LARISSA STAURENGO FERRARI

PARTICIPAÇÃO DA IL-33 / ST2 EM MODELO DE ARTRITE SÉPTICA EM CAMUNDONGOS

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito à obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof° Dr° Waldiceu Ap. Verri Jr.

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Londrina, 25 de Fevereiro de 2011.

À Deus,

aos meus pais, Sérgio e Giselda,

aos meus irmãos, Andressa e Gabriel

e ao meu noivo Rogério.

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"Sonhe com o que você quiser. Vá para onde você queira ir. Seja o que você quer ser, porque você possui apenas uma vida e nela só temos uma chance de fazer aquilo que queremos.Tenha felicidade bastante para fazê-la doce. Dificuldade para fazê-la forte. Tristeza para fazê-la humana. E esperança suficiente para fazê-la feliz."

Clarice Lispector

LISTA DE FIGURAS

LISTA DE ABREVIAÇÕES E SIGLAS

-/-	Animais que não expressam o receptor ST2
AIA	Artrite induzida por antígeno
AIC	Artrite induzida por colágeno
AP-1	Proteína ativadora 1
AR	Artrite Reumatóide
DMARDs	Drogas Anti-Reumáticas Modificadoras de Doenças
ERK	Quinase reguladora de sinal extracelular
GRK-2	Quinase-2 de receptores acoplados a proteína G
GM-CSF	Fator estimulador de colônias de granulócitos e macrófagos
Ia	Intra-articular
IFNγ	Interferon gama
Ig	Imunoglobulina
IL-17R	Receptor de IL-17
IL	Interleucina
IL-1ra	Antagonista do receptor d IL-1
IL-1RAcP	Proteína acessória do receptor da IL-1
IRAK-4	Quinase - 4 associada ao receptor de IL-1
LPS	Lipopolissacarídeo
LTA	Ácido Lipoteicóico
Linfócitos Th1	Linfócitos T helper 1
Linfócitos Th2	Linfócitos T helper 2
Map quinases	Proteínas-quinases ativadas por mitógeno
MHC II	Complexo de Histocompatibilidade II
MSU	Cristas de Urato Monossódico
Myd88	Gene preliminar da diferenciação mielóide (88)
NF-kB	Fator nuclear kappa –B
NK	Natural Killer
NKT	Natural Killer T
RANKL	Fator de ligação de NF-kB
S. aureus	Staphylococcus aureus
sSt2	Receptor solúvel para IL-33
ST2L	Receptor de membrana para IL-33

TLR	Receptor toll
TRAF-6	Fator-6 associado ao receptor de TNF
TNFα	Fator de Necrose Tumoral α
TNFR	Receptor do Fator de Necrose Tumora
UFC	Unidades Formadoras de Colônias

SUMÁRIO

1 INTRODUÇÃO

1.1 ARTICULAÇÕES E ARTRITE

Articulação ou juntura é a conexão entre duas ou mais peças esqueléticas (ossos ou cartilagens) e são construídas com a finalidade de proporcionar movimentos e sustentação mecânica em resposta à contração muscular. Sua anatomia está intimamente relacionada à função (MAITRA; ABBAS, 2005). Estas podem ser agrupadas em cavitárias ou diartroses, que conferem grande mobilidade aos ossos, ou ainda, em sólidas ou sinartroses, que permitem movimentos mínimos, as quais são subdivididas em três classes de acordo com o tecido conjuntivo que une as extremidades ósseas: sinastoses, sincondroses e as sindesmoses (MAITRA, ABBAS, 2005; JUNQUEIRA & CARNEIRO,2004).

As diartroses ou articulações sinoviais, cuja anatomia permite grandes movimentações aos ossos (MAITRA; ABBAS, 2005), estão localizadas nas extremidades dos ossos longos originados por ossificação endocondral (processo em que as células mesodérmicas tornam-se células produtoras de cartilagem antes da formação completa do osso), envolvidas por uma densa cápsula fibrosa que une tais extremidades, delimitando uma cavidade fechada, a cavidade articular (JUNQUEIRA & CARNEIRO, 2004). Essas articulações são revestidas por células superficiais cubóides ou sinoviócitos, que estão dispostas em uma a quatro camadas celulares, variando conforme a estrutura articular considerada. Em geral são constituídas por camadas externas, a camada fibrosa e camadas internas, a camada ou membrana sinovial. Dois tipos celulares são encontrados nessas articulações: os sinoviócitos do tipo fibroblastos, produtores de diferentes proteínas e as células com aspectos morfológicos e funcionais semelhantes aos macrófagos, que são fagocíticas e sintetizam o ácido hialurônico (MAITRA; ABBAS, 2005), o qual confere resistência a cartilagem.

A ausência de uma membrana basal no revestimento sinovial permite a comunicação e trocas entre o sangue e o líquido sinovial. O líquido sinovial é um filtrado plasmático, produzido pela membrana sinovial, caracterizado pela presença de proteases, colagenases, glicoproteínas como a lubricina (KILLINGSWORTH, 1982) e o ácido hialurônico, que faz a nutrição da cartilagem hialina articular, a qual é constituída de um tecido conjuntivo rico em fibras de

colágeno tipo 2, água e moléculas de grandes dimensões, proteoglicanos e condrócitos. A cartilagem hialina atua como amortecedor das pressões mecânicas exercidas nessa região e na resistência a desgastes (MAITRA; ABBAS, 2005; JUNQUEIRA & CARNEIRO, 2004; SILVA, 2005). As fibras de colágenos são dispostas perpendicularmente e quando próximas a superfície apresentam-se na horizontal, isso confere a capacidade à cartilagem resistir ao estresse de tração e transmitir cargas verticais. A água e as proteoglicanas conferem à cartilagem seu turgor e elasticidade, pois em situações de pressão, a água acumula-se no espaço articular para nutrir a cartilagem, já que esta é desprovida de vasos sanguíneos (MAITRA; ABBAS, 2005; JUNQUEIRA & CARNEIRO, 2004; SILVA, 2005). Os proteoglicanos interagem com colágeno e ácido hialurônico contribuindo para a organização e estabilidade matricial da cartilagem e na remodelação tecidual (HEINEFARD et al., 1999; SCHEDEL et al., 2004). Os condrócitos, são as células especializadas da cartilagem, encontram-se dispersos na matriz e sintetizam e digerem enzimaticamente os componentes (MAITRA; ABBAS, 2005; JUNQUEIRA & CARNEIRO, 2004; SILVA, 2005).

O processo de síntese e degradação dos constituintes da matriz, o “turnover” (MAITRA; ABBAS, 2005), é minuciosamente controlado, pois durante esse processo são secretadas enzimas proteolíticas que levam a destruição da cartilagem articular e induzem ao processamento de diferentes precursores de mediadores inflamatórios (PINTO et al., 2010), como por exemplo as metaloproteinases. As patologias que afetam as articulações ativam essas enzimas, reduzindo a produção de moléculas inibidoras, acelerando assim a degradação da matriz. Este processo é mediado por uma extensa rede de citocinas, tais como TNFα, IL-1β, IL-6, que desencadeiam o processo de degradação, resultando num processo inflamatório e destruição tecidual (BOISIER, 2010).

A artrite é uma condição que envolve o dano articular, caracterizado por reações inflamatórias das articulações diartrodiais. Existem diferentes formas de artrite, cada uma com um padrão específico de resposta inflamatória. Contudo, essas variações resultam na necessidade de abordagens terapêuticas diferentes para cada tipo de situação.

O tipo mais comum das patologias articulares é a osteoartrite, que caracterizada pela degeneração da cartilagem articular, associada a alterações ósseas subcondrais e inflamação sinovial (POOLE, 1999; SCHEDEL et al., 2004).

De etiologia multifatorial, estão envolvidos elementos genéticos e fatores ambientais (REVEL et al., 1988; KRENN et al., 1999). Os condrócitos desempenham um importante papel, produzindo citocinas pró-inflamatórias, como TNFα e IL-1β, que estimulam a produção de metaloproteinases catabólicas, as quais inibem a síntese de colágeno tipo 2 e proteoglicanas (MAITRA; ABBAS, 2005).

A artrite reumatóide é a forma mais comum de artrite e ainda, caracterizada por ser uma doença auto-imune potencialmente incapacitante. É uma patologia crônica que acomete as articulações associadas a danos dos sistemas metabólicos, neurológicos e cardiovasculares. É uma doença de patologia complexa, com um acúmulo massivo de células do sistema imunológico como linfócitos T e B e macrófagos, culminando na destruição articular (BRENNAN & MCINNES, 2008).

A artrite reumatóide juvenil é uma doença infantil do tecido conjuntivo, caracterizado pelo início súbito da artrite reumatóide antes dos dezesseis anos, com sintomas persistentes por mais de seis semanas (KIM, 2010). Apesar de compartilhar fatores patogênicos semelhantes à artrite reumatóide, a artrite reumatóide juvenil difere em alguns pontos como: a ausência de fator reumatóide; as grandes articulações são afetadas com maior frequência e início sistêmico mais frequente (MAITRA; ABBAS, 2005).

Outro tipo menos comum de artrite, as espondiloartropatias negativas ou espondiloartrites, que são doenças caracterizadas pela artrite periférica, associadas a desordens secundárias, normalmente infecções. É um grupo heterogêneo de patologias articulares que compartilham manifestações clínicas, radiológicas e associações familiares. Neste grupo estão incluídos: espondilite anquilosante, artrite psoríatica, artrite enteropática ou reativa e síndrome de Reiter (MAITRA; ABBAS, 2005; ZOCHLING et al., 2006; ROMERO-SANCHÉZ et al., 2010;).

A artrite gotosa é uma desordem caracterizada pelo acúmulo tecidual de cristais de urato monossódico (MSU), o ácido úrico, um produto resultante do metabolismo das purinas. Essa patologia articular está fortemente associada com condições de estresse oxidativo, como obesidade e hipertensão (HERSHFIELD et al., 2010). Os cristais de urato induzem um intenso processo inflamatório por ativarem o inflamassoma de NALP-3 em células fagocíticas, culminando na secreção de IL-1β, recrutamento de neutrófilos e geração de mediadores inflamatórios, incluindo radicais livres (MARTINON et al., 2006; SO,

2008). Essa cascata de eventos resulta na destruição da cartilagem articular mediada por citocinas. Um outro Outro tipo de patologia articular semelhantes a gota, a pseudogota, também conhecida como condrocalcinose, é caracterizada pela deposição de cristais de pirofosfato de cálcio. Ela acomete indivíduos com mais de 50 anos. A progressão da doença é semelhante à gota, diferindo apenas no tipo de cristal (MAITRA; ABBAS, 2005).

Por último, mas não menos importante, a artrite infecciosa, definida pela presença de microorganismos viáveis na cavidade articular, é uma doença progressiva e altamente destrutiva, mesmo após a eliminação do microorganismo (VAROGA et al., 2006) Qualquer microrganismo (bactérias, fungos, vírus) pode semear as articulações e proliferar-se culminando num processo inflamatório articular, durante uma disseminação hematogênica. As articulações podem ser infectadas pela inoculação direta desses microorganismos, ou consequentes a um processo infeccioso nos tecidos moles (MAITRA; ABBAS, 2005).

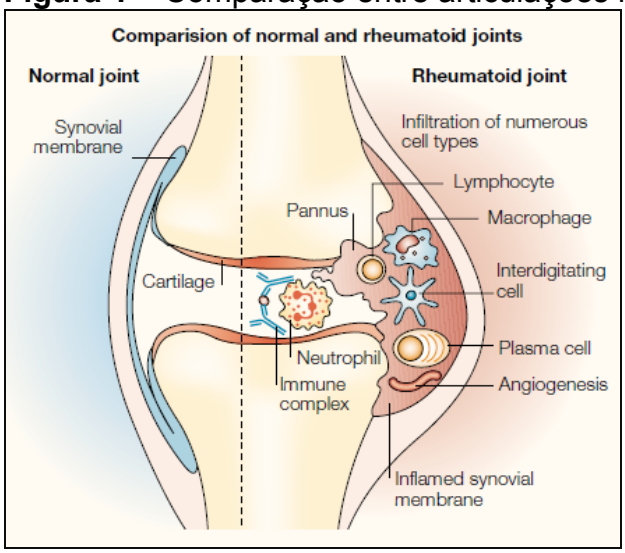
1.2 Artrite Reumatóide e as Citocinas Como Alvo Terapêutico

A artrite reumatóide é uma doença crônica inflamatória sistêmica, complexa, acompanhada de episódios inflamatórios agudos, associados principalmente a sensibilização articular e dor resultando em limitações dos movimentos (PINTO et al., 2010). Esta doença caracteristicamente engloba as pequenas articulações, das mãos, pés, sendo mais incidente nas articulações dos joelhos e acomete cerca de 1% da população mundial (BRENNAN & MCINNES, 2008).

Apesar de ser de origem desconhecida, o sistema imunológico desempenha um importante papel na sua cronicidade e progressão. A membrana sinovial na artrite reumatóide é caracterizada por um infiltrado inflamatório com linfócitos B e T ativados, neutrófilos e macrófagos, todos recrutados num intenso processo de neovascularização associado com linfoangiogênese (HARRIS, 1990; BRENNAN & MCINNES, 2008; PINTO et al., 2010). Sabe-se que há a participação de células da região articular, como os fibroblastos sinoviais, condrócitos e osteoclastos, que medeiam a destruição óssea e cartilaginosa. A atividade em conjunto dessas células mediadoras durante esse processo inflamatório, culmina na

formação do “pannus”, que é constituído de uma massa fibrocelular de sinóvia, caracterizada pela presença de células inflamatórias, tecido granulomatoso e principalmente fibroblastos (ZHAN et al., 2009) (Figura 1). Este tecido agressivamente invade o espaço articular, como resultado da degradação da matriz de colágeno, culminando em perda da função articular (MILLER et al., 2009).

Figura 1 – Comparação entre articulações normais e com artrite reumatóide.



Caracterização da articulação com artrite reumatóide e da intensa reação inflamatória mediada por células imunológicas e do tecido regional, culminando na formação do “pannus”.
Fonte: Feldman, 2002.

O intenso processo inflamatório crônico progressivo, mediado por células do sistema imunológico e células teciduais do hospedeiro, que acomete as articulações durante a artrite reumatóide é altamente regulado por uma extensa rede de citocinas, que são identificadas no tecido articular e soro de pacientes portadores de artrite reumatóide (FELDMAN, 2002; BRENNAN & MCINNES, 2008). Essa identificação contribuiu amplamente para determinação de moléculas-chaves envolvidas na patogênese dessa doença (BRENNAN & MCINNES, 2008). As citocinas são pequenas moléculas protéicas envolvidas na sinalização celular e podem ser secretadas por células da glia ou por diferentes células mediadoras do sistema imunológico.

