whether or not serum samples are reactive with SAg proteins.

617 [a] Human serum samples were obtained from infective endocarditis patients between 2006-2009,

618 New Royal Infirmary of Edinburgh. Bovine samples obtained from bovine mastitis cases, and

619 from an animal (cow 2211) without a history of *S. aureus* infection were provided by C. Smyth,

620 originally obtained from Teagasc Dairy Production Centre in Moorepark, Fermoy, Co. Cork.

621 [b] Data previously reported (14)

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626

627 **Table 3: Activation of Vβ subfamilies in response to RF122-encoded SAgs.**

SAg	Bovine Vβ [a, b, c]	SAg	Human Vβ [a, b, d]
SEC_{bov}	**15, 17**, 28	$SEC_{bov,}$	12, **13**, 14, **15, 17**, 20
SEI_{bov}	**1**, 10, 16	SEI	**1**, 5, 6, 23
$SElL_{bov}$	**1, 5**, 6, **7**, 10, **16**, 24	SElL	**1, 5, 7, 16**, 22, 23
$SElN_{bov}$	3, **9**, 16, 24, X	SElN	7, 8, **9,** 17
$SElX_{bov}$	3, 5, 8, 11, 16, 17, 24, X	$SElX_{bov}$	1, 6, 18, 21
TSST-1_{bov}	**2**, 4, 24, X	TSST-1	**2**
SEG_{bov}	3, 5, 13, 15, 17, X	SEG	3, 12, 13, 14, 15
$SElU_{bov}$	17	SElU	13, 14
$SElY_{bov}$	4, **7**, 10, 16, **24**, 28, X	$SElY_{bov}$	1, 3, 5, 6, **7**, 21, 22, 23, **24**
$SElZ_{bov}$	1, 3, 7, 11, 16, 17, 24, 28, X	$SElZ_{bov}$	13.2
$SElO_{bov}$	N/A	SElO	5, 7, 22

628 [a] Vβ subfamilies were named according to the classification of Arden *et al* (47).

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629 [b] Bovine and human Vβ subfamilies activated in response to the same SAg are highlighted in
630 bold.
631 [c] N/A, not applicable.
632 [d] HumVβ activation data was compiled from the references or this study for SElY and SElZ
633 (11, 12, 14, 15)
634
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841 ## Figure Captions

842 **Figure 1: RF122 encodes SAg from all four phylogenetic groups. a)** Maximum-
843 Likelihood phylogenetic tree of 26 superantigen protein sequences showing clustering of
844 SAgs into 4 general groups (1). Branches with more than 80% bootstrap support are marked
845 with black or grey circles. SAgs present in the RF122 strain are indicated by asterisks. **b)** A
846 circular representation of the genome of reference strain RF122 showing the location of SAg
847 genes.

848

849 **Figure 2: Bovine isolates of *S. aureus* typically encode 5 or more SAg genes**. Distribution
850 analysis of SAgs in bovine *S. aureus* isolates showing the repertoire of SAgs that are encoded.
851 Phylogeny is based on a core genome alignment and major clonal complexes are noted.
852 Coloured boxes indicate the presence of the SAg gene and are sorted according to association
853 with mobile genetic elements.

