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ARTHRITIS

Septic arthritis in an in vivo murine model induced by *Staphylococcus aureus*

A COMPARISON BETWEEN ACTIONS OF THE HAEMOLYSIN TOXIN AND THE EFFECTS OF THE HOST IMMUNE RESPONSE

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Aims

Staphylococcus aureus **is a major cause of septic arthritis, and in vitro studies suggest α 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 randomized into three groups to receive 1.0 to 1.4 × 10^7 colony-forming units (CFUs)/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% (SD 5.5%); p < 0.001) than DU1090 (28.9% (SD 16.0%); p = 0.009) and both were higher than controls (3.8% (SD 1.2%)). Histology revealed cartilage/bone damage with 8325-4 or DU1090 compared to controls (p = 0.010). Both infected groups lost weight (p = 0.006 for both) and experienced limb swelling (p = 0.043 and 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.

Conclusion

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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Keywords: Septic arthritis, Hla toxin, Cartilage, Chondrocyte, Staphylococcus aureus

Article focus

- Septic arthritis caused by *Staphylococcus aureus* is a highly destructive disease which can rapidly cause permanent damage to articular cartilage that initially may not be evident macroscopically.
- However, it is unclear what the effects of haemolysin (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

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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

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

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 tumour necrosis factor-α (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. matrix metalloproteinase (MMP)-13) and eventual 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 if effective future 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 patients who develop symptoms of osteoarthritis (OA) 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 predispose, initiate, or accelerate OA 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% to 65% of cases.[7,8] The laboratory 'wild-type' strain *S. aureus* 8325-4 is a well-characterized 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 α or α-toxin), Hlb (β-toxin), Hlg (γ-toxin), Hld (δ-toxin), 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 40 hours. By using 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-positive) with *S. aureus* DU1090 (Hla-negative) (with a vehicle control) into the stifle joint on chondrocyte viability and histological properties of joint tissues, together with the animal's gait, weight, and limb swelling. This study therefore permits a comparison between the deleterious actions of *S. aureus* Hla alone and with

any damaging secondary effects of the host's immune response.

Methods

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

Animals, randomization, and inoculation. A total of 75 adult male C57Bl/6 mice aged 12 weeks were used for these experiments following a power analysis as described in the Supplementary Material. On day 0, mice were randomized equally between three groups (see Supplementary Figure a) 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: 1) a 10 µl injection of 1.0 to 1.4 × 10^7 colony-forming units (CFUs)/ml of *S. aureus* 8325-4; 2) a 10 µl injection of the same concentration range of *S. aureus* DU1090; or 3) a control group (10 µl of phosphate-buffered saline (PBS)) (25 mice/group). The allocations of mice to the randomization 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. We have included an ARRIVE checklist to show that we have conformed to the ARRIVE guidelines.

Injection and dissection protocol. Injections using single-use 30 G needles were given into the right stifle joint of anaesthetized 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 (Figure 1a).

Confocal laser scanning microscope and imaging. An upright Zeiss LSM510 Axioskop (Carl Zeiss, UK) confocal laser scanning microsope (CLSM), with a ×10 numerical aperture (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 were labelled with propidium iodide (PI; red).[17-19] Images were obtained in the axial plane to visualize 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) ×100%) was determined within the region of interest ((ROI) 512 × 512 pixels, corresponding to 460.5 µm × 460.5 µm) using IMARIS software (version 8.0.2 Bitplane, Oxford Instruments, Switzerland) for live/dead cell counts.[17] Ten mice per group were killed for CLSM two days after injection.