Os tratamentos convencionais para portadores da artrite reumatóide são os glicocorticóides e imunossupressores. No entanto, essas drogas aliviam inicialmente os sintomas inflamatórios, como a dor, mas não desaceleram a progressão da doença. Outras abordagens mais recentes, como as drogas modificadoras de doenças reumáticas (DMARDs), metotrexato e sulfassalazina, que

podem retardar a destruição articular e danos estruturais, através da imunomodulação (MAINI et al., 2000; WOLLHEIM, 2001; SHAW et al., 2011) são associados com benefício parcial e significativa toxicidade (BRENNAN & MCINNES, 2008). Esses efeitos associados com o papel pleiotrópico das citocinas e por serem propensas a interagirem sinergicamente (GABAY & MCINNES, 2009), as tornam um importante alvo terapêutico.

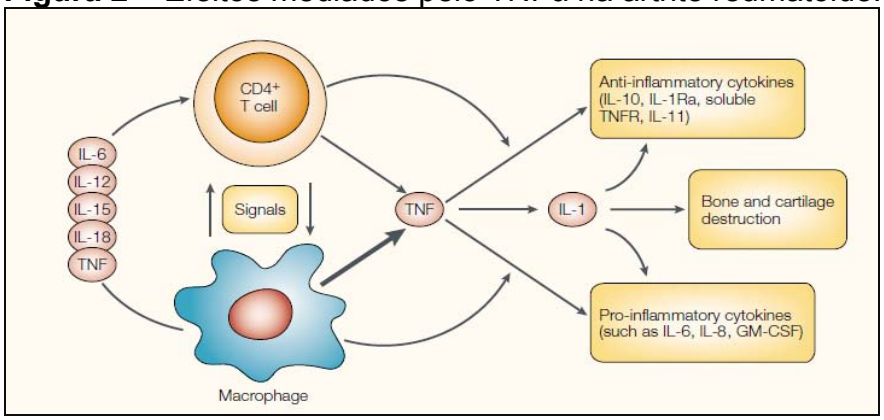
Diferentes citocinas desempenham um papel central na progressão da AR como as citocinas das superfamílias da IL-1, IL-6 e IL-12 e principalmente o TNFα. A participação desses mediadores na progressão da artrite reumatóide foi demonstrada em diferentes modelos experimentais e posteriormente confirmada clinicamente.

O TNFα exerce seu papel pró-inflamatório na artrite reumatóide após ligar-se a um de seus repecetores, o TNFR1 (p55) ou TNFR2 (p75), os quais são expressos em vários tecidos e células do sistema imunológico, respectivamente. Estes receptores tornam-se inibidores naturais quando são liberados na circulaçãov sanguínea, após clivagem enzimática (BOISIER, 2010). Sua atividade conduziu aos primeiros ensaios clínicos para o uso de biológicos nessa doenças. (FELDMANN, 2002; BRENNAN & MCINNES, 2008; GABAY & MCINNES, 2009).

No contexto da artrite reumatóide, o TNFα é uma citocina produzida primariamente por células do tecido sinovial e macrófagos ativados e induz a ativação de uma cascata de citocinas que leva ao aumento seqüencial de outras citocinas pró-inflamatórias, como IL-1, GM-CSF, IL-6 e IL-8 (ALVARO-GARCIA et al., 1990; HAWORTH et al., 1991; BUTLER et al., 1995; MIGITA et al., 1996), que de forma significativa contribuem para indução e manutenção da expressão do complexo de histocompatibilidade (MHC) de classe II em células apresentadoras de antígeno no tecido sinovial (BUTLER et al., 1995).

TNFα e seus receptores (TNFRs) foram identificados nos tecidos articulares com artrite reumatóide tanto em modelos experimentais murinos, como em humanos e de fato, foi observado que o uso de anticorpos para o TNFα diminuiu o dano e o processo inflamatório articular (WILIAMS et al., 1992). No entanto, a resposta parcial do bloqueio do TNF na artrite reumatóide e em muitos casos, a ineficácia (FELDMANM, 2002) e ainda, o risco de infecções recorrentes, como a artrite séptica (MOR et al., 2006) conduziu a busca de novas citocinas como alvo terapêutico para obtenção terapias mais adequadas.

Figura 2 – Efeitos mediados pelo TNFα na artrite reumatóide.



Representação esquemática da inter-relação de citocinas e interações celulares relacionadas ao TNF e a artrite reumatóide.
Fonte: Feldmann, 2002.

Recentemente, vem sendo sugerido à participação de células Th17 que secretam principalmente a IL-17, mas também IL-21 e IL-22. As células Th17 tem sua manutenção, ativação e expansão clonal induzidas pela IL-23 (LANGRISH et al., 2005) . A IL-17, apesar de ser produzida principalmente por células Th17, é também produzida por células T CD8+ de memória ativadas, NKT e linfócitos γδ (MILLS, 2008). A participação da IL-17 na artrite reumatóide é confirmada por estudos que demonstram que esta citocina orquestra diferentes funções imunes como a migração leucocitária (principalmente neutrófilos) (KOLLS & LIDEN, et al., 2004; PINTO et al., 2010), produção de mediadores pró-inflamatórios como TNFα, IL-1β e IL-6, quimiocinas e principalmente por atuar diretamente sobre os fatores pró-osteoclastogênicos através da estimulação da expressão do fator ligante do receptor ativador do fator nuclear kB (RANKL), o que leva a destruição da cartilagem e erosão óssea (KOTAKE et al., 1999; YAGO et al. 2009). Terapiad que visam bloquear a atividade da IL-17 e a sugerem como alvo terapêutico na AR encontra-se em ensaios clínicos iniciais de Genovese e colaboradores (2010), que avaliam efetivamente os efeitos do uso de imunobiológicos que visam bloquear a atividade da IL-17 e a sugerir como alvo terapêutico. Contudo, a IL-17 mostra-se importante durante o “clearance” de patógenos intracelulares e extracelulares, como fungos e bactérias, tanto gram-negativas quanto gram-positivas, como o Staphylococcus aureus (MAH et al. 2008), por induzirem a produção de quimiocinas e fatores de crescimento que levam a mobilização neutrofílica para o foco infeccioso (GAFFEN et al., 2008). Ademais, Heningsson e colaboradores (2010) verificaram que animais deficientes para IL-17 tem “clearance” bacteriano local limitado e desenvolvimento

de sinovite e erõses ósseas, após a inoculação de S. aureus. Esses achados sugerem que a terapia anti-IL-17 facilita infecções como a artrite séptica.

Vários membros da superfamília da IL-1 têm sido implicados na patogênese da artrite reumatóide. Identificado por Fontana e colaboradores (1982), a IL-1 foi a primeira citocina a ser encontrada nas amostras do líquido sinovial de pacientes com artrite reumatóide. A IL-1 é expressa ativamente na membrana sinovial com artrite reumatóide. A IL-1 exerce seus efeitos pró-inflamatórios principalmente na osteoclastogênese induzida por RANKL (KIM et al., 2009) e na liberação de metaloproteinases por fibroblastos e condrócitos articulares (VICENTI & BRINCKERHOFF, 2002). Além disso, a IL-1 induz a produção de outras citocinas pró-inflamatórias como a IL-17, mais especificamente neste caso, por participar em conjunto com outros mediadores na indução de linfócitos Th17 (NAKAE et al., 2003). Apesar desta citocina efetivamente contribuir para progressão da artrite reumatóide, terapias que visam o bloqueio da atividade da IL-1, como por exemplo, o Anakinra, um antagonista do receptor de IL-1 recombinante idêntico ao IL-1ra, com exceção de 1 aminoácido, tem sua eficácia controversa quando comparado às terapias anti-TNFα (FURST et al., 2005 ;BRENNAN & MCINNES, 2008). Consequentemente, novas terapias que visam à neutralização da atividade da IL-1 estão sendo desenvolvidas com o objetivo de serem mais eficazes.

Outro membro da superfamília da IL-1, a IL-18, identificado inicialmente como fator indutor da produção de IFNγ, é uma citocina altamente pró-inflamatória e um importante mediador tanto da resposta imunológica inata quanto adaptativa. Descrito por Gracie e colaboradores (1999) pela primeira vez na membrana sinovial de pacientes com artrite reumatóide, a IL-18 ativa e induz a produção de citocinas por células NK, macrófagos e neutrófilos e promove angiogênese e ainda medeia funções em células como os keratinócitos, osteoclastos e condrócitos (GRACIE et al., 2003). Em modelo murino de artrite induzida por colágeno (AIC) a ausência da IL-18 melhora a inflamação articular em camundongos AIC (PLATER-ZYBERK et al., 2001; WEY et al., 2001). A identificação e caracterização de sua atividade levaram a estudos por Tak e colaboradores (2006) que encontram-se em ensaio clínico de fase I que visam a IL-18 como alvo terapêutico na artrite reumatóide.

Estudos conduzidos por Kim e colaboradores (2005), com objetivo de identificar genes induzíveis pela IL-18, identificaram uma nova citocina, a IL-32,

produzida principalmente por células NK, linfócitos T, células epiteliais, monócitos sensibilizados com IL-2 ou IFNγ, e que desempenha um papel importante na resposta imunológica inata (DAHL et al., 1992; KIM et al., 1995). Tanto estudos in vitro como in vivo, também têm demonstrado a atividade da IL-32 na artrite reumatóide por ativar macrófagos sinoviais induzindo a produção de citocinas pró-inflamatórias como o próprio TNFα (JOOSTEN et al., 2006). Este dado é potencialmente importante considerando a participação do TNFα na fisiopatologia da artrite reumatóide como descrito anteriormente.

Recentemente, foi identificado o mais novo membro da superfamília da IL-1, a IL-33. Carriere e colaboradores (2007) e Xu e colaboradores (2008) identificaram a presença da IL-33 na membrana de pacientes com artrite reumatóide. Adicionalmente, foi observado que o tratamento com seu receptor solúvel (sST2) diminuiu a produção de citocinas pró-inflamatórias e a intensidade da artrite induzida por colágeno (AIC) experimental (LEUNG et al., 2004; XU et al., 2008).

Apesar dos consideráveis avanços no aspecto de estabelecer os potenciais alvos terapêuticos da extensa rede de citocinas que contribuem de forma individual ou sinérgica na artrite reumatóide, ainda há necessidade não atendida no sentido de chegar a terapias mais adequadas ao paciente de artrite reumatóide devido à variabilidade da resposta do tratamento com imunobiológicos em pacientes com artrite reumatóide. No caso das terapias anti-TNF, cerca de 30% dos pacientes não respondem de maneira adequada a esses imunobiológicos. Além da eficácia limitada, as terapias anti-TNF ou mesmo com outros imunossupressores propiciam um aumento da incidência de infeções nos pacientes com artrite reumatóide, como a artrite séptica (FAVERO et al., 2008; MA et al., 2009) (referencias). Assim, é essencial desvendar a participação dessas citocinas durante os processos inflamatórios em respostas inatas e adaptativas.

1.3 INTERLEUCINA-33

A IL-33 foi identificada por busca computacional em base de dados por estruturas semelhantes aos membros da família da IL-1 por Schimitz e colaboradores (2005). A IL-33 foi previamente descrita como produto do genen Dvs27, o qual é induzido por hemorragia subaracnóide experimental, e como o fator nuclear de vênulas endoteliais (NF-HEV), uma proteína expressa preferencialmente em células endoteliais descrito em 2003 (LIEW et al., 2010). Foi demonstrado que a IL-33 é o agonista do receptor previamente órfão da superfamília da IL-1, o ST2, também conhecido como T1, DER4 ou IL-1RL1 (SCHIMITZ et al., 2005). O gene st2 codifica três isoformas: uma forma ligada a membrana, denominada ST2L; uma forma solúvel, o sST2 e uma forma variante. O ST2 apresenta aproximadamente 38% de homologia com o receptor da IL-1 (YANAGISAWA et al., 1993), todavia, esse receptor é expresso preferencialmente em mastócitos (THOMASSEN et al., 1995) e células Th2 (XU et al, 1998; SCHIMITZ et al., 2005). Ademais, o ST2 pode negativamente regular a ativação de receptores do tipo Toll (TLR) por seqüestrar as moléculas adaptadoras MyD88 e Mal (BRINT et al. 2004; LIEW et al., 2005).

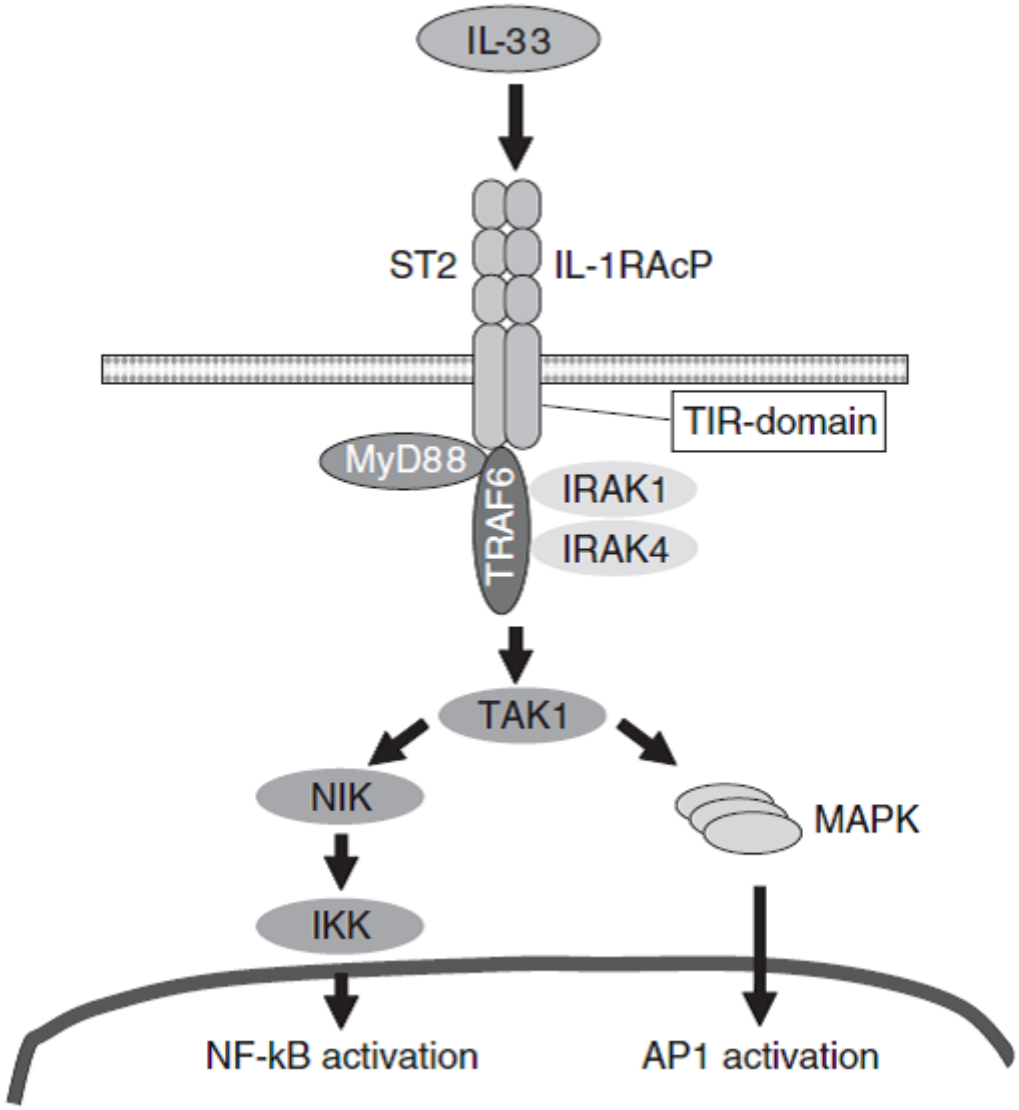
Assim como a IL-1 e IL-18, foi inicialmente proposto que a IL-33 seria transformada de pró-forma (pró-IL-33) para a forma ativa (IL-33) pela caspase-1 (SCHMITZ et al., 2005). Entretanto, novos estudos demonstraram que a efetividade da caspase-1 em clivar a IL-33 era limitada quando comparada a IL-1β e ainda foi identificada a participação de outras proteases, como a capase-7 e em menor quantidade pela caspase-3 (CAYROL et al., 2009; LUTHI et al., 2009). Hayakawa e colaboradores (2009) também demonstraram que a clivagem da pró-IL-33 pode ser mediada pela calpaína. Para aumentar a controvérsia sobre sua liberação, existem evidência que a caspase-1, na realidade, inativa a IL-33. A participação de caspases envolvidas na apoptose no processamento da IL-33 sugerem que ela seja liberada quando as células sofrem esse processo de morte programada.