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855 **Figure 3: RF122 SAgs are expressed *in vitro* and exhibit growth phase dependent**
856 **expression.** Transcription levels of RF122-encoded SAg genes from exponential and
857 stationary cultures, relative to 16S rRNA. Relative quantities of RF122 reverse-transcribed
858 mRNA normalized to the internal control *16SrRNA*, determined by qRT-PCR. Results shown
859 are the means of triplicate experiments and error bars indicate ± S.D.
860
861 **Figure 4: Proliferation of bovine T cell populations in response to stimulation with**
862 **RF122-encoded SAgs.** PBMC proliferation after 4 d exposure to **a)** RF122-8 supernatants
863 containing SAgs, and **b)** recombinant SAg proteins as indicated by the incorporation of [^{3}H]
864 thymidine. Results shown are the means of at least triplicate measurements from 2 animals ±
865 S.E.M
866
867 **Figure 5: RF122-encoded SAgs are able to stimulate all Vβ subsets of the bovine T cell**
868 **population.** Relative fold change in bovine Vβ expression after stimulation with RF122
869 SAgs. Bovine Vβ subfamilies were named according to the classification of Arden *et al*
870 (1995). The bTRBV analysed are functional genes tested previously (14). Bovine T cells
871 were stimulated with supernates from induced RF122-8α containing pALC2073::SAg
872 constructs (a-f) or purified recombinant proteins (g-j). Relative fold change in bovine Vβ
873 expression after stimulation with human and bovine alleles of SElY and SElZ. Results given
874 as mean fold change in expression ± SEM of 6 measurements, 3 each from two animals. *
875 Indicates expansion of a subfamily based on a significant increase from the baseline (p
876 <0.05). Expansion profiles of all 11 SAgs from RF122 are summarised (k)
877
878 **Figure 6: SAgs exhibit host-dependent functional activity. a)** PBMC proliferation after 4 d
879 exposure to bovine and human alleles of SElY and SElZ as indicated by the incorporation of
880 [^{3}H] thymidine. Results shown are the means of at least triplicate measurements from 3
881 donors ± SEM. Differences between proliferation induced by human and bovine variants of
882 these SAgs was assessed using Two-way ANOVA with Holm-Sidak's multiple comparisons

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883 test, asterisk denotes curves are significantly different (* p <0.05, ** p <0.01). **b)** Expansion

884 index of Vβ human $CD3^+$ cells after stimulation with human and bovine alleles of SElY and

885 SElZ. Expansion index was determined from the mean of three measures from 2 donors ±

886 SEM. * Indicates expansion of a subfamily based on a significant increase from the baseline

887 (p <0.05) and an expansion index > 1 **c)** Relative fold change in bovine Vβ expression after

888 stimulation with human and bovine alleles of SElY and SElZ. Results given as mean fold

889 change in expression ± SEM of 9 measurements, 3 each from three animals. * Indicates

890 expansion of a subfamily based on a significant increase from the baseline (p <0.05).

891

892 **Figure 7: SAgs promote clinical bovine mastitis.** Number of animals infected with RF122

893 or RF122-8, which exhibited evidence of clinical mastitis at any point during the 21 days of

894 the trail. Clinical mastitis in this experiment was defined as observable inflammation in any of

895 the four quarters of the cow's udder during the study.

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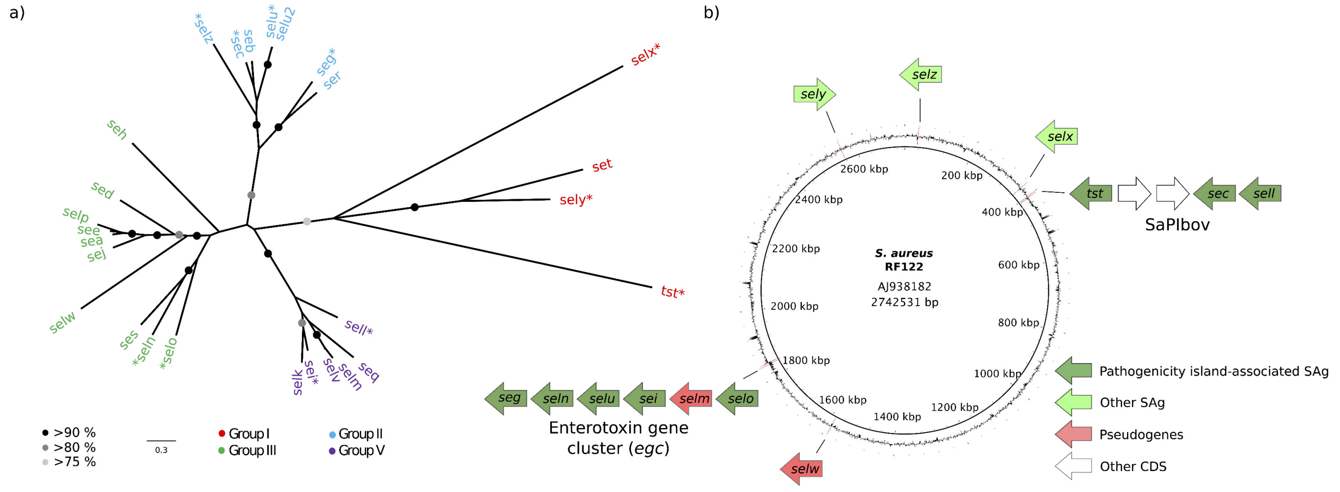

