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Septic arthritis in an in vivo murine model induced by Staphylococcus aureus; a comparison between actions of the Hla toxin and the effects of the host immune response.

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Septic arthritis in an *in vivo* murine model induced by *Staphylococcus aureus*; a comparison between actions of the Hla toxin and the effects of the host immune response.

Abstract

Aims

Staphylococcus aureus is a major cause of septic arthritis and *in vitro* studies suggest alpha haemolysin (Hla) is responsible for chondrocyte death. We used an *in vivo* murine joint model to compare inoculation with wild type *S. aureus* 8325-4 with a Hla-deficient strain DU1090 on chondrocyte viability, tissue histology and joint biomechanics. The aim was to compare the actions of *S. aureus* Hla alone with those of the animal's immune response to infection.

Methods

Adult male C57Bl/6 mice (N=75) were randomised into 3 groups to receive 1.0-1.4x10^7cfu/mL of 8325-4, DU1090 or saline into the right stifle joint. Chondrocyte death was assessed by confocal microscopy. Histological changes to inoculated joints were graded for inflammatory responses along with gait, weight changes and limb swelling.

Results

Chondrocyte death was greater with 8325-4 (96.2±5.5%;P=0.000872) than DU1090 (28.9±16.0%;P=0.00912) and both were higher than controls (3.8±1.2%). Histology revealed cartilage/bone damage with 8325-4 or DU1090 compared to controls (P=0.010). Both infected groups lost weight (P=0.00643 for both) and experienced limb swelling (P=0.043;P=0.018 respectively). Joints inoculated with bacteria showed significant alterations in gait cycle with a decreased stance phase, increased swing phase and a corresponding decrease in swing speed.

Conclusions

Murine joints inoculated with Hla-producing 8325-4 experienced significantly more chondrocyte death than those with DU1090 which lack the toxin. This was despite similar immune responses indicating that Hla was the major cause of chondrocyte death. Hla-deficient DU1090 also elevated chondrocyte death compared to controls suggesting a smaller additional deleterious role of the immune system on cartilage.

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Article focus.

- Septic arthritis caused by *Staphylococcus aureus* is a highly destructive disease which can rapidly cause permanent damage to articular cartilage which initially may not be evident macroscopically.
- However, it is unclear what the effects of Hla, the key toxin produced by *S. aureus*, are on cartilage cell (chondrocyte) viability, tissue histology and joint biomechanics, compared to the actions of the inflammatory response by the host's immune system.
- Using an *in vivo* murine model of joint infection, we have used two strains of *S. aureus*, one which produces Hla (*S. aureus* 8325-4) and the other which does not (*S. aureus* DU1090) to test the hypothesis that the Hla toxin is the primary cause of deleterious effects of *S. aureus* infection.

Key messages.

- There was significantly more chondrocyte death in murine joints inoculated with Hla-producing *S. aureus* 8325-4 compared to the Hla-deficient strain *S. aureus* DU1090.
- The effect of the immune responses following inoculation of the two strains was equivalent demonstrating a small additional damaging effect of the immune response to *S. aureus* infection.
- In septic arthritis caused by *S. aureus*, the Hla toxin is a major cause of chondrocyte death, which may lead to subsequent cartilage and joint damage.

Strengths and limitations of this study.

- This study involved the controlled inoculation of bacteria directly into the joint of an *in vivo* murine model.
- The bacterial strains used allowed the determination of the effects of the Hla toxin separate from the inflammatory response on chondrocyte viability, tissue histology and joint biomechanics.

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- The effect of the immune response resulting from both *S. aureus* strains was relatively minor. However, which component(s) of bacteria and/or their secretions were responsible for this was unknown.
- The details of the immune response to the *S. aureus* strains in terms of changes in cytokines and chemokines in blood and local joints, and immune cells in synovial tissues, have not been studied and compared.

Key Words: septic arthritis; *Staphylococcus aureus*, Hla toxin, cartilage, chondrocyte

Introduction

Septic arthritis is a feared disease and a medical emergency because it can rapidly destroy joints if untreated, leading to significant morbidity and in some cases death[1]. It arises when organisms (predominantly bacteria) gain access to the synovial space where they produce a range of factors including toxins, enzymes and adhesins which either directly or indirectly cause cartilage destruction. These factors can have a direct effect on the viability of articular cartilage and resident chondrocytes, but additionally, bacterial invasion will initiate a strong host inflammatory response by the synoviocytes. This leads to the release of potent pro-inflammatory cytokines e.g. interleukin-1β (IL-1β), interleukin 6 (IL-6) and TNF-α[2] resulting in tissue oedema, hyperaemia and migration of inflammatory cells into the joint space. The cytokines may also have an indirect effect on cartilage health through their action on chondrocytes leading to the production/release of degradative enzymes (e.g. MMP-13) leading to cartilage breakdown[3] which develops over a longer time period. The pathogenesis of acute septic arthritis is thus multifactorial and the combined presence of bacterial virulence factors and immune response will contribute to cartilage damage[4] however, their relative contributions are unknown. Separating out the contributions of the various factors and their time course following infection is challenging but essential for effective therapeutic strategies are to be adopted.

