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Impacto do uso de droga imunomoduladora no tratamento da artrite séptica por *Staphylococcus aureus*

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"Se você nunca falhou, você nunca tentou algo novo".

Albert Einstein

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Sumário

SUMÁRIO

Resumo

RESUMO

Staphylococcus aureus é considerado o agente etiológico mais importante da artrite séptica. Neste contexto, o objetivo geral deste trabalho foi investigar o potencial artritogênico de cepas padrão de *S. aureus* produtoras de superantígenos (SAgs) e testar a eficácia terapêutica da associação entre antibiótico e imunomodulador neste modelo. Camundongos C57BL/6 machos foram inicialmente infectados pelo plexo retro-orbital com as diferentes cepas. O desenvolvimento de artrite e sua gravidade foram acompanhados por 14 dias, através das seguintes determinações: peso corporal, escore clínico e incidência de artrite, alterações histopatológicas, número de unidades formadoras de colônias, expressão local de citocinas e subpopulação células por PCR em tempo real e produção de citocinas por células esplênicas. Os resultados foram organizados em 3 manuscritos. No primeiro, comparamos a capacidade artritogênica de 5 cepas produtoras de SAgs. Constatamos que as cepas ATCC 19095 SEC^+, N315 ST5 $TSST\text{-}1^+$ e S-70 $TSST\text{-}1^+$ determinaram artrite grave, intermediária e discreta, respectivamente. No segundo trabalho caracterizamos a artrite causada pela cepa ATCC 19095 SEC^+. Este tipo de artrite se caracterizou por elevado escore clínico associado à proliferação sinovial, inflamação intensa, formação de "*pannus*", destruição da cartilagem e erosão óssea. A análise da expressão local de fatores de transcrição indicou expansão acentuada de células Th_1 e Th_{17} nas lesões. No último manuscrito, avaliamos o efeito terapêutico da combinação do antibiótico bactericida cloxacilina com o imunomodulador tacrolimus. A administração destas duas drogas foi nemos eficaz no controle da doença do que o antibiótico administrado isoladamente.

Palavras-chave: Artrite séptica, *Staphylococcus aureus,* Cloxacilina, Tacrolimus, IL-17

Abstract

ABSTRACT

Staphylococcus aureus is considered the more important etiological agent of septic arthritis. In this context, the main objective of this work was to investigate the arthritogenic capacity of standard *S. aureus* strains, producer of superantigens (SAgs). We also tested the therapeutic effectiveness of an association antibiotic and an immunomodulatory drug in this model. C57BL/6 male mice were initially infected by retro-orbital plexus with different strains. Development and severity of arthritis were followed during 14 days, through determination of body weight, clinical score and incidence of arthritis, histopathological changes, determination of colony forming units, evaluation of local infiltration of Th subsets by real time PCR and cytokine production by spleen cells. The results were organized in three manuscripts. In the first one we compared the arthritogenic ability of 5 *S. aureus* strains. We found that the strains ATCC 19095 SEC^+, N315 ST5 $TSST\text{-}1^+$ and S-70 $TSST\text{-}1^+$ determined severe, intermediate and discrete arthritis, respectively. In the second study we characterized the arthritis caused by the ATCC 19095 SEC^+ strain. This type of arthritis was characterized by high clinical scores associated with synovial proliferation, intense inflammation, "*pannus*" formation, cartilage destruction and bone erosion. In the final manuscript we evaluated the therapeutic potential of the combination bewteen cloxacilin (bactericidal antibiotic) with tacrolimus (immunomodulator). The administration of these two drugs was less effective in controlling the disease than the antibiotic alone.

Key-word: Septic Arthritis, *Staphylococcus aureus,* Cloxacilin, Tacrolimus, IL-17

Introdução

1. INTRODUÇÃO

1.1.Características gerais da artrite séptica

A artrite é uma doença inflamatória que acomete de forma mais evidente as articulações, mas que também pode comprometer outros tecidos do corpo humano. As alterações locais mais relevantes incluem hiperplasia do tecido sinovial e danos estruturais na cartilagem, tecido ósseo e ligamentos (Bremell *et al*., 1991). Estas alterações serão descritas com mais detalhes posteriormente.

Existem diferentes tipos de artrite sendo a artrite reumatoide, que apresenta caráter autoimune, a mais conhecida (Imboden, 2009). Outro tipo de artrite é a séptica, também conhecida como artrite infecciosa. Apesar de menos comum, este tipo de comprometimento das articulações pode apresentar-se como uma patologia muito agressiva. A incidência de artrite séptica (AS) em países industrializados tem sido estimada em seis casos por 100.000 habitantes por ano. Já no grupo de pacientes com artrite reumatóide ou portando próteses, esta incidência sobe para 70 pacientes (Tarkowski, 2006). A incidência é dependente do sexo e da idade do paciente, apresenta frequência mais elevada nos homens nas faixas de 0-15 anos e acima de 55 anos (Nade, 2003).

Conceitualmente, a AS é descrita como uma lesão em articulações desencadeada pela colonização da articulação por um agente infeccioso seguida de destruição articular acelerada (Levine & Siegel, 2003). A adesão da bactéria no tecido articular seguida de sua multiplicação no espaço sinovial resulta em inflamação por acúmulo de leucócitos no líquido articular (Nade, 2003). A destruição da cartilagem é mediada por substâncias derivadas do patógeno, como, por exemplo, toxinas e também por mediadores tóxicos liberados por células do próprio hospedeiro como espécies reativas de oxigênio, metaloproteinas e enzimas lisossômicas (Wright & Nair, 2010). Este processo

destrutivo é iniciado por degradação da proteoglicano do hospedeiro, seguida por quebra do colágeno o que ocorre algumas horas após a infecção. A destruição permanente da cartilagem articular e da matriz óssea podem ocorrer em poucos dias (Shirthiff & Mader, 2002).

A análise histopatológica das articulações permite observar alterações típicas deste processo inflamatório. As alterações mais conhecidas foram observadas em modelos experimentais de artrite séptica e serão descritas a seguir. A figura 1 (Strand *et al*., 2007) ilustra as principais alterações histopatológicas as quais são comumente encontrados tanto na artrite reumatoide como na séptica.

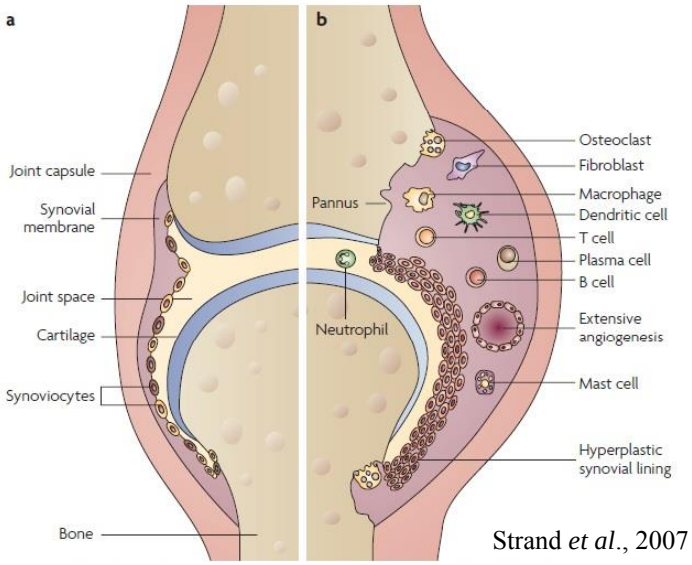

Figura 1. Representação esquemática de uma articulação normal e outra com artrite reumatóide. (a) articulação normal sem comprometimento das estruturas. (b) articulação com artrite. A membrana sinovial torna-se hiperplásica, infiltrada por células inflamatórias e ocorre a formação de "*pannus*".

A região do osso onde ocorre o movimento da articulação é envolto por uma cápsula, que é constituída por duas membranas, uma externa bem resistente e uma interna, chamada de membrana sinovial. A membrana sinovial é constituída por células

endoteliais específicas é ricamente vascularizada e sua função é produzir e reabsorver o líquido sinovial e prover nutrientes aos tecidos intracapsulares (Junqueira & Carneiro, 2004). A sinóvia é composta de 2 a 3 camadas de células especializadas denominadas sinoviócitos. Os sinoviócitos formam uma membrana que circunda o espaço sinovial. A hipertrofia da sinóvia é observada em camundongos experimentalmente infectados com *S. aureus* LS-1 (Bremell, *et al*., 1992).

A resposta do organismo à presença de agentes infecciosos na articulação é o aparecimento de edema, hiperemia e inflamação aguda local. Polimorfonucleares neutrófilos são vistos dentro dos vasos que irrigam a membrana sinovial, fora dos vasos e dentro do espaço sinovial. Estudos tem mostrado que outras subpopulações celulares incluindo macrófagos e linfócitos T contribuem de forma significativa para esta patologia (Tarkowski, 2006).

O *pannus* é uma estrutura típica da artrite; acredita-se que seja um exsudato inflamatório composto de macrófagos, fibroblastos e também por outras células inflamatórias (Furuzawa *et al*., 2008). Apesar de mais estudada na artrite reumatoide, esta estrutura também ocorre na artrite séptica (Ringe & Sittinges, 2009). O *pannus* é entendido como um tecido que acaba causando destruição da cartilagem e reabsorção óssea, pois invade a cavidade articular e cresce sobre a cartilagem e o osso (Robbins & Contran, 2010).

O processo de remodelação óssea envolve dois tipos celulares que são os osteoblastos e os osteoclastos. Os osteoblastos são células ósseas mononucleadas, de origem mesenquimal que estão localizadas na superfície dos ossos (Mackie, 2003; Cerri, 2005). São responsáveis por sintetizar a parte orgânica da matriz óssea (colágeno tipo I, osteocalcina, osteopontina, proteoglicanos, fosfoproteínas e citocinas) e também os fatores essenciais para a osteoclastogênese, ou seja, controlam a formação e a

reabsorção óssea. Os osteoclastos são membros da família de monócitos e macrófagos (Teitelbaum, 2000; Marriott, 2004). Quando recrutados para uma região da superfície óssea para realizar a reabsorção óssea, os osteoclastos se diferenciam em células móveis, gigantes, multinucleadas e extensamente ramificadas (Sodek & Mckee, 2000). Secretam ácido (íons H^+), colagenase e outras enzimas que atuam localmente digerindo a matriz orgânica e dissolvendo os cristais de sais de cálcio, realizando sua função de reabsorção óssea (Teitelbaum, 2000; Katagiri & Takahashi 2002; Garlet *et al*., 2006).

1.2. *Staphylococcus aureus*

A espécie *Staphylococcus aureus* é constituída por cocos Gram-positivos que medem de 0,5 a 1,5 μm de diâmetro, são imóveis, não formadores de esporos, catalase positivos e anaeróbios facultativos (Tortora *et al*., 2005). Esses micro-organismos se encontram amplamente distribuídos na natureza e nos animais são encontrados principalmente na pele e nas membranas mucosas. Em humanos adultos, o *S. aureus* se localiza preferencialmente nas narinas (Kloos & Bannerman, 1995).

Os estafilococos geralmente mantem uma relação benigna ou simbiótica com seus hospedeiros. Contudo, se a barreira cutânea for rompida, esses micro-organismos podem apresentar comportamento patogênico (Koneman *et al.*, 1997). Acredita-se que a interação com o tecido ósseo e desencadeamento da artrite séptica ocorra a partir da via hematogênica (Tarkowski, 2006). As propriedades artritogênicas do *S. aureus* têm sido atribuídas a alguns fatores de virulência que medeiam a interação da bactéria com o tecido ósseo. Dentre eles se destacam os sorotipos da cápsula polissacarídica, a adesina para colágeno e o fator "*clumping*" (Switalski *et al.*, 1993; Nilsson *et al.*, 1997; Josefsson *et al.*, 2001). Além disto, o *S. aureus* secreta um grande número de enzimas e toxinas como, as hemolisinas (α, β, γ, δ,) e a leucocidina. A combinação das

hemolisinas α e γ contribui com a capacidade artritogênica desta bactéria (Nilsson *et al.*, 1999). Outro fator de virulência do *S. aureus* que merece destaque no contexto da AS é a produção de superantígenos (SAgs) (McCormick *et al.*, 1993). Exemplos de SAgs são as enterotoxinas (A, B, C $_{(1\text{-}3),}$ D, E e G), a toxina da síndrome do choque tóxico (TSST-1) e as exfoliatinas A e B. Estas proteínas são capazes de estimular elevado porcentual de linfócitos T que expressam determinadas sequências Vβ nos receptores para antígenos (TCRs). Este processo é acompanhado de produção de várias citocinas tanto a partir de células T (IFN-γ, TNF-β, IL-2) quanto de monócitos (IL-1 e TNF-α). Esta elevada produção de citocinas desencadeia intensa resposta inflamatória, acarretando lesões em vários tecidos do hospedeiro. A contribuição dos SAgs derivados de *S. aureus* para a artritogenicidade tem sido claramente evidenciada (Abdelnour, *et al*., 1994). O próprio DNA bacteriano também é sugerido como um fator artritogênico uma vez que pode, em função da abundância de, sequencias CpG, determina produção local de citocinas pró-inflamatórias (Tarkowski, 2006).

1.3.Artrite séptica por *Staphylococcus aureus*

O *S. aureus* é descrito como a bactéria mais prevalente nos casos de artrite séptica humana e também como causador de lesões artríticas mais graves (Tarkowski, 2006). O comprometimento da articulação ocorre a partir de uma infecção em outro local do organismo, sendo a bacteremia e a sepse as mais usuais (Edwards & Massey, 2011). Este tipo de artrite é uma condição tipicamente aguda, mas que pode se tornar crônica.

Várias espécies de animais desenvolvem artrite causada por *S. aureus* de forma espontânea e poderiam ser utilizados como modelos experimentais. A inoculação do *S. aureus* por via intravenosa em camundongos tem sido a rota preferencial para o desenvolvimento da doença. Esta via permite que a bactéria se adapte ao ambiente

dentro do hospedeiro, que sobreviva aos componentes microbicidas existentes no soro e que, por fim, se dissemine até o tecido sinovial e penetre em várias estruturas até alcançar a cavidade articular. O desenvolvimento da artrite séptica é também dependente do inóculo. Neste caso, as concentrações recomendadas variam entre 7.10^6 até 2.10^7 UFC por animal (Tarkowski *et al.*, 2001).

Diversas linhagens de camundongos têm sido utilizadas para estudar a AS tais como: NRMI; C57BL/6 e 1295V e BALB/c (Hultgren *et al.*,1999; Shaw *et al.*, 2008; Narita *et al.*, 2010). Na maioria dos casos a doença é desencadeada por inoculação intravenosa da bactéria. A inoculação intra-articular de peptideoglicano purificado de *S. aureus* em várias linhagens de camundongos (BALB/c, C57BL/6, C3H/HeN, C3H/HeJ e CB17) também desencadeou artrite típica.

Outro aspecto de extrema relevância é a escolha da cepa de *S. aureus* a ser utilizada. Apesar do *S. aureus* ser a bactéria mais prevalente na artrite infecciosa humana, nem todas as cepas desta bactéria são artritogênicas. Tarkowski *et al.* (2001) contribuíram de forma significativa para a padronização deste modelo. Este grupo tem utilizado, sistematicamente, a cepa bacteriana denominada LS-1, descrita como causa de artrite espontânea em várias linhagens de camundongos.

1.4. Resposta imune associada à artrite séptica

Diferentemente da artrite reumatóide cuja imunopatogênese é bastante estudada, a artrite séptica tem recebido menor atenção. A resposta imune inata ao *S. aureus* é similar à resposta contra outras bactérias extracelulares e envolve, principalmente, resposta inflamatória, fagocitose e ativação do sistema complemento. Esta resposta é iniciada pelo recrutamento dos polimorfonucleares (PMNs) e macrófagos ao sítio da

infecção por quimiotaxia mediada tanto por fatores derivados do hospedeiro como do agente infeccioso (Deleo *et al.,* 2009).

A fagocitose é uma etapa crítica na remoção do *S. aureus* e requer interação entre moléculas presentes na superfície do patógeno (PAMPs) com receptores presentes na superfície das células fagocíticas, como, por exemplo, os receptores do tipo Toll (TLRs). Estes receptores também medeiam transdução de sinais intracelulares que aumentam a atividade microbicida das células fagocíticas. A relevância destes receptores tem sido comprovada em experimentos com animais deficientes em TLR-2, os quais são mais suscetíveis à infecção por *S. aureus* do que os animais normais (Takeuchi *et al*., 2000).

A infecção por *Staphylococcus* é caracterizada por um influxo local muito acentuado de PMNs. Apesar dos PMNs eliminarem esta bactéria de forma eficiente, estas células podem também lesar o tecido do hospedeiro pela liberação dos produtos antibacterianos. Anticorpos contra *S. aureus* estão presentes em praticamente todos os indivíduos e existem relatos mostrando que os títulos de anticorpos específicos aumentam após infecção (Dryla *et al.*, 2005). Entretanto, estes anticorpos e a memória imunológica parecem ser insuficientes para prevenir infecções subsequentes por esta bactéria. Recentemente foi demonstrado que o *S. aureus* determina ativação de células dendríticas via TLR2-MyD88, promovendo diferenciação de células Th_1 e Th_{17}.

Parte da virulência do *S. aureus* tem sido atribuída à sua capacidade de evasão frente aos mecanismos de defesa do hospedeiro. O *S. aureus* secreta, por exemplo, proteínas que inibem a ativação do complemento e a quimiotaxia dos PMNs. Também produz substâncias que neutralizam os peptídeos antimicrobianos dos grânulos dos PMNs e que inibem a opsonização por anticorpo e complemento. Além disto, são

capazes de produzir vários tipos de SAgs os quais desencadeiam anergia e imunossupressão (Foster, 2005).

A resposta imune inata forneceria proteção contra o agente infeccioso e impediria o subsequente aparecimento das lesões artríticas associadas ao *S. aureus* (Tarkowski, 2006). Protocolos que mimetizam defeitos na imunidade inata tais como depleção seletiva de neutrófilos, inibição seletiva na produção de óxido nítrico e depleção do sistema complemento, mostraram que deficiências destes componentes determinam quadros mais graves de artrite (Verdrengh & Tarkowski, 1997; Sakiniene, *et al.*, 1997; Sakiniene, *et al.,* 1999).

Por outro lado, evidências experimentais indicam que a resposta imune específica tem participação relevante no processo destrutivo das articulações. Por exemplo, a eliminação de linfócitos T do hospedeiro diminui de forma significativa a gravidade da artrite séptica nas infecções por *S. aureus* (Bremell *et al.*, 1994). Este processo foi mediado preferencialmente pelas populações de linfócitos T CD_4^+ ativadas pelos SAgs, pois a eliminação de toda a subpopulação CD_4^+ ou da subpopulação TCD_4^+ $V\beta_2$ determinou queda na gravidade da artrite séptica (Abdelnour, *et al.,* 1994). A participação das células apresentadoras de antígenos (APCs) na artrite séptica também tem sido avaliada. Estudos constataram que a ausência de moléculas MHC de classe II, as quais são fundamentais tanto na apresentação de antígenos convencionais como de SAgs, estava associada à proteção contra o desenvolvimento da artrite (Tarkowski, 2006).

Neste contexto de proteção e lesão, as citocinas, que fazem parte tanto da imunidade inata quanto específica, tem um papel fundamental e complexo na artrite séptica. Desempenham papel protetor permitindo não só a chegada de células fagocíticas no local da infecção como também sua ativação e morte subsequente das

bactérias. Por outro lado, a produção destas moléculas determina efeito deletério local, associado com inflamação prolongada. Classicamente as citocinas mais investigadas na artrite séptica associada ao *S. aureus* incluem IL-1, TNF-α, IL-6, IL-8, IL-12, IFN-γ, IL-4 e IL-10 (Kimura *et al.*, 1997; Gjertsson *et al.*, 2002; Palmqvist *et al.*, 2005; Zhao *et al.*, 1996; Hultgren *et al.*, 1998; Hultgren *et al.*, 2001). A contribuição da IL-17A na artrite séptica vem sendo investigada só mais recentemente. Um único trabalho, publicado por Henningsson *et al*. (2010), indicou que a IL-17A teria um papel relevante, impedindo o desenvolvimento da artrite.

1.5.Tratamento da artrite séptica

A eficácia e a rapidez do tratamento da AS são fundamentais para evitar a destruição irreversível das articulações. A base deste tratamento é, sem dúvida, a antibioticoterapia, que deve ser iniciada com a administração parenteral de antibióticos de amplo espectro tais como cefalosporinas e vancomicina (Tarkowski, 2006).