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Tree scale: 0.01

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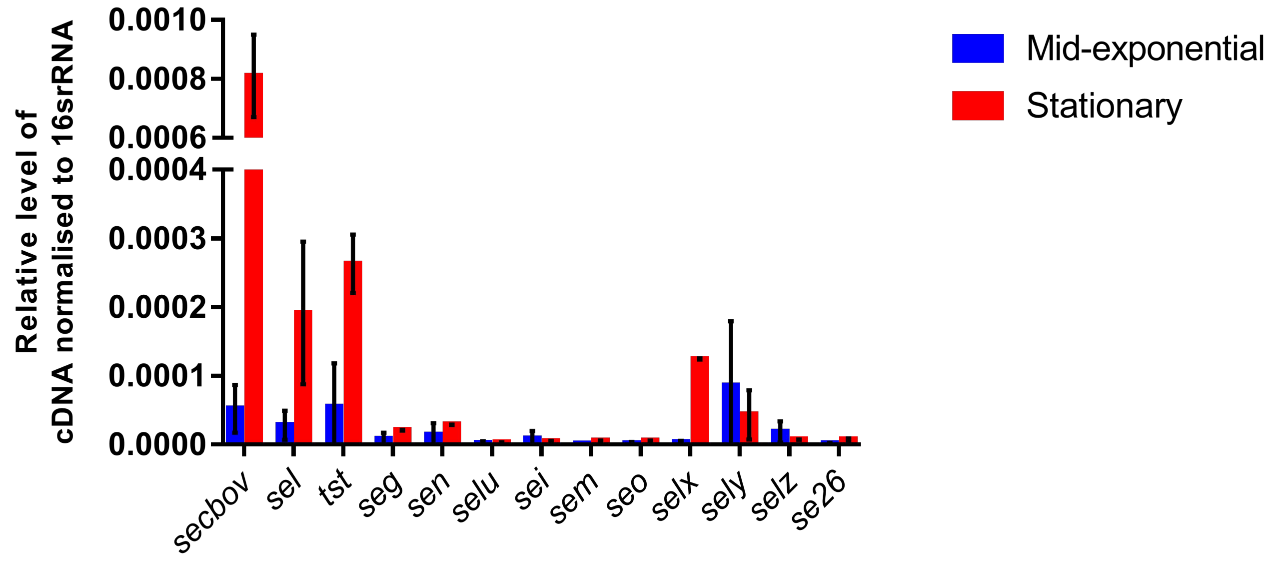