While antibiotic treatment with early and aggressive joint lavage appears to have a satisfactory initial outcome, evidence suggests that in the long term, there may be a proportion of

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patients who develop symptoms of osteoarthritis in their previously infected joints[5]. This suggests that damage to the cartilage and/or chondrocytes which was not initially evident, might have occurred leading to progressive and inevitable deterioration of cartilage. As cartilage is incapable of effective repair once damaged, this could pre-dispose, initiate or accelerate osteoarthritis development[6].

Although many different types of bacterial species have been implicated in septic arthritis, the predominant causative organism is *Staphylococcus aureus*, accounting for 40-65% of cases[7,8]. The laboratory 'wild-type' strain *S. aureus* 8325-4 is a well-characterised prophage-cured derivative of strain NCTC8325[9,10] and a valuable resource for *S. aureus* research[11]. *S. aureus* 8325-4 produces large amounts of Hla (also known as alpha or α-toxin), Hlb, Hlg, Hld, protein A, lipase hyaluronate, staphylokinase, metalloproteinase, serine proteinase, coagulase, nuclease and acid phosphatase but does not produce the enterotoxins PVL (Panton-Valentine leukocidin) or TSST-1 (Toxic Shock Syndrome Toxin-1)[12-14].

Of all the toxins and other factors produced by *S. aureus* 8325-4, Hla has been implicated as having the dominant catastrophic effect on chondrocyte viability[15]. Using a bovine cartilage explant model, and by assessing *in situ* chondrocyte viability using confocal laser scanning microscopy (CLSM), Smith *et al*.,[15] demonstrated that *S. aureus* 8325-4 which produces all the haemolysins and other toxins, rapidly reduced chondrocyte viability with >45% dead after 40hrs. By utilising a selection of isogenic mutants originating from *S. aureus* 8325-4, Smith *et al*.[15] identified Hla as the dominant toxin primarily responsible for chondrocyte death and showed that there was minimal cell death from the other haemolysins/toxins. One of the isogenic mutants of *S. aureus*, labelled DU1090, was particularly useful as it is identical to 8325-4 other than an inability to produce Hla[15]. An inactivated Hla gene which carries an associated erythromycin resistance marker (*hla*::Emr)[16], allows selective growth of DU1090 in antibiotic-loaded culture media.

In this investigation, we have used a murine *in vivo* model of septic arthritis, to compare joint infection by direct inoculation of *S. aureus* 8325-4 (Hla +ve) with *S. aureus* DU1090 (Hla –ve) (with a vehicle control) into the stifle joint on chondrocyte viability, histological properties of joint tissues together with the animal's gait, weight and limb swelling. This study therefore permits a

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comparison between the deleterious actions of *S. aureus* Hla alone and with any damaging secondary effects of the host's immune response.

Materials and methods

Bacterial strains

Cultures were prepared and selective growth of DU1090 performed in antibiotic (erythromycin; 10µg/mL) - loaded culture media as described[15].

Animals, Randomisation and Inoculation

Seventy-five adult male C57Bl/6 mice aged 12wks were utilised for these experiments following a Power analysis as described (see Supplemental Information). On day 0, mice were randomised equally between 3 groups (**see Supplemental Fig.1**) by a technician not involved in the research and given a unique identification code. The technician opened a sealed envelope for each mouse and then drew up the following experimental solutions into separate unlabelled Harrison syringes (i) a 10µL injection of 1.0-1.4x10^7 colony forming units (cfu)/mL of *S. aureus* 8325-4 or (ii) a 10µL injection of the same concentration range of *S. aureus* DU1090 or (iii) a control group (10µL of PBS) (25 mice/group). The allocations of mice to the randomisation groups were recorded and were not accessible until the final analysis of results. The research team were blinded to the allocations until after final data analysis.

Injection and Dissection protocol

Injections using single-use 30G needles were given into the right stifle joint of anaesthetised mice *via* the transpatellar approach under an operating microscope. Inoculation methods were trialled on cadaveric mice stifle joints with methylene blue to confirm correct needle placement prior to live procedures. For the preparation of cartilage samples, it was important to avoid any dissection close to the articular surface. Careful aseptic techniques to remove the proximal soft tissues, releasing attachments from the periosteum and then inverting the quadriceps and patella, produced confocal images with negligible chondrocyte death **(Fig. 1A).**

Confocal Laser Scanning Microscope (CLSM) and Imaging

An upright Zeiss LSM510 Axioskop (Carl Zeiss, Welwyn Garden City, UK) CLSM, with a x10 NA=0.3 objective, was used to acquire optical sections of cartilage. *In situ* living chondrocytes were labelled with 5-chloromethylfluorescein diacetate (CMFDA; green) whereas dead chondrocytes

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were labelled with propidium iodide (PI; red)[17-19]. Images were obtained in the axial plane to visualise chondrocytes at the articular cartilage surface within the patellofemoral groove. The percentage (%) of dead chondrocytes ((number of dead cells/number of living and dead cells)x100%) was determined within the region of interest (ROI;512 x 512 pixels, corresponding to 460.5μm x 460.5μm) using IMARIS software (version 8.0.2 Bitplane, Oxford Instruments, Zurich, Switzerland for live/dead cell counts[17]. Ten mice per group were sacrificed for CLSM at 2d after injection.