A IL-33 ativa, com 18 KDa, compartilha 55% de similaridade entre humanos e roedores, com expressão em diferentes tecidos como: estômago, pulmão, medula espinal, cérebro e pele; e tipos celulares, como: células dendríticas e macrófagos em situações basais em roedores e após estímulo com TNFα e IL-1β

em humanos (SCHMITZ et al., 2005). Nos níveis protéicos a IL-33 é principalmente expressa em fibroblastos, células epiteliais e nas vênulas altamente endoteliais (SANADA et al., 2007). Evidências apontam a IL-33 como um fator de transcrição associado a cromatina de maneira semelhante a IL-1α e HMGB1. Por outro lado, a IL-33 é liberada pela lesão celular, apresentando papel como alarmina ao ativar células de maneira parácrina. (LIEW et al., 2010).

Em relação à sinalização, a IL-33 ao se ligar ao ST2, induz ao recrutamento das moléculas MyD88, IRAK, IRAK4 e TRAF6, o que resulta na ativação de MAP quinases (p38, JNK e ERK) e dos fatores transcricionais AP-1 e NF-kB (SCHMITZ et al., 2005). Todavia, para a IL-33 exercer seus efeitos in vivo, Chackerian e colaboradores (2005), demonstraram que a proteína acessória do receptor da IL-1 (IL-1RAcP) é essencial assim como o ST2, formando o complexo do receptor da IL-33 (ST2/IL-1RAcP). Ao ligar-se ao complexo receptor expresso principalmente em mastócitos e células Th2, como citado anteriormente, a IL-33 induz a produção de citocinas que são caracteristicamente Th2, como a IL-4, IL-5 e IL-13. Adicionalmente, foi demonstrado que a administração in vivo de IL-33 induz um quadro caracteristicamente Th2 por induzir a produção de altos índices de IgE e eosinofilia (SCHIMITZ et al., 2005). Ainda foi verificado que animais deficientes para o receptor da IL-33 (ST2) apresentam redução na resposta inflamatória Th2, como por exemplo a asma.

Figura 3 – Vias de sinalização da IL-33.



A IL-33 se liga a seu receptor heterodimérico (ST2/IL-1RacP). O domínio TIR recruta Myd88 e TRAF6 resultando na ativação de NF-kB ou AP-1.
Fonte: OBOKI et al., 2010.

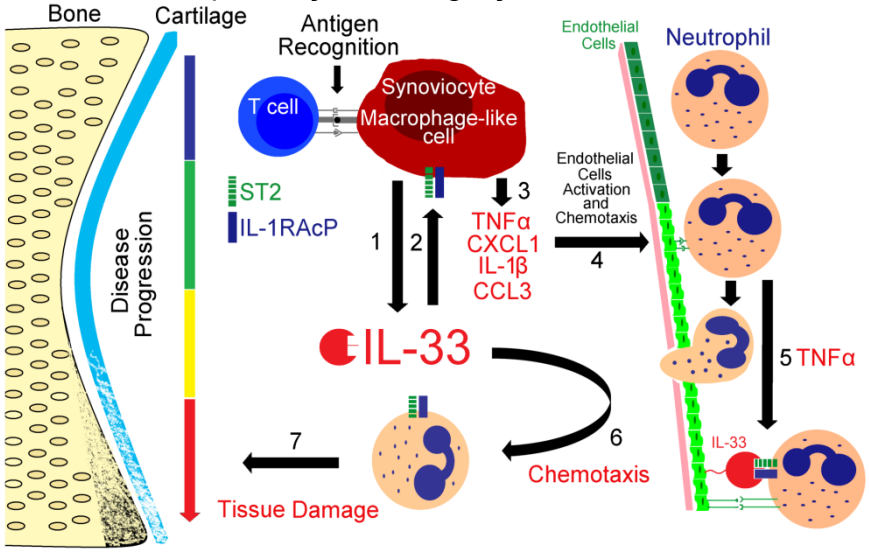
Além da participação em modelos inflamatórios, Sanada e colaboradores (2007) demonstraram que a IL-33/ST2 são ativados em resposta a estímulos mecânicos e apresentam efeito protetor em cardiomiócitos, evitando a hipertrofia mediada pela angiotensina II e fenilefrina. Adicionalmente, a IL-33 exerce efeitos protetores durante a aterosclerose por aumentar os níveis de citocinas Th2 (IL-4, IL-5 e IL-13) e marcadamente reduzir os níveis IFN-γ (padrão Th1) no soro e nos linfonodos (MILLER et al., 2010).

Em contrapartida, Verri e colaboradores (2008) demonstraram que em modelo de artrite auto-imune com animais imunizados com CFA (adjuvante

completo de Freund) o qual induz uma resposta inflamatória Th1, a IL-33 também tem um papel pró-inflamatório, inclusive induzindo a produção de IFNγ in vivo. Assim, sugeriu-se pela primeira vez a IL-33 como uma citocina pleitrópica, ou seja, sua atividade depende do ambiente de citocinas. Posteriormente, essa hipótese foi confirmada por outros grupos que detectaram que a IL-33 induz tanto respostas Th1 quanto Th2 em células polarizadas (SMITHGALL et al., 2008) e é responsável pelo desenvolvimento das lesões articulares em modelo de artrite (XU et al., 2008; PALMER et al., 2009) . A mesma induz a produção de IFNγ por células NK sugerindo uma participação em respostas inflamatórias inatas (BOURGEOIS et al., 2009) e Th17 (XU et al., 2008) . Adicionam a esses achados, diferentes autores que vem propondo a participação da IL-33 em modelos murinos distintos como artrite induzida por colágeno (XU et al., 2008) e por anticorpo (XU et al, 2010), e ainda, a deleção ou bloqueio com anticorpo específico para ST2 , em ambos os modelos para artrite reumatóide houve redução do desenvolvimento da doença por impedir a degranulação de mastócitos e produção de citocinas altamente pró-inflamatórias como IL-17, TNFα e IFNγ.

Além de mediar seus efeitos pró-inflamatórios através de mastócitos, em dados recentes foi demonstrado que IL-33, em artrite induzida por antígeno (AIA), participa no desenvolvimento da inflamação articular por induzir a migração de neutrófilos para o sítio inflamatório. Esse efeito da IL-33 é indireto por induzir a produção de outras citocinas e quimiocinas que são quimioatraentes para neutrófilos e/ou induzem o aumento da expressão de moléculas de adesão; e por um efeito direto via receptores ST2 por quimioatração de neutrófilos pela IL-33. É interessante que a expressão do receptor para IL-33 (ST2) em neutrófilos é induzida pelo TNFα. Assim, neutrófilos de pacientes que estão sob tratamento com terapia anti-TNF (p.ex. infliximab) tem redução da expressão de ST2 e redução da quimioatração exercida pela IL-33. Isto não ocorre quando os pacientes estão sob tratamento com outras terapias como o metotrexato (VERRI et al., 2010). Assim, a IL-33 exerce um papel pró-inflamatório na artrite reumatóide via recrutamento de neutrófilos que contribuirão para a lesão articular (Figura 3).

Figura 4 – Representação esquemática do mecanismo proposto para IL-33 na orquestração de migração de neutrófilos na artrite reumatóide.



(1) A IL-33 é produzida por sinoviócitos e células do tipo macrófagos, em resposta a estimulação antigênica. (2,3) A IL-33 atua de forma autócrina e induz a produção de outras citocinas e qumiocinas. (4) As citocinas e quimiocinas induzem a ativação de células endoteliais e quimiotaxia de neutrófilos. (5,6) A IL-33 pode diretamente atrair neutrófilos de camundongos sensibilizados com antígeno ou com artrite reumatóide pela ligação com ST2 em neutrófilos induzida pelo TNFα. (7) Os neutrófilos contribuem para a destruição articular.
Fonte: Verri et al., 2010.

Corroborando com esses dados, Matsuyama e colaboradores (2009) verificaram que fibroblastos do tipo sinoviócitos, mas não células do sangue periférico produzem IL-33 sob estimulação e que a produção de IL-33 no fluido sinovial de pacientes com artrite reumatóide é significativamente maior que quando comparadas aos pacientes com osteoartrite. Esse dado corrobora que existiria um gradiente de IL-33 para a articulação favorecendo o recrutamento de neutrófilos pela IL-33.

Na sepse, a IL-33 atua mantendo o recrutamento neutrofílico para o foco infeccioso, porém por um mecanismo diferente do que ocorre na artrite reumatóide. Na sepse ocorre a dessensibilização de receptores CXCR2 para quimiocinas em neutrófilos induzida pelo LPS (lipopolissacarídeo; componente da parede de bactérias gram-negativas), os quais são importantes para manutenção do direcionamento e recrutamento dos neutrófilos para o foco inflamatório e consequentemente para a eliminação do agente agressor. A IL-33 inibe a dessensibilização desses receptores induzida pelo LPS ou mesmo produtos de bactérias gram-positivas por regular negativamente a expressão de CXCR2 via GRK2. Assim, a inibição da atividade da IL-33 (em animais ST2 deficientes ou

tratados com ST2 solúvel) resulta numa diminuição da migração celular para o foco inflamatório, e a administração de IL-33 impede a dessensibilização induzida pelo LPS por diminuir a expressão de GRK2 (ALVES-FILHO et al., 2010). É importante ressaltar que a dessensibilização ocorre em uma fase da sepse na qual as bactérias ou seus produtos alcançam a corrente sanguínea e a princípio esse processo é necessário para manter os leucócitos ativos na circulação para que possam eliminar o agente infeccioso presente na circulação. Então, impedir a dessensibilização não é necessariamente algo totalmente positivo. Deve-se buscar o balanço entre o controle local e sistêmico da infecção, pois caso o foco local não seja controlado, com certeza evoluirá para um quadro sistêmico.

1.4 ARTRITE SÉPTICA INDUZIDA POR *Staphylococcus aureus*

A artrite séptica é caracterizada pela presença de microorganismos viáveis na articulação, que por sua vez, induzem resposta inflamatória local com lesão articular. As possíveis vias de semeação das articulações com bactérias incluem: hematogênica, consequentes a uma bacteremia ou sepse, osteomielite, via tecido adjacente, via procedimentos diagnósticos ou de tratamento, ou mesmo dano por punção ou trauma. A princípio, qualquer bactéria pode induzir a artrite séptica, porém a incidência com *Staphylococcus aureus* é de 80% (GOLDENBERG et al., 1998). A infecção por *S. aureus*, cujo principal fator de virulência é o ácido lipoteicóico (LTA), leva a danos articulares como inflamação sinovial, destruição cartilaginosa e óssea que culmina na destruição articular durante a progressão da artrite séptica (TARKOWSKY et al., 2001; TARKOWSKY et al., 2002). Contudo, fatores adicionais, após a eliminação bacteriana do hospedeiro, contribuem para progressão da artrite séptica atuando de forma relevante nos processos destrutivos (GOLDENBERG, 1985).

Reforçando esses dados, Varoga e colaboradores (2006) demonstraram a intensa participação do fator de crescimento endotelial vascular (VGEF) resultante da ativação de receptores do tipo Toll 2 (TLR2) nas amostras de fluídos sinoviais de pacientes com artrite séptica e pelo desafio in vitro de condrócitos com bactéria, como contribuintes para a artropatia destrutiva após infecção microbiana nas articulações. Adicionalmente, já foi demonstrada a

contribuição de metaloproteinases para a destruição óssea, sendo que a deficiência para MMP-7 reduz a intensidade da artrite séptica (severidade clínica e histologia), mas não o crescimento do Staphylococcus aureus (GJERTSSON et al., 2004).

A incidência de artrite séptica é 40% maior em pacientes com artrite reumatóide devido ao tratamento com drogas modificadoras de doenças (DMARDs) ou imunossupressores, que deixam o paciente mais suscetível à infecções (MOR et al., 2006). Diversos trabalhos vêm demonstrando tanto de forma clínica ou experimental, o aumento da susceptibilidade dos indivíduos tratados com terapias anti-TNF (ex. infliximab, etanercept) à infecções, principalmente tuberculose e artrite séptica, que são infecções oportunístas consequentes da ausência de TNFα, uma citocina que desempenha funções importantes no combate è infecções (HULTGREN et al., 1998; CUNNANE et al., 2003; ELLERIN et al., 2003; KROESEN et al., 2003).

Assim, a busca de novos tipos de abordagens terapêuticas em potencial torna-se necessárias, para que ocorra uma imunorregulação, sem alterar a resposta inata do hospedeiro, o que consequentemente levaria ao desenvolvimento do processo infeccioso nas articulações.

Seguindo essa linha de raciocínio, o foco desse trabalho é avaliar a participação da IL-33, por meio de animais deficientes para o receptor desse mediador, o ST2, e ainda comparar as análises a animais TNFR1-/- e IL-17R-/- na artrite séptica induzida por S. aureus. Esse protocolo permitirá avaliar se terapias anti-IL-33, seriam mais adequadas do que terapias atuais como anti-TNF ou sob investigação como anti-IL-17, as quais comprometem à defesa do hospedeiro contra infecções.

