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Predictors of outcome and immunological markers in patients with Systemic lupus erythematosus

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Dedicatória

Em memória do meu pai

Aos meus filhos

À minha família

Agradecimentos

Aos doentes,

A cujo bem-estar se dedicam os nossos esforços diários

Ao meu Orientador, Professor Dr. Fernando P. Fonseca,

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A todos os Investigadores colaboradores,

Que tornaram possível a realização destes trabalhos

Resumo

Introdução

O Lúpus Eritematoso sistémico (LES) representa o paradigma das doenças autoimunes multissistémicas, apresentando um largo espectro de manifestações clínicas que potencialmente envolvem quase todos os órgãos e sistemas, com um curso clínico crónico e gravidade clínica variada, entre ligeira a severa e com risco de vida. Existe uma necessidade fundamental por cumprir de identificar preditores de prognóstico que permitam a individualização da monitorização e intervenções terapêuticas para os doentes com LES.

Objetivos

Identificar preditores de prognóstico em doentes com LES, através dos objetivos específicos desta tese: (1) avaliar o desempenho dos critérios de classificação ACR e SLICC para a identificação de doentes com LES; (2) avaliar o efeito dos critérios de classificação preenchidos à data de diagnóstico de LES e outros preditores no prognóstico a longo prazo em termos de dano irreversível e mortalidade; (3) identificar preditores clínicos para agudizações da atividade clínica do LES; (4) contribuir para o conhecimento das relações entre marcadores imunológicos e a atividade clínica do LES.

Métodos

Efetuámos um estudo observacional transversal de 2055 doentes com diagnóstico clínico de LES, seguidos em 17 centros e integrados nos registos nacionais de Portugal e Espanha; a sensibilidade dos critérios de classificação ACR e SLICC foi comparada através do teste de McNemar; a sensibilidade dos dois sistemas de classificação foi ainda analisada em 5 subgrupos definidos de acordo com a duração da doença.

Realizámos um estudo prospetivo de coorte, incluindo 192 doentes com LES avaliados desde a data de diagnóstico e seguidos até 10 anos na *CHUC Lupus Clinic*; analisámos através de regressão multivariada de Cox o prognóstico a 10 anos, em termos de dano irreversível e mortalidade, em grupos definidos de acordo com os critérios de classificação cumpridos à data de diagnóstico (critérios ACR ou apenas os critérios SLICC) e ajustando para potenciais confundidores definidos à data de diagnóstico.

Conduzimos um estudo prospetivo de coorte incluindo 202 doentes com LES seguidos até 24 meses na *CHUC Lupus Clinic* ao longo de 1083 consultas; analisámos potenciais preditores clínicos de agudizações da atividade do LES, aplicando regressão multivariada de Cox ajustada a potenciais confundidores e com estimativa dos *hazard ratios* dos preditores.

Efetuámos estudos transversais incluindo dois grupos de doentes com LES, um com doença clinicamente ativa e outro com doença inativa, recrutados na *CHUC Lupus Clinic* e um grupo de indivíduos saudáveis recrutados no mesmo local; colhemos uma amostra de sangue periférico de cada participante, que foi processada através de protocolos de imunofenotipagem e analisada com citometria de fluxo multiparamétrica, para identificar relações entre marcadores imunológicos em células B, Th17 e NK e a classificação de LES e seus estados de atividade clínica.

Resultados

O estudo transversal do desempenho dos critérios de classificação demonstrou que a sensibilidade para o diagnóstico clínico de LES é mais elevado com o sistema de classificação SLICC do que com o ACR (93,2% e 85,6%, respetivamente, $p<0,0001$). Entre os doentes que não cumpriam os critérios ACR, 62,8% preencheram os critérios SLICC. Os pacientes com duração de doença até 5 anos apresentaram a maior diferença em sensibilidade entre o sistema SLICC e ACR de classificação (respetivamente 89,3% e 76,0%, $p<0001$).

O estudo prospetivo de coorte de LES inicial e seguimento até 10 anos, mostrou que os doentes que preenchiam à data de diagnóstico os critérios ACR de classificação apresentaram ao longo do seguimento mais casos de nefrite lúpica do que aqueles cumprindo apenas critérios SLICC (35,1% e 13,8%, respetivamente, $p<0,01$), mas menos casos de síndrome anti-fosfolípido trombótico (4,5% e 17,2%, respetivamente, $p<0,01$). Os modelos multivariados de Cox não mostraram diferenças entre grupos no risco de dano irreversível nem de mortalidade.

No estudo prospetivo de coorte com seguimento a 24 meses, os modelos multivariados de Cox demonstraram que o risco de agudizações clínicas do LES é mais de 2 vezes, 4 vezes e três vezes mais elevado para os doentes com diagnóstico de LES até aos 25 anos, com nefrite lúpica prévia ou sob terapêutica com imunossupressores à data de inclusão, respetivamente.

Nos estudos transversais de imunofenotipagem, a análise da linhagem de células B demonstrou que a expressão diferencial de BAFFR, CD81 e CD38 nas células B de transição permite a identificação de dois principais *clusters*: o *cluster* 1, que integrou todos os indivíduos saudáveis e 79% dos doentes com LES clinicamente inativo, enquanto o *cluster* 2 incluiu apenas doentes com LES e 82% daqueles com doença clinicamente ativa. A análise das células Th17 mão mostrou diferenças significativas na frequência de Th17 entre o grupo de controlos e o de doentes com LES, nem entre pacientes com doença clinicamente inativa ou ativa. A análise das células NK revelou menor número e frequência de células NK em doentes com LES comparativamente aos controlos, independentemente da atividade clínica da doença; uma frequência mais baixa de células NK $CD56^{dim}$ expressando CXCR3 revelou ser um marcador de LES clinicamente ativo (12,5% e 24,1% no grupo com doença ativa e inativa, respetivamente, $p<0,01$).

Conclusões

Os critérios de classificação SLICC apresentam maior sensibilidade e podem permitir estabelecer a classificação como LES mais precocemente no curso da doença do que o prévio sistema de classificação ACR. Os doentes preenchendo à data de diagnóstico qualquer dos dois sistemas de classificação não apresentam diferenças no prognóstico a longo prazo em termos de dano irreversível nem de mortalidade. Os doentes com diagnóstico de LES até aos 25 anos, com nefrite lúpica ou necessitando terapêutica imunossupressora, apresentam risco mais elevado de sofrer agudizações clínicas do LES; pacientes preenchendo à data de diagnóstico apenas os critérios de classificação SLICC podem apresentar maior risco de síndrome anti-fosfolípido trombótico: estes preditores clínicos fornecem uma base para individualizar estratégias de monitorização e tratamento de doentes com LES.

Os estudos de imunofenotipagem sugerem que os doentes com LES apresentam: hiperatividade de células B, com *clusters* de marcadores imunológicos em subtipos de células B que permitem diferenciar doentes com LES de indivíduos saudáveis e LES clinicamente ativo de inativo; hipoatividade das células NK e anomalias menos consistentes das células Th17. Fizemos prova do conceito que um painel de marcadores imunológicos pode providenciar uma base de validação biológica para definições clínicas de estados de atividade do LES.

Palavras-chave

Lúpus Eritematoso sistémico; Critérios de classificação; Investigação prognóstica; Estudos de coorte; Estados de saúde; Agudizações clínicas; linhagem de células B; Células Th17; Células NK

Resumo alargado em Português

Introdução

O Lúpus Eritematoso Sistémico (LES) é a doença autoimune sistémica mais representativa, apresentando um espectro de apresentações clínicas muito diversificado que compreende praticamente todos os órgãos e sistemas, um curso clínico crónico e grande variabilidade de severidade e prognóstico, de benigno a muito grave e com risco de vida. Na Europa, a prevalência do LES situa-se entre 20 a 97 casos por 100 000.[1-3] Os fatores epidemiológicos de risco para desenvolver LES incluem a idade, sexo, área geográfica de habitação e etnia; os mesmos fatores influenciam o prognóstico clínico.[4, 5] As mulheres são afetadas até 12 vezes mais frequentemente do que os homens, geralmente com início da doença em idade fértil. As manifestações clínicas mostram elevada heterogeneidade entre doentes, bem como no mesmo doente ao longo do curso crónico da doença, o que contribui para que o LES seja uma das doenças mais complexas de prever e tratar.[6, 7] A doença segue habitualmente um curso clínico caracterizado por períodos alternados de agudização e de quiescência. O curso global da doença, a altura em que ocorrerão as agudizações clínicas, bem como a sua gravidade, a resposta aos vários regimes terapêuticos imunossupressores existentes quanto à sua capacidade de prevenir e tratar as agudizações clínicas, são parâmetros prognósticos muito difíceis de prever em populações de doentes e ainda mais no doente individual, tornando extraordinariamente complexo o processo de decisão clínica. Adicionalmente, os doentes com LES apresentam elevado risco de desenvolver comorbilidades, particularmente infeções e complicações cardiovasculares, bem como sequelas irreversíveis nos órgãos envolvidos pela doença.[8] As manifestações do LES requerem frequentemente tratamentos intensivos e prolongados, que se associam a risco elevado de efeitos secundários. O tratamento baseia-se na combinação de hidroxicloroquina (um anti-malárico com efeitos imunomoduladores que o tornam terapêutica basilar do LES), corticosteróides e imunossupressores (sintéticos e biotecnológicos), com grande heterogeneidade de necessidades terapêuticas entre doentes, bem como no mesmo doente ao longo do tempo, devido à variabilidade de envolvimento de órgãos e da respetiva severidade.[9] A efetividade do tratamento do LES é de importância fundamental, uma vez que cada episódio de agudização clínica pode potencialmente causar sequelas e reduzir a esperança de vida.[10, 11] A atividade clínica da doença, o acréscimo de sequelas irreversíveis, as comorbilidades e os regimes terapêuticos são determinantes fundamentais do prognóstico a longo prazo dos doentes com LES.[4, 12] Existe uma necessidade fundamental por cumprir de melhorar a capacidade de previsão do prognóstico clínico, de forma a individualizar as estratégias de monitorização e intervenção terapêutica mais ajustadas ao doente individual com LES.

As referências bibliográficas nesta seção são numeradas de acordo com a ordem de citação no corpo do texto principal da tese.

A etiopatogenia do LES envolve a inter-relação entre genes de suscetibilidade, fatores de risco hormonais e ambientais, que em conjunto promovem anomalias nas células imunes e moléculas envolvidas na apoptose, respostas imunes inatas e adaptativas.[4, 13-17] Estes fatores conduzem à rotura irreversível da tolerância imunológica central e periférica, manifestada por respostas imunológicas aberrantes contra auto-antigéneos nucleares, entre outros.[18] Estudos integrais do genoma de associação genética (*genome-wide association studies* – GWAS) identificaram mais de 40 *loci* genéticos de suscetibilidade para LES.[19-21] A suscetibilidade para a doença conferida por cada gene é limitada, regra geral com um risco relativo inferior a 2.[22, 23] Os genes de suscetibilidade estão ligados a três principais vias imunológicas: (1) a remoção de resíduos celulares apoptóticos; (2) a resposta imunológica inata a ácidos nucleicos através dos recetores *tool-like* (TLR) e subsequente ativação de vias de sinalização dependentes de interferon (IFN); (3) as vias de sinalização linfocitária nas células T e B. Mecanismos epigenéticos estão potencialmente implicados na suscetibilidade para LES e também na atividade imunológica e clínica da doença: estes incluem processos como a metilação de ADN, modificações pós-translacionais de histonas e RNA não codificantes que podem modificar de forma durável a expressão genética.[24] A hipometilação do ADN tipicamente causa hiperexpressão de genes e encontra-se globalmente aumentada em células T de doentes com LES ativo e mais especificamente em genes estimulados por IFN.[13, 25] Em indivíduos geneticamente predispostos, fatores ambientais atuam como desencadeantes da perda de tolerância a auto-antigéneos, conduzindo a autoimunidade. A jusante, os elementos que permitem a perda sustentada de tolerância imunológica, ampliação e manutenção da resposta autoimune são ainda pouco conhecidos. Os processos patogénicos são heterogéneos entre diferentes manifestações da doença, entre doentes e populações étnicas diversas. Um conceito chave na patogenia do LES é o desequilíbrio entre a produção e a eliminação de resíduos celulares apoptóticos: quando a carga de resíduos apoptóticos excede a capacidade de eliminação, estes acumulam-se e podem despertar respostas imunes anormais.[26] O aumento da produção de resíduos apoptóticos pode ser gerado pela exposição à radiação ultravioleta, por infeções e outros fatores de risco ambientais para LES. Anomalias das vias de eliminação de resíduos apoptóticos contribuem também para a falência dos mecanismos que usualmente previnem a ativação imunológica em resposta a resíduos celulares endógenos. Os neutrófilos são elementos chave das respostas inflamatórias que sofrem extrusão do material nuclear, formando redes extracelulares (*neutrophil extracelular traps* – NETs), uma fonte de mais ácidos nucleicos antigénicos no LES.[27] Os ácidos nucleicos (ADN e ARN) contidos nos resíduos apoptóticos podem estimular respostas inflamatórias através da ativação de recetores que reconhecem ADN e/ou ARN, nomeadamente os TLR, que são constituintes do sistema imunitário inato.[13] Os TLR são expressos em células imunitárias, incluindo as células dendríticas, células B e T e os macrófagos, bem como em células não imunes (nomeadamente nas células epiteliais e fibroblastos). Os TLR residem no retículo endoplasmático e incluem os TLR3, TLR7 e TLR8 – que reconhecem ARN – e o TLR9 que reconhece ADN. Os dados experimentais implicam mais solidamente um papel dos TL7 e TLR9 na suscetibilidade ao LES,

com evidência mais limitada sugerindo que os TLR3 e TLR8 poderão estar também implicados.[28] Os modelos conceptuais atuais acerca da patogenia do LES postulam que os TLR desempenham um papel central, através da sensibilização aos ácidos nucleicos endógenos, iniciando o processo de perda de tolerância e desencadeando uma intensa produção de IFN tipo I.[13]

As células dendríticas plasmacitóides (pDC) são as células imunes que produzem a maior quantidade de IFN tipo I após ativação dos TLR. A hidroxicloroquina, que é o fármaco basilar da terapêutica do LES, reduz a ativação dos TLR7 e TLR9 e a subsequente produção de IFN tipo I pelas pDC.[29] O IFN tipo I e outras citocinas promovem a diferenciação das células B e a perda de tolerância imunológica. O *B-cell activating factor* (BAFF, também conhecido por BLyS) é produzido pelas células dendríticas, monócitos e outros tipos celulares e desempenha um papel importante como potente ativador da proliferação, diferenciação e sobrevivência das células B, bem como da sua produção de auto-anticorpos em doentes com LES.[30] A sua expressão é estimulada pelo IFN tipo I. O BAFF solúvel é inibido pelo belimumab, um imunossupressor biotecnológico aprovado e em uso corrente no tratamento da atividade clínica do LES. Após ativação das células B, estas diferenciam-se, expandem-se e produzem auto-anticorpos, que contribuem para a resposta imunológica adaptativa. O IFN tipo I também induz a diferenciação de células dendríticas mieloides (mDC) e a sua apresentação de auto-antigéneos às células T $CD4^+$ em doentes com LES.[31] Desta forma, tanto as pDC como as mDC são consideradas centrais para a patogénese do LES.[13]

Diversas anomalias das células T e B são consideradas potencialmente centrais para a patogenia do LES, incluindo: perda da tolerância de células T; interações disfuncionais entre células T e B, conduzindo à estimulação de células B auto-reativas; diferenciação e expansão de células T produtoras de interleucina 17 (IL-17), uma citocina pró-inflamatória; deficiência e/ou disfunção de células T reguladoras (T_{reg}), que constituem um importante ponto de controlo contra linfócitos auto-reativos; desenvolvimento anómalo de células B, conduzindo à sua perda de tolerância imunológica e aumento da sobrevivência de clones auto-reativos.[13, 32-34] As células B desenpenham um contributo fundamental para o LES, através da sua resposta a auto-antigéneos, com produção de auto-anticorpos que constituem um marcador típico da doença, bem como através de outros mecanismos independentes de auto-anticorpos. Os auto-anticorpos ligam-se a auto-antigéneos, formando imunocomplexos que ativam o complemento e, através de ligação a recetores celulares Fc, promovem inflamação em órgãos alvo, como sejam a pele e os rins. Outras funções potenciais das células B na patogenia do LES incluem: a sua capacidade de apresentar péptidos auto-antigénicos a células T, a promoção de respostas inatas mediadas pela expressão de TLR; a produção de variadas citocinas pró-inflamatórias e reguladoras.[34] Recetores celulares de co-estimulação e respetivos ligandos, como sejam o CD40-CD40L e o ICOS-ICOSL são outros importantes determinantes das interações entre células B e T que também podem estar implicadas na patogenia do LES.[34]

A doença inicia-se com uma fase pré-clínica caraterizada por anomalias autoimunes inespecíficas, prosseguindo mais tarde para uma fase de autoimunidade mais específica da doença.[35] Podem tardar vários anos para que os doentes desenvolvam suficientes anomalias imunológicas e clínicas para permitir o diagnóstico de LES, embora alguns doentes se apresentem desde o início com uma doença bem diferenciada. Devido a esta instalação habitualmente insidiosa, a altura de início da doença é frequentemente incerta e o diagnóstico pode ser tardio, o que se reflete negativamente no prognóstico clínico. O diagnóstico clínico estabelecido por um médico experiente em LES permanece a referência, uma vez que não existem critérios de diagnóstico ou alguma característica patognomónica da doença.[36] O diagnóstico clínico fundamenta-se na identificação de um conjunto de manifestações clínicas e analíticas consistentes com o diagnóstico de LES, que podem surgir quer concomitantemente, quer cumulativamente, considerando em simultâneo a probabilidade de outras explicações alternativas para o quadro clínico.

Foram desenvolvidos critérios de classificação para LES, inicialmente propostos em 1971, revistos e adotados em 1982 pelo Colégio Americano de Reumatologia (ACR) e de novo revistos em 1997, requerendo o cumprimento cumulativo de pelo menos 4 do conjunto de 11 critérios definidos pelo ACR para classificar como caso de LES (tabela 1.1., página 8).[37] Os critérios de classificação foram desenvolvidos para assegurar uma definição de caso consistente de LES, fundamentalmente para efeitos de inclusão em estudos de investigação clínica e translacional e ainda em ensaios clínicos – como tal, destinam-se a ser aplicados a doentes nos quais um diagnóstico clínico de LES foi previamente estabelecido. No entanto, estes critérios são também comumente usados para auxiliar o processo de diagnóstico na prática clínica, apesar das suas limitações e dos potenciais vieses que implica a sua aplicação para esta finalidade.[36] Uma consideração importante é que estes critérios de classificação do ACR foram desenvolvidos e validados em grupos de indivíduos com diagnóstico de LES previamente bem estabelecido e de longa evolução, aumentando o risco de exclusão de doentes com LES mais precoce ou com expressão clínica menos multissistémica.[38]

O grupo de investigadores do *Systemic Lupus International Collaborating Clinics* (SLICC) desenvolveu uma revisão extensa dos critérios de classificação do ACR, utilizando para o efeito um grande conjunto de casos clínicos sumariados (tabela 1.1., página 8).[39] Este novo sistema de classificação mostrou melhor sensibilidade do que os critérios ACR (97% e 83%, respetivamente) e apesar de a especificidade ser menor (84% e 96%, respetivamente), permitiu menor número de erros de classificação do que o sistema de classificação do ACR, tendo como referência o diagnóstico clínico consensual de especialistas em LES. As principais inovações introduzidas nos novos critérios SLICC incluem: redefinição do critério de manifestações cutâneas, incluindo maior diversidade de erupções lúpicas agudas e também subagudas, anteriormente não valorizadas; redefinição do critério de lúpus neuropsiquiátrico,

As tabelas e gráficos são identificados nesta secção de acordo com o seu número e página de apresentação no corpo principal da tese.

que foi expandido para incorporar uma diversidade de manifestações neurológicas lúpicas que anteriormente não eram contabilizadas para efeitos de classificação; a adição dos baixos níveis de complemento sérico como um dos critérios imunológicos de classificação, refletindo a sua reconhecida importância na patogenia da doença, bem como a sua boa sensibilidade e especificidade para LES. Outro aspeto importante na aplicação dos critérios de classificação SLICC é que a nefrite lúpica com comprovação histopatológica passou a ser considerado como critério clínico suficiente para a classificação, desde que na presença de anticorpos anti-nucleares (ANA) ou anti-ADN de cadeia dupla (anti-dsDNA) séricos. Este sistema SLICC requer para classificação como caso de LES o cumprimento de pelo menos 4 entre 17 critérios, sendo pelo menos um clínico e um imunológico; a única situação de exceção é o caso referido de nefrite lúpica que permite estabelecer a classificação com um mínimo de 2 critérios.

A atividade clínica da doença é um parâmetro prognóstico fundamental na avaliação de doentes com LES. Foram desenvolvidos e validados diversos índices de quantificação da atividade do LES, incluindo: *European Consensus Lupus Activity Measure* (ECLAM); *British Isles Lupus Assessment Group Index* (BILAG); *Systemic Lupus Activity Measure* (SLAM); *Systemic Lupus Erythematosus Disease Activity Index* (SLEDAI, incluindo as versões SLEDAI-2K e SELENA-SLEDAI).[40-45] Um requisito fundamental na aplicação de qualquer um destes índices é que apenas as manifestações presentes no período avaliado que sejam atribuíveis à atividade do LES devem ser pontuadas. O SLEDAI-2k é um instrumento validado e o mais largamente utilizado na atualidade (tabela 1.2., página 9).[44, 46, 47] Para que uma manifestação seja pontuada no SLEDAI (com um peso específico pré-estabelecido), tem de ter estado presente em qualquer altura durante os últimos 10 dias (no SLEDAI original e versão do SLEDAI-2k a 10 dias) ou durante os últimos 30 dias (na versão do SLEDAI-2k a 30 dias) e ser atribuível com maior probabilidade a atividade do LES do que a qualquer outra causa alternativa (como sejam infeções ou sequelas). A utilização sistemática de pelo menos um destes índices na monitorização clínica da atividade da doença é altamente recomendável.[9] O seu uso auxilia o médico a quantificar a atividade da doença de uma forma estruturada, sistemática e objetiva em cada visita e na monitorização longitudinal, permitindo apreciar mudanças clinicamente relevantes e a resposta à terapêutica. Estes índices refletem melhoria e agravamento clínico e demonstraram ser preditores do acréscimo de dano irreversível e de mortalidade a longo prazo.[47, 48]

O acréscimo de dano irreversível de órgãos é outro parâmetro prognóstico fundamental em doentes com LES. O *SLICC/ACR Damage Index* (SDI) é o instrumento validado desenvolvido para quantificar o dano cumulativo em doentes com LES (tabela 1.3., página 10).[49] O dano irreversível em doentes com LES pode ser causado pela própria doença, pelos fármacos utilizados no seu tratamento, em particular os glucocorticóides, ou ainda por comorbilidades.[50-53] É de frisar que o SDI apenas pontua dano irreversível com instalação após o diagnóstico de LES, mas independentemente de considerações de atribuição a causas relacionadas ou não com o LES. O SDI registra danos em 12 órgãos ou domínios. Para assegurar

que o dano é de facto irreversível, para a generalidade dos itens é requerido que este esteja presente durante pelo menos 6 meses para ser pontuado no SDI. A pontuação no SDI é categórica, no entanto para alguns itens é especificado um peso >1. Demonstrou-se que o dano irreversível é um forte preditor de mais dano subsequente e de menor sobrevida.[54] Contudo, o SDI não inclui muitas comorbilidades importantes ou qualquer dano que tenha tido data de início prévia ao diagnóstico de LES, que podem ter um impacto muito significativo na qualidade de vida e sobrevida.

Um biomarcador é 'uma caraterística objetivamente mensurável e avaliada como indicador de processos biológicos normais, processos patogénicos ou respostas farmacológicas a intervenções terapêuticas'.[55] Os biomarcadores podem auxiliar na predição de diagnóstico, prognóstico, resposta terapêutica ou como um marcador substituto de um indicador clínico. Apesar da investigação intensiva e compreensão melhorada da imunopatogenia do LES, nenhum marcador demonstrou capacidade de substituir ou predizer apropriadamente os indicadores clínicos relevantes nesta doença.[56] Os anticorpos anti-dsDNA e os níveis séricos das frações do complemento C3 e C4 estão incluídos tanto em critérios de classificação do LES como em índices de atividade clínica; contudo, são marcadores imunológicos inespecíficos e pouco sensíveis, apenas valorizáveis no contexto de instrumentos compósitos que integram parâmetros clínicos.[37, 39, 46] O valor preditivo destes marcadores para indicadores de prognóstico é limitado, incluindo a previsão de envolvimento de órgãos major (como os rins), de agudizações clínicas do LES, dano irreversível ou mortalidade.[52, 56-61] Um grande número de biomarcadores putativos no sangue e urina forma investigados, consistindo na maioria em marcadores imunológicos relacionados com a atividade de vários tipos de células imunes envolvidas no LES, mas até agora nenhum emergiu como clinicamente útil.[56]

As recomendações para a monitorização e tratamento do LES baseiam-se no princípio de *treat-to-target*, que procura alcançar em todos os doentes um estado de remissão ou pelo menos de baixa atividade clínica da doença, estável e logo com prevenção de novos surtos de agudização.[11, 62] Ao alcançar e manter de forma sustentada ao longo do tempo os alvos clínicos é espectável que se melhore o prognóstico a longo prazo em termos de progressão do dano irreversível e mortalidade. Apesar de que o tratamento atual do LES melhorou dramaticamente a sobrevivência em comparação com o passado, alcançar de forma completa e prolongada a remissão clínica permanece um objetivo inalcançado para muitos doentes.[63] A complicar ainda mais a questão, foram propostas diversas definições para o alvo de remissão ou baixa atividade clínica, mas nenhuma delas é consensual, o que limita grandemente a implementação prática de estratégias *treat-to-target* na monitorização e tratamento do LES.[11, 64-69] No entanto, apesar das discrepâncias entre as definições clínicas propostas de alvo terapêutico, o SLEDAI é um parâmetro central na generalidade das definições recentemente propostas, com uma pontuação máxima de 4 estabelecida como o limiar máximo da remissão

ou da baixa atividade clínica: o desacordo entre especialistas diz respeito aos critérios adicionais requeridos (tabela 1.4., página 12).[70, 71]

Objetivos desta tese

O trabalho apresentado nesta tese tem quatro objetivos:

1. Avaliar o desempenho dos sistemas de classificação de LES - ACR (revistos em 1997) e SLICC (publicados em 2012) para LES.
2. Avaliar o impacto dos critérios de classificação (ACR ou apenas o SLICC) cumpridos à data do diagnóstico clínico de LES e outros preditores potenciais nos indicadores de prognóstico a longo prazo (constituição de dano irreversível e mortalidade) dos doentes com LES.
3. Identificar preditores clínicos de agudizações da atividade do LES.
4. Contribuir para o conhecimento das relações entre marcadores imunológicos e a atividade clínica do LES.

Populações de estudo

Nesta tese foram estudados doentes com LES de três coortes observacionais prospetivas:

1. Registo Português de Doenças Reumáticas (Reuma-pt). Este registo nacional prospetivo foi desenvolvido pela Sociedade Portuguesa de Reumatologia a partir de 2008. O registo de doentes com LES no contexto do Reuma.pt teve início em 2012, considera-se que inclui a grande maioria dos doentes com LES seguidos por Reumatologistas em Portugal e tem atualmente registados mais de 1600 doentes.[72] Dados multicêntricos desta coorte são apresentados no capítulo 2.
2. A coorte de doentes com LES do Centro Hospitalar Universitário de Coimbra (CHUC) é uma coorte prospetiva monocêntrica dos doentes seguidos na *CHUC Lupus Clinic*, uma consulta especializada no contexto do Serviço de Reumatologia do CHUC, desenvolvida e dirigida pelo autor desta tese e oficialmente inaugurada em 2005. Inclui atualmente mais de 400 doentes com LES, que estão também registados no Reuma.pt. Dados desta coorte são apresentados nos capítulos 2-7. Os estudos acerca de marcadores imunológicos, descritos nos capítulos 5-7, foram efetuados num

conjunto de 60 doentes com LES recrutados desta coorte (tabelas 1.5 e 1.6., páginas 14-15) e em controlos saudáveis recrutados no mesmo centro.

3. O Registo de Lúpus Eritematoso Sistémico da Sociedade Espanhola de Reumatologia (RELESSER) é um registo prospetivo nacional de base hospitalar, estabelecido desde 2011, é considerado ser representativo da população de doentes com LES seguidos por Reumatologistas em Espanha e inclui atualmente mais de 3500 doentes com LES.[73] Dados multicêntricos desta coorte são apresentados no capítulo 2.

A coorte de doentes com LES do CHUC foi a principal população estudada nesta tese. O capítulo 1 da tese apresenta uma introdução geral.

Sumário e discussão dos estudos publicados

Os estudos publicados e apresentados nos capítulos 2-7 são de seguida sumariados e integrados de acordo com os objetivos desta tese, com breve discussão e perspetivas futuras de aplicação e investigação. O capítulo 8 apresenta um sumário e discussão em inglês dos resultados dos estudos que integram esta tese.

Avaliar o desempenho dos sistemas de classificação ACR (1997) e SLICC (2012) para o diagnóstico clínico de LES

O diagnóstico e classificação do LES colocam grandes desafios, devido à extrema heterogeneidade de manifestações multissistémicas, bem como de vias imunopatológicas subjacentes. Os critérios de classificação para LES são de extrema importância para assegurar uma definição de caso consistente. Os mais recentes critérios de classificação SLICC demonstraram melhor desempenho para o diagnóstico clínico de LES, em comparação com os clássicos critérios de classificação ACR, graças à sua maior sensibilidade, quando validados num conjunto de cenários clínicos sumariados.[39]

Contudo, desconhecia-se se os critérios SLICC apresentariam o mesmo desempenho melhorado, em comparação com os critérios ACR, se aplicados a populações reais de doentes na prática clínica, que apresentam maior heterogeneidade. O conhecimento limitado acerca da validade externa dos critérios de classificação SLICC limitava muito a sua utilização alargada na prática clínica e em investigação.

O objetivo primário do estudo apresentado no capítulo 2 foi comparar a sensibilidade para o diagnóstico clínico de LES entre os sistemas de classificação ACR e SLICC, numa população multicêntrica e internacional representativa da população de doentes com LES em seguimento na prática clínica real. Adicionalmente, testámos a sensibilidade de cada um dos conjuntos de critérios de classificação em categorias de doentes estabelecidas de acordo com a duração da doença, para determinar qual dos sistemas de classificação permitiria classificar como LES mais precocemente no curso da doença.