Histology and Evaluation

Five mice per group were sacrificed at day 2 to observe histological evidence of localised joint infection to compare with CLSM results. A further five mice per group were sacrificed for histology after 7d of infection to ascertain whether any difference in chondrocyte death observed after 2d at the same time translated into increased histological evidence of cartilage damage. Following animal termination, whole limbs were fixed (4% formalin) overnight. Limbs were decalcified in ethylenediaminetetraacetic acid (EDTA) for 4wks on a shaking incubator at 40°C and pH=7, and the solution changed weekly. Following decalcification, limbs were embedded in paraffin wax and 10μm sections cut in the sagittal plane. Sections were stained with haematoxylin and eosin. Blinded grading of the tissue was undertaken using a modified protocol[20]. The parameters assessed were: (a) synovial hypertrophy or hyperplasia, (b) infiltration of inflammatory cells into the extra-articular space (c) pannus formation, and (d) cartilage and/or subchondral bone destruction. Each parameter was given a grade of 0 for normal appearance, 1 for mild changes, 2 for moderate change and 3 for severe change. An overall grade of the histological severity of septic arthritis was calculated as 0=normal, 1-4=mild, 5-8=moderate and 9-12=severe. Three sections for each animal were graded separately and averaged. The scores for each group were averaged to allow comparisons between groups and time points.

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Gait analysis

Five mice per group were used for gait analysis on days 0,1,2,3 and 7. Using the 'CatWalk' gait analysis system (Noldus, Wageningen, The Netherlands). At each time point, 3 runs for each mouse were performed and the mean value used in the analysis.

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Weight changes, limb swelling and confirmation of active infection.

The weight of each mouse was measured initially and then 2d following injection. The ten living animals in each group were weighed on days 4 and 7. Limb swelling was measured on days 0, 2, 4 and 7 for all mice alive at that time point. After 7d, the remaining ten mice in each group were sacrificed and the injected stifle joints opened under aseptic conditions to determine if viable bacteria were present. Samples from the stifle joint were spread onto tryptone soya agar (TSA) plates which were then cultured at 37°C overnight to assess the presence of active infection. Pulsed-field gel electrophoresis (PFGE) was performed[21-23] to confirm that the isolated bacteria were the same as the injected organisms (data not shown).

Statistics

These were performed using SPSS version 21 (IBM Inc., New York, USA). Comparisons of individual gait parameters with the pre-operative values within one group were tested *via* Student's paired t-test. To identify differences between groups at specific time points, one-way ANOVA with Tukey's *post-hoc* analyses were performed. The percentage chondrocyte death, percentage weight change and the percentage limb swelling were compared between groups using ANOVA with *post hoc* Tukey's test if data were parametric or Kruskal-Wallis test if non-parametric. Comparison of overall histology scores or the individual component scores (grouped into 4 categories: normal, mild, moderate, severe) was made using a Chi-squared test. Data were presented as means ± Standard Deviation (SD) with a significant difference accepted when $P<0.05$.

Results

Confirmation of active infection

There was no bacterial growth from the stifle joint cultures of any of the PBS-injected animals whereas those injected with *S. aureus* 8325-4 or DU1090 all had positive cultures. PFGE showed that isolates from the infected groups were *S. aureus* 8325-4 or DU1090 confirming active infection for mice injected with bacteria at end of the 7d experimental period (data not shown).

Chondrocyte viability

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In the control (PBS) group, viability was very high (>95%) demonstrating that techniques for joint dissection, cartilage isolation and fluorescence labelling were effective and not associated with iatrogenic injury **(Fig.1A(a); Fig. 1B).** Some (~30%) chondrocyte death occurred after 48hrs following injection with DU1090 **(Fig.1A(b); Fig. 1B),** however there were no viable chondrocytes (>95% dead) in the cartilage of the patellofemoral groove in the *S. aureus* 8325-4 group (Fig.1A(c)). There were differences between each group (P=0.000764) and chondrocyte death was higher in joints infected with *S. aureus* 8325-4 (P=0.000873) or DU1090 (P=0.000687) compared to controls. Additionally, there was considerably more cell death caused by 8325-4 compared to DU1090 (**Fig.1B**; P=0.000576).

Joint Histology

The histology in the controls from days 2 and 7 did not show any features of septic arthritis (**Fig.2(a);** Table 1). However, in animals infected with 8325-4 or DU1090 on day 2 there was an intense inflammatory response localised to the stifle joint with evidence of extensive infiltration of inflammatory cells within the joint and synovial tissues, pannus formation and synovial hyperplasia. The invading cells had the appearance of neutrophils with a smaller number of macrophages visible. There was no statistically significant difference in the scores for synovitis, infiltrate or pannus between 8325-4 and DU1090 at either time point (Table 1; $P \geq 0.05$ for all comparisons). There was, however, a higher rate of cartilage/bone destruction on day 2 between animals infected with 8325-4 compared to DU1090 (P=0.010) but by day 7 the difference was not significant (P=0.362). Typical histological appearances are illustrated in **Fig. 2(a-c).**

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Weight change

There was no significant difference between the percentage weight change between groups at day 2 (P=0.06). There were, however, differences at day 4 (P=0.00617) and day 7 (P=0.00913; **Fig. 3A).** Tukey's *post-hoc* comparison showed that there was a difference between PBS and both infected groups at day 4 and at day 7 (P=0.00732 for all comparisons). There was no difference between DU1090 and 8325-4 at day 4 (P=0.70) or day 7 (P=0.79).