Histology and evaluation. Five mice per group were killed at day 2 to observe histological evidence of localized joint infection to compare with CLSM results. A further five mice per group were killed for histology after seven days of infection to ascertain whether any difference in chondrocyte death observed after two days 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 four weeks on a shaking incubator at 40°C and at a pH of 7, and the solution changed weekly. Following decalcification, limbs were embedded in paraffin wax and 10 µm 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: 1) synovial hypertrophy or hyperplasia, 2) infiltration of inflammatory cells into the extra-articular space, 3) pannus formation, and 4) cartilage and/or subchondral bone destruction. Each parameter was given a grade of 0 for normal appearance, 1 for mild change, 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 to 4 = mild, 5 to 8 = moderate, and 9 to 12 = severe. Three sections for each animal were graded separately and the means averaged. The scores for each group were averaged to allow comparisons between groups and timepoints.

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, the Netherlands). At each timepoint, three runs for each mouse were performed, and the mean value used in the analysis.

Weight changes, limb swelling, and confirmation of active infection. The weight of each mouse was measured initially and then two days 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 timepoint. After seven days, the remaining ten mice in each group were killed 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)[21–23] was performed to confirm that the isolated bacteria were the same as the injected organisms (data not shown).

Statistical analysis. Statistical analysis was performed using SPSS version 21 (IBM, USA). Comparisons of individual gait parameters with the preoperative values within one group were tested via paired *t*-test. To identify differences between groups at specific timepoints, one-way analysis of variance (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

