UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Ciências Médicas

NÍVEIS DE INTERFERON ALFA E FATOR DE NECROSE TUMORAL ALFA EM PACIENTES COM LÚPUS ERITEMATOSO SISTÊMICO JUVENIL: ASSOCIAÇÕES COM MANIFESTAÇÕES CLÍNICAS

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“A curiosidade é mais importante do que o conhecimento.”

(Albert Einstein)

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ABREVIATURAS

aCL- Anti-cardiolipina

ACR- American College of Rheumatology

ANA- Anticorpo antinuclear

Anti-dsDNA- Anti-DNA de fita dupla

Anti-Sm- Anti-Smith

APC- Células apresentadoras de antígenos

BAI- Inventário de ansiedade de Beck

BDI- Inventário de depressão de Beck

CDI- Inventário de depressão infantil

CMV- Citomegalovírrus

DNA- Ácido dexoxirribonucléico

DP- Desvio padrão

EBV- Epstein-Bar

ELISA- Enzyme-Linked Immunoabsorbent Assay

ENA- Anticorpos contra antígenos extraídos do núcleo

Fator de necrose tumoral- TNF-α

FCM- Faculdade de Ciências Médicas

HC- Hospital de Clínicas

HLA- Antígeno leucocitário humano

IFIG- Genes induzidos por INF-α

IFNAR- Receptor de Interferon

IL- Interleucina

Interferon alfa- INF-α

LA- Anticoagulante lúpico

LCR- Líquido cefalorraquidiano

LES- Lúpus eritematoso sistêmico

LES NP- Lúpus neuropsiquiátrico

LESj- Lúpus eritematoso juvenil

mAb- Anticorpo monoclonal

NMDA- Anticorpos contra o receptor N-metil-d-aspartato

NP- Neuropsiquiátrico

NZB- New Zealand Black

NZW- New Zealand White

PCR- Proteína C reativa

PTX- Pentoxifilina

RNA- Ácido ribonucléico

RNP- Ribonucleoprotéico

RPM- Rotação por minuto

SLEDAI- Systemic Lupus Erythematosus Disease Activity Index

SLICC/ACR (DI)- Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index

SNC- Sistema nevoso central

SNP- Single-nucleotide polymorphism

TCLE- Termo de consentimento livre e esclarecido

TNFR1/TNFR2- Receptor de fator de necrose tumoral

UV- Radiação ultravioleta

VHS- Velocidade de hemossedimentação

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Sumário

Resumo

Lúpus Eritematoso Sistêmico (LES) é uma doença autoimune, crônica e mutissistêmica, caracterizada por períodos de atividade e remissão. O interesse em identificar biomarcadores que se correlacionem com a atividade sistêmica do LES e que possam predizer um envolvimento orgânico futuro é crescente. O presente estudo, de característica transversal, teve como objetivo avaliar os níveis de interferon alfa (INF-α) e fator de necrose tumoral alfa (TNF-α) em pacientes com LES juvenil (LESj) (início da doença≤16 anos), familiares de primeiro grau e indivíduos sadios não aparentados e elucidar sua associação com a atividade da doença, dados laboratoriais e de tratamento. Foram selecionados pacientes consecutivos com LESj acompanhados na Unidade de Reumatologia Pediátrica da UNICAMP entre 2009/2010. Manifestações clínicas, laboratoriais e medicação em uso foram avaliadas. A atividade da doença [SLE Disease Activity Index (SLEDAI)], dano cumulativo [Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI)] foi determinado para cada paciente no dia da coleta de sangue. Os transtornos de humor foram determinados através dos inventários de Depressão (BDI) e Ansiedade (BAI) de Beck. A dosagem das citocinas foi realizada por ELISA (*Enzyme Linked Immuno Sorbent Assay*). Níveis séricos de INF-α e TNF-α estavam aumentados no LESj quando comparado a familiares de primeiro grau e indivíduos sadios não aparentados. Níveis séricos de INF-α e de TNF-α foram significativamente maiores em pacientes com doença ativa (p=0,031; p=0,014, respectivamente). INF-α (r=0,33; p=0,012) e TNF-α (r=0,39; p=0,002) correlacionaram-se com SLEDAI. INF-α foi significativamente maior em pacientes com anti-dsDNA positivo (p=0,011), pacientes com vasculite cutânea (p=0,001), pacientes com rash malar (p=0,032) e em pacientes sem medicação (p=0,035). Níveis séricos de INF-α correlacionaram-se com níveis de C3 (r=0,34; p=0,032) e idade (r=-

0,17; p=0,025). Níveis séricos de TNF-α foram significativamente maiores em pacientes com nefrite (p=0,009) e em pacientes com depressão (p=0,001). De acordo com nossos resultados, INF-α e TNF-α estiveram associados com a atividade da doença e poderiam ser considerados biomarcadores para avaliar atividade, porém estudos longitudidinais são necessários para determinar se o aumento dessas citocinas pode prever períodos de atividade em pacientes com LESj.

Abstract

Systemic lupus erythematosus (SLE) is a chronic, multisystemic, relapsing and remitting autoimmune disease. The interest in identifying biomarkers that correlate with the SLE systemic activity and that can predict a future organ involvement is growing. The present cross-sectional study aimed to assess the levels of interferon alpha (IFN-α) and tumor necrosis factor alpha (TNF-α) in pediatric SLE patients (disease onset ≤16 years), first-degree relatives and healthy unrelated controls and to elucidate its association with the activity disease, laboratory data and treatment. We selected consecutive pediatric SLE patients followed at the Pediatric Rheumatology Unit of UNICAMP between 2009/2010. Clinical, laboratory, disease activity [SLE Disease Activity Index (SLEDAI)], cumulative damage [Systemic Lupus International Collaborating Clinics / American College of Rheumatology Damage Index (SDI)] and current drug exposure were evaluated. Mood disorders were determined through the Depression (BDI) and Anxiety (BAI) Becks Inventory. The measurement of cytokines was performed by ELISA (Enzyme Linked Immuno Sorbent Assay). Serum levels of INF-α and TNF-α were increased in pediatric SLE patients ($p \leq 0.05$) when compared to first-degree relatives and unrelated healthy controls. Serum levels of INF-α and TNF-α were significantly higher in patients with active disease ($p=0.031$; p=0,014, respectively). INF-α ($r=0.33$; $p=0.012$) and TNF-α ($r=0.39$; $p=0.002$) correlated with SLEDAI. INF-α was significantly higher in patients with positive anti-dsDNA ($p=0.011$), patients with cutaneous vasculitis ($p=0.001$), patients with malar rash ($p=0.032$) and patients without medication ($p=0.035$). Serum levels of IFN-α correlated with C3 levels ($r=0.34$; $p=0.032$) and age ($r=-0.17$; $p=0.025$). Serum levels of TNF-α were significantly higher in patients with nephritis ($p=0.009$) and in patients with depression ($p=0.001$). According to our results, IFN-α and TNF-α were associated with

disease activity and could be considered biomarkers to assess disease activity in pediatric SLE patients, however longitudinal studies are needed to determine if increase of these cytokines may predict flares in pediatric SLE patients.

1. Introdução

1.1 Definição

O lúpus eritematoso sistêmico (LES) é uma doença do tecido conjuntivo com manifestações clínicas diversas, caracterizada por períodos de remissão e exacerbação, com participação intensa do sistema imunológico (Dubois, 1964; West, 1994; Ruiz-Irastorza, 2001; Rahman, 2008; O'Neill, 2010).

1.2 Epidemiologia

A taxa de incidência do LES é de 1 a 10 por 100.000 pessoas/ano e a taxa de prevalência em geral, varia de 20 a 70 por 100.000 habitantes (Siegel & Lee, 1973; Petri, 2002). Quanto às diferentes raças, observa-se a freqüência de 1 para cada 250 mulheres negras nos Estados Unidos da América; 22,4 para cada 100.000 asiáticos e 10,3 para cada 100.000 caucasianos (Fessel, 1974; Hopkinson, 1994; Johnson, 1995; Alarcon, 2001; Petri, 2002; O'Neill, 2010). Entretanto, apresenta-se como uma doença rara entre os negros africanos (Molina, 1997; Molokhia, 2001). No Brasil, observa-se uma frequência maior entre caucasóides, principalmente na região sudeste do país (Chahade, 1995).

Apesar de surgir geralmente na segunda e terceira década de vida, o LES pode se manifestar em qualquer idade, predominantemente no sexo feminino (Dubois e Tuffanelli, 1964; Siegel e Lee, 1973; Petri, 2002). Aproximadamente 15% a 20% dos diagnósticos são feitos na infância (Hashimoto, 1987; Pande, 1993; Cervera, 1993; Costallat & Coimbra, 1994; Tucker, 1995; Rood, 1999; Carreño, 1999; Klein-Gitelman, 2002; Mok, 2005; Gómez, 2006; Tucker, 2008; Ramirez Gomez, 2008; Hoffman, 2009; Feng, 2010; Livingston, 2011). Nas crianças, a relação entre sexo feminino e masculino é de 1,4 a 5,8:1; nos adultos varia de 8:1 a 13:1; nos indivíduos de idade mais avançada,

esta relação é de 2:1 (Hashimoto, 1987; Pande, 1993; Cervera, 1993; Costallat & Coimbra, 1994; Tucker, 1995; Press, 1996; Font, 1998; Rood, 1999; Marini, 1999; Carreño, 1999; Huemer, 2001; Klein Gitelman, 2002; Huang, 2004; Mok, 2005; Gómez, 2006; Tucker, 2008; Ramírez Goméz, 2008; Hoffman, 2009 Feng, 2010; Livingston, 2011; Pineles, 2011).

1.3 Critérios classificatórios do LES

Não existem critérios definitivos para o diagnóstico do LES. O Colégio Americano de Reumatologia (ACR) definiu critérios classificatórios de LES, segundo os quais são necessários no mínimo quatro critérios clínicos e/ou laboratoriais entre onze (Tan, 1982), após cuidadosa investigação e exclusão de doenças infecciosas e neoplásicas, entre outras. Estes critérios foram revisados em 1997, e o item "presença de células LE", constante do critério "alterações imunológicas", foi excluído, e o teste falso positivo para sífilis foi substituído pela presença de anticorpos antifosfolípides (Hochberg, 1997) (Tabela 1). Apesar de amplamente utilizados, estes critérios têm suas limitações e novas mudanças estão sendo estudadas (Petri M, 2011).

Tabela 1. Critérios revisados para a classificação de LES (Hochberg, 1997)

Critério	**Observações**
Rash malar	Eritema fixo sobre as eminências malares e/ou pregas naso-labiais
Lesão discóide	Placas eritematosas, elevadas e circulares, com escamação aderente, comprometimento dos pelos e cicatrização com atrofia
Fotossensibilidade	*Rash* cutâneo resultado da exposição à luz solar, observadas por médico
Úlceras orais	Ulceração oral e/ou em nasofaringe, geralmente dolorosa, observadas por médico

Artrite	Não erosiva de 2 ou mais articulações
Serosite	Pleurite Pericardite
Doença renal	Proteinúria maior que 0,5 g/dia Leucocitúria, na ausência de infecção Hematúria dismórfica Cilindros celulares
Envolvimento do sistema nervoso central (SNC)	Convulsão Psicose
Alterações hematológicas	Anemia hemolítica (Bilirrubinemia indireta, LDH elevada, Coombs direto positivo) Leucopenia menor que 4.000/mm^3 Linfopenia menor que 1.500/mm^3 Plaquetopenia menor que 100.000 /mm^3
Alterações imunológicas	Anticorpos Anti-dsDNA Anticorpos Anti-Sm Anticorpos antifosfolípide [anticardiolipina (aCL) IgG/IgM; anticoagulante lúpico (LA)]
Anticorpos antinucleares (ANA)	Título ≥1/80 de ANA por imunofluorescência ou um ensaio equivalente a qualquer ponto no tempo, na ausência de drogas conhecidas por induzirem ANA

1.4 Apresentação clínica no LES adulto

As apresentações clínicas do LES variam desde manifestações mucocutâneas a manifestações do SNC, como convulsões e psicose. Sintomas constitucionais como fadiga, perda de peso e febre são frequentemente observados e tem um impacto significativo na qualidade de vida dos pacientes (Tench, 2000; Rahman, 2008).

O envolvimento cutâneo no LES é muito comum, afetando até 90% dos pacientes. Além do *rash* malar e das lesões discóides, a fotossensibilidade é

frequentemente observada. Alopécia é frequentemente transitória associada à atividade da doença, mas pode ocasionar cicatrizes quando associada a lesões discóides. Úlcera oral recorrente, especialmente no palato mole é também uma característica de doença ativa (Manson, 2006; Rahman, 2008; Renner, 2009; Kuhn, 2011).

Artralgia e mialgia acometem a maioria dos pacientes. Artrite afeta, geralmente as pequenas articulações da mão e não evoluindo para erosões. A clássica "Artropatia de Jaccoud", resulta em deformidade e incapacidade funcional significativa, embora não causada por artrite destrutiva (Manson, 2006; Rahman, 2008; Ball, 2011).

As manifestações renais afetam cerca de 30% dos pacientes com LES (Rahman, 2008; O'Neill, 2010). O desenvolvimento da nefrite lúpica é mais comum nos primeiros anos da doença. A nefrite lúpica é caracterizada por proteinúria (> 0,5 g/24 horas), presença de sedimento urinário (hemácias dismórficas, leucócitos) e ainda achados histológicos. A revisão dos critérios de classificação (Weening, 2004), desenvolvido pela Sociedade Internacional de Nefrologia e da Sociedade de Patologia Renal foi atualizada (Weening, 2004). Como o envolvimento renal é muitas vezes assintomático, o exame de urina regular e monitoramento da pressão arterial tornam-se cruciais (O'Neill, 2010).

As alterações hematológicas incluem anemia, trombocitopenia e leucopenia. Doença hematológica grave pode ocorrer, mas é relativamente rara (Sultan, 2003; Rahman, 2008). A anemia geralmente é normocítica e normocrômica, e surge dependendo da gravidade e duração da doença (Sultan, 2003). Trombocitopenia, definida quando a contagem de plaquetas está inferior a 150.000 células/mL, é um achado frequente no LES. O grau é variável. A trombocitopenia transitória muitas vezes aparece durante uma fase de exacerbação da doença sem causar tendência hemorrágica

(Sultan, 2003; Rahman, 2008). Leucopenia é comum e pode resultar de doença ativa ou devido à reação a drogas (Sultan, 2003; Rahman, 2008).

Pleurite, causando dor no peito, tosse e falta de ar é a manifestação pulmonar mais comum no LES (Paran, 2004). Embora os sintomas possam estar relacionados diretamente à atividade da doença, embolia pulmonar deve ser sempre considerada, principalmente naqueles que têm anticorpos antifosfolípides positivos. As infecções são comuns, e qualquer lesão parenquimatosa deve ser tratada como infecciosa até que se prove o contrário (Paran, 2004; Torre, 2011).

As complicações cardíacas incluem pericardite, doenças valvares, endocardite de Libman-Sacks, miocardite, cardiomiopatia, doenças da artéria coronária e distúrbios da condução (Yeh, 2007). Vinte e cinco porcento dos pacientes com LES apresentam envolvimento cardiovascular em algum momento da doença. As complicações cardiovasculares representam a terceira maior causa de morte nestes pacientes, embora nem todas as doenças cardiovasculares sejam de natureza inflamatória de fato; uma significativa porção é devido à aterosclerose (Urowitz, 1997; Jacobsen, 1998).

As manifestações neuropsiquiátricas (NP) ocorrem em até 75% dos pacientes. No entanto, a frequência dessas manifestações é muito variável, dependendo do tipo de manifestação incluída e do método usado para avaliação (Johnson, 1968; Costallat, 1990; Costallat, 1994; West, 1994; Hanly, 1992; Postal, 2011). Lúpus neuropsiquiátrico (LES NP) é, muitas vezes, de difícil diagnóstico. Em 1999, o ACR elaborou um consenso para a terminologia e definição das síndromes NP que ocorrem no LES (ACR, 1999), com a participação de reumatologistas, neurologistas, psiquiatras, entre outros, e definiu 19 síndromes mais prevalentes (Tabela 2). Estes critérios foram posteriormente validados apresentando uma sensibilidade de 91% e especificidade de 46% (Ainiala, 2001). A baixa especificidade se deu devido à presença de ansiedade, cefaléia,

depressão leve, distúrbio cognitivo leve e polineuropatia não confirmada por eletroneuromiografia (Ainiala, 2001). Quando estas manifestações foram excluídas, observou-se uma especificidade de 93% (Ainiala, 2001).

Sintomas de depressão e ansiedade são comumente relatados em pacientes com LES e é, provavelmente, devido ao déficit físico e ao estresse de viver com uma doença crônica (Seawell, 2004; Postal, 2011). Pacientes com transtornos de depressão e ansiedade, muitas vezes sentem vergonha de assumir publicamente os seus sintomas; alguns métodos de avaliação, como questionários podem ser úteis na identificação desses sintomas nos pacientes (Bachen, 2009).

Tabela 2: Manifestações neuropsiquiátricas segundo o ACR (ACR,1999)

Sistema Nervoso Central	**Sistema Nervoso Periférico**
Meningite asséptica	Síndrome de Guillain-Barré
Estado confusional agudo	Disfunção autonômica
Ansiedade	Neuropatia craniana
Doença cerebrovascular	Mononeuropatia
Disfunção cognitiva	Miastenia grave
Síndrome desmielinizante	Plexopatia
Cefaléia	Polineuropatia
Transtorno de movimento (Coréia)	
Transtorno do humor	
Mielopatia	
Psicose	
Convulsão	

1.5 Apresentação clínica no LES juvenil

O LES juvenil (LESj) (início da doença ≤16 anos) muitas vezes apresenta manifestações clínicas mais agudas e graves quando comparado ao LES de início

adulto. Envolvimento renal (50% a 67%), neurológico (22-95%) e hematológico (77%), além de febre e linfadenopatia são mais frequentes em crianças quando comparado com ao LES de início adulto (Font, 1998; Carreño, 1999; Sibbitt, 2002; Brunner, 2008; Ramírez Gómez, 2008; Hoffman, 2009; Mina, 2010). Pleurite, presença dos anticorpos anti-Sm, anti-Ro/SSA e anti-La/SSB são igualmente frequentes no LES juvenil e de início adulto (Font, 1998; Mina, 2010). Artrite, fotossensibilidade, lesões discóides, por outro lado, são mais frequentemente observadas no LES de início adulto (Font, 1998, Hoffman, 2009; Carreño, 1999). Em relação à atividade da doença, pacientes juvenis têm uma doença significativamente mais ativa, não só no início da doença, mas também ao longo do tempo quando comparado com LES de início adulto (Tucker, 1995; Hersh, 2009).

1.6 Patogênese

Duas características principais dos indivíduos que desenvolvem LES são a produção de auto-anticorpos e o *clearance* prejudicado de corpos apoptóticos. O LES é uma doença multifatorial, incluindo fatores genéticos, ambientais e hormonais. Além disso, alteração nas linhagens de células B e T também contribui para o desenvolvimento da doença (Ruiz-Irastorza, 2001; Rahman, 2008; O'Neill, 2010). De maneira simplificada, os mecanismos envolvidos na patogênese são: susceptibilidade genética, fatores ambientais, produção de auto-antígenos, sistema neuroendócrino, produção de auto-anticorpos e hiperatividade de células B e T. A seguir, cada item será detalhado.

1.6.1 Susceptibilidade genética

A probabilidade de desenvolvimento do LES em gêmeos monozigóticos e dizigóticos é de 24-57% e 2-5%, respectivamente, indicando que a genética tem um

papel importante na patogênese do LES (Rhodes, 2008; Muñoz, 2010). Genes do antígeno leucocitário humano (HLA), particularmente HLA-DRB1e HLA-DQB1 têm sido associados à susceptibilidade ao LES (Reveille 1991; Walport, 1982; Muchinechi, 1998; Gladman, 1999; Wakeland , 2001; Graham, 2002; Smikle, 2002; Farabosco, 2006; Graham, 2007; Fu, 2011). O perfil HLA-DRB1*0301 tem sido associado à susceptibilidade em indivíduos latino-americanos (Fu, 2011). Análises sorológicas específicas mostram que tanto o HLA-DR3 como o HLA-DR2 também são fatores de risco (Fu, 2011). Já o HLA-DR3-DQ2 é um haplótipo que tem forte associação no desenvolvimento do LES em caucasianos (Fu, 2011).

1.6.2 Fatores ambientais

Há evidências de que a exposição à radiação ultravioleta (UV) altera a química do ácido desoxirribonucléico (DNA) e sua localização, bem como a disponibilidade dos antígenos ribonucleiprotéicos (RNP) e Ro (Sontheimer, 1996). Outro fator ambiental envolvido é a exposição a determinados vírus como o Epstein-Bar (EBV) e citomegalovírus (CMV). Após infecção, ocorre um mecanismo chamado de mimetismo molecular entre os antígenos próprios e externos, seguido da ativação inespecífica de linfócitos T e B, resultando na liberação de auto-antígenos mais imunogênicos (Zandman-Goddard, 2008).

1.6.3 Produção de auto-antígenos

O mecanismo de ativação induzida pela morte celular programada (apoptose) é provavelmente uma das principais fontes de auto-antígenos no LES (Levine, 1999; Mevorach, 2010; Rastin, 2011). Uma célula em apoptose desenvolve vesículas de superfície resultantes dos antígenos que se deslocam do núcleo para a membrana celular. Perto da superfície das células, o antígeno pode ativar a resposta imune. Células

em apoptose são encontradas continuamente em indivíduos sadios, mas em pacientes com LES este mecanismo se torna patogênico, devido ao aumento na quantidade e duração de células apoptóticas em circulação (Herrmann, 1998; Gaipl, 2006). Há evidências substanciais de que o *clearance* de células apoptóticas é prejudicado em pacientes com LES (Herrmann, 1998).

1.6.4 Sistema neuroendócrino

Anormalidades na função do hipotálamo e/ou hipófise contribuem para a patogênese do LES. Foi observado que alguns pacientes têm hiperprolactinemia e outros têm níveis inadequados do hormônio antidiurético (Chrousos, 1995; Jara, 2001; Méndez, 2004).

1.6.5 Produção de auto-anticorpos

Os anticorpos ANA são frequentes em pacientes com LES, originalmente descritos em 1957 através de um ensaio de imunofluorescência com o tecido do fígado de roedores como substrato (Ippolito, 2011; Radic, 2011). Mais de 90% dos pacientes com LES têm ANA positivo. Valores de 1/80 ou maiores são aceitos como títulos significativos. Embora sensíveis estes auto-anticorpos não são específicos para o LES (Ippolito, 2011).

Anti-dsDNA é um auto-anticorpo altamente específico para o LES, presente em até 70% dos pacientes, mas em menos de 0,5% dos indivíduos sadios ou pacientes com outras doenças autoimunes (Isenberg, 1985; Ippolito, 2011). Entre os pacientes que têm títulos elevados de anti-dsDNA e doença clinicamente quiescente, 80% têm a doença que se torna clinicamente ativa dentro de 5 anos após a detecção de títulos elevados deste auto-anticorpo (Ng, 2006).