2 OBJETIVO

Avaliar a participação da IL-33 em um modelo de artrite séptica por Staphylococcus aureus em comparação com a participação do TNFα e IL-17.

2.1 OBJETIVOS ESPECÍFICOS

1.A) Avaliar pelo experimento de dose resposta de Staphylococcus aureus em modelo de monoartrite infecciosa induzida pela inoculação local (fêmur-tibial), a dose que atinge níveis significativos de hiperalgesia mecânica, edema e migração leucocitária.

2.B) Avaliar a hiperalgesia mecânica, edema e migração leucocitária em camundongos ST2-/-, TNFR1-/- e IL-17R-/- que receberão injeção intra-articular de S. aureus. A hiperalgesia mecânica e edema serão avaliados até o 28º dia após a inoculação local de S. aureus e a migração leucocitária será avaliada após o sacrifício dos animais no 28º dia após a inoculação.

3 RESUMO DO ARTIGO 1

A preparação da suspensão bacteriana e o intervalo de confiança de UFC nessa suspensão é um importante procedimento utilizado em laboratórios como métodos para avaliação de respostas inflamatórias e pode ser obtido por diferentes métodos, tais como diuições seriadas e pela análisa visual da turbidez através da escala de McFarland. Nós investigamos a influência do armazenamento da suspensão de Staphylococcus aureus na viabilidade de bactérias e sua influência na inflamação induzida por essa suspensão. O armazenamento da suspensão de S. aureus a 8 º C por 24 h diminuiu a viabilidade bacteriana não só em suspensões preparadas por diluições seriadas, mas também ao seguirmos a escala de McFarland 0,5. O aumento do tempo de armazenamento reduziu o número de UFC de S. aureus. Como conseqüência da viabilidade bacteriana reduzida, foi detectada redução do recrutamento de leucócitos em um modelo de peritonite bacteriana. Em modelo de artrite séptica foi detectada redução da hiperalgesia mecânica, edema e recrutamento de leucócitos. Esses resultados demonstram que o armazenamento da suspensão bacteriana afeta a viabilidade bacteriana e também a resposta inflamatória "in vivo". Uma solução possível para determinar/estimar o número de UFC é o uso da escala de McFarland , que permitirá a elaboração e utilização de uma suspensão bacteriana no mesmo dia para testes in vivo e assim, evita-se a diminuição da viabilidade bacteriana e a influência de resultados experimentais.

Palavras-Chave: Escala de McFarland. *Staphylococcus aureus*. Migração celular.

4 ARTIGO PARA PUBLICAÇÃO 1

Este é um trabalho realizado no Laboratório de Dor, Neuropatia, Câncer e Inflamação, formado pelo artigo científico: Influence of bacterial suspension storage in inflammatory response in mice. Larissa Staurengo-Ferrari, Jacina Santos Pelayo, Rubia Casagrande, Halha Ostrensky Saridakis, Waldiceu Aparecido Verri Júnior.

As formatações do artigo seguem as normas da revista African Journal of Pharmacy and Pharmacology (AJPP) (Anexo 1).

Influence of bacterial suspension storage in the inflammatory response in mice.

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Abstract

The preparation of bacterial suspension is an important procedure used in laboratories for inflammatory evaluation protocols and can be obtained by different methods such as CFU (colony forming unities), needs storage counting and McFarland scale turbidity (does not need storage). We investigated the influence of storage of Staphylococcus aureus suspension as bacterial viability and its influence in bacteria-induced inflammation. . The increase of time of storage reduced the S. aureus CFU. As a consequence of reduced bacterial viability, it was detected reduced leukocyte recruitment in a model of bacterial peritonitis, and reduced mechanical hyperalgesia, edema and leukocyte recruitment in septic arthritis. These results demonstrate that storage of bacterial suspension affected bacterial viability and also the inflammatory response in vivo, raising the importance of standard procedures for bacterial suspension preparation. A conceivable approach would be to determine the number of CFU at a specific McFarland's scale degree, which will allow the preparation and use a bacterial suspension in the same day for in vivo testing and avoiding reduced bacterial viability.

Keywords: Edema. Inflammation. McFarland's Scale. Neutrophil. Septic arthritis. Staphylococcus aureus.

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INTRODUCTION

The estimated number of viable cells by determining the number of colony forming unities (CFU) in a bacterial suspension is of great importance in bacteriology, immunology, and inflammation/infection studies in general. There are different methodologies for estimating the number of CFU in a suspension of bacterial cells such as serial dilutions and plating, and using the McFarland's Scale with visual or nephelometric reading.

The serial dilutions and plating to establish CFU (Brock et al., 1994) is a lengthy process in which the suspension is prepared followed by plating a partial volume and counting the CFU after 24h, meanwhile the suspension is held at 8ºC until use. The McFarland's Scale (which is basically a nephelometric scale) is a methodology for analysis of visual turbidity of a suspension and is used to estimate the number of bacteria in a given suspension. It consists essentially of a series of standard dilutions containing barium chloride precipitated in sulfuric acid, and the different turbidities indicate degrees of McFarland's scale. This process is useful to correlate the number of bacteria dispersed in isotonic solutions (saline or PBS) (McFarland et al., 1907). Therefore, comparing the turbidity of bacteria dispersed in saline or PBS with the standards of McFarland's Scale, it is possible to obtain a good estimation and optimization of process to determine the number of viable bacteria within the same day of preparation of the bacterial suspension avoiding the storage of sample.

Staphylococcus aureus is a prominent gram-positive human pathogen, with an ability to produce systemic infections (Berends et al., 2010) and also is the major contributor to osteomyelitis, invasive endocarditis and septic arthritis (Bannan et al., 1999). Infection by these organisms induces acute and chronic inflammatory response, causing tissue edema and the associated pain (Whaley and Burt, 1996) and leading to pyogenic or suppurative inflammation (Okoli et al., 2008). This bacteria is highly efficient phagocytosed by neutrophils culminating in an intense inflammatory response involving a complex cascade of cellular events to eradicate pathogens via oxidative and non-oxidative mechanisms (Anwar et al., 2009).

As bacterial suspensions of S. aureus is used in different inflammatory models such as infectious sepsis and septic arthritis (Crosara-Alberto et

al., 2002; Guo et al., 2009) or in different methodologies in laboratories, and there is evidence that temperature and time of storage can influence the viability of gram-positive bacterias in suspension, even as Clostridium sporogenes (Mah et al., 2010), we investigated the effect of storage of bacterial suspension of S. aureus and the influence this process in inflammatory models in vivo .

MATERIALS AND METHODS

Animals: Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21º C. All testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina. All efforts were made to minimize the number of animals used and their suffering.

Staphylococcus aureus suspension preparation: S. aureus was obtained from ATCC (American Type Culture Collection, U.S.A.) number 6336. Twenty four hours before each experiment samples of bacteria were cultured in blood agar medium at 37º C. The bacterial suspension was centrifuged and the pellet was ressuspended in sterile phosphate-buffered saline (PBS) to fit 0.5 McFarland Scale, visual comparison, for administration to the animals and serial dilutions to confirm bacterial viability. The number of colony forming units (CFU) of the bacterial suspension was determined through serial dilution and plating on BHI agar dishes viable CFU determined after 24 h.

Peritonitis Model and neutrophil migration: The peritonitis was induced through intraperitoneal injection of S. aureus suspension. The animals received bacterial suspension or saline into the peritoneal cavity in a volume of 500 µL. The quantity of bacteria injected was 5 x 108 CFU/ml. Neutrophil migration was assessed 6h after i.p. injection of bacterial suspension. The peritoneal cavities of mice were then washed with 2mL of saline containing 10 mM EDTA (Verri et al., 2007). The exudates were collected by aspiration for determination of the total number of leukocytes in a Neubauer chamber diluted in Turk's solution. Differential cell counts were determined in slices stained by the Rosenfelt method (Verri et al., 2007).

Induction of bacterial arthritis and neutrophil migration: The septic arthritis was induced by local injection in the femur-tibial joint of mice of S. aureus suspension. As control other animals received 10 µL of sterile saline. The mice were sacrificed at the 15th day after inoculation of bacteria and the synovial cavity of knee joint was washed twice with 5 µL saline contained 10 mM EDTA (Rocha et al., 2008). The total number of infiltrating cells was determined in a Neubauer Chamber diluted in Turk's solution and the differential counts were performed in slices stained by the Rosenfeld method.

Evaluation of articular hyperalgesia: The articular hypernociception of the femur–tibial joint was evaluated using a previous method (Verri et al., 2008; Pinto et al., 2010) with modification. In a quiet room, mice were placed in acrylic cages (12 x 10 x 17 cm high) with a wire grid floor 15–30 min before testing for environmental adaptation. Stimulations were performed only when animals were quiet, did not display exploratory movements or defecation, and were not resting on their paws. In these experiments, an electronic pressure-meter was used. It consists of a hand-held force transducer fitted with a polypropylene tip (IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). For this model, a large tip (4.15 mm2) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the femur–tibial joint followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the hind paw. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until three subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g) were obtained. The flexion-elicited mechanical threshold was expressed in grams (g).

Evaluation of joint edema: The edema of femur-tibial joints was evaluated by measurement of the transverse diameters of femur-tibial joints using an analogical caliper (Mitutoyo Corp., Kanagawa Japan). Values of femur-tibial joint thickness are expresses as the difference between the diameter measure before (basal) and after induction of articular inflammation in millimeters (Valerio et al., 2009).

Statistical analysis: Statistical analyses were performed using GraphPad Prism (La Jolla, 5 CA). Results are presented as means ± SEM of 2 independent experiments. The "n" in the legends refers to the number of mice used

in the experimental group of each experiment. The differences between the experimental groups were compared by ANOVA (one-way) and individual comparisons were subsequently made with Tukey's post hoc test. The level of significance was set at $P < 0.05$.

RESULTS

Standardization of the number of S. aureus CFUs in 0.5 McFarland's scale suspension.

S. aureus suspension was prepared from colonies incubated overnight at 37oC on blood agar plates. At three different days, suspensions were prepared in accordance with the 0.5 of McFarland's scale. The suspensions were diluted sequentially for subsequent plating in triplicate with a fixed volume to determine the number of CFU in each suspension after 24 h incubation at 37oC. The number of bacteria was considered within the range of 30 to 300 CFU per plate, as previously described (Brock et al., 1994). The mean of three experiments performed in triplicate was 1.38 x 108/mL (Table 1). This value was considered the mean CFU in 0.5 degree of McFarland's scale and was used for subsequent experiments. There was no significant difference between the experiments.

Effect of storage in S. aureus suspension viability.

To examine the effect of storage in the viability of bacteria, a bacterial suspension of S. aureus which was prepared fitting 0.5 of McFarland Scale, was plated for serial dilution at 0h (before storage) and 24h after storage at 8oC, which is a usual temperature in laboratory routine. The number of viable bacteria was determined by plated serial dilution and counting the number of CFU 24h after plating. Total number of CFU per plate was considered valid if ranging between 30 and 300 CFU per plate (Brock et al., 1994) (Fig.1). It was detected that the storage for 24h reduced the number of viable S. aureus either the suspension was prepared using the McFarland's scale (Fig.1). Thus, these results indicate that storage of S.

aureus at 8oC for 24h reduces the number of viable bacteria in the suspension prepared according McFarland's Scale..

Effect of bacterial suspension storage in mice peritonitis.

S. aureus suspension was prepared according to 0.5 value of McFarland's scale, and 5 x 108 CFU/ml were administrated via intraperitoneal route (i.p.) immediately (0h) or after being stored for 24 hours at 8ºC (Fig. 2). Saline was used (500 µl/cavity) as negative control. There was a significant increase of total leukocytes (Fig. 2A) and total neutrophils (Fig. 2B) in the peritoneal cavity of mice that received 0 h S. aureus suspension compared to control group. On the other hand, the total leukocytes (Fig. 2A) and total neutrophils (Fig. 2B) were reduced in 24 h S. aureus suspension compared to 0h S. aureus suspension. Furthermore, there was no statistical difference between control group and 24h S. aureus suspension group (Fig. 2A and 2B).

Effect of storage of bacterial suspension in the S. aureus-induced septic arthritis in mice.

S. aureus suspension was prepared according to 0.5 value of McFarland's scale, and 1 x 107 CFU were injected intra-articularly (i.a., 10 µl) in the femur-tibial joint of mice at 0 h (before storage) and 24 h (after storage at 8oC) (Fig. 3). The i.a. injection of both suspension induced significant mechanical hyperalgesia (Fig. 3) and edema (Fig. 4) in mice. Nevertheless, the i.a. administration of 0 h S. aureus suspension induced significantly greater hyperalgesia (Fig. 3) and edema (Fig. 4) compared to 24 h S. aureus suspension. Concerning cellular recruitment, it was observed that i.a. administration of 0 h S. aureus suspension induced significant recruitment of total leukocytes (Fig.5A), neutrophils (Fig. 5B) and mononuclear cells (Fig. 5C) to the knee joint of mice compared to saline group and 24 h S. aureus suspension.

DISCUSSION

An important question in bacteriology and in studies involving bacteria is determining how many living bacteria are in a sample, and its effect upon in vivo investigations. Herein, it was demonstrated that storage of bacterial suspension for 24 h to determine the number of bacteria by serial plating reduces the number of viable Staphyloccocus aureus in the sample, resulting in reduced bacterial load in vivo. As a consequence, there is reduced inflammation (e.g. pain, edema and leukocyte recruitment). An interesting and reliable approach to determine bacterial counts in a sample is the use of McFarland's scale to estimate the number of CFU and avoiding the reduction of S. aureus viability that affects bacterial count-related in vivo inflammation.

It is a common/standard procedure in laboratory routine to determine the number of viable bacteria by preparing a bacterial suspension, which follows two separated procedures. In one procedure, this suspension undergoes serial dilutions followed by plating and determinations of the total number of colony-forming unities (CFU) (Richards et al., 1977; Madrid et al., 1999). The number of CFU must be in the range of 30-300 (Brock et al., 1994). In the second procedure, the bacterial suspension is stored at approximately 8oC for 24 h until the result of bacterial CFU counting is determined in the first procedure. Then, the bacterial suspension can be used.

An interesting alternative to the CFU counting method is the use of McFarland's scale in which the number of bacteria is estimated by the turbidity of the suspension (McFarland et al., 1907). This procedure can be performed using equipments to determine the optical density or by visual comparison with a standard scale. In the present study, we used the visual comparison with a standard scale to ensure the use of a method with maximal simplicity and low cost avoiding the need of equipment. The number of viable bacteria in the suspension was confirmed by CFU counting using random suspension and 0.5 McFarland's degree scale. It was observed a reduction in the number of viable S. aureus CFU by plating the suspension for 24 h of storage compared to 0 h (before storage). Therefore, storage at 8oC reduces S. aureus viability either using a random suspension or 0.5 McFarland's scale degree suspension, indicating that the CFU counting method with 24 h storage will result in reduced S. aureus load. Indeed, temperature and time of

storage also affects other bacteria such as Vibrio spp (Matches et al., 1971) and Clostridium sporogenes (Mah et al., 2010). This fact can be atribuitted to damage of the cytoplasmic membrane as well as to outer membrane of bacteria (Souzu, 1980).