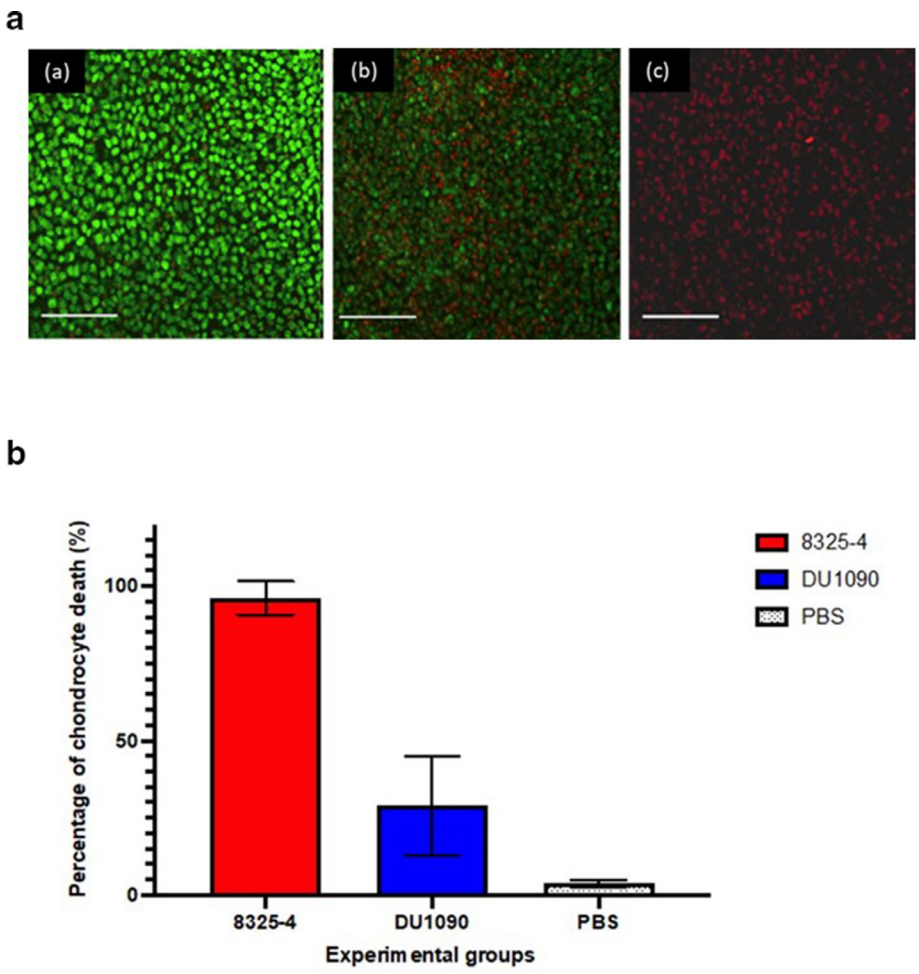

Fig. 1

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 in the Methods section, 48 hours after injection with (a) phosphate-buffered saline (PBS), (b) *Staphylococcus aureus* DU1090 (haemolysin (Hla) toxin-deficient), or (c) *S. aureus* 8325-4 (Hla toxin-expressing). Scale bar = 100 µm. 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% (standard deviation (SD) 5.5%), 28.9% (SD 16.0%) for DU1090 group, and 3.8% (SD 1.2%) for the PBS control. Data shown are means and standard deviations (SDs) for five separate animals for each group.

if data were parametric, or Kruskal-Wallis test if non-parametric. Comparison of overall histology scores or the individual component scores (grouped into four categories: normal, mild, moderate, severe) was done using a chi-squared test. Data were presented as means and standard deviations (SDs), 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 the end of the seven-day experimental period (data not shown).

Chondrocyte viability. 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 (Figures 1a and 1b). Some (~30%) chondrocyte death occurred after 48 hours following injection with DU1090 (Figures 1a and 1b), however there were no viable chondrocytes (> 95% dead) in the cartilage of the patellofemoral groove in the *S. aureus* 8325-4 group (Figure 1a). There were differences between each group ($p < 0.001$, one-way ANOVA with post-hoc Tukey's test) and chondrocyte death was higher in joints infected with *S. aureus* 8325-4 ($p < 0.001$, one-way ANOVA with post-hoc Tukey's test) or DU1090 ($p < 0.001$, one-way ANOVA with post-hoc Tukey's test) compared to controls. Additionally, there was considerably more cell death

caused by 8325-4 compared to DU1090 (Figure 1b; $p < 0.001$, one-way ANOVA with post-hoc Tukey's test).

Joint histology. The histology in the controls from days 2 and 7 did not show any features of septic arthritis (Figure 2a and Table I). However, in animals infected with 8325-4 or DU1090 on day 2, there was an intense inflammatory response localized 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 timepoint (Table I; $p \geq 0.05$ for all comparisons, one-way ANOVA with post-hoc Tukey's test). There was, however, a higher rate of cartilage/bone destruction on day 2 between animals infected with 8325-4 compared to DU1090 ($p = 0.010$, chi-squared test) but by day 7 the difference was not significant ($p = 0.362$, chi-squared test). Typical histological appearances are illustrated in Figures 2a to 2c.

Weight change. There was no significant difference between the percentage weight change between groups at day 2 ($p = 0.060$). There were, however, differences at day 4 ($p = 0.006$) and day 7 ($p = 0.009$, one-way ANOVA with Tukey's post-hoc comparison; Figure 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 ($p = 0.007$ 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 (Figure 3b). There was a significant difference between the two bacteria-infected groups and the control at day 2 ($p = 0.014$) that was maintained to day 7 ($p = 0.026$, one-way ANOVA with Tukey's post-hoc comparison). Tukey's post-hoc comparison showed that there was a difference between PBS and both 8325-4 ($p = 0.043$) and DU1090 ($p = 0.018$) at day 2, but at day 7 only the difference between PBS and 8325-4 remained significant ($p = 0.040$). There was no difference in limb swelling between DU1090 and 8325-4 at day 2 ($p = 0.912$) or day 7 ($p = 0.992$; Figure 3b).

Gait results. The animal's running speed (crossing distance of 20 cm/time duration) showed no difference between groups ($p = 0.211$, paired *t*-test). Animals infected with DU1090 and 8325-4 were thought to require an increase in the number of steps to complete the runs compared to controls, but this was not significant ($p = 0.187$, paired *t*-test). Animals injected with 8325-4 or DU1090 showed alterations in their gait cycle with a decreased stance phase, increase in swing phase, and a corresponding decrease in swing speed for the right hind limb (Figure 4). The infected mice also avoided placing the right hind limb in a single stance. The differences were significant between infected (8325-4 or DU1090) and control mice ($p = 0.0341$, one-way ANOVA), but there was no significant difference between 8325-4 and DU1090 groups for any parameter.