Este foi um estudo observacional transversal, que incluiu 2055 doentes com diagnóstico clínico de LES seguidos em 17 centros em Portugal e Espanha e registados nos respetivos registos nacionais Reuma.pt ou RELESSER (tabela 2.1., página 24). A sensibilidade dos critérios de classificação foi comparada através do teste de McNemar.

Verificámos nesta população que a sensibilidade para o diagnóstico clínico de LES é mais elevada com os critérios SLICC do que com os critérios ACR de classificação (93,2% e 85.6%, respetivamente, p <0,0001) (tabela 2.3., página 26). Entre o grupo de doentes com LES não cumprindo os critérios ACR, 62,8% puderam ser classificados através dos critérios SLICC. Além disso, no grupo com doença mais precoce, até 5 anos após a instalação clínica, constatou-se a maior diferença na sensibilidade entre os critérios SLICC e ACR (respetivamente 89,3% e 76,0%, p <0.0001) (tabela 2.3., página 26).

Neste estudo, demonstrámos que os critérios de classificação SLICC apresentam maior sensibilidade e podem permitir classificar mais casos de LES mais precocemente no curso da doença do que com os clássicos critérios ACR, numa grande população de doentes que é provavelmente representativa da população geral de doentes com LES. Este estudo providencia uma muito necessária validação externa dos critérios de classificação SLICC. Uma vantagem adicional que também verificámos foi que os critérios SLICC apresentam capacidade de classificar como LES maior número de doentes apresentando envolvimento de órgãos *major*, nomeadamente com nefrite lúpica, bem como apresentam maior sensibilidade para o lúpus neuropsiquiátrico, dada a definição mais alargada deste tipo de envolvimento. Além disso, neste estudo apenas 1,6% dos doentes que cumpriam critérios ACR não eram classificáveis também através dos critérios SLICC. Desta forma, o sistema de classificação SLICC contribui grandemente para minimizar o problema clinicamente frequente de doentes com um diagnóstico clínico estabelecido de LES, mas não classificáveis como tal através da aplicação dos critérios clássicos ACR – designados frequentemente como casos de LES 'incompleto'. No entanto, a especificidade do sistema SLICC apresenta limitações, pelo que para minimizar os erros, os critérios de classificação devem ser aplicados a doentes com um diagnóstico clínico prévio de LES.

Impacto dos critérios de classificação preenchidos à data de diagnóstico de LES e outros preditores no prognóstico a longo termo de dano irreversível e mortalidade

A aplicação dos critérios de classificação SLICC, em vez dos clássicos critérios ACR, para definição de caso de LES em ensaios clínicos e estudos observacionais irá permitir a inclusão de uma maior proporção de doentes com diagnóstico clínico de LES, como concluímos no estudo apresentado no capítulo 2. Tal pode conduzir ao recrutamento de populações de estudo com um espectro clínico mais alargado e potencialmente mais heterogéneo, com doença mais precoce, assim como doentes previamente catalogados como portadores de 'lúpus incompleto' e mesmo como doença indiferenciada do tecido conjuntivo.

Contudo, como resultado, é possível que as populações de doentes selecionadas por aplicação dos critérios SLICC em vez dos critérios ACR para definição de casos de LES poderiam apresentar diferenças no fenótipo da doença, evolução clínica, necessidades de monitorização clínica, estratégias de tratamento e resposta à terapêutica, resultando por fim em diferente prognóstico a longo prazo, definidos pela progressão de dano irreversível e mortalidade. Se fosse esse o caso, a comparabilidade entre estudos que recrutem a população de estudo utilizando um ou outro dos sistemas de classificação seria problemática.

O objetivo do nosso estudo apresentado no capítulo 3 foi investigar se existem diferenças prognósticas em termos de desenvolvimento de dano irreversível de órgãos e de mortalidade até 10 anos após o diagnóstico, entre doentes preenchendo à data de diagnóstico clínico de LES os critérios de classificação clássicos do ACR e aqueles doentes que cumpriam apenas os mais recentes critérios de classificação SLICC. Além do potencial efeito preditor dos critérios de classificação, testámos o valor preditivo de outras co-variáveis para o prognóstico a longo prazo de dano e mortalidade.

Este foi um estudo observacional numa coorte prospetiva aberta, monocêntrica, que incluiu 192 doentes desde a data de diagnóstico até ao máximo de 10 anos (tabelas 3.1. e 3.2., páginas 36 e 37). A avaliação de potenciais preditores para cada um dos parâmetros prognósticos – ocorrência de dano irreversível e morte – foi efetuada através de métodos de análise de sobrevivência, univariada e multivariada, com curvas de Kaplan-Meyer, testes log-rank e regressão multivariada de Cox (com estimativa das curvas de sobrevivência ajustadas e *hazard ratios* com intervalos de confiança a 95%). Os preditores potenciais determinados à data de diagnóstico para ambos os marcadores prognósticos a longo prazo foram: os critérios de classificação cumpridos (ACR ou apenas os SLICC), sexo, idade, nefrite lúpica, lúpus neuropsiquiátrico, pontuação no SLEDAI-2k, medicação com prednisolona, anticorpos anti-fosfolípido, anticoagulante lúpico, anticorpos anti-dsDNA, anticorpos anti-Sm, hipertensão arterial e tabagismo.

Observámos neste estudo que à data de diagnóstico 30,2% dos 192 doentes apenas preenchiam os critérios SLICC de classificação, enquanto os restantes 69,8% cumpriam os critérios ACR. De entre os doentes que cumpriam os critérios ACR, 97,8% também satisfaziam os critérios SLICC. Os doentes que satisfaziam os critérios ACR, comparativamente aos que apenas preenchiam os critérios SLICC, apresentaram durante o seguimento maior frequência de nefrite lúpica (35,1% e 13,8%, respetivamente, p <0,01), mas menor frequência de síndrome anti-fosfolípido trombótico (4,5% e 17,2%, respetivamente, p <0,01). Os modelos multivariados de Cox não revelaram diferenças no risco de desenvolver dano irreversível ou morte entre os grupos definidos de acordo com os critérios de classificação cumpridos à data de diagnóstico do LES (tabela 3.3. e figura 3.1., páginas 38 e 39). A idade à data de diagnóstico demonstrou ser um preditor significativo para dano e morte, enquanto o lúpus neuropsiquiátrico foi preditivo apenas para dano. Não se demonstrou nenhum valor preditivo para as outras co-variáveis. Os doentes satisfazendo os critérios ACR à data de diagnóstico necessitaram mais frequentemente durante o seguimento de tratamento com prednisolona e imunossupressores (tabela 3.2., página 37).

Em conclusão, este estudo não mostrou que existam diferenças no prognóstico a longo prazo em termos de dano irreversível e mortalidade, desde a data do diagnóstico de LES até 10 anos de seguimento, entre doentes preenchendo ao início, os critérios de classificação ACR ou SLICC. Contudo, verificaram-se diferenças importantes no fenótipo clínico e imunológico entre os doentes identificados por um ou outro dos sistemas de classificação, o que resultou em necessidades de monitorização e tratamento diferentes. Estas diferenças devem ser consideradas no desenho e interpretação de ensaios clínicos, estudos observacionais e na prática clínica.

Os resultados dos estudos apresentados nos capítulos 2 e 3, no seu conjunto, demonstram que os mais recentes critérios de classificação SLICC apresentam vantagens relevantes para a classificação do LES: (1) permitem a inclusão da maioria dos pacientes com diagnóstico clínico de LES; (2) autorizam a classificação mais precocemente no curso da doença; (3) incluem uma maior proporção dos doentes que apresentam envolvimento de órgãos *major*; (4) estendem a classificação de LES para um espectro mais alargado da doença, nomeadamente de um grupo de doentes com preponderância de síndrome anti-fosfolípido secundário, que é uma manifestação potencialmente muito severa. Como resultado, o conjunto de casos definidos com os critérios de classificação SLICC são provavelmente mais representativos do LES na população geral do que o grupo identificado com os clássicos critérios ACR. Adicionalmente, a aplicação do sistema de classificação SLICC não enviesa a estimativa do prognóstico a longo prazo; contrariamente contribui para facilitar a identificação de casos, o que terá um expectável impacto positivo na monitorização e tratamento mais apropriado e atempado dos doentes com LES. A adoção futura dos critérios SLICC como sistema de referência para

classificação e definição de caso irá permitir o recrutamento em investigação de amostras mais representativas da população geral com LES.

Apesar das vantagens do sistema de classificação SLICC em comparação com o ACR, conforme demonstrado nos nossos estudos, este mantém limitações, particularmente quanto à especificidade, o que impede a sua implementação como critérios de diagnóstico. A preferência por um ou outro destes sistemas de classificação continua a ser ainda controversa, conforme discutido num artigo de opinião publicado na revista *Nature Reviews Rheumatology* a propósito do nosso estudo apresentado no capítulo 2.[87] O nosso estudo apresentado no capítulo 3 e agora publicado irá contribuir para resolver questões prévias acerca de estratificação prognóstica relacionada com os critérios de classificação. O desenvolvimento de critérios de diagnóstico para LES permanece uma necessidade não satisfeita, a requerer futura investigação.[36] Na nossa perspetiva, a possibilidade de construir critérios de diagnóstico ou novos critérios de classificação com especificidade significativamente melhorada irá requerer uma evolução importante no paradigma de conceção da patogenia do LES, que permita identificar biomarcadores de LES com adequada sensibilidade, especificidade e cuja aplicação seja praticável na clínica.[189]

Identificação de preditores clínicos de agudizações do LES

No capítulo 3, avaliámos potenciais preditores à data do diagnóstico de LES para o prognóstico a longo prazo, em termos de dano irreversível e de mortalidade. Durante o curso crónico do LES, o nível de atividade clínica e a ocorrência de agudizações são indicadores fundamentais para a monitorização dos doentes. Índices validados de quantificação da atividade da doença, como o SLEDAI, devem ser utilizados no seguimento longitudinal na prática clínica e em investigação, para quantificar a atividade da doença e apreciar as suas alterações clínicas e resposta ao tratamento. A atividade persistentemente elevada da doença e as agudizações clínicas, quantificadas por estes índices, são, preditores reconhecidos do risco de progressão de dano irreversível e de mortalidade. [48, 50, 91] Consequentemente, a prevenção de agudizações e o atingimento de um estado de baixa atividade clínica ou mesmo de remissão prolongada são objetivos centrais da monitorização e tratamento dos doentes com LES.

A capacidade de prever o risco de agudizações do LES é crucial para permitir otimizar a monitorização e instituir as medidas terapêuticas preventivas mais ajustadas ao doente individual. Contudo, os estudos anteriormente realizados não tinham identificado preditores clínicos nem biomarcadores fiáveis para o risco de agudização clínica do LES.[74]

O objetivo do estudo apresentado no capítulo 4 foi identificar preditores clínicos de agudização do LES.

Este foi um estudo observacional de coorte prospetiva que seguiu até ao máximo de 24 meses, 202 doentes com LES da coorte de LES do CHUC (tabela 4.1., página 49). As agudizações do LES foram definidas como um aumento na pontuação do SLEDAI-2k ≥4 pontos. Como medida da atividade da doença ao longo do seguimento, calculámos a média do SLEDAI-2k ajustada ao tempo (AMS) para cada doente e comparámos o AMS entre os pacientes com e sem episódios de agudização clínica, através de teste t.[105] Os potenciais preditores clínicos para agudização, presentes à data de inclusão (sexo, idade à data de diagnóstico, LES severo requerendo tratamento com imunossupressores, nefrite lúpica, pontuação no SLEDAI-2k, duração da doença desde o diagnóstico, tratamento com hidroxicloroquina, tratamento com glucocorticóides sistémicos) foram testados através de análise de sobrevivência (incluindo análise univariada com curvas de Kaplan-Meyer e testes log-rank, seguida por análise multivariada com modelos de regressão de Cox para estimativa das curvas ajustadas de sobrevivência e *hazard ratios* com intervalos de confiança a 95%).

Neste estudo, verificámos que dos 202 doentes com LES incluídos e seguidos ao longo de 1083 consultas, 16,8% apresentaram agudizações da doença. A AMS foi mais elevada ao longo do seguimento nos doentes que apresentaram agudizações em comparação com o grupo sem agudizações (6,3 e 3,1, respetivamente). A análise multivariada permitiu identificar preditores clínicos de agudização: idade de diagnóstico do LES ≤25 anos, existência prévia de nefrite lúpica e necessidade de terapêutica com imunossupressores. Em concreto, em qualquer ponto no curso clínico do LES, o risco de agudizações da doença nos 24 meses seguintes é mais de 2 vezes, 4 vezes e três vezes mais elevado nos doentes cujo diagnóstico de LES foi estabelecido até aos 25 anos, naqueles com envolvimento renal pelo LES ou necessitando de manter medicação imunossupressora, respetivamente (figuras 4.1.-4.3, páginas 50-52). Igualmente importante, não encontrámos evidência de um risco menor de agudizações associado a uma baixa atividade da doença nem com duração mais prolongada do LES.

Este estudo é importante, pois além da identificação de preditores clínicos e de fácil aplicação na prática clínica, demonstrámos conceptualmente a exequibilidade de estratificar os doentes de acordo com o risco de agudizações. Em termos práticos, os doentes que se apresentam com LES até aos 25 anos de idade apresentarão ao longo do curso da doença com maior risco de agudizações; se durante o curso clínico desenvolverem nefrite lúpica ou outro envolvimento de órgão grave que requeira o tratamento com imunossupressores, estas condições passam a tipificar de forma dominante o maior risco de agudizações. Adicionalmente, é muito importante ter em conta que o risco de agudizações não se reduz com a duração progressiva da doença, nem caso os doentes alcancem pontualmente uma baixa atividade do LES. As implicações para o desenho de ensaios clínicos, particularmente naqueles que tiverem como objetivo a redução de ocorrência de agudizações, são que este conjunto de preditores clínicos pode ser incluído nos critérios de inclusão, de forma a recrutar uma população de estudo de maior risco, de forma a aumentar a eficiência do

ensaio. Na prática clínica, os doentes com estes preditores de risco de agudização devem ser monitorizados mais frequentemente e deve procurar-se implementar nestes casos estratégias preventivas eficientes de forma a alcançar e manter de forma estável uma baixa atividade da doença ou idealmente um estado de remissão clínica. No entanto, demonstrámos também que as atuais estratégias terapêuticas imunossupressoras são ineficientes para a prevenção de agudizações. O desenvolvimento de estratégias *'treat-to-target'* eficientes e de imunossupressores efetivos permanece uma necessidade não satisfeita para os doentes com LES.[11]

Independentemente da definição utilizada para as caracterizar, as agudizações clínicas são uma medida de transição de um nível mais baixo para outro de maior atividade clínica do LES num ponto do tempo subsequente. Durante um determinado período de tempo, os pacientes que sofram agudizações têm maior probabilidade de apresentar um nível médio de atividade da doença mais elevado, conforme verificámos no nosso estudo. Níveis persistentemente elevados de atividade clínica ao longo do tempo estão associados a maior progressão de dano irreversível.[54] Inversamente, estados de remissão ou de baixa atividade clínica sustentados ao longo do tempo associam-se a menor progressão de dano.[65, 66, 69]

A manutenção de remissão clínica estável, sem agudizações, durante pelo menos 2 anos consecutivos foi verificada ser a menor duração de remissão associada a redução da progressão do dano, num estudo recentemente publicado e realizado em doentes caucasianos seguidos na Clínica de LES de Pádua.[65] Entre os 293 doentes seguidos neste estudo de coorte, 78,8% mantiveram um estado de remissão clínica durante pelo menos 2 anos, o que é consistente com o verificado no nosso estudo. Pelo contrário, um estudo de coorte numa grande população de doentes com LES Norte-Americanos seguidos na *Hopkins Lupus Clinic*, verificou que nestes pacientes a remissão prolongada era rara, com a maioria sofrendo agudizações com um tempo médio para o agravamento clínico de apenas 3 meses.[63] A necessidade de terapêutica imunossupressora mais intensiva à data de inclusão foi o principal preditor de LES clinicamente ativo, o que é consistente com os nossos resultados. As diferenças entre estes estudos são provavelmente devidas a múltiplos fatores, em particular a elevada proporção de Afro-Americanos na coorte dos EUA (que em média apresentam maior gravidade clínica do LES, nomeadamente maior frequência de nefrite lúpica) e as diferentes definições de remissão aplicadas.[71] A definição de critérios de remissão e de baixa atividade clínica é um foco de investigação emergente no LES, permanecendo as várias propostas recentemente publicadas controversas e requerendo validação robusta.

Contribuir para o conhecimento das relações entre marcadores imunológicos e a atividade clínica do LES

Os objetivos clínicos fundamentais a longo prazo para os doentes com LES são minimizar a constituição e progressão de danos irreversíveis e maximizar a sobrevivência. No capítulo 3, estudámos potenciais preditores de prognóstico presentes desde a data de diagnóstico de LES. Durante o seguimento clínico, a prevenção de agudizações e a manutenção sustentada de remissão, ou pelo menos de um estado de baixa atividade da doença, são objetivos importantes que contribuem para melhorar o prognóstico a longo prazo.[70] A estratificação do risco de agudizações clínicas é de importância central para a implementação de estratégias de monitorização e tratamento individualmente ajustadas e mais eficientes. No estudo apresentado no capítulo 4, identificámos preditores clínicos de agudização, que contribuem para este objetivo na prática clínica.

Contudo, ainda não existe uma definição consensual e consistentemente validada de remissão clínica ou de estado de baixa atividade da doença, apesar dos progressos recentes.[64, 65, 69, 70] Uma dificuldade fundamental para alcançar esta definição é a inexistência de um padrão de referência objetivo para a sua derivação e validação. Embora o LES se associe a disfunção alargada dos vários tipos de células do sistema imunitário inato e adaptativo, nenhum biomarcador ou conjunto de biomarcadores emergiu como referencial fiável para a atividade da doença.[56] Apesar disso, é expectável que o perfil de anomalias imunológicas em pacientes com LES clinicamente ativo comparativamente àqueles com LES quiescente seja diferente e, assim sendo, a sua caracterização pode providenciar uma base biológica para a definição clínica de remissão.

Os doentes com LES são portadores de variantes genéticas de suscetibilidade associadas a anomalias de células e moléculas envolvidas na resposta imunitária, que subsequentemente desenvolvem perda sustentada da tolerância, com reatividade autoimune crónica: os marcadores resultantes desta disfunção imunológica inerente à etiopatogenia da doença estarão provavelmente presentes mesmo em fases de remissão clínica do LES. Em contraste, a ativação de respostas autoimunes e de inflamação em órgãos alvo conduz a doença clinicamente ativa: as anomalias imunológicas associadas podem ser reversíveis quando a doença entrar em inatividade clínica.

O objetivo dos estudos descritos nos capítulos 5-7 foi contribuir para o conhecimento das relações entre marcadores imunológicos e a atividade clínica do LES.

As células B desempenham um papel crucial na patogenia do LES, com falência dos mecanismos de tolerância central e periférica, particularmente ao nível das células B de transição – *näive*, o que conduz à expansão de células B autorreativas.[17] Celulas B

autorreativas e produção de auto-anticorpos são tipicamente encontradas muito antes do aparecimento de manifestações clínicas do LES.[117] Comprovando o papel importante da atividade das células B na atividade clínica do LES, um anticorpo monoclonal anti-BAFF solúvel – belimumab - é eficaz no tratamento da atividade clínica do LES, pelo menos em alguns doentes.[190, 191]

O objetivo do estudo apresentado no capítulo 5 foi identificar padrões fenotípicos em subtipos de células B do sangue periférico que permitissem distinguir pacientes com LES com doença clinicamente inativa daqueles com doença clinicamente ativa, comparando também com indivíduos saudáveis.

Este foi um estudo transversal em 41 doentes com LES (incluindo 24 com doença clinicamente inativa e 17 com doença ativa) e em 28 controlos saudáveis emparelhados para o sexo e idade (tabela 5.1., página 60). A amostra de sangue de cada participante foi submetida a permeabilização intracitoplasmática seguida de marcação por imunofluorescência direta (com combinações de anticorpos monoclonais anti-CD20, -CD27, -CD19, -CD45, -CD81, -BAFFR, -CD38) e análise por citometria de fluxo para identificação dos subtipos de células B (imaturos transicionais, näive, memória e plasmablastos) e expressão em cada subtipo dos marcadores de superfície, em termos de percentagem de células com expressão e quantificação por intensidade média de fluorescência (MFI). [192, 193] Foi aplicada análise de componentes principais para identificar *clusters* de marcadores imunofenotípicos descriminantes dos grupos de participantes e comparação entre grupos aplicando testes de Kruskal-Wallis, Mann-Whitney-U e Chi^2.

Neste estudo, descobrimos que os doentes com LES clinicamente inativo, comparativamente aos controlos saudáveis, apresentaram um número e percentagem de células B entre os linfócitos totais semelhante, mas com uma distribuição anormal dos subtipos de células B: a frequência de células B de transição é mais elevada (10,1% e 4,1%, respetivamente, $p<0,0001$) e a de células B memória é mais baixa (22,5% e 34,4%, respetivamente, $p<0,001$). Os doentes com LES clinicamente ativo, comparativamente ao grupo com LES quiescente, apresentaram uma contagem e frequência de células B mais baixa (1,1% e 2,8%, respetivamente, $p<0,01$) e uma distribuição anormal dos subtipos funcionais, mais notavelmente com maior número e frequência de plasmablastos (3,0% e 0,9%, respetivamente, $p<0,05$). O resultado central deste estudo foi obtido através da análise de componentes principais, que identificou dois *clusters* de expressão diferencial de marcadores de superfície nas células B de transição que permitiram uma boa discriminação entre os grupos de controlos e doentes e entre o grupo com LES em atividade clínica e com doença clinicamente inativa: o *cluster* 1 integrando todos os controlos saudáveis e 79% dos doentes com LES inativo, enquanto o cluster 2 apenas integrou doentes com LES, incluindo 82% do grupo com doença clinicamente ativa (figura 5.1., página 63). Adicionalmente, nos 12 meses subsequentes à colheita de sangue, dos doentes que se apresentavam com doença ativa,

melhoraram para doença clinicamente inativa todos os classificados no *cluster* 1, mas apenas 21,4% dos integrados no cluster 2. Não se identificaram *clusters* com valor discriminativo nos restantes subtipos de células B.

Em conclusão, neste estudo provámos o conceito que um painel de biomarcadores pode providenciar uma validação biológica para definições clínicas de estados de atividade clínica do LES: verificámos que a expressão combinada de BAFFR, CD81 e CD38 nas células B de transição permite boa discriminação entre grupos de LES com doença ativa e inativa. São necessários mais estudos, em populações de validação diferentes e de maior dimensão para confirmar estes resultados e eventualmente construir um painel de biomarcadores com valor discriminativo mais elevado. A investigação deve também focar-se noutras vias imunológicas adicionais que poderão necessitar ser integradas no painel, dada a heterogeneidade imunopatológica entre diferentes manifestações clínicas do LES e populações de doentes.

A interleucina 17 (IL-17), em particular a IL-17A e o principal tipo celular que a produz, as células T *helper* 17 (Th17), desempenham um papel fundamental na patogénese de diversas doenças reumáticas inflamatórias e autoimunes, como a psoríase, a artrite psoriática e a espondilite anquilosante, conforme demonstrado pela eficácia de anticorpos monoclonais anti-IL-17A em ensaios clínicos no tratamento destas doenças.[194] Existe evidência sugerindo um papel da IL-17 e das células Th17 na patogenia do LES: níveis elevados de IL-17 e de células produtoras de IL-17 foram encontrados nos órgãos alvo em doentes com LES, nomeadamente nos rins e pele com doença ativa.[143, 145, 195] Nos órgãos alvo, a IL-17 pode promover o processo inflamatório através da indução da produção local de quimocinas e citocinas e do recrutamento de outras células inflamatórias.[196, 197] Além disso, a IL-17 contribui para estimular as células B, atuando em sinergia com o BAFF.[198] Contudo, os estudos prévios que avaliaram no sangue periférico os níveis de IL-17 e de células Th17 em doentes com LES reportam resultados díspares.[196, 199-201]

O objetivo principal do estudo apresentado no capítulo 6 foi comparar a frequência e caraterísticas funcionais das células Th17 no sangue periférico de indivíduos saudáveis, de doentes com LES portadores de doença clinicamente inativa e com doença ativa. Este foi um estudo observacional transversal em 34 doentes com LES (incluindo 19 com doença inativa e 15 com doença clinicamente ativa) e 15 controlos saudáveis emparelhados para o sexo e idade (tabela 6.1., página 76). Foi efetuada uma colheita de sangue periférico a cada participante, sendo as amostras de sangue total processadas com estimulação *in vitro* e de seguida submetidas a permeabilização intra-citoplasmática, marcação por imunofluorescência e finalmente aquisição dos dados por citometria de fluxo, para determinar a frequência de células Th17, a expressão (MFI) de IL-17 e a frequência de células expressando IL-2, TNF-α e IFN-γ. Os grupos de participantes foram comparados aplicando testes Mann-Whitney-U.

Neste estudo, não encontrámos diferenças significativas na frequência de células Th17 entre indivíduos saudáveis e doentes com LES, nem entre pacientes com doença clinicamente ativa ou inativa. A expressão de IL-17 foi menor nos doentes com LES em comparação com os controlos saudáveis, mas sem diferenças significativas entre grupos definidos pelo estado de atividade clínica (tabela 6.2., página 78). Adicionalmente, verificámos que além de IL-17, a maioria das células Th17 podem expressar as citocinas pro-inflamatórias caraterísticas das células Th1, nomeadamente IL-2, TNF-α e/ou IFN-γ, constituindo oito subtipos funcionais diferentes, definidos de acordo com o conjunto de citocinas produzidas; no entanto, o padrão de distribuição destes subtipos funcionais foi relativamente similar entre grupos de indivíduos saudáveis e de doentes com LES, independentemente do estado de atividade clínica.

Em conclusão, o nosso estudo não suporta a hipótese que a frequência e estado funcional das células Th17 no sangue periférico possa ser um biomarcador do estado de atividade clínica em doentes com LES. É possível que a frequência de células produtoras de IL-17 em órgãos alvo se correlacione com a atividade da doença, mas devido às dificuldades de obtenção de amostras de tecido na prática clínica, como sejam amostras seriadas de biópsias renais, esse não constituiria um biomarcador útil na prática clínica.[159] É possível que em alguns doentes, mas não em todos, os níveis séricos de IL-17 se correlacionem com a atividade clínica do LES, o que justificaria os resultados discordantes dos estudos publicados.[202-204]

As células *Natural Killer* (NK) são um componente importante para as respostas imunitárias inatas, que poderão desempenhar quer funções reguladoras, quer um papel promotor de doença no LES.[205] Os polimorfismos genéticos do *killer cell immunoglobulin-like receptor* (KIR), expressos pelas células NK, foram reconhecidos como um fator de suscetibilidade para o LES.[206] As células NK humanas no sangue periférico apresentam dois subtipos funcionais, definidos de acordo com a densidade de expressão do marcador de superfície CD56: as células NK $CD56^{dim}$ são as mais frequentes, expressam elevados níveis de KIR e exercem citotoxicidade celular através da libertação de perforina e granzima dos seus grânulos citoplasmáticos; as células NK $CD56^{bright}$ produzem abundantemente citocinas e quimocinas pro-inflamatórias, incluindo IFN-γ e TNF-α, mas apresentam menor capacidade citotóxica. A expressão pelas células NK de recetores de quimocinas, como o CXCR3, promove a sua migração para locais de inflamação, podendo assim contribuir para a atividade clínica do LES.[175] O CD57 é um marcador de diferenciação das células NK e anomalias das células $CD57^{+}$ foram associadas a doenças autoimunes sistémicas.[207] Contudo, a contribuição das células NK para a patogenia do LES e respetiva atividade da doença está por clarificar.

O objetivo do estudo apresentado no capítulo 7 foi comparar a frequência e caraterísticas funcionais dos subtipos de células NK no sangue periférico, entre indivíduos saudáveis, doentes com LES e doença clinicamente inativa e aqueles com doença ativa. Este foi um estudo observacional transversal, incluindo 44 doentes com LES (26 com doença inativa e 18

com LES clinicamente ativo) e 30 controlos saudáveis emparelhados para a idade e sexo (tabela 7.1., página 92). Foi colhida uma amostra de sangue periférico a cada participante, que foi marcada por imunofluorescência e analisada por citometria de fluxo para determinar a frequência de células NK $CD56^{dim}$ e $CD56^{bright}$ e a respetiva expressão de CD57, CXCR3, granzima B, perforina, IFN-γ e TNF-α. A comparação dos grupos de participantes foi efetuada aplicando testes de Mann-Whitney-U.

Este estudo revelou um menor número e frequência de células NK nos doentes com LES em comparação com os indivíduos saudáveis, independentemente do estado de atividade da doença, sem diferenças na distribuição dos subtipos $CD56^{dim}$ e $CD56^{bright}$ (tabela 7.2., página 93). Os doentes com LES clinicamente ativo apresentaram uma menor frequência de células NK $CD56^{dim}$ expressando CXCR3 (12,5% e 24,1% no grupo com LES clinicamente ativo e inativo, respetivamente, $p<0,01$), concomitantemente com níveis mais baixos de expressão de CXCR3 (MFI) nessas mesmas células. As células NK $CD56^{dim}$ de ambos os grupos de doentes com LES expressavam menor quantidade de granzima B em comparação com o grupo de controlos saudáveis, enquanto os doentes com LES clinicamente ativo apresentaram maior frequência de células NK $CD56^{dim}$ expressando perforina, em comparação com o grupo de doentes com doença inativa. A análise do subtipo de células NK $CD56^{bright}$ mostrou níveis mais elevados de expressão (MFI) de IFN-γ nos doentes com LES, independentemente do estado de atividade da doença, em comparação com os indivíduos saudáveis, enquanto a frequência de células NK $CD56^{bright}$ expressando TNF-α era mais baixa nos doentes com LES clinicamente ativo.

Em conclusão, este estudo demonstrou no sangue periférico menor número e frequência de células NK e expressão alterada de marcadores funcionais nos seus subtipos $CD56^{dim}$ e $CD56^{bright}$ em doentes com LES comparativamente a indivíduos saudáveis; identificámos como potenciais candidatos a biomarcadores diferenciadores do estado de atividade clínica do LES, a expressão de CXCR3 nas células NK $CD56^{dim}$ e de TNF-α nas células NK $CD56^{bright}$. Estudos muito recentemente publicados corroboram os nossos achados.[208-210]

No seu conjunto, os estudos de imunofenotipagem por citometria de fluxo de vários tipos de células imunes no sangue periférico em doentes com LES e indivíduos saudáveis que integram esta tese (capítulos 5-7), sugerem: (1) hiperatividade de células B nos doentes com LES, com *clusters* de marcadores de subtipos funcionais permitindo distinguir os doentes com LES dos indivíduos saudáveis e ainda diferenciar entre si os grupos de doentes com LES de acordo com o estado de atividade da doença; (2) um défice de frequência e funcional de células NK; (3) distúrbios menos consistentes nas células Th17 dos doentes com LES.