S. aureus was chosen because it is a bacterium with clinical relevance. There is participation of S. aureus in septic arthritis (Tarkowski et al., 2001; Tarkowski et al., 2002) and sepsis originated in the peritoneum (Bannan et al., 2009). S. aureus infection induces inflammatory signs of pain, edema and recruitment of leukocytes. Therefore, it was investigated whether there is reduced inflammation by administrating of stored (24 h) compared to non-stored (0 h) S. aureus suspension using 0.5 McFarland's degree. The non-stored S. aureus suspension induced significantly greater recruitment of total leukocytes and neutrophils in the peritonitis model compared to stored S. aureus suspension.

In the septic arthritis mode, it was also possible to determine that non-stored S. aureus suspension induced significantly greater intensity of joint mechanical hyperalgesia, edema, and total leukocyte, mononuclear cells and neutrophils recruitment to the knee joint of mice compared to stored S. aureus suspension. Thus, in different sites of infection and different inflammatory parameters were affected by the storage of S. aureus suspension.

The inflammatory phenomena are at some extend interconnected . For instance, neutrophils are important leukocytes recruited in acute inflammation that contribute to the development of mechanical hyperalgesia by further producing nociceptive mediators such as prostaglandin E2 (Cunha et al., 2008; Guerrero et al., 2008; Ting et al., 2008; Verri et al., 2009). In this sense, reduced bacterial load will result in reduced activation of resident cells (e.g. macrophages) that will produce reduced amounts of chemotatic mediators (e.g. TNFα, IL-1β and chemokines) resulting in reduced neutrophil recruitment, edema and pain (Verri et al., 2006; Verri et al., 2007; Verri et al., 2010). Recruited neutrophils are also important for bacterial clearance by phagocytosis and production of microbicidal oxygen and nitrogen species. Thus, reduced bacterial load will result in reduced neutrophil recruitment and activation (Crosara-Alberto et al., 2002).

This study raises implications concerning the interpretation of data. In sepsis, there is a failure (reduced) of neutrophil recruitment to the inflammatory foci because neutrophils are activated in the circulation by bacteria and bacterial products. Bacterial products such as lipopolysaccharide (LPS) down-regulate the

expression of chemokine receptors (e.g. CXCR2) via GRK, therefore, there is no gradient towards inflammatory foci (Alves-Filho et al., 2010). It is possible to induce peritonitis, sepsis or septic arthritis by administrating a bacteria suspension. However, if the bacterial load is excessive, it is possible to induce septic shock instead of peritonitis since the resident cells and recruited cells will not be able to control infection in the inflammatory foci because the bacterial load is supra-maximal (Alves-Filho et al., 2010). In this sense, using a bacterial dose based on a literature study that used CFU counting plus storage, but using McFarland's scale will result in a significantly greater inflammation that can eventually lead to loose of local control of infection and sepsis. Other implications are that pharmacological treatments might be ineffective if the bacterial load is supra-maximal, or the window between negative (PBS or saline) and positive controls (bacteria) is too small to allow detection of reduction by a testing treatment.

Concluding, the present results suggest that standardization of bacterial counts is an important step in bacteriology and related areas, and that storing or not the sample has implications such as the intensity of inflammation obtained in vivo, and the consequent interpretations of data. Accordingly, the use of the MacFarland scale is a good instrument for process optimization and also to reliably estimate the number of bacteria in a sample.

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FIGURE LEGENDS

Table 1: Mean values of CFU in suspensions prepared according to McFarland scale at different days. A suspension of culture of Staphylococcus aureus ATCC (American Type Culture Collection, U.S.A.) number 6336 in Blood Agar at 37ºC for 24hours was prepared in PBS to fit the 0.5 of McFarland Scale. Serial dilutions of the suspension were prepared and immediately plated. After 24 hours of incubation at 37ºC it was determined the number of viable bacteria (colony forming unity – CFU). The number of bacteria was considered within the range of 30 to 300 CFU per plate, as previously described. There was no significant difference between experiments.

Fig. 1. Storage of Staphylococcus aureus suspension diminishes its viability. S. aureus ATCC (American Type Culture Collection, U.S.A.) number 6336 were cultured in blood agar medium at 37º C for 24 hours. A bacterial suspension was prepared fitting 0.5 of McFarland Scale. The number of colony forming units (CFU) was determined through serial log dilution and plating on BHI agar dishes using non-stored (0 h) and stored (24 h) bacteria suspension samples. The storage was at 8oC for 24 h. n = 3, representative of two separated experiments. *P<0.05 compared to 0 h suspension. t test.

Fig. 2. Storage of S. aureus suspension diminishes inflammatory response in the peritoneal cavity of mice. Mice received an intraperitoneal injection of 5 x 108 CFU of S. aureus /ml suspension according to 0.5 of McFarland scale or PBS (0 h, 500 µl). The same suspension was stored at 8oC for 24 h and administered in another group of mice at the same dose. The total number of leukocytes (A) and neutrophils (B) into the peritoneal cavity were quantified 4 h after stimulus injection. n = 7, representative of two separated experiments. *P < 0.05 compared to saline and #P < 0.05 compared to suspension 0 hour. One-way ANOVA followed by Tukey's test.

Fig. 3. Storage of S. aureus suspension reduced mechanical articular hyperalgesia. The bacterial suspension of S. aureus (107 CFU) or saline (10 µl) was injected in the femur-tibial joint of mice. The articular hyperalgesia was evaluated with an electronic pressure meter over 15 days. Data are means ± SEM (n =5), representative of two separated experiments. * P < 0.05 compared to saline group, # P < 0.05 compared to saline group and stored bacterial suspension (24 h). One-way ANOVA followed by Tukey's t test.

Fig. 4. Storage of S. aureus suspension reduced joint edema in mice. The bacterial suspension of S. aureus (107 CFU) or saline (10 µl) was injected in the femur-tibial joint of mice. The articular edema was evaluated with an analog caliper during 15 days. Data are means ± SEM (n =5), representative of two separated experiments. * $P < 0.05$ compared to saline group, # $P < 0.05$ compared to saline group or bacterial suspension (24 h). One-way ANOVA followed by Tukey's t test.

Fig. 5. Storage of S. aureus suspension reduced leukocyte migration to the knee joint of mice. The bacterial suspension of S. aureus (107 CFU) or saline (10 µl) was injected in the femur-tibial joint of mice. Total leukocytes (A), neutrophil (B) and mononuclear cells (C) counts were determined using Newbauer chamber and Rosenfelt stained slices 15 days after bacterial suspension injection. Data are means ± SEM (n =5), representative of two separated experiments. * $P < 0.05$ compared to saline group, # $P < 0.05$ compared to saline group or bacterial suspension (24 h). One-way ANOVA followed by Tukey's t test.

Table 1

McFarland Scale	Means of Values (CFU x 10^8 / ml)
	1.37
	1.49
	1.29
Mean of Means ± SEM	**1.38 ± 0.05**

Figure 1

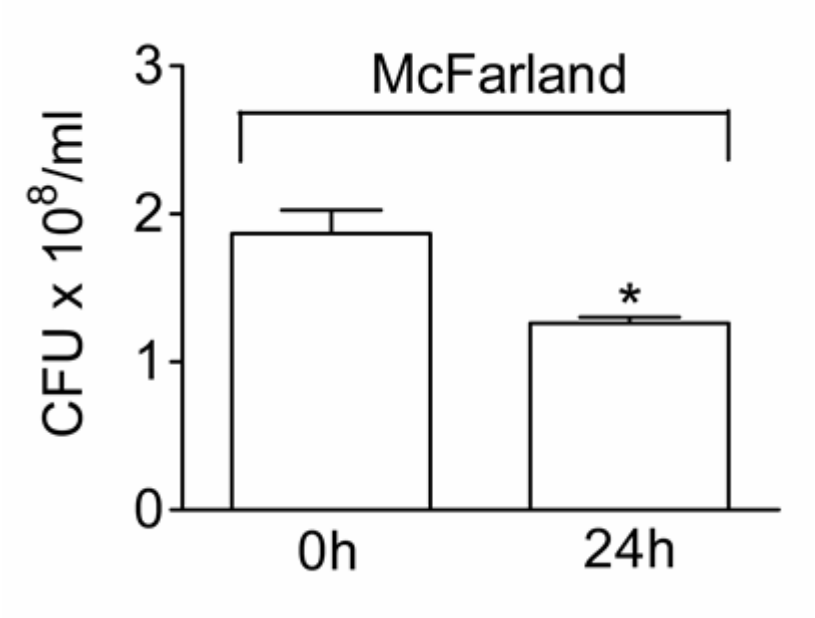


Figure 2

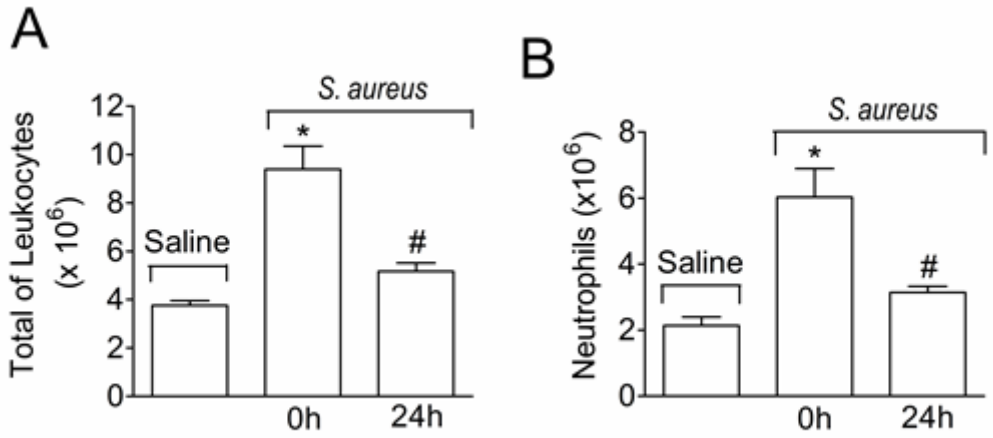


Figure 3

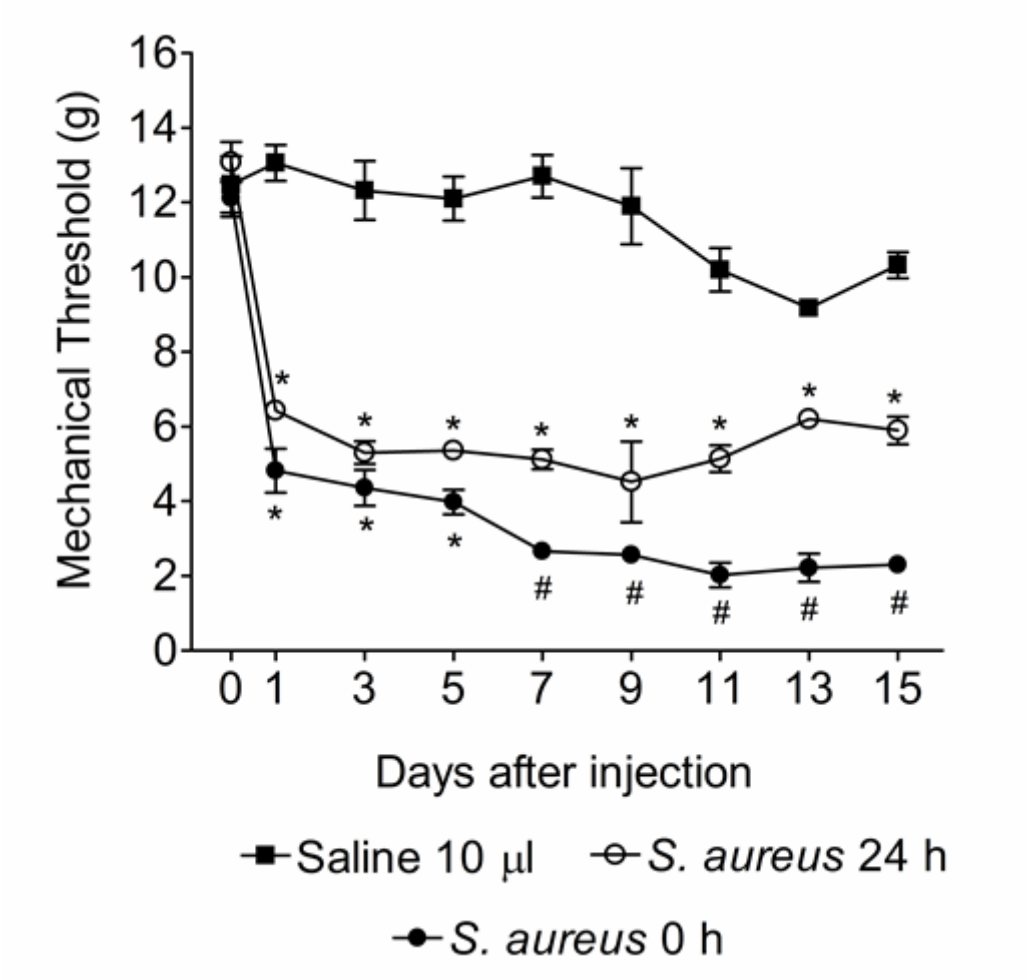

Mechanical Threshold (g)
16
14
12
10
8
6
4
2
0
0 1 3 5 7 9 11 13 15
Days after injection
Saline 10 μl
S. aureus 24 h
S. aureus 0 h