Discussion

We have used an in vivo murine septic arthritis model to compare the effects of joint inoculation with *S. aureus* 8325-4, which produces the potent toxin Hla, against that resulting from injection with *S. aureus* DU1090, which does not produce the toxin. This allowed us to separately compare the effects of Hla with the inflammatory response on chondrocyte viability, joint histology, weight change, limb swelling, and gait. Two major findings emerged. First, Hla-expressing *S. aureus* 8325-4 caused significantly higher levels of chondrocyte death than the Hla-deficient strain DU1090. Second, chondrocyte death occurred following injection with the Hla-deficient *S. aureus* DU1090, suggesting a detrimental effect of the immune response either directly or indirectly on chondrocytes. This suggests that chondrocyte death caused by *S. aureus* infection was not the result of a single pathway, but has a multifactorial pathogenesis.

It was proposed that mice infected with *S. aureus* 8325-4 or DU1090 would mount a similar immune response and exhibit equivalent manifestations of septic arthritis. Injection with either strain of *S. aureus* resulted in the characteristic appearance of septic arthritis with weight loss (Figure 3a), limb swelling (Figure 3b), and gait changes (Figure 4) not seen in controls. Histological analysis demonstrated profound changes within the joint in both experimental groups with an infiltrate of predominantly neutrophils and macrophages (Table I; Figure 2). These results, demonstrating no difference between *S. aureus* 8325-4 or DU1090 groups, showed that the effects 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 immune response to septic arthritis. The CLSM results indicated that chondrocyte death was an early event in septic arthritis (Figures 1a and 1b) occurring within 48 hours 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 two days the histological analysis graded the cartilage damage between 'normal' and 'mild'. This suggests that using 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