O LES é uma doença com elevada heterogeneidade das vias imunopatológicas dominantes entre diferentes manifestações clínicas, doentes e populações de origem étnica e geográfica distinta: tal é ilustrado pelos resultados dos ensaios clínicos com belimumab, que demonstrou

eficácia clínica num conjunto limitado de envolvimentos de órgão e em menos de 60% dos doentes.[102, 191] Notavelmente, no grupo de doentes com níveis séricos elevados de BAFF a taxa de respondedores foi superior.[211] Noutro subgrupo de doentes, a atividade da doença estará provavelmente mais dependente de outras vias imunológicas diferentes da atividade das células B, particularmente a ativação de respostas dependentes do IFN tipo I. Foi demonstrado que a expressão transcriptómica de genes induzíveis por IFN tipo I (assinatura genética do IFN) está aumentada em células mononucleares do sangue periférico de muitos doentes com LES.[212] Além disso, um ensaio clínico em doentes com LES testando o anticorpo monoclonal anti- IFN-α, sifalimumab, mostrou eficácia, tendo os doentes com elevada assinatura genética do IFN uma maior probabilidade de ser respondedores.[213] Num estudo recente e muito inovador, Banchereau e colaboradores analisaram longitudinalmente o perfil transcriptómico de sangue total em 158 doentes pediátricos com LES e controlos saudáveis.[214] Neste estudo aplicaram uma estratégia de análise similar à do nosso estudo em células B, com o objetivo de identificar *clusters* de marcadores transcriptómicos correlacionados com a classificação como LES e com os estados de atividade clínica da doença. Identificaram uma elevada assinatura genética do IFN como o marcador melhor correlacionado com a classificação do LES, integrado num *cluster* que também incluiu hiperexpressão de marcadores moleculares de neutrófilos, de inflamação, do ciclo celular, eritropoiese e histonas e ainda hipoexpressão de células NK, células linfoides B e T e marcadores de síntese proteica, que no conjunto do cluster apresentou uma elevada correlação com os critérios de classificação para LES (R^2 =0,94). Este estudo identificou sete *clusters* de marcadores transcriptómicos imunológicos que melhor se correlacionaram com subgrupos de doentes com LES clinicamente ativo, com cada grupo caraterizado por uma combinação diferente de assinaturas imunológicas transcriptómicas, incluindo aumento de marcadores de plasmablastos, assinatura de IFN, linhagem mielóide/neutrófilos e linhagem linfóide; por outro lado, os transcriptos de células NK mostraram-se negativamente correlacionados. A assinatura transcriptómica de plasmablastos foi particularmente forte em Afro-Americanos e foi globalmente a melhor correlacionada com a atividade da doença nesta população pediátrica com frequência elevada de nefrite lúpica. No entanto, dada a grande heterogeneidade das vias imunológicas envolvidas no LES, verificaram que as assinaturas de plasmablastos ou de IFN isoladamente não permitiam, em dois terços dos casos, identificar os doentes com atividade clínica do LES. Além disso, este estudo não avaliou preditores moleculares de agudização clínica do LES. Os resultados deste estudo são consistentes com os nossos próprios, apresentados nos capítulos 5-7, nomeadamente quanto à correlação positiva de marcadores de linhagem de células B, correlação negativa da linhagem de células NK e sem relação clara das células Th17 com os estados de atividade clínica do LES. De forma importante, demonstrou que um painel integrado de marcadores transcripcionais pode fiavelmente correlacionar-se com a classificação de LES e com os estados de atividade clínica da doença. Em investigação futura, tais painéis podem ser aplicados para comparar grupos de doentes preenchendo diferentes critérios de classificação do LES, de forma a explorar

potenciais diferenças na imunopatogenia e marcadores celulares e/ou moleculares que possam vantajosamente ser integrados em sistemas de classificação, reforçar a validação e otimizar os critérios de classificação.[189] Uma estratégia similar pode ser aplicada para redefinir e providenciar uma validação molecular de critérios clínicos de remissão do LES – estes devem identificar doentes apresentando baixas assinaturas de células B/plasmablastos e IFN, entre outros marcadores trancripcionais imunológicos integrando um *cluster* consistente com um estado de doença quiescente. Tal pode requerer a integração no painel de informação adicional, como seja: epigenética, proteonómica, imunofenotipagem celular, autoanticorpos e citocinas séricas. Entre os marcadores serológicos, níveis elevados de BAFF mostraram ser preditores de agudizações clínicas nas 52 semanas subsequentes.[61, 211] Desta forma, os doentes classificados como estando num estado de remissão clínica devem apresentar níveis séricos baixos de BAFF, para aumentar a probabilidade de manterem essa remissão estável, que é uma condição importante para otimizar o prognóstico a longo prazo em termos de não progressão do dano irreversível e de maximização da sobrevida.[65, 71] Estes painéis moleculares combinados serão complexos e de difícil aplicabilidade regular na prática clínica, pelo menos no futuro próximo; em vez disso, eles devem ser aplicados no contexto de investigação translacional para auxiliar a identificação e validação (ao providenciar uma fundamentação molecular) de painéis de preditores clínicos de prognóstico e estratificação de doentes apropriados para estratégias terapêuticas individualizadas. Como exemplo desta abordagem, no estudo apresentado no capítulo 4, identificámos a nefrite lúpica e a necessidade de medicação imunossupressora com sendo preditores clínicos de agudização do LES: verificou-se mais recentemente que estes preditores clínicos se associam a níveis séricos elevados de BAFF ≥2 ng/mL, que é por sua vez um marcador molecular de agudizações do LES.[211]

Uma estratégia de importância crítica para a derivação, validação e otimização de modelos preditivos de prognóstico clínico dos doentes com LES é a aquisição estruturada e a conjunção de dados clínicos de alta qualidade em coortes prospetivas multicêntricas de grande dimensão de vários registos nacionais.[215] Aplicámos com sucesso tal estratégia no estudo descrito no capítulo 2, que integrou as bases de dados de doentes com LES de grandes dimensões dos registos nacionais Reuma.pt e RELESSER. Tencionamos expandir este modelo em investigação futura para modelação de preditores clínicos de prognóstico dos doentes com LES.

Comentários finais

O trabalho apresentado nesta tese teve como principais objetivos avaliar potenciais preditores clínicos de prognóstico de doentes com LES e contribuir para o conhecimento das relações entre marcadores imunológicos e a atividade clínica do LES. Concretizámos quatro

contribuições principais para melhorar a classificação e monitorização dos doentes com LES e compreender o perfil imunológico associado a diferentes estados de atividade clínica da doença: (1) contribuímos para melhorar a classificação do LES, através da clarificação de qual dos sistemas de classificação do LES em uso corrente tem maior sensibilidade, sendo assim capaz de definir como casos de LES uma maior proporção dos pacientes com diagnóstico clínico na população geral, especialmente em fases mais precoces do curso clínico do LES – o que é crucial para melhorar o prognóstico clínico, através do início atempado de monitorização e intervenção apropriadas; (2) demonstrámos que os doentes classificados à data do diagnóstico clínico de LES por qualquer dos dois sistemas de classificação atualmente em uso apresentam similar prognóstico clínico a longo prazo – o que sublinha de forma fundamental a necessidade de providenciar a todos e o mais precocemente possível as estratégias de monitorização e tratamento *treat-to-target* apropriadas para otimizar o prognóstico a longo prazo; (3) identificámos preditores clínicos de agudização do LES e quantificámos, pela primeira vez, o risco relativo a eles associados, o que apresenta importante aplicabilidade na prática clínica para instituir individualmente nos casos apropriados uma monitorização apertada e medidas preventivas, de forma a otimizar o controlo da atividade clínica do LES com manutenção estável de um estado de remissão ou pelo menos de baixa atividade da doença e assim melhorar o prognóstico clínico a longo prazo; (4) contribuímos para o conhecimento acerca das relações entre marcadores imunológicos e a atividade da doença, nomeadamente através da caracterização em doentes com LES com e sem atividade clínica, comparativamente a indivíduos saudáveis, de anomalias imunofenotípicas em distintas linhagens de células imunes das respostas imunológicas inata (células NK) e adaptativa (células B e Th17) envolvidas na patogenia do LES – o diferente perfil de marcadores imunofenotípicos em pacientes em estados de doença clinicamente ativa comparativamente à inativa, conforme demonstrámos claramente na linhagem de células B, pode providenciar uma base para validação biológica e otimização de definições clínicas de remissão. Uma definição de remissão clínica do LES válida e, logo, que possa ser consensualmente reconhecida, é crucialmente necessária para providenciar um alvo uniforme e robusto para a monitorização e tratamento do LES, de modo a otimizar o prognóstico clínico a longo prazo destes doentes.

Abstract

Background

Systemic lupus erythematosus (SLE) is the prototypic multisystem autoimmune disease, with a broad spectrum of clinical presentations encompassing almost all organs and a chronic course, which can vary from mild to life-threatening. There is a major unmet need for outcome prediction enabling tailoring management and therapeutic interventions for SLE patients.

Objectives

To improve outcome prediction in SLE, the specific aims of this thesis were: (1) to evaluate the performance of the ACR and the SLICC classification criteria sets for SLE; (2) to evaluate the effect of the classification criteria fulfilled at time of SLE diagnosis and other predictors on long-term outcomes of damage and mortality; (3) to identify clinical predictors for SLE flares of disease activity; (4) to increase knowledge about the relationships between immunological markers and SLE disease activity.

Methods

We conducted a cross-sectional observational study of 2055 patients with a clinical diagnosis of SLE followed at 17 centers and registered in the Portuguese and Spanish national registries; the sensitivity of the ACR and SLICC classification criteria was compared using the McNemar's test; the sensitivity of the two classification sets was further examined in five subgroups defined according to disease duration.

We conducted a prospective inception cohort study of 192 SLE patients from time of diagnosis and followed up to 10 years at the CHUC Lupus Clinic; we assessed with multivariate Cox models the 10-year outcomes of damage and mortality, according to SLE classification status (fulfilling the ACR or only the SLICC criteria) at inception, and adjusting for potential baseline confounders.

We conducted a prospective cohort study of 202 SLE patients followed up to 24 months at the CHUC Lupus Clinic over 1083 visits; we evaluated potential clinical predictors for disease activity flares with multivariate Cox regression models adjusting for potential confounders factors and estimating hazard ratios.

We conducted cross-sectional studies of two groups of SLE patients, one with clinically active and another with inactive disease recruited at the CHUC Lupus Clinic and a group of healthy subjects enrolled at the same site; one peripheral blood sample was collected from each

participant and analyzed with flow cytometry multiparametric immunophenotyping protocols to define relationships between immunological markers in B cells, Th17 cells and NK cells and SLE classification and disease activity status.

Results

The cross-sectional study on performance of classification criteria showed that the sensitivity for SLE clinical diagnosis was higher with the SLICC than with the ACR classification criteria (93.2% versus 85.6%, $p<0.0001$). From patients not fulfilling the ACR criteria, 62.8% could be classified with the SLICC. Patients within 5 years since disease onset presented the largest difference in sensitivity between the SLICC and the ACR criteria (respectively 89.3% and 76.0%, $p<0.0001$).

In the 10-year prospective inception cohort study, patients meeting the ACR criteria compared to those with only the SLICC criteria at inception presented during follow-up with more cases of lupus nephritis (35.1% versus 13.8%, $p<0.01$), but less thrombotic antiphospholipid syndrome (4.5% versus 17.2%, $p<0.01$). The Cox models showed no significant differences in risk for damage or death between groups.

In the 24-month prospective cohort study, the multivariate Cox models demonstrated that the risk of flare was more than two-fold, four-fold and three-fold higher for patients with SLE diagnosis up to 25 years, previous lupus nephritis or baseline immunosuppressant treatment, respectively.

In the cross-sectional immunophenotyping studies, analysis of B cell subsets showed that differential expression of BAFFR, CD81 and CD38 in the transitional B cells allowed identifying two major clusters: the cluster 1 integrated all healthy subjects and 79% of SLE patients with clinically inactive disease, while in the cluster 2 there was only patients with SLE and 82% of those with clinically active disease. The analysis of Th17 cells showed no significant differences in the frequency of Th17 among healthy subjects and SLE patients, as well as among patients with clinically inactive and active disease. The analysis of NK cells showed a lower number and frequency of NK cells in SLE patients as compared to healthy subjects, regardless of disease activity status, and a lower frequency of $CD56^{dim}$ NK cells expressing CXCR3 was a marker of clinically active SLE (12.5% versus 24.1% in the active and inactive SLE group, respectively, $p<0.01$).

Conclusions

The SLICC classification criteria are more sensitive and may allow a SLE classification earlier in the disease course than the previous ACR criteria. Patients fulfilling at inception either of the classification criteria present no differences in the major long-term outcomes of organ damage and mortality. Patients with a SLE diagnosis before age 25, lupus nephritis or immunosuppressant treatment/severe SLE present higher risk for flares of disease activity;

patients fulfilling at inception only the SLICC classification criteria may present higher risk of thrombotic antiphospholipid syndrome: these clinical predictors thus provide a basis for tailoring management strategies of SLE patients.

Immunophenotyping studies suggested that SLE patients present: an upregulation of B cells, with subset clusters able to differentiate SLE patients from healthy subjects and clinically active from inactive SLE; a downregulation of NK cells, and less clear changes of Th17 cells. We provide proof-of-concept that a panel of immunological markers may provide a basis for biologic validation of clinical definitions for SLE disease activity states.

Keywords

Systemic lupus erythematosus; Classification criteria; Outcomes research; Cohort studies; Health status; Symptom Flare up; B-lymphocyte subsets; Th17 cells; NK cells

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Chapter 1

General introduction

Background

Systemic lupus erythematosus (SLE) is the prototypic multisystem autoimmune disease, with a broad spectrum of clinical presentations encompassing almost all organs and a chronic course, which can vary from mild to life-threatening. In Europe, prevalence ranges from 20 to 97 per 100 000.[1-3] The prevalence, clinical presentation and course of SLE are affected by age, gender, geographical factors and ethnicity.[4, 5] Women are affected up to twelve times more frequently than men, and disease onset occurs most frequently during childbearing age.

The clinical manifestations of SLE are extremely diverse and variable both between patients and in the same person over time, making it one of the most complex diseases to predict, manage and investigate.[6, 7] The disease usually follows a relapsing and remitting course. The overall course of the disease, the time of occurrence and severity of disease activity flares, and the effectiveness of immunosuppressant regimens to prevent and treat these flares are difficult to predict in SLE populations and even more so at the individual patient level. Additionally, SLE patients have a high chance of developing co-morbidities such as infections and irreversible organ damage.[8] Disease manifestations frequently require intensive and prolonged treatments that are risk-bearing. Treatment is based on a combination of glucocorticoids, antimalarials and synthetic and biologic immunosuppressants, which differ greatly between patients and over time in the same patient due to the variety and severity of organ systems involvement.[9] Effective treatment in SLE is of paramount importance, since each disease activity flare adds to irreversible damage and increases the risk of mortality.[10, 11] Disease activity, irreversible organ damage accrual, comorbidities and treatment regimens are major determinants of long-term prognosis.[4, 12] All these aspects make the management of SLE a very complex and demanding exercise of trying to balance the risk of medication with the well-being and safety of the patient in the short and longterm. Ideally, this exercise would be informed by the consideration of valid predictors of patient outcomes, allowing the clinician to tailor monitoring and treatment for each patient and over time. However, there is an outstanding paucity of such markers and this represents one of the most important unmet needs in this area of medicine.

Etiopathogenesis

The etiopathogenesis of SLE involves the interplay of susceptibility genes, hormonal and environmental risk factors, and abnormalities in immune cells and molecules involved in apoptosis, innate and adaptive immune responses.[4, 13-17] These factors lead to an irreversible breakdown of immunological tolerance manifested by aberrant immune responses against endogenous nuclear and other self-antigens.[18] Over 40 genetic loci were identified through

genome-wide association studies (GWAS) as associated with SLE.[19-21] Susceptibility to SLE conferred by each gene is limited, generally with a relative risk less than 2.[22, 23] The susceptibility genes are connected with three main immune pathways: (1) apoptotic waste clearance; (2) nucleic acid innate sensing by Toll-like receptors (TLR) and downstream interferon (IFN) signaling pathways; (3) lymphocyte signaling within T or B cells. Epigenetic mechanisms are also potentially implicated in SLE susceptibility and disease activity: these include processes such as DNA methylation, post-translational histone modifications, and non coding RNAs that can durably modify gene expression.[24] DNA hypomethylation, which typically causes overexpression of genes, was found globally increased in T cells from patients with active SLE and specifically in IFN-stimulated genes.[13, 25] In genetically predisposed individuals, environmental factors act as triggers of loss of tolerance to self antigens, eliciting autoimmunity. Downstream elements that drive a self-sustained loss of tolerance, spreading and maintenance of autoimmunity are still poorly understood. Pathogenic processes are heterogeneous across different disease features, patients, populations and ethnic groups. A key concept in the pathogenesis of SLE is an imbalance between production of apoptotic waste and its disposal: when the burden of apoptotic cells exceeds clearance rate, the accumulated apoptotic debris can illicit abnormal immune responses.[26] Increased apoptotic cell load can be generated by exposure to ultraviolet light, infections and other environmental risk factors for SLE. Abnormal apoptotic pathways contribute to failure of the mechanisms that usually prevent immune activation in response to endogenous cellular debris. Neutrophils are key inflammatory participants that extrude nuclear material, forming neutrophil extracellular traps (NETs), a source of yet more nucleic acid antigens in SLE.[27] Nucleic acids (DNA, RNA) contained in apoptotic debris can stimulate an inflammatory response through activation of nucleic-acid recognition receptors, such as Toll-like receptors (TLR), which are constituents of the innate immune system.[13] The TLRs are expressed in immune cells, including dendritic cells (DC), B and T cells, and macrophages, as well as nonimmune cells (epithelial cells, fibroblasts). TLRs reside in the endoplasmic reticulum, with TLR3, TL7 and TLR8 recognizing RNA, while TLR9 senses DNA. Experimental data support more strongly a role for TLR7 and TLR9 in SLE susceptibility, with more limited evidence implicating TLR3 and TLR8.[28] Current models of SLE pathogenesis postulate a central role for TLRs in which they engage self nucleic acids, drive loss of tolerance and induce a strong type I interferon (IFN) production.[13]

The plasmacytoid DCs (pDC) are immune cells that produce the highest levels of type I IFN after activation by TLRs. Hydroxychloroquine, a cornerstone of SLE treatment, reduces TLR7/9 activation and Type I IFN production by pDC.[29] Type I IFN and other cytokines promote B-cell differentiation and loss of tolerance. B-cell activating factor (BAFF or BLyS) is upregulated by type I IFN and is an important driver of B-cell activation, survival and autoantibody production in SLE.[30] BAFF is the target of belimumab, a drug approved for treatment of SLE. Once activated, B cells mature, expand and secrete antibodies, which

enhance the adaptive immune response. Type I IFN also induces differentiation of conventional myeloid DC (mDC) and auto-antigen presentation by these cells to CD4-positive T cells in SLE.[31] Thus, both pDCs and mDc are thought to be pivotal to the disease process in SLE.[13]

T-cell and B-cell abnormalities are considered central to the pathogenesis of SLE and include: loss of T-cell tolerance; aberrant T-cell to B-cell interactions leading to stimulation of autoreactive B cells; differentiation and expansion of pro-inflammatory interleukin-17 (IL-17) producing T-cells; defective or deficient regulatory T-cells (T_{reg}), that represent an important checkpoint against autoreactive lymphocytes; aberrant development of B-cells, leading to a break in B-cell tolerance and increased survival of autoreactive clones.[13, 32-34] B-cells contribute to SLE through the production of antibodies against auto-antigens, the autoantibodies that constitute a hallmark of the disease, but also through autoantibody-independent mechanisms. Immune complexes formed between autoantibodies and their respective self antigens activate complement and bind Fc receptors, driving inflammation at target tissues, such as the skin and kidney. Other roles of B-cells in SLE include their ability to present autoantigenic peptides to T cells, to drive innate responses mediated by the expression of TLRs, and to produce a large array of proinflammatory and regulatory cytokines.[34] Co-stimulatory cell receptors and ligands, such as CD40-CD40L or ICOS-ICOS ligand are important determinants of B-cell-T-cell interactions that may be implicated in SLE.[34]

Diagnosis and classification

The disease process starts with an early preclinical phase characterised by nonspecific autoimmune abnormalities and proceeds to a more disease-specific autoimmunity phase.[35] It may take years for patients to develop clinical and immunological disturbances that allow the diagnosis of SLE, although some patients present from inception with a full-blown disease. For these reasons, time of disease onset is often uncertain and diagnosis may be delayed, with a negative impact in patient outcomes. The “gold-standard” is a clinical diagnosis established by a clinician experienced in SLE, since there are no diagnostic criteria or any pathognomonic features.[36] The clinical diagnosis is established based on the identification of several clinical and analytical features consistent with the diagnosis of SLE, either concomitantly or cumulatively, while carefully considering other alternative explanations.

Criteria for SLE classification were developed by the American College of Rheumatology (ACR) in 1971, revised in 1982 and last updated in 1997 and require cumulative fulfillment of at least 4 of the 11 criteria for classifying as SLE (table 1.1.).[37] Classification criteria were developed to ensure a consistent case definition of SLE for inclusion in clinical research and

randomized controlled trials: they are intended to be applied to patients in whom a clinical diagnosis of SLE had been previously established. Nonetheless, these criteria are often used to assist diagnosis despite caveats about their use for this purpose.[36] Importantly, the ACR criteria were developed and validated in patients with longstanding established disease and may exclude patients with early or limited disease.[38]

The Systemic Lupus International Collaborating Clinics (SLICC) group undertook a revision of the ACR criteria using 'real-case patient scenarios' (table 1.1.).[39] The resulting classification system showed a better sensitivity than the ACR criteria (97% versus 83%), at the cost of lesser specificity (84% versus 96%). Importantly, it resulted in fewer misclassifications with regard to the "gold-standard" expert clinical diagnosis. Main differences introduced by the SLICC criteria include: one of the cutaneous criteria includes a larger number of acute and subacute manifestations as they largely overlap; the scope of neurologic manifestations was also expanded but still counted as a single criterion and low complement was added to the immunologic criteria, reflecting its importance to disease pathogenesis. Another important aspect is that applying the SLICC criteria, biopsy-proven lupus nephritis, in the presence of positive ANA or anti-dsDNA antibodies, is sufficient to classify a patient as having SLE. The SLICC system requires for SLE classification the fulfillment of at least 4 of the 17 listed criteria, with at least one being clinical and one immunological disorder or positive ANA criteria, or alternatively a biopsy-proven lupus nephritis with positive ANA or anti-dsDNA antibodies.

Disease assessment

Disease activity is a fundamental outcome measure in the evaluation of patients with SLE. Several validated activity indices have been developed, including the European Consensus Lupus Activity Measure (ECLAM), the British Isles Lupus Assessment Group Index (BILAG), the Systemic Lupus Activity Measure (SLAM), and the SLE Disease Activity Index (SLEDAI, with SLEDAI-2K and SELENA-SLEDAI versions).[40-45] A crucial issue in the application of any of these indexes is that only features attributable to active SLE should be scored. The SLEDAI-2k is a validated instrument and widely used (table 1.2).[44, 46, 47] For an item to be scored in SLEDAI with the indicated weight, the manifestation must have been present at any time within the past 10 days (original SLEDAI) or 30 days (SLEDAI-2k 30-day version) and be attributed to lupus disease activity. The use of at least one of these indices for monitoring disease activity is highly recommended for optimal follow-up and treatment of patients with SLE.[9] It helps the clinician to quantify disease activity in an objective and standardized way at each visit and to appreciate changes and response to treatment in longitudinal follow-up. These indices were shown to reflect actual changes in disease activity and to be predictors of damage and mortality.[47, 48]

The accrual of irreversible organ damage is another fundamental outcome in patients with SLE. The SLICC/ACR Damage Index (SDI) is a validated instrument designed to evaluate this domain (table 3).[49] SLE-related damage may be due to the disease itself or to drug treatment, particularly glucocorticoids.[50-53] Of note, the SDI scores only irreversible damage starting after the SLE diagnosis, but regardless of its attribution to SLE-related or non-related causes. SDI records damage in 12 organs or domains; furthermore, to ensure that the damage recorded is irreversible, most features are required to be present for at least 6 months before being scored. The scoring in SDI is categorical, however a weight >1 is attributed to some features. Organ damage is a strong predictor of further damage accrual and increased patient mortality.[54] However, the SDI does not consider many important comorbidities or any damage starting before the SLE diagnosis, even though they may significantly influence patients' quality of life and survival.

Biomarkers in SLE

A biomarker is 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention'.[55] Biomarkers can assist in predicting diagnosis and prognosis, assessing therapeutic responses or as a surrogate substitute for a clinical endpoint. Despite intense research and improved understanding of the immunopathogenesis of SLE, no single immunological parameter has emerged as a suitable biomarker for disease activity or for prediction of clinical outcomes in SLE.[56] The serum levels of anti-dsDNA antibodies and C3 and C4 complement components are included in both the SLE classification criteria and disease activity measures; however they lack specificity and sensitivity, and thus can only be valued alongside clinical characteristics as part of composite measures.[37, 39, 46] They have a limited value as predictors for clinical outcomes, including major organ involvement (such as renal involvement), flares of disease activity, organ damage or mortality.[52, 56-61] A multitude of putative biomarkers in blood and urine have been investigated, most of them being immunological markers for activity of the various immune cell types involved in SLE, but none emerged so far as clinically useful.[56]

Table 1.1. The revised ACR and the SLICC classification criteria for SLE [37, 39]

Criteria	*ACR criteria* (1997 update)	*SLICC criteria* (2012)
Skin	**1. Malar rash** (fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds) **2. Discoid rash** (erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occur in older lesions) **3. Photosensitivity** (skin rash as a result of unusual reaction to sunlight, by patient history or physician observation)	**1. Acute cutaneous lupus** (lupus malar rash, do not count if malar discoid ; bullous lupus ; toxic epidermal necrolysis variant of SLE ; maculopapular lupus rash ; photosensitive lupus rash), or **subacute cutaneous lupus** (non-indurated psoriasiform and/or annular polycyclic lesions that resolve without scarring) **2. Chronic cutaneous lupus** (classic discoid rash: localised or generalised ; hypertrophic verrucous lupus ; lupus panniculitis profundus ; mucosal lupus ; lupus erythematosus tumidus ; chillblain lupus ; discoid lupus/lichen planus overlap) **3. Non-scarring alopecia**
Ulcers	**4. Oral or nasopharyngeal ulceration**	**4. Oral or nasal ulcers**
Synovitis	**5. Non-erosive arthritis** (involving ≥2 peripheral joints, characterised by tenderness, swelling or effusion)	**5. Inflammatory synovitis** (in ≥2 joints: a. Characterised by swelling or effusion, or b. Tenderness and ≥30 min of morning stiffness)
Serositis	**6.** Any of: a. **Pleuritis** (convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion); b. **Pericarditis** (documented by ECG or rub or evidence of pericardial effusion)	**6.** Any of: a. Typical **pleurisy** (lasting >1 day, or pleural effusions, or pleural rub) b. Typical **pericardial** pain (pain with recumbency improved by sitting forward, for >1 day), or pericardial effusion, or pericardial rub or pericarditis by electrocardiography
Renal disorder	**7.** Any of: a. Persistent **proteinuria** >0.5 g/day, or >3+ if measurement is not performed ; b. **Cellular casts**: red cell, haemoglobin, granular tubular or mixed	**7.** Any of: a. **Urine protein/creatinine (or 24 h urine protein**) representing ≥500 mg of protein/24 h, or b. **Red blood cell casts**
Neurological disorder	**8.** Any of: a. **Seizures**; b. **Psychosis** (in the absence of offending drugs or known metabolic derangements)	**8.** Any of: a. **Seizures**; b. **Psychosis**; c. **Mononeuritis multiplex**; d. **Myelitis**; e. **Peripheral or cranial neuropathy**; f. **Cerebritis** (acute confusional state)
Haematological disorder	**9.** Any of: a. **Haemolytic anaemia** (with reticulocytosis) ; b. **Lymphopenia** (<1500/mm^3); c. **Thrombocytopenia** (<100.000/mm^3)	**9. Haemolytic anaemia** **10. Leucopenia** (<4000/mm^3), or **lymphopenia** (<1000/mm^3) at least once **11. Thrombocytopenia** (<100 000/mm^3) at least once
Immunological disorder	**10.** Any of: a. **Anti-DNA antibody** to native DNA in abnormal titer; b. **Anti-Sm** (presence of antibody to Sm nuclear antigen) ; c. Positive finding of **antiphospholipid antibodies** (based on: 1. an abnormal serum concentration of IgG or IgM anticardiolipin antibodies ; 2. a positive test result for SLE anticoagulant ; or 3. a false-positive serological test for syphilis, known to be positive for ≥6 months and confirmed by negative *Treponema pallidum* immobilisation or fluorescent treponemal antibody absorption test)	**12. Anti-dsDNA** above laboratory reference range (except ELISA: twice above laboratory reference range) **13. Anti-Sm** **14. Antiphospholipid antibody** positivity: lupus anticoagulant, false-positive test for syphilis (rapid plasma reagin), anticardiolipin (medium or high titer IgG, IgM, or IgA), or anti-β2 glycoprotein 1 (positive IgG, IgM, IgA) **15. Low complement**: low C3, or low C4, or low CH50 **16. Direct Coombs' test** (in the absence of haemolytic anaemia)
Antinuclear antibody	**11.** Abnormal titre of **ANA** (by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with drug-induced lupus)	**17. ANA** (above laboratory reference range)

Table 1.2. The Systemic Lupus Erythematosus Disease Activity Index-2K (SLEDAI-2K) [44]