Como descrevemos anteriormente, a destruição das articulações se deve principalmente à resposta imune exacerbada do hospedeiro. Portanto, só matar as bactérias não impede a evolução deste processo patológico. Por esta razão, a associação de corticoides com antibióticos é o tratamento de escolha como tem sido descrito em modelos experimentais e pacientes (Sakiniene *et al.*, 1996). Apesar desta associação diminuir a inflamação articular e a mortalidade, a mesma não previne, de forma significativa, o processo de reabsorção óssea que ocorre nas articulações (Verdrengh *et al.*, 2006). Neste contexto, vários fármacos têm sido testados com o objetivo de impedir, de forma mais eficaz, o processo de ativação local da resposta imune. Exemplos destas drogas incluem o uso de antioxidantes (Sakiniene & Collins, 2002) e administração de bifosfonato para reduzir a reabsorção óssea (Verdrengh *et al.*, 2007). Outra

possibilidade explorada é minimizar o efeito dos SAgs. As alternativas nesta área são inúmeras e incluem diminuir a produção de SAgs *in vivo*, neutralizar sua atividade inflamatória após secreção, bloquear a interação com MHC classe II ou TCR e induzir tolerância ao SAgs (Tarkowski, 2006).

1.6. Imunorregulação por tacrolimus

O tacrolimus é uma droga imunossupressora, que é produzida pela bactéria *Streptomyces tsukubaensis* (Pritchard, 2005). Foi originalmente aprovada pelo FDA para uso em transplantes de fígado, mas seu uso foi estendido a outros tipos de transplantes e posteriormente a várias outras patologias, tais como colite ulcerativa, eczema, uveíte e vitiligo. Quimicamente, o tacrolimus é um macrolídeo e sua atividade imunossupressora tem sido atribuída, em grande parte, à inibição do processo de ativação dos linfócitos T. A ativação dos linfócitos T via TCR é iniciada pelo aumento do nível de cálcio intracelular, que ativa a calcineurina via calmodulina. Por sua vez, a calcineurina desfosforila o fator de transcrição NF-AT, o qual fica livre e se desloca até o núcleo celular. No núcleo o NF-AT aumenta a transcrição de genes que codificam IL-2 e outras citocinas resultando em proliferação de linfócitos T. O tacrolimus previne a desfosforilação do NF-AT e bloqueia a proliferação dos linfócitos T (Proft & Fraser, 2003).

Efeitos inibidores adicionais mediados pelo tacrolimus sobre linfócitos T têm sido descrito tais como aumento de apoptose e indução de células T reguladoras (Migita & Eguchi, 2001; Kogina *et al*., 2009). Este efeito indutor de apoptose foi demonstrado em um modelo experimental com um SAg de *S. aureus* (SEB) e foi mediado por diminuição da expressão de BcL-xL (Migita *et al.*, 1999). Esta droga também inibe a função de macrófagos ativados e promove sua apoptose. Estes efeitos têm sido

relacionados com a atividade terapêutica observada na colite (Yoshino *et al.*, 2010). Além disto, o tacrolimus também foi capaz de inibir a liberação de mediadores pró-inflamatórios e a infiltração de leucócitos ativados em um modelo de inflamação induzido por carragenina (Vigil *et al.*, 2008).

A eficácia da associação entre tacrolimus e antibiótico foi recentemente comprovada em processos inflamatórios de peritonite séptica experimental (Assfalg *et al.*, 2010). No entanto, a eficácia de uma associação similar na artrite séptica não tem sido estudada. Um único trabalho mostrou que o tacrolimus foi capaz de inibir o desenvolvimento da artrite induzida por colágeno e potencializada por superantígeno (SEB) em camundongos (Takaoka *et al.*, 1998).

1.7. Racional do projeto

O *S. aureus* é o principal agente etiológico da artrite séptica (AS) e acredita-se que os superantígenos (SAgs) produzidos por esta bactéria contribuam para o desenvolvimento desta doença. Os estudos em pacientes humanos são dificultados não só pela rapidez com a qual as lesões se desenvolvem nas articulações, mas também pela dificuldade em se obter amostras do local afetado. Outro agravante desta doença é a complexidade associada ao tratamento. Além da necessidade de um antibiótico para frear a multiplicação do agente infeccioso, é necessário a associação com um substância imunomoduladora que impeça a inflamação exagerada, que é a causa principal da lesão articular. Neste sentido, propomos testar a associação de um antibiótico com tacrolimus, uma droga imunomoduladora e que ainda não foi testada na artrite séptica. A complexidade desta patologia vem sendo esclarecido por estudos em modelos animais, sendo camundongos infectados com a cepa LS-1 produtora de TSST-1, o modelo mais empregado. Neste contexto, nosso trabalho se propõe a testar várias cepas padrão de *S.*

aureus produtoras de SAgs. Esta abordagem permitirá determinar se outras cepas de *S. aureus,* além da LS-1 TSST-1, apresentam capacidade artritogênica e também se as características histológicas da lesão permitem sua utilização como um modelo experimental adicional. Além disto, esta proposta permitirá determinar se a associação de tacrolimus com antibiótico é recomendada para tratamento de AS.

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Objetivos

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o potencial artritogênico de diferentes cepas padrão de *S. aureus* e a eficácia terapêutica da associação entre antibiótico e imunomodulador na artrite séptica experimental.

3.2 Objetivos Específicos

1. Comparar o potencial artritogênico de cinco cepas padrão de *S. aureus* produtoras de superantígenos, considerando incidência da doença, escore clínico e alterações histopatológicas.

2. Caracterizar a doença causada pela cepa mais artritogênica e também avaliar a participação local de subpopulações de células T e de citocinas no processo inflamatório.

3. Avaliar a eficácia da administração combinada de cloxacilina e tacrolimus em camundongos com artrite séptica experimental

Resultados e Discussão

4. RESULTADOS E DISCUSSÃO

Os resultados e a discussão dos dados obtidos encontram-se apresentados na forma de artigos científicos.

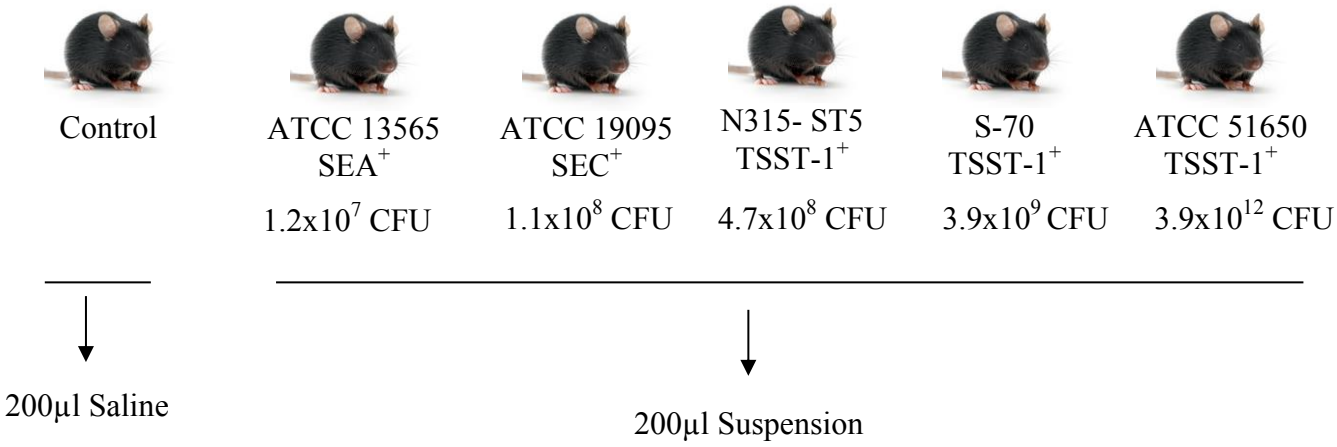
4.1. Artigo científico I: Differential arthritogenicity of *Staphylococcus aureus* strains isolated from biological samples (Submetido à Revista BMC Infectious Diseases – ID: 2006792852978369)

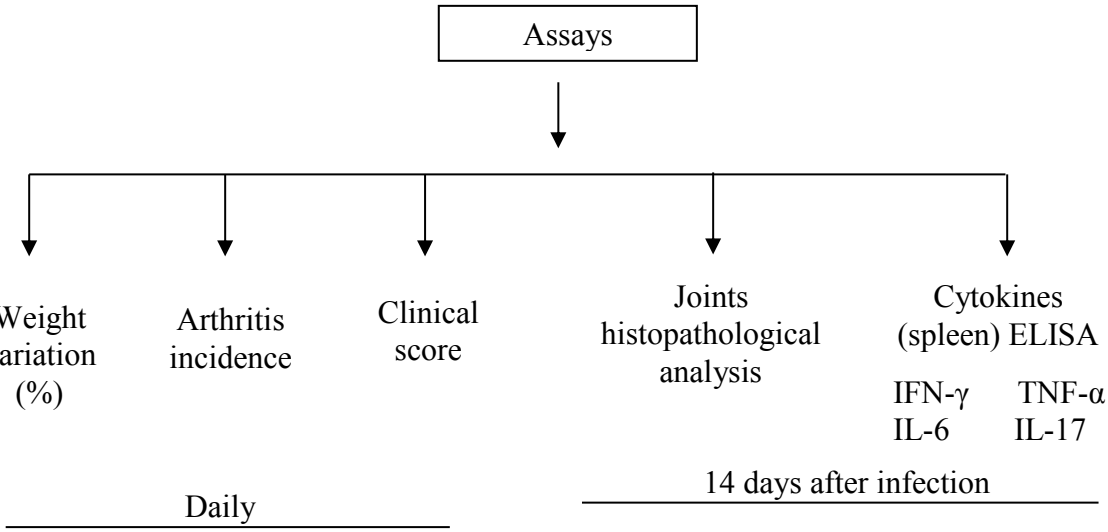
4.2. Artigo científico II: Septic arthritis triggered by enterotoxin C producer *Staphylococcus aureus* is associated with strong Th1 and Th17 expansion

4.3. Artigo científico III: Tacrolimus does not improve cloxacillin efficacy in experimental septic arthritis

Artigo científico I

Artigo científico I: Differential arthritogenicity of *Staphylococcus aureus* strains isolated from biological samples





Artigo científico I: Differential arthritogenicity of *Staphylococcus aureus* strains isolated from biological samples

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Abstract

Background

Staphylococcus aureus is the most common agent of septic arthritis that is characteristically a severe, rapidly progressive and erosive disease. Superantigens produced by *S. aureus* are considered the major arthritogenic factors. In this study we compared the arthritogenic potential of five superantigen-producing staphylococcal strains.

Methods

Male C57BL/6 mice were intravenously infected with ATCC 19095 SEC^+, N315 ST5 $TSST\text{-}1^+$, S-70 $TSST\text{-}1^+$, ATCC 51650 $TSST\text{-}1^+$ and ATCC 13565 SEA^+ strains. Clinical parameters as body weight, arthritis incidence and clinical score (arthritic index 0 to 3 scale) were daily evaluated. Joint histopathological analysis and spleen cytokine production were evaluated at the 14^{th} day after infection.

Results

Weight loss was observed in all infected mice. ATCC 19095 SEC^+, N315 ST5 $TSST\text{-}1^+$ and S-70 $TSST\text{-}1^+$ were arthritogenic, being the highest scores observed in ATCC 19095 SEC^+ infected mice. Intermediate and lower clinical scores were observed in N315 ST5 $TSST\text{-}1^+$ and S-70 $TSST\text{-}1^+$ infected mice, respectively. The ATCC 13565 SEA^+ was lethal causing death of 85% of the animals after 48h. Arthritis triggered by the ATCC 19095 SEC^+ strain was characterized by accentuated synovial proliferation, inflammation, *pannus* formation, cartilage destruction and bone erosion. Similar joint alterations were found in N315 ST5 $TSST\text{-}1^+$ infected mice, however they were strikingly more discrete. Only minor synovial proliferation and inflammation were triggered by the S-70 $TSST\text{-}1^+$ strain. The lowest levels of TNF-α, IL-6 and IL-17 in response to *S. aureus* stimulation were found in cultures from mice infected with the less arthritogenic strains (S-70 $TSST\text{-}1^+$ and ATCC 51650 $TSST\text{-}1^+$).

The highest production of IL-17 was detected in mice infected with the most arthritogenic strains (ATCC 19095 SEC^+ and N315 ST5 TSST-1^+).

Conclusions

Together these results demonstrated that *S. aureus* strains isolated from biological samples were able to induce a typical septic arthritis in mice. Interestingly, the most arthritogenic strain was originally isolated from a human leg abscess. These results also suggest that the variable arthritogenicity of these strains was at least in part, related to their differential ability to induce IL-17 production.

Key-words: *Staphylococcus aureus*, Septic arthritis, IL-17

Background

Staphylococcus aureus is a major cause of bacteremia, which frequently leads to infective endocarditis, metastatic abscess formation, toxic shock syndrome, gastroenteritis, pneumonia, osteomyelitis and septic arthritis [1]. The development of these secondary infections is due to bacterial dissemination from the blood into surrounding tissues and is associated with significantly increased morbidity and mortality [1]. Even though all these secondary infections are serious, septic arthritis calls attention because it is a rapidly progressive and highly erosive disease of the joints in which both host and bacterial factors are of pathogenic importance [2]. The most important risk factor for septic arthritis (SA) is undoubtedly pre-existing joint disease, especially rheumatoid arthritis and prosthetic joint surgery [2]. The mortality rate in septic arthritis patients is already elevated; 5-20% of adults with septic arthritis may die as a consequence of their systemic infection. However, in a group of patients having RA and infected with *S. aureus* in more than one joint, the risk of mortality increases to

50% because of the combination of delayed diagnosis, the state of immunosuppression caused by the therapy, older age and also the polyarticular involvement [2].

One of the hallmarks of septic arthritis is the massive inflammation that precedes bone destruction. The infection by *S. aureus* is accompanied by a rapid recruitment of polymorphonuclear granulocytes and activated macrophages soon followed by T cells [3]. Although monocytes and macrophages are important in the clearance of bacteria, they also play a pivotal role in the destructive inflammation within the joint [4]. The involvement of proinflammatory cytokines in the pathogenesis of *S. aureus* infection has been reported. This bacteria can induce cytokines such as TNF-α, IFN-γ, IL-1, IL-2, and IL-6 [5]. Cytokines released from macrophages as TNF-α, IL-1β and IL-6 have been classically pointed as the major player of the severe inflammation that precedes cartilage and bone destruction in septic arthritis. These molecules stimulate osteoclast differentiation and bone resorption in a synergistic fashion [6]. TNF-α that is considered the most osteoclastogenic cytokine, activates NF-kB which in turn is associated with the survival of osteoclasts [7].

The role of IL-17 in SA is not well established. However, a possible deleterious role is highly supported by many reports in the areas of rheumatoid and osteoarthritis [8]. IL-17A appears to play a key role in host defense against local *S. aureus* infections by inducing the production of neutrophil-mobilizing chemokines and growth factors and the subsequent mobilization of neutrophils [9,10].

S. aureus strains can produce a number of different components that may contribute to virulence and arthritogenicity, including surface-associated adhesins, capsular polysaccharides, "*clumping*" factor A (ClfA) exoenzymes, and exotoxins [11,12,13]. Some of the toxins produced by *S. aureus* are called superantigens (SAgs) because they are endowed with the ability to activate various T cell clones,

independently of their specificity. These SAgs mediate T cell activation in a very distinctive way from conventional antigens. These molecules are able to bind to class II MHC molecules on antigen presenting cells and stimulate a large T cell population comprising all clones that share certain variable regions in the Vβ chain [14]. They cause fever, hypotension and other acute toxic-shock-like symptoms by inducing the release of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1 and IL-12 [15,16].

Several studies indicate that experimental arthritis in mice is the best model to study this pathology because of the striking resemblances between the murine and human immune systems [17]. The characteristics of the murine model closely mirror changes seen in human septic arthritis, especially with regard to the high frequency and severity of periarticular bone erosivity [3]. The most employed *S. aureus* strain used to trigger experimental septic arthritis is the LS-1 that is a TSST-1 producer strain [4, 17, 18]. The main objective of this work was to compare the arthritogenic capacity of various superantigen-producing *Staphylococci* considering disease incidence, clinical scores histopathological alterations and cytokine production.

Methods

Experimental design

Mice were infected with different *S. aureus* strains and were daily evaluated by a clinical follow up that included weight determination, disease incidence and individual clinical scores. Fourteen days after infection they were euthanized and submitted to histopathological and immunological analysis. Cellular immunity was checked considering cytokine production by spleen cells stimulated with *S. aureus* and Concanavalin A (ConA). Non-infected animals were included as a control group.

Animals

Male C57BL/6 mice (8-10 weeks old) were purchased from PUSP-RP (USP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Medical School, Univ. Estadual Paulista).

***S. aureus* strains and culture conditions**

The following SAg producer ATCC strains were used: ATCC 19095 SEC^+ (isolated from a leg abscess of a patient in Albert Merritt Billings Hospital, University of Chicago), N315 ST5 TSST-1^+ (isolated from the pharyngeal smear of a japanese patient) S-70 TSST-1^+ (isolated from a bovine abcess), ATCC 51650 TSST-1^+ (isolated from a wound of a patient with nonmenstrual toxic shock syndrome, Vancouver, British Columbia, Canada), and ATCC 13565 SEA^+ (isolated from a ham involved in food poisoning). Before each experiment, bacteria were cultured in blood agar plates (Merck) for 24h at 37ºC in order to confirm their purity and to determine their morphology and specific color. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37ºC for 24h. Bacteria were collected by centrifugation, washed three times and resuspended in cold sterile saline, as described by França *et al*., (2009). The bacterial suspensions were prepared according to the McFarland nephelometer nº 0.5. The exact amount of live bacterial cells was determined by further enumeration of the number of colony forming units (CFU) on agar plates.

Arthritis induction

Disease was induced according to the methodology described [18]. However the infection was performed through the retro-orbital route instead of the caudal vein as originally described. Each animal was infected with 0.2 mL of a *S. aureus* suspension made in physiological saline and control mice were injected with 0.2 mL of this diluent. According to the CFU, the following amounts of bacteria were actually used for initial infection, table 1.

Clinical evaluation

Mice were individually analyzed and joints were inspected every day. The mean number of arthritic limbs per animal was registered. Arthritis was defined as a visible joint erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis, a clinical scoring (arthritic index) was carried out, using a system where macroscopic inspection yielded a score of 0–3 points for each limb (1 point = mild swelling and/or erythema; 2 points = moderate swelling and erythema; 3 points = marked swelling and erythema). The arthritic index was constructed by dividing the total score (number of arthritic limbs) by the number of animals used in each experimental group. The overall condition of the mice was evaluated by assessing body weight according to the methodology described in literature [19].

Histopathological examination

Joint histopathological examination was done 14 days after infection. After fixation by 10% formaldehyde, the joints were decalcified for 8 weeks in 18% EDTA. After confirmation by a radiographical procedure that joints were decalcified they were washed, dehydrated and embedded in paraffin. Serial sections with 5μm thickness were

cut and stained with haematoxilin and eosin. The sections were qualitatively evaluated in relation to the presence of inflammatory infiltrates, hyperplasia of the synovial membrane, *pannus* formation, cartilage destruction and bone erosion. The predominance of polymorphonuclear or mononuclear cells in the joints was also evaluated.

Cytokine quantification

Control and infected animals were euthanized 14 days after infection. Spleen cells were collected and adjusted to $5x10^6$cells/mL. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum, 20mM glutamine and 40 IU/mL of gentamicin). Cultures were stimulated with a standardized preparation of *S. aureus* (Pansorbin from Calbiochemical) or ConA (Sigma-Aldrich). Pansorbin is a suspension of heat killed and formalin-hardened *S. aureus* Cowan I cells and was used at a final dilution of 1:2500 (v:v); ConA was used at a final concentration of 10μg/mL of cell culture. Cytokine levels were evaluated 48h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IFN-γ BD OptEIA Sets (Becton Dickinson) and IL-6, IL-17 and TNF-α Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instruction.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between infected groups were made by Student's test or one way ANOVA with Tukey test for parameters with normal distribution. Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results

Weight loss

Body weight was daily checked. The percentage of weight variation after 3, 7 and 14 days of infection is illustrated in figure 1. As can be observed, in spite of some variation, infection with all strains usually determined a significant weight loss.