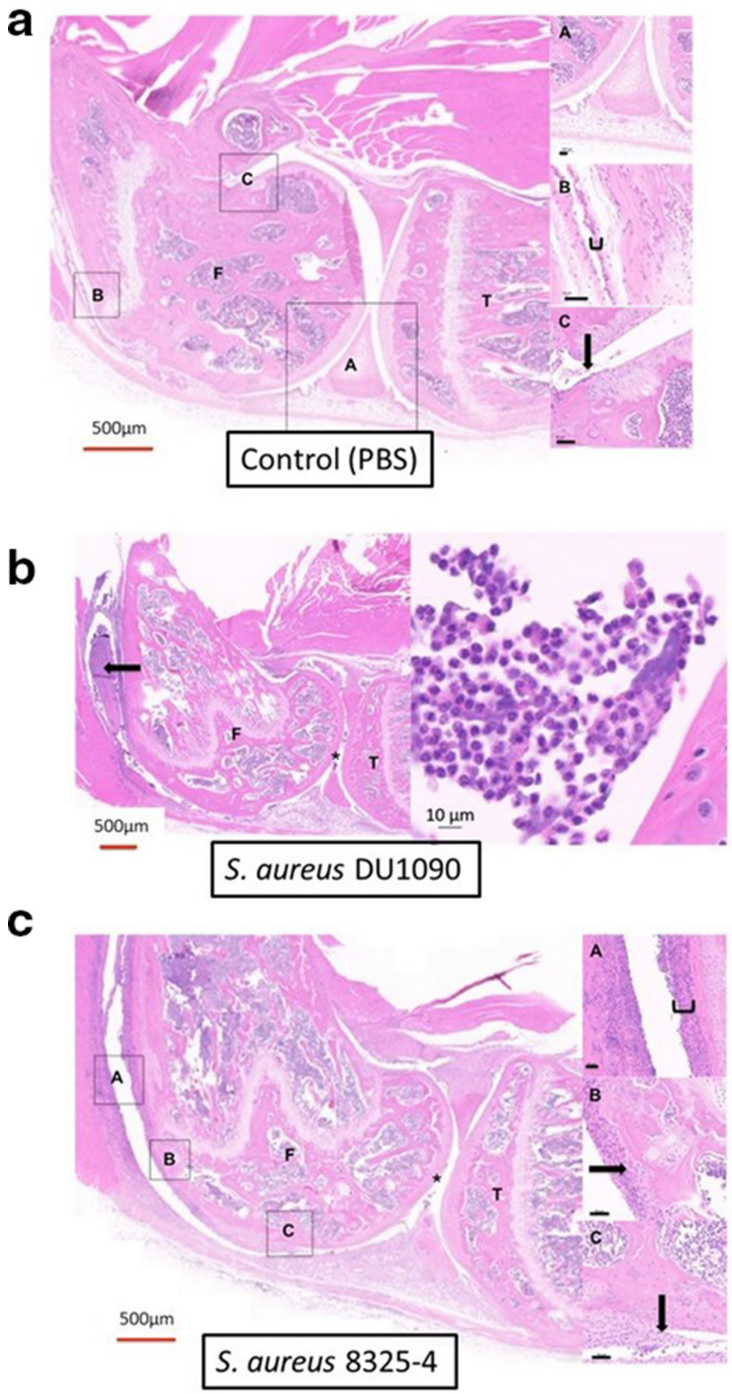

Fig. 2

Histological appearance of the right stifle joints of mice following injection with or without bacteria. a) A control (phosphate-buffered saline (PBS)) joint seven 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; and C = normal cartilage-synovium interface with absence of pannus formation. Red scale bar = 500 µm, black scale bars = 50 µm. b) A joint two days following injection with *Staphylococcus 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 = 500 µm. c) Appearance seven days 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). Magnification: ×60.

Table I. 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 *Staphylococcus aureus* 8325-4, *S. aureus* DU1090, or control (phosphate-buffered saline) was performed. An overall grade of the histological severity of septic arthritis was assessed as 0 = normal, 1 to 4 = mild, 5 to 8 = moderate, and 9 to 12 = severe.

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)

Values are given as mean (standard deviation).
PBS, phosphate-buffered saline.

Hla-deficient strain DU1090. In our model, chondrocyte death caused by 8325-4 was rapid and almost universal (> 95%) within 48 hours. However, the Hla-deficient strain DU1090 was not without effect on chondrocyte viability. Thus, after the same time period, chondrocyte death was increased by approximately 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 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. 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 OA.[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 (Figures 1a and 1b). 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 (300 mOsm; 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 900 mOsm with sucrose and including 5 mM $MgCl_2$ 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 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 neutralizing 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