Aproximadamente 70% dos pacientes com lesões subagudas possuem anticorpo anti-Ro (SSA), que pode estar associado a anticorpos anti-La (SSB) (Castellino, 2007). O anti-Ro e o anti-La são imunoglobulinas específicas contra as proteínas do RNA, sendo que o anti-La normalmente coexiste com o anti-Ro, raramente sendo encontrado sozinho. Além disso, a presença de anti-Ro e anti-La, ou ambos durante a gravidez confere um risco de 1 a 2% maior de bloqueio cardíaco fetal (Buyon, 2003).

Os anticorpos anti-receptores N-metil-D-aspartato (NMDA), NR2a e NR2b têm sido observados em pacientes com manifestações NP. Embora anticorpos anti-NR2 tenham sido estudados em pacientes com LES, apenas anticorpos anti-NR2 no líquido cefalorraquidiano (LCR), e não no soro, estão associados com manifestações NP difusas no LES (Arinuma, 2008). Os anticorpos anti-receptores NMDA no LCR acometem o SNC independente de eventos trombóticos ou vasculite (DeGiorgio, 2001). Estes anticorpos no LCR foram associados com manifestações NP em geral (Yoshio, 2006) e manifestações NP difusas (Arinuma, 2008); já no soro, foram descritas associações com distúrbio cognitivo (Omdal, 2005), depressão (Omdal, 2005), défcit de memória recente e de aprendizado (Omdal, 2005).

Proteína P ribossomal é um pentâmero composto por 3 fosfoproteínas diferentes, formando o monômero P0 e os dímeros P1 e P2. Ela desempenha um papel importante em todas as etapas da síntese protéica. A presença de anticorpos anti-P pode estar associada ao comprometimento do SNC (Tzioufas, 2000; Abdel-Nasser, 2008; Hirohata, 2007; Briani, 2009), rins (Hulsey , 1995; Chindalore , 1998; Monova, 2001) e/ou danos no fígado (Koren, 1993; Arnett, 1995; Koscec, 1997).

1.6.6 Hiperatividade de células B e T

Os mecanismos de hiperatividade de células B e T envolvem a produção de auto-antígenos, que está relacionada ao aumento da apoptose e ao *clearance*

prejudicado de corpos apoptóticos fornecidos continuamente pelo dano tecidual. Um único antígeno inicia uma resposta, mas na ausência do mecanismo de tolerância imunológica, a resposta imune torna-se ininterrupta, envolvendo mais células B e T com especificidade relacionada ao antígeno inicial, até que ambas sejam ativadas por antígenos múltiplos, muitos dos quais são antígenos próprios (Chan, 1999; Gaipl, 2006). Outro mecanismo importante sobre hiperatividade é a expressão aumentada de moléculas de superfície que participam da ativação de células em ambas as populações de células (células B e T) (62). Auto-anticorpos podem ativar células T e ajudam na sua diferenciação. O estímulo aumentado na diferenciação e maturação de células T leva a uma produção anormal de citocinas em pacientes com LES (Chan, 1999; Gaipl, 2006).

As citocinas são proteínas de baixo peso molecular, produzidas por diferentes células do sistema imunológico inato e adaptativo (Yap, 2010). Elas mediam a ativação e regulação funcional do sistema imunológico através da ligação aos receptores de superfície celular, desempenhando um papel fundamental na diferenciação, maturação e ativação de várias outras células (Wozniacka, 2006; Yap, 2010).

No LES, o perfil de citocinas pode determinar alguns aspectos disfuncionais do sistema imunológico e o envolvimento de vários órgãos. O LES é uma doença heterogênea quanto à apresentação, gravidade da doença e resposta ao tratamento. Perfis alterados de citocinas podem ser responsáveis por essas variações observadas na prática clínica (Yap, 2010). A seguir, serão descritas duas citocinas envolvidas na patogênese da doença, o interferon alfa (INF-α) e o fator de necrose tumotal alfa (TNF-α).

1.7 Interferon alfa

A ação do interferon (INF) sobre as células do sistema imunológico começou a ser estudada aproximadamente há 40 anos (Banchereau, 2006). Em 1979, níveis anormais de INF foram encontrados no soro de pacientes que sofrem de várias doenças

autoimunes (Hooks, 1979), como artrite reumatóide, síndrome de Sjögren e posteriormente confirmado no LES (Preble, 1982).

O sistema INF tipo I engloba não somente fatores moleculares e celulares envolvidos na produção de INF-I, tais como genes INF-I e suas proteínas, como também células-alvo afetadas pelo INF-I (Koutouzov, 2006; Rönnblom, 2008). A família do gene INF-I é composta por 17 genes; 13 genes codificam INF-α e um gene específico para cada subtipo: IFN-β, IFN-ω, IFN-κ e IFN-ε (Rönnblom, 2008). Estes genes e seus produtos são semelhantes estruturalmente e funcionalmente (Rönnblom, 2008). Genes INF-I são induzidos por células expostas a vírus ou à ácido ribonucléico (RNA) de fita dupla e interagem com o mesmo receptor, o receptor de IFN-α/β (IFNAR) (Doly, 1998; Bogdan, 2000). As diferenças, no entanto, são observadas nos produtos pós-interação com o IFNAR (Doly, 1998; Bogdan, 2000).

1.7.1 INF-α no LES

Os genes codificadores de INF-α estão associados ao desenvolvimento do LES, sugerindo um importante papel na etiologia da doença. Além disso, alteração da expressão desses genes e aumento nos níveis séricos de INF-α são frequentemente encontrados em pacientes com LES (Koutouzov, 2006; Rönnblom, 2008). INF-α está muito associado a determinadas manifestações clínicas no LES, sendo um alvo promissor para intervenções terapêuticas (Yoo, 2010).

A implicação do IFN-α na patogênese do LES inclui fatores genéticos (polimorfismos e superexpressão gênica) e indução a doença por tratamento com INF-α (Kirou, 2010). Em indivíduos sadios, durante a infecção viral, as células dendríticas de secretam IFN-α. Após contenção da infecção, a produção autócrina de IFN-α é bloqueada (Bogdan, 2000). Nos pacientes com LES, a expressão gênica anormal pode impedir o encerramento da produção de IFN-α (Rönnblom, 2008).

Além disso, imuno-complexos contendo RNA ou DNA são capazes de induzir células dendríticas a produzirem IFN-α (39). Em indivíduos geneticamente susceptíveis, células B expressando auto-anticorpos reativos não são removidas (Yurasov, 2005). Isso ocorre devido ao *clearance* prejudicado de corpos apoptóticos. O material nuclear exposto estimula células B auto-reativas levando a secreção de auto-anticorpos e a formação de imuno-complexos. Estes imuno-complexos e corpos apoptóticos, por fim estimulam as células dendríticas a produzirem cada vez mais moléculas de INF-α (Yurasov, 2005; Kirou, 2010).

Uma vez que o limiar de expansão e ativação de células imunes é atingido, a doença se autoperpetua. Esse quadro pode piorar devido a fatores exógenos, que induzem a apoptose local e/ou aumentam a sobrevivência de células B, induzidas pela radiação UV e por infecções que desencadeiam respostas de células Th1 e a produção de INF-α, comumente observados em períodos de atividade da doença (Shlomchik, 2001).

1.7.2 Estudos em modelos animais

O uso de modelos animais fundamenta as pesquisas clínicas. Embora existam vários modelos animais para o LES, nenhum deles parece reproduzir integralmente a doença humana. Os modelos espontâneos mais conhecidos são *New Zealand Black* (NZB), *New Zealand White* (NZW), LMR, BXSB e SWR (Liu, 2006). Estudos realizados em modelos animais nos permitiram entender melhor o papel patogênico do INF-I no LES (Braun, 2003; Santiago-Raber, 2003; Hron, 2003; Mathian, 2005; Schwarting, 2005). Iremos detalhar os estudos com INF-α.

Uma linhagem de camundongos NZB congênicos sem a α cadeia do receptor para IFN-α/β (IFN-α/βR), comum a várias espécies foi criada. Comparados aos controles, camundongos NZB homozigotos sem IFN-α/βR tiveram uma redução

significativa nos títulos de anticorpos anti-eritrócitos e de anti-dsDNA, anemia hemolítica, nefrite e também queda de mortalidade (Santiago-Raber, 2003). Nos camundongos heterozigotos sem IFN-α/βR, essas reduções foram intermediárias. A melhora da doença pode ser observada através da redução da esplenomegalia e de outros subconjuntos de células imunes, incluindo células B-1, que são as principais produtoras de anti-eritrócitos. Diminuição de subconjuntos de células B, na proliferação de células T e na maturação de células dendríticas também foi observada. Estes achados sugerem que IFN-α/β são importantes mediadores na patogênese do LES em modelos animais, e que a redução da sua atividade no homólogo humano pode ser benéfica (Santiago-Raber, 2003).

Ao contrário do estudo acima, que usou modelos de baixa/moderada severidade da doença, um trabalho estudou o papel do IFN-α em camundongos [NZB×NZW (B/W)] F1, um modelo que muito se assemelha ao LES humano (Mathian, 2005). Demonstrou-se que anti-dsDNA apareceu logo após o 10º dia do início do tratamento com IFN-α. Proteinúria e morte causada por glomerulonefrite ocorreu em todos os camundongos tratados por aproximadamente 18 semanas. Todos os camundongos que não receberam o tratamento não apresentaram qualquer sinal de doença. IFN-α *in vivo* induz a uma superexpressão de estimuladores de linfócitos. Todos os efeitos provocados por IFN-α foram dose-dependente. Camundongos NZB × NZW F1 infundidos com IFN-α purificado de modelos animais também mostram aceleração no desenvolvimento do LES. Assim, a expressão prolongada de IFN-α *in vivo* induz a um fenótipo de LES letal em animais susceptíveis (Mathian, 2005).

1.7.3 Estudos em humanos

Estudos suportam o papel pró-inflamatório do INF-α em pacientes com LES (Hooks, 1979; Ytterberg, 1982; Kim, 1987; Baechler, 2003; Dall'era, 2005, Kirou, 2005; Niewold, 2007; Niewold, 2008; Niewold, 2008; Zhang, 2010; Weckerle, 2011).

Polimorfismos genéticos nos genes diretamente envolvidos na via de ativação do IFN-α têm sido associados com a susceptibilidade ao LES, incluindo IRF5 e IRF7 (Boule, 2004; Honda K, 2005). Além disso, estudos também demonstraram níveis alterados de IFN-α em familiares de primeiro grau de pacientes com LES em comparação com indivíduos sadios não aparentados (Niewold, 2008; Niewold, 2008), sugerindo que a alta nos níveis séricos de IFN-α possa ser um fator de risco hereditário para o LES.

A superexpressão de genes indutores de IFN-α em todas as células mononucleares do sangue periférico de pacientes com LES é denominada de "assinatura de IFN" (Obermoser, 2010). Essa "assinatura de IFN" em pacientes adultos com LES foi mencionada em dois estudos independentes (Baechler, 2003; Kirou, 2004). O primeiro estudo, com base no perfil genético das células mononucleares de sangue periférico, não só identificou uma "assinatura de IFN" em 50% dos pacientes com LES, mas também associou sua intensidade com a gravidade da doença (Baechler, 2003). O segundo estudo identificou, por meio da reação em cadeira da polimerase em tempo real, uma expressão coordenada de genes codificadores de IFN-α presentes nas células mononucleares de sangue periférico de pacientes com LES (Kirou, 2004).

Dois estudos demonstraram através da metodologia de *microarray* a superexpressão de genes indutores de IFN-α em células mononucleares do sangue periférico de pacientes com LES quando comparada a de indivíduos sadios (Baechler, 2003; Crow, 2003). Embora essa descoberta suporte o papel no INF-α na patogênese do

LES, ela não nos permite inferir se o IFN-α é uma causa ou um produto secundário da doença (Baechler, 2003; Crow, 2003).

Em relação a estudos clínicos, alguns indivíduos que receberam INF-α recombinante humano para tratamento de infecção viral crônica ou doença maligna desenvolveram LES (Ronnblom, 1990; Niewold, 2005). Os sintomas do LES são transitórios, desaparecendo com a descontinuação do tratamento. A indução ao LES pelo tratamento com IFN-α suporta o papel desta citocina na quebra da tolerância imunológica, e esta quebra em alguns indivíduos susceptíveis resulta num fenótipo específico de LES (Niewold, 2005). Tomados em conjunto, estes dados sugerem fortemente que o IFN-α está envolvida na patogênese do LES.

1.8 Fator de necrose tumoral alfa

O fator de necrose tumoral alfa (TNF-α) é expresso como um trímero na superfície celular e na forma solúvel após a ativação de macrófagos e células dendríticas. O papel do TNF-α na patogênese do LES permanece controverso, pois tem sido descrito seu papel tanto protetor como deletério em diferentes modelos animais (Yap, 2010).

1.8.1 TNF-α no LES

TNF-α é uma citocina pró-inflamatória e está diretamente envolvida no processo de apoptose (Aringer, 2003), e na patogênese de várias doenças reumáticas, como artrite reumatóide (Joseph, 2010). No LES, o papel do TNF-α ainda é controverso (Yap, 2010). Compreender como o sistema imunológico integra as propriedades pleiotrópicas do TNF-α é um desafio, em especial em doenças como o LES. TNF-α exerce funções imuno-regulatórias e pró-inflamatórias em células do sistema imune inato e adaptativo (Aringer, 2003) (Figura 1).

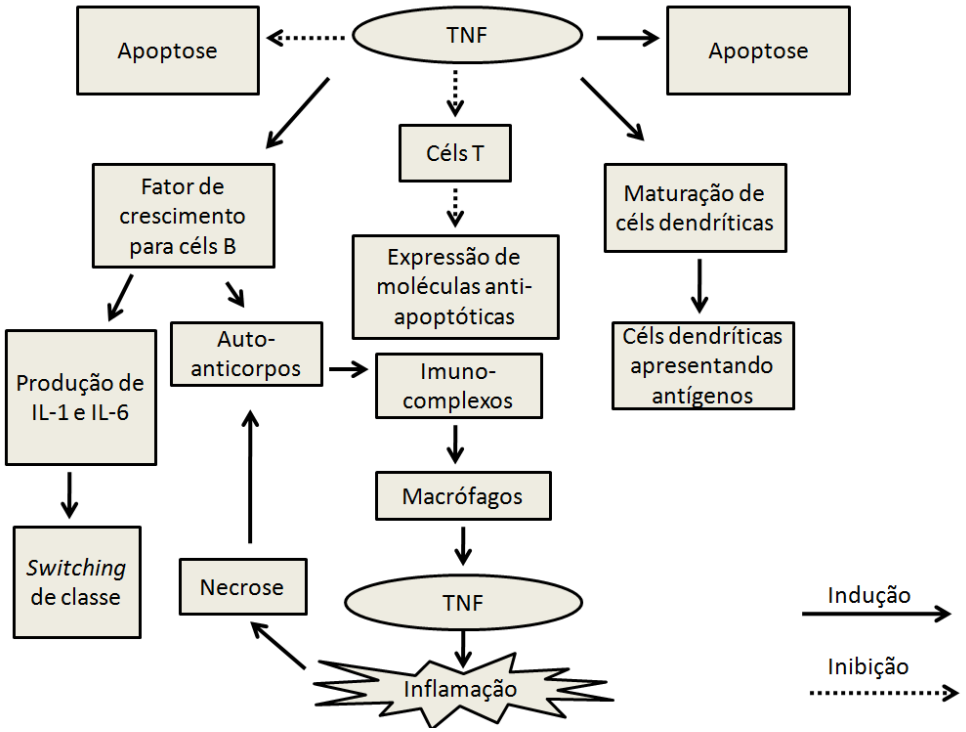

Figura 1: Funções imuno-regulatórias do TNF. TNF atua como um fator de crescimento para as células B, induzindo a produção de interleucina (IL)-1 e IL-6. Isto estimula o *switching* de classe, envolvidos na produção de anticorpos. TNF leva a hiporeatividade de células T e a expressão de moléculas anti-apoptóticas. Além disso, pode promover maturação de células dendríticas, que atuam como células apresentadoras de antígenos. Imuno-complexos são formados por auto-anticorpos e antígenos. Estes imuno-complexos estimulam macrófagos a expressarem TNF, promovendo inflamação. A inflamação pode ser uma fonte de morte celular (necrose), induzindo a formação de novos auto-anticorpos (Postal, 2011).

1.8.2 Estudos em modelos animais

O TNF-α tem sido estudado em diversos modelos animais, como nas espécies *New Zealand Black*, especialmente na espécie *New Zealand White* (NZB/W) (Jacob 1988), *Medical Research Laboratory lymphoproliferation mice* (MRL/lpr) (Yokoyama, 1995), e nos camundongos C3H.SW (Segal, 2001).

Descobertas anteriores mostraram uma produção diminuída de TNF-α nos camundongos NZB/W associada ao desenvolvimento de manifestações graves da doença, como nefrite (Jacob, 1988). Camundongos NZB/W TNF-α competentes mostram pouca atividade de doença (Kontoyiannis, 2000), sugerindo, portanto, que a deficiência de TNF-α é um gatilho importante para desenvolvimento da doença nessa espécie (Kontoyiannis, 2000).

Camundongos NZB/W que receberam doses relativamente elevadas de TNF-α no início da vida mostraram um atraso no início da doença, no entanto, isso não impediu que a doença surgisse (Gordon, 1989; Jacob, 1991). Além disso, ficou comprovado que a aplicação de TNF-α após o início da doença foi prejudicial, e que o tratamento anti-TNF é um benefício terapêutico em ratos propensos ao LES (Brennan, 2001; Segal, 2001). Embora os resultados preliminares demonstrem um efeito protetor do TNF-α contra a autoimunidade, este último leva à hipótese de que o TNF-α estimula a destruição de órgãos já afetados pelo LES (Jacob, 1992; Aringer, 2002).

Outro estudo mostrou efeitos benéficos quando altas doses de TNF-α foram aplicadas, mesmo depois do desenvolvimento de nefrite, no entanto, sem proteção a longo prazo contra a doença (Gordon, 1989). Mesmo nos camundongos NZB/W, em que TNF-α pareceu ser protetor, essa citocina apresentou um papel benéfico e prejudicial concomitantemente (Gordon, 1989).

Em contraste com os achados em camundongos NZB/W, o *tnf* é altamente expresso no soro e no tecido renal de camundongos MRL/*lpr*. Os níveis de TNF-α ainda se correlacionam com o grau de inflamação do órgão (Yokoyama, 1995). Em camundongos MRL/*lpr*, perda da função renal ocorre entre o 3º- 4º mês de idade e progride rapidamente, resultando em morte durante o 5º- 6º mês de vida. A terapia anti-TNF mostrou-se benéfica para a espécie MRL/*lpr* (Yokoyama, 1995).

O bloqueio do TNF-α proporcionou uma melhora na artrite, nefrite, pneumonite e leucopenia em outros modelos animais, tais como nos camundongos C3H.SW (Segal, 2001). LES experimental foi induzido em camundongos C3H.SW *naive* por injeção de anti-dsDNA monoclonal humano (mAb). Duas semanas após injeções de reforço, o tratamento com mAb anti-TNF-α ou pentoxifilina (PTX) foi iniciado, por um período de 6 semanas. A produção de TNF-α foi determinada 3 e 7 meses após a indução da doença, e os animais experimentais foram também acompanhados clinicamente. Ambos os protocolos de tratamento reduziram a produção de duas citocinas pró-inflamatórias e o título de anticorpos anti-dsDNA foi significativamente menor nos animais tratados com qualquer um dos dois protocolos. A deleção do TNF-α nas fases iniciais do LES experimental por mAb anti-TNF-α ou PTX melhorou o estado clínico dos animais (Segal, 2001).

1.8.3 Estudos em humanos

Há evidências substanciais que demonstram o papel pró-inflamatório do TNF-α em pacientes com LES com base em estudos que analisaram polimorfismos do gene TNF-α, expressão gênica, e níveis séricos de TNF-α.

Do ponto de vista genético, uma série de estudos sugere que polimorfismos do gene TNF-α estão associados a susceptibilidade ao LES. A maioria dos estudos foi realizada utilizando polimorfismos de microssatélites e polimorfismos de único nucleotídeo (SNP) localizados na região promotora nas posições -308 (Fugger, 1989; Tomita, 1993; Wilson, 1993; Danis, 1995; Rudwaleit, 1996; Sullivan, 1997; Wang, 1999; Rood, 200; van der Linden, 2001; Zuniga, 2001; May, 2002; Azizah, 2004; Parks, 2004; Correa, 2005; Suarez, 2005; McHugh, 2006; Guarnizo-Zuccardi, 2007; Jiménez-Morales, 2009; Lin, 2009; Santos, 2011) e 238 (D'Alfonso, 1996; Rudwaleit, 1996; Chen, 1997; Parks, 2004; Correa, 2005; McHugh, 2006; Hirankarn, 2007).

Alguns estudos genéticos observaram uma associação entre determinados polimorfismos e manifestações clínicas (Hajeer, 1997, Azizah, 2004; Hirankarn, 2007). *Rash* malar, lesão discóide, úlceras orais, serosite e distúrbios hematológicos foram associados ao polimorfismo -308A/G em pacientes com LES de Taiwan (Lin, 2009). Fenômeno de Raynaud tem sido associado ao polimorfismo -863A em pacientes tailandeses (Hirankarn, 2007).

Muitos estudos têm descrito uma expressão gênica anormal de TNF-α em células mononucleares do sangue periférico e em células da medula óssea de pacientes com LES (Alvarado-de la Barrera, 1998; Pitidhammabhorn, 2006; Zhu, 2007; Lee, 2009; Wozniacka, 2008). Todos estes estudos foram baseados em pequenos grupos e analisaram a expressão do gene TNF-α em relação à atividade da doença (Alvarado-de la Barrera, 1998; Pitidhammabhorn, 2006; Zhu, 2007; Lee, 2009; Wozniacka, 2008).

Vários estudos têm analisado os níveis séricos de TNF-α em pacientes com LES (Studnicka-Bencke, 1996; Gabay, 1997; Jones, 1999; Gómez, 2004; Mahmoud, 2005; Pitidhammabhorn, 2006; Sabry, 2006; Al-Mutairi, 2007; Wozniacka, 2008). Esses níveis são notoriamente mais elevados em pacientes com LES adulto quando comparados a indivíduos sadios não aparentados (Studnicka-Bencke, 1996; Gabay, 1997; Jones, 1999; Gómez, 2004; Mahmoud, 2005; Pitidhammabhorn, 2006; Sabry, 2006; Al-Mutairi, 2007; Wozniacka, 2008). Estudos determinaram uma associação entre o aumento desses níveis séricos com atividade da doença (Studnicka-Bencke, 1996; Sabry, 2006; Gabay, 1997; Mahmoud, 2005). No entanto, em um estudo prévio, os níveis de TNF-α foram maiores em pacientes com doença inativa em comparação com pacientes com doença ativa e controles, sugerindo que, TNF-α também poderia ser um fator protetor em pacientes com LES (Gómez, 2004).