Arthritis incidence

The kinetics of arthritis development was very similar in the groups infected with ATCC 19095 SEC^+ and N-315 ST5 $TSST\text{-}1^+$. Both groups already presented clinical disease signs (edema and/ or erythema) 24 hours after infection. They also reached around 80% of disease incidence. The S-70 $TSST\text{-}1^+$ infected group presented a delayed clinical manifestation that occurred only at the 5^{th} day of infection. A 100% disease incidence was observed in this group at the 10^{th} day of infection. The ATCC 51650 $TSST\text{-}1^+$ strain did not trigger any sign of arthritis during 14 days that was the planned experimental period. These results are shown in figure 2a. The ATCC 13565 SEA^+ was lethal causing death of 85% of the animals in 48h (not shown).

Clinical scores

Clinical scores are shown in figure 2b. The highest clinical scores were observed in the group infected with the ATCC 19095 SEC^+ strain. In spite of a certain degree of variation, these high scores were maintained until the 14^{th} after initial infection. Even though hind paws and forepaws were affected, the hind paws presented higher scores (not shown). Lower clinical scores were observed in ATCC N315 $TSST\text{-}1^+$ and S-70 $TSST\text{-}1^+$ infected mice. The ATCC 51650 $TSST\text{-}1^+$ did not provoke any clinical sign of arthritis. The ATCC 13565 SEA^+ strain was lethal to 85% of the animals, but the

remaining 15% did not develop any clinical sign of arthritis. Only ATCC 19095 SEC$^+$ strain occasionally triggered nodose tail.

Histopathological analysis

The normal histological appearance of mice joints is shown in figure 3a, 3a'. A very thin synovial layer, a marked joint cavity, an intact cartilage and a preserved bone structure can be observed (figure 3a, 3a'). After 14 days of infection the histological aspect of swollen joints from forepaws was very distinct in the three experimentally infected groups. Mice infected with the ATCC 19095 SEC$^+$ strain presented a severe arthritis characterized by a marked proliferation of synovial tissue and a huge inflammatory process. The inflammatory infiltrate was localized in the synovial cavity but it also penetrated into both, cartilage and bone structures. A striking *pannus* formation occupied almost the entire joint cavity. Cartilage and bone erosion were also observed in many areas. (figure 3b, 3b'). Synovial hyperplasia, inflammation, *pannus* formation and cartilage and bone erosion were also detected in mice infected with the N-315 ST5 TSST-1$^+$ but in lesser extension. (figure 3c, 3c'). As shows in figure 3d, 3d', only minor degrees of synovial proliferation and inflammation were detected in the joints of mice infected with the S-70 TSST-1$^+$ strain. Arthritis in the hind paws of ATCC 19095 SEC$^+$ infected animals was even more severe than in the forepaws. In this case the synovial space was entirely occupied by inflammatory infiltrates and bone tissue was severely eroded (figure 4b, 4b'). Various cell types as polymorphonuclear cells, mononuclear cells and fibroblast like cells were observed. Granuloma-like structures were also visualized in these joints. Histopathological changes were not detected in the hind paws of animals infected with N-315 ST5 TSST-1$^+$ or S-70 TSST-1$^+$ *S. aureus* strains as can be observed in figures 4c and 4d.

Cytokine production

The cellular immune response was evaluated by the production of cytokines by spleen cells stimulated with fixed *S. aureus* Cowan strain I or ConA. High levels of IFN-γ were produced by cells stimulated with *S. aureus*. Even though these levels were generally similar to the ones produced by cells from control (non infected) mice, the ATCC 19095 SEC^+ and S-70 $TSST\text{-}1^+$ groups produced significantly higher amounts than the ATCC 51650 $TSST\text{-}1^+$ group (figure 5a). Stimulation with ConA triggered elevated IFN-γ production by all experimental groups including, as expected, the control not infected group (figure 6a). All groups produced TNF-α when the cells were stimulated with *S. aureus*, being the levels found in the control, ATCC 19095 SEC^+ and ATCC N315 $TSST\text{-}1^+$ similarly elevated. The levels of this cytokine were, however, significantly lower in the groups S-70 $TSST\text{-}1^+$ and ATCC 51650 $TSST\text{-}1^+$ (figure 5c). Production of IL-6 induced by *S. aureus* was significantly lower in the groups infected with *S. aureus* S-70 $TSST\text{-}1^+$ (figure 5b). A very similar profile was observed when the cultures were stimulated with ConA, i.e., significantly lower levels of IL-6 being produced by mice infected with S-70 $TSST\text{-}1^+$ and ATCC 51650 $TSST\text{-}1^+$ strains in comparison to ATCC N315 $TSST\text{-}1^+$ (figure 6b). The profile of IL-17 production was similar in the cultures stimulated with *S. aureus* and ConA, being its levels higher in cultures from ATCC N315 $TSST\text{-}1^+$ and ATCC 19095 SEC^+ groups as can be observed in figure 5d and 6c, respectively. The most striking differences were, however, observed in the cultures stimulated with *S. aureus*, in this case IL-17 levels were significantly higher in the groups ATCC N315 $TSST\text{-}1^+$ and ATCC 19095 SEC^+ in comparison to all other groups (figure 5d).

Discussion

Septic arthritis is an infectious disease that affects the joints. Due to its fast evolution, even with prompt therapy, it can cause irreversible joint damage and even death [2]. *S. aureus* is the most common causative agent of this disease and it has been believed that superanting production plays a pivotal role in arthritogenicity [2, 16]. The main goal of this work was to compare the arthritogenic potential of superantigen-producing *S. aureus* strains considering disease incidence, clinical score and histopathological alterations. Cytokine production was also evaluated to get some insight into possible differences among the various strains. After an initial screening process C57BL/6 male animals were chosen. BALB/c (male and female) and C57BL/6 female animals did not develop disease (not shown). Male C57BL/6 mice were then infected by the retro-orbital plexus and body weight and clinical scores were daily checked until day 14 when animals were euthanized for histopathological and immunological evaluations. The 5 strains caused a significant weight loss, indicating that infections by all strains were effective as has been shown for other *S. aureus* strains [20].

Arthritis incidence and clinical scores varied among *S. aureus* strains. ATCC 19095 SEC^+ and N315 ST5 TSST-1^+ were the most arthritogenic ones. They triggered earlier symptoms and the highest levels of incidence. The ATCC 19095 SEC^+ strain was associated with the highest clinical scores. The S-70 TSST-1^+ infected group presented a delayed clinical manifestation and lower clinical scores whereas the ATCC 51650 TSST-1^+ strain did not trigger any sign of arthritis during 14 days. The ATCC 13565 SEA^+ was lethal causing death of 85% of the animals after 48h (not shown).

No direct relationship was found between arthritogenicity and bacterial inoculum. By comparing bacterial inoculum with the degree of arthritis severity, we can conclude

that the differential arthritis severity was not associated with distinct bacterial concentrations. In addition, the ATCC 13565 SEA^+ strain that killed the animals was injected in the same concentration as the arthritogenic strains.

Concerning this differential arthritogenicity among *S. aureus* strains we would like to highlight two aspects. This is the first demonstration that these strains that were originally isolated from biological samples can cause septic arthritis in mice. In addition these results indicate that SAg production was not enough to elicit arthritis. One of the TSST-1 strains (ATCC 51650) was not able to induce septic arthritis even though it has been used in the range of concentration considered adequate by other author [17]. This finding is in accordance with the idea that induction of arthritis during *S. aureus* infection is most likely triggered by the concerted action of multiple events as SAgs activating T lymphocytes, exposure to peptidoglycans/capsular polysaccharides, surface-associated adhesins, "*clumping*" factor A and free bacterial DNA [2, 11, 12, 13].

The histopathological analysis of the joints revealed the presence of synovial proliferation, *pannus* formation and inflammatory infiltrates in the joint cavity. These findings are in accordance with the most consensual features of SA caused by *S. aureus* [21]. Cartilage and bone erosion were also present, mainly in joints from animals infected with the ATCC 19095 SEC^+ strain. This evolution from the inflammatory process to cartilage and bone erosion is very relevant because it mimicries the human situation during SA by *S. aureus*. In this case, 25-50% of the patients progress to bone destruction and irreversible loss of joint function [22,23,24].

TNF-α, IL-6 and IL-17 are being described as some of the most relevant mediators in SA immunopathogenesis [25,26]. Involved we checked their production in spleen cell cultures stimulated with a particulate *S. aureus* antigen. Interestingly, the

lowest levels of these three cytokine were found in cultures from mice that presented the lowest clinical scores. In addition, differently from IFN-γ, TNF-α and IL-6, IL-17 was not produced by spleen cells from normal mice. The highest production of IL-17 was observed in infection associated with the more arthritogenic strains, i.e., ATCC 19095 SEC^+ and N315 ST5 TSST-1^+ *S. aureus*. The role of IL-17 in mediating joint destruction is very well studied in rheumatoid arthritis. It has been described that this cytokine promotes bone and joint damage through induction of matrix metalloproteinases and osteoclasts [24, 27]. IL-17 also induces production of IL-6 and IL-8 by rheumatoid arthritis synovial fibroblasts via NF-kB and PI3-kinase/ Akt-dependent pathways [28]. IL-17 induces production of other proinflammatory cytokines and chemokines such as TNF-α, IL-1β, CXCL1, CXCL5 [29,30]. These cytokines have potent effects on the process of bone remodeling by stimulating the proliferation and differentiation of osteoclast progenitors in to mature osteoclasts in the presence of osteoblasts [31, 32].

Contrasting with this well established role of IL-17 in RA, the role of IL-17 in *S. aureus*-induced arthritis is not well known. To our knowledge, this higher production of IL-17 in mice infected with the most arthritogenic strains is being described for the first time. Even though this cytokine has been associated with protection in animals immunized with "clumping" Factor A and also with local host defense during *S. aureus*-induced arthritis, its arthritogenic contribution in SA is still not disclosed. In this sense, these results strongly support the possibility that higher IL-17 inducer strains are endowed with stronger arthritogenic ability [25, 33].

Conclusions

Together these results demonstrated that *S. aureus* strains isolated from biological samples were able to induce a typical septic arthritis in mice. Interestingly, the most arthritogenic strain was originally isolated from a human leg abscess. These results also suggest that the variable arthritogenicity of these strains was at least in part, related to their differential ability to induce IL-17 production.

Abbreviations

ATCC - American Type Culture Collection

BHI- Brain heart infusion

CFU - Colony forming units

ConA - Concanavalin A

EDTA- Ethylenediamine tetraacetic acid

MCH - Major histocompatibility complex

NF-kB - factor nuclear kappa B

RA- Rheumatoid arthritis

RANKL - Receptor activator of nuclear factor kappa-B ligand

SA- Septic arthritis

SAgs – Superantigens

SEC - Staphylococcal enterotoxin C

SEA - Staphylococcal enterotoxin A

SAC - *S. aureus* Cowan strain I (Pansorbin)

TSST - Toxic shock syndrome toxin

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This study was conceived by PMCM and AS. All authors contributed to carry out the experiments, read and approved the final manuscript.

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Figures

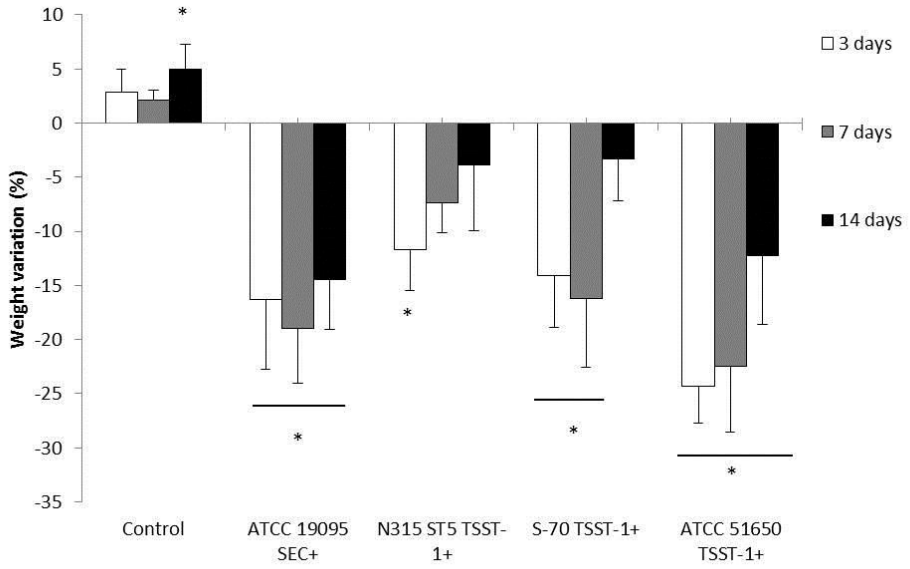


Figure 1. Weight variation in C57BL/6 mice infected with *S. aureus* strains. Animals were infected with ATCC 19095 SEC$^+$, N315 ST5 TSST-1$^+$, S-70 TSST-1$^+$ or ATCC 51650 TSST-1$^+$. Weight variation at 3, 7 and 14 days after infection is illustrated. Data is presented by mean ± SE of 6 mice. * represents the difference between initial weight and the weight in each time interval. $p< 0.05$.

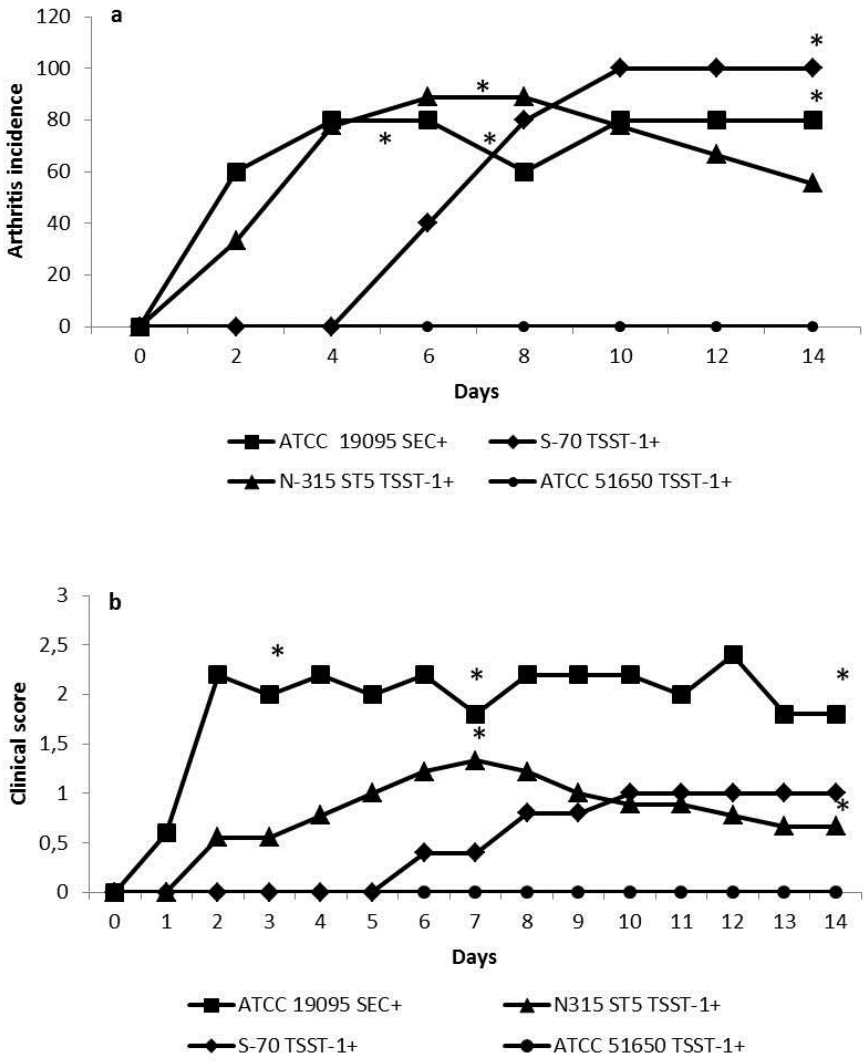


Figure 2. Arthritis development in C57BL/6 mice infected with S. *aureus* strains. Animals were infected with ATCC 19095 SEC$^+$, N315 ST5 TSST-1$^+$, S-70 TSST-1$^+$ or ATCC 51650 TSST-1$^+$. (a) Arthritis incidence and (b) clinical score were daily evaluated. Data is presented by mean ± SE of 6 mice. * represents the difference with the group infected by the non arthritogenic ATCC 51560 TSST-1$^+$ strain. $p< 0.05$.

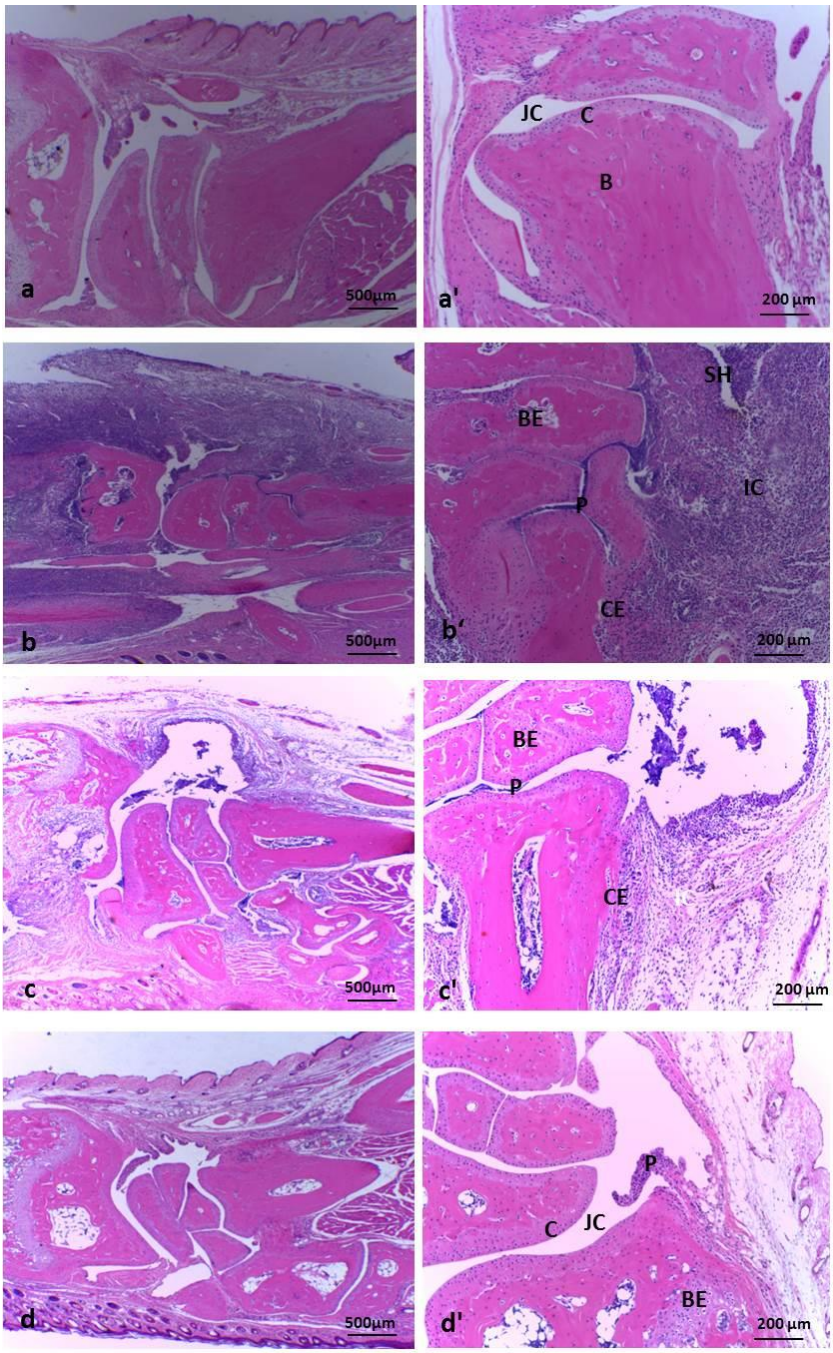


Figure 3. Histopathological analysis in forepaw joints from C57L/6 mice infected with *S. aureus* strains. (a, a') non infected control. (b, b') infected with ATCC 19095 SEC^{+}. (c, c') infected with N315 ST5 $TSST\text{-}1^{+}$. (d, d') infected with the S-70 $TSST\text{-}1^{+}$. (a), (b), (c), (d) and (a'), (b'), (c '), (d') represent histopathological micrographics with 4x and 10x magnification, respectively. JC, joint cavity; C, cartilage; B, bone; SH, synovial hyperplasia; BE, bone erosion; CE cartilage erosion; P, *pannus* formation and IC, inflammatory cells. Panel is representative of 6 animals/group.