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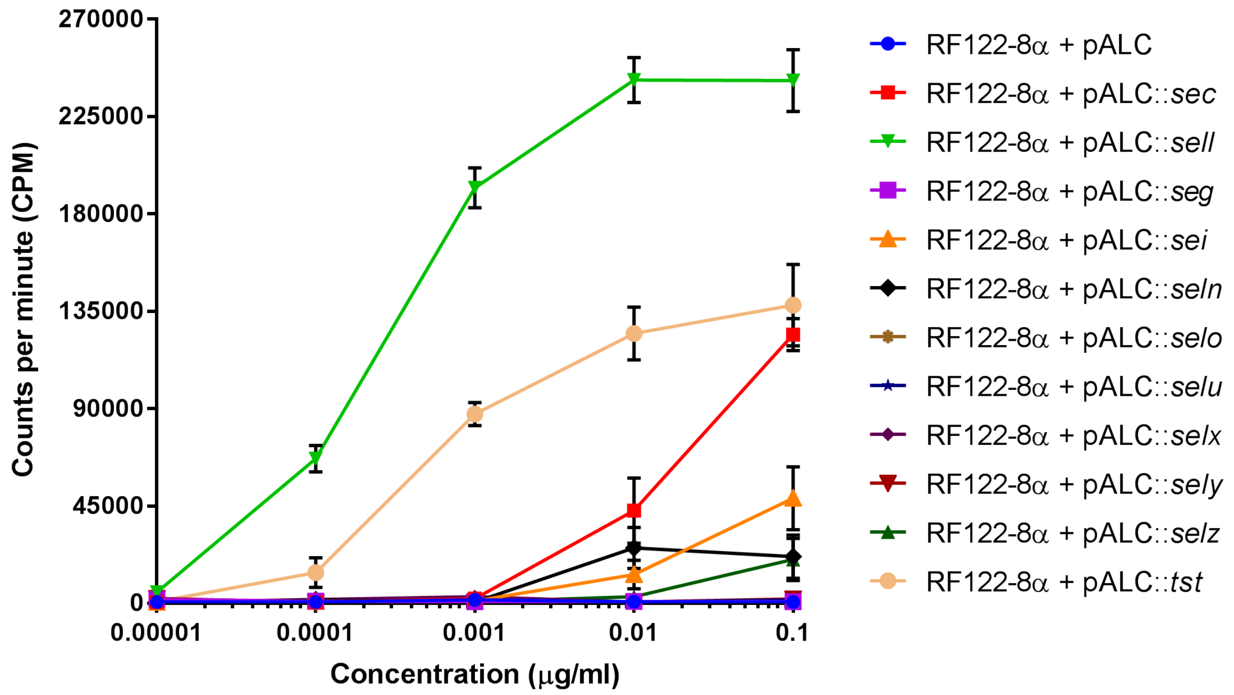

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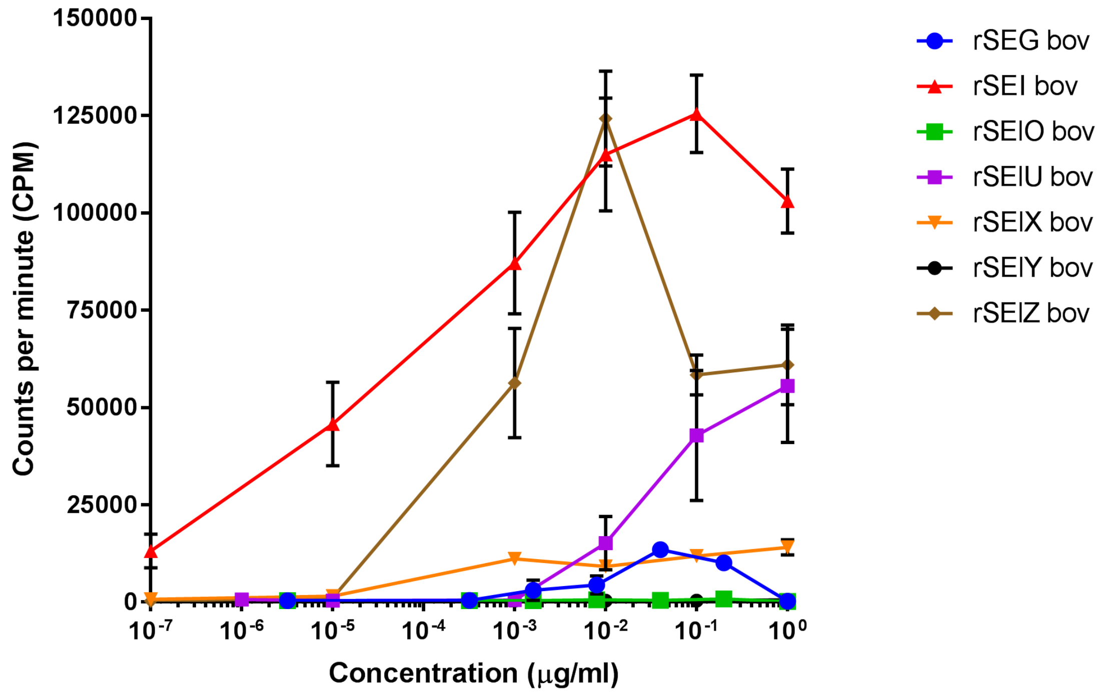