2. Objetivos

2.1 Objetivo geral

2.1.1 Determinar os níveis e a importância clínica do INF-α e do TNF-α em pacientes com LESj.

2.2 Objetivos específicos

2.2.1 Determinar os níveis de INF-α em:

- pacientes com LESj
- familiares de primeiro grau (sem histórico pessoal de doença autoimune)
- indivíduos sadios não aparentados, sem histórico de doença autoimune pessoal e na família

2.2.2 Correlacionar os níveis de INF-α com:

- Manifestações clínicas
- Manifestações laboratoriais

2.2.3 Dosar os níveis de TNF-α em:

- pacientes com LESj
- familiares de primeiro grau (sem histórico pessoal de doença autoimune)
- indivíduos sadios não aparentados, sem histórico de doença autoimune pessoal e na família

2.2.4 Correlacionar os níveis de TNF-α com:

- Manifestações clínicas
- Manifestações laboratoriais

3. Pacientes e Métodos

3.1 Tipo do estudo

Trata-se de um estudo transversal, com grupo controle.

3.2 Seleção dos pacientes

Foram selecionados 60 pacientes consecutivos com LESj, acompanhados no ambulatório de Reumatologia e de Reumatologia Pediátrica da Faculdade de Ciências Médicas da UNICAMP cujas manifestações clínicas e laboratoriais foram rotineiramente estudadas de acordo com protocolo já estabelecido.

3.2.1 Critérios de inclusão

1. Foram incluídos pacientes com diagnóstico de LES segundo os critérios estabelecidos pelo ACR (Tan, 1997) e acompanhados rotineiramente nos ambulatórios de Reumatologia da UNICAMP
2. Pacientes com idade de início da doença ≤ 16 anos
3. Pacientes que tinham, no mínimo, 6 meses de acompanhamento nos ambulatórios de Reumatologia da UNICAMP

3.2.2 Critérios de exclusão

1. Pacientes que não concordaram em participar da pesquisa

3.3 Seleção dos familiares de primeiro grau

Este grupo foi constituído por familiares de primeiro grau dos pacientes com LESj. Os familiares de primeiro grau, acompanhantes dos pacientes com LES na visita clínica, foram convidados a participar do estudo após a consulta clínica e a coleta de sangue foi realizada no mesmo dia, não necessitando outras visitas ao HC/UNICAMP.

Foram incluídos indivíduos sadios, sem infecção na data da coleta de sangue e sem histórico pessoal de doença autoimune.

3.4 Seleção dos indivíduos sadios não aparentados

O grupo controle foi constituído por indivíduos sadios com idade e distribuição de gênero semelhante ao grupo de pacientes com LES, que não apresentaram infecções na data da coleta de sangue e que concordaram em participar do projeto de pesquisa. Os indivíduos sadios pertenciam à mesma região geográfica (Campinas e região), sendo estes amigos de pacientes e pesquisadores e profissionais do hospital. Foram excluídos indivíduos com doenças autoimunes e antecedentes familiares de doença autoimune.

3.5 Termo de consentimento livre e esclarecido

Todos os pacientes e voluntários foram previamente informados e assinaram o termo de consentimento livre e esclarecido (TCLE), aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas (FCM) - UNICAMP (nº617/2009).

3.6 Análise clínica-laboratorial

Manifestações pregressas foram analisadas através da revisão do prontuário médico. Foram analisadas as seguintes manifestações clínicas, laboratoriais e de tratamento: presença de adinamia; emagrecimento (> 4 kg); febre (≥37,8° C); artrite (não erosiva em duas ou mais articulações periféricas, vista pelo médico); necrose asséptica (documentada em pacientes sintomáticos por radiografia simples, cintilografia ou ressonância magnética); deformidades articulares (geralmente redutíveis vistas pelo médico); eritema malar (eritema fixo sobre as eminências malares e/ou pregas naso-labiais); lesões discóides (Placas eritematosas, elevadas e circulares, com a presença de escamas queratóides aderidas); alopécia; úlcera oral e/ou nasal (ulceração oral e/ou em nasofaringe, geralmente dolorosa, observadas por médico); fotossensibilidade (*rash*

cutâneo resultado da exposição à luz solar, relatado na história clínica ou observada por médico); nefrite (definida pela presença de proteinúria maior que 0,5 g/l em 24 horas, aumento progressivo de creatinina sérica ou ainda alterações histopatológicas quando compatíveis com nefrite lúpica, segundo critérios da Organização Mundial de Saúde); hipertensão arterial (níveis pressóricos maiores que recomendados para a idade); síndrome nefrótica (proteinúria maior que 3 g/l em 24 horas); serosite (presença de pleurite, pericardite ou ambas documentada no exame clínico e por imagem); outras manifestações pulmonares como hipertensão pulmonar, pneumonite e hemorragia pulmonar; outras manifestações cardíacas como miocardite, endocardite própria do LES e infarto do miocárdio; miopatia (revelada por fraqueza muscular, alterações enzimáticas, alterações da biópsia muscular e /ou da eletromiografia).

Outros fatores avaliados foram: envolvimento intestinal, hepático, e do sistema retículo-endotelial, presença de tromboembolismo pulmonar e alterações oculares e a presença do fenômeno de Raynaud.

Os seguintes exames, solicitados rotineiramente no diagnóstico e monitoramento do LES foram realizados de acordo com as técnicas utilizadas no Laboratório de Patologia Clinica e no Laboratório de Investigação em Alergia e Imunologia/UNICAMP. Foram considerados: leucopenia (<4000 células/mm^3); linfopenia (<1500 células/mm^3); anemia hemolítica (Bilirrubinemia indireta, LDH elevada, Coombs direto positivo); trombocitopenia (< 100000 células/mm^3); FAN (por imunofluorescência indireta, positivo em títulos maiores que 1/80); anticorpo anti-dsDNA (por imunofluorescência indireta com *Crithidia luciliae* como substrato) (Harris, 1987); anticorpo anti-cardiolipina (por método imunoenzimático); anticoagulante lúpico (por TTPA e Russel) (Brandt, 1995). Anticorpos contra antígenos extraídos do núcleo (ENA), incluindo Ro (SSA), La (SSB) e Sm foram detectados por

um método padronizado de ELISA (ORG 506 ENAscreen- ORGENTEC Diagnostika GmbH). Toda investigação clínica foi realizada por um reumatologista capacitado.

3.7 Análise da atividade de doença e dano

A atividade da doença foi avaliada pelo *Systemic Lupus Erythematosus Disease Activity Index* (SLEDAI) e doença foi considerada ativa quando a somatória de pontos do SLEDAI foi igual e/ou superior a três pontos (Bombardier, 1992; Yee, 2011). O dano cumulativo foi avaliado através de um questionário especificamente desenvolvido para este fim, o *Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index* (SLICC/ACR-DI) (Gladman, 1997). O SDI consiste em 38 itens e a pontuação pode variar de 0 a 47 pontos. Foi considerada a presença de dano se a pontuação foi igual e/ou superior a 1.

3.8 Avaliação dos transtornos de humor

Todos os indivíduos completaram os Inventários de Ansiedade (BAI) (Beck, 1988; Cunha, 2001) e Depressão de Beck (BDI) (Beck, 1961; Cunha, 2001). Para pacientes com menos de 16 anos foi aplicado o Inventário de Depressão Infantil (CDI) (Kovacs, 1985; Cunha, 2001). Essas escalas consistem em 21 itens, cada um descrevendo um sintoma comum a ansiedade/ depressão. O entrevistado foi convidado a avaliar o quanto ele ou ela foi incomodado por cada sintoma durante o mês passado em uma escala de 4 pontos variando de 0 a 3. Os itens são somados para obter uma pontuação total que pode variar de 0 a 63. Os valores de corte utilizados para o BDI são: 0-13: sem depressão/mínimo; 14-19: depressão leve; 20-28: depressão moderada e 29-63: depressão severa e para o BAI: 0-7: não/nível mínimo de ansiedade; 15/08: ansiedade leve; 16-25: ansiedade moderada; 26-63: ansiedade severa. No caso do CDI, o valor de corte é 17. Acima deste valor é considerado depressão.

3.9 Tratamento

Foram consideradas as medicações prescritas na data da coleta da amostra sanguínea do paciente. As medicações consideradas foram prednisona, hidroxicloroquina e outras drogas imunossupressivas (azatioprina, ciclofosfamida, ciclosporina, metotrexato e micofenolato mofetil).

3.10 Investigação laboratorial

Foi coletado um total de 8 mL de sangue venoso de cada paciente, no centro de coleta, em conjunto com os exames de rotina solicitados regularmente para avaliação de atividade de doença. A coleta de sangue dos familiares/controles foi realizada em no mesmo dia da coleta do paciente.

As amostras de sangue, após coagulação em temperatura ambiente de 30 minutos, foram centrifugadas, aliquotadas e conservadas a –80°C, para posterior análise no Laboratório de Reumatologia FCM/UNICAMP.

Foram dosados os níveis de INF-α e TNF-α pelo método de *Enzyme Linked Immuno Sorbent Assay* (ELISA), conforme previamente apresentado na literatura e descritos a seguir.

3.10.1 Técnica de ELISA

A amostra utilizada foi soro, a partir de sangue total, colhido em tudo seco com gel. Esperamos a amostra de sangue total coagular, a temperatura ambiente por 30 minutos. O tubo com a amostra de sangue foi centrifugado a 4000 rpm por 10 minutos.

PREPARO DE REAGENTES:

1. Solução de lavagem: 100 mL do concentrado foram diluídos em água destilada e/ou deionizada para obtenção de um volume final de 1000 mL.

2. Substrato: O substrato liofilizado foi reconstituido com 6,0 mL de diluente de substrato, 10 minutos antes do uso.

3. O amplificador liofilizado foi reconstituido com de 6,0 mL de diluente do amplificador, 10 minutos antes do uso.

4. Padrão: A solução padrão foi reconstituída com o diluente Calibrador, segundo as especificações impressas no rótulo do frasco do padrão para produzir uma solução estoque.

    a. Tubos de polipropileno foram utilizados para montagem da curva de calibração. 500 µL de Calibrador Diluente foram pipetados em cada tubo. A solução estpque foi utilizada para produzir uma série de diluição. Cada tubo foi homogeneizado cuidadosamente antes da próxima transferência. O padrão diluído serviu de padrão elevado. O Calibrador Diluente serviu como padrão zero (0 pg/mL).

PROCEDIMENTO:

1. Todos os reagentes, amostras e padrões de trabalho foram preparados previamente, conforme indicado nas seções anteriores.

2. 50 µL de Diluente de amostra foram adicionados em todos os poços.

3. 200 µL de solução padrão, amostra ou controle foram adicionados em seus respectivos poços. A microplaca foi coberta com a fita adesiva fornecida pelo kit. A microplaca foi incubada por 3 horas à temperatura ambiente.

4. Lavagem

    a. O líquido dos poços foi removido por aspiração ou por inversão da microplacaplaca, descartando o conteúdo.

    b. O excesso de líquido foi retirado, segurando firmemente a microplaca, pressionando-a em toalha de papel limpa, por 5 vezes.

c. Foram colocados 400 µL de tampão de lavagem em cada poço da microplaca, utilizando uma pipeta multicanal automática.

d. O líquido dos poços foi removido por aspiração ou por inversão da microplacaplaca, descartando o conteúdo.

e. Os passos b, c, d foram repetidos por 5X para um total de seis lavagens.

5. Foram adicionados 200 µL de Conjugado em cada poço. A microplaca foi coberta com a fita adesiva fornecida pelo kit. A microplaca foi incubada por 2 horas à temperatura ambiente.
6. A lavagem (passo 5) foi realizada novamente.
7. Foram adicionados 50 µL de Sustrato em cada poço. A microplaca foi coberta com a fita adesiva fornecida pelo kit. A microplaca foi incubada por 1 hora à temperatura ambiente. Após incubação a placa não foi lavada.
8. Foram adicionados 50 µL de Amplificador em cada poço. A microplaca foi coberta com a fita adesiva fornecida pelo kit. A microplaca foi incubada por 30 minutos à temperatura ambiente. A adição do Amplificador iniciou o desenvolvimento da cor da solução. Após incubação a placa não foi lavada.
9. Foram adicionados 50 µL de solução de parada em cada poço. A adição da solução de parada não afetou a cor dos poços.
10. A densidade óptica de cada poço foi determinada por uma leitora de microplacas ajustada para 490 nm, no prazo de 30 minutos depois de colocada solução de parada.

3.10.2 Obtenção de resultados

Após obtenção da média das duplicatas (absorbância), foi subtraída a média do padrão zero. A curva padrão foi desenhada a partir da densidade óptica (absorbância) e

as concentrações dos padrões já conhecidos. Os dados puderam ser linearizados por log/log.

Para determinar a concentração de cada citocina de cada amostra, primeiro, encontrou-se o valor da absorbância no eixo-y e estendeu-se uma linha horizontal para a curva padrão. No ponto de intersecção, estendeu-se uma linha vertical para o eixo-x e leu-se a concentração correspondente a citocina.

3.11 Análise estatística

Para a determinação dos resultados foi utilizado o teste de normalidade de Shapiro-Wilk. Para variáveis de distribuição normal foi utilizado o teste T. Para as variáveis não-normais foram utilizados o teste de Kruskal-Wallis para comparar os níveis das citocinas entre os 3 grupos, o teste de Mann–Whitney para comparar as variáveis categóricas e as correlações de Perason e Spearman para correlacionar as variáveis contínuas [níveis séricos das citocinas e SLEDAI, SLICC/ACR (DI), BDI e BAI]. Para todas as análises, $p<0,05$ foi considerado estatisticamente significativo.

4. Resultados

4.1 Capítulo 1 Associações clínicas e sorológicas associadas ao INF-α em pacientes com LESj

4.1.1 Dados demográficos

Dos 60 pacientes selecionados, incluímos 57 (54 mulhers) pacientes com LESj com idade média de 17,33 anos [desvio padrão (DP)±4,50 anos; intervalo 9-37]. Três amostras não apresentaram quantidade suficiente de soro para a dosagem do INF-α, por isso a redução de 3 pacientes no total. O tempo de doença foi de 4,71 anos (DP±4,57; intervalo 0-26 anos). Sessenta e quatro familiares de primeiro grau (59 mulheres) com idade média de 39,95 anos (DP±5,66; intervalo 28-52) foram incluídos. O grupo controle foi constituído por 57 voluntários sadios (52 mulheres) com idade média de 19,30 (DP±4,97 anos; intervalo 6-30 anos) (Tabela 3). Pacientes e indivíduos sadios não aparentados foram estatisticamente pareados por idade e sexo. Familiares de primeiro grau eram significativamente mais velhos conforme esperado ($p<0,05$).

Tabela 3: Características demográficas e clínicas dos indivíduos incluídos no estudo com INF-α

Parâmetro	**Pacientes LESj N=57**	**Familiares de primeiro grau N=64**	**Indivíduos sadios não aparentados N=57**
Sexo Feminino	54 (94,7%)	59 (92,18%)	52 (91,22%)
Idade (anos)	17,33±4,50 (intervalo 9-37)	39,95±5,66* (intervalo 28-52)	19,30±4,97 (intervalo 6-30)
Tempo de doença (anos)	4,71 ±4,57 (intervalo 0-26)	-----	-----

SLEDAI Doença Ativa N=30 Doença Inativa N=27	4,43±4,94 8,37±3,80 0,39±0,80	-----	-----
SLICC/ACR-DI	0,50±0,82	-----	-----
PCR (mg/dL)	0,44±0,72	-----	-----
VHS (mm/h)	29,69±31,28	-----	-----
INF-α (pg/mL)	13,84±8,46 *	10,36±6,04	11,68±6,66

*p≤0,05

4.1.2 Características clínicas, laboratoriais e tratamento

No momento de inclusão no estudo, 30 (52,6%) pacientes com LESj aprsentavam doença ativa (SLEDAI ≥3), com pontuação média de SLEDAI de 8,37 (DP±3,80; intervalo 3-18). Pacientes com doença inativa [N= 27 (47,4%)] apresentavam uma média de pontuação de SLEDAI de 0,39 (DP±0,80; intervalo 0-2). A média de proteína C reativa (PCR) dos pacientes na data da coleta foi de 0,44 (DP±0,72) e da velocidade de hemossedimentação (VHS) foi de 29,69 (DP±31,28). Nenhuma correlação entre PCR e/ou VHS e níveis de INF-α ou SLEDAI foi observada. Nefrite (29,8%), *rash* malar (7%), alopécia (5,2%), vasculite cutânea (5,2%) e serosite (3,5%) foram as manifestações clínicas observadas.

Depressão foi identificada em 8 (14,0%) pacientes e em nenhum controle sadio ou familiar de primeiro grau. Depressão leve foi identificada em 5 (8,7%) pacientes e 3 (5,2%) pacientes apresentavam depressão moderada/severa. Ansiedade foi observada em 22 (38,5%) pacientes com LESj. Treze (22,8%) pacientes apresentavam ansiedade leve e 9 (15,7%) apresentaram ansiedade moderada/severa.

Na data da coleta de sangue, 8 (13,3%) pacientes estavam sem qualquer medicação. Trinta e nove (68,4%) pacientes estavam em uso de prednisona, 32 (53,3%) hidroxicloroquina e 22 (36,6%) pacientes estavam em uso de outras drogas imunossupressoras (Tabela 4).

Tabela 4: Medicação em uso pelos pacientes na data da coleta de sangue para dosagem do INF-α

Tratamento	**Pacientes N=57**
Sem medicação	8 (14%)
Prednisona	39 (68,4%)
Hidroxicloroquina	32 (56,1%)
Imunossupressor	22 (38,6%)
Azatioprina	11 (19,3%)
Ciclofosfamida	2 (3,5%)
Ciclosporina	4 (7%)
Metotrexato	1 (1,7%)
Micofenolato mofetil	4 (7%)

4.1.3 Dosagem dos níveis séricos de INF-α

O nível sérico médio de INF-α foi 13,84±8,46 pg/mL nos pacientes com LESj em comparação com 10,36±6,04 pg/mL (p=0,012) em familiares de primeiro grau e 11,68±6,66 pg/ml nos indivíduos sadios não aparentados (p=0,043). Nenhuma diferença significativa entre familiares de primeiro grau e indivíduos sadios não aparentados foi observada (p=0,484) (Gráfico 1).

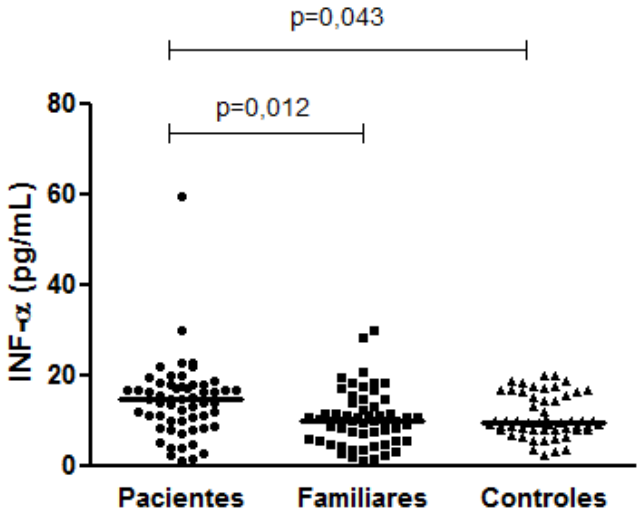

Gráfico 1: Níveis de INF-α nos três grupos avaliados

Níveis séricos de INF-α foram significativamente maiores em pacientes com anti-dsDNA positivo (p=0,011),em pacientes com vasculite cutânea (p=0,001) em pacientes com *rash* malar (p=0,032).

Em pacientes com LESj em atividade (SLEDAI≥3), os níveis de INF-α foram significativamente mais elevados (p=0,031). Níveis de INF-α estavam significativamente mais elevados em pacientes sem medicação (média=13,01; DP±6,09) quando comparados aos pacientes em uso de medicação (média=21,59; DP±16,02; p=0,035). Ao analisar cada um dos medicamentos individualmente, níveis mais elevados de INF-α foram observados em pacientes que não estavam em uso de prednisona (média=20,07; DP±14,65) quando comparados aos pacientes em uso de prednisona (média=12,95, DP±6,19, p=0,042). Níveis de INF-α correlacionaram-se diretamente com os níveis de C3 (r=0,34; p=0,032) e com o SLEDAI (r=0,43; p=0,012) e, indiretamente, com a idade dos pacientes (r= -0,17; p=0,025).

Nenhuma associação entre os níveis de INF-α e outra variável clínica, laboratorial (hematológica ou imunológica) e com SLICC/ACR (DI) foi observada. Não

houve diferença estatística nos níveis de INF-α entre pacientes com e sem imunossupressores, hidroxicloroquina ou outros.

4.2 Capítulo 2 Associações clínicas e sorológicas associadas ao TNF-α em pacientes com LESj

4.1.1 Dados demográficos

Foram incluídos 60 (57 mulheres) pacientes consecutivos com LESj com idade média de 17,85 anos (DP±3,91 anos; intervalo 9-37). O tempo de doença foi de 5,38 anos (DP±4,25; intervalo 0-26 anos). Foram inclcuídos 64 familiares de primeiro grau (59 mulheres) com idade média de 39,95 anos (DP±5,66; intervalo 28-52) e 57 (52 mulheres) indivíduos sadios não aparentados com idade média de 19,30 (DP±4,97 anos; intervalo 6-30 anos). Pacientes e indivíduos sadios não aparentados foram estatisticamente pareados por idade e sexo (Tabela 5). Familiares de primeiro grau eram significativamente mais velhos conforme esperado (p<0,05).

Tabela 5: Características demográficas e clínicas dos indivíduos incluídos no estudo com TNF-α

Parâmetro	**Pacientes LESj N=60**	**Familiares de primeiro grau N=64**	**Indivíduos sadios não aparentados N=57**
Sexo Feminino	57 (95%)	59 (92,18%)	52 (91,22%)
Idade (anos)	17,85±3,89 (intervalo 9-37)	39,95±5,66* (intervalo 28-52)	19,30±4,97 (intervalo 6-30)
Tempo de doença (anos)	5,38±4,25 (intervalo 0-26 anos)	-----	-----
SLEDAI Doença Ativa N=30 Doença Inativa N=30	4,28±4,88 8,24±4,09 0,55±0,89	-----	-----
SLICC/ACR-DI	0,48±0,81	-----	-----

PCR (mg/dL)	0,48±0,75	-----	-----
VHS (mm/h)	36,29±46,74	-----	-----
TNF-α (pg/mL)	4,47 ± 8,95 *	2,33 ± 2,38	1,83±1,82

*p≤0,05

4.2.2 Características clínicas, laboratoriais e tratamento

No momento de inclusão no estudo, 30 (50%) pacientes com LESj apresentavam doença ativa (SLEDAI≥3), com pontuação de SLEDAI de 8,24 (DP±4,09; intervalo 3-18). Os 30 pacientes (50%) inativos apresentaram pontuação média de SLEDAI de 0,55 (DP±0,89; intervalo 0-2). A média de PCR dos pacientes na data da coleta foi de 0,48 (DP±0,75) e de VHS foi de 36,29 (DP±46,74). Nenhuma correlação entre PCR e/ou VHS e níveis de INF-α ou SLEDAI foi observada. Nefrite ativa (33,3%), *rash* malar novo (6,6%), alopécia (5,0%), vasculite cutânea (5,0%) e serosite (3,3%) foram as manifestações clínicas observadas.