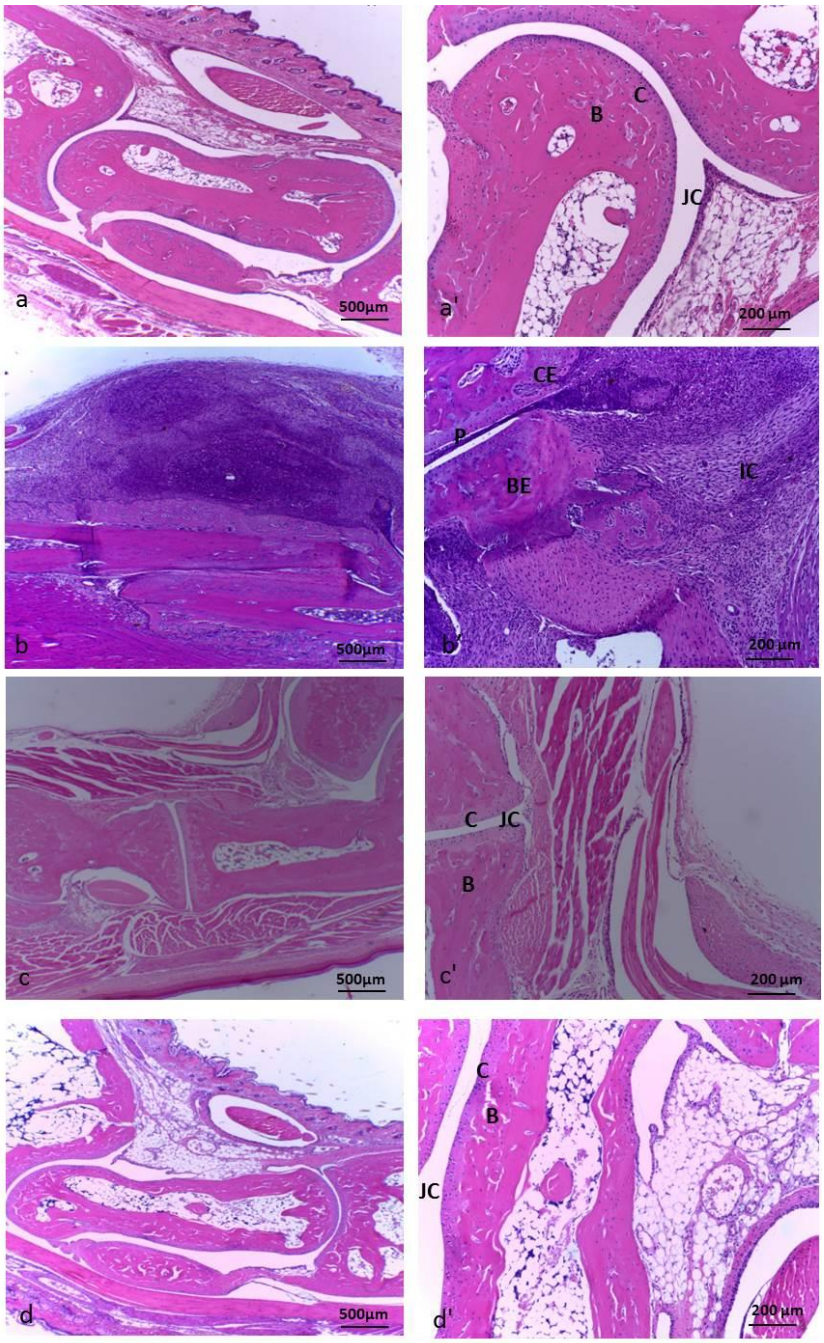


Figure 4. Histopathological analysis in hind paw joints from C57L/6 mice infected with *S. aureus* strains. (a, a') non infected control. (b, b') infected with ATCC 19095 SEC^{+}. (c, c') infected with N315 ST5 TSST-1^{+}. (d, d') infected with the S-70 TSST-1^{+}. (a), (b), (c), (d) and (a'), (b'), (c '), (d') represent histopathological micrographics with 4x and 10x magnification, respectively. JC, joint cavity; C, cartilage; B, bone; BE, bone erosion; CE cartilage erosion; P, *pannus* formation and IC, inflammatory cells. Panel is representative of 6 animals/group.

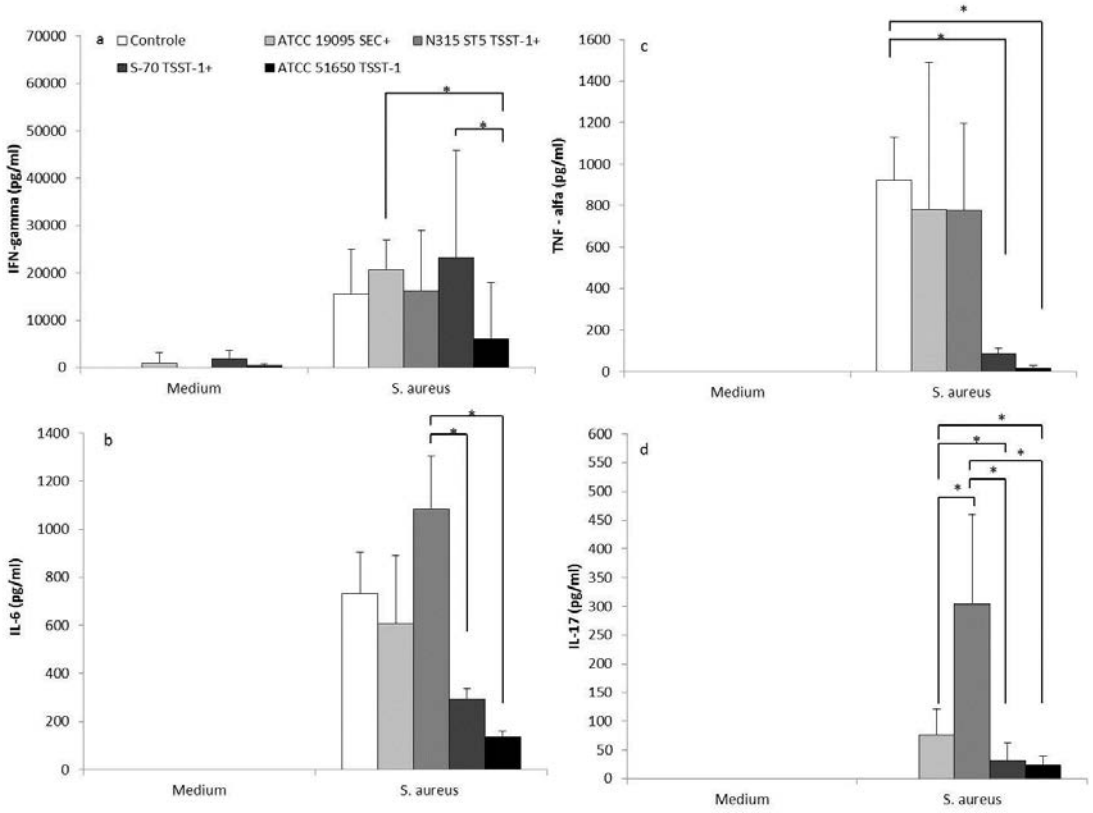


Figure 5. Production of cytokines by spleen cell cultures stimulated with *S. aureus.* C57BL/6 mice were infected with ATCC 19095 SEC$^+$, ATCC N-315 ST5 TSST-1$^+$, ATCC S-70 TSST-1$^+$, and ATCC 51650 TSST-1$^+$. IFN-γ (a), IL-6 (b), TNF-α (c) and IL-17 (d) were quantified in culture supernatants by ELISA after 48h of incubation. Data is presented by mean±SE of 6 mice. * represents statistical difference between the two indicated groups. $p<0.05$

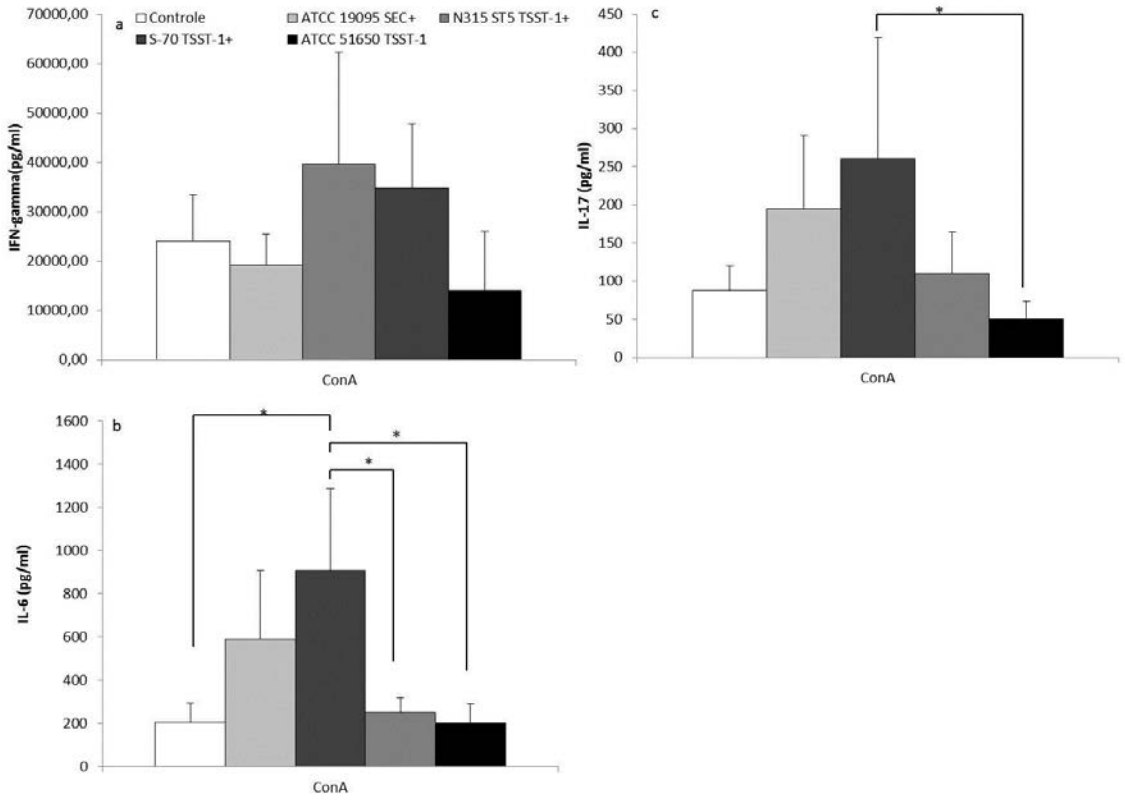


Figure 6. Production of cytokines by spleen cell cultures stimulated with ConA. C57BL/6 mice were infected with ATCC 19095 SEC$^+$, ATCC N-315 ST5 TSST-1$^+$, ATCC S-70 TSST-1$^+$, and ATCC 51650 TSST-1$^+$. IFN-γ (a), IL-6 (b), and IL-17 (c) were quantified in culture supernatants by ELISA after 48h of incubation. Data is presented by mean±SE of 6 mice. * represents statistical difference between the two indicated groups. $p<0.05$.

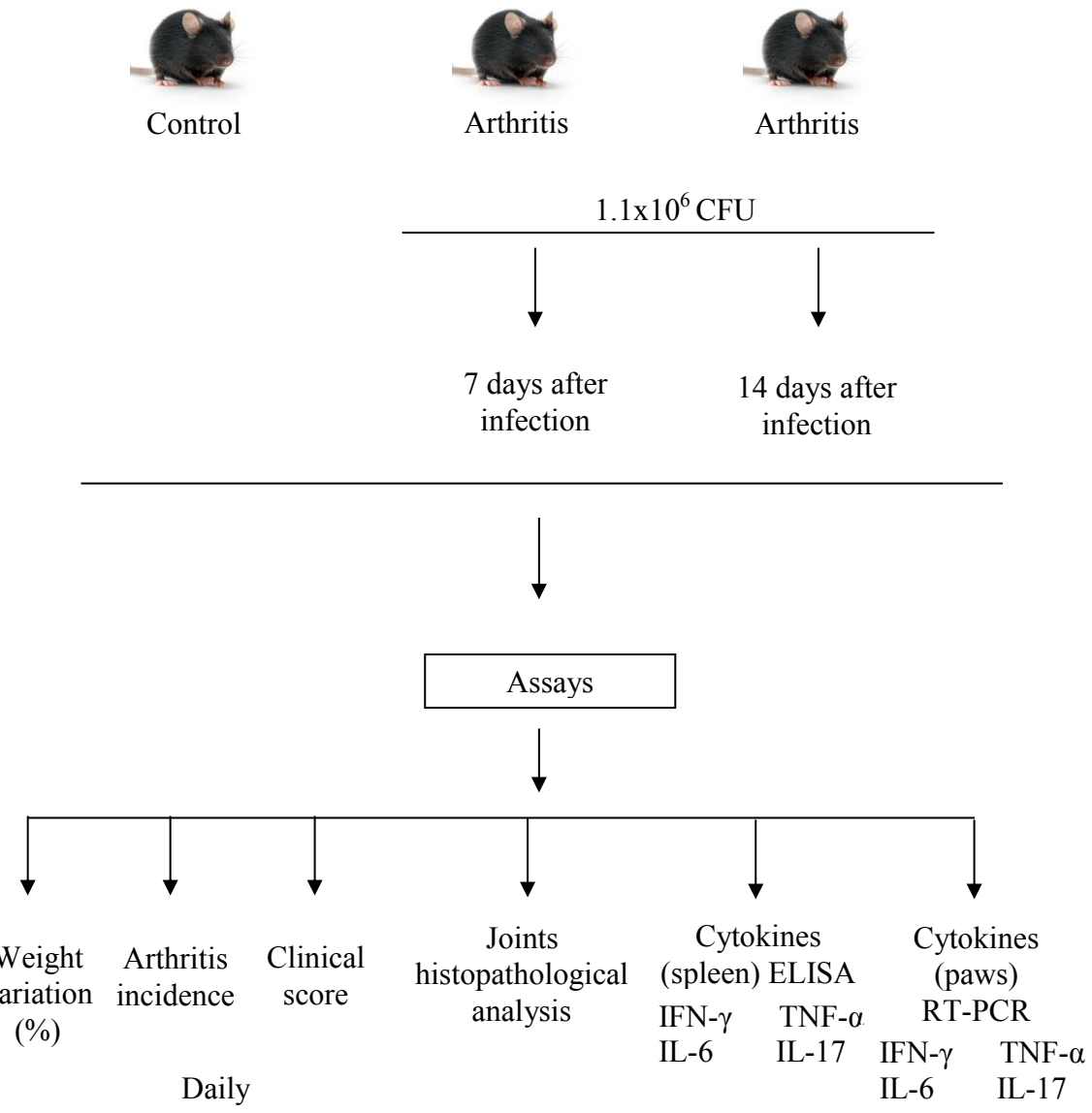
Table

Table 1. Superantigen producing strains and the number of colony forming units

Strain	CFU
ATCC 19095 SEC^+	$2.2x10^7$
N315 ST5 $TSST\text{-}1^+$	$9.4x10^7$
S-70 $TSST\text{-}1^+$	$7.8x10^8$
ATCC 51650 $TSST\text{-}1^+$	$7.8x10^{11}$
ATCC 13565 SEA^+	$1.3x10^7$

Artigo científico II

Artigo científico II: Septic arthritis triggered by enterotoxin C producer *Staphylococcus aureus* is associated with strong Th_1 and Th_{17} expansion



Septic arthritis triggered by enterotoxin C producer *Staphylococcus aureus* is associated with strong Th_1 and Th_{17} expansion

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Abstract

Staphylococcus aureus is the most common causative agent of septic arthritis that is a severe, rapidly progressing and erosive disease. Superantigens produced by *S. aureus* are considered major arthritogenic factors. In this study we determined the arthritogenicity of an enterotoxin C producer *S. aureus* and we also evaluated the contribution of T cell subsets and their cytokines to the inflammatory process. Male C57BL/6 mice were intravenously infected with the ATCC 19095 SEC^+ *S. aureus* strain and clinical parameters as body weight, clinical score and disease incidence were daily evaluated during 14 days. Cytokine production by splenocytes and cytokine mRNA expression by joint tissue were evaluated 7 and 14 days after infection by enzyme linked immunosorbent assay and real-time PCR, respectively. Local histopathological analysis and quantification of T cell subsets were also evaluated at both periods. ATCC 19095 SEC^+ strain caused a very severe experimental arthritis in C57BL/6 mice. Infected animals lost weight, achieved high clinical scores and a 100% disease incidence. Histopathological analysis revealed inflammation, *pannus* formation and bone erosion. Arthritis appearance and aggravation were associated with increased levels of arthritogenic cytokines. Th_1 and Th_{17} cells predominated over Th_2 and Treg cells in the articular lesions. These findings indicate that this *S. aureus* strain triggers a very typical infectious arthritis that can be further explored as an experimental model to investigate immunopathogenesis of human septic arthritis.

Key-words: Enterotoxin C, *S. aureus*, septic arthritis, bone erosion, cartilage erosion

Introduction

Septic arthritis (SA), also called infectious arthritis, is an inflammatory disease of the joints that is started by an infectious agent. Typically, SA involves one large joint as the knee or the hip but it can also commit any other joint. The most common causative organism in both, children and adults SA, is *Staphylococcus aureus* (Howard-Jones *et al.,* 2013; Clerc *et al.,* 2011). SA by *S. aureus* is generally considered a secondary infection, i.e., the bacteria exits the bloodstream and enters the surrounding tissues. A number of strategies as endothelial attachment, transcytosis, paracytosis and bacteria transportation by professional phagocytes have been described as putative mechanisms to allow *S. aureus* to disseminate from the blood to other tissues (Edwards & Massey 2011).

The local damage in SA results from a complex combination of bacterial invasion, host inflammation and tissue ischemia. Bacterial enzymes and toxins are directly harmful to cartilage that can also be damaged by lysosomal proteases and reactive oxygen species released by neutrophils (Ross, 2005). Even though not fully understood, the contribution of a cytokine net to bone and cartilage destruction is highly supported by numerous investigations in both, human and experimental models (Mal *et al.,* 2013; Henningsson *et al.,* 2012; Varoga *et al.,* 2009).

Numerous virulence factors have been associated with the well-established *S. aureus* arthritogenicity. The bacterium is able to express staphylococcal adhesins as collagen adhesin and clumping factor (a molecule that mediates fibrinogen-dependent clumping of staphylococci) which allow it to adhere to joint structures. Formylated peptides can be also released by the bacterium allowing the recruitment of neutrophils into synovial tissue, that substantially contribute to joint destruction (Switalski *et al.,* 1993; Patti *et al.,* 1994; Palmqvist *et al.,* 2005; Gjertsson *et al.,* 2012).

In addition, *S. aureus* produces and secretes a large number of enzymes and toxins that have been implicated in infectious arthritis (Tarkowski, 2006). A subset of these molecules displays superantigenic properties, i.e., they possess the unique ability to activate a large number of T lymphocytes expressing certain Vβ sequences. As the human genome encodes approximately 50 TCR Vβ elements, it has been estimated that these superantigens can activate up to 20% of the T cell pool (Proft & Fraser, 2003). This Vβ recognition is simultaneously associated to binding to antigen presenting cells via MHC class II molecules. These interactions are able to trigger T cell proliferation and a massive cytokine release by both cell types (Baker & Acharya, 2004; Grumann *et al.,* 2013). The contribution of superantigens to SA has been clearly observed in experimental arthritis (Bremell & Tarkowski, 1995; Abdelnour *et al.,* 1994; Abdelnour *et al.,* 1994) Even though the TSST-1 superantigen have been more frequently (47%) found in the synovial fluid of patients with SA, enterotoxin C was also found in 39% of the cases (Noorbakhsh *et al.,* 2013).

In this context, we used an experimental murine model to study the arthritogenicity of an enterotoxin C *S. aureus* strain. We also analysed the contribution of T cell subsets and their cytokines to the local inflammatory and destructive process.

Material and Methods

1. Experimental design

Mice were infected with an enterotoxin C producer *S. aureus* strain and were sacrificed 7 and 14 days after infection. They were evaluated by clinical follow-up (body weight, arthritis incidence and clinical score) and also by histopathological analysis of the hind and forepaws. Cellular immune response was checked by cytokine production by spleen cells stimulated with *S. aureus* and also by mRNA expression in

joint samples. The local contribution of distinct T cell subsets (Th_1, Th_2, Th_{17} and Tregs) to immunopathogenesis was determined by real-time PCR. Non-infected animals were included as a control group.