Weight	Descriptor	Definition
8	**Seizure**	Recent onset, exclude metabolic, infectious or drug causes
8	**Psychosis**	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Includes hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganised or catatonic behaviour. Exclude uraemia and drug causes
8	**Organic brain syndrome**	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features, inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes
8	**Visual disturbance**	Retinal changes of SLE. Include cytoid bodies, retinal haemorrhages, serous exudate or haemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection, or drug causes
8	**Cranial nerve disorder**	New onset of sensory or motor neuropathy involving cranial nerves
8	**Lupus headache**	Severe, persistent headache; may be migrainous, but must be non-responsive to narcotic analgesia
8	**Cerebrovascular accident**	New onset of cerebrovascular accident(s). Exclude arteriosclerosis
8	**Vasculitis**	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter haemorrhages or biopsy or angiogram proof of vasculitis
4	**Arthritis**	>2 Joints with pain and signs of inflammation (i.e., tenderness, swelling or effusion)
4	**Myositis**	Proximal muscle aching/weakness, associated with raised creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis
4	**Urinary casts**	Haem-granular or red blood cell casts
4	**Haematuria**	>5 Red blood cells/high-power field. Exclude stone, infection or other cause
4	**Proteinuria**	>0.5 g/24 h
4	**Pyuria**	>5 White blood cells/high-power field. Exclude infection
2	**Rash**	Inflammatory type rash
2	**Alopecia**	Abnormal, patchy or diffuse loss of hair
2	**Mucosal ulcers**	Oral or nasal ulcerations
2	**Pleurisy**	Pleuritic chest pain with pleural rub or effusion, or pleural thickening
2	**Pericarditis**	Pericardial pain with at least one of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation
2	**Low complement**	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory
2	**Increased DNA binding**	Increased DNA binding by Farr assay above normal range for testing laboratory
1	**Fever**	>38°C. Exclude infectious cause
1	**Thrombocytopenia**	<100 000 platelets × 10^9/L, exclude drug causes
1	**Leucopenia**	<3000 white blood cells × 10^9/L, exclude drug causes

Table1.3. The SLICC/ACR Damage Index for SLE [49]

Item	Score
Ocular (either eye by clinical assessment)	
Any cataract ever	1
Retinal change or optic atrophy	1
Neuropsychiatric	
Cognitive impairment (eg, memory deficit, difficulty with calculation, poor concentration, difficulty in spoken or written language, impaired performance level) or major psychosis	1
Seizures requiring treatment for 6 months	1
Cerebrovascular accident ever (score 2 if >1)	1, 2
Cranial or peripheral neuropathy (excluding optic)	1
Transverse myelitis	1
Renal	
Estimated or measured glomerular filtration rate <50%	1
Proteinuria >3.5 g/24 h	1
End-stage renal disease (dialysis or transplantation)	3
Pulmonary	
Pulmonary hypertension (right ventricular prominence, or loud P2)	1
Pulmonary fibrosis (physical and radiographical)	1
Shrinking lung (radiograph)	1
Pleural fibrosis (radiograph)	1
Pulmonary infarction (radiograph)	1
Cardiovascular	
Angina or coronary artery bypass	1
Myocardial infarction ever (score 2 if >1)	1, 2
Cardiomyopathy (ventricular dysfunction)	1
Valvular disease (diastolic murmur, or systolic murmur >3/6)	1
Pericarditis for 6 months or pericardiectomy	1
Peripheral vascular	
Claudication for 6 months	1
Minor tissue loss (pulp space)	1
Significant tissue loss ever (eg, loss of digit or limb) (score 2 if >1 site)	1, 2
Venous thrombosis with swelling, ulceration or venous stasis	1
Gastrointestinal	
Infarction or resection of bowel below duodenum, spleen, liver or gallbladder ever, for any cause (score 2 if >1 site)	1, 2
Mesenteric insufficiency	1
Chronic peritonitis	1
Stricture or upper gastrointestinal tract surgery ever	1
Chronic pancreatitis	1, 2
Musculoskeletal	
Muscle atrophy or weakness	1
Deforming or erosive arthritis (including reversible deformities)	1
Osteoporosis with fracture or vertebral collapse	1
Avascular necrosis (score 2 if >1)	1, 2
Osteomyelitis	1
Tendon rupture	1
Skin	
Scarring chronic alopecia	1
Extensive scarring of panniculum other than scalp and pulp space	1
Skin ulceration (excluding thrombosis, for >6 months)	1
Premature gonadal failure	1
Diabetes (regardless of treatment)	1
Malignancy (excluding dysplasia) (score 2 if >1 site)	1

Management & outcomes

Recommendations for the management of SLE are based in treat-to-target principles, meaning that they aim to achieve a state of remission or at least of low disease activity, measured by a validated SLE disease activity index, and prevention of new disease flares.[11, 62] Achieving and sustaining these targets over time is expected to improve the long-term outcomes of irreversible organ damage and mortality. Although treatment of SLE has dramatically improved patient survival, prolonged and complete remission has remained elusive for many patients.[63] To further complicate this matter, several definitions were proposed for the target of remission or low disease activity state, but none is broadly accepted, which impairs the practical implementation of a treat-to-target strategy in the management of SLE.[11, 64-69] Despite differences between these proposed treatment targets, SLEDAI is a central endpoint for most recent definitions, with a maximum score of 4 as the cutoff for remission or low disease activity: disagreement among experts concerns differences in additional requirements (table 1.4.).[70, 71]

Unmet needs

There is a fundamental unmet need for reliable predictors of outcomes in patients with SLE, allowing the clinician to tailor monitoring and treatment strategies for each patient and over time. Unmet needs start with the issue of SLE diagnosis and classification: for diagnosis, the gold-standard is the expert clinical diagnosis and there are two alternative classification criteria – which one should be used? Limitations of the ACR criteria are well recognized, but lack of confidence in the new SLICC criteria remains, due to lack of validation studies in real-life clinical practice. Furthermore, the SLICC criteria may allow to classify as SLE patients previously labelled as incomplete lupus or undifferentiated connective tissue, that possibly have milder outcomes – should we stratify patients according to the SLE classification criteria fulfilled, in order to adjust for potential differences in outcome? Studies on predictive value of SLE classification criteria for long-term outcomes are needed. During the disease course of patients with SLE, the ability to identify patients at higher risk of flare is crucial to optimize monitoring and treatment, but still an unmet need – studies to identify reliable predictors of flare are much needed, especially for pragmatic clinical predictors that can be easily applied in the clinical practice. A broadly accepted definition of remission or low disease activity state to serve as target for SLE management remains elusive, despite recent progress. A major constraint to this objective is the lack of a gold-standard for the derivation and validation of such a definition. In our view, the profile of immunological abnormalities in clinically active and inactive SLE patients is expected to be different and its characterization may provide a basis for the biologic validation of clinical definitions of remission.

Table 1.4. Overview of most recently proposed definitions of remission and low disease activity in SLE

Authors, year	Designation	SLEDAI threshold	Additional requirements	Treatments allowed
Franklyn et al, 2016[69]	Lupus Low Disease Activity Score (LLDAS)	SLEDAI-2k ≤4	• No lupus disease activity in renal, CNS, cardiopulmonary, gastrointestinal systems; no hemolytic anaemia, vasculitis or lupus fever • PGA (0-3) ≤1 • No new feature of disease activity compared with previous visit	• Antimalarials • Immunosuppressants, including approved biologic agents, at well tolerated standard maintenance dose • Prednisolone≤7.5 mg/day
Zen et al, 2017[65]	Clinical Remission on/off corticosteroids	SLEDAI-2k ≤4	• Only items allowed positive in SLEDAI are serological: low complement (C3, C4); positive anti-dsDNA • No hemolytic anaemia, vasculitis or gastrointestinal lupus disease activity	• Antimalarials • Immunosuppressants • Prednisolone 1-5 mg/day (on corticosteroids) • No prednisolone (off corticosteroids)
Van Vollenhoven et al, 2017[64]	Remission on/off therapy	SLEDAI-2k ≤4	• Only items allowed positive in SLEDAI are serological: low complement (C3, C4); positive anti-dsDNA • PGA (0-3) ≤0.5	• Antimalarials • Immunosuppressants, including approved biologic agents, at stable dose (on therapy) • No immunosuppressants (off therapy) • Prednisolone 1-5 mg/day (on therapy) • No prednisolone (off therapy)

PGA: Physician Global Assessment of disease activity in a visual analog scale from 0-3; CNS: central nervous system

Aims of this thesis

The work presented in this thesis addresses four objectives:

1. To evaluate the performance of the ACR (1997) and the SLICC (2012) classification criteria sets for SLE.
2. To evaluate the value of the classification criteria fulfilled at time of SLE diagnosis and other parameters as predictors of long-term outcomes of damage and mortality.
3. To identify clinical predictors for SLE flares of disease activity.
4. To increase our knowledge about the relationships between immunological markers and SLE disease activity.

Study populations

The work reported in this thesis included patients with SLE from three prospective observational cohorts:

1. The Rheumatic Diseases Portuguese Register (Reuma.pt) of SLE patients. Reuma.pt is a nationwide prospective registry of patients with rheumatic diseases, developed and launched by the Portuguese Society of Rheumatology in 2008. The SLE registry within Reuma.pt was established in 2012 and now includes over 1600 SLE patients.[72] Multicenter data from this cohort is presented in chapter 2.
2. The Registry of Systemic Lupus Erythematosus Patients of the Spanish Society of Rheumatology (RELESSER) is a nationwide hospital-based prospective registry of SLE patients, established in 2011, now including over 3500 SLE patients.[73]. Multicenter data from this cohort is presented in chapter 2.
3. The SLE cohort of the *Centro Hospitalar Universitário de Coimbra* (CHUC) is a single center prospective cohort of patients followed at the tertiary CHUC Lupus Clinic, developed and led by the author of this thesis since 2005 and now includes over 400 SLE patients. These patients are also registered in Reuma.pt SLE register. Data from this cohort is presented in chapters 2-7. Studies on immunological markers, described in chapters 5-7, were performed in a subset of 60 SLE patients from this cohort (tables 1.5 and 1.6) and healthy control subjects recruited at the same site.

Table 1.5. Population included in the studies on immunological markers (chapter 5-7): Characterization of the SLE group with inactive disease at time of study blood collection according to most recently proposed definitions of remission and low disease activity in SLE (n = 31)

Characteristics	Proportion of patients
SLEDAI-2k ≤4 (score range)	100% (0-4)
Only items positive in SLEDAI are serological: low complement (C3, C4); positive anti-dsDNA	100%
PGA (0-3) ≤0.5 (score range)	100% (0-5)
No lupus disease activity in renal, CNS, cardiopulmonary, gastrointestinal systems; no hemolytic anaemia, vasculitis or lupus fever (%)	100%
No new features of disease activity compared with previous visit (%)	100%
Immunosuppressants, including approved biologic agents, at well tolerated standard maintenance dose (% taking)	32.3%
Prednisolone (% taking, dose range)	12.9% (5-7.5 mg/day)
Prednisolone≤5 mg/day (%)	96.8%
Clinical Remission (Zen *et al*, 2017)[65]	96.8%
• on corticosteroids	9.7%
• off corticosteroids	87.1%
Remission (van Vollenhoven *et al*, 2017)[64]	96.8%
• on therapy	9.7%
• off therapy	87.1%
Lupus Low Disease Activity Score (LLDAS) (Franklyn *et al*, 2016)[69]	100%

PGA: Physician Global Assessment of disease activity in a visual analog scale from 0-3; CNS: central nervous system.

Table 1.6. Population included in the studies on immunological markers (chapter 5-7): Characterization of the SLE group with clinically active disease at time of study blood collection (n = 29)

Characteristics	
SLEDAI-2k (median score, range)	8 (6-24)
PGA (0-3) (median score, range)	1.5 (1.0-2.3)
Immunosuppressants (% taking)	65.5%
Prednisolone (% taking)	86.2%
Prednisolone (median dose, range)	10 (5-40 mg/day)

PGA: Physician Global Assessment of disease activity in a visual analog scale from 0-3

Performance of the ACR (1997) and the SLICC (2012) classification criteria for SLE

The diagnosis and classification of SLE poses great challenges, due to the extreme heterogeneity of the manifestations of this disease. Classification criteria for SLE are of utmost importance to ensure a consistent case definition. The newer SLICC classification criteria showed an improved performance for SLE clinical diagnosis as compared to the ACR criteria, mostly due to an increased sensitivity, when applied to a set of abstracted patient scenarios.[39]

However, it was not known if the SLICC criteria sustain an increased sensitivity for SLE compared to the most widely used ACR criteria, if applied to a more heterogeneous real-life SLE population. Limited knowledge about the performance of the SLICC criteria greatly impaired their clinical use.

The primary aim of the study presented in chapter 2 is to compare the sensitivity of the ACR and SLICC classification criteria sets for SLE clinical diagnosis in a real-life, multicenter, international SLE population. In addition, we tested the sensitivity of each classification criteria set across categories of SLE duration, to determine which set of criteria allows an earlier SLE classification in the disease course.

Effect of the classification criteria fulfilled at time of SLE diagnosis and other predictors on long-term outcomes of damage and mortality

The SLICC classification criteria present higher sensitivity for SLE compared to the ACR criteria, as we conclude in chapter 2. Furthermore, we also showed that the SLICC criteria might be useful in classifying SLE earlier in the disease course, as compared to the ACR criteria. Hence, use of the SLICC criteria might allow enrollment of a broader spectrum of SLE

patients in clinical trials and other research studies, with earlier SLE as well as those previously classified as 'incomplete' lupus or undifferentiated connective tissue disease.

However, the possibility that SLE populations selected with the SLICC criteria present a different (possibly milder) prognosis in terms of clinical course, response to treatment, irreversible damage or survival had never been tested. If this was the case, comparability of studies using one or another of the classification criteria for patient selection would be questionable.

The aim of the study presented in chapter 3 is to investigate whether there are differences in damage and mortality outcomes up to 10 years after diagnosis, between patients satisfying the ACR classification criteria for SLE and those fulfilling only the SLICC criteria. In addition, we tested the effect of other patient covariates in predicting long-term damage and survival.

Identification of clinical predictors for SLE flares of disease activity

During the disease course, SLE clinical activity is a fundamental outcome measure in the evaluation of patients with SLE. For quantifying disease activity in clinical practice and research studies, validated indices such as SLEDAI should be used. In longitudinal follow-up, these indices allow the appreciation of clinical changes and of response to treatment. Flares of disease activity can be measured as longitudinal changes in SLEDAI score. High disease activity and flares measured by these indices were shown to be predictors of damage and mortality.[48, 50] Accordingly, prevention of flares and attainment of a stable low disease or remission state is a major objective of the clinical management of SLE patients.

In clinical practice, the ability to identify patients at risk of flare in the next few months is crucial to optimize monitoring and preventive treatment. However, previous research efforts have not been successful in identifying clinical or biomarker predictors of flare which are reliable enough for use in clinical practice.[74]

The study presented in chapter 4 aimed to identify clinical predictors of SLE flare.

Knowledge about the relationships between immunological markers and SLE disease activity

The fundamental long-term objectives of the clinical management of SLE patients are to minimize irreversible organ damage and maximize survival. In chapter 3, we studied potential predictors for these outcomes present from the inception time of SLE diagnosis. During follow-up management of SLE patients, preventing flares of disease activity and maintaining a state of remission, or at least of low disease activity, is an important target that can arguably improve these long-term objectives.[70] Establishing the risk of clinical flares is of paramount importance in order to tailor monitoring and treatment strategies for individual patients. In the study presented in chapter 4, we identified clinical predictors of flare that can contribute to this aim in clinical practice.

However, a broadly accepted definition of remission or low disease activity state to serve as target for SLE management remains elusive, despite recent progress.[64, 65, 69, 70] A major constraint to this objective is the lack of a gold-standard for the derivation and validation of such a definition. Although SLE is associated with widespread dysfunction of innate and adaptive immune cells, no single biomarker has emerged as a reliable surrogate for disease activity.[56] Nevertheless, the profile of immunological abnormalities in clinically active and inactive SLE patients is expected to be different and its characterization may provide a basis for the biologic validation of clinical definitions of remission.

Patients with SLE are carriers of susceptibility genes associated with abnormalities in immune cells and molecules, which develop a self-sustained loss of immune tolerance and chronic maintenance of autoimmunity: resulting markers of immunological dysfunction are likely to be present even in clinically quiescent phases of the disease. In contrast, enhanced autoimmune responses and target organ inflammation are the hallmarks of clinically active disease: associated immunological abnormalities may be reversible when SLE becomes clinically inactive.

The aim of the studies described in chapters 5-7 is to increase knowledge about the relationships between immunological markers and SLE disease activity.

The peer-reviewed published original articles integrating this thesis are presented in chapters 2-7.

A summary and general discussion about the findings of this thesis is presented in chapter 8.

A summary of this thesis in Portuguese is provided in the forepart.

Chapter 2

Classification of Systemic lupus erythematosus: Systemic Lupus International Collaborating Clinics versus American College of Rheumatology criteria.

A comparative study of 2055 patients from a real-life, international SLE cohort.

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Arthritis Care Res (Hoboken) 2015; 67(8): 1180-5

Abstract

Objectives

The new SLICC 2012 classification criteria (SLICC'12) aimed at improving the performance of SLE classification over the ACR 1997 criteria (ACR'97). However, the SLICC'12 need further external validation. Our objective was to compare the sensitivity for SLE classification between the ACR'97 and the SLICC'12 sets in a real-life, multicenter, international SLE population.

Methods

Cross-sectional observational study of patients with a clinical diagnosis of SLE followed at the participating Rheumatology centers and registered in the Portuguese and Spanish national registries. The sensitivity of the two classification sets was compared using the McNemar's test. The sensitivity of ACR'97 and SLICC'12 was further examined in five subgroups defined according to disease duration.

Results

We included 2055 SLE patients (female =91.4%; Caucasian =93.5%; age at disease onset =33.1±14.4; age at SLE diagnosis =35.3±14.7; age at the time of the study =47.4±14.6) from 17 centers. The sensitivity for SLE classification was higher with the SLICC'12 than with the ACR'97 (93.2% versus 85.6%, $p<0.0001$). Of 296 patients not fulfilling the ACR'97, 62.8% could be classified with the SLICC'12. The subgroup of patients with <5 years since disease onset presented the largest difference in sensitivity between the SLICC'12 and the ACR'97 (89.3% vs. 76.0%, $p<0.0001$); this difference diminished with longer disease duration and it was no longer significant for patients with >20 years of disease.

Conclusions

The SLICC'12 were more sensitive than the ACR'97, in real-life clinical practice in SLE. The SLICC'12 may allow patients to be classified as SLE earlier in the disease course.

Introduction

Systemic lupus erythematosus (SLE) poses great challenges to diagnosis and classification, due to its extremely heterogeneous multi-systemic manifestations.[4] Classification criteria are of utmost importance to ensure a consistent case definition for clinical research and randomized clinical trials. The SLE classification criteria set most commonly used is the one established by the American College of Rheumatology (ACR) in 1982 and updated in 1997 (ACR 1997 criteria - ACR'97).[37, 75] Despite the fact that the ACR'97 performed well, problems with these criteria are recognized, in particular a limited sensitivity against the "gold-standard" of SLE expert clinical diagnosis.[76] Other major concerns with the ACR'97 include: the inability to classify patients with only biopsy-proven lupus nephritis; the redundancy of photosensitivity with skin rashes; not considering several clinically relevant integument and nervous system lupus manifestations as well as important immunologic tests, namely complement fractions and anti-ß2-glycoprotein I. Consequently, patients included in clinical trials and other clinical research studies with SLE defined according to ACR'97 may not be representative of the real spectrum of the disease. To address these problems, the 'Systemic Lupus International Collaborating Clinics' research group (SLICC) recently proposed a new classification criteria set (SLICC 2012 criteria – SLICC'12, herein).[39] Sensitivity of the ACR'97 and of the SLICC'12 for clinically diagnosed SLE as "gold-standard" was 83% and 97%, respectively, in the original validation set of patient scenarios.[39] Inversely, specificity was reduced from 96% with the ACR'97 to 84% with the SLICC'12.[39] Taken together, the SLICC'12 criteria resulted in fewer misclassifications of SLE compared to ACR'97.

However, it is not known if the SLICC'12 sustains an increased sensitivity for SLE compared to the ACR'97, if applied to a more heterogeneous real-life SLE population. The primary aim of this study is to compare the sensitivity for SLE clinical diagnosis of the ACR'97 and SLICC'12 classification criteria sets in a real-life, multicenter, international SLE population.

Patients and Methods

Study population

We aimed to include all patients with a clinical diagnosis of SLE followed at the participating hospital-based rheumatology departments. Data collection was performed through the respective national Portuguese or Spanish registries (Reuma.pt and RELESSER, respectively).[77, 78] The clinical diagnoses of SLE were established by an attending rheumatologist experienced

in SLE and did not require the fulfillment of the SLE classification criteria. However, RELESSER excluded from registration patients with ≤2 criteria from the ACR'97 set.[37] Patients signed a written informed consent to participate in the study. At study closure, at least 70% of all the patients with a clinical diagnosis of SLE identifiable in the administrative or clinical databases of each participating center were included.[78] The inclusion period was from October 27, 2011 to June 30, 2013.

Study design and data collection

This was a cross-sectional observational study. Co-primary endpoints were the proportion of patients cumulatively fulfilling each of the SLE classification criteria sets (ACR'97 and/or SLICC'12) at the time of this study.

Variables assessed for each participant included: gender, ethnicity, age at onset of SLE (defined as age at first clinical manifestation attributable to SLE, as established by the attending rheumatologist), age at SLE clinical diagnosis (defined as age at SLE diagnosis, regardless of classification criteria but implying that the attending rheumatologist starts intent-to-treat care for SLE), age at enrollment in this study, SLE duration since disease onset and from diagnosis, medication and cumulative fulfillment of each SLE criterion included in the ACR'97 and SLICC'12 sets. Data for each variable were obtained from direct patient evaluation and from review of hospital records. The patients' data were recorded in the respective national registry. Anonymised data from the participants were extracted from the registries and collected in an Excel spreadsheet (Redmond, WA: Microsoft Corp.).

Both registries guarantee confidentiality of the participants' data and comply with the applicable national laws for data protection. This project adheres to the principles of the Declaration of Helsinki and obtained approval by the participating centers' Research Ethics Committees.

Statistical analyses

For each patient, we scored as a dichotomous variable the fulfillment of the ACR'97 and of the SLICC'12 classification criteria sets. The sensitivity for SLE classification of each set was calculated. We compared the proportion of cases in the study population fulfilling the ACR'97 and SLICC'12 criteria using the McNemar's test.

The sensitivity of each criterion from the ACR'97 and SLICC'12 sets for SLE was calculated. To examine the sensitivity of the two sets according to disease duration, we categorized the study population into five subgroups from disease onset to the enrollment in this study (up to

5 years, >5-≤10, >10-≤15, >15-≤20, and more than 20 years). For each subgroup, the sensitivity of the ACR'97 and SLICC'12 classification criteria was compared applying the McNemar's test. We applied a Chi-square or Fisher's exact test (as appropriate) to test for differences in sensitivity of each classification criteria set across categories of SLE duration and also to compare medication across subgroups. The statistical level of significance considered for all tests was ≤0.05. Analyses were done using SPSS Statistics version 19.0 (Armonk, NY: IBM Corp.).

Results

We included 2055 patients with a clinical diagnosis of SLE, followed at 17 hospital-based rheumatology clinics (12 Portuguese and 5 Spanish). Four centers (2 in Portugal and 2 in Spain) included from 200 to 351 patients and five centers included <50 patients each. Characteristics of the study population are presented in Table 2.1. and 2.2. A significantly higher proportion of these patients fulfilled the SLICC'12 classification criteria than the ACR'97 set (93.2% versus 85.6%, p<0.0001). In this study, 94.6% of the patients satisfied at least one of these SLE classification criteria sets and 92.3% were treated with antimalarials and/or immunosuppressants during follow-up. There was no significant difference in the proportion of patients treated with antimalarials comparing the subgroup fulfilling the ACR'97 and those fulfilling only the SLICC'12.

Table 2.1. Characteristics of the SLE study population.

Characteristic	Value
Number of SLE patients	2055
Participating centres (n)	17
Ethnicity (% European Caucasian)	93.5%
Female gender (%)	91.4%
Age at study entry (mean± SD, years)	47.4±14.6
Age at SLE onset (mean± SD, years)	33.1±14.4
Age at SLE diagnosis (mean± SD, years)	35.3±14.7
SLE duration since diagnosis (median, IQR, years)	10.3 (12.0)
SLE duration since onset (median, IQR, years)	12.1 (12.3)

IQR=Interquartile range; SD=Standard deviation

Table 2.2. Sensitivity of each SLICC'12 and ACR'97 criterion for SLE in the study population.

SLICC'12 Criteria	Sensitivity (%)	ACR'97 Criteria	Sensitivity (%)
1. Acute cutaneous lupus	67.4	1. Malar rash	44.2
2. Chronic cutaneous lupus	12.9	2. Discoid rash	10.3
3. Oral or nasal ulcers	35.4	3. Oral or nasal ulcers	35.4
4. Non-scarring alopecia	28.8	4. Photosensitivity	50.0
5. Synovitis	72.5	5. Arthritis	72.5
6. Serositis	23.0	6. Serositis	23.0
7. Renal	29.4	7. Renal	29.4
8. Neurologic	8.6	8. Neurologic	6.1
9. Hemolytic anemia	11.1	9. Hematological	67.0
10. Leukopenia (<4000 cells/mm^3) or Lymphopenia (<1000 cells/mm^3)	47.1	a. Hemolytic anemia	11.1
11. Thrombocytopenia (<100,000 cells/mm^3)	19.2	b. Leukopenia (<4000 cells/mm^3)	41.8
12. ANA	98.9	c. Lymphopenia (<1500 cells/mm^3)	47.7
13. Anti-dsDNA	74.3	d. Thrombocytopenia (<100,000 cells/mm^3)	19.2
14. Anti-Sm	15.2	10. Immunologic abnormalities	82.1
15. Antiphospholipid antibodies	35.2	a. Anti-dsDNA	74.3
16. Low complement	71.0	b. Anti-Sm	15.2
17. Direct Coombs' test	13.9	c. Anti-phospholipid antibodies	31.3
		11. ANA	98.9

Applying the SLICC'12 criteria resulted in the addition of 186 SLE cases as compared to the ACR'97. Isolated biopsy-proven lupus nephritis with positive antinuclear or anti-double-stranded DNA antibodies accounted for 10 of such cases. Conversely, 1.6% (n=29) of patients fulfilling the ACR'97 failed to be classified with the SLICC'12: In 18 cases, because of photosensitive malar rash scored as just one criterion in SLICC'12; in three due to loss of the lymphopenia criterion because of the lower cutoff (1000/mm3) with the SLICC'12; in four

additional cases because of both of the aforementioned issues, and yet another four failed the immunological criterion.

The proportion of missing data for each criterion from the ACR'97 and SLICC'12 was less than 4%, except for the direct Coombs' test which was not available in 36.3% of cases. The SLICC'12 acute cutaneous lupus criterion was fulfilled by 67.4% of patients, whereas 62.9% scored the ACR'97 malar rash and/or the photosensitivity criteria ($p<0.0001$). A higher proportion of patients scored the SLICC'12 chronic cutaneous lupus criterion compared to the ACR'97 discoid rash criterion ($p<0.0001$). More patients scored for the neurologic criterion with SLICC'12 compared to the ACR'97 ($p<0.0001$). Beyond ANA positivity, significantly more patients scored at least one of the other immunologic abnormalities in the SLICC'12 compared to the ones included in the ACR'97 criterion (89.6% and 82.1%, respectively, $p<0.0001$). This difference was mostly due to the inclusion of low complement levels in the SLICC'12. The sensitivity of the individual SLICC'12 and ACR'97 criteria for SLE classification in the study population is presented in Table 2.2.

The sensitivity for SLE classification increased with longer disease duration, for both criteria sets ($p<0.0001$). The subgroup of patients with <5 years of disease duration presented the largest gain in sensitivity of SLICC'12 compared to ACR'97 (89.3% versus 76.0%, $p<0.0001$); this difference diminished as disease duration increased and it was no longer significant for the subgroup with >20 years of disease duration (Table 2.3.).

Table 2.3. Comparison of classification set performance according to categories of SLE duration.

SLE duration since onset	Sensitivity of ACR'97 criteria (%)	Sensitivity of SLICC'12 criteria (%)	Difference in sensitivity	p
Any duration	85.6	93.2	7.6	<0.0001
≤5 Years	76.0	89.3	13.3	<0.0001
5-10 Years	82.0	90.3	8.3	<0.0001
10-15 Years	87.7	94.9	7.2	<0.0001
15-20 Years	91.9	98.2	6.3	<0.0001
>20 Years	94.3	96.9	2.6	0.0963

Discussion

Our study confirms that the SLICC'12 are more sensitive than the ACR'97, in a large group of patients representing real-life clinical practice in SLE and thus provides a further external validation of the SLICC classification criteria. Furthermore, our results suggest that the SLICC'12 may allow a SLE classification earlier in the disease course.

For the original derivation and validation work, SLE patients and control subjects with relevant non-SLE diagnosis were selected from highly specialized lupus clinics and the "gold standard" SLE clinical diagnosis was established by an expert committee reviewing abstracted patient scenarios.[39] In our study we aimed to include a multicenter representative sample of the real-life SLE population participating in interventional and observational studies in Spain and Portugal.[78-80] The large population included in this study is also likely to be representative of the general population of SLE patients, as most individuals with a clinical suspicion of SLE regardless of disease severity are likely to be referred to the participating centers in these countries.

Possibly the most controversial change brought by the SLICC'12 is ending the "double counting" of photosensitive malar rash as two criteria as allowed with the ACR'97.[36, 81] In fact, we found this to be the most frequent cause for losing SLE classification by SLICC'12 when achieved by ACR'97. Nonetheless, in our study this caused a loss of classification with SLICC'12 in a very small proportion of patients. In a study in the LUMINA cohort, proportion of SLE patients not classified with SLICC'12 whilst satisfying the ACR'97 due to this issue was larger.[81] However, it must be noted that fulfilling the ACR'97 criteria was a pre-condition to be included in the LUMINA cohort. Furthermore, data for additional cutaneous features as well as other clinical and immunological manifestations newly included in SLICC'12 had not been obtained in those patients in a systematic manner. These are likely sources of bias limiting the interpretation of those results. In our study, data regarding the clinical and immunologic parameters newly included in SLICC'12 were obtained purposely, with a very low proportion of missing data; the Coombs' test was the only exception.

The arthritis criterion was substantially redefined from the ACR'97. In the SLICC'12 it has an exclusively clinical definition and may be established even without detection of joint swelling. This definition requires a substantial expertise in rheumatologic evaluation to correctly differentiate lupus arthritis from other conditions such as fibromyalgia. In this study all patients were evaluated by rheumatologists and the proportion fulfilling the ACR'97 and SLICC'12 arthritis definition was the same.