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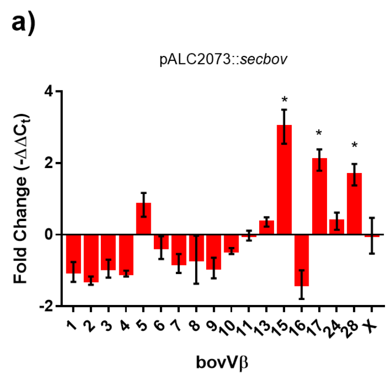

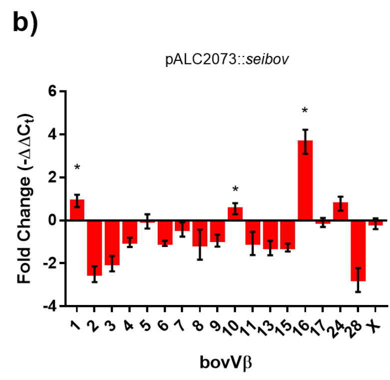

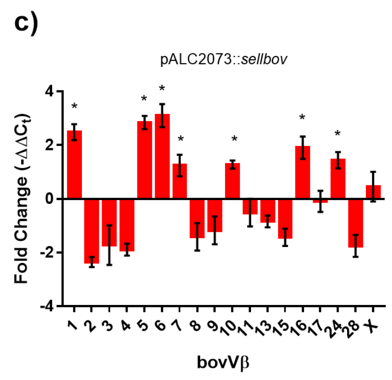

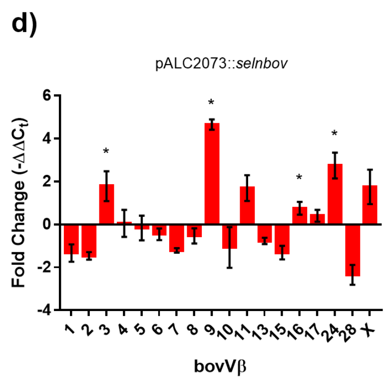

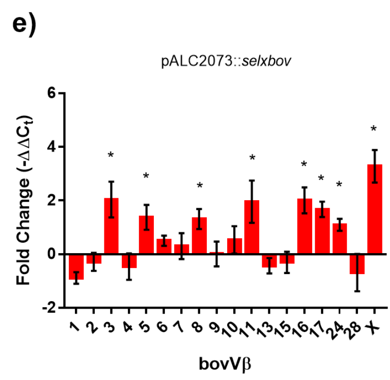

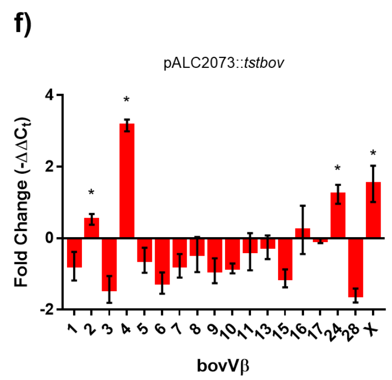