2. Animals

Male C57BL/6 mice (8-10 weeks old) were purchased from PUSP-RP (USP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Medical School, Univ. Estadual Paulista).

3. *S. aureus* strains and culture conditions

The ATCC 19095 SEC^+ was originally isolated from a leg abscess of a patient in Albert Merritt Billings Hospital, University of Chicago. Before each experiment, bacteria were cultured in blood agar plates (Merck) for 24h at 37ºC in order to confirm their purity and to determine their morphology and specific color. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37ºC for 24h. Bacteria were collected by centrifugation, washed three times and resuspended in cold sterile saline, as described by França *et al.,* (2009). The bacterial suspensions were prepared according to the McFarland nephelometer nº 0.5. The exact amount of live bacterial cells was determined by further enumeration of the number of colony forming units (CFU) on agar plates.

4. Arthritis induction and clinical evaluation

Disease induction was performed according to the classical methodology described by Bremell *et al*. (1991). However, the original caudal vein infection route was replaced by the retro-orbital via. Each animal was infected with 0.2 mL ($1.1x10^6$ CFU) of a *S. aureus* suspension made in physiological saline; control mice were injected with 0.2 mL of physiological saline. All mice were individually checked out every day and the mean number of arthritic limbs per animal was registered. Arthritis was defined as visible joint erythema and/or swelling of at least one joint or both. To evaluate the intensity of arthritis, a clinical scoring (arthritic index) was carried out, using a system where macroscopic inspection yielded a score of 0–3 points for each limb (1 point = mild swelling and/or erythema; 2 points = moderate swelling and erythema; 3 points = marked swelling and erythema). The arthritic index was obtained by dividing the total score (number of arthritic limbs) by the number of animals used in each experimental group.

5. Histopathological examination

Histopathological analysis of the joints was comparatively performed after 7 and 14 days of infection. Briefly, fore and hind paws were fixed with 10% formaldehyde and embedded in paraffin after decalcification with 18% EDTA. Serial sections with 5µm thickness were cut and stained with haematoxilin and eosin. The sections were qualitatively evaluated in relation to the presence of inflammatory infiltrates, hyperplasia of the synovial membrane, cartilage destruction and bone erosion. Predominance of mononuclear or polymorphonuclear cells was also assessed.

6. Cytokine quantification

Animals were euthanized 7 and 14 days after infection and spleen cells were adjusted to $5x10^6$ cells/mL. Cells were cultured in RPMI medium supplemented with 5% of fetal calf serum, 20mM of glutamine and 40 IU/mL of gentamicin in the presence of *S. aureus* Cowan I (SAC), from Calbiochem-Behring La Jolla, CA. This *S. aureus* suspension was used at a final dilution of 1:2500(v/v) in the culture. Cytokine levels were evaluated 48h later, in culture supernatants, by enzyme-linked immunosorbent assay (ELISA) using IFN-γ BD OptEIA Sets (Becton Dickinson) and IL-6, IL-17 and TNF-α Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instruction.

7. Cytokine quantification by mRNA expression

Hind paws were collected, fragmented and stored in RNAlater (Ambion, Austin, TX) solutions. Samples were submitted to molecular analyses as previously described by Garlet *et al.* (2010). Total RNA was extracted from samples by using TRIZOL reagent (Life Technologies, Grand Island, NY), and complementary DNA was synthesized by using 3 µg RNA in a reverse transcription reaction as described elsewhere by Menezes *et al*., (2008) and Garlet *et al*., (2003). Real-time polymerase chain reaction (PCR) quantitative mRNA analysis was performed in an Viia7 instrument (Life Technologies Corporation) by using TaqMan system chemistry (Life Technologies Corporation), 100 nmol/L specific taqman primers inventoried primers, and 2.5 ng cDNA (or 5 ng DNA) in each reaction. The standard PCR conditions were 95ºC (10 minutes), followed by 40 cycles of 94ºC (1 minute), 56ºC (1 minute), and 72ºC (2 minutes). The relative levels of gene expression were determined by using the

cycle threshold method in reference to glyceraldehyde-3-phosphate dehydrogenase, and β-actin expression, as described elsewhere (Menezes *et al*., 2008; Garlet *et al*., 2003).

8. Statistical analysis

Data were expressed as mean ± SE. Comparisons between infected groups were made by Student's test and ANOVA. Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results:

1. Weight variation

As shown in figure 1a, the normal control group presented a small weight gain after 7 and 14 days. At these same time periods, the SEC^+ infected groups presented a significant weight loss.

2. Arthritis incidence and clinical score

The majority of the infected animals presented arthritis characterized by edema and erythema, being the incidence after 7 and 14 days, 90% and 100%, respectively (figure 1b). Some animals already presented swollen joints (clinical scores around 0.5) after 24 hours. These scores steadily increased and reached 3.0 at day 5. These high clinical score were maintained until day 14 that was the last evaluated period (figure 1c). At 7 days fore and hind paws were similarly affected. Even though both paws showed higher scores by day 14, the clinical scores were always higher in the hind paws (figure 1d).

3. Correlation between histopathological alterations and clinical scores

Histopathological analysis done in joint samples from distinct clinical scores considered the following criteria: presence of synovial hyperplasia, visualization of joint cavity, degree of inflammation, *pannus* formation, cartilage destruction and also the occurrence of bone erosion. Generally histopathological alterations occurred in both, fore and hind paws and they became more accentuated in higher scores. The clinical appearance of a normal or arthritic forepaw presenting scores 1, 2 and 3 is shown in figures 2a, 2b, 2c and 2d respectively. Their corresponding histopathological characteristics are illustrated in figures 2a', 2b', 2c' and 2d' respectively. The same analysis was done in hind paws and is illustrated in figure 3. As expected, a very thin synovial layer, a marked joint cavity, an intact cartilage and a preserved bone structure can be observed in samples derived from normal mice (figure 2a' and 3a') . In samples from score 1 there is already an evident synovial hyperplasia and an incipient *pannus* formation, together with the presence of inflammatory infiltrates. In spite of these alterations we can still clearly see the joint cavities and the almost entirely preserved cartilage and bone structures (figure 2b' and 3b'). The most striking histopathological alterations detected in scores 2 and 3 included the presence of a conspicuous *pannus* formation that is occupying almost the entire joint cavity and also a huge increase in the amount of inflammatory cells (figure 2c', 2d', 3c'and 3d'). Mononuclear and polymorphonuclear cells are present among inflammatory cells a clear predominance of PMN cells is observed only at 14 days (figure 4d). Cartilage and bone erosion are clearly observed in these two scores. All theses findings are more pronounced in score 3. Aggravation of histopathological findings was clearly observed when hind paw samples from 7 days were compared to 14 days; this finding is illustrated in figures 4a and 4b.

4. Cytokine evaluation

Spleen cells from normal and infected mice (7 and 14 days) were stimulated with *S. aureus* and the production of TNF-α, IL-6, IFN-γ and IL-17 was evaluated 48h after incubation. Normal and infected mice produced similar amounts of TNF-α at 7 days, however infected animals produced significantly higher levels of this cytokine at 14 days comparing to the 7 days time period (figure 5a). The prolife of IL-6 production presented exactly this same pattern as illustrated in figure 5b. Production of IFN-γ was always higher in the infected group. Even though there was an increased production of IFN-γ at the 14 day of infection, this difference was not statistically different from the values observed after 7 days. Spontaneous production of this cytokine was detected in non stimulated cultures from infected but not from normal mice (figure 5c). IL-17 production was also significantly higher after 14 days of infection in comparison to 7 days (figure 5d). Differently from the other 3 cytokines, IL-17 was not produced by spleen cells from normal mice. The production of these same cytokines was also evaluated in arthritic lesions by real-time PCR. Their corresponding mRNA was already raising 7 days after infection; however, only IL-17mRNA levels were significantly elevated in comparison to the amounts found in the non infected control group (figure 6d). On the other hand, mRNA expression for all 4 cytokines after 14 days of infection was significantly increased in comparison to both, normal mice and 7 days of infection (figure 6a, 6b, 6c and 6d).

5. Quantification of T cell subsets

T cell subsets (Th_1, Th_2, Th_{17} and Treg) were quantified by the mRNA expression of their corresponding transcription factors. tBET, RORg and $GATA_3$ mRNA levels were higher in infected mice at both time periods (figure 7a, 7b, 7c and 7d). tBET

mRNA was the only factor that showed significant increase when 14 days expression was compared to the one observed at 7 days of infection (Figure 7a). A very discrete and variable increment was detected in FOXp3 mRNA levels in samples from infected animals (figure 7c).

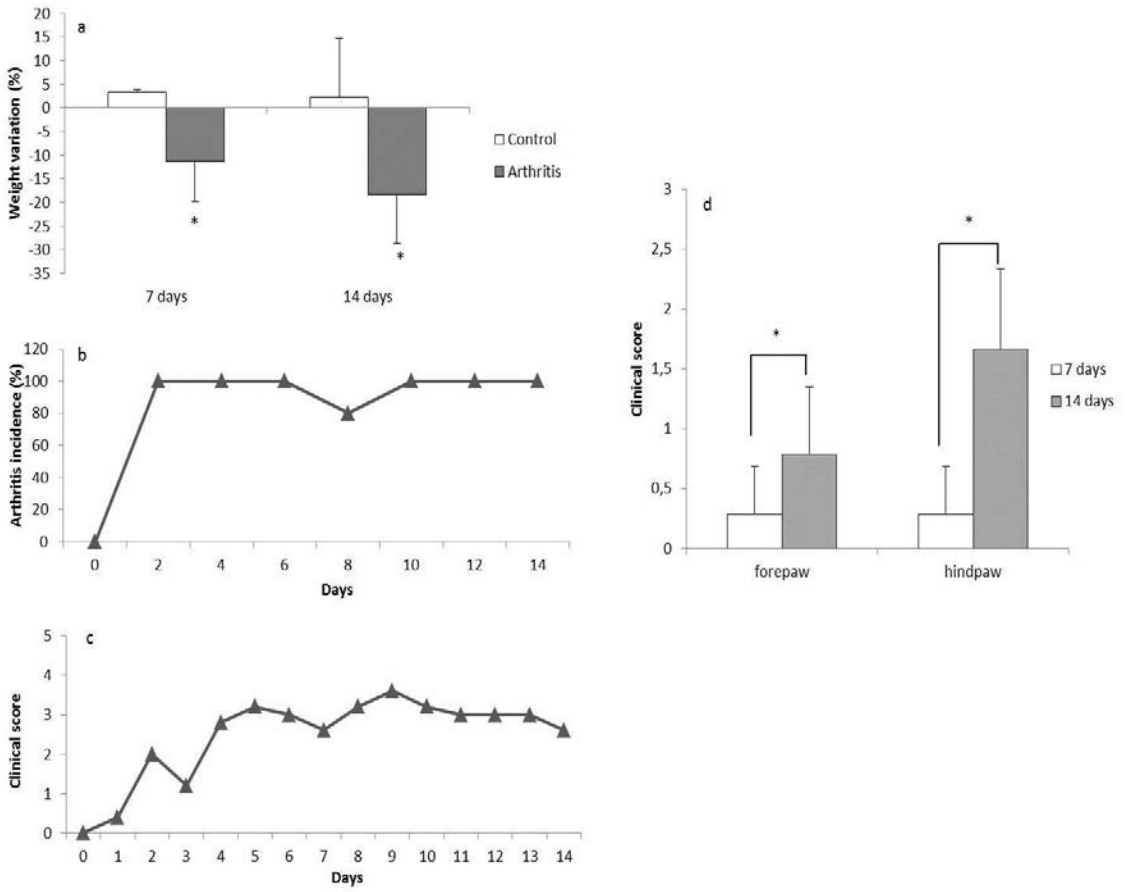

Figure 1. Arthritis development in C57BL/6 mice infected with enterotoxin C producer *S. aureus.* (a) Weight variation after 7 and 14 days, (b) Arthritis incidence, (c) Total clinical score and (d) Fore and hind paws clinical scores. Data were daily evaluated and were presented by mean ± SE of 6 mice and is representative of two independent experiments. * represents significant differences ($p < 0.05$).

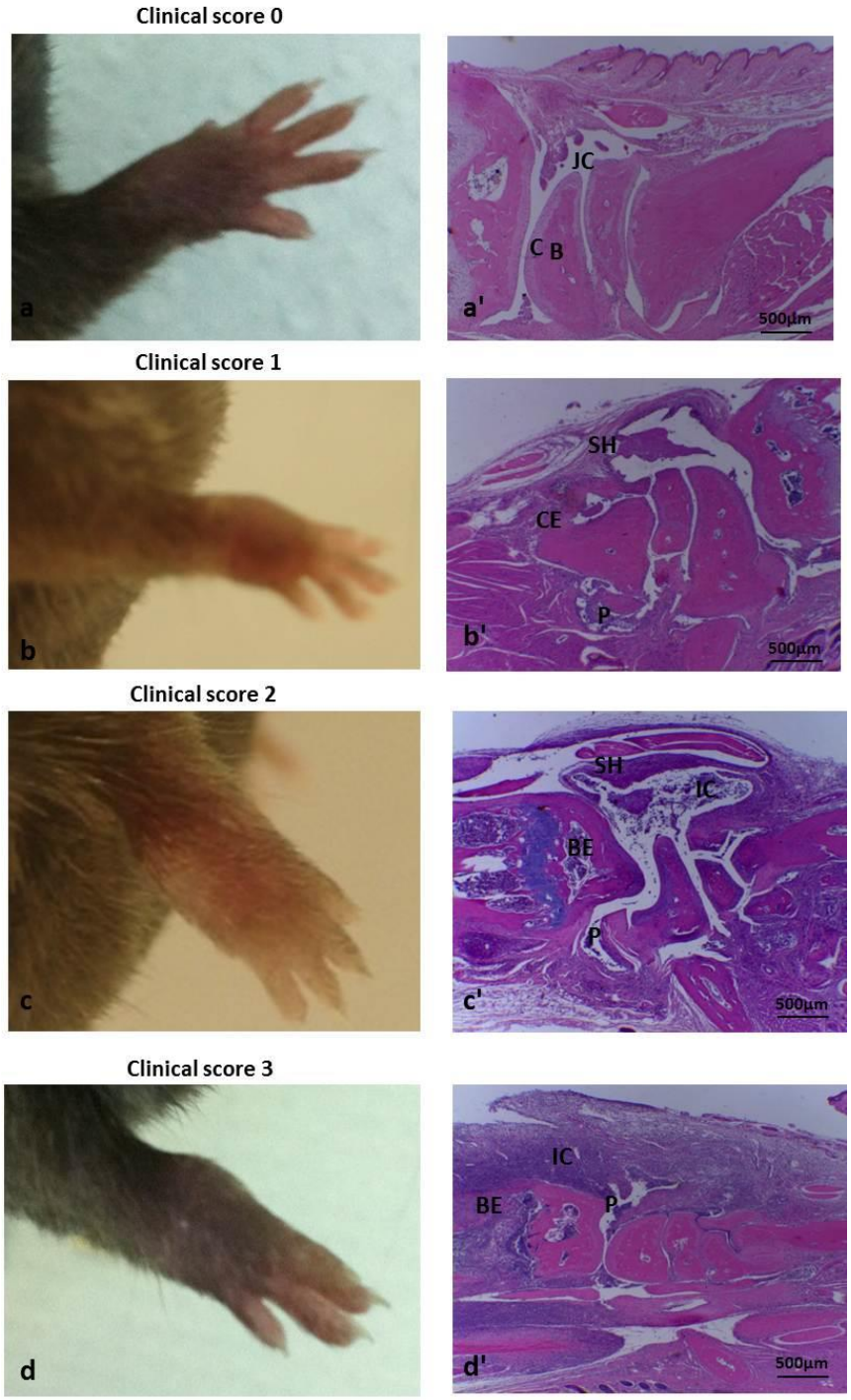

Figure 2. Clinical scores and corresponding histopathological analyses of forepaws from C57BL/6 mice infected with enterotoxin C producer *S. aureus* strain. (a, a') clinical score 0 in normal mice; (b, b') clinical score 1; (c, c') clinical score 2; (d, d') clinical score 3 in arthritis mice. Histopathological micrographics are shown with 4x magnification. JC, joint cavity; C, cartilage; B, bone; SH, synovial hyperplasia; BE, bone erosion; CE cartilage erosion; P, *pannus* formation and IC, inflammatory cells. Panel is representative of 6 animals/group.

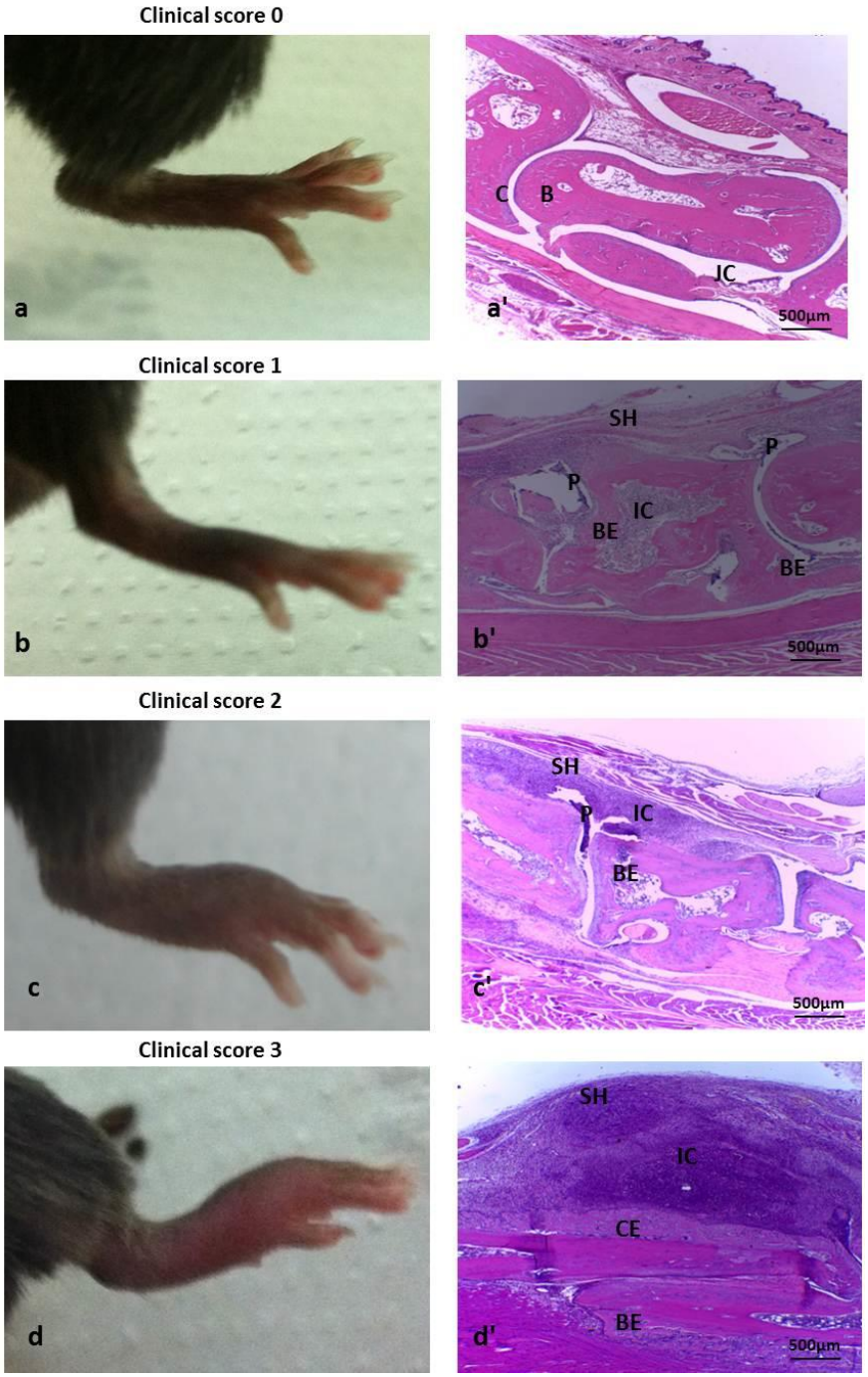

Figure 3. Clinical scores and corresponding histopathological analyses of hind paws from C57BL/6 mice infected with enterotoxin C producer *S. aureus* strain. (a, a') clinical score 0 in normal mice; (b, b') clinical score 1; (c, c') clinical score 2; (d, d') clinical score 3 in arthritis mice. Histopathological micrographics are shown with 4x magnification. JC, joint cavity; C, cartilage; B, bone; SH, synovial hyperplasia; BE, bone erosion; CE cartilage erosion; P, *pannus* formation and IC, inflammatory cells. Panel is representative of 6 animals/group.