Limb swelling

Measurements of the anterior to posterior diameter of the right stifle joint showed that animals injected with bacteria experienced significantly more limb swelling than the PBS controls **(Fig. 3B).**

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2 There was a significant difference between the two bacterial-infected groups and the control at day
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4 2 (*P*=0.014) that was maintained to day 7 (*P*=0.026). Tukey's *post-hoc* comparison showed that
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6 there was a difference between PBS and both 8325-4 (*P*=0.043) and DU1090 (*P*=0.018) at day 2
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8 but at day 7 only the difference between PBS and 8325-4 remained significant (*P*=0.040). There
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10 was no difference in limb swelling between DU1090 and 8325-4 at day 2 (*P*=0.912) or day 7
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12 (*P*=0.992; **(Fig. 3B).**
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14 ***Gait results***
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17 The animal's running speed (crossing distance of 20cm/time duration) showed no difference
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19 between groups (*P*=0.211). Animals infected with DU1090 and 8325-4 were thought to require an
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21 increase in the number of steps to complete the runs compared to controls, but this was not
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23 significant (*P*=0.187). Animals injected with 8325-4 or DU1090 showed alterations in their gait
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25 cycle with a decreased stance phase, increase in swing phase and a corresponding decrease in
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27 swing speed for the right hind limb (**Fig. 4**). The infected mice also avoided placing the right hind
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29 limb in a single stance. The differences were significant between infected (8325-4 or DU1090) and
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31 control mice (*P*=0.0341) but there was no significant difference between 8325-4 and DU1090
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33 groups for any parameter.
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35 **Discussion**
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38 We have utilised an *in vivo* murine septic arthritis model to compare the effects of joint
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40 inoculation with *S. aureus* 8325-4 which produces the potent toxin Hla, with that resulting from
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42 injection with *S. aureus* DU1090 which does not produce this toxin. This allowed us to separately
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44 compare the effects of Hla with the inflammatory response on chondrocyte viability, joint histology,
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46 weight change, limb swelling and gait. Two major findings emerged. Firstly, Hla-expressing *S.*
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48 *aureus* 8325-4, caused significantly higher levels of chondrocyte death than the Hla-deficient strain
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50 DU1090. Secondly, chondrocyte death occurred following injection with the Hla-deficient *S. aureus*
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52 DU1090 suggesting a detrimental effect of the immune response either directly or indirectly on
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54 chondrocytes. This suggests that chondrocyte death caused by *S. aureus* infection was not the
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56 result of a single pathway but has a multifactorial pathogenesis.
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59 It was proposed that mice infected with *S. aureus* 8325-4 or DU1090 would mount a similar
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immune response and exhibit equivalent manifestations of septic arthritis. Injection with either

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strain of *S. aureus* resulted in the characteristic appearance of septic arthritis with weight loss **(Fig. 3A), limb swelling (Fig. 3B) and gait changes (Fig. 4)** not seen in controls. Histological analysis demonstrated profound changes within the joint in both experimental groups with an infiltrate of predominately neutrophils and macrophages (Table 1; **Fig.2).** These results, demonstrating no difference between *S. aureus* 8325-4 or DU1090 groups, showed that the effect of the immune response in the two infection groups were similar, supporting our hypothesis.

It was anticipated that infected mice would have reduced chondrocyte viability resulting from the combined effect of *S. aureus* toxins and the action of the immune response to septic arthritis. The CLSM results indicated that chondrocyte death was an early event in septic arthritis **(Fig. 1A&B)** occurring within 48hrs of infection. However, differentiating between the severities of joint damage between the two strains of *S. aureus* through macroscopic observations (weight, limb swelling, gait changes), may not be sensitive enough. Thus, despite the death of almost all (>95%) chondrocytes in the superficial regions of cartilage in the joint infected with *S. aureus* 8325-4, after 2d the histological analysis graded the cartilage damage between 'normal' and 'mild'. This suggests that utilising chondrocyte viability as assessed here as an outcome measure, may allow a more accurate and sensitive analysis of the early events in septic arthritis.

In view of the potent nature of Hla on bovine chondrocytes[17] it was expected that joints infected with the Hla-producing strain *S. aureus* 8325-4 would exhibit higher levels of chondrocyte death than those infected with Hla-deficient strain DU1090. In our model, chondrocyte death caused by 8325-4 was rapid and almost universal (>95%) within 48hrs. However, the Hla-deficient strain DU1090 was not without effect on chondrocyte viability. Thus, after the same time period, chondrocyte death was increased by ~28% compared to the control (3.8%) suggesting a deleterious action by other *S. aureus* toxins and/or the effect of the host's immune response, directly or indirectly on chondrocytes.