Figure 4

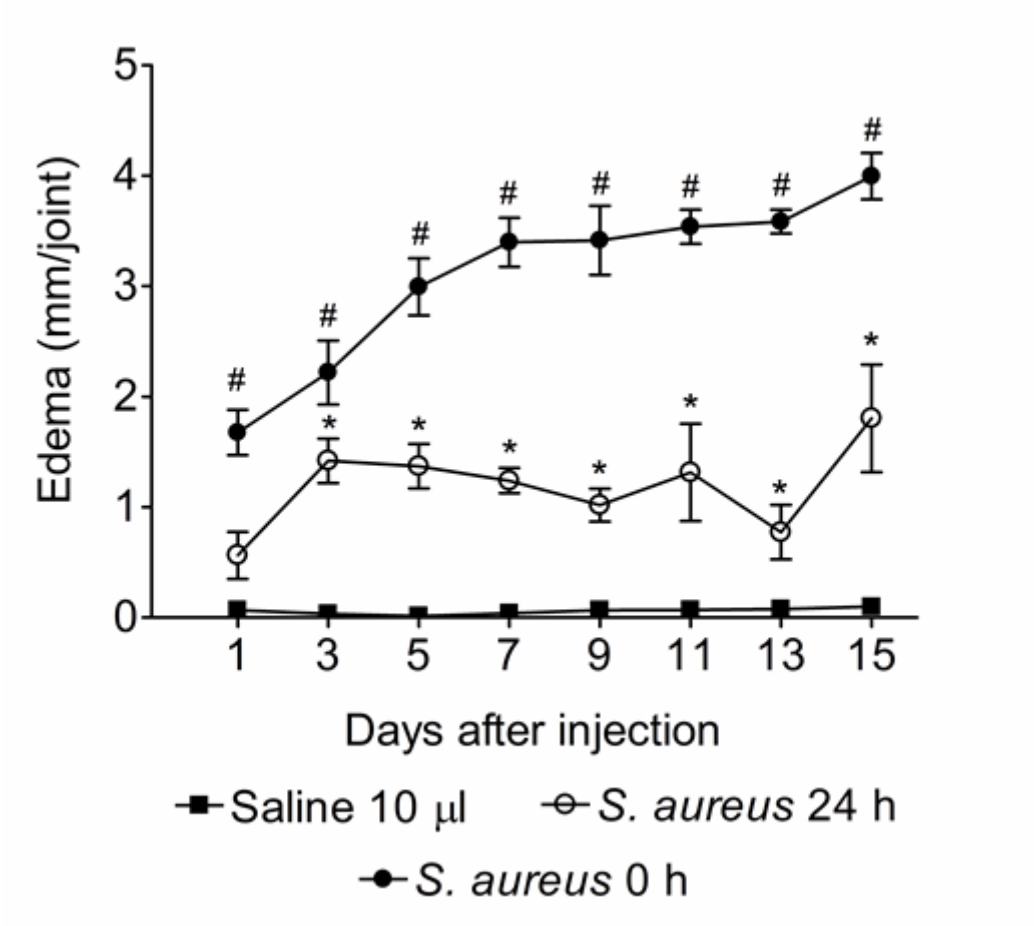
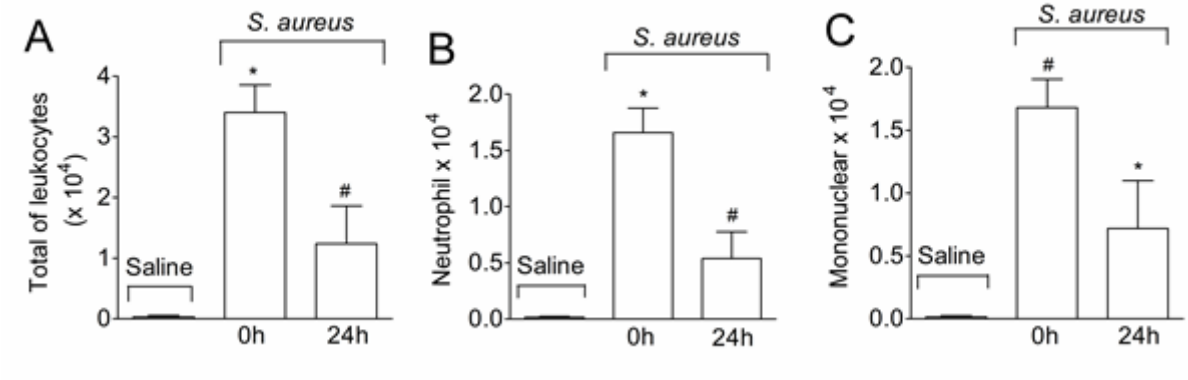


Figure 5



5 RESUMO DO ARTIGO 2

A IL-33 é uma citocina da família da IL-1, e assim como a IL-1 e a IL-18, tem efeitos pró-inflamatórios. Tem sido demonstrado que a IL-33 participa no desenvolvimento da inflamação articular por induzir a migração de neutrófilos para o sítio inflamatório tanto de forma direta, como de forma indireta. Em modelo animal de sepse induzida pela perfuração do ceco, a ativação de receptores tipo Toll promove a dessensibilização dos receptores para quimiocinas como o CXCR2, os quais são necessários para manutenção do direcionamento de neutrófilos para o foco infeccioso, a IL-33 inibe essa dessensibilização, mantendo o recrutamento de neutrófilos para o foco infeccioso. Uma doença que de certa forma engloba características dessas duas condições é a artrite séptica. Assim, o presente estudo teve por objetivo avaliar a participação da IL-33 na fisiopatogenia da artrite séptica, avaliando especificamente os parâmetros inflamatórios: dor, edema e migração celular. Para isso, foi preparada em salina suspensão de Staphylococcus aureus ATCC (American Type Culture Collection, USA) número 6336, a partir do padrão 0.5 da escala de McFarland. A suspensão foi inoculada na articulação fêmur-tibial de animais Balb/c (WT) e ST2-/-, C57BL/6 (WT), TNFR1-/- e IL-17R-/-pesando de 20-25g (n=6). Foram utilizadas doses de 1 x 10 5 CFU, 1 x 106 CFU, 1 x 107 CFU por articulação, num volume de 10 ul. O controle negativo recebeu 10 ul de salina (NaCl 0,9%). A hiperalgesia mecânica (dor) e edema foram avaliados antes da administração das bactérias (tempo 0h) e depois em dias alternados até o 28º dia após a inoculação. Os camundongos foram sacrificados no 28º dia para avaliação do infiltrado leucocitário (leucócitos totais e contagem diferencial). A administração de bactérias induziu aumento dose-dependente da hiperalgesia mecânica, edema e migração celular para o foco infeccioso. A dose de 107 CFU/10µl atingiu os maiores índices de hiperalgesia mecânica, edema e migração celular, sendo esta escolhida para o tratamento dos animais deficientes para o receptor da IL-33 (ST2-/-). Os animais ST2-/- que receberam 107 CFU/10µl de Staphylococcus aureus apresentaram diminuição da hiperalgesia, edema e migração de leucócitos em comparação com os animais Balb/c. Por outro lado, os animais TNFR1-/- e IL-17R-/- apresentaram aumento nesses parâmetros inflamatórios. A partir desses dados, sugere-se que a IL-33 participa no desenvolvimento da dor, edema e migração de leucócitos na monoartrite séptica induzida pela administração intra-articular de Staphylococcus aureus e que a inibição da atividade da IL-33 poderia reduzir o desenvolvimento de inflamação na artrite séptica. Por outro lado, a inibição da atividade do TNFα e IL-17 resultaria em piora da artrite séptica. Assim, o trabalho sugere principalmente que a IL-33 é uma citocina importante para a resposta inflamatória na monoartrite bacteriana e que a inibição da sua atividade reduz a inflamação, não interferindo na resposta local contra a bactéria. Ademais, a inibição da atividade da IL-33 poderia ser um alvo terapêutico na artrite reumatóide com menos efeitos colaterais do que as terapias anti-TNF (infliximab, etanercept) e terapias recém sugeridas, como a anti-IL-17, pois na primeira os pacientes não ficariam suscetíveis às infecções como nas últimas.

Palavras-chaves: IL-33. Artrite reumatoide. Artrite séptica. Dor. Edema e migração celular.

6 ARTIGO PARA PUBLICAÇÃO 2

Este é um trabalho realizado no Laboratório de Dor, Neuropatia, Câncer e Inflamação, formado pelo artigo científico: IL-33 RECEPTOR DEFICIENCY AMELIORATES SEPTIC ARTHRITIS IN MICE. STAURENGO-FERRARI, L.1; CARDOSO, R.D.R.; PELAYO, J.S.; XU, D.; LIEW, F.Y.; CUNHA, F.Q; SARIDAKIS, H.O.; VERRI, W.A., Jr.

As formatações do artigo seguem as normas da revista: Annals of Rheumatic Diseases (ARD) (Anexo 2).

EXTENDED REPORT

IL-33 RECEPTOR DEFICIENCY AMELIORATES SEPTIC ARTHRITIS IN MICE.

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Short title: IL-33 receptor deficiency reduces septic arthritis.

Key words: Hyperalgesia, Inflammation, Septic Arthritis, IL-33, ST2.

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Abbreviations: RA (Rheumatoid arthritis); S. aureus (Staphylococcus aureus); LPS (Lipopolysaccharide); GRK2 (G protein–coupled receptor kinase-2); DMARDs (Disease-modifying antirheumatic drugs); TNFR1 (TNFα receptor); IL-17R (IL-17 receptor); M. tuberculosis (Mycobaterium tuberculosis).

Word Count: 2.605 words

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ABSTRACT

Objectives: IL-33 is a cytokine of IL-1 family, which signals through its receptor ST2 and induces pleiotropic responses depending on cytokine milieu. For instance, it mediates neutrophil recruitment in rheumatoid arthritis, and sepsis. The septic arthritis has features or links rheumatoid and sepsis since RA patients are more susceptible to septic arthritis. Herein, we investigated the involvement of IL-33 in the pathophysiology of septic arthritis. **Methods and Results:** The results showed that administration of Staphylococcus aureus in the femur-tibial joint of mice caused a dose-dependent increase in the mechanical hyperalgesia, edema and cell migration to the infection site. The mechanical hyperalgesia, edema and cell migration (total of leucocytes; neutrophils and mononuclear cells) were diminished in ST2 deficient (-/-) mice compared to Balb/c mice during the 28 days evaluated. On the other hand, TNFR1-/- and IL-17R-/- mice exhibited increased inflammatory response compared to WT. **Conclusion:** The results suggest that IL-33 participates in the development of pain, edema and leukocyte migration in septic monoarthritis induced by intra-articular administration of S. aureus. Furthermore, anti-IL-33 therapy seems to be more adequate than anti-TNF or anti-IL-17 therapies for RA since would not favor the development of septic arthritis.

INTRODUCTION

IL-33 is a novel cytokine of IL-1 family that includes IL-1 and IL-18. However, unlike IL-1 and IL-18, which mainly promote T-helper 1 (Th1)- associated responses, IL-33 predominantly induces the production of Th2 cytokines (IL-5 and IL-13) and increases the levels of serum Ig. [1] IL-33 binds the heterodimeric receptor complex consisting of ST2 (IL-1RL1) and IL-1 receptor accessory protein. [1-3] ST2 is expressed on TH2 cells and mast cells and has a key role in TH2 effector functions. [4, 5] Moreover, ST2 can negatively regulate TLR activation via seques¬tration of the TLR signaling components myeloid differentiation factor-88 (MyD88) and Mal.[6]

Recently, it has been demonstrated that IL-33 also induces innate, Th1 and Th17 inflammatory responses [7-10]. This pleiotropic cytokine has been involved in the pathogenesis of a variety of rheumatic, immune regulation and in inflammatory diseases. However, in sepsis, IL-33 has also considered a critical cytokine capable of activating neutrophils during protective host response against polimicrobial infection [10]. As such, it offers novel therapeutic potential. [7,8,11] Accordingly, IL-33 and ST2 are detected in the synovial tissue and serum of rheumatoid arthritis patients. [4,7] In murine models of rheumatoid arthritis, IL-33 contributes to the development of articular mechanical hyperalgesia [7] and increases the inflammatory responses by inducing neutrophil recruitment to the knee joints. [12] This neutrophil recruitment is indirect by IL-33-induced production of chemotatic cytokines and chemokines, and direct because neutrophils express elevated levels of ST2 in rheumatoid arthritis in response of TNFα, therefore, being chemoattracted by IL-33. [13]

At some extend, septic arthritis has features or links rheumatoid arthritis since there is increased incidence of septic arthritis in rheumatoid arthritis patients. [14] This may be due to several reasons, such treatment with immunosuppressive, disease-modifying antirheumatic drugs (DMARDs) and biological therapies may decrease the immune function required for protection from pathogens predisposes to bacterial joint colonization, such [14,15] current anti-TNF (e.g. infliximab, etanercept) therapies [14] and experimental targeting IL-17 therapies [16]. These facts have implications for identifying novel targets in disease to avoid

the development of infection such as septic arthritis that is inflammatory processes accompanied by erode articular damage, bone and joint destruction leading to irreversible loss of joint function [17].

Considering the evidence described above of dual role of IL-33 as pathogenic or host protective factor in autoimmune or microbial settings, we addressed the involvement of IL-33 in the pathogenesis of septic arthritis induced by local administration (femur-tibial; knee joint) of Staphylococcus aureus in mice, which is the most common pathogen in septic arthritis. [18] Specifically, it was evaluated the effect of IL-33 receptor deficiency (ST2-/-) in the septic arthritis-induced articular inflammatory pain (referred to as mechanical hyperalgesia), edema and leukocyte migration.

Compelling evidence indicates a critical role for TNFα [17,18] and IL-17 [19-22] in sustaining the inflammatory response in several models of arthritis, we compared the results obtained with ST2-/- mice to TNFR1-/- and IL-17R-/-mice.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, ST2-/-, TNFR1-/-, IL-17R-/- from Faculty of Medicine of Ribeirao Preto (FMRP) were maintained in the Universidade Estadual de Londrina (UEL). Mice were housed in standard clear plastic cages with free access to food and water, and a light/dark cycle (12:12 h) at 21 °C. The Ethics Committee on Animal care and handling procedures of the UEL approved this study.

Staphylococcus aureus suspension preparation. S. aureus was obtained from ATCC (American Type Culture Collection, U.S.A.) number 6336. Twenty four hours before each experiment samples of bacteria were cultured in blood agar medium at 37ºC. The bacterial suspension was centrifuged and the pellet was ressuspended in sterile phosphate-buffered saline (PBS) to fit 0.5 McFarland Scale for administration to the animals and serial dilutions to confirm bacterial viability. The number of colony forming units (CFU) according to the McFarland scale was previously standardized. [23]

Induction of septic arthritis. The septic arthritis was induced by local injection of Staphylococcus aureus suspension in the femur-tibial joint of mice. As control other animals received 10 µL of sterile saline. The animals were evaluated over 28 days after injection.

Evaluation of articular hyperalgesia. The articular hyperalgesia of the femur-tibial joint was evaluated over 28 days in mice as previously reported method. [20] In quiet room, mice were placed in acrylic cages with a wire grid floor 15–30 min before testing for environmental adaptation. The test consisted of evoking hind-paw flexion reflex with a hand-held force transducer adapted with a 4.15 mm2 polypropylene tip. The end point was characterized by paw to induce flexion of the femur–tibial joint followed by paw withdrawal. After the paw withdrawal the intensity of the pressure was automatically recorded, and the value for the response was obtained by averaging three measurements. The flexion-elicited mechanical threshold was expressed in grams (g).