Na data de coleta de sangue, 8 (13,3%) pacientes não estavam em uso de nenhuma medicação imunossupressora. Quarenta e dois (70%) pacientes estavam em uso de prednisona, 32 (53,3%) de hidroxicloroquina e 29 (48,3%) pacientes estavam em uso de outras drogas imunossupressoras (Tabela 6).

Tabela 6: Medicação em uso pelos pacientes na data da coleta de sangue para dosagem do TNF-α

Tratamento	**Pacientes N=60**
Sem medicação	8 (13,3%)
Prednisona	42 (70%)
Hidroxicloroquina	32 (53,3%)
Imunossupressor	29 (48,3%)

Azatioprina	15 (25%)
Ciclofosfamida	2 (3,3%)
Ciclosporina	5 (8,3%)
Metotrexato	1 (1,6%)
Micofenolato mofetil	6 (10%)

Depressão foi identificada em 10 (16,7%) pacientes e em nenhum controle saudável ou familiar de primeiro grau. Depressão leve foi identificada em 5 (8,3%) pacientes e 5 (8,3%) pacientes apresentavam depressão moderada/severa. Ansiedade foi observada em 21 (35%) pacientes com LESj. Doze (20%) pacientes apresentavam ansiedade leve e 9 (15%) ansiedade moderada/severa.

4.2.3 Dosagem dos níveis séricos de TNF-α

Níveis de TNF-α foram significativamente maiores em pacientes com LESj (p=0,037) quando comparados a familiares de primeiro grau e indivíduos sadios não aparentados (p=0,004) (Tabela 5). Não houve diferença significativa nos níveis de TNF-α entre os familiares de primeiro grau e indivíduos sadios não aparentados (p=0,711) (Gráfico2).

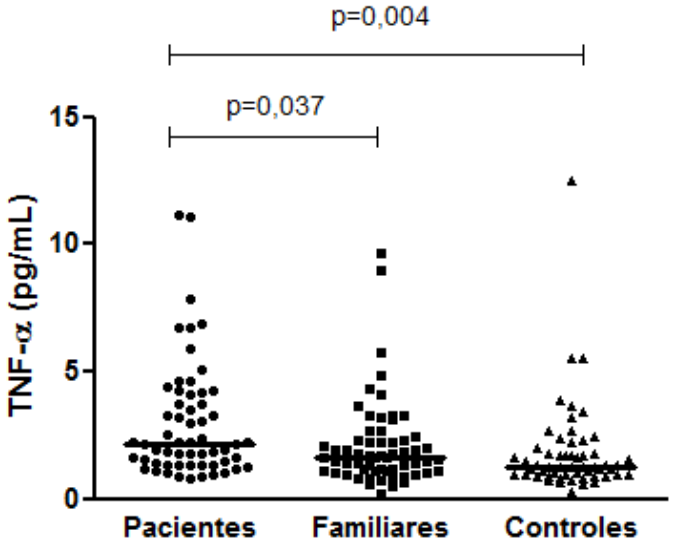

Gráfico 2: Níveis de TNF-α nos três grupos avaliados

Níveis de TNF-α (p=0,014) foram significativamente maiores em pacientes com doença ativa (SLEDAI≥3), quando comparados a pacientes com doença inativa. Além disso, níveis de TNF-α correlacionaram-se diretamente com pontuações de SLEDAI (r=0,39, p=0,002).

TNF-α (p=0,009) foi significativamente maior em pacientes com nefrite ativa quando comparados a pacientes sem nefrite (Gráfico 3).

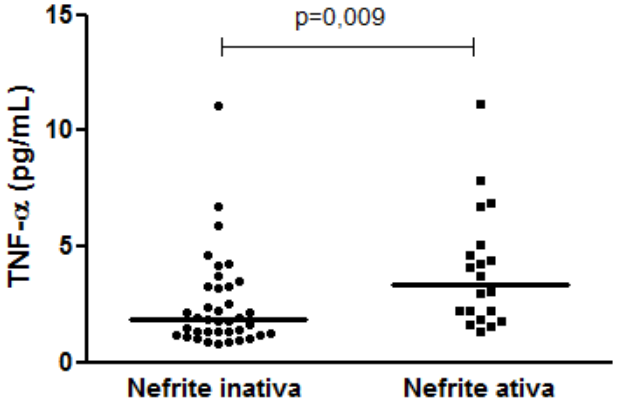

Gráfico 3: Associação dos níveis de TNF-α e a presença de nefrite

TNF-α também foi significativamente maior em pacientes com depressão (p=0,01) quando comparados aos pacientes sem depressão (Gráfico 4).

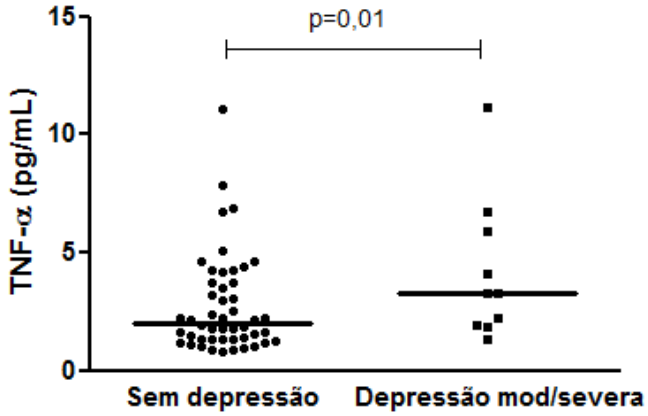

Gráfico 4: Associação dos níveis de TNF-α e depressão

Não houve associação entre os níveis de TNF-α e outras variáveis clínicas, laboratoriais e SLICC/ACR (DI). Além disso, nenhuma diferença nos níveis de TNF-α entre pacientes com e sem medicação foi observada.

5.Discussão

As citocinas são proteínas de baixo peso que desempenham um papel fundamental no desequilíbrio imunológico observado nas doenças autoimunes. O aumento dos níveis de citocinas pró-inflamatórias tem um papel importante na patogênese do LES (Yap, 2010). Níveis aumentados de citocinas em pacientes com LES podem promover exacerbação da resposta inflamatória, da apoptose e na produção de auto-anticorpos que além de iniciar , podem também manter a atividade da doença ao longo do tempo (Yap, 2010).

5.1 INF-α

A primeira anormalidade no perfil de citocinas observada no LES foi o aumento do nível sérico de INF-α, uma citocina com funções antivirais e imunoregulatórias

(Hooks, 1979). A contribuição do IFN-α para o LES pode ser explicada com base em mecanismos distintos, no entanto, relacionados. Em indivíduos geneticamente susceptíveis, precursores de células B expressando auto-anticorpos reativos não são removidos (Rönnblom, 2003; Golding 2010). Isto, provavelmente, devido à quebra de tolerância imunológica, promovendo o aumento de células apoptóticas; o material nuclear estimula células B auto-reativas levando a secreção de anticorpos e formação de imuno-complexos (Rönnblom, 2003; Zhang, 2010). Esses imuno-complexos e corpos apoptóticos estimulam as células dendríticas plasmocitóides a produzirem INF-α. (Rönnblom, 2003; Zhang, 2010).

Níveis séricos aumentados de INF-α os são frequentemente encontrados em pacientes com LES (Hooks, 1979; Ytterberg,1982; Kim, 1987). Outros estudos, através da análise da expressão gênica, demonstraram um aumento na atividade de INF-α. Um aumento na atividade de INF-α, ou seja, maior ativação dos genes induzidos por INF-α resulta em um aumento sérico desta citocina (Baechler, 2003; Dall'era, 2005; Kirou, 2005; Niewold, 2007; Niewold, 2008; Niewold, 2008). Em nosso estudo observamos aumento do nível sérico de INF-α em pacientes com LESj quando comparados a familiares de primeiro grau e aos indivíduos sadios não aparentados. Nossos dados suportam os resultados de estudos anteriores que demonstraram níveis mais altos de INF-α no soro de pacientes com LES adulto (Hooks, 1979; Ytterberg, 1982; Kim, 1987).

A via de ativação do IFN-α no LES está intimamente envolvida na patogênese do LES. Terapias (ensaios clínicos) direcionadas contra o IFN-α estão atualmente em andamento, na tentativa de diminuir a ativação desta via (Yoo, 2010; Rönnblom, 2010). Além disso, a via de ativação do IFN-α pode identificar um subgrupo de pacientes com LES com manifestações clínicas distintas, ajudando a prever possíveis prognósticos e

tratamentos mais específicos para cada paciente. Um dos pontos mais importantes é que a mudança nos níveis de IFN-α pode refletir na atividade da doença e, assim, ajudar no manejo clínico da doença (Obermoser, 2010). Em nosso estudo, INF-α foi significativamente maior em pacientes com SLEDAI ≥3 quando comparados a pacientes com doença inativa. Além disso, observamos uma correlação direta entre a pontuação de SLEDAI e níveis de INF-α, sugerindo que o INF-α poderia ser um biomarcador para a atividade da doença no LESj. Resultados semelhantes foram observados no LES adulto (Hooks, 1979; Ytterberg,1982; Kim, 1987; Baechler , 2003; Dall'era, 2005; Kirou, 2005).

Estudos anteriores sugerem um importante papel de IFN-α na imunopatogênese do LES (Hooks, 1979; Ytterberg,1982; Kim, 1987; Baechler , 2003; Dall'era, 2005; Kirou, 2005; Niewold, 2007; Niewold, 2008; Niewold, 2008). Há uma associação entre IFN-α e as múltiplas características clínicas e sorológicas de doença (Dall'era, 2005; Zhang, 2010). Estudos já demonstraram associações entre INF-α e manifestações cutâneas e renais (Dall'era, 2005; Kirou, 2005). A relação entre o aumento da expressão de genes induzidos por INF-α (IFIGs) e a presença de mainfestações clínicas mais graves, como envolvimento de SNC e renal foi observado em outro estudo (Baechler, 2003). Em nossa coorte, observamos um aumento nos níveis IFN-α em pacientes com manifestações cutâneas.

Nossos dados também mostraram um aumento nos níveis de INF-α em pacientes com LESj com anti-dsDNA positivo e também uma correlação direta entre INF-α e níveis de C3, porém nenhuma associação com doença renal foi observada. Aumento da expressão de genes codificadores de IFN-α em células mononucleares do sangue periférico tem sido associado com a presença de nefrite lúpica, manifestações cutâneas, e presença de anti-Ro/SSA, anti-Sm, anti-RNP e anti-dsDNA (Baechler, 2003;

Weckerle, 2011). Anti-dsDNA tem sido associado com nefrite lúpica (Bastian, 2007), enquanto anti-Ro/SSA tem sido associado a manifestações cutâneas (Sontheimer, 1982). Ainda não está claro se a associação entre IFN-α e as manifestações cutâneas e renais em estudos anteriores é primária ou secundária devido a uma associação entre auto-anticorpos e IFN-α (Weckerle, 2011). Nós não observamos associações entre INF-α e outros auto-anticorpos, como anti-Ro/SSA, anti-Sm ou anti-RNP.

Familiares de pacientes com LES têm maior risco de desenvolver doenças autoimunes, como o LES (Niewold, 2007; Niewold, 2008). A predisposição genética para alteração da via de ativação do IFN-α em familiares pode explicar o risco hereditário aumentado dos familiares de primeiro grau dos pacientes. Uma possível variabilidade genética na via de sinalização do IFN-α tem sido sugerida devido à presença de polimorfismos de SNPs nos genes codificadores de IFN-α (IRF5 e TYK2) (Boule, 2004; Graham, 2006; Bauer, 2009; Rullo, 2010; Garcia-Romo, 2011) em pacientes com LES, embora o impacto destes polimorfismos na atividade da doença *in vivo* não seja conhecido (Niewold, 20073; Rullo, 2010). Não observamos diferença significativa entre os níveis séricos de IFN-α de familiares de primeiro grau e indivíduos sadios não aparentados. No entanto, o pequeno número de indivíduos pode ter afetado os resultados.

Encontramos uma correlação indireta entre os níveis de INF-α e a idade dos pacientes. Achados semelhantes foram relatados no LES adulto, bem como em indivíduos sadios não aparentados (Niewold, 2008). Não é claro na literatura se os níveis séricos aumentados de IFN-α observados em pacientes com LES mais jovens é a causa ou o resultado da atividade da doença, mas esta correlação pode explicar as diferentes manifestações clínicas e sorológicas entre pacientes com LESj e pacientes com LES adulto.

Em relação à medicação, observamos níveis mais elevados de INF-α em pacientes sem medicação. Nenhum dos pacientes apresentou qualquer evidência de atividade da doença no momento da avaliação. No entanto, estudos longitudinais são necessários para determinar se o aumento nos níveis de INF-α pode prever períodos de atividade no futuro.

Estudos já demostraram uma diminuição significativa na expressão dos IFIGs em pacientes que receberam pulso de glicocorticóides (Kirou, 2005; Shodell, 2003). Dados sugerem que o tratamento com pulso de glicocorticóides pode diminuir o número de células produtoras de INF-α, reduzindo, transitoriamente o estímulo a expressão dos IFIGs, levando a uma diminuição nos níveis séricos de INF-α (Shodell, 2003).

5.2 TNF-α

Em nosso estudo observamos um aumento dos níveis séricos de TNF-α em pacientes com LESj quando comparados aos familiares de primeiro grau e aos indivíduos sadios não aparentados, como já observado em pacientes com LES adulto (Studnicka-Bencke, 1996; Gabay, 1997; Jones, 1999; Gómez, 2004; Mahmoud, 2005; Pitidhammabhorn, 2006; Sabry, 2006; Al-Mutairi, 2007; Wozniacka,2008).

Embora vários estudos tenham analisado níveis de TNF-α em pacientes com LES adulto, o seu significado clínico é menos claro (Studnicka-Bencke, 1996; Sabry, 2006; Al-Mutairi, 2007). A associação entre TNF-α e a presença de anti-dsDNA (Studnicka-Bencke, 1996), nefrite (Sabry, 2006) e envolvimento pulmonar (Al-Mutairi, 2007) foi demonstrada em estudos anteriores.

Vários estudos com pacientes com LES adulto têm mostrado um aumento nos níveis de TNF-α em pacientes com doença ativa (Studnicka-Bencke, 1996; Gabay, 1997; Jones, 1999; Gómez, 2004; Mahmoud, 2005; Sabry, 2006). No entanto, essa associação nunca fora antes estudada em um grupo de pacientes com LESj. Observamos

em nossa coorte um aumento dos níveis de TNF-α em pacientes com doença ativa, além de uma correlação positiva entre a pontuação de SLEDAI, sugerindo que o TNF-α pode ser um biomarcador para a atividade da doença no LES.

Níveis significativamente mais altos de TNF-α foram observados em pacientes com nefrite quando comparados aos pacientes sem nefrite. Nefrite lúpica é um protótipo de lesão renal induzida por imuno-complexos (Masutani, 2001). Na nefrite lúpica, o padrão de lesão glomerular está relacionado essencialmente com a presença de anticorpos (anti-dsDNA e anti-C1q) e com a formação de imuno-complexos. Esses imuno-complexos são depositados na superfície do tecido, induzindo a resposta inflamatória, através da ativação de moléculas de adesão no endotélio. Esta resposta leva ao recrutamento de leucócitos pró-inflamatórios. Lesão renal resulta da ativação e dano das células glomerulares, do infiltrado de macrófagos e da presença de citocinas (Gigante, 2011).

A alteração no processo de apoptose desempenha um papel importante no desenvolvimento da nefrite lúpica proliferativa (Studnicka-Bencke, 1996; Gloor, 1998). Níveis mais elevados de TNF-α também foram observados em um estudo anterior que comparou nefrite lúpica ativa com nefrite inativa (Sabry, 2006), e em outros estudos com nefropatias não lúpicas, incluindo nefropatias membranosa e síndromes nefrótica (Ihm, 1997; Lionaki, 2009). Estes resultados suportam a hipótese de que TNF-α pode desempenhar um papel patogênico na indução ou manutenção da disfunção da barreira glomerular em doenças renal (Lionaki, 2009).

Linhas de pesquisa suportam o papel do TNF-α na nefrite lúpica, comprovando que há uma melhoria importante da nefrite nos pacientes que estão em uso da terapia bloqueadora de TNF-α (Dean, 2000; Aringer, 2004; Aringer, 2007; Aringer, 2008; Zhu, 2010). Em particular, nefrite pode permanecer em remissão de longo prazo após apenas

algumas infusões de Infliximab (Zhu, 2010). Apesar do aumento de auto-anticorpos contra cromatina em pacientes tratados com terapia bloqueadora de TNF-α, estes foram transitórios e sem consequências patológicas (Mageed, 2002). É importante considerar algumas limitações deste estudo, como o pequeno número de pacientes com LES (7 pacientes) incluídos e o curto período de seguimento (4-10 semanas) (Aringer, 2007).

Apesar de apenas 17% dos pacientes apresentarem depressão, observamos um aumento nos níveis de TNF-α em pacientes com depressão moderada/severa quando comparados aos pacientes sem depressão/leve.

Nos últimos 20 anos, desde os relatos iniciais de interações neuro-imunológicas na depressão, vários estudos têm demonstrado uma clara associação entre a ativação do sistema imunológico, os níveis de citocinas pró-inflamatórias, e os sintomas psiquiátricos (Mikova, 2001; Tuglu, 2003; Dowlati, 2010). TNF-α exerce seu efeito biológico, principalmente pela ligação aos receptores TNFR1 e TNFR2, causando a ativação das cascatas de sinalização que mediam diferentes efeitos intracelulares (Baud, 2001). No cérebro, TNFR1 parece mostrar um padrão de expressão constitutiva enquanto TNFR2 se expressa, principalmente sob condições de estímulo (Baud, 2001). As maiores concentrações de receptores de TNF-α no cérebro são encontradas em várias regiões envolvidas na regulação do humor e do funcionamento cognitivo como o hipotálamo, hipocampo e áreas do córtex cerebral (Khairova, 2009). Apesar do vínculo associativo entre neuro-inflamação e transtornos de humor ser amplamente aceito, mais estudos são necessários para estabelecer a relação causa-efeito (Kaster, 2011).

Embora uma literatura considerável associe o TNF-α à patogênese de detrminadas doenças degenerativas como a doença de Alzheimer (Clark, 2010) e esclerose múltipla (Mikova, 2001), além de associar com outros tipos de depressão como a depressão atípica (Yoon, 2011), transtorno depressivo maior (Mikova, 2001;

Tuglu, 2003; Kim, 2007; Himmerich , 2008; Dowlati, 2010), essa associação não foi relatada no LES até agora.

Em relação à medicação, não observamos diferença significativa nos níveis de TNF-α entre pacientes com ou sem medicação. Dados da literatura sugerem que os antimaláricos interferem na liberação de TNF-α por células humanas e de camundongos, embora seu modo exato de ação não seja totalmente compreendido (Wozniacka, 2006). Outro estudo mostrou que a hidroxicloroquina tem sido eficaz na redução de atividade e dano da doença, redução de ocorrência de eventos vasculares e trombóticos e até mesmo eficaz no aumento da sobrevida (Alarcón, 2007).

6. Conclusões

1. Níveis séricos de INF-α estão significativamente mais elevados em pacientes com LESj quando comparados a familiares de primeiro grau e indivíduos sadios não aparentados. Nenhuma diferença significativa entre familiares de primeiro grau e indivíduos sadios não aparentados foi observada.
2. INF-α está associado com atividade da doença, presença do anticorpo anti-dsDNA, níveis de C3 e manifestações cutâneas (*rash* e vasculite cutânea) em pacientes com LESj. O INF-α se correlacionou com SLEDAI e inversamente com a idade dos pacientes com LESj.
3. Níveis de TNF-α estão significativamente mais elevados em pacientes com LESj quando comparados a familiares de primeiro grau e indivíduos sadios não aparentados. Nenhuma diferença significativa entre familiares de primeiro grau e indivíduos sadios não aparentados foi observada.
4. TNF-α está associado com atividade da doença, nefrite e depressão em pacientes com LESj. O TNF-α se correlacionou com SLEDAI.

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8. Apêndices

8.1 Artigos submetidos

8.1.1 Apêndice 1- Artigo submetido à revista Lupus

Th1/Th2 cytokine profile in childhood-onset systemic lupus erythematosus

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Abstract

Objective: To determine the serum levels of Th1 (IL-12, INF-γ,TNF-α) and Th2 (IL-5, IL-6 and IL-10) cytokines in childhood-onset SLE, first-degree relatives and healthy controls. To elucidate their association with disease activity, laboratory and treatment features. **Methods:** We included 60 consecutive childhood-onset SLE patients (mean age 17.85±3.89), 64 first-degree relatives (mean age 39.95±5.66) and 57 healthy (mean age 19.30±4.97) controls. Controls were age and sex-matched to SLE patients. SLE patients were assessed for clinical and laboratory SLE manifestations, disease activity (SLEDAI), damage (SDI) and current drug exposures. Mood disorders were determined through Becks Depression (BDI) and Anxiety Inventory (BAI). Th1 (IL-12, INF-γ,TNF-α) and Th2 (IL-5, IL-6 and IL-10) cytokines levels were measured by ELISA and compared by non-parametric tests. **Results:** Serum TNF-α (p=0.004), IL-6 (p=0.007) and IL-10 (p=0.03) levels were increased in childhood-onset SLE patients when compared to first-degree relatives and healthy controls. TNF-α levels were significantly increased in patients with active disease (p=0.014) and correlated directly with SLEDAI scores (r=0.39; p=0.002). IL-12 (p=0.042) and TNF-α (p=0.009) levels were significantly increased in patients with nephritis and TNF-α in patients with depression (p=0.001). No association between cytokine levels and SDI scores or medication was observed. **Conclusion:** Th1 cytokines may play a role in the pathogenesis of neuropsychiatric and renal manifestations in childhood-onset SLE. The correlation with SLEDAI suggests that TNF-α may be a useful biomarker for disease activity in childhood-onset SLE, however longitudinal studies are necessary to determine if increase of this cytokine may predict flares in childhood-onset SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, multisystemic autoimmune disease predominantly affecting women of childbearing age (1). Approximately 10–20% of all cases of SLE occur in the first two decades of life (1-4). In childhood-onset patients the female-to-male ratio is 4:3 with disease onset during the first decade of life, 4:1 during the second decade when compared to 9:1 ratio in adult-onset SLE (5-7).