Two previous studies have used isogenic mutants of *S. aureus* in murine models of septic arthritis and reported increased cartilage damage by Hla-producing strains[24,25]. However, these investigations induced septic arthritis *via* haematogenous infection and reported differences in the rates and timings of septic arthritis onset between strains. It was unclear from their results whether the increased cartilage damage was due to an enhanced ability of Hla-producing strains to

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establish arthritis or as a direct effect of Hla itself on cartilage. Neither study was able to isolate Hla as the major cause of cartilage damage. In contrast, the initiation of septic arthritis by joint inoculation as performed here, allowed a direct comparison between *S. aureus* 8325-4 and DU1090 without the confounding influence of a variable onset of septic arthritis. The results strengthen those of Smith *et al.,*[15] who demonstrated a catastrophic influence of Hla on chondrocyte viability in a bovine cartilage model. The importance of maintaining viable chondrocytes is essential for cartilage survival. For example, drying animal joints *in vivo* leads to chondrocyte death and subsequent cartilage damage similar to that observed in osteoarthritis[26].

In contrast to Smith *et al.,*[15] who reported minimal chondrocyte death caused by *S. aureus* strains incapable of producing Hla, the present study demonstrated significant cell death following *S. aureus* DU1090 infection *in vivo* (**Fig. 1A & B**). Crucially, the major difference would be the absence of an immune response in the explant model of Smith *et al.,*[15]. This is almost certainly the cause of the enhanced chondrocyte death in the Hla-deficient strain DU1090 observed in our *in vivo* murine model. Thus, it is probable that the chondrocyte death reported here with the Hla-deficient strain was primarily due to an acute inflammatory response rather than the presence of the other toxins produced by this strain.

Our results demonstrating a rapid and damaging effect of Hla on chondrocyte viability, may have clinical relevance for septic arthritis treatment caused by *S. aureus*[7,8]. Current treatment is a combination of intravenous antibiotics and removal of contaminated synovial fluid either through surgical washout or serial aspirations. However, a recent study suggested that deleterious effects of Hla can be rapidly suppressed by a small modification to standard saline (300mOsm; 0.9% NaCl) typically used for joint washout. Using the rabbit haemolysis assay for determining Hla potency, Liu and Hall[27] reported that raising osmolarity of saline to 900mOsm with sucrose and including 5mM $MgCl_2$ could markedly reduce the deleterious effect of Hla. This could provide a cheap, rapid and benign option for immediate protection against Hla during surgical washout.

The identification of the dominant role for Hla causing chondrocyte damage/death during septic arthritis arising from *S. aureus* infection, suggests other potential therapeutic targets for cartilage protection. The receptor for Hla on eukaryotic cells has been identified as A-disintegrin and metalloprotease 10 (ADAM10)[28-30]. Binding of Hla to ADAM10 receptors on chondrocytes

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leads to cell membrane pore formation and influx of Na^+ and water, causing cell lysis[31]. An example of Hla-targeted therapy lies in the choice of antibiotics as shown by Miller *et al.,*[32] whereby a bacteriostatic antibiotic linezolid, showed rapid curtailing of Hla-induced haemolysis in the rabbit erythrocyte assay. The action of linezolid was faster compared to the bacteriolytic antibiotic penicillin. This may be because of the rapid inhibitory effect of linezolid on Hla production whereas the bacteriolytic action of penicillin is slower and/or releases a pool of Hla from dying/dead bacteria. Future studies should aim to identify treatments capable of blocking the action of *S. aureus* Hla e.g., through neutralising antibodies or selective blockade of ADAM10.

The possibility that the inflammatory response is a cause of chondrocyte death may offer some explanation for the improved outcomes seen in children given steroids following washout for septic arthritis[33,34]. However, there is concern regarding uncontrolled infection following steroid administration and with a mortality of 9.2% from septic arthritis (13 inpatient deaths out of 142 patients) seen in a skeletally-mature population (unpublished observations). Thus, it would be premature to suggest that such an intervention is adopted routinely in clinical practice. There are other novel and targeted therapies such as antimicrobial peptides and matrix metalloproteinase-based therapy with direct bactericidal and immunomodulatory effects which show promising early results[35]. However, they will require further work before they are clinically applicable. In any event, the present work utilised an Hla-producing and an Hla-deficient strain of *S. aureus* in an *in vivo* model, and identified two distinct harmful mechanisms on chondrocyte viability during *S. aureus* septic arthritis, (a) rapid cell death produced by Hla, and (b) a delayed inflammatory response also resulting in chondrocyte death. The present recognition of these events may identify future therapeutic targets and protect cartilage from the short and long-term effects of *S. aureus* septic arthritis.

Conflict of interest statement

The authors have no Conflicts of Interests to declare.

Author contributions

Acknowledgements

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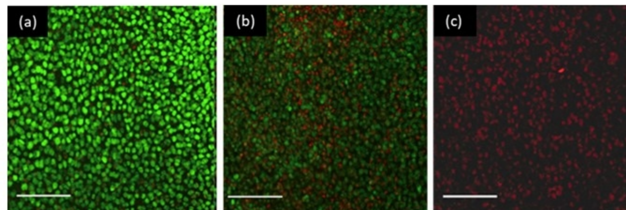
Figure 1

(A)



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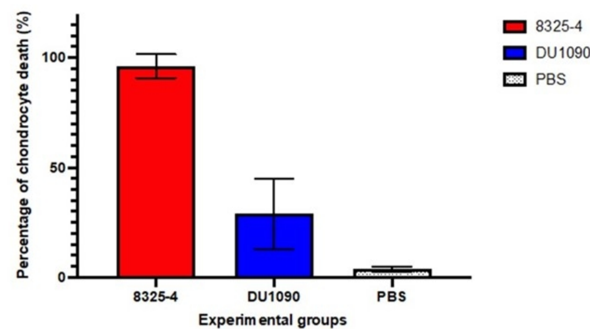