Evaluation of joint edema. The femur-tibial joints edema was evaluated over 28 days by measurement of the transverse diameters of femur-tibial joints using an analog caliper (Mitutoyo Corp., Kanagawa Japan). Values of femur-tibial joint thickness are expresses as the difference between the diameter measure before (basal) and after induction of articular inflammation in millimeters. [24]

***In vivo* leukocyte migration.** Mice received S. aureus in femur-tibial joint or sterile saline were sacrificed at the 28th day after inoculation of bacteria and the synovial cavity of knee joint was washed twice with 5 µL saline contained 10 mM EDTA. [25] The total number of infiltrating cells was determined in a Neubauer Chamber diluted in Turk's solution. Differential cell counts were determined in cytocentrifuge Rosenfeld stained slices (Cytospin 4; Shandon, Pittsburg, PA). Differential cell counts were performed with a light microscope and the results were expressed as the number (mean ± SEM) of neutrophils per cavity. [25]

Statistical analysis. Statistical analyses were performed using GraphPad Prism (La Jolla, 5 CA). Results are presented as means ± SEM of 2 independent experiments. The "n" in the legends refers to the number of mice used in the experimental group of

each experiment. The differences between the experimental groups were compared by ANOVA (one-way) and individual comparisons were subsequently made with Tukey's post hoc test. The level of significance was set at $P < 0.05$.

RESULTS

S. aureus induces articular mechanical hypernociception, edema and leukocyte recruitment in a dose-dependent manner.

Staphylococcus aureus is the microorganism most frequently associated with bacterial arthritis, [26,27] which results in synovial inflammation, cartilage and bone destruction. [28] The suspension of S. aureus (105-107 CFU [colony forming unities]/10 µL) induced articular mechanical hyperalgesia (pain, detected as decrease of mechanical threshold), edema and leukocyte recruitment in a dose-dependent manner which peaked with 107 CFU/10 µL per joint (Fig.1 A-C). The articular mechanical hyperalgesia and edema was significant from 1 day after injection. The articular mechanical hyperalgesia showed a peak on day 13 and was maintained until day 28, where observed the leukocyte recruitment, showing the interdependence between the pro-nociceptive response (hyperalgesia) and leukocyte migration. A dose 107 CFU/10 µL per joint was chosen for subsequent experiment.

IL-33/ST2 mediates S. aureus-induced articular mechanical hypernociception in mice.

IL-33/ST2 mediates antigen-induced articular hyperalgesia. [7] We therefore investigated the role of this cytokine in articular mechanical hyperalgesia induced by S. aureus. The S. aureus-induced articular mechanical hyperalgesia was significantly reduced in ST2-/- mice compared to WT mice (note that increase of mechanical threshold indicates reduced hyperalgesia) (Fig. 2A), suggesting the involvement of the IL-33/ST2 pathway in septic arthritis hyperalgesia. Interestingly, up to 9 days after infection, WT and ST2-/- mice presented similar responses, and thereafter ST2-/- mice presented reduced hypernociception compared to WT (Fig 2A). This result indicates the possibility that ST2-/- mice respond better to infection, and therefore, presented reduced hypernociception in the chronic phase of septic

arthritis. On the other hand, TNFR1-/- and IL-17R-/- mice showed a significant increase of articular mechanical hyperalgesia compared to WT controls (Fig. 2B-C). This difference was observed since first day of infection, suggesting the development of chronic septic arthritis in these animals more severe than in ST2-/- mice.

IL-33/ST2 mediates S. aureus-induced edema in mice.

Edema is also observed in septic arthritis, [29, 30] therefore, we also evaluated whether IL-33 receptor deficiency would also result in reduction of joint edema or IL-33 role is specific in pain development. The S. aureus induced articular edema was significantly reduced in ST2-/- mice compared to WT control (Fig. 3 A), suggesting the involvement of the IL-33/ST2 pathway in septic arthritis edema. There was an early reduction of edema in ST2-/- mice, which was even more evident in the chronic phase of septic arthritis reaching similar levels of the vehicle control group (Fig 3A). Concerning TNFR1-/- and IL-17R-/- mice, these groups presented a significant increase of articular edema compared with WT control mice, this increase was evident after 9th day on infection and maintained until the end of the period observed (Fig 3B-C). These results might be related to the impaired bacterial elimination in TNFR1-/- and IL-17R-/- mice, indicating a protective and important function of normal TNFα and IL-17 signaling in the development of S. aureus – induced septic arthritis.

IL-33/ST2 mediates S. aureus-induced leukocyte recruitment to the knee joint of mice.

The leukocyte recruitment is observed in the joints with septic arthritis. [31-34] We therefore, determined the association IL-33/ST2 in S. aureus induced-leukocyte recruitment. The intra-articular injection of S. aureus induced reduced total leukocyte, neutrophil and mononuclear cells recruitment to the knee joint of ST2-/- mice compared to WT mice (Fig. 4A). It is unlikely that this reduced inflammation in the chronic phase of septic arthritis has as consequence the reduction of bacterial elimination since TNFR1-/- and IL-17R-/- mice, presented increased total leukocytes, neutrophils and mononuclear cells in the knee joint as

well as hypernociception and edema (Fig 4B-C). As well as in pain, this effect was not observed, suggesting that despite the increased inflammatory response in these animals it perishes not be sufficient to eliminate the infection.

DISCUSSION

Rheumatoid arthritis patients are at particular risk for septic arthritis development, most likely because joint disease predisposes to bacterial joint colonization and the rheumatoid arthritis treatments with corticosteroids, disease-modifying antirheumatic drugs (DMARDs) and biological therapies (i.e. anti-TNF therapies, such infliximab) decrease the immune function required for protection against pathogens. [14,15] Therefore, it is important to search for novel therapies for auto-immune diseases that do not damp the host innate responses predisposing to, for instance, septic arthritis. Here in, it was shown that absence of IL-33 / ST2 signaling ameliorates joint pain, edema and leukocyte recruitment in septic arthritis, and opposing results were observed in TNFR1-/- and IL-17R-/- mice.

The painful joints are a common symptom of septic arthritis [35]. There is interdependence between decrease in the nociceptive threshold (pro-nociceptive response, pain) and the neutrophil migration. [14]. [36-39] Indeed, in this study we observed in Balb/c mice that the increase in the mechanical articular hyperalgesia produced by S. aureus-induced septic arthritis occurs concurrently with an increase in the number of neutrophil that infiltrated the joint, suggesting an association between these two events.

Cytokines are the most important substances involved in the induction of inflammatory events of RA, but are also critical in the protective response against bacterial infection. The current idea is that the IL-33 plays a pivotal role in physiopatology of RA [2, 5-8] and in different models of infection [10, 40]. This hypothesis is supported by studies that have shown that this cytokine mediates neutrophil recruitment in rheumatoid arthritis, [13] and in cecal ligation and puncture-induced sepsis, [10] raising the question as to whether this cytokine plays a pathogenic or protective role in S.aureus induced-septic arthritis. Further supporting this question we have shown that IL-33 mediates inflammation in septic arthritis. The present data demonstrated that ST2-/- mice unlike TNFR1-/- and IL-17R-/- mice, showed a minor articular mechanical hyperalgesia. This result suggests that

abrogation of IL-33/ST2 signaling ameliorates the host response against infection and confirms the important role of TNFα [41] and IL-17 [42] in response to bacterial infections, including S. aureus.

Considering that IL-33 is produced by endothelial cells and can increase in ST2-dependent manner the endothelial cell permeability in vitro. [42] It is conceivable that it could contribute to the regulation of vascular permeability during inflammation, and the resulting edema. This effect could explain involvement of the IL-33/ST2 pathway in septic arthritis edema, since ST2-/- mice showed decrease in joint edema in S. aureus induced- septic arthritis.

The intense leukocyte infiltration is observed in joints with septic arthritis, almost neutrophilic, [37] but has previously demonstrated that cells of acquired immune system, i.e., T and B lymphocytes are presents in S. aureus induced-septic arthritis [31,34]. Moreover, the IL-33 is a potent chemoattractant for neutrophils. [10,13] Additionally, in this study we observed a impairment in the number of leukocytes that infiltrated joints in ST2-/-. This reduction in the cellular recruitment was consistent with the reduction in hyperalgesia since recruited neutrophils can contribute to hyperalgesia by further producing nociceptive mediators. [36-38]

Taken together these results indicate that absence of IL-33/ST2 signaling pathway ameliorates the inflammatory process, that could be a consequence of better host response against infection, unlike TNFR1-/- and IL-17R-/- mice, which presented prolonged inflammatory response that might be related to the impaired bacterial elimination by the host and the presence of TNFα and IL-17-independent mechanisms maintaining an inflammatory response that is not able to eliminate the infection agent. In agreement, TNFR1/2-/-, anti-TNF treated, IL-17R-/- and IL-17A-/- mice do not properly control bacterial infection. [43,44]

Corroborating our data, Wieland et al. (2009) demonstrate that ST2-/- mice display a normal host defense against pulmonary infection with Mycobacterium tuberculosis. Similarly, IL-17 seems dispensable for protection against M. tuberculosis infection. [41] On the other hand, the treatment of patients with monoclonal anti-TNF antibodies has led to increase in incidence of M. tuberculosis induced-infection. [48]

In sepsis is a response characterized by widespread inflammation and multiple organ failure, and the signaling pathway IL-33/ST2 is important to

maintenance of neutrophil recruitment to sites of infection for the clearance of the pathogens. [10] Once at the infectious focus, neutrophils do not need to continue moving because they are already in the site of infection responding to bacteria. They must respond by producing antimicrobial products. In fact, it is logical to suppose that TLRs agonists down-modulate neutrophil trafficking to keep these cells at the site of infection. [49,50] This response may be occurring in septic arthritis and could explain the mechanistic difference between sepsis and septic arthritis (systemic versus local infection). Another explanation for this fact is the presence of IL-4, this cytokine promotes bacterial growth in joints [29] and IL-33 was described to induce Th2-associated cytokines such as IL-4, IL-5 and IL-13. [1]

To mimic the current standard anti-TNF therapy and address the importance of IL-17 [rheumatoid arthritis is a Th17 associated disease and for being a therapy recently suggested (Drug in phase I clinical trials)] [51], it was also addressed the development profile of septic arthritis in TNFR1-/- and IL-17R-/- mice. Opposing to the results observed with ST2-/- mice, an inflammatory response in TNFR1-/- and IL-17R-/- was exacerbated. Therefore, it is likely that anti-IL-33 therapies might be more suitable for rheumatoid arthritis treatment than anti-TNF and anti-IL-17 therapies since would not favor the development of septic arthritis and rather optimize host defense against infection.

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COMPETING INTERESTS. None.

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LEGENDS

Figure. 1. Staphylococcus aureus induces septic arthritis and articular inflammation. Staphylococcus aureus (American Type Culture Collection, U.S.A.) number 6336 (105-107 CFU/10 µL) or saline (10 µL) was injected in the femur-tibial joint of Balb/c mice. Mechanical hyperalgesia (A) and edema (B) were evaluated at different time points after inoculation with an electronic pressure meter and caliper, respectively. Total leukocytes, monocuclear cells and neutrophil counts (C) were determined using Newbauer chamber and Rosenfelt stained slices 28 days after bacterial injection. Data are mean ± SEM, n=6 per experiment, representative of 2 separate experiments.* P < 0.05 compared to saline group, # P < 0.05 compared to saline group and 105-106 CFU dose of bacteria (panels A,B) or saline (panel C).

Figure. 2. IL-33 mediates articular hyperalgesia in S. aureus–induced septic arthritis. S. aureus (107 CFU/10 µl) was injected in the femur-tibial joint of Balb/c, C57 (WT) and ST2-/- , TNFR1-/-, IL-17R -/- mice. Control mice (Vehicle) received saline. Mechanical hyperalgesia was determined over 28 days after inoculation with an electronic pressure meter. Data are mean ± SEM, n=6 per experiment, representative of 2 separate experiments. * P < 0.05 compared to saline group (panels A-C), # P < 0.05 compared to WT group (panels A-C).

Figure. 3. ST2 deficiency reduces edema in S. aureus-induced septic arthritis. S. aureus (107 CFU/10 µl) was injected in the femur-tibial joint of of Balb/c, C57 (WT) and ST2-/- , TNFR1-/-, IL-17R -/- mice. Control mice (Vehicle) received saline. Articular edema was evaluated over 28 days after inoculation with a caliper. Data are mean ± SEM, n=6 per experiment, representative of 2 separate experiments. * P < 0.05 compared to saline group (panels A-C), # P < 0.05 compared to WT group (panels A-C).

Figure. 4. IL-33/ST2 mediates leukocyte recruitment in S. aureus-induced septic arthritis. S. aureus (107 CFU/10 µL) was injected in the femur-tibial joint of Balb/c, C57 (WT) and ST2-/- , TNFR1-/-, IL-17R -/- mice. Control mice (Vehicle) received saline. Total leukocytes, neutrophil and mononuclear cells counts were determined using Newbauer chamber and Rosenfeld stained slices 28 days after bacterial injection. Data are means ± SEM (n=6), representative of two independent experiments. * $P < 0.05$ compared to saline group, # $P < 0.05$ compared to WT group.