Childhood-onset SLE often presents more acute and severe disease features than adult-onset SLE. Renal (50% to 67%), neurological (22-95%) and hematological (77%) involvement, in addition to fever and lymphadenopathy are more frequently observed in children when compared to adult-onset SLE (8-13). Equally frequent in pediatric and adult-onset SLE are serositis, anti-Smith (anti-Sm), anti-Ro, and anti-La antibodies (8, 9). Arthritis, photosensitivity, discoid lesions, on the other hand, are more frequently observed in adult-onset SLE (9, 10, 12). In relation to disease activity, pediatric SLE patients have a more active disease not only at disease onset, but also over time when compared to adult-onset SLE (14,15).

The impact of SLE on children is often profound, and a satisfactory outcome in this age group is not a 5 or 10-year survival, but a survival that more closely approximates the normal lifespan (16). The awareness that SLE in childhood is a potentially fatal disease, that atypical presentations are very common, and that aggressive treatment should be introduced early in the course of the disease, has significantly improved survival in the childhood-onset SLE cohorts (14-16). Over the last decades, morbi-mortality rates have significantly dropped in pediatric patients in a similar patter as in adults SLE patients (14, 17).

Independently of the age of onset, there is strong evidence supporting the role of cytokine in the pathogenesis of SLE (18). Although antibody production, driven by Th2

lymphocytes and immune complex formation are key features in SLE, recent evidences have suggested that Th1 lymphocytes have an important pathogenic role in SLE (18,19). The main cytokines associated with cellular immunity (Th1) are interleukin (IL) 12, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), while IL-5, IL-6 and IL-10 are associated with the production of antibodies and induction of humoral immunity (Th2) (19). Not only is the cytokine profile in SLE different when compared to healthy controls, it also varies according to disease phenotypes and disease activity (18).

Serum IL-12, INF-γ, TNF-α, and IL-5, IL-6 and IL-10 and the relation between these Th1 and Th2 cytokines have been studied in adult-onset SLE (19-22). However, the role of these cytokines in childhood-onset SLE has never been investigated. The aim of our study was to determine the serum levels of Th1 (IL-12, INF-γ, TNF-α) and Th2 (IL-5, IL-6 and IL-10) cytokines in childhood-onset SLE patients, first-degree relatives and healthy controls. In addition we evaluated their association with disease activity, laboratory and treatment features.

Patients and methods

Subjects

Sixty consecutive childhood-onset SLE patients, recruited from the Pediatric Rheumatology Outpatient Clinic of State University of Campinas were included in this study. Patients were included in the present study if they: (i) fulfilled at least four criteria of American College of Rheumatology (ACR) (23); (ii) were below 16 years of age at disease onset; and (iii) had a follow-up duration of at least 6 months.

Sixty- four first-degree relatives and 57 healthy controls without history of any chronic disease (including auto-immune diseases) were included as a control group. The healthy volunteers were matched by age and gender to the patients.

This study was approved by the ethics committee at our institution, and informed written consent was obtained from each participant and/or legal guardian.

Clinical features

All patients had their medical histories, clinical and serological characteristics evaluated at study entry according to the ACR (23). Features included in this protocol were age at onset of disease (defined as the age at which the first symptoms clearly attributable to SLE occurred), age at diagnosis (defined as the age when patients fulfilled four or more of the 1982 revised criteria for the classification of SLE (23), and follow-up time (defined as the time from disease onset until May 2010).

All clinical manifestations and laboratory findings were recorded at the day of blood withdrawal. Nephritis was diagnosed on the basis of proteinuria exceeding 0.5 g/L with abnormal urinary sediment and/or histological findings. Nephrotic syndrome was defined as proteinuria in excess of 3.0 g/day. Hematological alterations were ascribed to lupus only in the absence of bone-marrow suppression (leukopenia <4000 cells/mm^3; thrombocytopenia <100,000 cells/mm^3; hemolytic anemia). We also

analyzed the presence of malar rash, discoid lesions, subacute cutaneous lesions, cutaneous vasculitis, photosensitivity, oral ulcers, arthritis and serositis. Neurological and psychiatric involvement was defined according to ACR (24).

Treatment prescribed at time of blood withdrawal, as well as its adverse events related to drug use, was recorded. Doses of oral and parenteral corticosteroids were analyzed and converted to the equivalent doses of prednisone.

Laboratory studies

Antinuclear antibodies (ANA) were determined by indirect immunoflurescence using using HEp-2 cells as substrate, and regarded as positive if higher than 1/80. Anti-double stranded DNA (dsDNA) antibodies were determined by indirect immunoflurescence using *Crithidia* as substrate and considered positive if higher than 1:10. Precipitating antibodies to extractable nuclear antigens (ENA), including Ro (SSA), La (SSB), and Sm were detected by a standardized ELISA method, and considered positive if higher than 1/40. Rheumatoid factor (RF) was detected by nefelometry, and regarded as positive if higher than 10. Anticardiolipin antibodies (aCL) of the IgG and IgM isotypes were measured by an ELISA method (25). The lupus anticoagulant (LA) activity was detected by coagulation assays in platelet-free plasma obtained by double centrifugation, following the recommendation of the subcommittee on LA of the Scientific and Standardization Committee of the International Society of Thrombosis and Homeostasis (26). These measurements were carried out twice, at an interval of 12 weeks.

Disease Activity and damage

Disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (27). SLEDAI scores range between 0 and 105. Scores of ≥3 were considered active disease (28). Active nephritis was diagnosed on the basis of

renal items of the SLEDAI (proteinuria exceeding 0.5 g/L, abnormal urinary sediment, low complement levels).

Cumulative SLE-related damage in all patients was determined using the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI) (29) at time of blood withdrawal. SDI score range from 0 to 47. Damage was considered if scores ≥1 (29).

Mood evaluation

All subjects completed the Beck Depression (BDI) (30) and Beck Anxiety Inventory (BAI) (31) at study entry. For patients under sixteen years old, Children's Depression Inventory (CDI) was applied. These scales consist of 21 items, each describing a common symptom of depression/anxiety. The respondent is asked to rate how much he or she has been bothered by each symptom over the past month on a 4-point scale ranging from 0 to 3. The items are summed to obtain a total score that can range from 0 to 63. The cutoffs used for the BDI are: 0–13: no/minimal depression; 14–19: mild depression; 20–28: moderate depression; and 29–63: severe depression and for the BAI: 0-7: no/minimal level of anxiety; 8-15: mild anxiety; 16-25: moderate anxiety; 26-63: severe anxiety. The cutoff used for CDI is 17.

Cytokines assays

A blood sample was collected from all participants, centrifuged at 3000 rpm for 15 min after being allowed to clot for 30 min at room temperature. Sera were separated as soon as possible from the clot of red cells after centrifugation to avoid TNF-α production by blood cells that falsely could increase its values (32). Separated sera were kept in aliquots at −80°C until the time of assay. None of the samples was taken during an episode of a severe bacterial infection requiring hospitalization because TNF-α could be increased due to a secondary cause (33).

Commercially available kits from R&D Systems (London, UK) were used for the measurement of serum INF-γ, TNF-α, IL-5, 6, 10 and 12 levels by enzyme-linked immunosorbent assay (ELISA), carried out in accordance with the manufacturer's instructions. The minimum detectable dose (MDD) was less than 8.0pg/mL for INF-γ, 0.29pg/mL for IL-5, 0.039 pg/mL for IL-6 and less than 3.9 pg/mL for IL-10. For IL-12, MDD was typically less than 0.5 pg/mL. The detection range for TNF-α was 0.5–32 pg/ml with a sensitivity of 0.106 pg/ml (high-sensitivity human TNF-α kit).

Statistical analysis

Kruskal-Wallis Test was used to compare cytokines levels between groups. Spearman's correlation was used to correlate continuous variables (e.g. cytokines levels and SLEDAI, SDI, BDI and BAI scores). Cytokine levels and categorical variables were compared by Mann–Whitney *U* test. For all analyses, a p-value < 0.05 was considered statistically significant.

Results

Demographics

We included 60 consecutive childhood-onset SLE patients. Fifty-seven (95%) were female with mean age of 17.85 years [Standard deviation (SD) ±3.91years; range 9-37]. Disease duration was 5.38 years (SD±4.25; range 0-26 years). Sixty-four first-degree relatives [59 women; mean age of 39.95 years (SD±5.66; range 28-52)] agreed to participate in the study. The control group consisted of 57 healthy controls (52 women) with a mean age of 19.30 (SD±4.97 years; range 6-30 years). Patients and healthy controls were statistically comparable in terms of age and sex (Table 1).

Clinical, laboratory, and treatment features

All patients had disease onset before the age of 16 and clinical and laboratory manifestations at disease onset are shown in Table 2. At time of study entry, 30 (50%) childhood-onset SLE patients had active disease (SLEDAI ≥3) with mean SLEDAI scores of 8.24 (SD±4.09, range 3-18). The 30 (50%) inactive patients had a mean SLEDAI score of 0.55 (SD±0.89 range 0-2)]. Active nephritis (33.3%), new malar rash (6.6%), new alopecia (5.0%) and cutaneous vasculitis (5.0%) were the clinical manifestations more frequently observed (Table 2).

At time blood withdrawal, 8 (13.3%) patients were not taking any immunosuppressant medication. Forty-two (70%) patients were receiving prednisone, 32 (53.3%) hydroxychloroquine and 22 (36.6%) patients were receiving other immunosuppressive drugs (Table 2).

Depression was identified in 10 (16.7%) patients and in no healthy control or first-degree relatives. Mild depression was identified in 5 (8.3%) patients and 5 (8.3%) patients had moderate/severe depression. Anxiety was observed in 21 (35%) childhood-onset SLE patients. Twelve (20%) patients had mild and 9 (15%) had moderate/severe anxiety.

Cytokines assays

Sera TNF-α, IL-6 and IL-10 levels were significantly increased in childhood-onset SLE when compared to first-degree relatives and healthy controls (Table 3). No significant difference in serum TNF-α, IL-6 and IL-10 levels was observed between first-degree relatives and healthy controls. No significant difference in serum levels of INF-γ, IL-5 and IL-12 was observed among childhood-onset SLE, first-degree relatives and healthy controls. TNF-α levels ($p=0.014$) were significantly increased in patients with active disease (SLEDAI≥3) when compared to patients with inactive disease. In addition, TNF-α levels correlated directly with SLEDAI scores ($r=0.39$; $p=0.002$).

Although IL-6 was increased in patients with active disease when compared to patients with inactive disease, no statistically significance was noted.

IL-12 ($p=0.042$) and TNF-α ($p=0.009$) levels were significantly increased in patients with active nephritis when compared to patients without nephritis. IL-6 levels were significantly increased in patients with dysmorphic hematuria ($p=0.003$) and IL-10 in patients with positive dsDNA ($p=0.02$). An indirect correlation between the TNF/IL-10 ratio and dsDNA was observed ($r=-0.45$; $p=0.001$).

TNF-α levels were significantly increased in patients with depression ($p=0.01$) when compared to patients without depression. IL-10 levels had a negative correlation with the severity of depression ($r=-0.45$; $p=0.013$).

No association between other SLEDAI variables or SDI scores and IL-12, INF-γ, TNF-α, IL-5, IL-6 and IL-10 levels was observed. In addition, no difference in these cytokine levels in patients with and without medication was observed.

Discussion

Cytokines are low-weight proteins that play a key role in immunological dysregulation observed in autoimmune diseases. The increased levels of proinflammatory cytokines are believed to play a key role in the pathogenesis of SLE (34). Higher cytokine levels in SLE patients may promote inflammatory response, apoptosis and autoantibody production that not only initiate, but may also maintain SLE disease activity over time (34).

The main cytokines associated with cellular immunity (Th1) are IL-12, IFN-γ and TNF-α. IL-12 is a proinflammatory cytokine that induces IFN-γ, favoring the differentiation of Th1 cells, and maintaining a link between innate and adaptive response (35). IL-12-induced IFN-γ mediates many of the pro-inflammatory activities of IL-12, whereas the ability of IL-12 to favour a Th1 response exemplifies its function as an immunoregulatory cytokine that bridges innate resistance and adaptive immunity. So, IL-12 has been shown to have an important role in the Th1 response that sustains organ-specific autoimmunity in several mouse experimental models, and to be instrumental in resistance to many infections, particularly with bacteria and intracellular parasites (36).

INF-γ is a dimeric glycoprotein with 146 amino acids subunits (37). INF-γ participates in the activation of macrophages in both the innate and the adaptive immune response. TNF-α has a central role in inflammation, induces the expression of other pro-inflammatory molecules, chemotactic cytokines and adhesion molecules and has been intensely investigated in rheumatic diseases (38-40).

IL-5, IL-6 and IL-10 are Th2 secreted cytokines (18). IL-5 preferentially activates B1 cells to produce natural antibodies cross-reactive to self antigens (41). IL-6 is produced by antigen presenting cells (APC) such as macrophages, dendritic cells, and

B cells, although its secretion may also be found in T- and B-lymphocytes (19). Its production is triggered by IL-1, IL-2, and TNF-α but dampened by IL-4, IL-10, and IL-13. One of the most important effects of IL-6 is to induce the maturation of B lymphocytes into plasma cells and augment the immunoglobulin secretion (19). IL-10 is produced mainly by monocytes and lymphocytes. It blockades the activation of APC, down-regulates the expression of costimulatory molecules and, thereby, blunts T cell activation and TNF-α secretion (42). IL-10 boosts B cell proliferation and immunoglobulin class switching resulting in enhanced antibody secretion with the capacity to enter extravascular compartments and promote inflammation in SLE (42).

IL-10 is also a cytokine secreted by type-1 T regulatory (Tr1) cells (43,44). The main cytokines produced by Tr1 cells are IL-10 and transforming growth factor beta (TGF-β), which downregulate immune responses mediated by naive and memory T cells (45,46). Interestingly, Tr1 cells produce these cytokines in the absence of significant levels of IL-2 or IL-4, which are potent T-cell growth factors (47).

In our study we observed increased TNF-α, IL-6 and IL-10 levels in childhood-onset SLE when compared to healthy controls and first-degree relatives, as previously observed in adult-onset SLE patients (18-22,48-54).

Although several studies have analyzed TNF-α levels in adult-onset SLE patients, the clinical significance is less clear (19, 20,48,50-54). In addition to increased TNF-α levels in patients with active disease, we observed a positive correlation between SLEDAI scores, suggesting that TNF-α could be a biomarker for disease activity in SLE. Several studies (19,50-52,54) analyzing adult-onset SLE have shown higher TNF-α levels in SLE patients with active disease. However, this association was never studied in a childhood-onset cohorts.

We also observed significantly higher levels of TNF-α in patients with nephritis when compared to patients without nephritis. SLE nephritis is a prototype of immune-complex induced kidney damage (55). In SLE nephritis, the pattern of glomerular injury is primarily related to dsDNA and anti- C1q antibodies and the formation of immune complexes. These immune complexes are deposited on the tissue surface, inducing inflammatory response by activating adhesion molecules on endothelium. This response leads to the recruitment of pro inflammatory leukocytes. Renal injury results from activated and damaged glomerular cells, infiltrating macrophages, and cytokines (56).

Recently, it has been shown that dysregulated apoptosis is also an important factor for developing proliferative lupus nephritis (54,57). Higher TNF-α levels were also observed in one previous study that compared active SLE nephritis with inactive nephritis (20), and in other non-SLE nephropathies, including membranous nephropathies and nephritic syndromes (58,59). These findings support the hypothesis that TNF-α may play a pathogenic role in the induction or maintenance of glomerular barrier dysfunction in renal diseases (59).

The involvement of TNF-α in lupus nephritis is further supported by the improvement of lupus nephritis under TNF-α blocking therapy (60-64). In particular, nephritis may remain in long-term remission after just a few infusions of infliximab (64). Although an increase in the autoantibody response to chromatin was observed in patients treated with TNF-α blocking therapy, these were transient and without pathologic consequences (65). It is important to consider some limitations of the study analyzing TNF-α blocking therapy, such as the small number of SLE patients included (7 patients) and the short follow up period (4–10 weeks) (63).

We also detected higher levels of IL-12 in patients with nephritis. Previous studies have demonstrated that increased IL-12 production was associated closely with

renal disease in parallel with Th1 polarization and increased IFN-γ solubilization in vitro (66,67). In addition, the increase in urinary IL-12 apparently reflected both its serum and its glomerular accumulation (66,67).

In SLE patients it has been shown that elevated production of IL-10 is capable of promoting generation of dsDNA antibodies (68), as observed in our study. Although SSA and SSB were also reported to be associated with high TNF/low IL-10 genotype (69), we did not find such association in our cohort. In addition, IL-6 was demonstrated to be highly expressed in kidneys and to be significantly increased in the serum SLE patients with nephritis (20). We did not find an association between IL-6 and nephritis; however we observed that patients with dysmorphic hematuria had increased levels of serum IL-6.

Although depression was identified in only 17% of our cohort, we observed increased levels of TNF-α in patients with moderate/severe depression when compared to patients with no/mild depression.

In the past 20 years since the initial reports of neural-immune interactions in depression, several studies have shown a clear association between activation of the immune system, levels of proinflammatory cytokines, and psychiatric symptoms (70-72). TNF-α exerts its biological effect mainly by binding to tumor necrosis factor receptor 1 (TNFR1) and receptor 2 (TNFR2), causing activation of complex signaling cascades that mediate different intracellular effects (73). In the brain, TNFR1 seems to show a constitutive pattern of expression whereas TNFR2 is mainly expressed under stimulatory conditions (73). The highest concentrations of TNF-α receptors in the brain are found in several regions involved in mood regulation and cognitive functioning like the hypothalamus, hippocampus, and areas of the cerebral cortex (74). Although an associative link between neuroinflammation and mood disorders is widely accepted,

further studies are necessary to establish the cause-effect relationship (75). Several studies connect cytokines with the pathogenesis of depression in Alzheimer's disease (76), in atypical depression (77), in major depressive disorder (70-72, 78,79), and in multiple sclerosis (70), however this association has not been reported in SLE so far.

We did not observed correlation between depression and other cytokines studied, however we found a negative correlation between IL-10 levels and the severity of depression. There is no data in the literature exploring this correlation between depression and IL-10 levels so far.

Hydroxychloroquine has been shown to reduce the probability of flares, the accrual of damage, to possibly protect patients with SLE from the occurrence of vascular and thrombotic events and even to increase survival (80). Literature data suggests that antimalarials interfere with TNF-α release from human and murine cells, although their exact mode of action is not fully understood (34). In one previous study (34) chloroquine was shown to lower TNF-α levels, however in our study we did not observe differences in TNF-α levels between hydroxychloroquine users and non-users.

The pathogenesis of SLE is a combination of multifactorial, genetic and environmental influences, which lead to an irreversible break in immunologic self-tolerance (81). SLE family members are at higher risk of developing not only SLE, but also other autoimmune diseases (81-83). However, IL-12, INF-γ, TNF-α, IL-5, IL-6 and IL-10 levels have never been studied in first-degree relatives. In our study we did not observe any difference in the IL-12, INF-γ, TNF-α, IL-5, IL-6 and IL-10 levels of first-degree relatives when compared to healthy controls.

To the best of our knowledge, this is the first study to evaluate Th1 (IL-12, INF-γ,TNF-α) and Th2 (IL-5, IL-6 and IL-10) cytokines in childhood-onset SLE. Th1 cytokines may play a role in the pathogenesis of neuropsychiatric and renal

manifestations in childhood-onset SLE. The correlation with SLEDAI suggests that TNF-α may be a useful biomarker for disease activity in childhood-onset SLE, however longitudinal studies are necessary to determine if increase of this cytokine may predict flares in childhood-onset SLE.

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Table 1: Demographic and clinical characteristics of patients and controls included in the study

Parameter	Childhood-onset SLE patients N=60	First-degree relatives N=64	Healthy controls N=57
Sex			
Female	57 (95%)	59 (92.18%)	52 (91.22%)
Age (years)	17.85±3.89	39.95±5.66*	19.30±4.97
Disease duration (years)	5.38±4.25	-----	-----
SLEDAI	4.28±4.88	-----	-----
Active disease N=30	8.24±4.09		
Inactive disease N=30	0.55±0.89		
SDI	0.48±0.81	-----	-----

*$P \leq 0.0\ 5$

Table 2: Clinical, laboratory and treatment features at day of blood withdrawal

Manifestations	Patients N=60
Clinical features	
Alopecia	3 (5%)
Malar Rash	4 (6.6%)
Nephritis	20 (33.3%)
Neurologic manifestations	21 (35%)
Serositis	2 (3.3%)
Vasculitis	3 (5%)
Laboratory features	
Anticardiolipine or LA	13 (21.6%)
Anti-SM	9 (15%)
Anti-SSA/Ro	8 (13.3%)
dsDNA	25 (41.6%)
Leukopenia	2 (6.7%)
Thrombocytopenia	2 (6.7%)
Treatment	
No medication	8 (13.3%)
Prednisone	42 (70%)
Hydroxychloroquine	32 (53.3%)
Immunosuppressive drugs	29 (48.3%)
Azathioprine	15 (25%)
Ciclophosphamide	2 (3.3%)
Cyclosporine	5 (8.3%)
Methotrexate	1 (1.6%)
Mycophenolate mofetil	6 (10%)

dsDNA: double-stranded DNA, LA: lupus anticoagulant

Table 3: Cytokines sera levels of the individuals included in the study

Sera Levels (pg/mL)	**Childhood-onset SLE patients N=60**	**First-degree relatives N=64**	**Healthy controls N=57**
Th1 Cytokines			
IL-12	3.52±7.97	1.87±1.55	1.79±1.75
INF-γ	9.62±6.44	9.29±3.81	9.41±4.38
TNF-α	4.47 ± 8.95*	2.33 ± 2.38	1.83±1.82
Th2 Cytokines			
IL-5	3.98±5.07	2.72±1.83	2.69±2.16
IL-6	2.81±2.84*	2.02±2.03	1.46±1.93
IL-10	26.11±62.73*	11.70±31.63	9.69±19.94

*$P \leq 0.0\ 5$

8.1.2 Apêndice 2- Artigo submetido à revista Journal of Biomedicine and Biotechnology

Type I Interferon in the pathogenesis of Systemic Lupus Erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by the production of autoantibodies and the formation of immune complexes, leading to chronic inflammation. Complex genetic disorders are at the origin of the disease, leading to the escape of auto-reactive B-lymphocytes. Autoimmune B-cells are not sufficient for the disease to occur and additional mechanisms such as T-cell are clearly involved in the pathogenesis of SLE. Type I Interferons (INFs) are involved in multiple aspects of lupus etiology and pathogenesis. Leading observations in patients and data in SLE-prone mice have now established a key role of INFs in SLE. Several systemic clinical symptoms and laboratory findings can indeed be interpreted as downstream effects of a high IFN production, and point to this cytokine as a link between the expansion of autoimmune B-cells and the stimulation of other components of the immune system. These insights can now be transplanted to the clinic and designate IFN as a new promising therapeutic target.