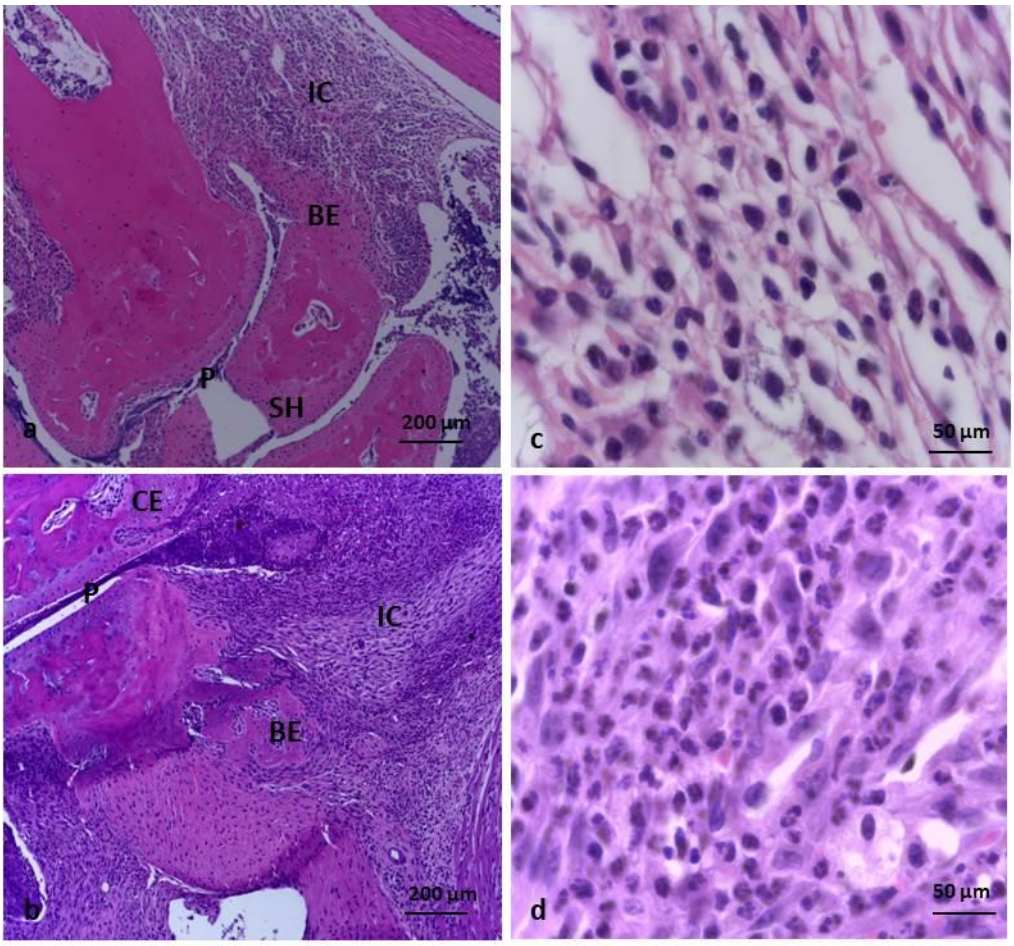

Figure 4. Disease aggravation after 14 days of infection. Histopathological analyses done after 7 (a) and 14 days (b). Mixed inflamatory infiltrate containing mononuclear and polymorphonuclear cells (c) after 7 days and predominant polymorphonuclear infiltrate after 14 days (d)**.** Histopathological micrographics are shown with 10x (a,b) and 40x (c,d) magnification. IC, inflammatory cells; BE, bone erosion; CE, cartilage erosion; SH, hyperplasic the membrane synovial; P, *pannus* formation. Panel is representative of 6 animals/group.

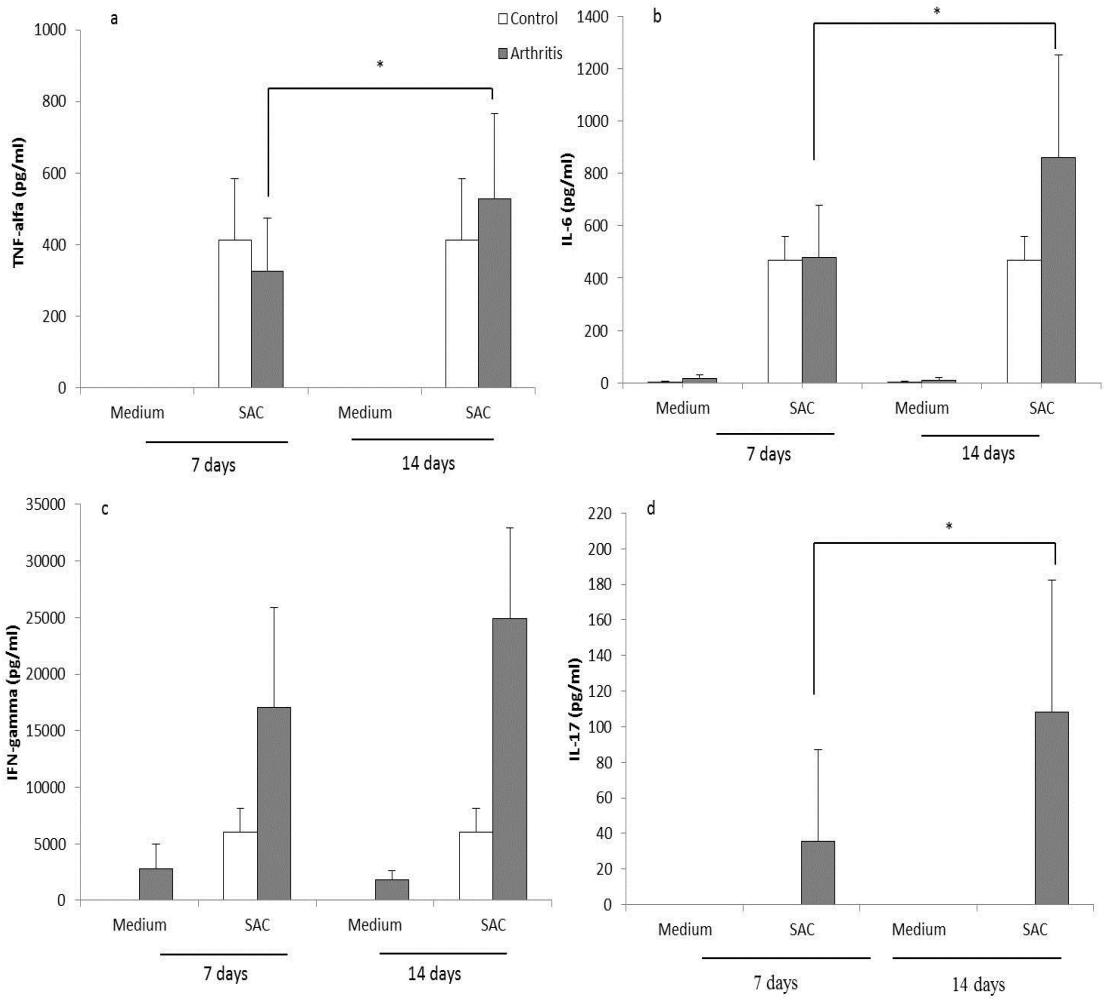

Figure 5. Production of cytokines by spleen cells from mice with arthritis. C57BL/6 mice were infected with enterotoxin C producer *S. aureus* and TNF-α (a), IL-6 (b), IFN-γ (c) and IL-17 (d) production were assayed in spleen cells cultures stimulated *in vitro* with *S. aureus* (SAC). Data were presented by mean±SE of 6 mice and is representative of two independent experiments. *represents significant difference between 7 and 14 days ($p<0.05$).

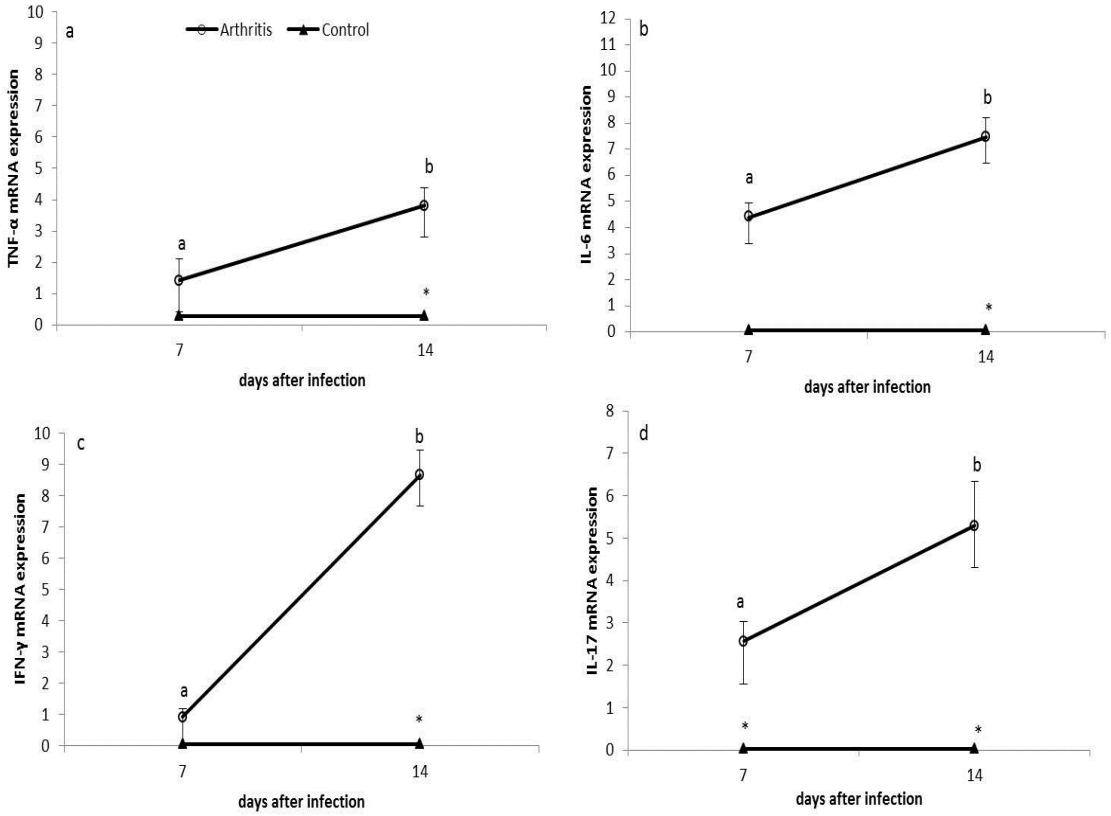

Figure 6. TNF-α, IFN-γ, IL-6 and IL-17 mRNA in mice with arthritis. C57BL/6 mice were infected with enterotoxin C producer *S. aureus*. Hind paws from experimental and control groups were collected to analyze mRNA expression that was quantified by real-time PCR by using the SYBR Green system. (a) TNF-α; (b) IL-6; (c) IFN- γ and (d) IL-17 production. * represents the difference between the negative control and the arthritic group; letters represent the difference between 7 and 14 days ($p < 0.05$).

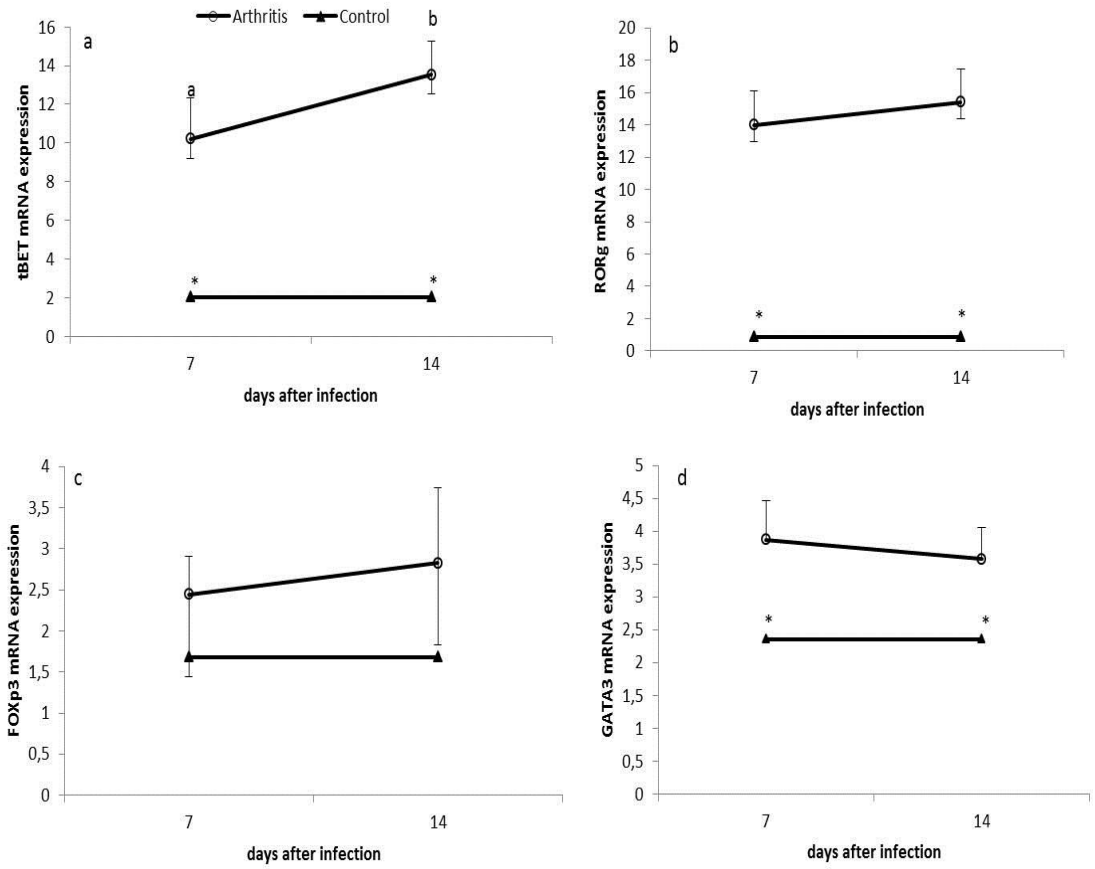

Figure 7. Expression of Th subset transcription factors in mice with arthritis. The levels of tBET, RORg, Foxp3 and GATA3 mRNA were evaluated in the inguinal LNs (a–d, respectively). The results are presented as mean±SE of the target mRNAs with normalization to β-actin obtained in 6 mice/group. * represents the difference between the normal and the arthritic group; letters represent the difference between 7 and 14 days ($p<0.05$).

Discussion

Septic arthritis caused by *S. aureus* is known as the most severe form of inflammatory joint pathology, leading to an extensive cartilage and bone destruction in a few days. Most of the experimental models used to study the involved immunopathogenesis and the effects of therapeutical products are obtained by inoculation of the *S. aureus* LS-1 strain. This strain is widely described as able to cause septic arthritis in different mouse strains (Bremell *et al*., 1991; Tarkowski *et al*., 2001). The ability of *S. aureus* LS-1 to produce the superantigen (SAg) TSST-1 is also well recognized and has been linked to its arthritogenic properties (Sakiniene *et al.,* 1999; Sakiniene & Tarkowski, 2002).

In the present study we characterized the development of septic arthritis in C57BL/6 mice infected with an *S. aureus* strain that produces the enterotoxin C (SEC^+). Male mice were infected with $1.1x10^6$ CFU and daily inspected during 14 days. Weight loss was observed after 7 and 14 days of infection and indicated an effective infection at both periods as has been already described by other authors (Bremell & Tarkowski 1995; Tarkoswisk, 2006). A very high arthritis incidence, reaching almost a 100% was observed during the experimental period. The clinical scores also remained significantly elevated. Together with histopathological findings, these results suggest that infection with the SEC^+ strain was able to trigger a septic arthritis condition very similar to the classical infectious arthritis caused by the LS-1 strain (Bremell *et al.,* 1991; Jonsson *et al*., 2004). Accordingly to our knowledge this is the first demonstration that *S. aureus* SEC^+ is highly arthritogenic. There are no reports about the colonization prevalence of human beings with this type of strain, even though it is usually isolated from animals (Marr *et al.,* 1993). Differently from the other enterotoxins, this SAg binds outside the

binding groove on the flanking helix from the α chain (Redpath *et al.*, 1999). There are also no reports on the potential of this *S. aureus* strain to cause human arthritis.

A detailed histopathological analysis indicated that hind paws were more affected than fore ones. With this analysis we also detected a very clear correlation of clinical scores and histopathological alterations in both, fore and hind paws. In the more elevated scores (2 and 3) we observed the presence of a conspicuous *pannus* formation, an accentuated infiltration of inflammatory cells and also cartilage and bone erosion. All these findings were, as expected, much more accentuated in samples obtained from animals with score 3.

Interestingly, aggravation of histopathological alterations, including bone erosion, observed at the analysis done 14 days after infection coincided with higher production of TNF-α, IL-6, IFN-γ and IL-17 by spleen cells stimulated with *S. aureus*. This arthritogenic cytokine profile found in the spleen was also present in local lesions what was ascertained by real-time PCR in samples from joint tissue. The elevated levels of TNF-α are consistent with the relevant role of this cytokine in the immunopathogenesis of septic arthritis. TNF-α is being found in synovial and blood samples from experimental and human septic arthritis (Zhao *et al.,* 1996; Osiri *et al.,* 1998; Talebi-Taher *et al.,* 2013). A more direct approach revealed that arthritis frequency was clearly higher in the wild-type mice compared with the TNF/LT alpha-deficient ones. Additionally, histopathological examination showed lower frequency of synovity and erosivity in these knockout animals (Hultgren *et al.,* 1998). More recently the role of this cytokine in joint destruction has been explored as a therapeutical target. Fei *et al.,* (2011) demonstrated that simultaneous TNF-α inhibition and antibiotic therapy mitigates staphylococcal arthritis. The elevated systemic and local production of IFN-γ in our model is also supported by other author's findings. Zhao *et al*., (1998) observed

that IFN-γ neutralization decreased both, frequency and severity of SA. The detection of elevated IFN-γ levels in spleen cultures stimulated with *S. aureus* and the local increment of tBET mRNA expression suggest that bacteria specific Th_1 cells are contributing to the inflammatory process. This possibility is reinforced by the histopathological data. A predominant mononuclear infiltrate was encountered in the joints at the 7th day of infection.

Even though the literature concerning IL-17 involvement in SA is very scarce, there is reason to believe that it plays a relevant role too. This possibility is mainly based on the crucial role of this immune mediator in rheumatoid arthritis. In this pathology IL-17 not only elicits inflammation but also triggers cartilage damage and bone erosion (Schett & Gravallese, 2012). Our findings suggest that Th_{17} and IL-17 greatly contributed to joint destruction. The significant augmentation of both, Th_{17} cells and IL-17 production that were more pronounced at the 14th day of infection, were concurrent with a change from mononuclear to polymorphonuclear preponderance. There were also clear indications of increased local bone resorption, cartilage degradation, and intense inflammation with increased *pannus* formation in this period. These findings are in accordance with the role of IL-17 in the control of *S. aureus* infections. This cytokine activates neutrophils by inducing the production of chemokines and pro-inflammatory cytokines, such as TNF-α, IL-6, CXCL1, CXCL5, and therefore exacerbates the inflammatory response and tissue damage. Studies done in RA, that shares some histopathological features with SA, indicate that this cytokine stimulates the differentiation stimulates the differentiation of bone-resorbing osteoclasts, thereby stimulating local bone resorption (Fonseca *et al.,* 2009; Schett & Gravallese, 2012).

We initially attributed IL-17 production to Th_{17} cells as has been widely accepted (Sarkar *et al.,* 2010). However, other cell sources as invariant NK T lymphocytes, γδ T cells and neutrophils could be also contribute to its elevated levels (Raifer *et al.,* 2012). These results shed some light to this complex and still challenging disease.

Considering that the infection of C57BL/6 mice with the *S. aureus* SEC^+ strain triggered a very characteristic SA, we believe that it can be explored as an additional model to study this pathology. In addition, these results show that the destructive phase of the disease, dominated by PMN cells is preceded by expansion of Th_1 and TH_{17} subsets.