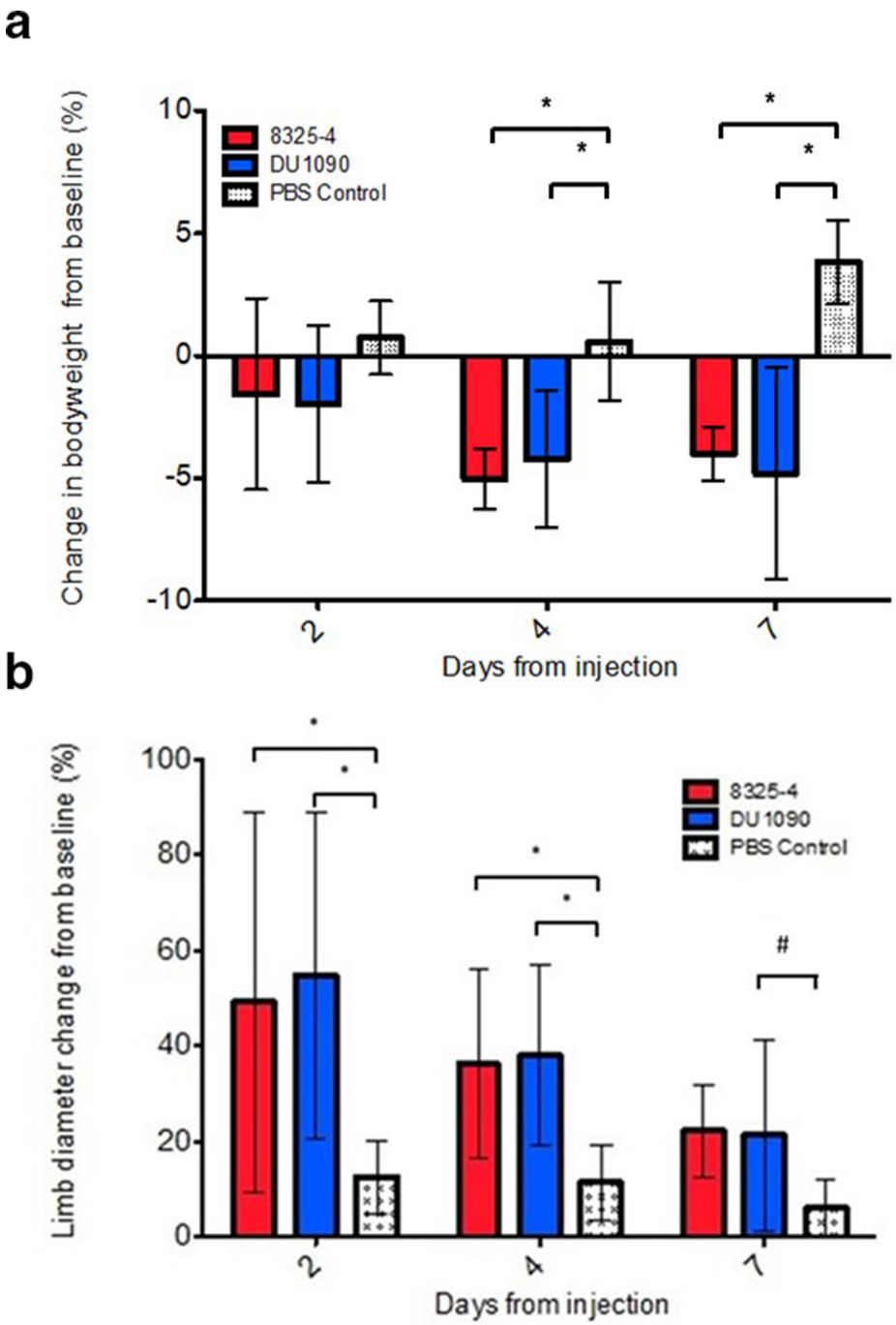

Fig. 3

The change in murine bodyweight and limb diameter following joint injection with *Staphylococcus aureus* 8325-4, *S. aureus* DU1090, or phosphate-buffered saline (PBS). a) Percentage change in body weight two, four, and seven 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) (one-way analysis of variance). b) Percentage change in anterior to posterior limb diameter at two, four, and seven 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 (#) indicates a significance between PBS and 8325-4 only. For both panels, data shown are means and standard deviations for ten animals.

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 that show promising early results.[35] However, they will require further work before they are clinically applicable. In any event, the present work used