Figure 1. In situ chondrocyte viability in the articular surface of murine cartilage in the patellofemoral groove following joint inoculation. The images shown in Panel (A) are representative confocal images of fluorescently-labelled living (green) or red (dead) in situ chondrocytes labelled as described (see Materials and methods) 48hrs after injection with (a) PBS, (b) S. aureus DU1090 (Hla toxin deficient) or (c) S. aureus 8325-4 (Hla toxin expressing). Scale bar = 100µm. Panel (B) shows the % of chondrocyte death on day 2 following joint injection with S. aureus 8325-4, S. aureus DU1090 or PBS. Chondrocyte death at day 2 for S. aureus 8325-4 group was 96.2±5.5%; for DU1090 group was 28.9±16.0% and for the PBS control 3.8±1.2%. Data shown are means ± S.D. for N=5 separate animals for each group.

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Figure 2

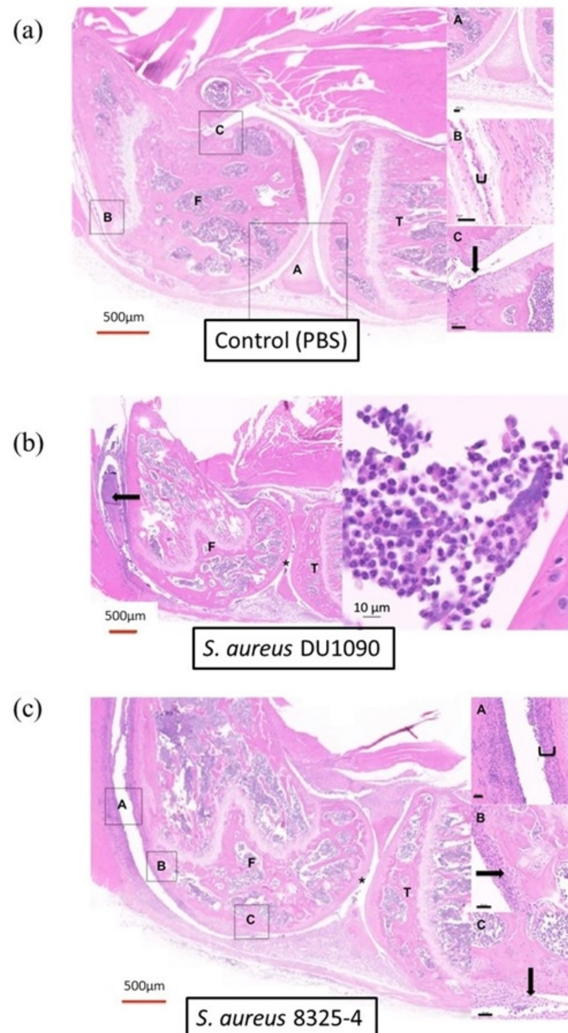

Figure 2. Histological appearance of the right stifle joints of mice following injection with or without bacteria. Panel (a) shows the histology of a control (PBS) joint 7 days following injection illustrating normal joint architecture F=femur, T=tibia. A=normal articular cartilage with no evidence of inflammatory cells within the joint space; B=single layer of synovium; C=normal cartilage-synovium interface with absence of pannus formation. Red scale bar = 500µm, black scale bars = 50µm. Panel (b) shows the histology of a joint 2 days following injection with S. aureus DU1090 including a magnified view of the inflammatory infiltrate. There was an increased joint space secondary to effusion and the presence of inflammatory cells in the joint (horizontal arrow). There was mild synovial hypertrophy visible either side of this and very early pannus formation. The inflammatory infiltrate was dominated by neutrophils. Red scale bar = 500µm. Panel (c) displays the appearance 7d after joint injection with S. aureus 8325-4. Images on the right: (A), (B) and (C) are magnified from the main picture on the left. There was: (A) severe synovial hypertrophy (block), (B) bone erosions (horizontal arrow) and (C) pannus formation (downward arrow). Despite this, the majority of the articular surface appeared to be macroscopically intact (asterisk).