This paper provides an overview of the pathogenesis, clinical aspects and management of INF in SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic and multisystemic disease resulting from defects in innate and adaptive immune system (1-4). The pathogenesis of SLE is not completely understood, however both genetic and environmental factors are important determinants for the different phenotypes observed (3). SLE is characterized by loss of tolerance to nuclear antigens, however a heterogeneous course of the disease is observed not only regarding clinical manifestations, but as well as disease severity (4,5). It has become evident over the past decade, that the autoantibody responses characteristically observed in SLE, such as anti-double-stranded (ds) DNA and anti-Sm, as well as certain clinical manifestations, such as nephritis, are due to the overproduction of type I interferon (IFN-I) (6-8).

The importance of IFN-I in autoimmunity is evident in the association between autoimmune manifestations and IFN-α treatment observed in hepatitis C infection or chronic myelogenous leukemia (9-11). Antinuclear antibodies (ANA) have been found in up to 22% of patients treated with IFN-α (9) and the onset of autoimmune diseases, such as SLE, rheumatoid arthritis, have been reported after IFN-α therapy (8).
In this paper, we will give a brief overview of the role of the type I IFN system in the pathogenesis of SLE. In addition, we will discuss recent data that established type I IFNs as an important therapeutic target in SLE.

Type I INF system

The type I IFN system comprises the molecular and cellular players involved in type I IFN production and their downstream effects, such as type I IFN genes and proteins, cells producing type I IFNs, and target cells affected by the IFNs (2,7).The human type I IFN gene family consists of 17 genes, 13 genes encoding IFN-α subtypes and single genes for IFN-β, IFN-ω, IFN-κ and IFN-ε (3). These genes and their products

show similarities in structure and functions (3). These are induced by cells exposed to virus or double-stranded RNA (dsRNA) and they interact with the same receptor, the IFN- α/β receptor (IFNAR) (12,13) (Figure 1). Differences, however, are observed in the post-IFNAR level (12,13).

The major IFN-producing cells (IPCs) were early on designated natural IFN-producing cells (NIPC), now known as dendritic cells (DCs) (10). DCs are the initiators and regulators of immune responses (14). DC differentiation can be classically divided into two distinct pathways (15): myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (16).

Tissue-resident mDCs express receptors, such as TLRs, nucleotide binding oligomerization domain (NOD) proteins, and lectins, to sense pathogens at the mucosal surfaces or at sites of tissue damage (14). mDCs can also be activated by ICs through the activating FcgRIIa (17, 18). Once activated, they migrate to the lymph node via afferent lymphatic system (14).

pDCs circulate in the blood and lymphoid organs. Upon viral exposure, these cells secrete large amounts cytokines, such as INF (16). pDCs express TLR7 and TLR9. Chromatin-containing and snRNPs-containing ICs are internalized by pDCs via FcgRIIa and reach the endosomal compartment where they activate TLR9 and TLR7, respectively, leading to secretion of cytokines including IFN-ab (19-22). Although pDC population constitutes only 0.1% of the peripheral blood mononuclear cells (PBMCs), each cell has the capacity to produce as many as 109 IFN-α molecules in 12 hours (23).

Two specific blood dendritic cell antigen markers were identified in pDCs: BDCA-2 and BDCA-4 (24). These antigens are specific for pDCs in peripheral blood, although BDCA-2 is lost as the cells mature and BDCA-4 also appears on

differentiating monocyte-derived and $CD34^+$ cell-derived DCs (24,25). BDCA-2 is a type II C-type lectin that can internalize antigen for presentation to T cells (25). BDCA-2 molecule inhibits the IFN-α production by pDCs triggered by a wide variety of IFN-α inducers. It is intriguing that such pDCs still can mature into efficient antigen presenting cells despite downregulated IFN-α production (25). Therefore, BDCA-2 molecule and any endogenous or microbial ligands may have an important role in immune activation by favoring Th2 immune response. It is possible that BDCA-2 ligation can cause inhibition of other functions of pDCs, such as interleukin-12 production (24,25).

BDCA-4 is shown to be identical to neuropilin-1(NP-1), a neuronal receptor belonging to the class-3 semaphorin subfamily, and a receptor on endothelial and tumor cells for vascular endothelial growth factor (VEGF-A). In blood and bone marrow, BDCA-4/NP-1 is exclusively expressed on pDCs, but it may be found on primarily follicular B helper memory T cells (T_{FH}) in tonsils, for example (26).

Viral DNA or RNA are the typical activators of type I IFN production, and secreted IFNs act on the type I IFN receptor (IFNAR) on target cells and induce production of proteins that inhibit viral replication (2). Five of the ten human TLRs (TLR3, 4,7, 8 and 9) mediate type I IFN gene transcription, and these receptors are expressed either on the cell surface (TLR4) or in the endosome (TLR3, 7, 8, 9). TLR3 is activated by double-stranded RNA (dsRNA), TLR7 and TLR8 by single-stranded RNA (ssRNA) and TLR9 by unmethylated CpG-rich DNA. In addition, there are nucleic acid sensors in the cytosol that can mediate IFN production. These include the DNA binding protein DNA-dependent activator of IFN regulatory factors (DAI) and the two RNAhelicases RIG-I and Mda5 (2, 27,28).

The pleiotropic functions of type I IFNs on the immune system

The type I IFNs have mainly been considered as antiviral proteins, because they are produced during viral infections and confer viral resistance in target cells (29). However, these IFNs also exert prominent immunoregulatory effects and might act as key cytokines in the innate immune system and in the adaptive immune responses (29).

Type I IFNs have an important role in lymphocytic development, T cells, B cells, and NK cells. Type I IFNs are able to promote survival and differentiation of antigen-activated Th1 cells, due to their ability to activate signal transducer and activator of transcription 4 (STAT4) and maintain expression of a functional interleukin-12R. Type I IFNs are also involved in maturation of antigen-presenting monocyte-derived DCs and stimulation of B lymphocytes (30). In spite of its growth-promoting effect on mature lymphocytes, IFN-α is involved in the impairment T- and B-cell development at the pro-T and pro-B, interleukin-7-responsive, stages in the thymus and bone marrow, respectively (31). IFN-α is an endogenous regulator of lymphopoiesis as it is produced by resident bone marrow macrophages (31).

Type-I IFNs are also an important adjuvant during the primary immune response by augmenting the intensity of antigen-specific memory T-cells (32). It has also been suggested that repeated exposures to type-I IFNs would promote the survival of polyclonal memory T-cells. Moreover, type-I IFNs induce cross-priming of $CD8^+$ T cells, and this effect involves direct stimulation of pDCs by IFNs (33).

In B-lymphocytes, both IFN-α and IFN-β act as costimulators of growth induced by mitogenic lipopolysaccharide or anti-IgM. Type-I IFNs enhance humoral immunity and promote isotype switchinig of B-cells *in vivo*. This response was long-lasting and mediated by pDCs. Most recent work demonstrated that T- and B-cells were direct targets for type-I IFNs, as the antibody response was greatly impaired in mice with selective deletion of IFNAR in T or B-cells (34). Consistently with the above results,

type-I IFNs secreted by virus-infected pDCs stimulate plasma cell differentiation and antibody secretion in synergy with IL-6 (35).

Type I IFNs are potent stimulators of natural killer (NK) cell cytotoxicity, although they do not stimulate these cells for IFN-γ production (36). Indeed, mice deficient for the transcription factor IFN regulatory factor-1 have normal NK cell development, yet greatly reduced NK cell-mediated cytotoxicity (37).

Pleiotropic effects of type I IFNs on memory T-cells and DCs generate optimal conditions to stimulate isotype switching in B-cells (33). Given that type I IFNs also act directly on B-cells to stimulate their maturation into plasma cells, they are extremely potent to trigger humoral and cellular arms of the immune system, in as much as it may induce direct NK- or $CD8^+$ T-cell-mediated cytotoxicity (36,37).

Type I INFs in SLE

The implication of IFN-I in SLE pathogenesis comes from multiple pieces of evidence, including genetic, gene expression association studies, and induction of SLE by interferon treatment (38).

Upon viral infection, pDCs from healthy individuals secrete IFN for few hours, followed by other cytokines such as TNF, which shuts down autocrine IFN- α/β production (12). Data suggest a key role for pDCs, and the IFN-α that they produce, in the pathogenesis of SLE. Genetic and abnormal expression in SLE might prevent the shutdown of IFN production. For example, the increased amounts of soluble TNF receptors in SLE serum might contribute to sustained IFN- α/β production by blocking TNF.

In addition, SLE-derived immune complexes (IC) containing either RNA or DNA are able to induce IFN-α in pDCs (39). In genetically susceptible individuals, B cell precursors expressing self-reactive antibodies are not removed (40). This would be

that due to mismanagement of naturally occurring apoptotic cells, nuclear material stimulates autoreactive B-cells leading to antibody secretion and the formation of ICs. These ICS and apoptotic bodies stimulate pDCs to produce type I IFNs. The latter enhance antigen presentation by DC to T-cells while promoting memory T-cell expansion and survival. A Th1 response ensures B-cells to switch from the production of polyspecific IgM to high-affinity anti-self IgG. These bind to self-antigens, fix complement and exert a pathogenic role by complement-mediated lysis and by restimulating DC to produce type I IFNs (2,29,39).

On the other hand, cytokines produced by T-cells and especially IFN-γ activate monocytes to produce inflammatory cytokines and chemokines such as Monokine-induced by IFN-γ (MIG/CXCL9) (41). This chemokine is indeed a major pDC attractant that helps $CXCR3^+$ pDC to migrate into T-cell zones of secondary lymphoid organs were they produce type I IFNs and directly interact with T-cells and myeloid DC (41).

Once a threshold has been reached for immune-cell expansion and activation, the disease becomes self-perpetuated. It may worsen due to exogenous factors that induce local apoptosis and/or augment B-cell survival such as interleukin-10 induced by ultra violet (UV) light, and infections that trigger Th1 T-cell response or type I IFNs production, commonly observed in flares of the disease (7).

Animal Studies

Several murine lupus models exist, though none of them appears to fully reproduce human SLE disease (42). The best known spontaneous models arise on New Zealand Black (NZB), New Zealand White (NZW), MRL, BXSB, and SWR backgrounds (42). Studies conducted on animal models of SLE have allowed us to further understand the pathogenic role of type I IFNs (IFNα/β) in SLE (43-47). In a first study, the effect of type I IFNs on the development of the lymphoproliferative disorder

in Fas-defective *lpr* mice was evaluated (43). It reported that sustained injection of polyinosinic (poly I:C), a potent inducer of type I IFNs, in B6 *lpr* mice resulted in worsening of nephritis, higher titers of autoantibodies, a 10-fold increase in serum Ig and accumulation of activated lymphocytes. Moreover, introducing a null mutation for the IFN-I-Receptor gene into the *lpr* background resulted in an important decrease of ICs deposition in the kidney and reduced lymphadenopathy (43).

Similar results were observed in NZB mouse models (44). A congenic NZB mice lacking the α-chain of IFN-α/βR, the common receptor for the multiple IFN-α/β species was created. Compared to controls, homozygous IFN-α/βR deleted NZB mice had significantly reduced anti-erythrocyte autoantibodies, hemolytic anemia, anti double-stranded DNA (dsDNA) autoantibodies, nephritis, and mortality. In the heterozygous-deleted mice, these reductions were intermediate. The disease-ameliorating effects could be observed by reductions in splenomegaly and in other immune cell subsets, including B-1 cells which are the major producers of anti-erythrocyte autoantibodies. Decreases in B cells subsets, T cell proliferation and stimulatory activity *in vitro* and *in vivo*, and DCs maturation *in vitro* were also detected. These findings suggest that type I IFNs are important mediators in the pathogenesis of murine lupus, and that reducing their activity in the human counterpart may be beneficial (44).

Contrary to the above studies that used SLE models of low to moderate severity, one work studied the role of IFN-α in NZB×NZW F1 (B/W) mice, a model that resembles human SLE (45). It showed that *in vivo* adenovector-mediated delivery of murine IFN-α resulted in pre-autoimmune [New Zealand Black (NZB) X New Zealand White (NZW)] F1, but not in normal, mice, in a rapid and severe disease with all characteristics of SLE. Anti-dsDNA appeared as soon as day 10 after initiation of IFN-α

treatment. Proteinuria and death caused by glomerulonephritis occurred in all treated mice ~18 weeks, at a time when all untreated NZB×NZW F1 did not show any sign of disease. IFN-α *in vivo* induced an overexpression of B lymphocyte stimulator in circulation at similar levels in both the pre-autoimmune and the normal mouse strains. All effects elicited by IFN-α were dose dependent. NZB×NZW F1 infused with purified murine IFN-α also showed acceleration of SLE. Thus, prolonged expression of IFN-α *in vivo* induces early lethal lupus in susceptible animals (45).

Although all the above reported studies strongly suggest that IFN-α is pathogenic in murine models of SLE, another study using lupus-prone mice of the MRL background suggested the opposite (46). A congenic lupus-prone MRL/CD95*lpr/lpr* (MRL/*lpr*) mice lacking the type I IFN receptor (IFN-RI), type II IFN receptor (IFN-RII), or both, were derived. As expected, deficiency for IFN-RII protected MRL/*lpr* mice from the development of significant autoimmune-associated lymphadenopathy, autoantibodies, and nephritis. However, deficiency for the IFN-RI deteriorated lymphoproliferation, autoantibody production, and end organ disease. Animals doubly deficient for IFN-RI and IFN-RII developed an autoimmune phenotype intermediate between wild-type and IFN-RII deficient animals, all correlating with an ability of type I IFNs to suppress MRL B cell activation. Thus, type I IFNs protected against both the humoral and end organ autoimmune syndrome of MRL/*lpr* mice, independently of IFN-γ. These findings warrant caution in the use of type I IFNs therapy in autoimmune diseases and suggest further investigation into the interplay between the types I and II IFNs during the source of autoantibodies (46).

Another work indirectly confirmed the study cited above (47). MRL/*lpr* mice with mild or advanced lupus-like disease were treated with IFN-β. For determining the effects of IFN-β treatment in SLE, MRL-*Faslpr* mice were injected with IFN-β. MRL-

Faslpr mice with mild and advanced disease were the first animals to receive the treatment. IFN-β was highly effective in prolonging survival and ameliorating the renal function, proteinuria, splenomegaly, and skin lesions, serologic (autoantibodies and cytokines), and histologic parameters of the lupus-like disease in mice that had mild and advanced disease. Decreased T cell proliferation and infiltration of leukocytes into the kidney, decrease in IgG3 isotypes and a reduction in nephrogenic cytokines were identified. IFN-β treatment of lupus nephritis in MRL-*Faslpr* mice is remarkably beneficial and suggests that IFN-β may be an important therapeutic candidate for subtypes of human lupus (47).

Human studies

There is substantial evidence to show that type I INFs have a similar proinflammatory role in SLE patients (1, 48-57). There is an association between IFN-α and multiple clinical and serological features observed in SLE (55,56). Increased expression of IFN-α-induced genes in PBMC has been associated with presence of lupus nephritis, proteinuria, cutaneous manifestations, presence of anti-Ro, anti-Smith (anti-Sm), anti-RNP, and anti- dsDNA antibodies (52,57). It remains unclear whether the association between IFN-α and cutaneous and renal disease manifestations in previous studies is primary, or secondary due to an association between autoantibodies and IFN-α (57).

Another important finding is SLE family members are at higher risk of developing not only SLE, but also other autoimmune diseases (48,51). A heritable predisposition to increased activation of the IFN-α pathway in SLE families could explain some of the burden of both SLE and non-SLE autoimmunity in the population. Possible genetic variability in endogenous IFN-α signaling has been suggested by the association of single nucleotide polymorphisms (SNPs) in the IFN-α pathway genes

IRF5 and TYK2 (58-61) with SLE, although the impact of these polymorphisms on IFN-α activity *in vivo* is not known (48,59).

Moreover, an IFN signature in adult SLE was mentioned in two independent studies (52,62). The first was identified by means of DNA chip analysis and real-time PCR, a coordinate expression of IFN-α—but not IFN-γ- induced genes in PBMC from SLE patients (62). The second study, based on gene profiling of PBMC, not only identified an IFN signature in 50% of SLE patients but linked its intensity to the severity of disease (52). Taken together, these data strongly suggest that IFN-α is involved in SLE pathogenesis.

Therapeutic strategies targeting type I INF

The current standard of care of SLE involves the use of corticosteroids and immunosuppressive agents that are widely acknowledged to cause unacceptable adverse events with long-term use (63). IFN-α became an important target since that type I IFNAR knockout mice have reduced disease activity, led to the development of a therapeutic agent targeting the type I IFN system (44,64-68). A phase I clinical trial using a single injection of anti-IFN-α monoclonal antibody (mAb) in SLE patients showed that there was a dose-dependent inhibition of type I IFN-inducible genes in both peripheral blood and skin biopsies, as well a reduction in clinical disease activity (69). However, neutralizing type I IFN antibodies may trigger an IFN-like response in endothelial cells and PBMCs *in vitro* (70). As adequate type I IFN production is critical in the response to certain pathogens, especially viruses, clinicians may have to pay attention to the possibility of viral infection. Therefore, selective inhibition of the abnormal and continuous IFN-α production in SLE without the risk of infection would be the most attractive therapeutic strategy (71).

There are a few clinical trials with anti-IFN-α in SLE underway. One of them is a phase II, multicenter, open-label, dose-escalation study to evaluate the safety and tolerability of multiple subcutaneous doses of MEDI-545, a fully human anti-IFN-α monoclonal antibody in SLE patients. The patients were scheduled to take MEDI- 545 at four different doses (72). Another phase II, multicenter, open-label, dose-escalation study to evaluate safety and tolerability of IV or SC Dose of MEDI-545 in japanese SLE patients is ongoing, but patients are currently recruiting (73).

There is another phase II study with moderately to severely active SLE patients, whose aim is to evaluate the efficacy and safety of recombinant human anti-IFN-α monoclonal antibody (Rontalizumab), compared with placebo (74). This study is ongoing, but finished recruiting participants.

In addition, there has been an alternative way to block IFN. A Phase I-II study proposes that active immunization with IFN-α kinoid (IFN-K) may induce a polyclonal antibody response (75). The aim of this study is to evaluate the safety of IFN-K in patients with mild to moderate SLE. It will also measure the induction of anti-IFN-α antibodies and evaluate the clinical impact on SLE disease (75).

Conclusion

Hopefully, as our understanding of SLE pathogenesis grows, we will be able to target therapeutic interventions to the immune mechanisms that cause the disease manifestations in an individual patient. Such new therapies may be effective in various manifestations with lower toxicity, and without wide suppression of the immune system. Before we apply anticytokine targeting therapy, we need to develop novel biomarkers to monitor disease activity, and more clearly identify patients who are able to show a favorable response to treatment.

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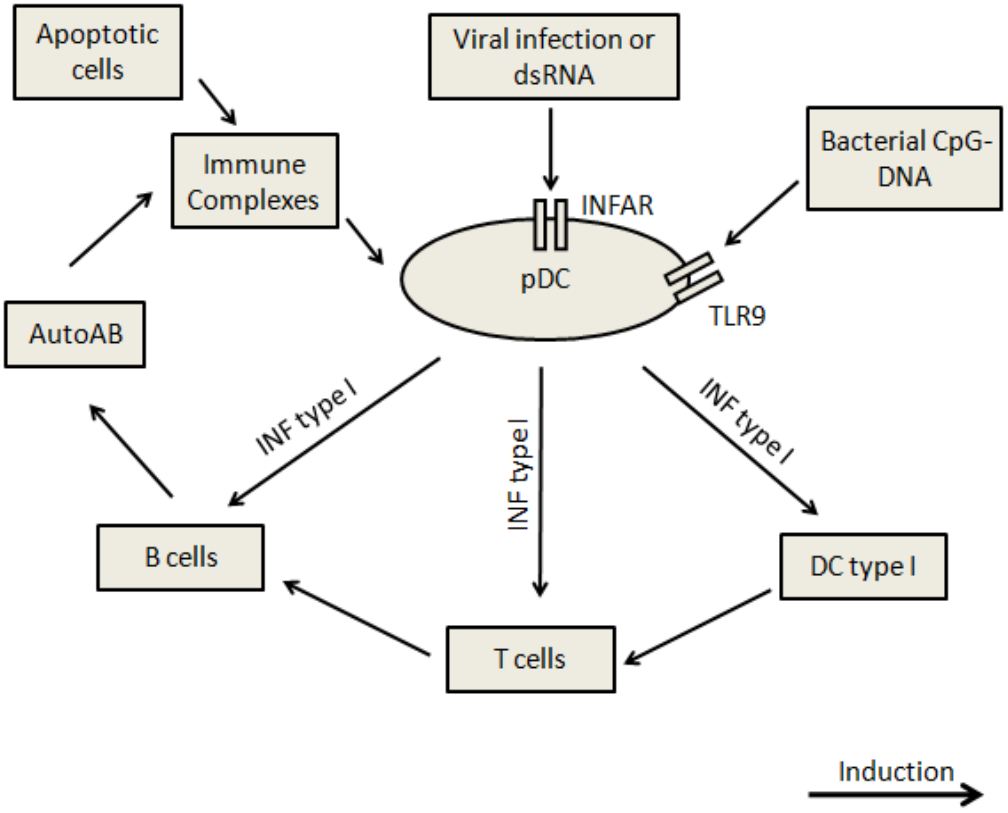
Figure 1: Inducers of type I INFs



IFNs type I are produced by plasmacytoid dendritic cell (pDC) as a consequence of viral or double-strande RNA (dsRNA) and also by bacterial CpG-DNA. INFs type I produced promotes denditric cells 1(DC1) development, T cell activation and autoantibody production by B cells. Apoptotic bodies and autoantibodies form immune complexes (ICs) that act as endogenous IFN-α inducers and cause a prolonged IFN-α production.

8.2 Artigos publicados

8.2.1Apêndice 3- Artigo publicado na revista Cytokine

The role of Tumor Necrosis Factor alpha (TNF-α) in the pathogenesis of systemic lupus erythematosus

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Running title: **TNF-α in SLE**

Keywords: Tumor necrosis factor alpha (TNF-α), polymorphism, mRNA, systemic lupus erythematosus

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Abstract

The Tumor Necrosis Factor alpha (TNF-α) is a pleiotropic cytokine that produces different stimuli in various physiological and pathological conditions. TNF-α contributes importantly to the development of T cells, B cells, and dendritic cells. However, TNF-α is also a potent inflammatory mediator and apoptosis inducer. The significance of the TNF-α involvement in the pathogenesis of systemic lupus erythematosus (SLE) remains controversial. From the genetic standpoint, a number of studies suggest that the TNF-α gene polymorphism is involved in the susceptibility of SLE. Moreover, there is a close association between the TNF-α gene expression and clinical manifestations. In addition, the increased serum level of TNF-α is observed in SLE patients and associated with disease activity and certain systemic manifestations. Treatment with anti-TNF agents is, however, controversial in SLE since induction of antinuclear antibodies, anti-dsDNA, anticardiolipin antibodies, and cases of drug-induced lupus have been observed in rheumatoid arthritis patients. In this context, this study reviewed the importance of TNF-α in the pathogenesis of SLE.