The increased scope of clinical and immunological manifestations included in SLICC'12 may allow fulfillment of SLE classification earlier in the disease course. In our study, the subgroup

analysis supports this possibility, as the improvement in sensitivity of SLICC'12 over ACR'97 was greater in those patients with shorter disease duration. On the contrary, a study in the LUMINA and GLADEL cohorts showed that the proportion of patients fulfilling the ACR'97 earlier in the disease course was larger than for the SLICC'12. However, as noted, this study had no data on several clinical and immunological manifestations newly included in SLICC'12.[81]

Our study has some limitations. It included mostly European Caucasian patients, recruited from adult rheumatology clinical settings and this does not guarantee comparable performance of classification criteria in other ethnic groups or for pediatric cases.[81, 82] Another limitation is the exclusion of patients fulfilling ≤2 ACR'97 criteria from entering the Spanish registry. This study was not designed to determine which set of criteria allows an earlier SLE classification: An observational longitudinal study of an inception cohort of patients suspected of having lupus and related disorders will be better suited for that. Finally, we did not aim to compare specificity of these classification criteria; the original SLICC'12 derivation and validation work and subsequent studies suggest that specificity may be better with the ACR'97.[39, 81, 82]

The SLICC'12 greatly contributes to reduce the frequent issue of "incomplete lupus" cases, not fulfilling the classification criteria. However, the SLICC'12 were not tested for purposes of diagnosis. Development of diagnostic criteria for SLE to use in the clinical practice remains an important unmet need.[36, 83]

The use of the SLICC'12 criteria in interventional and observational studies will allow the inclusion of a larger proportion of patients with a clinical diagnosis of SLE. The possibility of simultaneously applying the ACR'97 does not seem justifiable: that would add unnecessary complication as almost all patients fulfilling the ACR'97 will also be positively classified with SLICC'12. For studies where the specificity of SLE classification is a dominant issue, the ACR'97 may be considered.

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Chapter 3

Risk of damage and mortality in SLE patients fulfilling the ACR or only the SLICC classification criteria.

A 10-year, inception cohort study.

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Lupus 2017. doi: 10.1177/0961203317731534. (Epub ahead of print: Sept 21, 2017)

Abstract

Objectives

To compare damage and mortality, from inception up to 10-year follow-up, between SLE patients meeting at baseline the 1997 ACR criteria or only the 2012 SLICC classification criteria.

Methods

Patients fulfilling the ACR and/or the SLICC classification criteria for SLE were enrolled at inception and followed-up to 10 years at an academic lupus clinic. Damage was defined as SLICC Damage Index (SDI) score ≥1. We assessed with multivariate Cox models the damage and mortality outcomes, according to SLE classification status at inception, adjusting for potential baseline confounders.

Results

We recruited 192 patients (69.8% fulfilling at inception the ACR criteria and 30.2% only the SLICC criteria). During follow-up, 24.0% of patients accrued organ damage and 4.2% died. Patients meeting ACR criteria compared to those with SLICC criteria alone presented during follow-up with more cases of lupus nephritis (35.1% versus 13.8%, $p<0.01$), but less thrombotic antiphospholipid syndrome (4.5% versus 17.2%, $p<0.01$). The Cox models showed no significant differences in risk for damage [HR (95% CI) 0.991 (0.453-2.167)] or death [HR (95% CI) 0.694 (0.107-4.506)] between groups.

Conclusions

The SLE classification status at inception identified different patterns of clinical phenotype, but did not influence damage accrual or mortality up to 10-year follow-up.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic multiorgan autoimmune chronic disease with extremely heterogeneous clinical manifestations in terms of symptoms and signs, organ system involvement, clinical course and response to treatment. In general, SLE follows a waxing and waning clinical course that frequently leads to irreversible organ damage and a decreased survival.[4] There are no validated diagnostic criteria and as a result, SLE remains largely a clinical diagnosis, requiring considerable expertise from the clinicians. Classification criteria for SLE were developed to ensure a consistent and standardized disease definition for patient selection into clinical trials and observational studies. Most published trials used the ACR classification criteria, developed in 1971 and revised in 1982 and 1997.[37, 75] A new set of validated criteria was proposed in 2012 by the SLICC research group that presented a higher sensitivity but less specificity for SLE compared to the ACR revised criteria.[39] A large multicenter observational study confirmed the increased sensitivity with the SLICC criteria.[84] This study also showed that the SLICC criteria might be useful in classifying SLE earlier in the disease course: within five years from disease onset, 89.3% of patients fulfilled the SLICC criteria versus 76.0% for the ACR revised criteria.[84] Hence, use of the SLICC criteria might allow enrollment of a broader spectrum of SLE patients in research studies, with earlier SLE as well as those previously classified as 'incomplete' lupus or undifferentiated connective tissue disease.[85] The European Medicines Agency (EMA) advises, in its most recent guideline on SLE clinical trials, the use of either the ACR or the SLICC classification criteria for patient selection to treatment trials.[86] The possibility that SLE populations selected with the SLICC criteria present a different (possibly milder) prognosis in terms of clinical course, response to treatment, irreversible damage or survival has never been tested. If this was the case, comparability of studies using one or another of the classification criteria for patient selection would be questionable.[87]

The aim of this study was to investigate if there are differences in damage and mortality outcomes up to 10 years after diagnosis, between patients satisfying the ACR classification criteria for SLE and those fulfilling only the new SLICC criteria.

Methods

Study population

Eligible patients were all subjects with a newly established clinical diagnosis of SLE at the CHUC Lupus Clinic (a University hospital-based lupus clinic), that fulfilled at inception the ACR-revised and/or the SLICC classification criteria for SLE.[37, 39, 80] The inclusion period for this study was from January 1, 2002 up to March 31, 2015. Patients were included if they had: (i) at least two visits to the clinic or died after their baseline inclusion visit, and (ii) made at least one visit during each 12-month period of follow-up after SLE diagnosis. These requirements were established to ensure availability of the core data needed for statistical modeling from all participants. Patients whose diagnosis at inception was changed during follow-up were excluded. Baseline was defined as the time of visit when the SLE clinical diagnosis was first established. Data were collected as part of standard of care clinical assessments. Patient data were inserted in the SLE national registry overseen by the Portuguese Rheumatology Association (www.reuma.pt), after patients provided written informed consent.[77, 84] This project adheres to the principles of the Declaration of Helsinki and obtained approval from the CHUC research ethics committee.

Study design and data collection

Patients were enrolled into a prospective, open, inception cohort study. Co-primary study outcomes were: (i) Development of any irreversible organ damage, and (ii) death, from inception up to 10 years of follow-up. Damage was defined as accrual in the SLICC Damage Index (SDI) from a score =0 at inception to ≥1.[49] Time of death was registered and survival status was cross-checked for patients lost to follow-up in the National Health Service registry, which provides universal coverage and mandatory death register at time of death certification. The SDI was evaluated at each visit to the lupus clinic.

Fulfillment of the ACR classification criteria was verified at baseline visit. Fulfillment of the SLICC classification criteria at inception was verified at time of clinical SLE diagnosis for those patients diagnosed after January 1, 2012 and retrospectively for the inception time point for those with earlier diagnoses. At inception, data were collected on age, gender, ethnicity, SLE clinical and immunological manifestations, SLEDAI-2k score, systemic steroid use for SLE manifestations, past and present smoking habits (yes/no) and hypertension (systolic blood pressure >140 mmHg and/or diastolic blood >90 mmHg and/or taking drugs for hypertension). These variables were used as covariates to adjust for potential predictors of more severe disease and increased damage accrual.[52, 54] The definitions of SLE manifestations provided by the ACR classification criteria were used whenever applicable. Lupus nephritis was biopsy-

proven in all cases. Blood, urine and biopsy analysis were centrally performed at the University Hospital laboratory. Anti-nuclear antibodies (ANA) were tested by indirect immunofluorescence assay on HEp-2 substrates (IIFA), with positivity considered at ≥1:160 dilution. Anti-dsDNA testing was done using Farr radioimmunossay with positivity defined as an assay result above the laboratory reference range and confirmed by positive *Crithidia luciliae* immunoflurescence test (CLIFT).[39, 88]

Patients were followed at the CHUC Lupus Clinic according to standard of care, with data available for this study through to July 1, 2015. For each patient, data were considered from inception to last registered visit, up to 10 years of follow-up. During follow-up and at each visit, in addition to SDI scoring and survival, we recorded data on new incident SLE manifestations, SLEDAI-2k score, SLE treatment (antimalarials, steroids and immunosuppressants) and secondary antiphospholipid syndrome.[46, 89]

Statistical analyses

Descriptive statistics were used to summarize patient data at the inception time point and also cumulative follow-up data from inception thorough to the last study visit. For each patient we scored the fulfillment of the SLE classification criteria at inception as a dichotomous variable: a) Patient fulfills the ACR classification criteria (regardless of status on SLICC criteria), or b) Patient fulfills the SLICC classification criteria, but not the ACR set. Patient data in the two groups defined by this inception SLE classification status were compared using Mann-Whitney U or Chi-square tests, as appropriate.

Identification of predictors for each of the study outcomes, (i) SDI ≥1 and (ii) death up to 10 years from SLE inception diagnosis was done through univariate and multivariate survival analysis. First, we performed univariate analysis with Kaplan-Meyer curves and Log-Rank tests to assess differences between groups defined by each predictor. The baseline factors tested in univariate analysis as potential predictors for the outcomes were: SLE classification status, gender, SLE diagnosis at younger age (categorized as ≤25 or >25 years old), SLE diagnosis at older age (categorized as ≤50 or >50 years old), lupus nephritis, neurolupus, baseline SLEDAI-2k score (categorized as <10 or ≥10, corresponding to mild/moderate versus high disease activity, respectively), baseline prednisolone daily dosage (categorized as <20 versus ≥20 mg/day), any antiphospholipid antibodies (positive/negative), lupus anticoagulant, anti-dsDNA antibodies (positive/negative), anti-Sm antibodies (positive/negative), hypertension, and ever tobacco smoking. In a second stage, we used multivariate Cox regression analyses to determine if the 10-year SDI damage status and mortality differed according to the SLE classification status at inception, after adjusting for potential confounders. The baseline values of the following covariates were selected *a priori* and included in the Cox models for damage and for mortality: SLE classification status, gender, age at SLE diagnosis (years), lupus nephritis, neurolupus, SLEDAI-2k score, prednisolone daily dosage, any antiphospholipid antibody (positive/negative), lupus

anticoagulant, anti-dsDNA antibodies (positive/negative), anti-Sm antibodies (positive/negative), hypertension and ever smoking. The adjusted survival curves and hazard ratios (HR) with 95% confidence intervals (CI) are presented for each model. Multicollinearity was checked by the standard error of each covariate included in the models. Proportional hazard assumption was verified with log-minus-log plots. Multivariate analysis was also applied to investigate the predictive value of early SDI damage (up to 12 months from inception) for the 10-year SDI damage status and mortality. Statistical tests were two-sided, and p values below 0.05 were considered statistically significant. Analyses were performed with SPSS Statistics, V.23.

Results

Patients' characteristics at inception

A total of 224 patients had their diagnosis of SLE established at our clinic during the study inclusion period. From these, 29 were excluded for not fulfilling any of the classification criteria at inception and 3 others for losing the SLE clinical diagnosis during follow-up (at baseline, one fulfilled the ACR and two only the SLICC classification criteria). The remaining 192 patients were included in this study (98% were Caucasian).

At inception, 30.2% of the subjects fulfilled the SLICC classification criteria alone, while the other 69.8% fulfilled the ACR criteria. Characteristics of these 2 groups at baseline are presented in table 3.1. From the patients fulfilling the ACR criteria, 97.8% also satisfied the SLICC criteria. The group of patients fulfilling the SLICC criteria alone was less likely to present lupus nephritis but more frequently carried antiphospholipid antibodies at inception. The group of patients fulfilling the ACR criteria presented, on average, a higher disease activity and was more frequently treated at baseline with steroids. However, from those treated with steroids, the median daily prednisolone dose was lower in the patients with ACR versus those with SLICC-only criteria [respectively 10 mg, interquartile range (IQR) 7.5-25, and 20 mg, IQR 10-40, ($p<0.0001$)].

Patient outcomes during follow-up

During a median follow-up of 6 years, 24.0% of patients developed organ damage and 4.2% died. Cardiovascular damage was the most frequent (19.0% of patients); second in frequency were malignancy, ocular and skin damage (14.3% for each category), followed by renal, pulmonary and musculoskeletal (11.9% for each). Neuropsychiatric damage emerged in 9.5% of cases. Diabetes, gastrointestinal and peripheral vascular damage jointly accounted for the remaining cases (14.3% of patients). Median age at death was 61 years (range 28-90 years).

Causes of death were: malignancy (3 cases), stroke (3 cases), lupus disease activity and infection (1 case each).

Table 3.1. Baseline patient demographics and disease characteristics according to the SLE classification criteria status at inception.

	With ACR criteria	With SLICC criteria alone	p
n	134	58	-
Median age (IQR)	34.0 (25-49)	36.5 (26.8-52)	n.s.
Gender (% female)	83.6%	81.0%	n.s.
Median SLEDAI-2k score (IQR)	6 (4-8)	4 (3-6)	<0.0001
Lupus nephritis	28.4%	8.6%	<0.01
Neurolupus	3.7%	1.7%	n.s.
Anti-phospholipid antibodies	19.7%	39.3%	<0.01
Lupus anticoagulant	13.7%	35.7%	<0.001
Anti-dsDNA antibodies	91%	86.2%	n.s.
Anti-Sm antibodies	11.2%	5.2%	n.s.
Prednisolone treatment	71.3%	41.5%	<0.01
High blood pressure	20.1%	20.7%	n.s.
Smoking (ever)	22.6%	37.5%	<0.05

IQR=Interquartile range; n.s. =not significant

Comparison of follow-up data from the two groups of patients is shown in table 3.2. Over time, 7.3% were lost to follow-up, in half of cases because of emigration/change in place of residence. During follow-up, 7 (12.1%) patients fulfilling the SLICC criteria alone evolved to meet the ACR set. Cumulative incidence of lupus nephritis was higher in those fulfilling the ACR criteria at inception. Conversely, SLE patients classified only thorough the SLICC criteria developed more frequently secondary antiphospholipid syndrome (one patient had pregnancy losses; all other cases presented only thrombotic events). Both groups of patients were systematically treated with hydroxychloroquine (95.8% of all patients). At time of last visit, patients attained, on average, a low SLEDAI-2k score, without difference between groups. However, a larger proportion of patients with positive ACR criteria at inception required treatment with systemic steroids and immunosuppressants during follow-up. At last visit, 42.5% and 22.8% of the patients from the ACR and SLICC-only criteria group were receiving prednisolone, respectively ($p<0.01$), with a median daily dose of 5 mg in both groups.

Table 3.2. Comparison of follow-up data and outcomes according to the SLE classification criteria status at inception.

	With ACR criteria	With SLICC criteria only	p
Median follow-up time, months (IQR)	84 (48-120)	60 (36-87)	<0.05
Lupus nephritis (%)	35.1	13.8	<0.01
Median SLEDAI-2k score at last visit (IQR)	2 (0-4)	2 (0-2)	n.s.
Prednisolone treatment (% ever)	77.6	49.1	<0.0001
Hydroxychloroquine (% ever)	96.3	94.7	n.s.
Immunosuppressants (% ever)	52.2	31.6	<0.01
Antiphospholipid syndrome (%)	4.5	17.2	<0.01

IQR=Interquartile range; n.s. =not significant

Analyses for relative risk of damage and mortality

The SLE patients fulfilling, at inception, either the ACR criteria or only the SLICC classification criteria did not present statistically significant differences in progression to irreversible damage, as scored in SDI, or in relative risk of death up to the first 10 years following the SLE diagnosis.

Patients aged 50+ years at inception presented both a higher risk of developing damage (p=0.037) and lower survival (p=0.001) compared to those with an earlier age at baseline. Patients with neurolupus at inception also presented higher risk of damage (p<0.0001), that was, in all such cases, due to epilepsy requiring long-term drug treatment. Neurolupus at inception did not affect mortality. Patients with hypertension at baseline presented a lower survival time (p=0.013), but no difference in damage accrual. None of the other baseline factors tested presented a significant relationship with damage.

The risk of any organ damage scored in the SDI and the patient survival up to 10 years after the diagnosis, according to the SLE classification status at inception, was analyzed in two separate multivariate Cox models, adjusting for the predefined potential confounders (table 3.3. and figure 3.1.). In these Cox models, the two groups of patients presented no significant differences in risk of damage [HR (95% CI) 0.991 (0.453-2.167)] or death [HR (95% CI) 0.694 (0.107-4.506)]. Age at inception was a significant predictor for damage (p<0.05) and death (p<0.01). Neurolupus was predictive of damage (p<0.0001). No significant effect was found for other covariates.

Up to 12 months from inception, patients accrued any SDI item in 9.7% and 12.1% of cases within the ACR and the SLICC-only criteria groups, respectively (non significant difference). None of the patients presented early renal damage scored in SDI. Multivariate analysis for the

outcome defined as any new SDI damage accrual from 1 year up to 10 years of follow-up was done, including as independent variables the inception classification group, SDI damage at 1 year (yes/no), gender, and age at inception. Neither the SDI (any damage) at 1 year (95% CI 0.156-2.607) nor the SLE classification groups (95% CI 0.790-6.731) were predictive. A similar multivariate analysis done for the death outcome did not show any predictive value of SDI at 1 year (95% CI 0.071-6.442) or the SLE classification group (95% CI 0.177-3.767).

Table 3.3. Baseline factors in the Cox model for damage accrual up to 120 months from diagnosis.

Variable	Hazard ratio (95% CI)	P value
SLE classification status (ACR criteria)	0.991 (0.453-2.167)	n.s.
Gender (male)	0.542 (0.213-1.382)	n.s.
Age at diagnosis, years	1.025 (1.001-1-048)	0.041
Lupus nephritis (yes)	1.273 (0.496-3.266)	n.s.
Neurolupus (yes)	9.996 (3.043-32.836)	0.00015
SLEDAI-2k score	1.027 (0.951-1.108)	n.s.
Prednisolone daily dosage, mg	1.003 (0.984-1.022)	n.s.
Antiphospholipid antibodies (any positive)	2.028 (0.666-6.176)	n.s.
Lupus anticoagulant (positive)	0.588 (0.157-2.207)	n.s.
Anti-dsDNA antibodies (positive)	0.736 (0.245-2.212)	n.s.
Anti-Sm antibodies (positive)	0.420 (0.094-1.872)	n.s.
Hypertension (yes)	1.060 (0.470-2.390)	n.s.
Ever smoking (yes)	1.565 (0.732-3.347)	n.s.

n.s. =not significant

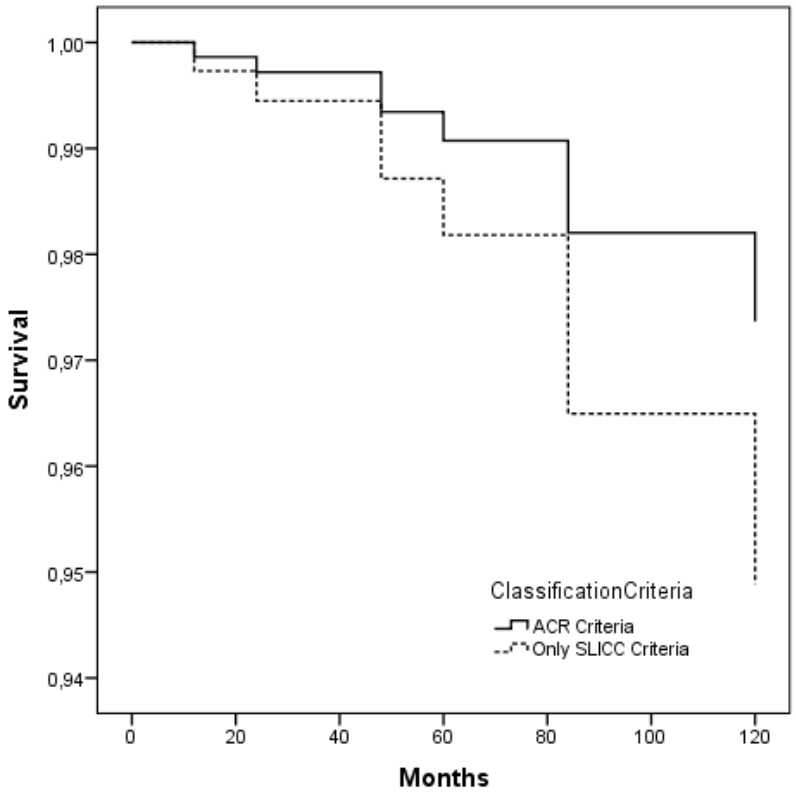

Figure 3.1. Survival estimates, according to the classification criteria status at inception.

Discussion

We prospectively compared damage accrual and mortality from SLE diagnosis up to 10-year of follow-up, in patients either fulfilling at inception the ACR criteria or the SLICC classification criteria alone. We found that there were no differences between these two groups in damage scored in SDI or in mortality. However, there were significant differences in clinical and immunological phenotype and treatment requirements.

The ACR criteria were able to classify most patients presenting with lupus nephritis, and this difference persisted throughout follow-up. It should be noted that all patients with lupus nephritis fulfilling the ACR criteria also fulfilled the SLICC criteria at inception; in the group fulfilling only the SLICC criteria at inception, there were five additional cases of lupus nephritis at baseline and a total of eight over the follow-up. Thus, 14.5% of patients developing lupus nephritis could only be classified as SLE at baseline with the SLICC criteria. Conversely, the SLICC criteria classified an additional group of patients presenting more

frequently with anti-phospholipid antibodies and developing more thrombotic events during follow-up. In accordance, treatment requirements of these two groups during follow-up were not equal. This difference in the SLE clinical and immunological phenotype captured by the two sets of classification criteria is, to the best of our knowledge, a novel finding. Previous studies suggest that positivity of antiphospholipids changes the clinical pattern, prognosis and management requirements of SLE. [90, 91]

The proportion of positive anti-dsDNA in our patients is higher than in most SLE cohorts.[39, 54] However, the prevalence of anti-dsDNA positivity across different cohorts is variable, which is due to the large number of different methodologies and cutoff level of positivity considered. In this study, we applied the tests recommended to achieve the best specificity.[88] Other cohorts report a positivity rate similar to ours; for example, in a multicenter study assessing the diagnostic accuracy of different anti-dsDNA assays in 223 SLE patients fulfilling the ACR criteria and 216 controls, the Farr assay presented a sensitivity of 95% and a specificity of 90%.[92]

The proportion of SLE patients developing any organ damage in our inception cohort is comparable to that reported from a London academic centre (27.1% with SDI >0 at a median follow-up of 6 years).[48] It is lower than that reported in the SLICC Inception Cohort (51.1% with SDI>0 at 6 years of follow-up), a multiethnic cohort from centers in 4 continents.[54] Both these studies included only patients fulfilling the ACR classification criteria for SLE. Caucasians living in Europe, that constitute almost all patients in our study population, were found in the SLICC inception cohort to present lower risk of damage accrual compared to patients of other ethnicities or from outside Europe.[54] In addition, treatment with antimalarials, prescribed from inception to almost all patients in our cohort, was associated with a reduced risk of damage accrual and increased survival.[48, 52, 54, 93, 94]

It is possible that, over a longer term, differences in damage accrual may emerge between these two groups of patients. Lupus nephritis, use of glucocorticoids and immunosuppressants, more frequent in the patients fulfilling ACR criteria, were previously associated with increased damage accrual.[48, 52, 54, 95] On the other hand, the group of patients fulfilling the SLICC criteria alone presented more frequently with lupus anticoagulant and antiphospholipid syndrome, both associated with higher damage accrual and mortality in SLE patients.[12, 52, 96]

In our cohort the estimated survival rate at 10 years was over 95%, which is consistent with recent studies.[12, 89, 97, 98] The accrual of organ damage, scored with the SDI, has been shown to be a strong predictor of mortality in SLE patients.[50, 53, 54, 98, 99] A study from Rahman *et al.* in the Toronto SLE cohort showed that early damage at 12 months from inception was a predictor for 10-year mortality.[98] This was not the case in our cohort. However, that Toronto cohort was recruited from 1970 to 1987, while our cohort started in 2002. Hence, our patients benefited from improved standard of care strategies, namely for lupus nephritis, systematic

prescription of antimalarials and restricted use of glucocorticoids. Early renal damage was the only SDI damage item associated with mortality in the Toronto study, while in our cohort none of the patients presented it up to 12-months from inception. It is possible that survival curves may diverge after a follow-up longer than 10 years. Doria *et al*. reported that in patients with lupus nephritis compared to those without, a higher mortality becomes apparent only 10-15 years after the diagnosis of SLE.[12] Ruiz-Irastorza *et al*. found that antiphospholipid syndrome is a predictor of mortality at 15-year follow-up of an inception cohort of SLE patients.[96] The fact that, in our cohort, lupus nephritis and antiphospholipid syndrome were differently distributed in the two patient groups may have contributed to level out potential differences in mortality.

The survival analysis modeling in this study adjusts for different follow-up time among patients in an open prospective cohort such as ours; however it did not account for potential differences in total SDI scores or in individual items of SDI. The setting of this study is at a single, tertiary center with a homogeneous Caucasian population, and, thus, cannot account for influence from different ethnic or geographical location factors. An important strength of this study is that our center prospective cohort is designed to admit any patient with a clinical SLE diagnosis and not limited to those fulfilling a classification criteria set.

In conclusion, we found no differences in major outcomes of organ damage and mortality up to 10-year follow-up between SLE patients fulfilling, at inception, either the ACR criteria or the SLICC classification criteria alone. However, there were differences in the SLE clinical and immunological phenotype captured by the two sets of classification criteria, resulting in heterogeneous management requirements. These differences should be taken in consideration in the design and interpretation of clinical studies as well as in clinical practice.

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Chapter 4

Identification of clinical predictors of flare in Systemic lupus erythematosus patients: A 24-month prospective cohort study.

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Rheumatology (Oxford) 2014; 53(1): 85-9 *

* Editors' Choice article of the month

Abstract

Objectives

Systemic lupus erythematosus (SLE) has a relapsing-remitting course with disease activity flares over time. This study aims to identify clinical predictors of SLE flare.

Methods

This prospective cohort study over 24-months included all SLE patients on follow-up at one academic lupus clinic. Flare was defined as an increase in SLEDAI2k score ≥4 points. Baseline clinical and demographic parameters were compared using survival analysis for time-to-flare outcome with univariate Log-Rank tests. Variables with significant differences were further evaluated as predictors with multivariate Cox regression models adjusting for potential confounding/contributing factors and Hazard Ratio (HR) calculation.

Results

A total of 202 SLE patients were included. Over follow-up, 1083 visits were documented and 16.8% of patients presented flares. In multivariate analysis the following parameters emerged as flare predictors: SLE diagnosis up to 25 years of age (HR=2.14, p=0.03); lupus nephritis previous to baseline visit (HR=4.78, p<0.0001) and immunosuppressor treatment/severe SLE (HR=3.22, p<0.001). Baseline disease activity, disease duration and treatment with prednisone or hydroxycloroquine were not predictive factors.

Conclusions

Patients with SLE diagnosis before age 25, lupus nephritis or immunosuppressor treatment/severe SLE present higher HR for flare, suggesting need for tighter clinical monitoring. Current immunosuppressive strategies seem to be inefficient in providing flare prevention.

Introduction

Systemic lupus erythematosus (SLE) has a relapsing-remitting course, with patients experiencing disease activity flares over time.[4] Aiming at flare reduction, hydroxychloroquine (HCQ) is standard treatment for most SLE patients during the whole disease course and conventional immunosuppressors are given to those with severe organ involvement.[100, 101] New biologic agents might further reduce flares but pose challenges regarding appropriate case selection.[102] In clinical practice, the ability to identify patients at risk of flare in the next few months is crucial to optimize monitoring and preventive treatment.

However, previous research efforts have not been successful in identifying clinical or biomarker predictors of flare which are reliable enough for use in clinical practice.[74, 103, 104]

This study aims to identify clinical predictors of SLE flare. Eventually, most SLE patients will develop a flare; therefore the more relevant question is not whether, but how soon it may occur. Thus, we applied survival analysis to identify predictors of flare.

Methods

All patients fulfilling the American College of Rheumatology classification criteria for SLE on regular follow-up at a single academic Lupus Clinic were included.[37] This specialized clinic was established in 2005 at the University Hospital of Coimbra Rheumatology Department. Referrals come in equal parts from Primary care Units and other Departments from this and other hospitals from a geographical area of one third of the Country and with a population of about two million. This is an ethnically homogeneous population with more than 90% native Caucasian. The Lupus Clinic is the main care provider for SLE patients managed in long-term follow-up. Patients gave written informed consent according to the Declaration of Helsinki and the hospitals' ethics committee approved the protocol. Regular follow-up was defined as at least 2 visits 2-6 months apart, and no absence from clinic exceeding 12 consecutive months, during the study period, from June 1, 2009 to January 31, 2012.

Study design was a prospective cohort study with outcome defined as time to first flare from baseline up to 24-months follow-up. Patients were included at their first visit to the Clinic after study start (baseline) and allowing for new participants entering at any time during the study period. All patients were assessed by the same rheumatologist at each visit (every 1 to 6 months), disease activity being scored according to SLEDAI2k.[46] Flare was defined as an increase in SLEDAI2k ≥4 points from inclusion.[46, 47] At baseline, demographic data, cumulative

SLE organ involvement and medication were registered. Immunosuppressive treatment at baseline was assumed as intent-to-treat marker for severe SLE. As a summary measure of disease activity over time, we calculated the time-adjusted mean SLEDAI2k (AMS) over follow-up for each patient.[105]

Statistical analysis

Clinical and demographic parameters at study entry were evaluated as potential predictors for flare outcome in survival analysis. For each patient, time (in months) from study entry to first flare event was determined and in those without flares during follow-up, observation was censored at time of last visit up to 24 months after baseline. Duration of observation period was variable, as participants could be added or lost to follow-up over the study period. Analysis was carried out in 2 steps. First, we applied univariate analysis with Kaplan-Meyer curves and Log-Rank tests to assess differences between groups defined by predictor. The factors tested in univariate analysis were: (1) Gender; (2) Age at SLE diagnosis (categorized as juvenile/young adult if diagnosis ≤25 years old or >25 years); (3) Severe disease defined as use of immunosuppressors (except steroids) at baseline (yes/no); (4) Previous biopsy-proven lupus nephritis (yes/no); (5) Baseline SLEDAI2k score (categorized as low/mild activity with score <4 and moderate/high if score ≥4); (6) Time since SLE diagnosis (categorized in up to- or more than- 2, 5, 10 years); (7) HCQ user status at baseline and (8) Steroid user status at study entry. At the second step, variables with significant differences were further evaluated as predictors in multivariate Cox proportional hazards models. Each significant predictor identified in the univariate analysis was entered in a Cox multivariate model with variables we considered to be potential confounders. Non-significant covariates were excluded from the models with backward stepwise procedures in order to calculate adjusted Hazard Ratios (HR) for flare. Proportional hazard assumption was verified with log-minus-log plots.

The AMS was compared between those with and without flares, with a two-tailed independent samples t-test, after Kolmogorov-Smirnov test for normal distribution. Chi^2 tests were used to compare distribution of categorical variables across two groups. All tests were two-sided with a risk at 5%. Statistical analyses were performed using IBM SPSS Statistics version 19.