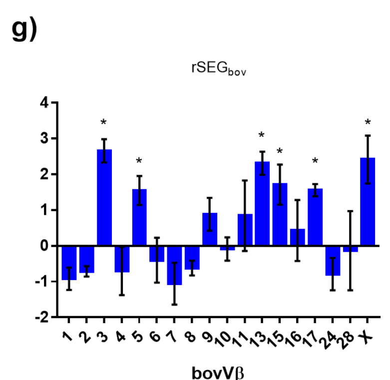

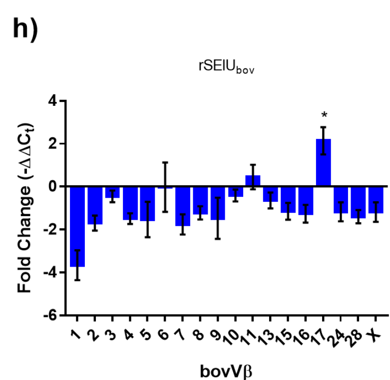

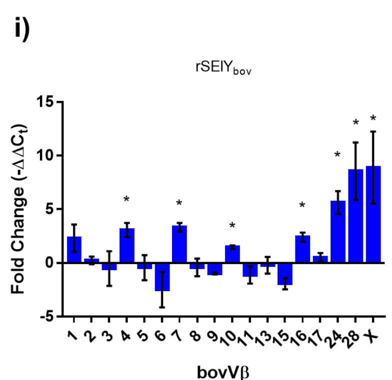

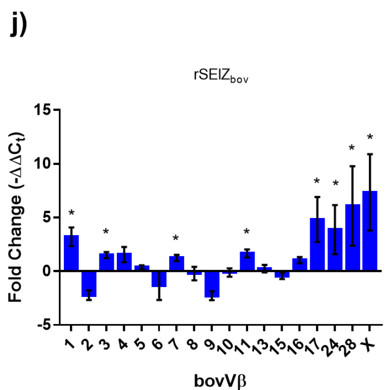

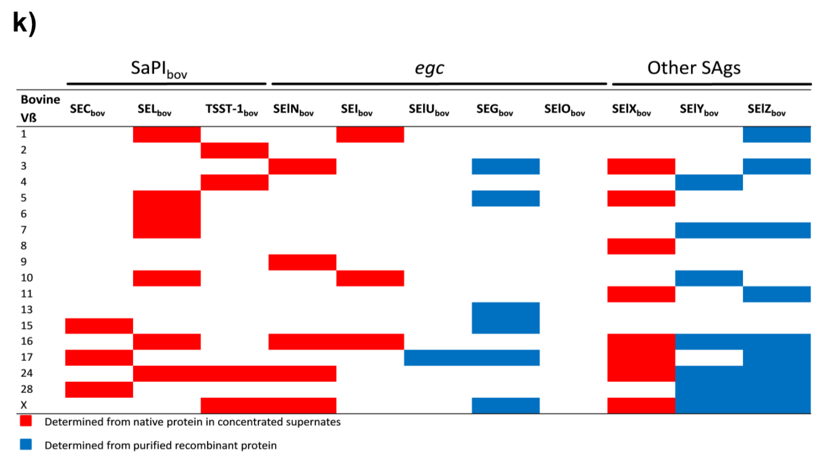

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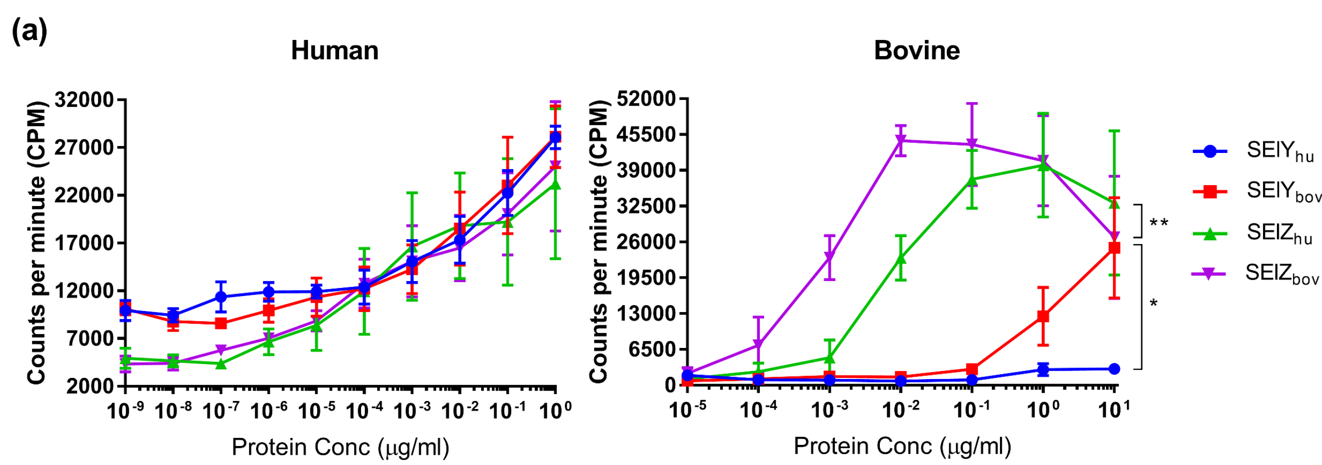