Introduction

Systemic lupus erythematosus (SLE) is systemic autoimmune disease in which a complex interaction between the innate and adaptive immune system is observed (1). A variety of abnormalities in cellular and humoral immune responses has been reported in both murine lupus models and SLE patients (2, 3). The immunopathology of SLE has traditionally been attributed to the tissues and organs' deposition of immune complexes and/or autoantibody-mediated damage. Although these mechanisms account for an important component of the inflammation observed, previously published data suggest that cytokines are also involved in tissue damage (3).

Cytokines are low-weight soluble proteins that are produced by different cells in the innate and adaptive immune system. They mediate activation or functional regulation of the immune system by binding to cell surface receptors. They play a pivotal role in the differentiation, maturation, and activation of various immune cells (1,3).

In SLE, these molecules are probably the product of endogenous or exogenous triggers of the autoimmune response, as well as the effort of the immune system cells to gain control over its activated component (1, 4). In addition, the cytokine profile may determine some of the dysfunctional aspects of the immune system and the involvement of organ systems. SLE is a heterogeneous disease regarding presentation, as for disease severity, response to treatment, and organ damage, among others. Different cytokine profiles may account for these variations observed in the clinical practice (1).

The knowledge of the cytokine profiles in SLE not only provides new insight into the pathogenesis of SLE but also sheds light on various clinical applications. Some cytokines, such as interleukin 6 (IL-6), interleukin 10 (IL-10), interferon alpha (INF-α), and tumor necrosis factor alpha (TNF-α) can serve as biomarkers to monitor disease

activity and predict disease severity (1, 5, 6). In addition, the manipulation of these cytokines may represent a potential therapeutic strategy for the treatment of SLE (1, 7).

The TNF-α may promotes a derangement in the immune regulation and could be the one factor potentially responsible for autoantibody induction. However, TNF-α is the most important proinflammatory cytokine, it is directly involved in apoptosis (8), and in the pathogenesis of several rheumatic diseases, such as rheumatoid arthritis (9). In SLE, the role of TNF-α is less clear. Therefore, the role of TNF-α in the pathogenesis of SLE was reviewed in the present study.

The physiological role of TNF-α

Understanding how the immune system integrates the pleiotropic properties of TNF-α is a challenge, particularly so in diseases like SLE. TNF-α has immunoregulatory and proinflammatory functions on a range of cells in the innate and adaptive immune system and is directly involved in apoptosis (8). Analyzes of the immunoregulatory functions and the different effects of TNF-α on B cells, T cells, and dendritic cells need to be considered (8) (Figure 1).

TNF-α is expressed as a trimer on the cell's surface and is found in a soluble form after the activation of macrophages and dendritic cells (1). Both, membrane-bound TNF and secreted TNF cooperate with the lymphotoxin development of secondary lymphoid organ structures, such as in the lymph nodes and Peyer's patches (8, 10-12). Deficient TNF production leads to the absence of both germinal centers and follicular dendritic cells (8, 10-12).While membrane-bound TNF confers the major structure of secondary lymphoid organs, soluble TNF appears to be involved in the generation of primary B-cell follicles (13).

In addition, TNF is a growth factor for B lymphocytes inducing the production of IL-1 and IL-6 (14). However, B lymphocytes are able to produce significant amounts

of TNF in an autocrine loop (15-17). Therefore, low levels of TNF-α are associated with abnormal lymphoid organogenesis and aberrant B-cell responses.

Furthermore, studies on normal human T lymphocytes demonstrated that TNF-α also plays a role in the T cell responses (18). Recombinant human TNF-α was demonstrated to enhance T cell proliferation in response to a variety of stimuli such as IL-2, alloantigen, or phorbol esters (18). Moreover, through nuclear factor kappa B (NF-κB) activation, TNF-α promotes upregulation of the major histocompatibility complex (MHC) molecules, interferon gamma (IFN-γ) production, and TNF receptor 2 (TNFR2) (18-20).

A distinction has been observed between short-term and long-term exposure of TNF-α on T-cells (8). Short-term stimulation of activated T lymphocytes with TNF-α results in further activation and proliferation of T-cells, and increased production of IFN-γ (18,2,22). Long-term TNF-α exposure induces a reversible loss of the surface T-cell receptor complex, leading to hyporesponsiveness of T-cell, without affecting the IL-2-mediated cell proliferation (8, 23, 24). This hyporesponsiveness can be reversed with anti-TNF therapy (24).

In SLE, the numbers of both myeloid and plasmacytoid dendritic cells (DC) are reduced in the peripheral blood. This reduction correlates with increased levels of soluble TNF receptors, thus suggesting the potential role of TNF in the observed DC alterations (6, 25,26). Immune complexes induce macrophages to produce high levels of TNF-α (27) and SLE is an immune complex-mediated disease showing large amounts of immune complexes deposited in tissue, especially in the glomeruli. Therefore, it has been suggested that the TNF-α expression observed in the tissues of SLE patients is associated with inflammation and consecutive tissue injury (8).

TNF-α binds to two cell-surface receptors (TNFR1 and TNFR2) (28). TNFR1 mediates most of the biological properties, such as apoptosis and activation of NF-κB (29) (Figure 2). Upon oligomerization, TNFR1 binds to the TNFR-associated death domain (TRADD), which serves as a platform to recruit at least three additional mediators, the receptor-interacting protein 1 (RIP-1), Fas-associated death domain (FADD), and TNF receptor-associated factor-2 (TRAF-2). TNFR1 transduces apoptotic and anti-inflammatory signals through the recruitment of FADD and subsequent recruitment and activation of Caspase 8, resulting in apoptosis (8, 30). TNFR1 also mediates anti-apoptotic and inflammatory responses through the recruitment of TRAF-2 and RIP-1 (15). In contrast, TNFR2 lacks a death domain and interacts directly with TRAF-2. TRAF-2 activates transcription factors such as NF-κB and stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), thereby promoting cell survival and differentiation as well as immune and inflammatory responses (30,31).

Thus, TNFR1 is involved in both apoptotic and anti-apoptotic signaling, whereas TNFR2 is involved only in the anti-apoptotic effect of TNF. In addition, TRADD, FADD, RIP-1, and TRAF-2 are important molecules in the apoptosis and in the inflammatory signaling pathways of TNF-α (32). Interestingly, the intracellular death-domain of TNFR1 tends to self-associate and support the receptor clustering, which unless prevented by intracellular factors leads to cell death. This indicates that there are additional levels of regulation (17).

TNF-α is, therefore, involved in apoptosis by two different mechanisms: a. via TNFR1 which contains an intracellular 'death-domain', or by TNF-α receptors (TNFR1 and TNFR2) in parallel using a synergistic combination of both, distinct, signaling pathways (8, 16,17).

Confusion regarding the mechanisms of action of TNF-α is due in part to the considerable range of physiological effects potentially mediated by this cytokine. TNF-α presents cytotoxic effects on different cell types while it modulates different activities including the induction of other cytokines and in particular the regulation of vascular adhesion and MHC molecules (16).

TNF-α in SLE

Animal studies

TNF-α has been studied in several animal models such as the New Zealand Black mice especially the New Zealand White (NZB/W) (33), Medical Research Laboratory lymphoproliferation mice (MRL/*lpr*) (41), and C3H.SW mice (37).

Previous findings showed diminished production of TNF-α in NZB/W mice associated to the development of severe disease manifestations, such as nephritis (33). TNF-α competent NZB/W mice only show modest lupus activity (34), thus, suggesting that the TNF-α deficiency is an important trigger for the disease and an important driver of lupus-like autoimmunity in this strain (31). NZB/W mice that received relatively high dosages of TNF-α early in the life showed a delay in the disease onset; however, this did not prevent the disease (33, 35, 36). Furthermore, the application of TNF-α after the disease onset proved to be harmful, and the anti-TNF treatment is of therapeutic benefit in lupus-prone mice (37,38). Although preliminary findings were interpreted as showing a protective effect of TNF-α against autoimmunity; the latter leads to the hypothesis that TNF-α stimulates the destruction of organs already affected during the pathogenesis of SLE (39,40). Another study showed similar beneficial effects of high-doses of TNF-α, even after nephritis had developed, however, with no long-term protection against the disease (36). Even in the NZB/W mice, in which TNF-α was thought to be protective, the cytokine presented a double role–beneficial and detrimental (36,41).

In contrast to the findings in NZB/W mice, *Tnf* is highly over expressed in both sera and renal tissue of MRL/*lpr* lupus mice and the levels of TNF-α correlate with the degree of inflammatory organ disease (41). The interaction of the *lpr* gene and the MRL strain background causes autoimmune renal disease. Neither the *lpr* gene alone nor the MRL background causes the fulminant autoimmune renal injury observed in MRL/*lpr* mice (41). In MRL/*lpr* mice, loss of renal function occurs at three to four months of age and progresses rapidly resulting in death at five to six months of age. Anti-TNF therapy has been shown to be beneficial in MRL/lpr lupus (41).

TNF blockade improved arthritis, pneumonitis, nephritis and leukopenia in additional lupus models such as in the C3H.SW mice (37). Experimental SLE was induced in naive C3H.SW mice by injection of the human anti-DNA monoclonal antibody (mAb) bearing the common idiotype, 16/6 Id. Two weeks after booster injections, treatment with either an anti-TNF-α mAb, or pentoxifylline (PTX) was started, for a period of 6 weeks. The production of TNF-α was determined 3 and 7 months after disease induction, and the experimental mice were also followed for disease manifestations. Both treatment protocols, with anti-TNF-α mAb and PTX, reduced the production of the two pro-inflammatory cytokines and the anti-dsDNA antibodies were significantly lower in mice treated with either protocol. Abrogation of TNF-α production in the early stages of experimental SLE by an anti-TNF-α mAb or PTX improves the clinical status of mice afflicted with this autoimmune disease (37).

Human studies

There is substantial evidence showing that TNF-α has a similar proinflammatory role in SLE patients based on studies that analyzed the TNF-α gene polymorphism (42), gene expressions (43), and TNF-α serum levels (44).

Genetic susceptibility to SLE

SLE has a strong genetic component, with a concordance of disease in 24–50% of monozygotic twins compared with 2–5% of dizygotic twins and siblings (45). From the genetic standpoint, a number of association studies suggest the involvement of TNF-α gene polymorphism in the susceptibility to SLE (Table 1). Most of the studies were performed using microsatellite and single-nucleotide polymorphism (SNP) in the promoter regions at positions –308 (46-65) and –238 (52, 57, 58, 60, 66-68).

The TNF-α is an immunologically relevant gene in the human leukocyte antigen (HLA) region encoding a proinflammatory cytokine. Five microsatellite markers have been described in the TNF region; the TNFa and TNFb, located upstream of the TNF-β gene, the TNFc, in the first intron of the TNF-β gene, and the TNFd and TNFe, downstream of the TNF-α gene (69). The TNF microsatellites a2, b3, and d2 alleles have been associated with photosensitivity and Raynaud's phenomenon (69).

Some genetic studies observed an association between specific polymorphisms and clinical features (47, 66, 69). Malar rash, discoid rash, oral ulcers, serositis, and hematological disorders were associated with the −308A/G polymorphism in a Taiwanese cohort (62). Raynaud's phenomenon has been associated with the –863A polymorphism a Thai cohort (66).

Studies in the TNF-α promoter polymorphism (-308) and with the TNFd1 allele determined susceptibility to SLE in different ethnic groups, including Caucasoid, South African, and Asian SLE patients (57,62,67-75). However, the associations with clinical or laboratory characteristics of the disease are still unclear.

Abnormal expression of the TNF-α gene mRNA in SLE patients

Many studies have described an abnormal expression of TNF-α in peripheral blood mononuclear cells (PBMC) and in bone marrow cells from SLE patients (15, 43,76-78) (Table 2). All of these studies were based on small cohorts and analyzed the

TNF-α gene expression regarding disease activity (14, 43,76-78). The expression of mRNA for the TNF adapter molecules TRADD, FADD, RIP-1, and TRAF-2 has been studied in one single study and correlated negatively with SLE disease activity (15).

Serum levels of TNF-α

Several studies have analyzed the TNF-α levels in SLE patients (5,6, 44,77-82). In these studies, TNF-α was found to be markedly increased when compared to healthy controls (5,6, 44,77-82). Although most studies have shown increased TNF-α levels in the sera, the clinical significance of this increase is less clear (77-82). Several studies analyzing the onset of SLE in adults showed higher TNF-α levels in SLE patients with active disease (5, 6, 44,79). However, in one pervious study, the TNF-α levels were higher in patients with inactive disease compared with patients with very active disease and controls, suggesting that, TNF-α could also be a protective factor in SLE patients (80).

TNF-α was found to be high in glomeruli, in all forms of lupus nephritis, and the level of TNF-α expression was correlated with renal inflammatory activity (6,83). SLE nephritis is a prototype of immune-complex induced kidney damage (84,85). High TNF-α levels were also observed in other non-SLE nephropathies, including membranous nephropathies and nephritic syndromes (86-88). These findings support the hypothesis that TNF-α may play a pathogenic role in the induction or maintenance of glomerular barrier dysfunction in renal diseases (88).

Although the primary mechanism in the pathogenesis of proteinuria in SLE is considered to be the deposition of immunoglobulins along with components of the complement system on the epithelial side of the glomerular basement membrane, a contributory role of cellular immunity is also implied by several studies (89,90). This role is supported by the evidence of increased expression of TNF-α in the glomeruli,

high urinary levels, and activation of the complement cascade together with and without certain TNF gene polymorphisms in the affected patients (86-88).

TNF-α is a major proinflammatory cytokine produced in response to various stimuli including glomerular and mesangial cells (91). Circumstantial data suggest that it may serve as important autocrine and paracrine factors in glomerular injury. Its effect on mesangial and glomerular epithelial cells and on the secretion of several mediators is of interest, particularly in the nephrotic syndrome, where the epithelial cell damage is the key pathologic finding (86,88). Accordingly, *in vitro* data suggest that TNF-α may have cytotoxic activity affecting cell adhesion to human endothelial cells (88).

TNF-α inhibitors

TNF-blocking therapy for SLE is highly controversial. The main concerns derive from data from experimental models and the induction of antinuclear antibodies (ANA), anti-dsDNA, and anticardiolipin antibodies (aCL) in cases of drug-induced lupus–like syndromes in patients treated with anti-TNF agents (92). Agents like etanercept, infliximab, and adalimumab have been approved for the treatment of rheumatoid arthritis, but have not demonstrated complete efficacy in SLE yet. Studies indicated that increased availability of apoptotic antigens after anti-TNF-α treatment might play a role in the autoantibody formation induced by the blockade (92).

Not all SLE related clinical manifestations respond to anti-TNF therapy. SLE patients with arthritis and nephritis are most likely to benefit from anti-TNF therapy when compared to patients with other clinical manifestations (92). However, larger and controlled clinical trials are needed to properly address the potential value of the TNF-blocking therapy in SLE.

The new anti –TNF therapies may provide an advantage to achieve rapid disease control and to minimize the corticosteroid usage. It is of interest that this autocrine

TNF production is induced by ligation of CD40, a potential therapeutic target in SLE (93,94), and is prevented by cyclosporin A, another therapeutic agent for SLE (95).

However, the role of these anti-cytokine treatments in the maintenance phase remains unclear (92). The long-term use of these cytokines as therapeutic targets may seem attractive, yet the possibilities of complications such as infection and malignancy must be considered.

Conclusion

In summary, TNF-α participates in the autoimmune process in diverse ways. A better understanding of the signaling mechanisms mediated by TNF-α should eventually lead to the development of small molecules that could successfully inhibit and modulate the biological activity of this cytokine in autoimmune diseases.

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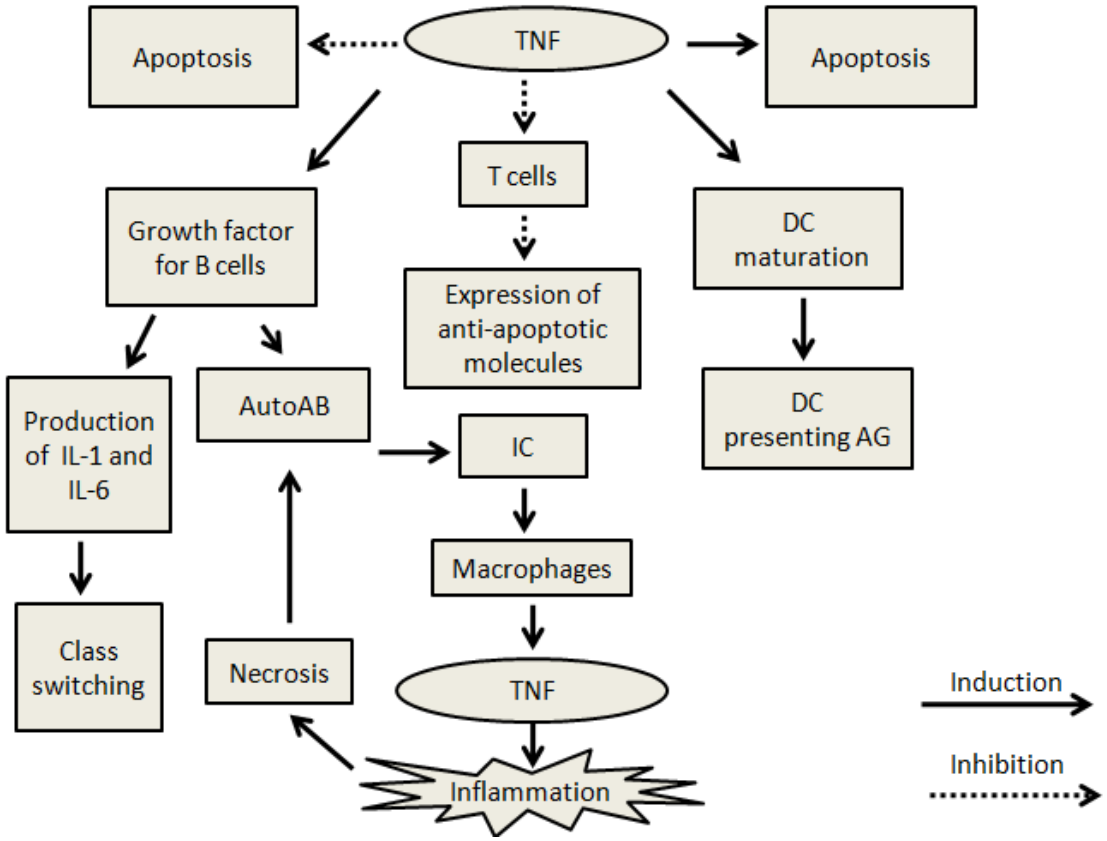
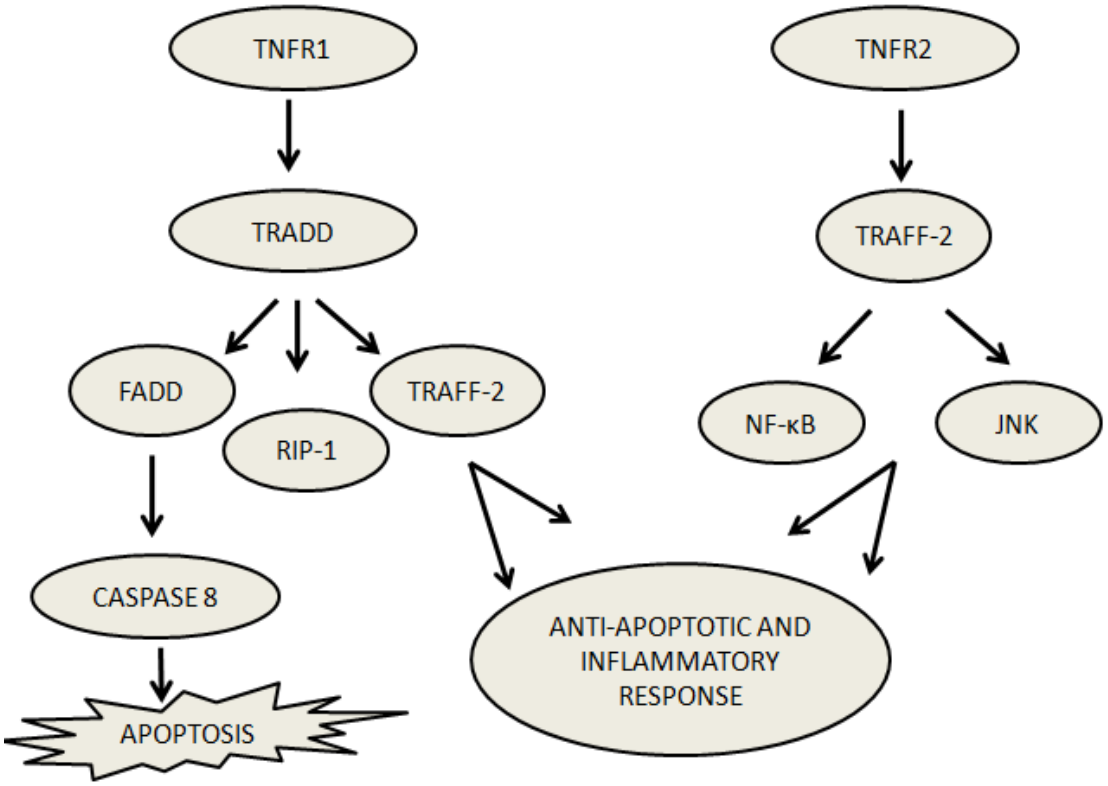
Figure 1: The immunoregulatory functions of TNF



Tumor necrosis factor (TNF) acts as a growth factor for B cells, inducing the production of IL-1 and IL-6. This stimulates the class switching, involved in the antibodies production. TNF leads to T-cell hyporesponsiveness and to the expression of anti-apoptotic molecules. Moreover, it may promote dendritic cell (DC) maturation, which acts as antigen-presenting cells. Immune complexes (IC) are formed by autoantibodies (autoAB) and antigens. These complexes stimulate macrophages to express TNF, promoting inflammation. The inflammation can be a source of cell death (necrosis), inducing the formation of new autoAB.

Figure 2: TNF surface receptors



There are two cell-surface receptors (TNFR1 and TNFR2). TNFR1 mediates apoptosis recruiting the TNFR-associated death domain (TRADD), which serves as a platform to recruit the receptor-interacting protein 1 (RIP-1), Fas-associated death domain (FADD), and TNF receptor-associated factor-2 (TRAF-2). Subsequently, the recruitment and activation of Caspase 8 results in apoptosis. TNFR1 also mediates anti-apoptotic and inflammatory responses through the recruitment of TRAF-2 and RIP-1. In contrast, TNFR2 interacts directly with TRAF-2, which activates NF-κB and JNK, thereby promoting anti-apoptotic and inflammatory responses.