Results

Baseline characteristics of the 202 patients included are presented in table 4.1. These accounted for 94% of the SLE patients attending the Lupus Clinic during the study period. An additional 12 patients attended the clinic just once or otherwise were not on regular follow-up and were excluded. Previous biopsy-proven lupus nephritis was the most frequent major organ involvement at baseline, affecting 45.7% of patients (WHO Class III-V: 82.4%). At baseline, 85.1% of patients were on HCQ treatment, 49% on glucocorticoids (median dose 5 mg/day) and 32.7% on immunosuppressive drugs for SLE, mostly as maintenance treatment for nephritis, severe arthritis or hematological involvement. At study entry, 55.4% of patients presented low/mild disease activity as defined by a SLEDAI2k score below 4, and 11.4% were serologically inactive as defined by normal C3 and C4 complement and anti-dsDNA levels.

A total of 1083 visits to the lupus clinic from these 202 patients were registered over median (IQR) follow-up of 24 (10) months. Mean interval between visits was 3.8 months. Flares were observed in 16.8% of patients. All but one flare occurred in patients with serological activity at baseline. All flares included increased activity in one or more organs and not purely serological activity. Organ involvement at time of first flare during follow-up included: nephritis in 60.0% of cases; arthritis (13.3%); mucocutaneous (13.3%); hematological (11.1%) and vasculitis (2.2%). The average AMS over follow-up time was higher for patients who experienced flares (6.3±3.4) than in those without flares (3.1±2.0) ($p<0.0001$; 95% CI 2.4-4.1).

The predictors of SLE flare identified by univariate analysis with Kaplan-Meyer curves and Log-Rank tests were: SLE diagnosis at younger age (≤25 years old) ($p=0.023$), severe disease defined as use of immunosuppressants at baseline ($p<0.001$) or previous lupus nephritis ($p<0.0001$). Other factors were not predictors: gender, baseline SLEDAI2k score or time since SLE diagnosis, HCQ or steroid user status. Steroid use was not a significant predictor when evaluated either as current versus no-user, or as daily dose above versus up-to 5 mg.

Groups defined according to the presence of each of the three flare predictors found to be significant in univariate analysis were compared: Patients with SLE diagnosis ≤25 years old were more likely to have nephritis at baseline (61.1% vs. 36.7%; $p<0.001$); those with previous nephritis were more frequently taking immunosuppressants ($p<0.0001$).

Multivariate analysis confirmed as predictors for flare outcome: a younger age at SLE diagnosis (≤25 years) (HR=2.14, 95% CI 1.09-4.19; $p=0.03$) (figure 4.1.); previous lupus nephritis at baseline (HR=4.78; 95% CI 2.08-10.98; $p<0.0001$) (supplementary figure 4.S1.); and baseline immunosuppressant treatment (HR=3.22, 95% CI 1.63-6.37; $p<0.001$) (supplementary figure 4.S2.). Due to multicollinearity concerns, the predictors were analyzed in three separate, alternative models. The following covariates were included in multivariate-

adjusted analysis: 1) for younger age at SLE diagnosis - gender and time since diagnosis (continuous variable); 2) for previous lupus nephritis and for baseline immunosuppressant use – gender, younger age at diagnosis, time since diagnosis (continuous variable), baseline SLEDAI2k (continuous variable), baseline use of HCQ and steroids. In all models, the covariates did not have a significant effect and were eliminated in the stepwise analysis.

Table 4.1. Baseline characteristics of the study population (N=202)

Age, mean (SD), years	41.9 (14.5)
Female gender (%)	86.6
Caucasian (%)	97.5
Age at SLE diagnosis, mean (SD), years	31.9 (13.5)
Time since SLE diagnosis, mean (SD), years	9.9 (7.8)
SLEDAI2k score, mean (SD)	4.3 (3.6)
Medication, % current users	
Prednisolone, [median daily dose, mg (IQR)]	49% [5.0 (5.0)]
Hydroxychloroquine	85.1
Immunosuppressants*	32.7

*Immunosuppressants: azathioprine, mycophenolate mofetil, calcineurin inhibitors, methotrexate, cyclophosphamide, rituximab.

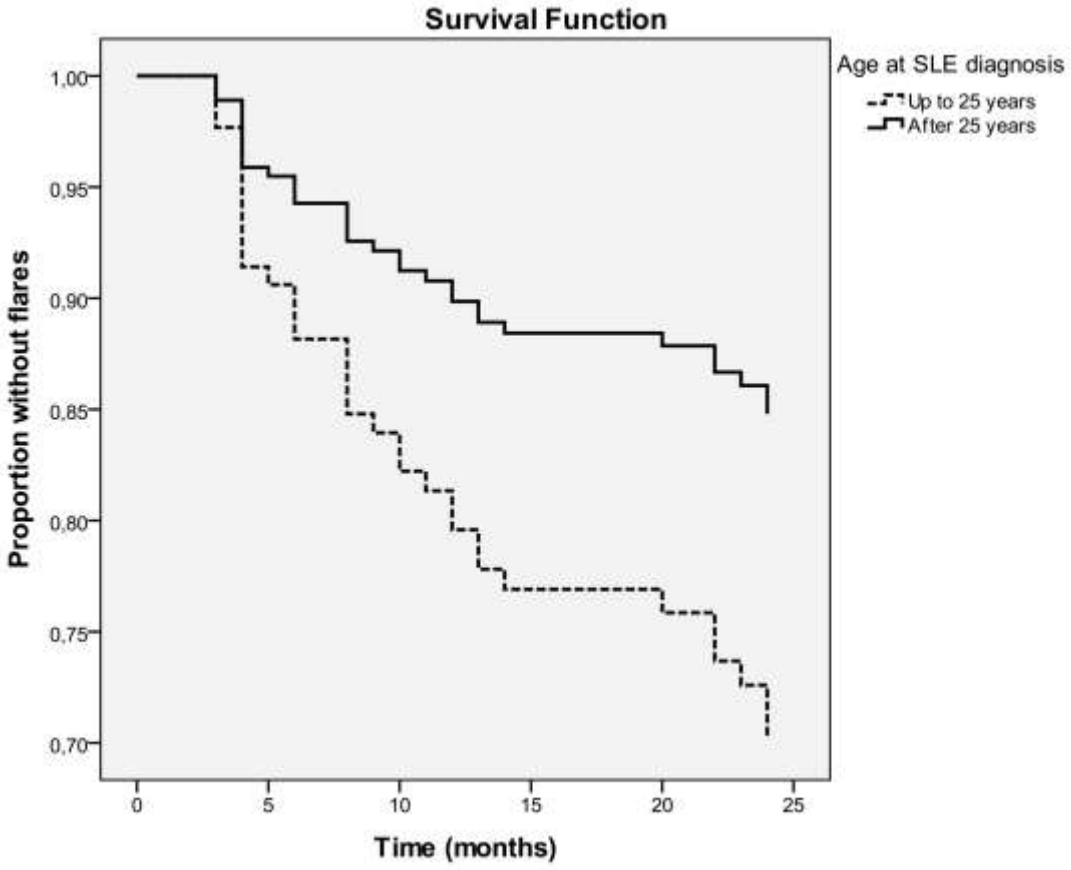
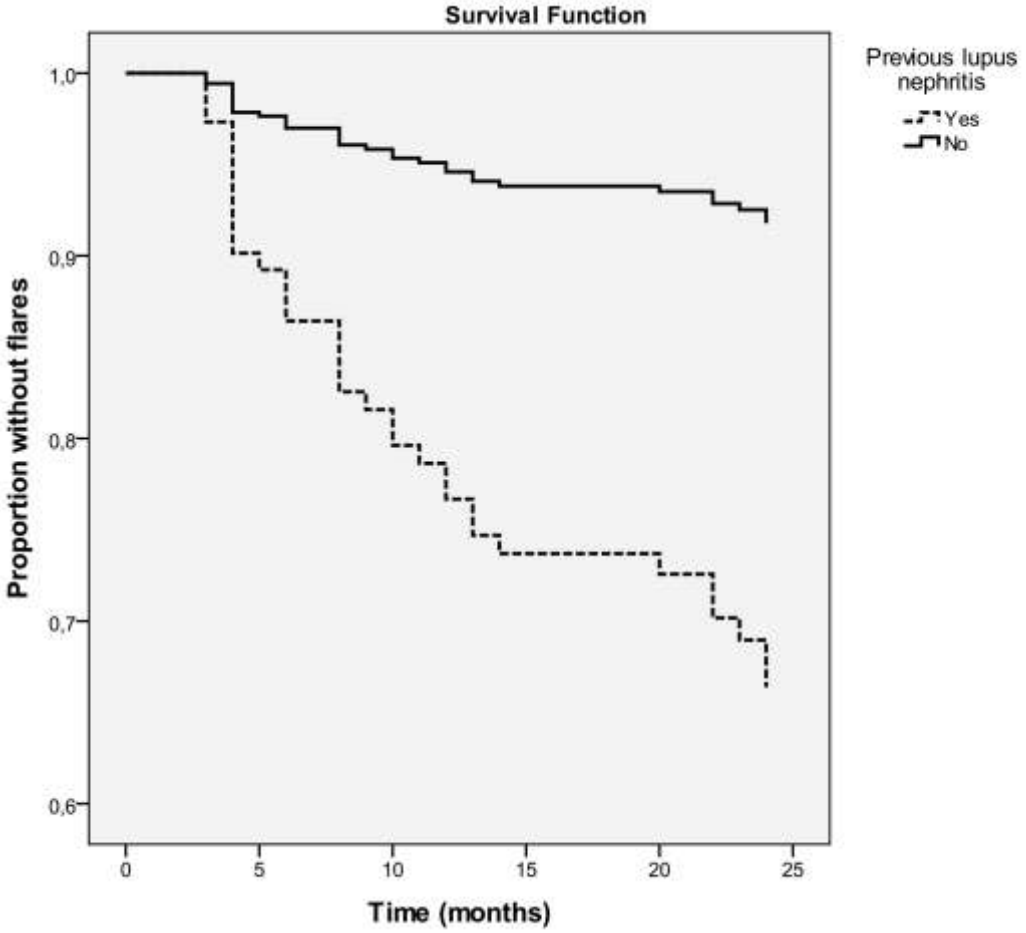
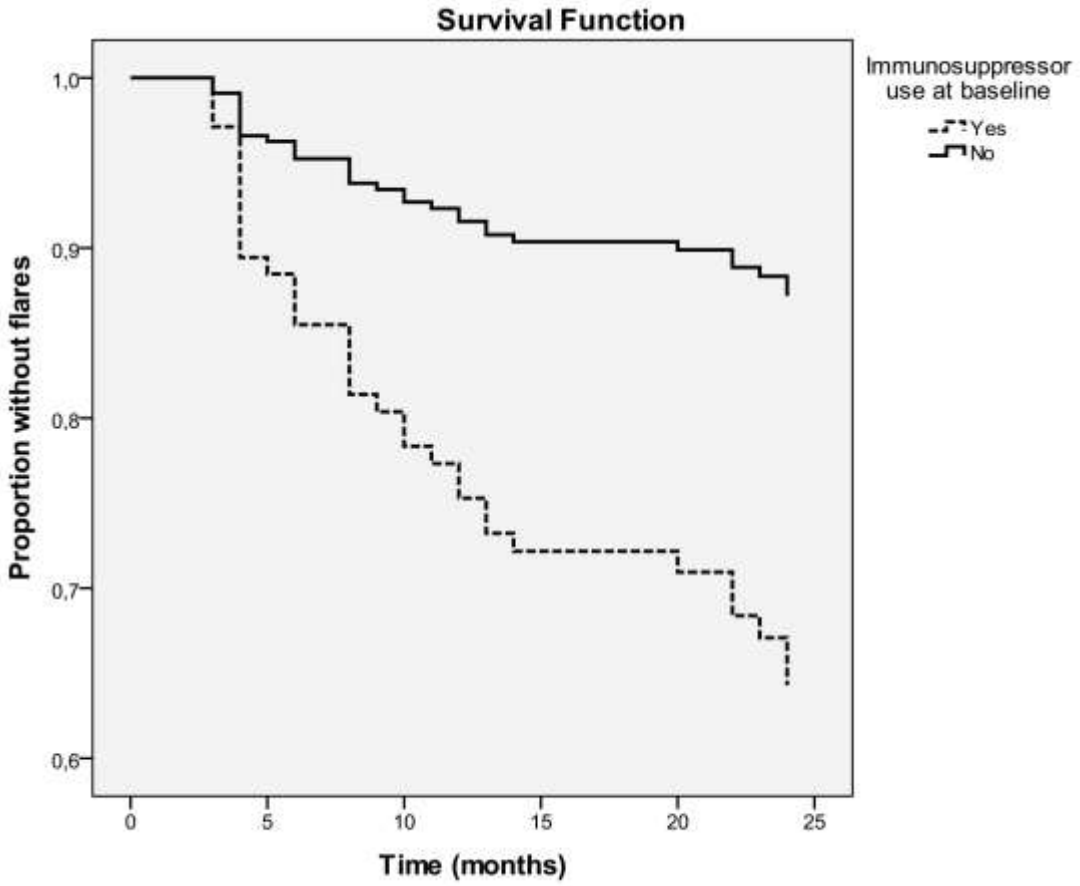


Fig. 4.1. Survival curve showing the flare-free proportion of patients according to categories of age at time of SLE diagnosis (Hazard Ratio =2.14).



Supplementary fig. 4.S1. Survival curve showing the flare-free proportion of patients according to categories of history of previous lupus nephritis at baseline (Hazard Ratio =4.78).



Supplementary fig. 4.S2. Survival curve showing the flare-free proportion of patients according to categories of treatment with or without immunosuppressants at baseline (Hazard Ratio =3.22).

Discussion

This study identified as clinical predictors for increased flare hazard: a younger age at SLE diagnosis (≤25 years), previous lupus nephritis and baseline immunosuppressant treatment/severe SLE. Specifically, at any time-point up to 24-month follow-up, the risk of flare was more than two-fold, four-fold and three-fold higher for patients with SLE diagnosis ≤25 years, previous lupus nephritis or immunosuppressant treatment, respectively. It found no evidence for a lower flare hazard associated with baseline low disease activity or longer SLE duration.

Flare is an important outcome in SLE, both in clinical practice and clinical trials, but challenging to measure. Existing instruments have different profiles of strengths and weaknesses with an overall moderate-to-good agreement to detect flares.[106] We applied SLEDAI2k, a simple, sensitive to change index and used a flare definition previously shown to represent a clinically meaningful increase in disease activity.[46, 47] The outcome event in this study was time to first flare. Time to first flare and difference in SLEDAI score was equally counted from study baseline. Patients may develop progressively an increase in disease activity, for example a new malar rash (SLEDAI score is 2) in the 2th observation month and a new pericarditis (SLEDAI score is 2) with ongoing rash at the 4th observation month. If we looked at SLEDAI as change over time, this same case would be classified variably as presenting a flare or not at the 4th month, depending on the absence or occurrence of an intermediate visit at the 2th month, respectively. Accounting for the SLEDAI change from baseline avoids this potential source of bias. Importantly, and differently from previous studies, we used a time-to-event flare outcome. In the setting of person-time data, with varying risk periods derived from a dynamic open cohort such as in this study, survival analysis methods are the most appropriate.[107-109] This use of Cox's Proportional Hazards Regression Model, a powerful statistical tool, offers a better opportunity to identify clinically relevant flare predictors. A similar approach was previously employed, to evaluate time to renal flare in the MAINTAIN trial, by Houssiau *et al*.[110] The same approach has been used in prospective cohort studies to evaluate the risk of SLE organ damage and mortality.[48, 111] We found a relatively low proportion of patients suffering flares as compared to other cohort studies.[4, 51, 74] It is likely that the use of a more sensitive flare instrument, as the BILAG or a lower cutoff for the SLEDAI2k increase, would identify a higher number of milder flares.[47, 106] The systematic treatment with HCQ in this study's cohort may also have contributed to the low flare rate and to explain why we didn't find lower flare HR with HCQ.[100] Patients with and without flares differed significantly in the adjusted mean SLEDAI2k over the follow-up time, which is a prognostic marker for irreversible damage accrual, coronary artery disease and mortality.[105]

An important contributor to flare hazard with SLE diagnosis at younger age was the higher prevalence of lupus nephritis in this subset of patients.[51, 112-114] The use of immunosuppressive treatment is a marker of severe disease. However, the fact that standard-of-care immunosuppressive medication is associated with higher flare hazard confirms that these drugs are not effective to suppress flares to the level of non-severe patients.[51, 107]

The limitations of this study include it being single-center based and the inexistence of a consensual definition of flare. Observational cohorts may differ systematically with regard to variables related to exposure or outcome, which questions the generalizability of our results and raises need for confirmatory studies in other settings and different ethnic backgrounds. Another concern was the multicollinearity between clinical predictors. We addressed this problem by developing three separate models. We think this option makes the most clinical sense, as the 3 identified clinical predictors should be regarded as alternative, to use one or another depending on the individual case: a patient presenting early with SLE is at increased risk of flare; if further on the disease course the patient develops nephritis or another organ involvement requiring immunosuppressants, any one of those will be the dominant clinical predictor for flare. The primary strength is the application of survival analysis, a powerful statistical method that allowed this novel demonstration of clinical predictors of flare.

In summary, our work suggests that SLE patients with diagnosis up to 25 years of age or previous lupus nephritis or severe disease requiring immunosuppressants, present larger flare hazard and might need tighter clinical monitoring and treatment.

Chapter 5

CD38, CD81 and BAFFR combined expression by transitional B cells distinguishes active from inactive systemic lupus erythematosus

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Abstract

Objectives

In view of its heterogeneous presentation and unpredictable course, clinical management of systemic lupus erythematosus (SLE) is difficult. There is a need for biomarkers and diagnostic aids to monitor SLE disease activity and severity prior to, during and after treatment. We undertook this study to search for unique phenotypic patterns in each peripheral blood (PB) B cell subset, capable of distinguishing SLE patients with inactive disease versus SLE patients with active disease versus controls by using an automated population separator (APS) visualization strategy.

Methods

Peripheral blood was collected from 41 SLE patients and 28 age- and gender-matched controls. We analyzed the cell surface markers expression (in a tube with CD20/CD27/CD19/CD45/CD38/CD81/BAFFR combination) on PB B cell subsets using principal component analysis, implemented with the APS software tool.

Results

The cluster analysis of immunophenotypic profiles of B cell subsets highlighted disease specific abnormalities on transitional B cells focused on the decreased expression of CD38, CD81 and BAFFR in transitional B cells.

Conclusions

Overall, our analysis indicates that active SLE can be distinguished from inactive SLE on the basis of a single tube analysis, that emerge as promising surrogate markers for disease activity. Further validation is needed with larger samples and prospective follow-up of patients.

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disorder, which presents a broad spectrum of symptoms and signs of disease, a diverse degree of severity, course and prognosis. Currently, the pathogenesis of SLE remains only partially understood and results of therapy are frequently unsatisfactory. Such disease complexity underlines the need for new biomarkers of disease activity, and it also highlights the need for further understanding the pathogenic mechanisms for the identification of new and more effective targeted therapies.[115] Studies conducted over the past 15 years indicate that B cells play a crucial role in SLE pathogenesis.[116] The presence of multiple autoantibodies reflects defective tolerance mechanisms leading to the activation of autoreactive B cells and the production of autoantibodies often long before the first expression of the disease.[117]

Although some intrinsic B cells abnormalities may be central to the disease process, the nature of the immune abnormalities resulting in these defects remains elusive. Notably, increased proportions of transitional B cells from peripheral blood have been described in patients with SLE, although bone marrow production and selection appeared to be normal. Since a significant proportion of the immature B cell repertoire is autoreactive, an assessment of the percentage of self- or poly-reactive B cells in early B cell populations has revealed two tolerance checkpoints: one at the immature to transitional junction and another one at the transitional to naïve junction.[118] Furthermore, several data suggest that B cell antigen receptor (BCR) ligation and B cell activation factor member of the tumor necrosis factor (TNF) family (BAFF) may play an essential role in the differentiation, survival and maturation of transitional B cells.[119] However, until now, few reports have evaluated the function of the interaction signaling through the colligation of the BCR and the complement (C3)-binding CD21/CD19/CD81 co-stimulatory complex which has been proposed as a co-receptor for CD38 in human B cells.[120] According to some studies in patients with autoimmune diseases, CD38 seems to participate in immunoglobulin (Ig) class switching and plays a role in distinct pathological situations, although little is known about its role in lupus development.[121] In addition, current knowledge has demonstrated the potential of BAFF to break immune tolerance when overexpressed.[122] Most importantly, some data indicate that elevated BAFF levels may correlate with SLE disease activity.[123]

Taking into account the above considerations, we decided to investigate the potential utility of these combined markers (CD19, CD38, CD81 and BAFFR) on each peripheral blood (PB) B cell subset to distinguish disease activity levels in SLE patients by using the automatic population separator (APS) graphical representation of the Infinicyt software.

Patients and Methods

Patients and controls

Sixty-nine adults were enrolled in the study, including 41 SLE patients and 28 age- and gender-matched healthy individuals (NC: 90 % female; 30 ± 6 years) recruited among healthy blood donors and research staff.

A convenience sample of consecutive patients fulfilling the 1997 American College of Rheumatology (ACR) classification criteria for SLE, with either active or inactive disease according to the criteria below, was recruited from a University Hospital-based Lupus Clinic.[37] Patients were evaluated according to the SLE Disease Activity Index 2000 (SLEDAI 2k) and classified as having active (SLEDAI 2k ≥5; 100 % female, 33 ± 11 years) or inactive disease (SLEDAI 2k<5; 84 % female, 33 ± 10 years).[43, 46] Table 5.1. summarizes the clinical and therapeutic data of all patients.

The number of subsequent flares and the SLEDAI score were measured during the study period. Follow-up was defined as at least two visits 2–6 months apart during 12-month period. Flare episode was defined as an increase in SLEDAI-2K score of ≥4 from the previous visit.[47]

Disease features cumulatively observed from the beginning of the disease until the time of the study were registered. Exclusion criteria: known or suspected ongoing infections and, for NC, any history of autoimmune disease or immunosuppressive therapy.

All participants were asked to provide a morning sample of peripheral blood which was processed fresh. Informed consent was obtained from all individual participants included in the study.

Table 5.1. Clinical features and active medication of SLE patients

	ISLE (n =24)	ASLE (n =17)
Mean SLEDAI 2k scores (±s.d)	1.6±0.9	9.7±3.2 *
Mean time since diagnosis (years; ±s.d.)	9.0±6.0	7.6±7.4
Cumulative clinical features [a]		
Lupus nephritis (%)	61.3	44.4
Neurolupus (%)	19.4	0 *
Lupus arthritis (%)	58.1	66.7
Hematological involvement (%)	87.1	100
Lupus skin disease (%)	74.2	77.8
Severe lupus (%) [b]	71	44.4 *
Anti-dsDNA antibodies (%) [c]		
Negative (<4.2 IU/ml)	42.3	11.1 *
Low positive (4.2-20 IU/ml)	32.3	11.1 *
Moderate positive (20-50 IU/ml)	22.6	22.2
High positive (>50 IU/ml)	6.5	55.6 *
Treatment (%)		
Hydroxychloroquine	87.1	94.4
Immunosuppressants [d]	32.3	66.7 *
Steroids	12.9	83.4 *
None to low dose (≤10 mg/day)	100	38.4
Moderate dose (10-30 mg/day)	0	27.8
High dose (>30 mg/day)	0	16.7

* Statistically significant differences were considered when $p<0.05$ for Mann–Whitney U and Chi-squared test. Standard deviation (s.d.)
[a] Clinical features defined as cumulative incidence since beginning of the disease until time of the study
[b] Severe lupus defined as cumulative major organ involvement
[c] Quantification at time of study blood sample collection
[d] Azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus, methotrexate, cyclophosphamide or rituximab

Flow cytometry analysis

Distribution of different B cell compartments was performed on erythrocyte-lysed and washed PB samples according to procedures which have been previously described in detail.[124] Briefly, all samples were stained and lysed using a direct immunofluorescence technique. PB white blood cells (WBC) were stained with the following monoclonal antibody (mAb) combinations: anti-CD20-PB (Pacific Blue; clone 2H7; BioLegend, San Diego, California, USA), anti-CD27-PC5 (phycoerythrin–cyanine 5; clone 1A4LDG5; Beckman Coulter; USA), anti-CD19-PC7 (phycoerythrin–cyanine 7; clone J3-119; Beckman Coulter, France), anti-CD45-KO (Krome Orange; clone J.33; Beckman Coulter), anti-CD81-APC-H7 (clone JS-81; BD Pharmingen), anti-BAFFR-PE (phycoerythrin; clone 11C1; BD Pharmingen, San Diego, California, USA) and anti-CD38-FITC (fluorescein isothiocyanate; clone HIT2, BD Pharmingen).

Cells were acquired on a FACS CantoTM II (BD) using FACSDiva software (BD), and 100,000 events were analyzed using Infinicyt 1.7 software (Cytognos, Salamanca, Spain).

B cell subsets were identified on the basis of the following immunophenotypic features: immature transitional ($CD19^+/CD20^+/CD27^-/CD38^+$), naïve ($CD19^+/CD20^+/CD27^-/CD38^-$), memory ($CD19^+/CD20^+/CD27^+/CD38^+$) and plasmablasts ($CD19^+/CD20^{-/+low}/CD27^{++}/CD38^{++}$). In each subset, the expression of CD19, CD20, CD38, CD81 and BAFFR were evaluated. Results represent the mean fluorescence intensity (MFI) for each marker within each cell compartment, as graphically displayed with the 3D automated population separator (APS) view—principal component 1 (PC1) versus PC2 versus PC3—of the Infinicyt™ software. As previously described in detail, in this APS view, each axis of a plot is represented by a different PC as a linear combination of parameters with distinct statistical weights.[125] Selected clusters were compared with each other to find significantly different bins in a search for cellular subsets that would serve as hallmarks of the cluster.

Statistical analysis

Multiple group comparisons were first performed by means of Kruskal–Wallis nonparametric test to determine whether any group differed from the others. Individual pairings were analyzed using a Mann–Whitney U test and Chi-squared (χ^2) test to determine significance. P values lower than 0.05 were considered statistically significant. SPSS version 21.0 software (SPSS Inc., Chicago, USA) was used to perform the statistical analyses.

Results

B cell subsets in healthy individuals and SLE patients

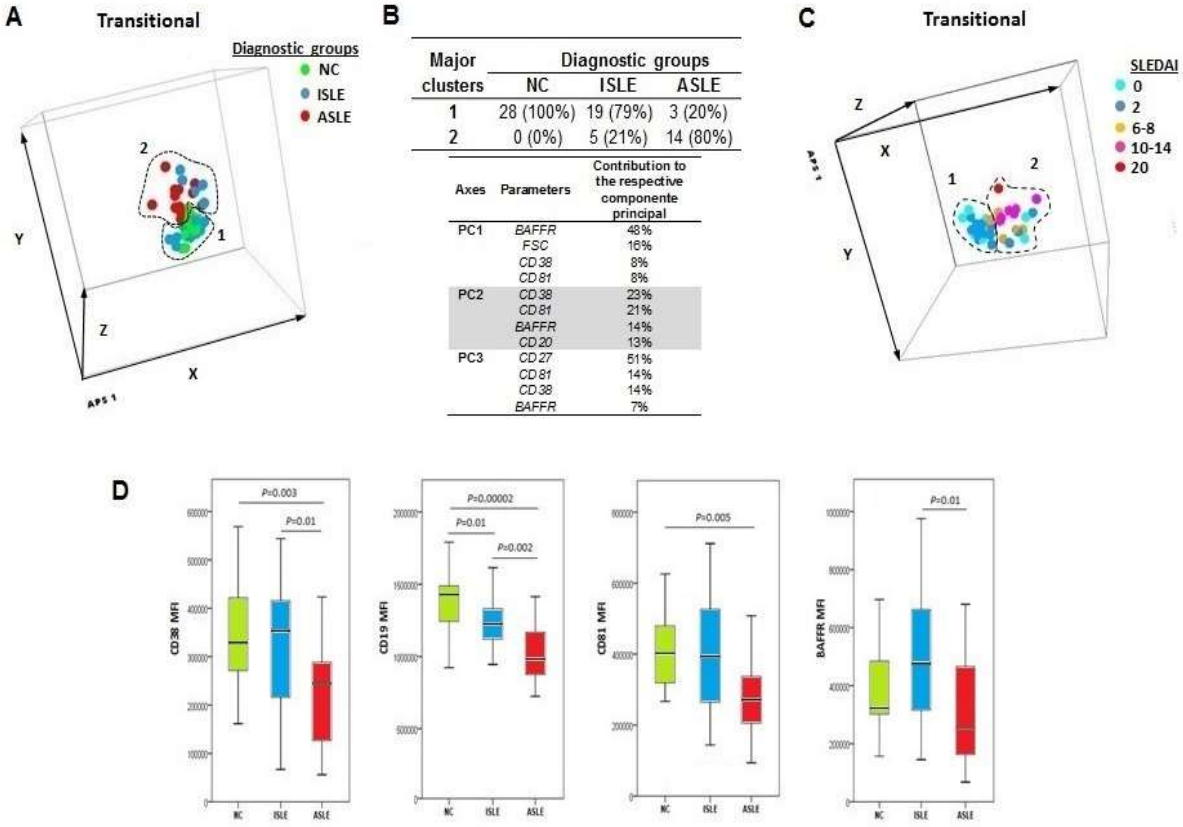
Lower absolute numbers of total B lymphocytes were found in active SLE patients when compared with healthy controls and inactive SLE patients ($p<0.05$). This was particularly reflected in the marked reduction in naïve and memory B cells, whereas both transitional B cells and plasmablasts were apparently not affected. Moreover, a higher frequency of plasmablasts, among B cells, was detected in active disease (3%) as compared with inactive disease (0.9%) and controls (1.3%). Regarding the pattern of B cell distribution in inactive SLE, a significant increase in the numbers of transitional B cells followed by a decrease in memory cells and plasmablasts was observed —additional data are given in supplementary table 5.S1.

To clarify the mechanisms underlying all these previous alterations, we further analyzed cell surface signal transduction molecules (CD19, CD20, CD38, CD81 and BAFFR) on B cell subsets from both SLE patients and healthy individuals—additional data are given in supplementary table 5.S2.

Differential expression of CD19, CD20, CD38, CD81 and BAFFR on B cell compartments from SLE patients versus normal controls

The mathematical tools used in the present study allowed the calculation of the complete immunophenotypic information derived from each PB B cell subset among SLE patients and NC. Herein, we demonstrated that with the analysis of transitional B cells, we could identify two major groups, as shown in Fig. 5.1a. In contrast, no clusters could be distinguished in the remaining B cells subsets (naïve, memory and plasmablasts)—additional data are given in supplementary figure 5.S1.