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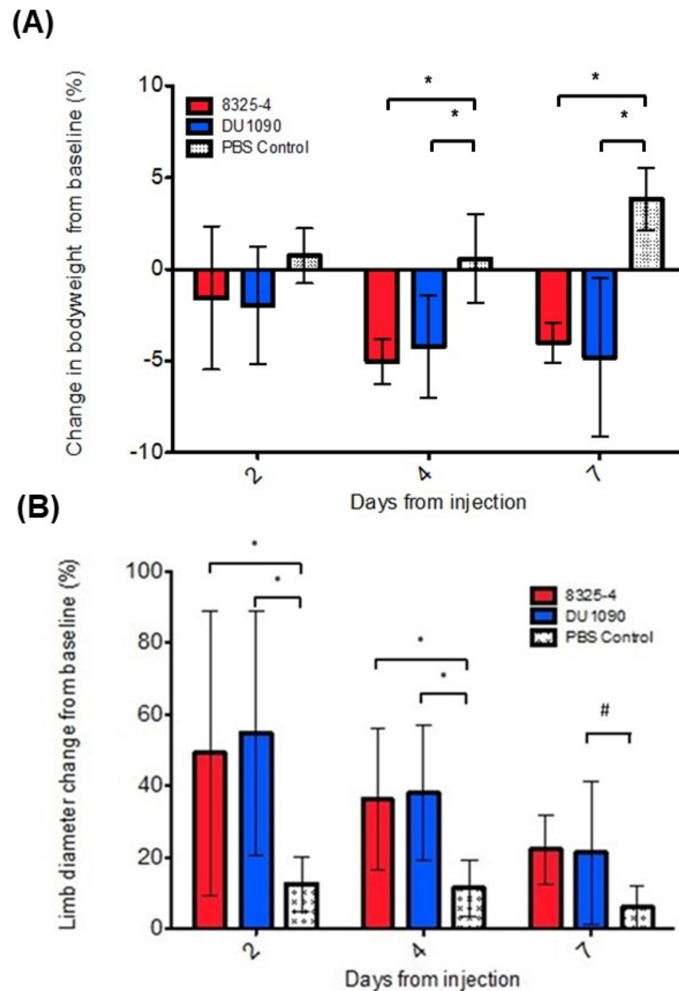

Figure 3. The change in murine bodyweight and limb diameter following joint injection with S. aureus 8325-4, S. aureus DU1090 or PBS. Panel (A) shows the % change in bodyweight 2, 4 and 7 days following injection with S. aureus 8325-4, S. aureus DU1090 or PBS. The asterisks indicate a significant difference between PBS control and both the 8325-4 and DU1090 groups (see text). Panel (B) shows the % change in anterior to posterior limb diameter at 2, 4 and 7 days following injection with S. aureus 8325-4, S. aureus DU1090 or PBS. The single asterisks indicate a significant difference between PBS and infected groups whereas the hash symbol (#) indicated a significance between PBS and 8325-4 only. For both panels, data shown are means ± S.D. for N=10 animals.

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Figure 4

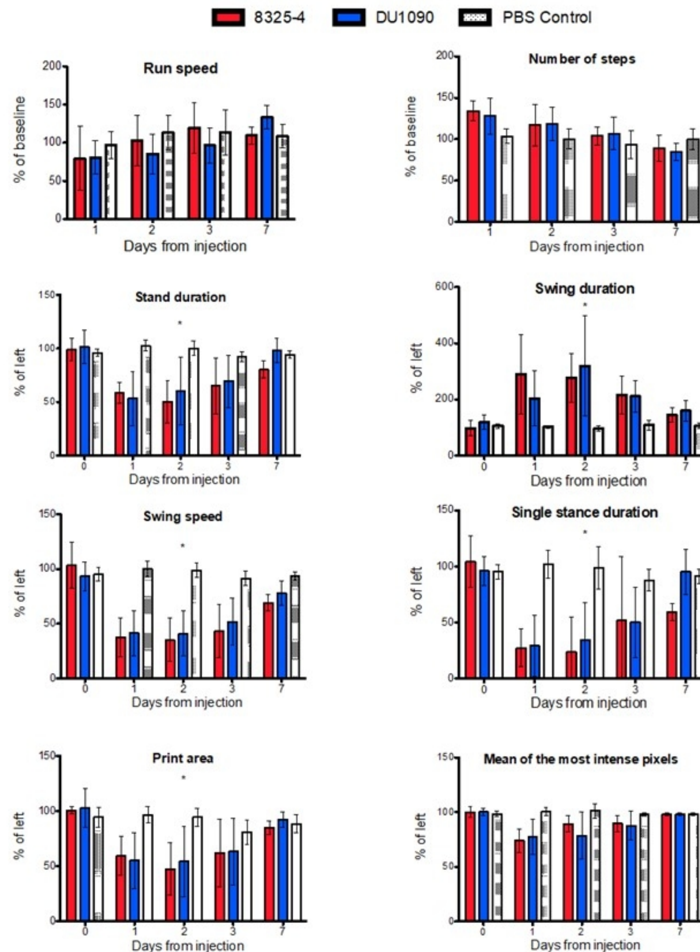

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Figure 4. Gait changes in mice for the three experimental groups over the course of the experiment following inoculation with or without bacteria. These were assessed using the 'CatWalk' automated gait analysis system (see Materials and Methods). The horizontal axis represented the number of days after the injection of the right stifle joint with either S. aureus 8325-4, DU1090 or PBS (control). The vertical axes were given as a percentage of the baseline (pre-inoculation, day 0 values) for the run speed and number of steps, or for the percentage of the left hind limb (stand/swing/single) stance duration, swing speed, print area of right-side foot, mean of most intense pixels. Statistics were only performed for comparison between groups on day 2. Asterisks denoted significant differences (P=0.01) between both experimental groups (8325-4 and DU1090) when compared to the PBS controls. Data shown are means ± S.D. for N=10 animals.