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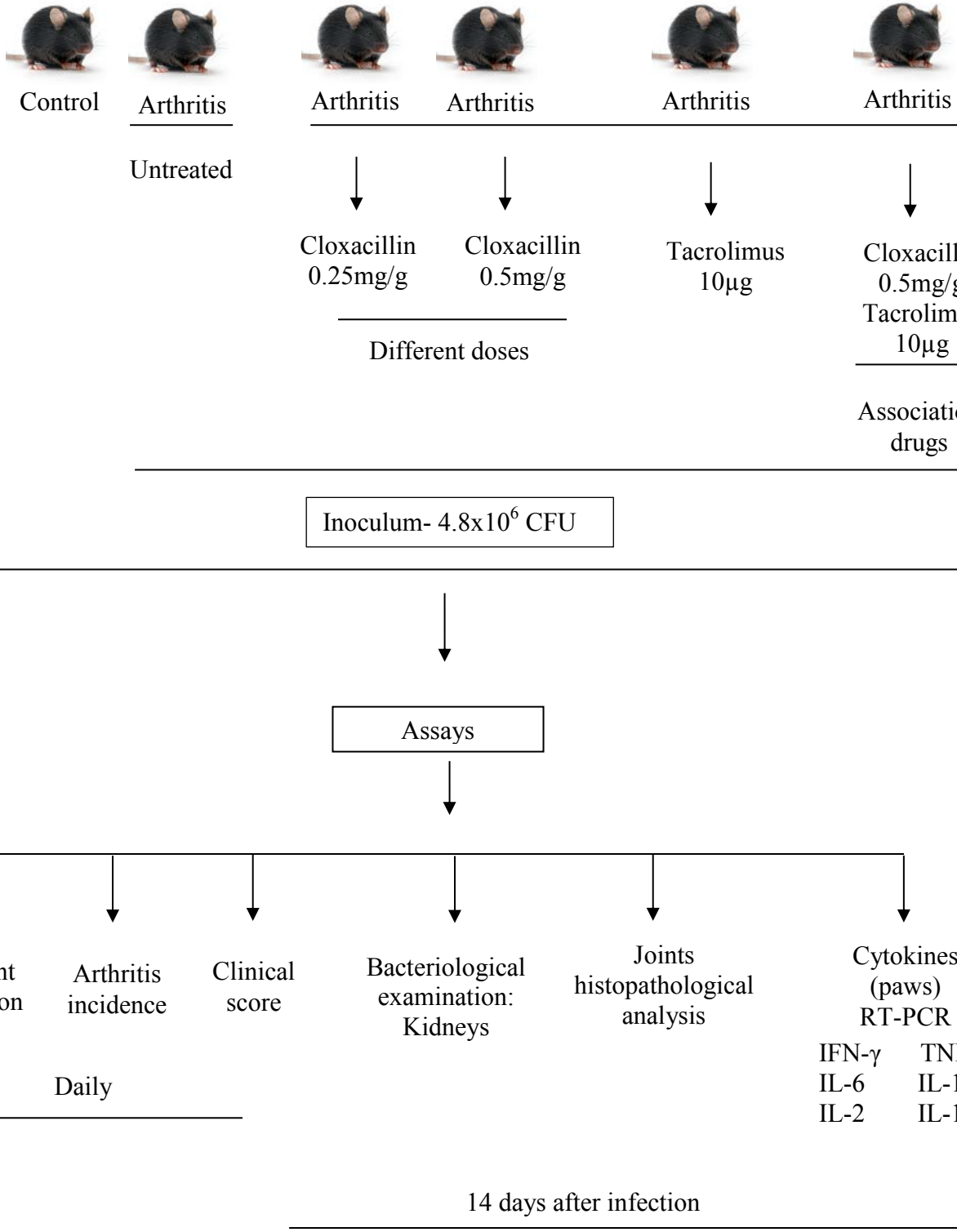
Artigo científico III

Artigo científico III: Tacrolimus does not improve cloxacillin efficacy in experimental septic arthritis



Artigo científico III: Tacrolimus does not improve cloxacillin efficacy in experimental septic arthritis

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Abstract

Although early antibiotic treatment of patients with septic arthritis eliminates bacteria, joint destruction can still occur due to the inflammatory response to infection. Septic arthritis treatment associated with *S. aureus* commonly involves the combined use of antibiotics to kill bacteria and immunomodulatory agents to simultaneously down regulate inflammation. In this context, we assessed the effect of the combined administration of tacrolimus and cloxacillin to mice with experimental arthritis. Male C57BL/6 mice were intravenously infected with the ATCC 19095 SEC^+ *S. aureus* strain and administration of the antibiotic cloxacillin started 3 days after infection and tacrolimus 1 day before and on days 1, 4, 7 and 10 after infection. Clinical parameters as body weight, clinical score and disease incidence were daily evaluated during 14 days. The number of colony forming units (CFU) was determined after 7 and 14 days of infection to evaluate the efficacy of treatment to eliminate bacteria. Arthritic lesions were also evaluated, after 14 days of infection, in relation to histopathological alterations and cytokine production by H&E and real-time PCR, respectively. Cloxacillin effectively decreased the CFU, reduced disease incidence and clinical score and also diminished histopathological alterations. Differently from expected, tacrolimus association with the antibiotic determined a slightly deleterious effect on arthritis severity.

Key-words: Septic arthritis, *Staphylococcus aureus,* cloxacillin, tacrolimus, IFN-γ

Introduction

Septic arthritis (SA) is a massive inflammation process that precedes bone destruction in the joints and that is started by an infectious agent. The most common and more pathogenic agent of SA is *Staphylococcus aureus* (Peacock *et al*., 2001). *S. aureus* can cause superficial skin infections such as abscesses and impetigo or serious invasive infections such infective endocarditis, toxic shock syndrome, gastroenteritis, pneumonia, osteomyelitis and septic arthritis (Edwards &, Massey 2011; Lowy, 1998; Peacock *et al*., 2001; Foster, 2005). It can also trigger food poisoning syndrome or foodborne outbreaks (Kérouanton *et al*., 2007). SA is generally considered a secondary infection, i.e., the bacteria exits the bloodstream and enters the surrounding tissues and is associated with significantly increased morbidity and mortality (Edwards &, Massey 2011).

Host and bacterial factors are considered of pathogenic importance during SA. The initial focus of joint destruction in *S. aureus* arthritis is the cartilage-synovium junction, with *pannus* formation and subsequent cartilage and bone destruction. There is an inflammatory process characterized by a rapid recruitment of polymorphnuclear granulocytes and activated macrophages soon followed by T cells (Bremell, 1992; Verdrengh *et al*., 2006). This process leads to irreversible loss of joint function and is associated with the production of cytokines like TNF-α, IL-1 and IL-17 (Campagnuolo *et al*., 2002; Kong *et al*., 1999).

The speed and accuracy of treatment is decisive for the outcome of SA. It is enough to have a suspicion of disease to initiate treatment while waiting for the final confirmation of the diagnosis. Even a limited delay in the treatment (days) may lead to irreversible joint destruction and an increased mortality rate. The basis of treatment for SA is unquestionably antibiotic therapy. It is imperative to start with broad-spectrum

antibiotics parenterally prior to any knowledge regarding the types of microorganism and their resistance patterns (Tarkoswik 2006). It has been described that concomitant corticosteroid and antibiotic treatment clearly down-regulates the severity of experimental SA and nephritis (Sakiniene *et al*., 1996; Verba *et al*., 1997).

Tacrolimus, formally known as FK506, is one of the calcineurin inhibitors and a subset of immunomodulators that potently inhibit lymphocyte proliferation and activation (Baumgart *et al.* 2003; Dieren *et al.,* 2006; Ogata *et al*., 2006). The best known application of their immunosuppressive capacities is in prevention of allograft rejection. In addition, systemic treatment with calcineurin inhibitors has been shown to be effective in the treatment of inflammatory bowel diseases. However, general systemic immune suppression, potential side-effects (insulin resistance, hypertension, neurological disorders) and potential toxicity (hepatotoxitcity and nephrotoxicity) limit the use of calcineurin inhibitors (Dieren *et al.*, 2010). In a model of inflammation induced by carrageenan indicate that tacrolimus has an important anti-inflammatory property, showing not only inhibition of proinflammatory mediators release but also inhibition of activated leukocyte infiltration into the site of inflammation. Most of the anti-inflammatory actions of tacrolimus were similar to those observed in animals treated with either indomethacin or dexamethasone (Vigil *et al*., 2008). Combination of tacrolimus and antibiotic was recently proven to be efficient in inflammatory processes in experimental septic peritonitis (Assfalg *et al*., 2010). In addition, a single study showed that tacrolimus was able to inhibit the development of collagen-induced arthritis potentiated by superantigen (SEB) in mice (Takaoka *et al*. 1998). The main objective of this work was to evaluate the efficacy of an association between a bactericidal antibiotic and tacrolimus in septic arthritis triggered by *S. aureus*.

Material and Methods

1. Experimental design

Mice were infected with enterotoxin C producer *S. aureus* strain and then treated with cloxacillin-alone, tacrolimus (FK506) or an association of cloxacillin and tacrolimus. Antibiotic administration started 3 days after infection and tacrolimus 1 day before and 1, 4, 7 and 10 after infection. Mice were daily evaluated by clinical follow-up (body weight, arthritis incidence and clinical score). The animals were euthanized 14 days after infection and the following parameters were assessed: number of colony-forming-units in the kidneys (CFU), local (arthritic lesions) cytokine production by real-time PCR and local histopathological analyses by H&E staining. Non-infected animals were included as a control group.

2. *S. aureus* strains and culture conditions

ATCC 19095 SEC^{+} was isolated from a leg abscess of a patient in Albert Merritt Billings Hospital, University of Chicago. Before each experiment, bacteria were cultured in blood agar plates (Merck) for 24h at 37ºC in order to confirm their purity and to determine their morphology and specific color. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37ºC for 24h. Bacteria were collected by centrifugation, washed three times and resuspended in cold sterile saline, as described by França *et al.,* 2009. The bacterial suspensions were prepared according to the McFarland nephelometer nº 0.5. The exact amount of live bacterial cells was determined by further enumeration of the number of colony forming units (CFU) on agar plates.

3. The arthritis model

Male C57BL/6 mice (8-10 weeks old) purchased from PUSP-RP (USP, São Paulo, SP, Brazil) were injected by the retro-orbital vein with an arthritogenic dose of ATCC 19095 SEC^{+}. Each animal was infected with 0.2 mL ($4.9x10^{6}$ CFU) of an *S. aureus* suspension made in physiological saline; control mice were injected with 0.2 mL of physiological saline. Mice were individually checked every day and the mean number of arthritic limbs per animal was registered. Arthritis was defined as visible joint erythema and/or swelling of at least one joint or both. To evaluate the intensity of arthritis, a clinical scoring (arthritic index) was carried out, using a system where macroscopic inspection yielded a score of 0–3 points for each limb (1 point = mild swelling and/or erythema; 2 points = moderate swelling and erythema; 3 points = marked swelling and erythema). The characterizations of these histopathological scores were clinically evaluated and characterized (1 point = evident synovial hyperplasia together with an incipient *pannus* formation, and also the presence of inflammatory infiltrates; 2 points = presence of *pannus* formation that is occupying almost the entire joint cavities, also a huge increase in the amount of inflammatory cells and cartilage and bone erosion; 3 points = presence stronger of *pannus* formation that is occupying almost the entire joint cavities, also a huge increase in the amount of inflammatory cells and cartilage and bone erosion).

4. Single and associated treatments

Cloxacillin (Sigma-Aldrich) was dissolved in sterile water and injected in mice every 24h during 10 days. This treatment begins at the third days of infection. Two antibiotic concentrations (0.25 and 0.5mg/Kg of body weight) were initially compared. The smaller dose was chosen to combine with tacrolimus. Tacrolimus (Sigma-Aldrich)

treatment included 5 doses: 1 day before and on days 1,4,7 and 10 after infection into the hind leg muscle. We employed 10µg/ animals during each one of these time points as was described by Kang *et al*. (2009). Tacrolimus was tested alone and in combination with cloxacillin.

5. Bacteria recovery

The two kidneys were aseptically removed after euthanasia and manually homogenized at 4ºC in sterile physiological saline. The homogenates were cultured in Bard-Park agar plates (Sigma-Aldrich) in serial dilutions and incubated for 24h at 37ºC greenhouse B.O.D. to estimate the bacterial load.

6. Histopathological analysis

Histopathological analysis of the joints was comparatively performed after 14 days of infection. Briefly, hind paws were fixed with 10% formaldehyde and embedded in paraffin after decalcification with 18% EDTA. Serial sections with 5µm thickness were cut and stained with haematoxilin and eosin. The sections were qualitatively evaluated in relation to the presence of inflammatory infiltrates, hyperplasia of the synovial membrane, cartilage destruction and bone erosion.

7. Cytokine quantification by mRNA expression

Hind paws were collected, fragmented and stored in RNAlater (Ambion, Austin, TX) solutions. Samples were submitted to molecular analyses as previously described by Garlet *et al.* (2010). Total RNA was extracted from samples by using TRIZOL reagent (Life Technologies, Grand Island, NY), and complementary DNA was synthesized by using 3 µg RNA in a reverse transcription reaction as described

elsewhere by Menezes *et al*., (2008) and Garlet *et al*., (2003). Real-time polymerase chain reaction (PCR) quantitative mRNA analysis was performed in an Viia7 instrument (Life Technologies Corporation) by using TaqMan system chemistry (Life Technologies Corporation), 100 nmol/L specific taqman primers inventoried primers, and 2.5 ng cDNA (or 5 ng DNA) in each reaction. The standard PCR conditions were 95ºC (10 minutes), followed by 40 cycles of 94ºC (1 minute), 56ºC (1 minute), and 72ºC (2 minutes). The relative levels of gene expression were determined by using the cycle threshold method in reference to glyceraldehyde-3-phosphate dehydrogenase, and β-actin expression, as described elsewhere (Menezes *et al*., 2008; Garlet *et al*., 2003).

8. Statistical analysis

Data were expressed as mean ± SE. Comparisons between groups were made by one way ANOVA with Holm Sidac method test for parameters with normal distribution, and Student test. Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results:

1. Effect of cloxacillin-alone treatment

Cloxacillin-alone treatment started at day 3 post infection, i.e., when very clear signs of arthritis were already present. Two doses were tested, 0.25 mg/Kg and 0.5 mg/Kg of body weight. Parameters as body weight, disease score and arthritis incidence were daily evaluated whereas CFU and cytokine production were checked at the 14^{th} day after infection. As expected from our previous experience, ATCC 19095 SEC^{+} infected mice (arthritis untreated group) presented a significant weight loss that is illustrated at figure 1a. Treatment with the antibiotic slightly decreased weight loss but

not in a significant way. Fourteen days after infection bacteria was still colonizing the host as is demonstrated by the significant amount of *S. aureus* recovered from kidney homogenates (figure 1b). Treatment with both cloxacillin doses determined a significant reduction in bacterial load compared to untreated animals (figure 1b). Arthritis in ATCC 19095 SEC^+ strain infected mice reached high clinical scores; around 2.5 at 8-9 days after infection. Disease incidence was also very elevated reaching a 100% incidence at this same time period. Clinical scores and incidence are illustrated at figures 2a and b, respectively. Treatment with the two cloxacillin doses modified arthritis profile reducing both clinical score and incidence. However, statistical analysis indicated that only the lower antibiotic concentration was able to significantly reduce incidence and disease severity as demonstrated in figures 2a and b.

2. Effect of combined treatment

The 0.25 mg/kg cloxacillin dose that reduced bacterial local more efficiently was choosen to be associated with 10 μg/kg of tacrolimus as suggested by the literature. Association of cloxacillin an tacrolimus did not avoid body weight loss (figure 3a) but it affected bacterial recovery. This association determined a significant reduction in bacterial load compared to untreated animals as demonstrated in figure 3b. The combined treatment modified arthritis profile, being the average clinical score lower in the treated group. In addition this drug association avoided the uprising in the clinical score that happened between days 9 and 10 in the arthritis control group and also in the cloxacillin-alone treated group. These findings are illustrated at figure 3c.

3. Effect of treatments on histopathological characteristics

The very well preserved articular cavity, cartilage and bone structures can be observed in the joint of a normal mice as shown in figure 4a. Contrasting with this normal appearance joints from mice infected 14 days earlier presented a severe arthritis characterized by evident synovial hyperplasia, a conspicuous *pannus* formation, a massive cellular infiltration and also evident cartilage and bone erosion (figure 4b). Cloxacillin treatment, independently of the dose, clearly decreased the severity of this process. These animals presented much less inflammation and synovia hyperplasia. *Pannus* formation and bone erosion were also very discrete (figures 4c and d). Joints from arthritic mice treated with tacrolimus, associated or not with the lower cloxacillin dose, presented an intermediate type of lesion considering severity. Even though they presented inflammatory infiltrates, synovial hyperplasia and cartilage and bone erosion, they were less impressive than the alterations observed in the arthritis positive control.

4. Local cytokine quantification

The local production of IFN-γ, TNF-α, IL-6 and IL-17 was evaluated by real-time PCR. The prolife of these four arthritogenic cytokines was the same. There was a significant up raise of their corresponding mRNA levels in the animals from the arthritis control group. On the other hand, treatments with cloxacillin, tacrolimus or association of these two drugs determined a significant drop in their level. There was no difference among the 3 treatment group in any of the studied cytokine.

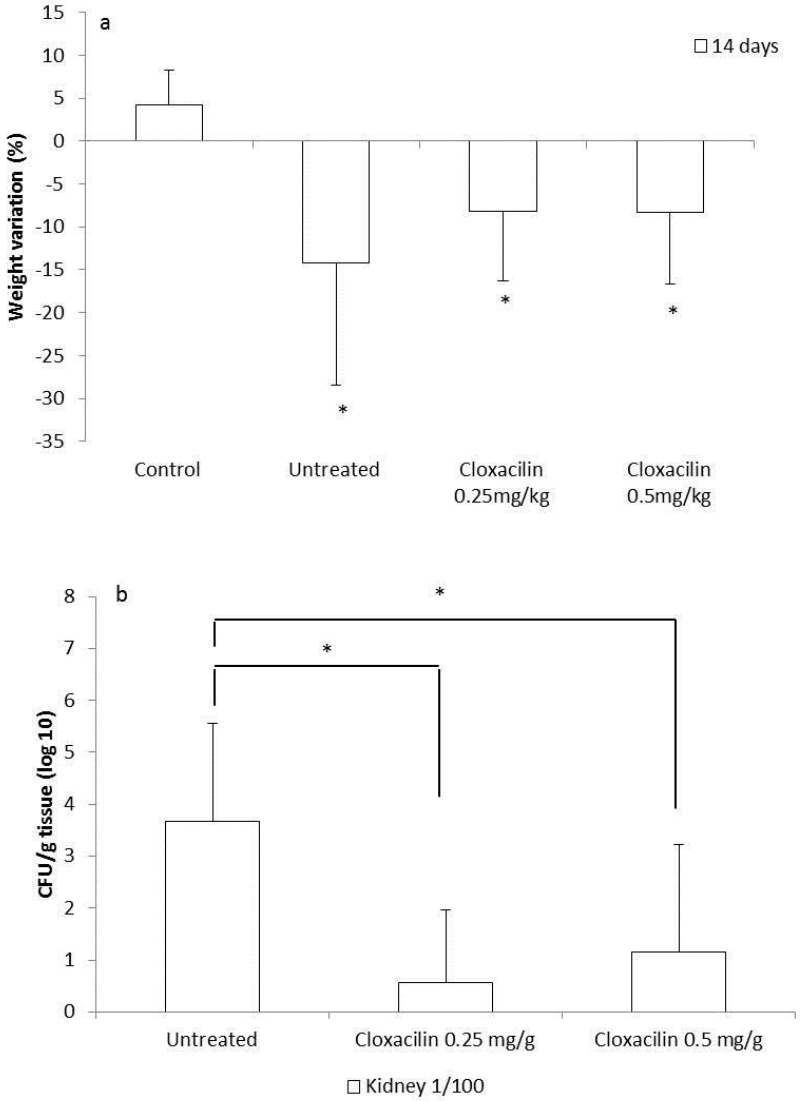

Figure 1. Effect of cloxacillin treatment on body weight and bacteria recovery in mice infected with enterotoxin C producer *S. aureus* strain. C57BL/6 mice were infected with *S. aureus* and treated, from the third day on, with cloxacillin during 10 days. Body weight variation (a) and number of colony forming units (CFU) recovered from the kidneys (b) after 14 days of infection. Data are presented by mean ± SE of 6 mice and is representative of two independent experiments. * represents significant differences ($p< 0.05$).