Regarding transitional B cells, Cluster 1 represented a total overlap between controls and the majority of SLE patients with inactive disease (79%), reflecting the intrinsic biological similarity of transitional B cells in these cases. Only 18% (three cases) of the SLE patients with active disease were included in Cluster 1 (Fig. 5.1a, b). Of note, the three patients with active disease assigned to Cluster 1 showed a relatively low SLEDAI (SLEDAI =8±0) compared with the remaining ASLE patients (mean SLEDAI =10±3.7) and all became inactive upon the 12-month follow-up period (Mean SLEDAI =3±1.1). On the other hand, Cluster 2 integrated 82% of all SLE patients with active disease and only 21% (5 cases) with inactive disease, these ones showing a SLEDAI =2 and a medical history of inactive nephritis and severe lupus. In addition, a flare episode during a follow-up of 12 months and an increased SLEDAI score (SLEDAI =6) was exclusively observed in one of these misclassified patients. Moreover, a reduction in SLEDAI score, although only six patients become inactive, was found 12 months after the study (mean SLEDAI =6±2.7) in active SLE patients included in this cluster — see additional data in supplementary table 5.S3.



Major clusters	Diagnostic groups		
	NC	ISLE	ASLE
1	28 (100%)	19 (79%)	3 (20%)
2	0 (0%)	5 (21%)	14 (80%)

Axes	Parameters	Contribution to the respective componente principal
PC1	*BAFFR*	48%
	FSC	16%
	CD38	8%
	CD81	8%
PC2	*CD38*	23%
	CD81	21%
	BAFFR	14%
	CD20	13%
PC3	*CD27*	51%
	CD81	14%
	CD38	14%
	BAFFR	7%

Fig. 5.1. Principal component analysis (three-dimensional X-Y-Z axis view of PC1 vs. PC2 vs. PC3, respectively) for comparison of SLE patients with inactive disease (ISLE), SLE patients with active disease (ASLE) and normal controls (NC) according to the expression of CD19, CD20, CD27, CD38, CD45, CD81, BAFFR as well as FSC and SSC parameters using the Infinicyt ™ software. Each circle represents the overall median position of an individual SLE patient and NC in the PC1 versus PC2 versus PC3 representation of the whole immunophenotypic profile of transitional B cell subset; overall, NC, ISLE and ASLE cases are distinguished by different colors (a). The most informative parameters contributing to the best discrimination between the two clusters are displayed in a decreasing order of percentage contribution to each of the principal component (b); the distribution of the SLEDAI among SLE patients is colored differently (c). Individual expression of CD19, CD38, CD81 and BAFFR on transitional B cells from SLE patients and NC (d). PC: principal component.

Supplementary table 5.S1. Absolute and relative numbers of the different maturation-associated B cell subsets in healthy individuals and patients with systemic lupus erythematosus.

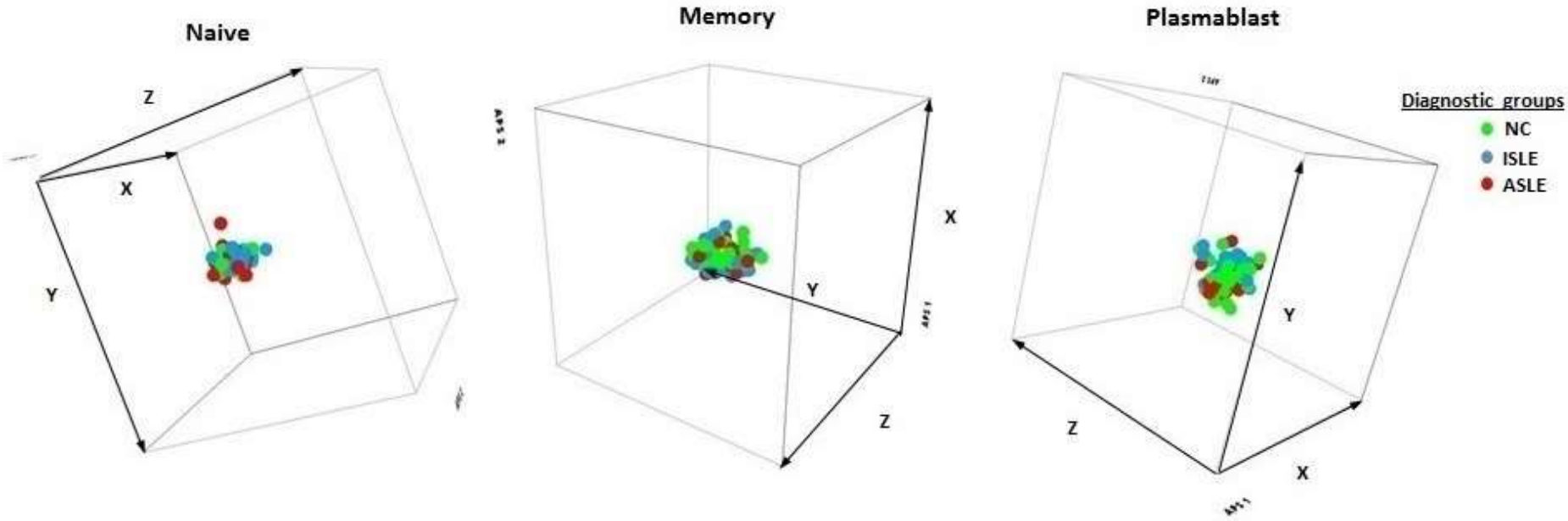
	Normal controls (NC)		Inactive SLE (ISLE)		Active SLE (ASLE)		p-value (NC vs ISLE)		p-value (NC vs ASLE)		p-value (ISLE vs ASLE)	
	Cells/µl	%	Cells/µl	%	Cells/µl	%	Cells/µl	%	Cells/µl	%	Cells/µl	%
Lymphocytes [1]	2166 (1340-5190)	30.5 (17.7-53)	1632 (580-3503)	25 (6-45.9)	837 (220-1630)	11.5 (2.4-48)	0.004	0.005	<0.00001	<0.00001	0.001	0.005
B cells [2]	188 (50-560)	2.9 (0.9-6.8)	172 (36-400)	2.8 (0.7-5.2)	68.3 (9.2-209)	1.1 (0.1-7.2)	NS	NS	0.0003	0.002	0.004	0.01
Transitional [2]	9 (3-37)	4.1 (2.2-9)	15 (1.5-70)	10.1 (1.6-22.5)	5.4 (0.1-25)	6.8 (1-32)	0.02	0.00003	NS	NS	0.002	NS
Naïve [2]	121 (30-356)	60.5 (43.3-74)	113 (17-276)	63.7 (46.4-76)	45 (1.9-166)	63.8 (20-82)	NS	NS	0.003	NS	NS	NS
Memory [2]	70 (18-204)	34.4 (19-51.4)	37 (8.8-154)	22.5 (10.7-44.1)	10 (0.4-35)	17.5 (4.3-38)	0.006	0.001	<0.00001	0.0002	0.02	NS
Plasmablasts [2]	2.4 (0.6-9)	1.3 (0.3-4.3)	1.4 (0.3-4.9)	0.9 (0.4-3.8)	2.6 (0.03-25)	3 (0.1-46)	0.005	NS	NS	0.04	0.00001	0.03

[1] Number of cells from total peripheral blood; [2] Number of cells from total peripheral blood B cells; p- values were determined by Mann Whitney U test; NS: no statistically significant differences. Results are expressed as median (range).

Supplementary table 5.S2. Expression levels of individual markers in the different maturation-associated B cell subsets from SLE patients and healthy individuals.

		B cell subsets							
		CD19 MFI				CD81 MFI			
		Transitional	Naïve	Memory	Plasmablast	Transitional	Naïve	Memory	Plasmablast
Participant group	**NC** (n =28)	15209 (10696-19153)	15893 (11614-37552)	17736 (13411-22906)	7467 (4473-11976)	4212 (475-8545)	1610 (949-2748)	1847 (930-3260)	3249 (970-6584)
	ISLE (n =24)	12529 (9996-21483)	13240 (9886-17735)	15721 (12280-20245)	5563 (556-8047)	4079 (1361-7750)	1652 (743-2836)	1766 (640-2875)	2583 (1099-3957)
	ASLE (n =17)	10947 (7542-17493)	10574 (8256-17231)	12341 (9833-20377)	4486 (2828-6120)	2630 (937-7268)	1606 (677-3312)	1716 (808-3101)	2703 (1406-4651)
p-value	NC versus ISLE	0.0005	0.0001	0.002	0.0001	NS	NS	NS	0.05
	NC versus ASLE	<0.00001	<0.00001	<00001	<0.00001	0.006	NS	NS	NS
	ISLE versus ASLE	0.004	0.004	0.002	0.0004	0.02	NS	NS	NS

NC: Normal controls; ISLE: patients with inactive SLE; ASLE: patients with active SLE; MFI: mean fluorescence intensity (range). p- values were determined by Mann Whitney U test; NS: no statistically significant differences.



Supplementary fig. 5.S1. Principal component analysis (3-dimensionalX-Y-Z axis view of PC1 vs PC2 vs PC3, respectively) for comparison of SLE patients with inactive disease (ISLE), SLE patients with active disease (ASLE) and normal controls (NC) cases according to the pattern of immunophenotypic markers (in a tube CD20/CD27/CD19/CD45/CD38/CD81/BAFFR combination) and FSC and SSC parameters, using the Infinicyt™ software. Overall, cases were clustered into groups distinguished by different colours.

Supplementary table 5.S3. Follow up data of misclassified SLE patients defined by Principal component analysis.

Patient group		Anti-dsDNA serum levels	Lupus nephritis	SLEDAI 2k score [1]	Number of lupus flares [2]	SLEDAI 2k score after 12 months
Cluster 1	Active SLE	Negative	Active	8	0	4
		Moderately positive	Never	8	0	2
		Negative	Never	8	0	4
Cluster 2	Inactive SLE	Moderately positive	In remission	2	0	2
		Low positive	In remission	2	0	2
		Low positive	In remission	2	0	2
		Moderately positive	In remission	2	1	6
		Low positive	In remission	2	0	3

[1] SLEDAI 2k scored at time of study blood collection; [2] Number of lupus flares in 12 month follow-up after the study blood collection; anti-dsDNA serum levels were evaluated at time of the study blood collection; lupus nephritis evaluated as cumulative incidence since SLE diagnosis and status of lupus nephritis activity at time of blood collection.

The dispersion observed within this latter group was proportional to the wide range of SLEDAI typically found in active disease. Moreover, even within Cluster 2, cases with higher SLEDAI (SLEDAI 10–14) appeared to cluster together, between those with lower SLEDAI (SLEDAI 5-8) and an isolated case with SLEDAI =20 (Fig. 5.1c). The most informative markers in this comparative analysis were: (1) CD38, CD81 and BAFFR for the identification of active SLE patients (Fig. 1b). Cluster 2 was mainly characterized by SLE patients with active disease that showed a marked decrease in CD19, CD38, CD81 and BAFFR expression on transitional B cells ($P<0.05$; Fig. 5.1d).

Discussion

Our findings describe a novel visualization strategy that combines expression of BAFFR, CD81 and CD38 on transitional B cells as a useful tool in the assessment of disease activity in SLE patients.

B cells from SLE patients display signaling defects that may underlie disease pathogenesis activity. Reportedly, complement receptor (CR) type 1 and type 2 are decreased on the surface of SLE B cells and their function appears to maintain B cell immune tolerance to self-antigens.[126]

Using a mathematical procedure for the immunophenotypic analysis of PB B cell populations, we evaluated the potential of B cell surface receptors in differentiating between SLE and healthy subjects.

Our visualization strategy revealed that the combined expression of BAFFR, CD81 and CD38 on transitional B cells had a greater weight in the discrimination of active SLE patients, clearly clustering together a major fraction of SLE patients with active disease (82%, 14 out of 17 active SLE patients), whereas most SLE patients with inactive disease were considered to be clearly different and closely related to controls (79%, 19 out of 24 inactive SLE patients). Of note, although eight cases were misclassified and could be viewed as ”failures” of the proposed procedure, it more likely reflected the need for additional markers to be included in this monoclonal antibody combination. Alternatively, it is also conceivable that misclassified patients are in progress toward a modification of their clinical disease activity status. In this sense, we also assessed the relationship between immunophenotypic profiles on well-defined subsets of peripheral blood B cells and the risk of further SLE flare-ups during 12-month follow-up. Notably, the only patient with inactive disease who had followed a flare episode was identified as a “misclassified” case within Cluster 2. Further long-term longitudinal studies on larger series of patients will be crucial to definitely clarify these hypotheses and define the precise value of this new tool in SLE. Noteworthy, the potential of this strategy is highlighted by successes obtained in the immunophenotypic screening and classification of individual patients into pre-established and well defined WHO diagnostic entities.[127] To the best of our knowledge, this is the first time that such a procedure, based

on information derived from phenotypic profiling of individual cells, is proposed in the disease stratification of SLE.

Overall, the results obtained showed that B cell surface receptors BAFFR, CD81 and CD38 are significantly affected in patients with active SLE, pointing to their possible involvement in the etiopathogenesis of the disease. Support for this concept comes from previous observations of decreased expression of CD19 and CD21 (CR2), a C3d receptor, as a result of subsequent interaction with circulating immune complexes (CICs) bearing C3 fragments in SLE patients.[128] Interestingly, the decreased expression of CD81, a negative regulator of B cell activation, and CD38 observed on transitional B cells from active SLE patients may suggest an enhanced early B cell activation or a more mature phenotype of transitional B cells. Although CD38 has been used extensively to classify various subpopulations of lymphocytes, in recent years, several publications also have linked CD38 with different pathologies, including autoimmune diseases. Indeed, apoptotic effect mediated by CD38 in immature cells is well described and absence of CD38 in lpr mice has been associated with an accelerated development of a lupus-like disease.[129] Furthermore, an increased acquisition of CD38 expression on memory B cells along with the expansion of plasmablasts observed in active SLE patients further supports the hypothesis of a stronger stimulation on SLE B cells and the subsequent generation of plasmablasts in active disease. Finally, it is well established that BAFF, through BAFFR, plays a key role in B cell activation and survival. It has been demonstrated that the chronically elevated overproduction of BAFF in SLE patients with active disease down-regulates BAFFR expression on transitional B cells.[130]

In conclusion, the present study demonstrates that the combined expression of BAFFR, CD81 and CD38 in transitional B cells may be used as a practical tool to disease classification of SLE. Further studies evaluating its efficiency in larger series of patients, where an extended follow-up monitoring is also included, are required to confirm these results.

Chapter 6

Th17 cells in systemic lupus erythematosus share functional features with Th17 cells from normal bone marrow and peripheral tissues

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* Luís Inês and Ana Henriques contributed equally to this work

Abstract

Objectives

This study was designed to investigate the functional heterogeneity of human Th17 and how their plasticity shapes the nature of immune cell responses to inflammation and autoimmune diseases, such as systemic lupus erythematosus (SLE).

Methods

We evaluated functional Th17 cell subsets based on the profile of cytokine production in peripheral blood (PB), bone marrow aspirates (BM) and lymph node biopsies (LN) from healthy individuals (n=35) and PB from SLE patients (n =34). Data were analyzed by an automated method for merging and calculation of flow cytometry data, allowing us to identify eight Th17 subpopulations.

Results

Normal BM presented lower frequencies of Th17 ($p = 0.006$ and $p = 0.05$) and lower amount of IL-17 per cell ($p=0.03$ and $p = 0.02$), compared to normal PB and LN biopsies. In the latter tissues were found increased proportions of Th17 producing TNF-α or TNF-α/IL-2 or IFN-γ/TNF-α/IL-2, while in BM, Th17 producing other cytokines than IL-17 was clearly decreased. In SLE patients, the frequency of Th17 was higher than in control, but the levels of IL-17 per cell were significantly reduced ($p < 0.05$). Among the eight generated subpopulations, despite the great functional heterogeneity of Th17 in SLE, a significant low proportion of Th17 producing TNF-α was found in inactive SLE, while active SLE showed a high proportion producing only IL-17.

Conclusions

Our findings support the idea that the functional heterogeneity of Th17 cells could depend on the cytokine microenvironment, which is distinct in normal BM as well as in active SLE, probably due to a Th1/Th2 imbalance previously reported by our group.

Introduction

Although it has been known for over 10 years that activated CD4+ T cells produce IL-17, the existence of a third subset of CD4+ effector T helper cells, named Th17 cells, has only recently been identified as an independent T cell lineage, distinct from classical Th1 and Th2 cells.[131-133] Nevertheless, a common developmental origin between human Th17 and Th1 cells has been described in various studies.[134-136] Accordingly, human Th17 clones appear to express IL-12Rß2 in addition to IL-23R, and the transcription factor Tbet in addition to RORγt, as well a remarkable proportion of human Th17 cells, produces both IL-17A and IFN-γ, or human Th17 clones can be induced to produce IFN-γ and upregulate T-bet expression when cultured in the presence of IL-12.[137] This close relationship between Th1 and Th17 cells is also marked in their capacity to cause T-cell-mediated inflammation and autoimmune disease. In fact, Th17 cells have been found to have a pathogenic role in several autoimmune disorders, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, inflammatory bowel disease, systemic sclerosis and systemic lupus erythematosus (SLE), which were previously thought to be mainly caused by Th1 responses.[138-145] Interestingly, studies show Th17 cells as more potent than Th1 cells in inducing disease, linking the cytokine IL-17 in coordinating tissue inflammation via the up-regulation of additional proinflammatory and neutrophil-recruiting cytokines and chemokines, suggesting a pro-inflammatory role in these conditions.[146] Moreover, in Crohn's disease, the existence of a remarkable number of double positive IFNγ+/IL17+ T cells, named Th17/Th1 cells, as well as the possibility to generate Th17/Th1 clones was reported.[147] These latter observations further support the concept that both Th1 and Th17 cells could contribute to the maintenance of inflammation in such disorders via the production of IFN-γ and IL-17.

However, whether human autoimmune disorders, including SLE, are prevalently Th1-mediated or Th17-mediated and how they might cooperate with one another to perpetuate the inflammatory response are still unclear.[144]

In the present study, we determined the relative frequency of Th17 cells (through their expression of IL-17) in peripheral blood samples (PB), bone marrow aspirates (BM) and lymph node biopsies (LN), simultaneously quantifying the frequency of these cells producing IL-2, TNF-α and IFN-γ. Using an automated method of integration of flow cytometry data from several multicolor stains of the same cell sample into a single multicolor staining data file, we described the functional heterogeneity of human Th17 cells, identifying eight different Th17 cell subpopulations.[148] Considering the well-defined role played by cytokines in the immune deregulation observed in autoimmune diseases, we compared the distribution of the eight identified Th17 subpopulations in a group of SLE patients and age-matched healthy control subjects, suggesting that their particular functional behavior may differ according to the microenvironment condition and participate dynamically in autoimmune response.

Taken together, our data shed new light on the nature of human Th17 cells and their close association with Th1 cells, strengthening the relevance of knowing how their great functional plasticity may influence the disease activity of SLE.

Methods

Patients and controls

We recruited patients fulfilling the 1997 American College of Rheumatology classification criteria for SLE followed at the Rheumatology Service of the University Hospital of Coimbra, Portugal.[37] After assessing disease activity at the time of evaluation according to the SLE Disease Activity Index (SLEDAI 2000), the PB samples from the 34 SLE patients were divided in two groups, one with clinically active (SLEDAI ≥5; n =15 comprising 86.7% female, mean age 32±11 years) and the other with inactive (SLEDAI <5; n =19 comprising 89.5% female, mean age 34±9 years) SLE.[43, 46, 47] The patients' active medication at the time of evaluation was recorded. The clinical features of SLE patients are presented in Table 6.1.

We recruited healthy individuals at the Histocompatibility Center of Coimbra from whom we obtained 10 bone marrow aspirate samples (70% female, mean age ± SD: 58.3±20.1 years), 15 peripheral blood samples (73% female, mean age ± SD: 33.8± 9.8 years) and 10 lymph node biopsies (50% female, mean age ± SD: 48±16.1 years); all were free from autoimmune disease and active infection and were not undergoing treatment with immunomodulatory drugs for any known condition. Bone marrow aspirate and peripheral blood samples were collected from healthy donors, while lymph node biopsies were collected from healthy donor victims of traffic accidents, and organs were received at the Histocompatibility Center of Coimbra to perform histocompatibility testing.

Pregnancy and age below 18 were exclusion criteria for participation. Samples from both controls and patients were sent to the laboratory, identified with a code number, blinding the laboratory to the disease status. The study protocol was approved by a local ethics committee, and all participants gave their signed informed consent.

Table 6.1. Clinical features in 34 patients with systemic lupus erythematosus (SLE)

	Inactive SLE (n =19)	**Active SLE** (n =15)
SLEDAI 2k score (mean±s.d.)	2.7±1.52	10.3±3.53
Time since diagnosis (years, mean±s.d.)	9±5	9±5
Disease manifestations [a]		
Lupus nephritis (%)	52.6	66.7
Neurolupus (%)	20	0
Lupus arthritis (%)	68.4	93.3
Hematological involvement (%)	84.2	93.3
Lupus skin disease (%)	52.6	80
Treatment at time of study (%)		
Hydroxychloroquine	94.7	80
Prednisolone	42.1	93.3
Daily dose (mean)	5.3	18.2
Immunossupressants [b]	31.6	53.3

[a] Cumulative incidence of SLE manifestations since diagnosis; [b] Mycophenolate mofetil, azathioprine, or cyclosporine A.

Flow cytometry data acquisition and analysis

Immunofluorescent staining of peripheral Th17 subsets after in vitro stimulation with PMA/ionomycin in the presence of Brefeldin A

We diluted 500 µL of each sample L/L (vol/vol) in RPMI-1640 medium (Gibco; Paisley, Scotland, UK), supplemented with 2-mML-glutamine. We added 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma, Saint Louis, MO, USA), 1 µg/mL of ionomycin (Boehringer Mannheim, Germany) and 10 µg/mL of Brefeldin A (Golgi plug-Sigma, Saint Louis, MO, USA) and incubated for 4 h at 37°C in a humidified incubator with 5% CO2 concentration. Each cultured sample was aliquoted and stained in three different tubes (200 µL/tube) using an intracytoplasmatic permeabilization and staining protocol in order to separately analyze the intracellular expression of IL-2, TNF-α and IFN-γ in IL-17-positive T cell subpopulations, within the $CD4^+$ T cell subset. All cell aliquots were stained with anti-IL-17 PE (clone 41802; R&D Systems, Europe) and separately with anti-IL-2 FITC (clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA), anti-TNF-α FITC (clone MAb11; BD Pharmingen, San Diego, C.A., USA) and anti-IFN-γ FITC (clone 4S.B3; BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions for fixation and permeabilization. These monoclonal antibodies (MAbs) were added to each tube after staining cells for surface expression of MAbs directed against T lymphocytes subsets—anti-CD3-PerCP (clone SK7; BD, San Jose, CA, USA) and anti-CD8 APC (clone SK1; BD, San Jose, CA, USA). Among positive CD3 cells, $CD4^+$ T cells were

identified after exclusion of the γδ-T cell subset according to their higher reactivity with anti-CD3 monoclonal antibody and typical light scatter. The election of CD8 instead of CD4 was made concerning the down-regulation of the CD4 co-receptor after in vitro stimulation, which is a fact that did not potentially influence the merging/calculating procedure since, in our study, five parameters were measured in common—forward light scatter (FSC), side light scatter (SSC), IL-17 PE, CD3 PerCP and CD8 APC—given a great security in the calculation of all the other parameters (IL-2 FITC, TNF-α FITC and IFN-γ FITC) in three separated four-color staining of the same cell sample.

Data acquisition was performed in two consecutive steps in a FACSCalibur flow cytometer (BDB, San Jose, CA) equipped with an argon ion laser and a red diode laser. In the first step, 2×104 events/test, corresponding to all nucleated cells present in the sample, were collected. To improve the sensitivity of the analysis of T cell subsets present at low frequencies in the different tissue samples, in the second step, information on $CD3^+$ cells and typical light scatter contained on a minimum of 3×105 events from the total sample cellularity was specifically stored through an electronic live gate. To identify the different T cell subsets and evaluate cytokine production, we used the Infinicyt™ software program (Cytognos, Spain). Results illustrate the percentage of positive cells within each cell subset or/and their mean fluorescence intensity (MFI).

Merging of flow cytometry data files and estimation of simultaneous cytokine production in a single file

As shown in Fig. 6.1., cytokine production was measured in a single multicolor data file obtained after merging the original four-color-staining (six-parameter) data files from each sample using the Infinicyt™ software program (Cytognos, Spain).[148] The basic concepts behind such strategies require: (1) merging of different data files corresponding to distinct aliquots of a sample; (2) virtual correction of differences between corresponding cell populations present in different aliquots of the same sample; and (3) calculation of immunophenotypic data measured in one sample aliquot to the corresponding/similar cells measured in the other aliquots of the same sample. In this sense, it required the inclusion of backbone reagents, such as anti-IL-17, -CD8 and -CD3, aimed at the identification of the cell population of interest (Th17 cells) in all MAb combinations to increase the reproducibility of the gating strategy used to select specifically these cell populations in a sample for the evaluation of their overall cytokine production (IL-2, TNF-α, INF-γ and IL-17). This strategy required strict supervision by an experienced operator since automated adjustment of gates between different data files may result in inappropriate detection of specific cell populations.

Sequential merging of all data files from several multicolor staining of the same cell sample was performed, and the calculation function of the Infinicyt™ software was based on nearest-neighbor statistical tools. Accordingly, in our study, five parameters were measured in

common: forward light scatter (FSC), side light scatter (SSC), IL-17 PE, CD3 PerCP and CD8 APC in three separated four color staining of the same cell sample; all other parameters (IL-2 FITC, TNF-α FITC and IFN-γ FITC) were measured only for that subset of cellular events corresponding to the specific three-color staining. Briefly, for each event, a vector in a three-dimensional space was built-up based on the data measured for the five common parameters (FSC, SSC, IL-17 PE, CD3 PerCP and CD8 APC). Then, the nearest neighbor for each individual event in a data file/sample aliquot was calculated as that event in another file/aliquot showing the shortest distance to it in the three-dimensional space generated by those parameters measured in common in both data files/sample aliquots. Then, for each individual event in a data file, those values obtained for each of the closest events in the other data files were assigned for each of those parameters not actually measured in the former event.

Statistical analysis

Data was analyzed using the non-parametric Mann–Whitney U test. Results were expressed as mean ± SD and median. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, USA), and differences were considered statistically significant when the p value was <0.05.

Results

Frequency of human Th17 cells in different samples from healthy individuals

As shown in Table 6.2., the frequency of Th17 among CD4 T cells in healthy individuals was significantly lower in the BM than that found in PB (p =0.006) and LN samples (p =0.02). The amount of IL-17 produced at single cell level (mean fluorescent intensity — MFI) was also markedly lower in the BM, when compared to PB (p =0.03) and LN (p =0.02) samples, with the amount of IL-17 produced being significantly higher in LN (p =0.003) than in PB (Table 6.2.).

Table 6.2. Frequency of Th17 and IL-17 production in tissues from healthy subjects and SLE patients

Participant group	**Healthy subjects**			**Inactive SLE**	**Active SLE**
Tissue	BM	LN	PB	PB	PB
Th17/Total CD4$^+$ T cells (%)	0.6±0.2	1.6±0.7	1.6±0.7	2.0±1.2	1.9±0.9
IL-17 MFI	63.9±25.5	157.4±50.9	90.0±20.2	62.8±20.7*	65.8±16.7*

* IL-17 MFI in PB is lower in SLE (active or inactive) versus healthy subjects (p<0.01)

Functional characterization of human Th17 cells in normal tissues using an automated flow cytometry method

To further our investigation of the functional activity of Th17 cells in different tissues in healthy individuals, we studied the percentage of cells producing different combinations of pro-inflammatory cytokines. As shown in Fig. 6.1., we identified eight distinct Th17 cell subpopulations in healthy subjects, according to their pattern of cytokine expression (TNF-α, IL-2 and INF-γ), using an automated flow cytometry method for merging and calculation of data files.

We found that the majority of normal BM Th17 cells did not produce type 1 cytokines, resulting in a large subpopulation of cells showing isolated IL-17 expression. In contrast, in both PB and LN samples, clearly distinct subpopulations of Th17 cells producing different cytokine combinations were identified with a consequent decrease in the frequency of the subpopulation producing isolated IL-17 ($p = 0.02$ and $p = 0.003$, respectively), a difference which was particularly marked in LN Th17 cells. The proportion of Th17 cell subpopulation simultaneously producing all studied cytokines was significantly higher in both peripheral tissues (PB: $p = 0.02$; LN: $p = 0.006$), especially in LN compared to BM. The Th17 cell subpopulation simultaneously producing TNF-α and IL-2 was significantly more represented in LN than in PB or BM (LN versus BM: $p = 0.003$; LN versus PB: $p = 0.005$; PB versus BM: $p = 0.02$). Those Th17 cells producing only TNF-α were also significantly increased in PB (PB versus BM: $p = 0.003$; PB versus LN: $p = 0.002$) but markedly decreased in LN when compared to BM ($p = 0.04$), and Th17 cells producing only IFN-γ were poorly represented in both peripheral tissue comparatively to BM (PB: $p = 0.02$ and LN: $p = 0.003$).