Table1: TNF-α promoter polymorphisms associated with susceptibility to SLE

Polymorphisms analyzed	References	Country	Sample Size	
			SLE patients	Healthy controls
–308 A	Fugger *et al*., 1989 (48)	Denmark	20	131
	Tomita *et al*., 1993 (49)	Japan	20	23
	Wilson *et al*., 1994 (50)	UK	81	168
	Danis *et al*., 1995 (51)	Australia	40	57
	Rudwaleit *et al*., 1996 (52)	UK South Africa	49 49	96 81
	Sullivan *et al*., 1997 (53)	USA (African Americans only)	88	64
	Wang *et al*., 1999 (54)	China	89	70
	Rood *et al*., 2000 (46)	The Netherlands	99	177
	van der Linden *et al*., 2001 (55)	The Netherlands	91	253
	May *et al*., 2002 (56)	Australia	47	44
	Parks *et al*., 2004 (57)	USA (Caucasians only)	86	203
	Azizah *et al*., 2004 (47)	Malaysia	70	59
	Correa *et al*., 2005 (58)	Colombia	100	430
	Suarez *et al*., 2005 (59)	Spain	192	343
	McHugh *et al*., 2006 (60)	UK	157	245
	Jiménez-Morales *et al*., 2009 (58)	Mexico	725	400
	Lin et al., 2009 (61)	Taiwan	154	154
	Santos et al., 2011 (65)	Portugal	115	152
–308	Goldstein & Sengar, 1993 (71)	Canada	91	91
	D'Alfonso *et al*., 1996 (67)	Iran	123	174
	Fong et al., 1996 (72)	China	67	89
	Chen *et al*., 1997 (68)	Taiwan	100	107

	Wang *et al*., 1998 (73)	China	51	187
	Zuniga *et al*., 2001 (64)	Mexico	51	55
	Parks *et al*., 2004 (57)	USA (African Americans only)	144	73
	Tobon et al., 2005 (74)	Colombia	113	65
	Guarnizo-Zuccardi et al., 2007 (63)	Colombia	120	102
	Lin *et al*.,2010 (75)	Taiwan	172	215
–238	D'Alfonso *et al*., 1996 (67)	Iran	123	174
	Rudwaleit *et al*., 1996 (52)	UK South Africa	49 49	96 81
	Chen *et al*., 1997 (68)	Taiwan	100	107
	Parks *et al*., 2004 (57)	USA (African Americans only) USA (Caucasians only)	144 86	73 203
–238G	Correa *et al*., 2005 (58)	Colombia	100	430
	McHugh *et al*., 2006 (60)	UK	157	245
	Hirankarn et al.,2007 (66)	Thailand	154	154
TNFa2, b3, d2	Hajeer *et al*., 1997 (69)	UK	91	109
TNFd1	Schotte *et al*., 2005 (70)	Germany	206	157

Table 2: TNF-α mRNA expression in SLE patients

References	Patients (N)	Controls (N)	Country	Associations
Zhu et al., 2007 (15)	51	17	China	The expression of mRNA for TNF adapter molecules TRADD, FADD, RIP-1 were negatively correlated with the SLE activity index (SELENA-SLEDAI).
Alvarado-de la Barrera et al., 1998 (76)	8	8	Mexico	The abnormal expression of genes regulating cell growth was correlated with the presence of auto reactive cells in the secondary lymphoid organs and peripheral blood of SLE patients.
Lee et al., 2009 (43)	11	6	Japan	TNF may repress the abnormal regulation by IFN-α in SLE whereas IFN-γ may have a synergistic effect.
Pitidhammabhorn et al., 2006 (77)	47	29	Thailand	The severity of SLE was found to correlate with the percentage of PBMC apoptosis. The degree of apoptosis correlated with the level of TNF-α in plasma.
Wozniacka et al., 2008 (78)	14	0	Poland	Significantly lower expression of IL-1b, IL-6, and TNF-α mRNAs was observed in irradiated skin samples after 3 months of chloroquine treatment.

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8.2.2 Apêndice 4- Artigo publicado na revista Clinics

Clinical and serologic manifestations associated to Interferon-α levels in childhood-onset systemic lupus erythematosus

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Abstract

Objective: To determine serum levels of interferon alpha (INF-α) in childhood-onset SLE patients, first-degree relatives and healthy controls. To elucidate their association with disease activity, laboratory and treatment features. Methods: We screened consecutive childhood-onset SLE patients followed in a longitudinal cohort at the pediatric rheumatology unit at the State University of Campinas between 2009/2010. All patients had disease-onset before the age of 16. Childhood-onset SLE were assessed for disease activity [SLE Disease Activity Index (SLEDAI)], damage [Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI)]. INF-α levels were measured by enzyme-linked immunoabsorbent assay (ELISA). Results: We included 57 childhood-onset SLE patients (mean age 17.33±4.50), 64 first-degree relatives (mean age 39.95±5.66) and 57 healthy (mean age 19.30±4.97) controls. Sera INF-α levels were significantly increased in childhood-onset SLE when compared to first-degree relatives and healthy controls. INF-α levels were significantly increased in patients with positive dsDNA antibodies, patients with cutaneous vasculitis, patients with new malar rash and in patients without medication. INF-α levels correlated with C3 levels, SLEDAI scores. In addition, we found an inverse correlation between patients' age and INF-α levels. Conclusion: INF-α may play a role in the pathogenesis of childhood-onset SLE, especially in cutaneous manifestations and dsDNA formation. Increased INF-α levels in patients not taking medication has to be followed in longitudinal studies to determine if rise in INF-α levels may predict SLE flares.

Introduction

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by diverse clinical manifestations that range from malar rash to renal involvement (1,2). Disorders in the immune system and abnormalities in cytokine productions have been described in patients with SLE (1). The pathogenesis of SLE is multifactorial and likely driven by a complex combination of genetic risk factors and environmental influences, which lead to an irreversible break in immunologic self-tolerance (3).

Around 20% of all SLE patients have disease onset prior to the age of 16 (4). Childhood-onset SLE has a different phenotype than adult-onset SLE. Renal (50% to 67%), neurological (22-95%) and hematological (77%) involvement, in addition to fever and lymphadenopathy are more frequently observed in children when compared to adult-onset SLE (4,5-11). In addition, childhood-onset SLE has a significantly more active disease not only at disease onset, but also over time when compared to adult-onset SLE (9). The outcome of childhood-onset disease is, however, worse than for adult-onset disease (4,12). The awareness that SLE in childhood is a potentially fatal disease, that atypical presentations are very common, and that aggressive treatment should be introduced early in the course of the disease, has significantly improved survival in the childhood-onset SLE cohorts (13,14).

There is strong evidence supporting the role of cytokine in the pathogenesis of SLE (15, 16). The first documented cytokine abnormality in SLE was an increased serum level of interferon (IFN), subsequently characterized as IFN-α, and produced mainly by leukocytes (17). Raised serum levels of IFN-α has been observed in adult-onset SLE, and levels correlate with both disease activity and severity (15, 17, 18). Associations between IFN-α levels and several markers of immune activation were also

observed, such as complement activation and double-stranded DNA (dsDNA) antibody titers (15). However, the role of INF-α in childhood-onset SLE has never been investigated.

The aim of our study was to determine the serum levels of INF-α in childhood-onset SLE patients, first-degree relatives and healthy controls. In addition, we evaluated the association of INF-α with disease activity, laboratory and treatment features.

Patients and methods

Subjects

Fifty-seven consecutive childhood-onset SLE patients followed at the Pediatric Rheumatology Outpatient Clinic of State University of Campinas were invited to participate in this cross-sectional study. Patients were included in the present study if they: (i) fulfilled at least four criteria of American College of Rheumatology (ACR) (19); (ii) were below 16 years of age at disease onset; and (iii) had a follow-up duration of at least 6 months.

Sixty-four first-degree relatives and 57 healthy controls without history of any chronic disease (including auto-immune diseases) were included as a control group. The healthy controls were matched for age, sex and demographic background. This study was approved by the ethics committee at our institution, and informed written consent was obtained from each participant and/or legal guardian.

Clinical features

All patients had their medical histories, clinical and serological characteristics entered at the time of SLE diagnosis in special computer database programs. Features included in this protocol were age at onset of disease (defined as the age at which the first symptoms clearly attributable to SLE occurred), age at diagnosis (defined as the age when patients fulfilled four or more of the 1982 revised criteria for the classification of SLE (19), and follow-up time (defined as the time from disease onset until May 2010).

All clinical manifestations and laboratory findings were recorded at disease onset, on a quarterly basis on follow-up and at the day of blood withdrawal. Nephritis was diagnosed on the basis of proteinuria exceeding 0.5 g/L with abnormal urinary sediment and/or histological findings. Nephrotic syndrome was defined as proteinuria in

excess of 3 g/day. Hematological alterations were ascribed to lupus only in the absence of bone-marrow suppression (leukopenia <4000 cells/mm^3; thrombocytopenia <100,000 cells/mm^3; hemolytic anemia). We also analyzed the presence of malar rash, discoid lesions, subacute cutaneous lesions, cutaneous vasculitis, photosensitivity, oral ulcers, arthritis and serositis. Neurological and psychiatric involvement was defined according to ACR (20).

Treatment prescribed at time of blood withdrawal, as well as its adverse events related to drug use, was recorded. Doses of oral and parenteral corticosteroids were analyzed and converted to the equivalent doses of prednisone.

Laboratory studies

Antinuclear antibodies (ANA) were determined by indirect immunoflurescence using mouse liver as substrate, and regarded as positive if higher than 1/80. dsDNA antibodies were determined by indirect immunoflurescence using *Crythidia* as substrate and considered positive if higher than 1:10. Precipitating antibodies to extractable nuclear antigens (ENA), including Ro (SSA), La (SSB), and Sm were detected by a standardized enzyme-linked immunosorbent assay (ELISA) method, and considered positive if higher than 1: 80. Rheumatoid factor (RF) was detected by nefelometry, and regarded as positive if higher than 10. Anticardiolipin antibodies (aCL) of the IgG and IgM isotypes were measured by an ELISA method (21). The lupus anticoagulant (LA) activity was detected by coagulation assays in platelet-free plasma obtained by double centrifugation, following the recommendation of the subcommittee on LA of the Scientific and Standardization Committee of the International Society of Thrombosis and Homeostasis (22). These measurements were carried out twice, at an interval of 12 weeks.

Disease Activity/Cumulative Damage Evaluation

Disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (23). SLEDAI consists of 24 weighted items grouped into 9 domains, or organ systems, as follows: central nervous system (assigned a weight of 8), vascular (weight of 8), renal (weight of 4), musculoskeletal (weight of 4), serosal (weight of 2), dermal (weight of 2), immunologic (weight of 2), constitutional (weight of 1), and hematologic (weight of 1). SLEDAI scores range between 0 and 105. Score of ≥3 was considered active disease (24).

Cumulative SLE-related damage in all patients was determined using the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI) at time of blood withdrawal. SDI score range from 0 to 47 (25).

INF-α assay

Peripheral venous blood was collected from each subject and allowed to clot at room temperature for 30 min. Samples were then centrifuged for 15 min at 3000 rpm. Separated sera were kept in aliquots at −80°C until the time of assay. None of the samples was taken during an episode of acute or chronic infection (26).

Commercially available kits from R&D Systems (London, UK) were used for the measurement of serum INF-α levels by ELISA, carried out in accordance with the manufacturer's instructions.

Statistical analysis

Analysis of variance with Tukey's pairwise post hoc comparisons were used to compare INF- α levels between groups. Spearman's correlation was used to correlate continuous variables (e.g. INF- α levels and SLEDAI, SDI). INF- α levels and categorical variables were compared by 2-sample t-test. For all analyses, a p-value < 0.05 was considered statistically significant.

Results

Demographics

We included 57 consecutive childhood-onset SLE patients. Fifty-four (94.7%) were female with mean age of 17.33 years [Standard deviation (SD) ±4.50 years; range9-37]. Disease duration was 4.71 years (SD±4.57; range 0-26 years). Sixty-four first-degree relatives with a mean age of 39.95 years (SD±5.66; range 28-52) were included. The control group consisted of 57 healthy volunteers (52 women) with a mean age of 19.30 (SD±4.97 years; range 6-30 years) (Table 1).

Patients and healthy controls were statistically comparable in terms of age and sex.

Clinical, laboratory, and treatment features

At time of study entry, 30 (52.6%) childhood-onset SLE patients had active disease (SLEDAI ≥3) with mean SLEDAI scores of 8.37 (SD±3.80, range 3-18). Inactive patients [N=27 (47.4%)] had a mean SLEDAI scores of 0.39 (SD±0.80 range 0-2)]. Active nephritis (28.3%), new malar rash (6.6%), new alopecia (5.0%) and cutaneous vasculitis (5.0%) were the clinical manifestations more frequently observed.

At time blood withdrawal, 8 (13.3%) patients were without any medication. Thirty-nine (68.4%) patients were receiving prednisone, 32 (53.3%) hydroxychloroquine and 22 (36.6%) patients were receiving other immunosuppressive drugs (Table 1).

Cytokine assay

The mean serum INF-α level was 13.84±8.46 pg/mL in childhood-onset SLE, compared to 10.36±6.04 pg/mL (p=0.012) in first-degree relatives and 11.68±6.66 pg/mL in healthy controls (p=0.043). No difference between first-degree relatives and healthy controls was observed (p=0.484) (Figure 1a).

INF-α levels were significantly increased in patients with positive dsDNA antibodies (p=0.011) (Figure 1b), patients with cutaneous vasculitis (p=0.001) patients with new malar rash (p=0.032) and disease activity (p=0.031). INF-α levels correlated directly with C3 levels (r=0.34; p=0.032), SLEDAI scores (r=0.43; p=0.012) and indirectly with age (r=- 0.17; p=0.025). INF-α levels were significantly higher in patients without medication (mean=13.01; SD±6.09) when compared to patients on medication (mean=21.59; SD±16.02; p=0.035) (Figure 1c). When analyzing each individual medication, higher levels of INF-α were observed in patients not taking prednisone (mean=20.07; SD±14.65) when compared to patients taking prednisone (mean=12.95; SD±6.19; p=0.042). No association between INF-α levels and other clinical, laboratory variable (hematological or immunological) and SDI scores was observed. No difference in INF-α levels was observed between patients with and without hydroxicloroquine or other immunosuppressants.

Discussion

Cytokines are low-weight proteins that play a key role in immunological dysregulation observed in autoimmune diseases. The increased levels of proinflammatory cytokines are believed to play a key role in the pathogenesis of SLE (27). Higher cytokine levels in SLE patients may promote inflammatory response, apoptosis and autoantibody production that not only initiates, but may also maintain SLE disease activity over time (16,27).

The first documented cytokine abnormality observed in SLE was an increased serum level of INF-α, a cytokine with both antiviral and immunoregulatory functions (17,28). The contributions of IFN-α to SLE can be explained based on several distinct, but related mechanisms. In genetically susceptible individuals, B cell precursors expressing self-reactive antibodies are not removed (15). Probably, due to a mismanagement of naturally occurring apoptotic cells, nuclear material stimulates autoreactive B-cells leading to antibody secretion and the formation of immune complexes. These immune complexes and apoptotic bodies stimulate plasmacytoid dendritic cells to produce INF-α. The latter enhance antigen presentation by denditric cells to T-cells while promoting memory T-cell expansion and survival (15,29).

Increased IFN-α serum levels are often found in SLE patients (3,17,18,30-34). In our study we observed increased serum INF-α in childhood-onset SLE patients when compared to healthy controls and first-degree relatives. Our data supports the results of previous studies that demonstrated higher INF-α levels in serum of adult-onset SLE patients (3,17,18,30-35).

The clinical significance of IFN-α pathway activation in SLE is multifaceted. IFN-α has been implicated in the pathogenesis of the disease, and therefore targeted therapies against IFN-α are currently in clinical trials (36,37). Besides, IFN-α activation

may identify a subset of SLE patients with potential diagnostic, prognostic and therapeutic implications. One of the most important points is the change in IFN-α activity levels may reflect change in disease activity and thus help clinical management of the disease (38). In our study, INF-α was significantly higher in patients with SLEDAI ≥ 3 when compared to patients with inactive disease. Furthermore, we observed a directly correlation between SLEDAI scores and INF-α levels, suggesting that INF-α could be a biomarker for disease activity in childhood SLE. Similar results have been observed in adult-onset SLE (3,17,18,30-35).

Previous studies suggest an important role of IFN-α in the immunopathogenesis of SLE (3,17,18,30-35). There is an association between IFN-α and multiple clinical and serological features of SLE (29, 35). We observed that IFN-α levels were increased in patients with cutaneous manifestations. Our data also showed increased INF-α levels in childhood-onset SLE with positive dsDNA and also a direct correlation between INF-α and C3 levels, however no association with renal disease was observed. Increased expression of IFN-inducible genes (IFIGs) in peripheral blood mononuclear cells (PBMC) has been associated with presence of lupus nephritis and proteinuria, cutaneous manifestations, and presence of anti-Ro, anti-Smith (anti-Sm), anti-RNP, and anti-dsDNA antibodies (32,39). Anti-dsDNA antibodies have been associated with lupus nephritis (40), and studies have linked anti-Ro antibody to lupus-related skin findings (41). It remains unclear whether the association between IFN-α and cutaneous and renal disease manifestations in previous studies is primary, or secondary due to an association between autoantibodies and IFN-α (39). We do not observed associations between INF-α levels and other antibodies like anti-Ro, anti-Sm or anti-RNP.

SLE family members are at higher risk of developing not only SLE, but also other autoimmune diseases (3,31). A heritable predisposition to increased activation of

the IFN-α pathway in SLE families could explain some of the burden of both SLE and non-SLE autoimmunity in the population. Possible genetic variability in endogenous IFN-α signaling has been suggested by the association of single nucleotide polymorphisms (SNPs) in the IFN- α pathway genes INF regulatory factor 5 (IRF5) and non-receptor tyrosine-protein kinase (TYK2) (42-45) with SLE, although the impact of these polymorphisms on IFN-α activity *in vivo* is not known (3,43) . We did not observe differences in serum IFN-α levels between first-degree relatives and controls. However the small number of individuals may have affected the results.

We found an inverse correlation between patients' age and INF-α levels. Similar findings have been reported in adult SLE, as well as in healthy controls, independent of menopause status (31). It is not clear whether higher serum IFN-α activity seen in young SLE patients is a cause or a result of disease activity, but this correlation may explain different clinical and serologic manifestations between childhood-onset and adult-onset SLE patients.

In addition, we observed higher INF-α levels in patients off medication. None of the patients had any evidence of disease activity at time of evaluation. Studies showed a dramatically decreased in the expression of IFN-inducible genes (IFIGs) in patients who received pulse glucocorticoid (GC) therapy (46,47). Data from others suggest that intravenous pulse GC treatment may decrease the numbers of IFN producing cells, transiently reducing the stimulus for IFIG expression (47).

Although previous studies have analyzed INF-α levels in childhood-onset SLE, none of these studies have analyzed clinical and laboratory features associated with increased INF-α levels (31,45). Serum INF-α activity showed to be higher in younger individuals in the SLE family cohorts, and this tendency was accentuated in affected individuals (31). In addition, the other study revealed that childhood-onset SLE patients

are potent inducers of IFN-α (45). Genomic approaches have shown that >95% of childhood-onset SLE displays a "INF signature" as measured by PBMC gene expression profiling (45,48). The PBMC transcriptional signature in childhood-onset SLE corresponds to neutrophil-specific genes, and differential expression of these genes correlates with disease activity (45). Notably, SLE neutrophils undergo accelerated spontaneous apoptosis *in vitro*, and SLE sera induce the apoptosis of healthy neutrophils, both of which correlate with disease activity (49). In addition, IFIG expression signature establishes subgroups of patients with severe SLE characterized by renal disease, complement activation and autoantibody production to RNA associated autoantigens (33, 50).

Genetic association studies of SLE patients identified several genes, amongst which components of the upstream and downstream pathways of INF (mainly type I) are the most frequently found including Signal Transducer and Activator of Transcription 4 (STAT4) and IRF5 (38, 51-53). STAT4 interacts with type I INF receptors and is directly involved in IFN signaling. IRF5 is a transcription factor which induces IFN transcription in response to Toll-like receptor (TLR) signaling. In fact, the IRF5 risk haplotype in SLE patients is associated with high serum IFN-α activity (34). These findings are in accordance with the fundamental observations showed in a previous study which identified gene expression profiling of SLE PBMCs (38). These experiments demonstrate a significant upregulation of INF-regulated gene transcripts in adult and childhood-onset SLE PBMCs (32,34). This characteristic is referred to as the "INF signature" and assessed as a new biomarker for disease activity (38).

In summary, our findings suggest that INF-α may play a role in the pathogenesis of childhood-onset SLE. Higher levels in younger children may explain different clinical and serologic manifestations when compared to older patients. Increased INF-α

levels in patients not taking medication has to be followed in longitudinal studies to determine if rise in INF-α levels may predict SLE flares.

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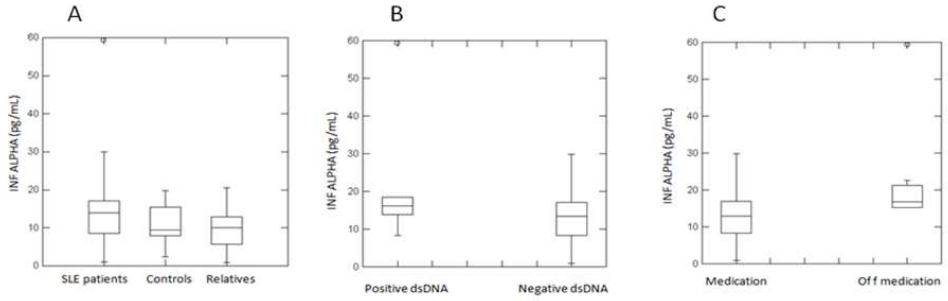
Table 1: Demographic and clinical characteristics of patients and controls included in the study

Parameter	Childhood-onset SLE patients N=57	First-degree relatives N=64	Controls N=57
Sex			
Female	54 (94.7%)	59 (92.18%)	52 (91.22%)
Age (years)	17.33±4.50 (range 9-37)	39.95±5.66* (range 28-52)	19.30±4.97 (range 6-30)
Disease duration (years)	4.71 ±4.57 (range 0-26)	-----	-----
SLEDAI	4.43±4.94	-----	-----
Active disease N=30	8.37±3.80		
Inactive disease N=27	0.39±0.80		
SDI	0.50±0.82	-----	-----
Treatment			
Off medication	8 (14%)		
Prednisone	39 (68.4%)		
Hydroxychloroquine	32 (56.1%)		
Immunosuppressive	22 (38.6%)		
INF-α (pg/mL)	13.84±8.46*	10.36±6.04	11.68±6.66

*P≤0.05

Figure 1. INF-α association in SLE. 1a- Analysis of variance with Tukey's pairwise post hoc comparisons between SLE, 1st degree relatives and controls; 1b-2-sample t-test in SLE patients with positive and negative dsDNA; 1c- 2-sample t-test in SLE patients with/without medication. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the 50th percentile, and the lines outside the boxes represent the minimum and maximum values.



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