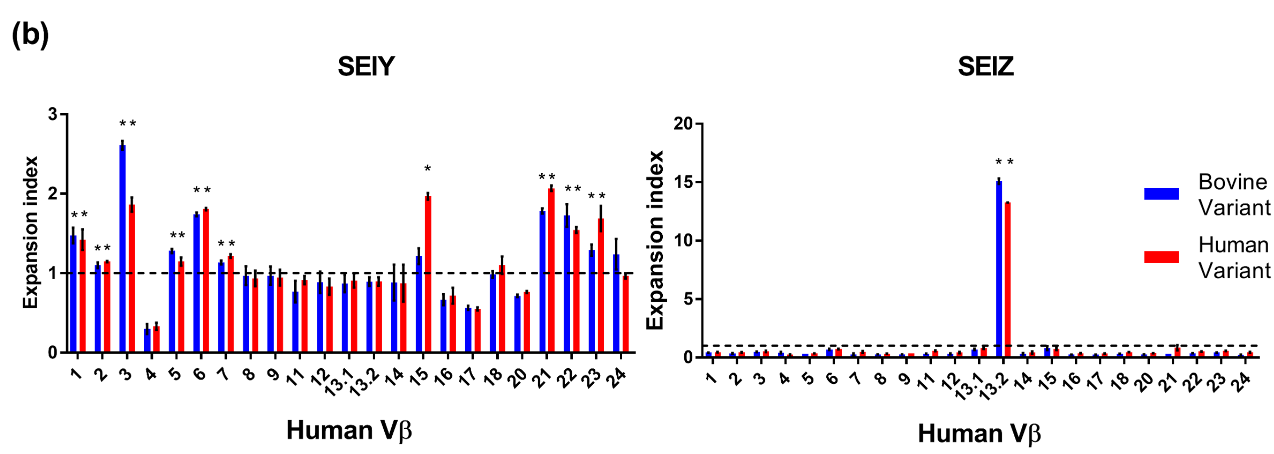

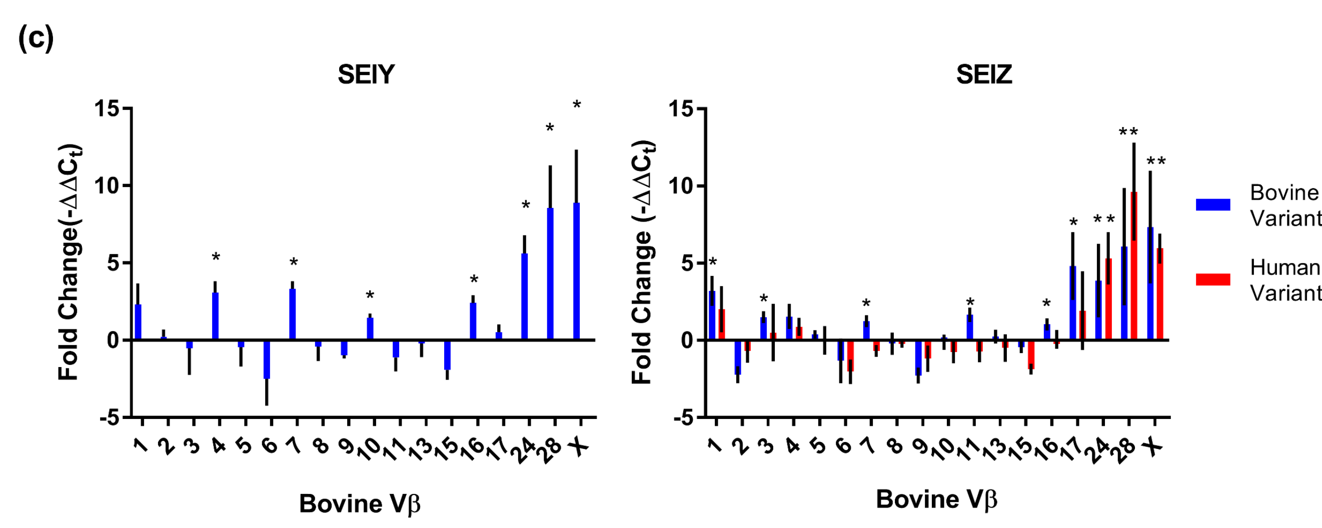

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