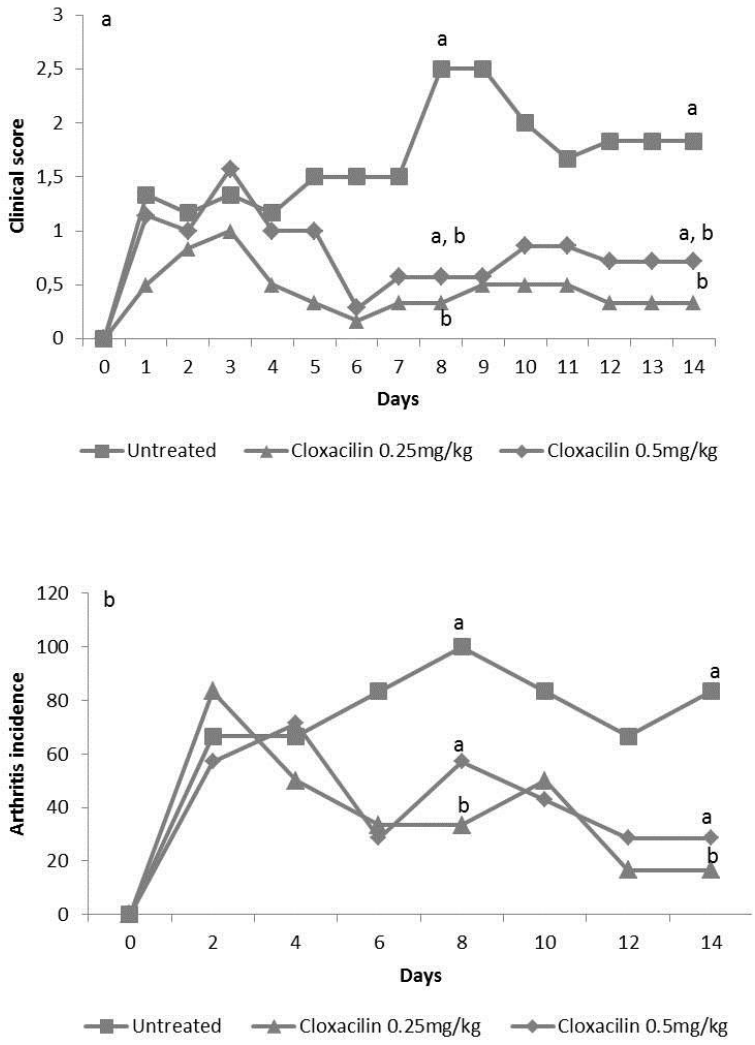

Figure 2. Effect of cloxacillin treatment on arthritis development. C57BL/6 mice were infected with enterotoxin C producer *S. aureus* and treated with 0.25 or 0.5 mg/Kg of the antibiotic. Clinical score (a) and arthritis incidence (b) were daily evaluated during 14 days. Data are presented by mean ± SE of 6 mice and is representative of two independent experiments. Different letters indicate statistical significance between the groups at the indicated time point ($p< 0.05$).

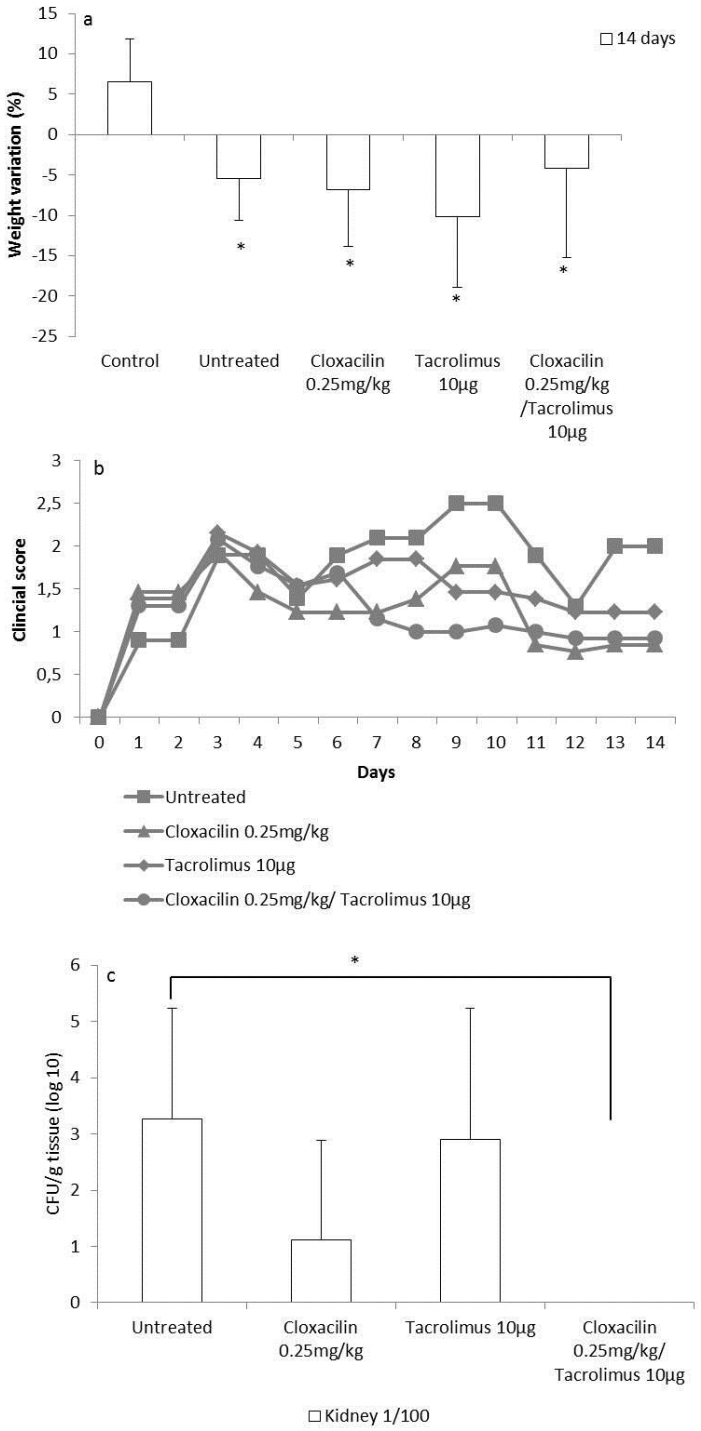

Figure 3. Effect of cloxacillin/ tacrolimus treatment on arthritis development. C57BL/6 mice were infected with enterotoxin C producer *S. aureus* and treated with cloxacillin plus tacrolimus. Body weight variation (a) and arthritis clinical scores (b) were daily evaluated. The number of colony forming units recovered from the kidneys (c) was determined 14 days after infection. Data are presented by mean ± SE of 6 mice and is representative of two independent experiments. * represents statistic difference between groups ($p < 0.05$).

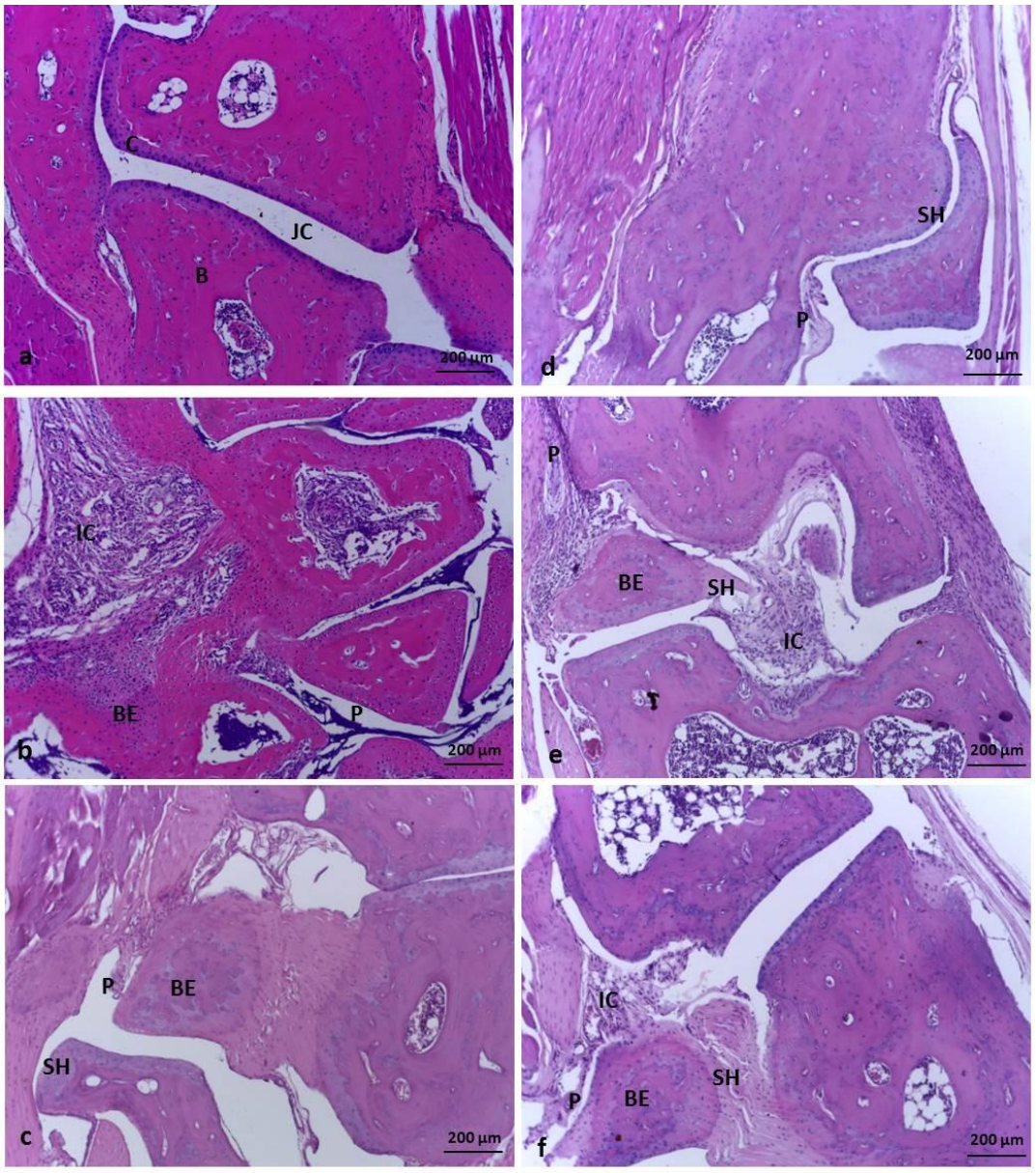

Figure 4. Effect of treatment with cloxacillin plus tacrolimus on septic arthritis triggered by enterotoxin C producer *S. aureus.* C57BL/6 were infected with *S. aureus* and treated with cloxacillin, tacrolimus on both drugs together. Histopathological analysis done after 14 days of infection showing micrographs of joints from normal mice (a); infected mice not treated (b); treated with the cloxacillin 0.5 mg/kg (c); treated with the cloxacillin 0.25 mg/kg (d); treated with the tacrolimus 10µg per animal (e) and treated cloxacillin 0.25 mg/kg plus tacrolimus 10µg per animal (f). Histopathological micrographics are shown with 10x magnification. Panel is representative of 6 animals/group. JC, joint cavity; C, cartilage; B, bone; SH, synovial hyperplasia; BE, bone erosion; P, *pannus* formation and IC, inflammatory cells.

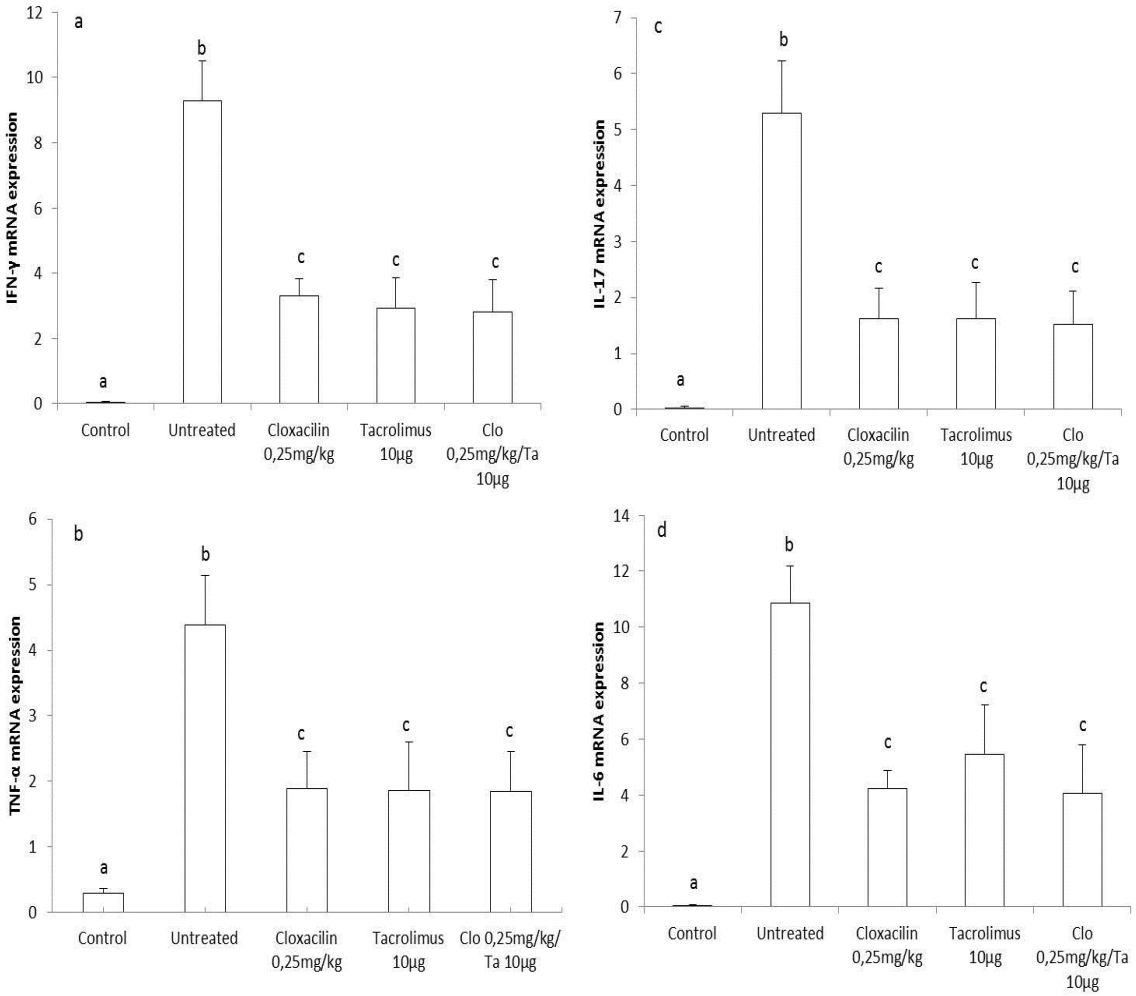

Figure 5. Effect of the combined treatment with cloxacillin and tacrolimus on local (arthritic lesions) cytokine production. C57BL/6 mice were infected with the enterotoxin C producer *S. aureus* and mRNA expression for IFN- γ (a), TNF-α (b), IL-17 (c) and IL-6 (d) was analysed 14 days later in the hind paws by real-time PCR. Data were presented by mean±SE of 6 mice. Different letters indicate statistical significance between the groups ($p< 0.05$).

Discussion

It is widely accepted that the concomitant treatment with antibiotic plus a corticosteroid can down-regulate the severity of experimental septic arthritis caused by *S. aureus* (Tarkowski, 2006). We used a murine model of hematogenously induced *S. aureus* arthritis to evaluate the effect of a combined treatment strategy, employing cloxacillin, that is a broad-spectrum bactericidal drug plus tacrolimus that regulates immune response at several stages (Pritchard, 2005).

As an experimental model we adopted C57BL/6 male mice infected with an enterotoxin C-positive *S. aureus* strain (SEC^{+}). We demonstrated before that this strain is highly arthritogenic for C57BL/6 male mice. In this work we confirmed the arthritogenicity of the enterotoxin C *S. aureus* strains. This bacteria was able to trigger a very typical clinical arthritis characterized by high disease incidence and elevated clinical scores. The histopathological analysis revealed that arthritic lesions included synovial hyperplasia, inflammation, *pannus* formation and erosion in both, cartilage and bone structures. These findings are very similar to the ones described in mice infected with the LS-1 classically strain (Bremell & Tarkowski, 1995; Gjertsson *et al.,* 2002; Vendrengh *et al.,* 2006).

Cloxacillin-alone treatment, that was started 3 days after infection, significantly decreased the amount of bacteria recovered from the kidneys. Cloxacillin was also able to significantly decrease arthritis incidence and clinical disease scores. Even though both doses had a similar effect, the smaller dose was more efficient. This clinical efficacy was expected and in accordance with the widespread use of cloxacillin to treat both, experimental and human infections provoked by *S. aureus* (Fei *et al*, 2011; Bru & Garraffo, 2012). Even though usually described as a lower toxicity antibiotic, cloxacillin is similar to flucloxacillin in structure and activity. As side effects as liver

injury for example, have been attributed to flucloxacillin (Andrews & Daly, 2008), we speculated that the less effective action of the higher cloxacillin dose in our model could be due to a toxic effect. Cloxacillin efficacy was expected and is probably due to its bactericidal effect. Its mechanism of action is based on its binding with enzymes implicated in the synthesis of peptidoglycan, main constituent of the bacterial wall (Pilly, 2009).

To evaluate the effect of the combined treatment of antibiotic plus immunosuppressor, we associated the lower cloxacillin dose (0.25 mg/kg of body weight) with 10 µg of tacrolimus that is the recommended dose suggested by the literature (Kang *et al.*, 2009). Mice treated with this drug association presented much less bacteria in the kidneys than animals treated only with cloxacillin. This finding suggests that tacrolimus has some antibacterial activity as demonstrated by Hung *et al.*, (2007). They also presented slightly lower clinical scores 9-10 days after infection, in comparison to the arthritic group treated only with cloxacillin. Even though these were not very accentuated effects, they were in accordance with previous findings that suggested to use tacrolimus as a new treatment approach in superantigen-associated diseases (Hauk & Leung, 2001). In addition, tacrolimus has also been explored as an adjunct treatment in rheumatoid arthritis (Kitahama *et al*., 2012) that presents many histopathological alterations in common with septic arthritis (Tarkowski, 2006). Tacrolimus efficacy as an immunossupressor has been mainly attributed to its dephosphorylating effect over the transcription factor called nuclear factor of activated T cells (NFAT) that is an important step preceding nuclear translocation of NFAT (Macian, 2005). As this nuclear factor intermediates the transcription of multiple cytokine and chemokine genes, it was possible that tacrolimus could modulate this experimental disease, at least partially, by decreasing cytokine secretion. Quantification

of mRNA in the lesions suggested, as expected, that high amounts of arthritogenic cytokines (IFN-γ, TNF-α, IL-6 and IL-17) were being locally transcribed an probably translated in infected mice. Cloxacillin-alone treatment significantly decreased their production what was consistent with the histopathological findings. Mice treated with the antibiotic presented very discrete signs of arthritis, showing that therapy efficiently killed the bacteria and avoided the ensuing destructive inflammatory reaction by the host. Treatment with tacrolimus alone was not able to decrease bacterial colonization neither arthritis development. Differently from expected, this drug was not able to contribute to arthritis control even though it was able to significantly reduce the production of all 4 proinflammatory cytokines. In addition mice simultaneously treated with cloxacillin and tacrolimus developed more serious lesions than animals treated only with the antibiotic.

The local cytokine mRNA levels in mice treated with the drug combination was, as the cloxacillin group, similarly low in comparison to the untreated infected group. These findings are difficult to reconcile with the inability of tacrolimus to act in concert with the antibiotic to control arthritis development. However, a similar behavior was described by Tilahun *et al.,* (2012). These authors demonstrated that despite its ability to suppress superantigen cytokine production, tacrolimus was not able to avoid a lethal staphylococcal enterotoxin-induced inflammatory response syndrome. Taken together, these results suggest that tacrolimus is not recommended to be associated to cloxacillin in septic arthritis treatment.

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