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Table 1

Group	Day	Synovitis	Infiltrate	Pannus	Cartilage/Bone erosion	Total score
8325-4	2	1.6 (0.9)	2.8 (0.4)	0.6 (0.9)	0.8 (0.4)	5.8 (1.5)
	7	2.6 (0.5)	1.4 (0.9)	2.2 (0.8)	1.8 (0.8)	8.0 (2.5)
DU1090	2	1.0 (0.0)	2.6 (0.5)	0.2 (0.4)	0.0 (0.0)	3.8 (0.4)
	7	2.2 (0.4)	2.4 (0.9)	1.8 (0.8)	1.2 (1.1)	7.6 (2.7)
PBS	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 1. Histology scores for the experimental groups at day 2 and day 7. Blinded grading of histological sections stained with haematoxylin and eosin from joints infected with *S. aureus* 8325-4, *S. aureus* DU1090 or control (PBS) were performed (see Materials and Methods). An overall grade of the histological severity of septic arthritis was assessed as 0 = normal, 1-4 = mild, 5-8 = moderate and 9-12 = severe. Values in brackets are standard deviations.

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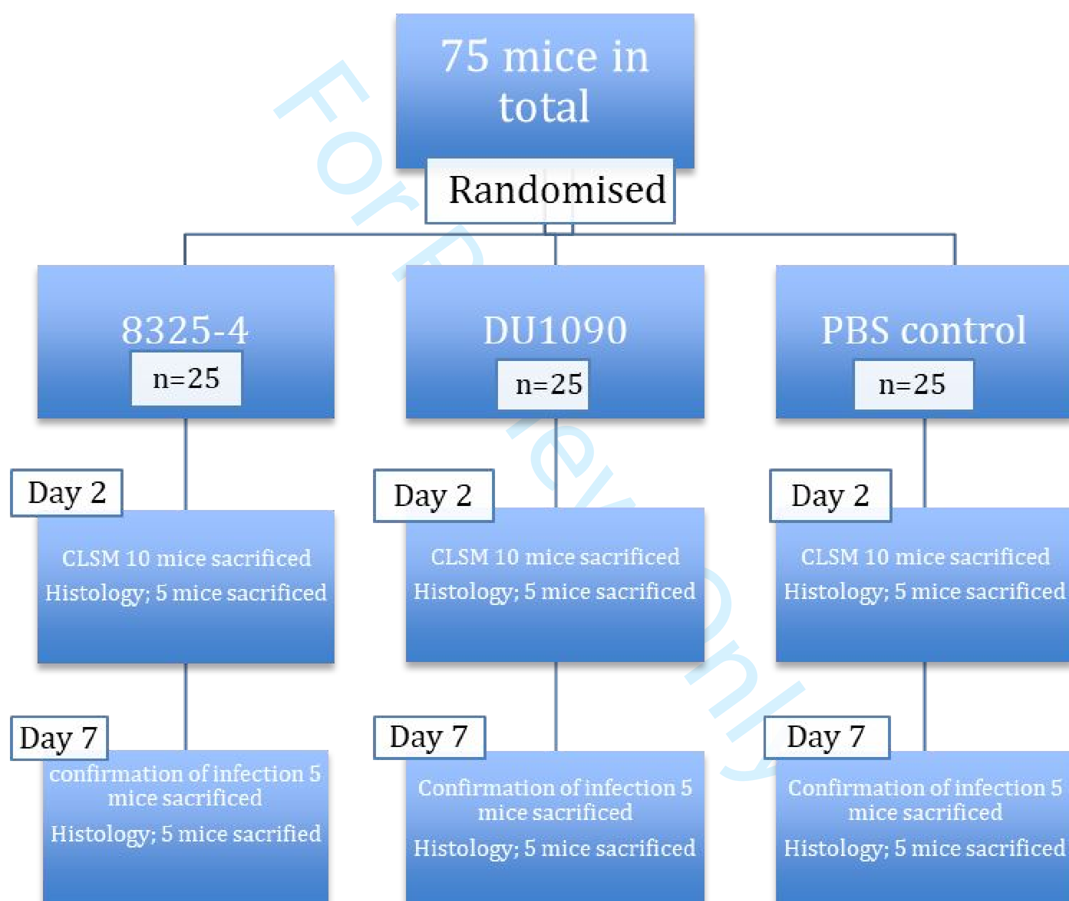
Supplementary Figure 1.

SUPPLEMENTARY Figure 1. A flowchart describing the randomisation of mice into the three experimental groups, number of mice sacrificed at each stage and the points at which histology of tissue was performed. (CLSM = confocal scanning laser microscopy).



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Supplementary Data

A power calculation was performed assuming a difference of 25% in chondrocyte viability between groups and a standard deviation of 10% within measurements for each group. To achieve a power of 90% with a type 1 error of 0.05 and type 2 error of 0.1, eight animals per group would be required for CLSM analysis. However, to account for potential loss of samples or a lower measured percentage difference ten animals/group were used for CLSM at a time point of 2 days after injection.

Five mice per group were used in order to observe for histological evidence of localised joint infection at the time the CLSM analysis was performed (day 2) and to compare the CLSM results with histological evidence of cartilage damage. A further five mice had histological analysis after 7 days of infection to ascertain whether any difference in chondrocyte death observed after 2 days translated into increased histological evidence of cartilage damage at this time. Histological analysis could not be performed on mice used for CLSM, therefore it was necessary to increase the group sizes by 10 animals. The experiment was not specifically powered to find a statistical difference in histology scores between groups as it was felt too many additional animals would be required and contradict the NC3R's principles.

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The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	

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