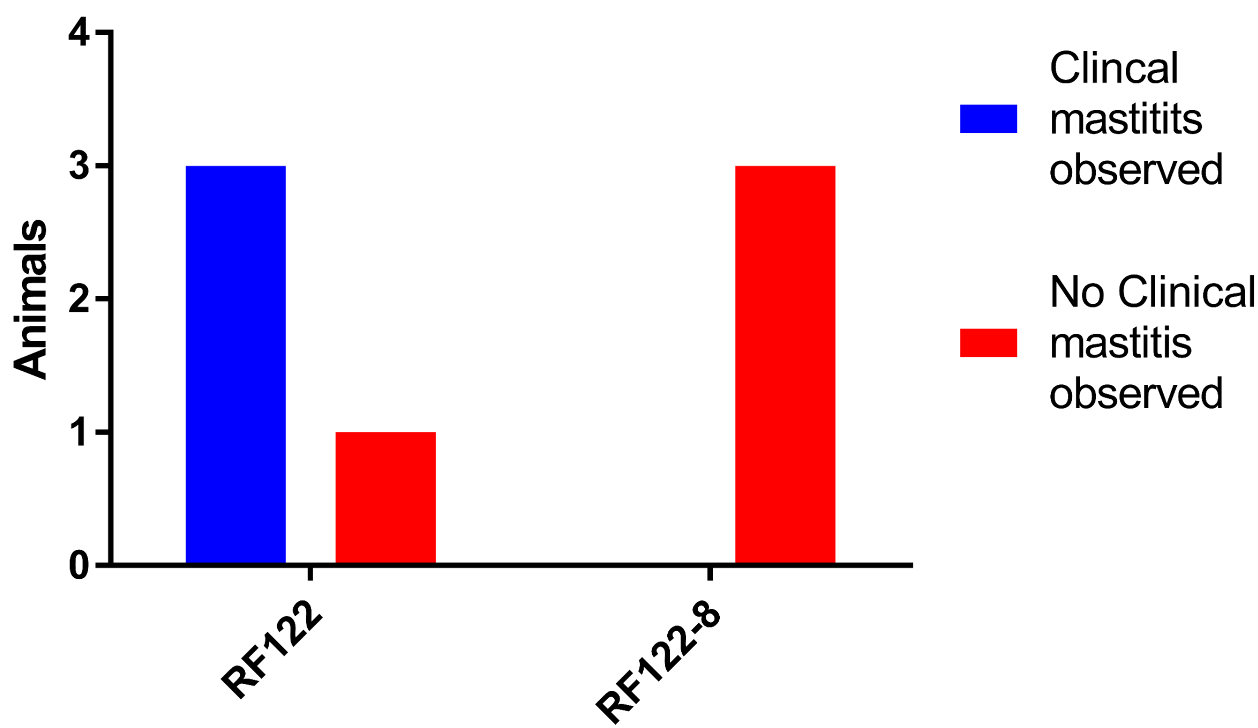

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