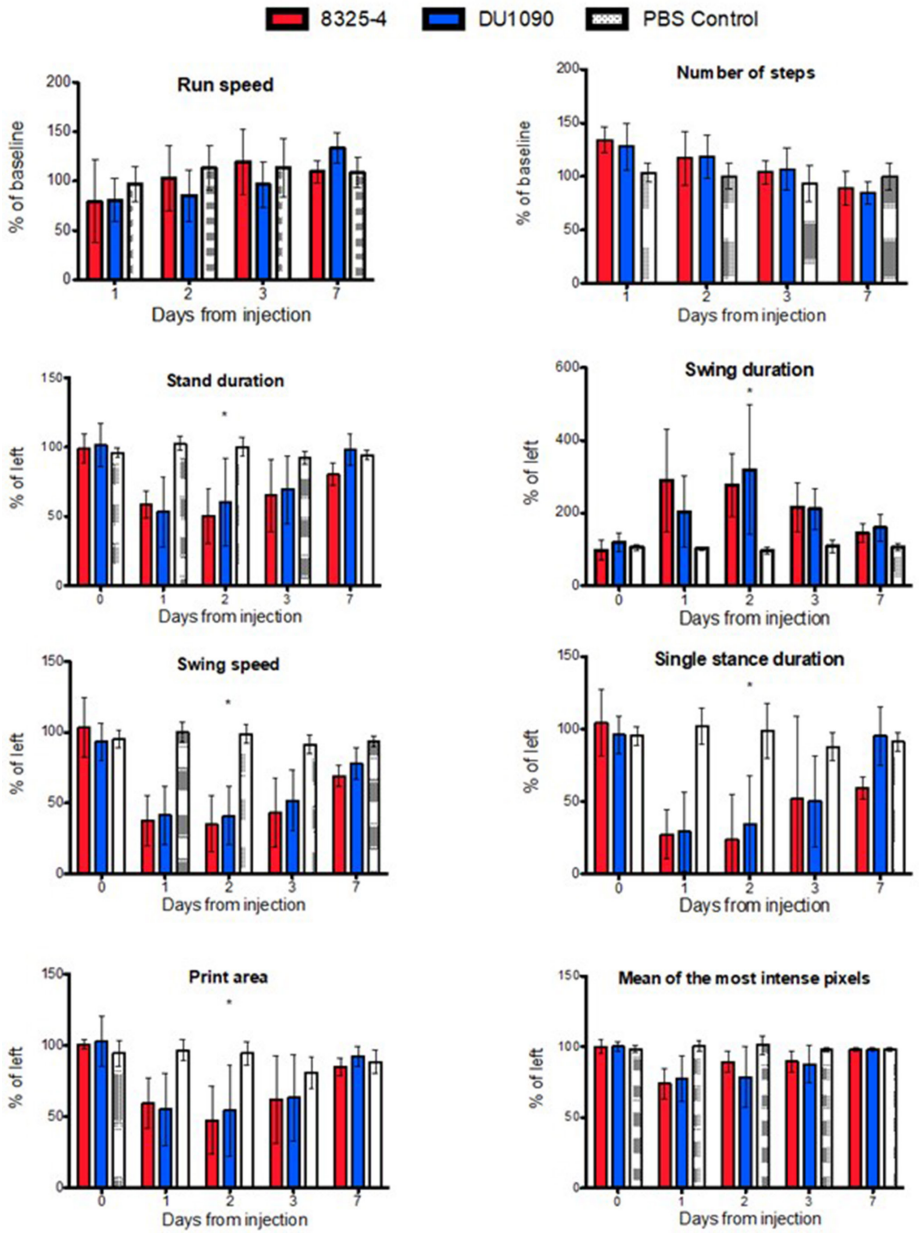

Fig. 4

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 Methods). The horizontal axis represents the number of days after injection of the right stifle joint with either *Staphylococcus aureus* 8325-4, DU1090, or phosphate-buffered saline (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 hindfoot, and mean of most intense pixels. Statistical analysis was only performed for comparison between groups on day 2. Asterisks denote significant differences ($p < 0.01$, one-way analysis of variance) between both experimental groups (8325-4 and DU1090) when compared to the PBS controls. Data shown are means and standard deviations for ten animals.

a Hla-producing and a 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: 1) rapid cell death produced by Hla, and 2) 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.

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Supplementary material

Flowchart illustrating randomization of mice into experimental groups, information on the power calculation, and ARRIVE checklist.

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