Figure 1

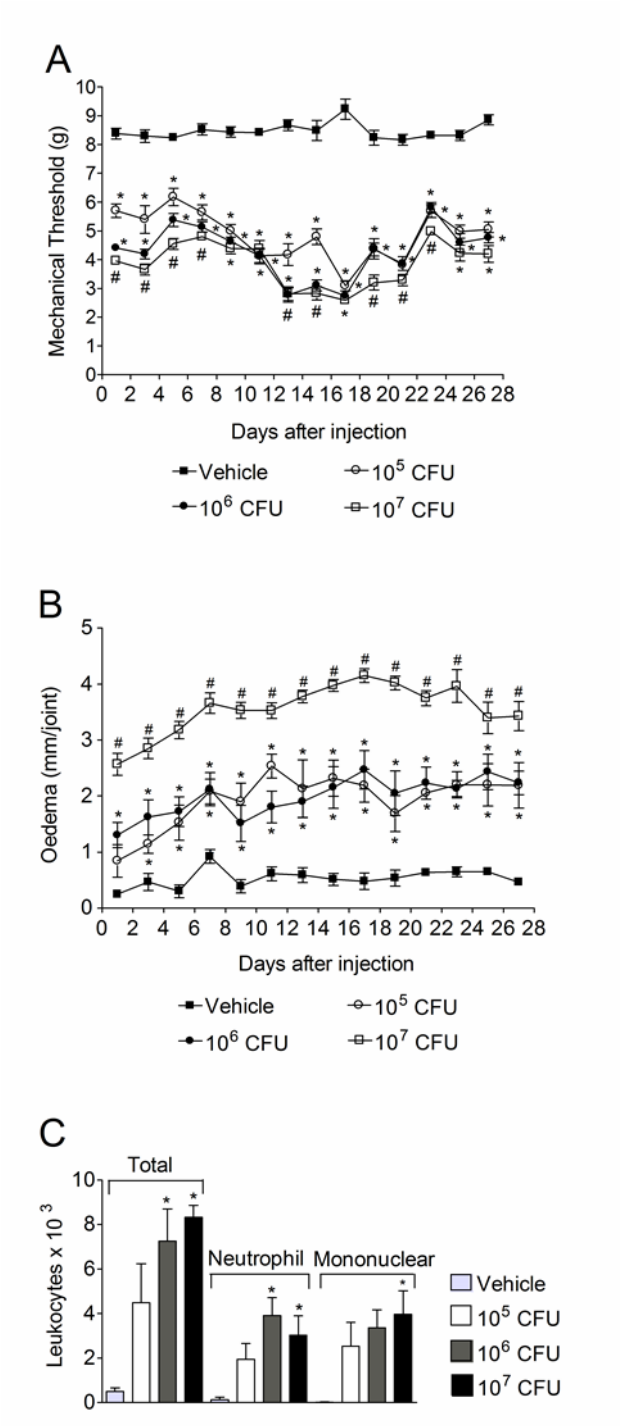

A
Mechanical Threshold (g)
Days after injection
Vehicle
10^5 CFU
10^6 CFU
10^7 CFU
B
Oedema (mm/joint)
Days after injection
Vehicle
10^5 CFU
10^6 CFU
10^7 CFU
C
Leukocytes x 10^3
Total
Neutrophil
Mononuclear
Vehicle
10^5 CFU
10^6 CFU
10^7 CFU

Figure 2

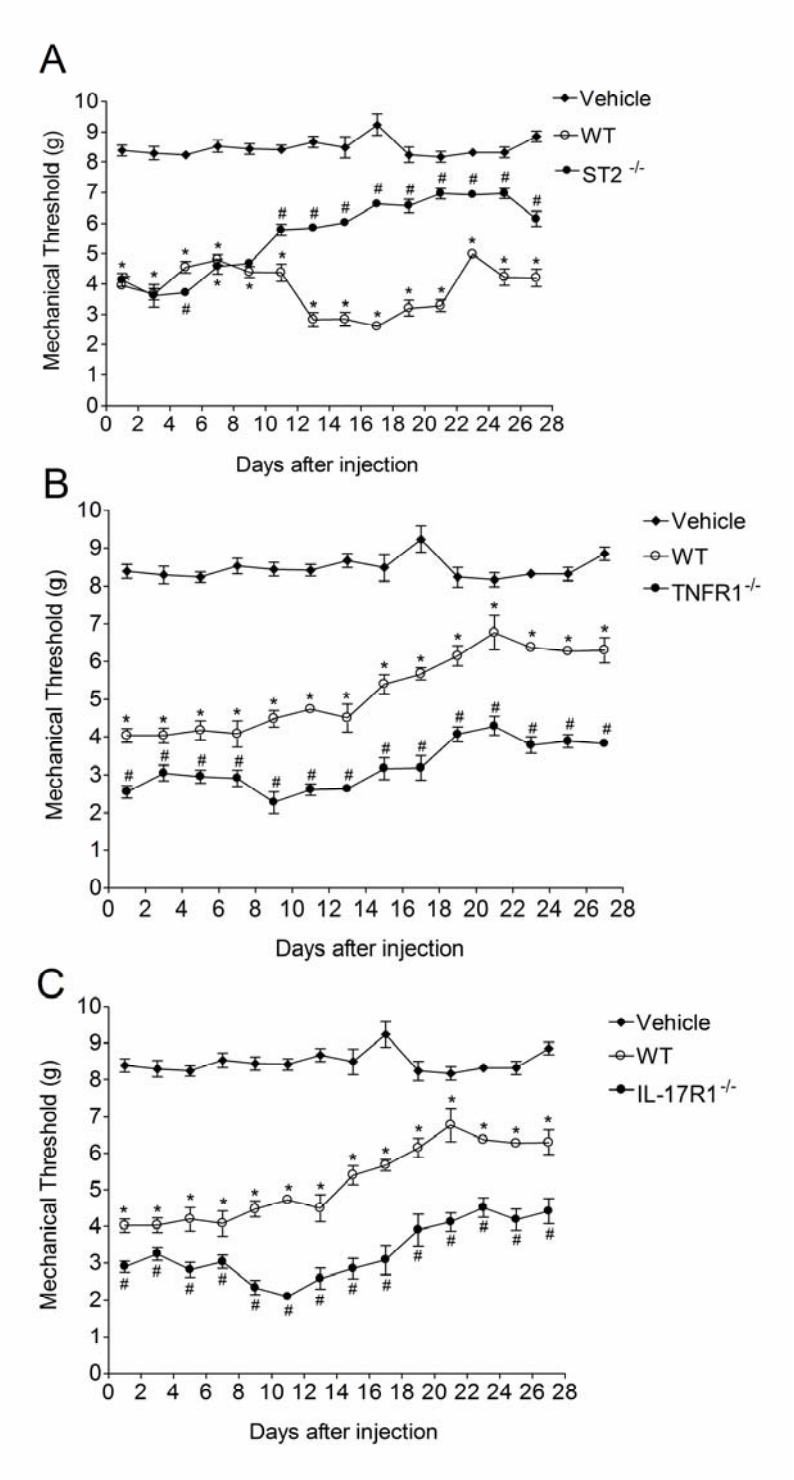

A
Mechanical Threshold (g)
Days after injection
Vehicle
WT
ST2 -/-
B
Mechanical Threshold (g)
Days after injection
Vehicle
WT
TNFR1-/-
C
Mechanical Threshold (g)
Days after injection
Vehicle
WT
IL-17R1-/-

Figure 3

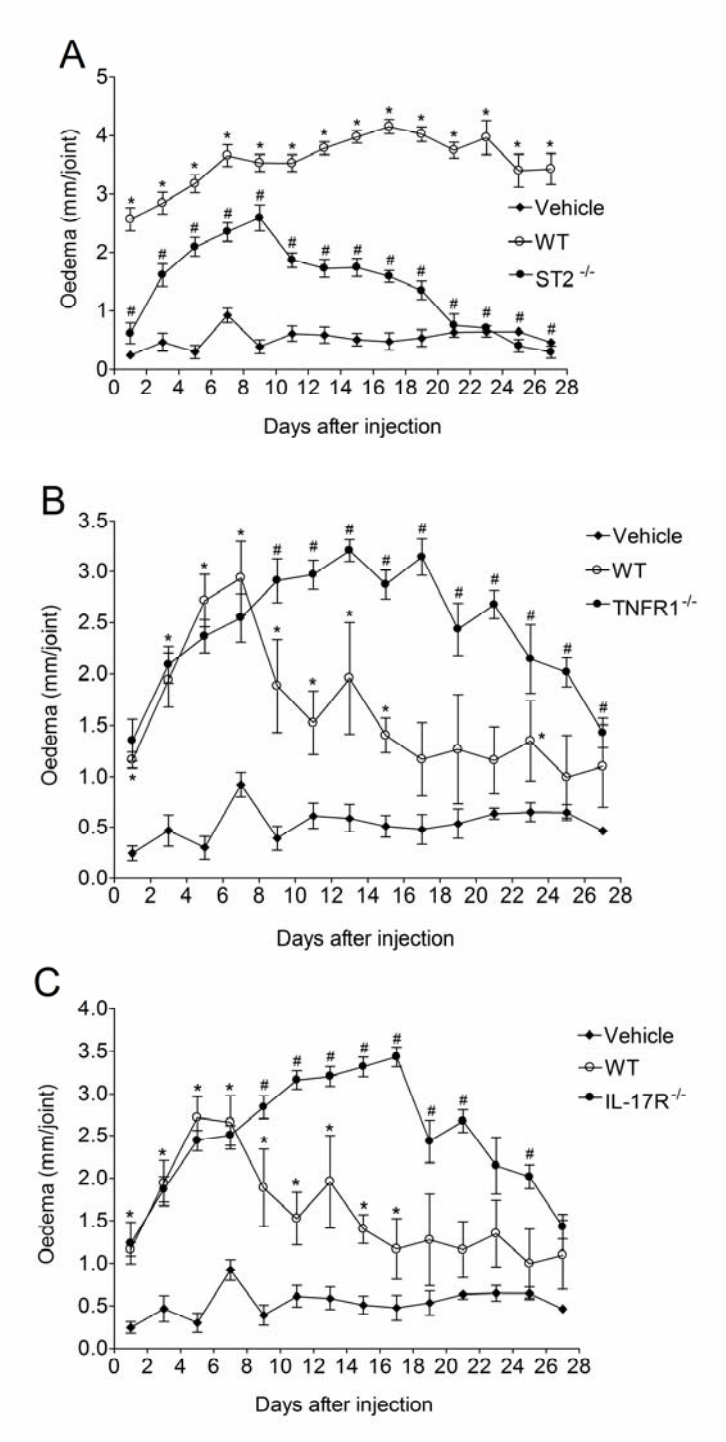
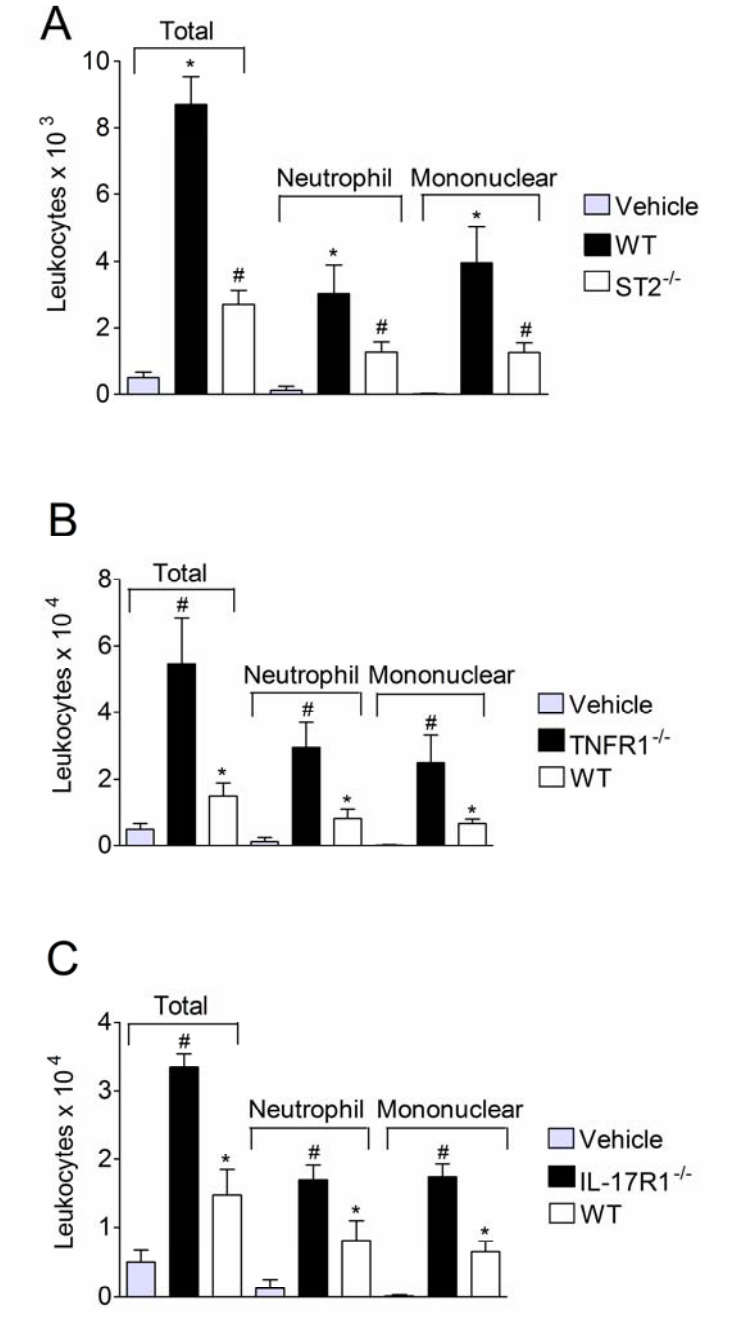

A
Oedema (mm/joint)
Days after injection
Vehicle
WT
ST2 -/-
B
Oedema (mm/joint)
Days after injection
Vehicle
WT
TNFR1 -/-
C
Oedema (mm/joint)
Days after injection
Vehicle
WT
IL-17R -/-

Figure 4


A
Total
Neutrophil
Mononuclear
Leukocytes x 10 3
Vehicle
WT
ST2-/-
B
Total
Neutrophil
Mononuclear
Leukocytes x 10 4
Vehicle
TNFR1-/-
WT
C
Total
Neutrophil
Mononuclear
Leukocytes x 10 4
Vehicle
IL-17R1-/-
WT

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8 ANEXOS

ANEXO A

INSTRUCTIONS FOR AUTHORS

The **African Journal of Pharmacy and Pharmacology (AJPP)** is an open access journal that provides rapid publication (monthly) of articles in all areas of Pharmaceutical Science. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published approximately one month after acceptance. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

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Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not

conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process. All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors within 3 weeks. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJPP to publish manuscripts within 8 weeks after submission.

Regular articles. All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present Addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.

Following the abstract, about 3 to 10 key words that will provide indexing The *Acknowledgments* of people, grants, funds, etc should be brief. *Tables* should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes.

Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. *Graphics* should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. *Tables* should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information
given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the
date to distinguish the works.

Examples:
Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.
Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615.

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ANEXO B

ABOUT ANNALS OF THE RHEUMATIC DISEASES

Annals of the Rheumatic Diseases (ARD) is an international peer review journal committed to promoting the highest standards of scientific exchange and education. It covers all aspects of rheumatology, which includes the spectrum of musculoskeletal conditions, arthritic disease, and connective tissue disorders. ARD publishes basic, clinical, and translational scientific research. Concise scientific communication is encouraged and peer reviewed proceedings of international meetings are featured. Educational papers include state of the art reviews, "how to" articles and educational cases that focus on problems faced in clinical practice. The journal was first published in 1939 and has an authorative global Editorial Board and a growing international readership.

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13 Koziol-Mclain J, Brand D, Morgan D, et al. Measuring injury risk factors: question reliability in a statewide sample. Inj Prev 2000;6:148-50.

Chapter in book

14 Nagin D. General deterrence: a review of the empirical evidence. In: Blumstein A, Cohen J, Nagin D, eds. Deterrence and incapacitation: estimating the effects of criminal sanctions on crime rates. Washington, DC: National Academy of Sciences 1978: 95-139.

Book

(personal author or authors) (all book references should have specific page numbers)
15 Howland J. Social norms and drunk driving countermeasures. In Graham JD, ed. Preventing automobile injury: new findings from evaluative research. Dover, MA: Auburn House Publishing Company 1988:163-96.

Abstract/supplement

16 Roxburgh J, Cooke RA, Deverall P, et al. Haemodynamic function of the carbomedics bileaflet prosthesis [abstract]. Br Heart J 1995;73 (suppl 2):P37.

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