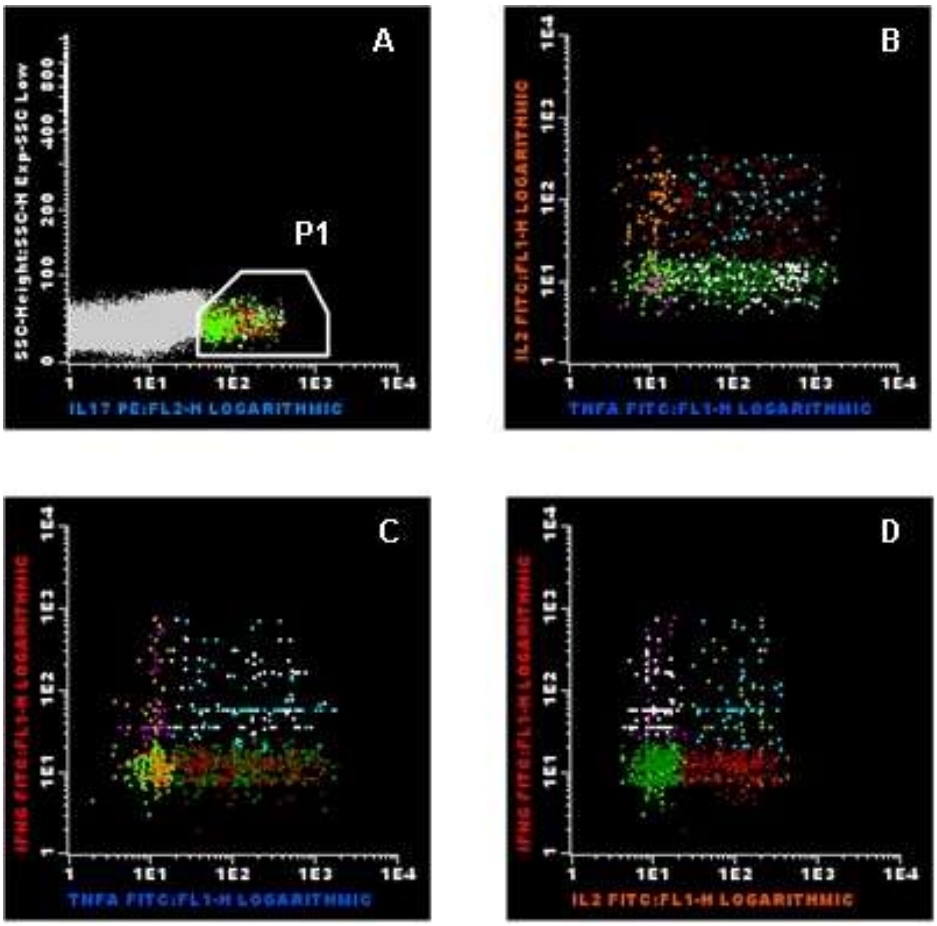

Fig. 6.1. Bivariate dot plot histograms illustrating the intracellular expression of IL-17, IL-2, TNF-α and IFN-γ in Th17 cells after the merging and calculation processes performed on a set of three original four-color-staining data files corresponding to three aliquots of a representative PB sample from a ASLE patient, according to five common parameters—forward light scatter (FSC), side light scatter (SSC), IL-17 PE, CD3 PerCP and CD8 APC—and stained separately with IL-2, TNF-α and IFN-γ. Firstly, a gate was set in the whole merged/calculated data file (e.g., for both actually measured and calculated events) to define T cells; this gate (CD3 gate) was set in FSC versus SSC and SSC versus CD3 bivariate dot plot histograms. In addition, two-dimensional dot-plot representations, corresponding to combinations of antibodies (IL2, TNF-α, INF-γ) conjugated with the same fluorochrome (FITC) but not obtained by direct staining of cells, were generated prior a "P1 gate" to the identification of "calculated Th17 cells" (a). Among the latter, eight "calculated Th17 subpopulations" were identified combining the three FITC-conjugated Mab (b–d).

Frequency of human Th17 cells in peripheral blood in SLE patients

We found a trend for a higher frequency of Th17 among CD4 T cells in both active and inactive SLE, but did not reach statistical significance (Table 6.2.). No differences were observed in the frequency of Th17 cells between active SLE and inactive SLE groups. However, analysis of the amount of IL-17 produced at single cell level (MFI) showed a significant decreased expression of IL-17 in Th17 cells of both SLE groups compared to normal controls (ASLE: p =0.006; ISLE: p =0.003) (Table 6.2.).

Functional characterization of human PB Th17 cells in SLE patients using an automated flow cytometry method

Since current evidence suggests that there is an increased expression of IL-17 in patients with a variety of autoimmune diseases and a possible developmental relationship between the Th17 and Th1 phenotypes, we compared the pattern of distribution of the eight different pro-inflammatory cytokine-producing Th17 cell subpopulations in PB samples from SLE and normal controls. Despite the Th17 cell heterogeneity observed in PB from SLE patients, the pattern of distribution of the different cytokine producing Th17 cell subpopulations observed was relatively similar to normal subjects. However, a significant decrease in the Th17 cell subpopulations producing only TNF-α and IFN-γ plus IL-2 was found in inactive SLE patients compared to normal controls ($p = 0.03$) and to active SLE groups ($p = 0.02$), respectively. Interestingly, we observed a higher proportion of Th17 cells producing IL-17 alone in active SLE patients comparatively to the other studied groups but as it occurs for other subpopulations, without significant differences between groups.

Discussion

In this study, we have applied a novel software program to map out the functional phenotype of Th17 cells according to their pro-inflammatory cytokine production profile, either in normal tissues or peripheral blood from SLE patient, to clarify the relevance of those different microenvironment conditions in Th17-cell differentiation.

Data published in the past few years suggest the existence of a novel subset of Th effectors that are distinct from the classic Th1 and Th2 and that have been named Th17 because of their ability to produce IL-17. Identified by IL-17 production, we showed in Table 2 that a clear decreased proportion of the Th17 cell population was observed in normal BM as compared to both normal peripheral tissues (PB and LN), with no significant differences between the latter groups. When we analyzed the amount of IL-17 expressed at the single-cell level, we observed a significant increase in both normal peripheral tissues, particularly in LN (Table 6.2.).

As measured by other studies, a remarkable proportion of Th17 cells also shares the ability to produce IFN-γ.[147, 149, 150] These cells, producing both IL-17 and IFN-γ, are usually named Th17/Th1. This new subset of IFN-γ-producing Th17 cells sharing features with both Th1 and Th17 challenged us to evaluate other pro-inflammatory cytokines that could raise new issues on the Th17 developmental and/or functional relationship with Th1 or even clarify its role in autoimmune disorders, such as SLE. In this sense, the use of an automated flow cytometry method allowed us to identify eight different merged/calculated Th17 subpopulations from the functional point of view. We also demonstrated that Th17 cells in peripheral tissues (PB

and LN) produce not only IFN-γ but also TNF-α and/or IL-2. BM Th17 cells showed a distinct profile of cytokine production, with the majority not secreting the type 1 cytokines studied. Moreover, a decreased amount of each cytokine was observed in normal BM as compared to PB and LN, without significant differences between those peripheral tissues (data not shown). Although predominately found in the lung and digestive mucosa, we demonstrated that Th17 cells can be observed in BM, PB and lymphoid tissues throughout the body. However, if Th17 cells represent terminally differentiated cells or whether they retain plasticity and can develop into another lineage, such as IL-2, TNF-α and/ or IFN-γ secreting Th1 cells, remains unproved.

To our knowledge, this is the first study that compares the relative distribution and functional activity of Th17 cells in normal BM, PB and LN according to cytokine production, and it is also the first application of an automated flow cytometry method capable of defining eight different Th17 subpopulations, based on distinct cytokine expression combinations, after in vitro stimulation. The demonstration of the reliability of this new statistical approach that may be used for the automated generation of flow cytometry data files containing information on single events about a virtually infinite number of parameters has already been done by Orfão *et al* and opens the door for all applications of multiparameter flow cytometry for which a large number of parameters are needed, provided the fact that the cell population (or cell populations) of interest could be identified with a relatively limited number of markers. Thus, by comparing the expression of four cytokines (IL-2, IL-17, TNF-α and IFN-γ), we demonstrated that the major Th17 subpopulation in BM only produces IL-17, while in normal peripheral tissues, this subpopulation was significantly smaller, particularly in LN. On the other hand, in normal PB, there was an increase in the Th17 subpopulations producing TNF-α plus IL-2 and only producing TNF-α, as well as a significant increase in the subset simultaneously producing all the four cytokines. In normal LN, this latter subpopulation was found even more represented, alongside the major Th17 subpopulation producing TNF-α plus IL-2 as compared to BM.

Our findings suggest a functional heterogeneity and plasticity of Th17 cells, which seems to reflect the different environmental conditions found in the three normal tissues studied. In fact, Th17 cells retained in the BM appear to show a reduced ability to produce IL-17 (Table 6.2.), and almost all do not express the type 1 cytokines studied, suggesting a protective environment with minimal contact with antigen and cytokine stimulation, and a strong fidelity lineage. In contrast, in peripheral tissues, Th17 cells can be dynamically induced to proliferate and become functional, activated according to various stimulus and interaction with antigen-APCs, resulting in a memory or effector phenotype characterized by an increased ability to co-produce cytokines, particularly in LN where "polyfunctional" Th17 cells could play an important homeostatic role. Thus, as also reported by others, our findings seem to support a notable flexibility between peripheral Th17 and Th1 cells or/and a shared early differentiation of Th1 and Th17 cells from naïve CD4+ T cell.[144, 151, 152] In this sense, as Th1 subsets are thought to be the crucial player for most of the organ specific autoimmune

diseases, these peripheral "Th17/Th1" subpopulations may be involved in the induction of autoimmunity and inflammatory reactions and represent highly pathogenic effector T cell subsets. Thus, once the functional profile of normal Th17 cells is defined, we focus our work in their evaluation in a group of SLE patients with different activity diseases using the automated method described previously.

In inactive SLE, the Th17 cells producing only TNF-α were significantly lower than in normal controls. Compared to controls and active SLE, in inactive disease, there was an overall increase in the proportion of Th17 subpopulation co-producing TNF-α/IL-2 with a consequently decrease in the proportion of Th17 expressing TNF-α alone or IFN-γ/IL-2. Interestingly, alterations in the frequency of pro-inflammatory cytokine-producing Th17 subpopulations, particularly the increased proportion of Th17 cells producing TNF-α/IL-2 observed in inactive SLE, might have important functional implications as these cytokines may exhibit a potent synergy with IL-17 for pro-inflammatory effects.[153]

In active SLE patients, we found a similar subpopulation distribution to that observed in normal PB, although showing a trend towards a higher proportion of Th17 cells producing IL-17 alone when compared to inactive SLE and normal controls, reflecting a decreased proportion of Th17 subpopulations expressing TNF-α/IL-2 and TNF-α. Actually, in line with the notable cytokine profile plasticity presented by normal peripheral Th17 cells and taking into account the enhancement of Th2 cell function found previously by us in active SLE patients, along with the various immunosuppressive drugs received by those patients, a suppressive effect may be exerted on Th17 polyfunctional effector phenotype, resulting in a decrease proportion of those subpopulations expressing TNF-α/IL-2 and TNF-α.[154-156] Especially, those findings may point to the strong fidelity lineage of Th17 in active disease SLE or even a terminal differentiation stage related with a repeated antigen challenge as compared to inactive SLE or normal control groups. Furthermore, consistent with other human studies that have demonstrated a lower number of Th1 cells in active SLE patients, our data suggest that the cytokine profile of Th17 cells may be essential for synergistically induced Th1-type cytokines and also potentially be distinct according to the pro-inflammatory environment.[157-159] Thus, the local cytokine milieu may induce new transcription factors and modify cytokine production even in fully polarized effector T-cell lineages. The data recorded here provide evidence to show that, when exposed to certain cytokine milieus, Th17 cells may acquire the capacity to express not only IL-17 but also different type 1 cytokines, such as IL-2, TNF-α and IFN-γ, thus favoring the shifting of these cells toward Th1 phenotype, which is particularly visible in those patients with inactive disease. In this regard, preliminary data suggest that these "Th17/Th1" cells appear to be differently influenced by environmental factors to exhibit dual Th1 and Th17 functions and to be related with disease activity in SLE patients, denoting their potential application in clinical trials as a therapeutic target.

In conclusion, it is conceivable that Th17 cells are responsive to local cytokine milieus and sufficiently flexible to acquire the ability to produce type 1 cytokines, contributing in a

pathologic condition to acute and chronic inflammation mainly in the context of Th1-initiated disease, herein creating a mixed “Th1/Th17” pathology and reduced dominance of Th1 in active SLE. Therefore, the data presented provide a detailed functional characterization of human Th17 isolated from PB of SLE patients, as well as from various normal tissues, using a new statistical approach for the automated generation of flow cytometry data files that clearly show its great utility in future studies that would complete the plasticity character of Th17 cells. In line with this, in the future, it would be important to determine in a large study, including also non-treated patients, if these eight T cell subsets, so-called Th1/Th17 cells, can be considered as distinct and stable lineage of CD4+ T effector cells that are committed to a certain lineage or whether they are an intermediate activation state destined to become true Th1 or Th17 cells and what might be their functional relevance in the clinical heterogeneity of SLE patients, namely, concerning organ involvement and monitoring patient treatment response.

Chapter 7

NK cells dysfunction in systemic lupus erythematosus: Relation to disease activity

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Abstract

Objectives

Through their cytotoxic capacities and cytokine production, natural killer (NK) cells modulate autoimmune diseases. However, their role in the pathogenesis of systemic lupus erythematosus (SLE) has not been extensively studied. The aim of this study was to analyze the immunophenotypic and functional characteristics of the two major NK cell subsets in SLE and relate them with disease activity.

Methods

Peripheral blood samples from 44 patients with active (n =18) and inactive SLE (n =26) and 30 controls were analyzed by flow cytometry to evaluate NK cell subsets, according to: the differential expression of CXCR3 and CD57; expression of granzyme B and perforin; and production of interferon gamma (IFN-γ) and tumor necrosis alpha (TNF-α), after PMA/ionomycin activation.

Results

A clear decrease in absolute and relative numbers of circulating NK cells was found in SLE, particularly in active disease, while the proportions of the major NK cell subsets were unaffected. Active SLE was associated with a reduced CXCR3 expression on both NK cell subsets and a lower frequency of $CD56^{dim}$ NK cells expressing CXCR3. Furthermore, granzyme B expression was decreased in both SLE groups, but the percentage of NK cells expressing granzyme B and perforin was higher, particularly in active disease. We found a significant decrease in the percentage of $CD56^{bright}$ and $CD56^{dim}$ NK cells producing TNF-α and of its expression on $CD56^{dim}$ NK cells in active disease, while IFN-γ expression on $CD56^{bright}$ NK cells was increased in both SLE groups.

Conclusions

Our findings suggest that NK cell subsets exhibit unique phenotypic and functional changes that are particularly evident in active SLE, and they may have the potential to affect the disease outcome.

Introduction

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease characterized by a wide array of clinical manifestations, from skin and mucosal lesions to severe injuries in the central nervous system, kidneys and other organs.[4, 18] The presence of high titers of autoantibodies against nuclear components (antinuclear antibodies), elevated circulating immune complexes and complement deficiency are the main characteristics of the disease.[18] However, the etiology of SLE remains unknown, and much of its pathogenesis is still to be unraveled. Nuclear antigens leading to antibody production in SLE are possibly exposed to the immune system due to disease-associated defects in the clearance of apoptotic cells.[160, 161] Previous research also demonstrated an over activation of the type I interferon (IFN) pathway in patients with SLE and several other autoimmune diseases.[162] Some of these studies have shown that the interferon alpha (IFN-α) production by plasmacytoid dendritic cells stimulated with RNA-containing immune complex can be upregulated by natural killer (NK) cells and inhibited by monocytes. These and other observations suggest that NK cells may participate in the regulation of SLE through several mechanisms, beyond their well-established role in host defense against malignancies and certain viruses.[163, 164] However, it remains controversial if NK cells have a disease-controlling or a disease promoting role in SLE.[165, 166]

Published evidence suggests that SLE is associated with decreased peripheral blood NK cell numbers and function.[167-169] However, it remains unclear whether these changes reflect primary defects involved in the disease pathogenesis or if they are a consequence of the disease process and its treatments. The former interpretation has been reinforced by recent studies documenting NK cell defects in treatment naïve and very early disease.[169, 170] The possible mechanisms proposed to underlie this impairment included the presence of anti-lymphocyte antibodies, an intrinsic NK cell defect and/or an NK cell deficiency. In humans, NK cells constitute approximately 10% of peripheral blood (PB) lymphocytes and include distinct subsets with disparate repertoires, location, function and developmental origin.[171] They can be split into two major subsets, based on the relative densities of CD56 surface expression. $CD56^{dim}$ NK cells comprise 90% of peripheral blood NK cells, have a high cytolytic capacity and secrete low levels of cytokines. Conversely, $CD56^{bright}$ NK cells are the main type of NK cells in secondary lymphoid tissue and sites of inflammation, secrete a greater number of cytokines, including IFNs and tumor necrosis factor alpha (TNF-α), and acquire cytotoxicity only after prolonged activation.[172] The predominant NK cell type in inflammatory lesions is $CD56^{bright}$, but NK cells $CD56^{dim}$ also express chemokine receptors that may lead them to sites of inflammation.[173] Chemokine receptors whose expression is inducible rather than constitutive, such as CXCR3 (receptor for interferon-γ–inducible 10 Kd protein - IP-10, also designated CXCL10), appear to play a relevant role in the control of inflammation.[174] In recent years, strong experimental and clinical evidence support the concept that CXCR3 is

involved in the development of autoimmune diseases, especially through the amplification of inflammation in target organs, thus causing a worsening of clinical manifestations.[175]
In this context, we investigated the expression of CXCR3, CD57, granzyme B, perforin, as well as relevant cytokines, such as interferon gamma (IFN-γ) and TNF-α, in NK cell subsets of patients with SLE, exploring their relationship with clinical disease activity.[176]

Methods

Patients and controls

Seventy-four participants were enrolled in the study, comprising 44 SLE patients, 18 with active disease (100% female; mean age, 33±11 years), 26 with inactive disease (84% female; mean age, 33±10 years) and 30 age- and gender matched controls (90% female; mean age±SD, 30±6 years). The study protocol was approved by the local ethics committee, and all participants gave their signed informed consent.
A convenience sample of 44 patients fulfilling the 1997 American College of Rheumatology classification criteria for SLE was recruited from a University Hospital-based Lupus Clinic.[37] Patients were evaluated according to the SLE Disease Activity Index 2000 (SLEDAI-2k) and classified as having active (SLEDAI-2k ≥5; n =18) or inactive disease (SLEDAI-2k <5; n =26).[43, 46, 47] The patient's medication at time of evaluation was recorded. Data regarding demographics, additional clinical parameters and medication at the time of sample collection were recorded (Table 7.1.).
The control group (NC) was composed of 30 healthy individuals. These participants were required to complete a brief questionnaire regarding previous or current medical conditions and medications. All were free from autoimmune disease, active infection and were not undergoing treatment with immunomodulatory drugs. All provided signed informed consent.

Flow cytometry data acquisition and analysis

Frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells and their expression of CD57 and CXCR3

Quantification of $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets and the expression of CD57 and CXCR3 in these cell subsets were performed in K3-EDTA PB samples using anti-CD56 phycoerythrin cyanine 7 (PC7) (clone N901 - NKH-1; Beckman Coulter, Marseille, France), anti-CD3 Pacific Blue (PB) (clone UCHT1; BD Pharmingen, San Diego, CA, USA), anti-CD57 fluorescein isothiocyanate (FITC; clone NK-1; BD Biosciences, San Jose, CA, USA) and anti-CXCR3

phycoerythrin (PE; clone 1C6/CXCR3; BD Pharmingen, San Diego, CA, USA). Samples were incubated for 10 min at room temperature in darkness. After this incubation period, a lyse and wash protocol was followed: ImunoPrep reagent (Beckman Coulter, Brea, CA, USA) was added in Beckman Coulter TQ-Prep (Beckman Coulter, Brea, CA, USA) instrumentation. Cells were washed twice with 2 ml of phosphate buffered saline (PBS) and resuspended in 0.75 ml of PBS (Gibco, Paisley, Scotland) before acquisition.

Frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells producing IFN-γ and TNF-α

In vitro stimulation of PB NK cells was performed using 500 µl of heparinized PB, diluted liter per liter (v/v), in RPMI-1640 medium (Gibco, Paisley, Scotland, UK), supplemented with 2 mM L-glutamine. Phorbol 12-myristate 13-acetate (50 ng/ml; Sigma, Saint Louis, MO, USA), 1 µg/ml of Ionomycin (Boehringer Mannheim, Germany)
and 10 µg/ml of Brefeldin A (Golgi plug Sigma, Saint Louis, MO, USA) were added, and the sample was incubated for 4 h at 37°C in a humidified incubator with 5% CO2 concentration. Each cultured PB sample was aliquoted and incubated with anti-CD56 PC7 (clone N901 (NKH-1); Beckman Coulter, Marseille, France), anti-CD3 Pacific Blue PB (clone UCHT1; BD Pharmingen, San Diego, CA, USA) and anti-CD8 Allophycocyanin (APC; clone B9.11, Beckman Coulter, Marseille, France). Then, an intracytoplasmatic permeabilization protocol with IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA) was followed and a staining protocol according to manufacturer's instructions in order to analyze the intracellular expression of IFN-γ FITC (clone 4S.B3; BD Pharmingen, San Diego, CA, USA) and TNF-α FITC (clone MAb11; BD Pharmingen, San Diego, CA, USA; clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA). Cells were resuspended in 0.75 ml of PBS (Gibco, Paisley, Scotland) before acquisition.

Frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells expressing granzyme and perforin

Quantification of $CD56^{dim}$ and $CD56^{bright}$ NK cells subsets expressing granzyme and perforin was performed in K3-EDTA PB samples using anti-CD56 PC7 (clone N901 (NKH-1); Beckman Coulter, Marseille, France), anti-CD3 PB (clone UCHT1; BD Pharmingen, San Diego, CA, USA) and anti-CD8 APC (clone B9.11, Beckman Coulter, Marseille, France) following an intracytoplasmatic permeabilization protocol with IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA) staining protocol according to manufacturer's instructions in order to analyze the intracellular expression of anti-granzyme B PE (clone CLBGB11; Pellicluster Amsterdam, Netherlands) and anti-perforin FITC (clone δG9; BD Pharmingen, San Diego, CA, USA). Cells were resuspended in 0.75 ml of PBS (PBS; Gibco, Paisley, Scotland) before acquisition. Samples were acquired on Navios flow cytometer (Beckman Coulter, Brea, CA, USA) using Navios acquisition Software (Beckman Coulter, Brea, CA, USA). Data were analyzed using Flow Cytometry Software Infinicyt 1.5 (Cytognos, Salamanca, Spain). Absolute

counts were calculated using a dual platform methodology (flow cytometry and haematological cell analyzer). The NK cells were defined as $CD3^-$ e $CD56^+$, and the two subpopulations of NK cells were defined according to the density of expression of CD56, high or low, $CD56^{bright}$ and $CD56^{dim}$, respectively (as shown in the Online resource 7.S1).

Statistical analysis

Results were expressed as the mean ± standard deviation. Differences between defined patient groups were assessed for statistical significance with the nonparametric Mann–Whitney U test. A Spearman's rank correlation was applied to detect the association between different study parameters. The p values below 0.05 were considered statistically significant. The SPSS version 15.0 software (SPSS Inc., Chicago, USA) was used to perform the statistical analyses.

Results

Population

The clinical features of patients are presented in Table 7.1.

Frequency and absolute number of NK cells and their major subsets in peripheral blood

Both the total number of NK cells and the percentage of lymphocytes in peripheral blood were significantly reduced in SLE patients (both active and inactive disease) than in controls. The difference was more pronounced in patients with active disease (Table 7.2). There were no statistically significant differences in the absolute number and frequency of NK cells between the active SLE and inactive SLE groups. The distribution of NK cells into the $CD56^{bright}$ and $CD56^{dim}$ NK subsets was similar in the three groups (Table 7.2.).

Table 7.1. Clinical findings in 44 patients with systemic lupus erythematosus (SLE).

	Inactive SLE (n=26)	**Active SLE** (n=18)
Mean SLEDAI-2k scores	1.6 ± 0.9	9.7 ± 3.2
Mean time since diagnosis	9.0 ± 6.0	7.6 ± 7.4
SLE clinical features		
Lupus nephritis	61.3%	44.4%
Neuropsychiatric lupus	19.4%	0%
Lupus arthritis	58.1%	66.7%
Haematological involvement	87.1%	100%
Mucocutaneous involvement	74.2%	77.8%
Severe Lupus*	71%	44.4%
Anti-dsDNA antibodies**		
Negative	42.3%	11.1%
Low positive	32.3%	11.1%
Moderately positive	22.6%	22.2%
High positive	6.5%	55.6%
Treatment		
Hydroxychloroquine	87.1%	94.4%
Immunossupressants***	32.3%	66.7%
Steroids****	12.9%	83.4%
Low dose	100%	38.9%
Moderate dose	0%	27.8%
High dose	0%	16.7%

Clinical parameters were evaluated from the beginning of the disease until the time of the study. *Severe Lupus defined as cumulative major organ involvement;**Anti-dsDNA antibodies: Negative, <4.2 IU/ml. Low positive, <20 IU/ml. Moderately positive, 20-50 IU/ml. High positive, <50 IU/ml; ***azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus, methotrexate, cyclophosphamide or rituximab; **** low dose, up to 10 mg/day; moderate dose, 10-30 mg/day; high dose, more than 30 mg/day; n = sample size.

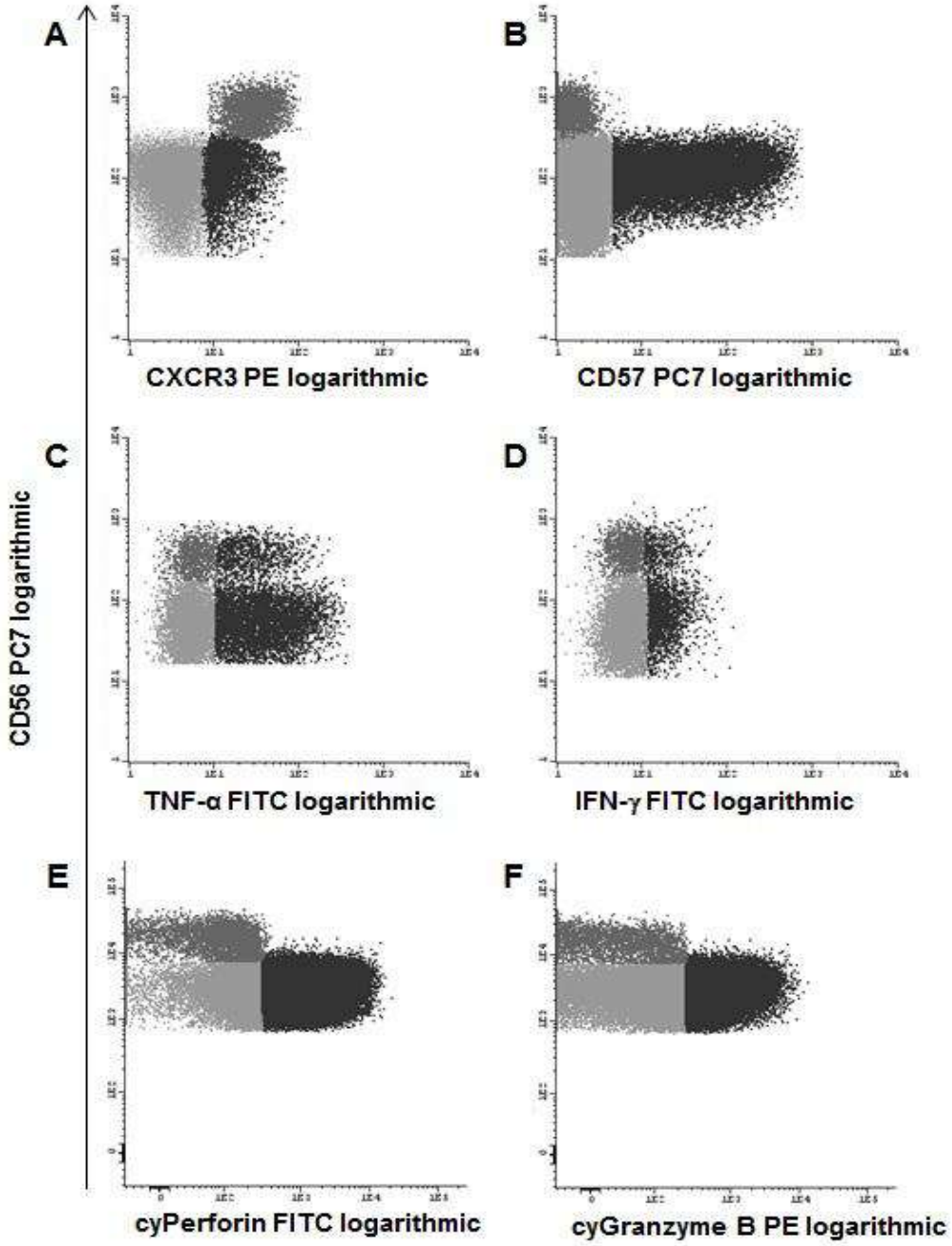
Table 7.2. Frequency (percentage) and absolute number (cells per microliter of blood) of circulating NK cells and of their two major subsets, among total NK cells, in healthy individuals and SLE patients and their frequency expressing CD57 and CXCR3, and expression levels of CXCR3 per cell.

		Normal Controls	Inactive SLE	Active SLE
WBC	**Cells/µl**	7,273±1,737	7,077±2,405	6,294±1,989 [b]
NK				
	%	3.3±1.8	1.8±1.0 [b]	1.4±1.2 [a]
	Cells/µl	240.0±150.0	120.0±80.0 [b]	90.0±60.0 [a]
CD56^{bright}				
	% NK	6.2±3.6	9.3±8.1	7.6±6.0
	Cells/µl	14.0±7.0	9.0±6.0 [b]	7.0±6.0 [a]
	% CXCR3	100	100	100
	MFI CXCR3	27.1±8.5	22.9±11.0	18.9±7.2 [b]
CD56^{dim}				
	% NK	93.8±3.6	90.7±8.0	92.4±6.1
	Cells/µl	220.0±150.0	110.0±70.0 [b]	80.0±60.0 [a]
	% CD57	40.5±16.6	35.0±16.9	28.7±14.3 [b]
	% CXCR3	31.0±21.3	24.1±12.4	12.5±8.6 [b,c]
	MFI CXCR3	18.5±6.2	17.2±7.1	13.3±1.9 [b,c]

Results are expressed as mean ±standard deviation. MFI = Mean Fluorescence Intensity of positive cells; % = Percent of cells from total PB; % NK = Percent of cells for total NK cells. NC, healthy individuals; ASLE: active disease; ISLE: inactive disease. Statistically significant differences were considered when $p < 0.05$ (Mann-Whitney U test): [a]ASLE versus NC; [b]ISLE versus NC; [c]ASLE versus ISLE.

Frequency of peripheral blood CD56^{bright} and CD56^{dim} NK cells expressing CD57 and CXCR3

The chemokine receptor CXCR3 was expressed in all CD56^{bright} NK cells and in a considerable fraction of CD56^{dim} NK cells (as shown in the online resource 7.S1. and Table 7.2.). In active disease, we observed a lower frequency of CD56^{dim} NK cells expressing CXCR3 than in inactive disease and controls ($p = 0.005$ and $p = 0.001$, respectively; Table 2). The expression of CD57 in these cells was also lower in active disease than in controls. Both NK cell subsets from active SLE bear CXCR3 at lower density than those from controls (CD56^{bright} NK cells, $p = 0.002$ and CD56^{dim} NK cells, $p = 0.003$). The difference between active and inactive disease was also significant for CD56^{dim} NK cells ($p = 0.015$; Table 7.2.).



Online Resource 7.S1. Representative bivariate dot plots illustrating the gating used for inmunophenotypic identification of both CD56 NK cell subsets and the analysis of their surface membrane levels of CXCR3 and CD57, and cytoplasmatic levels of IFN-γ, TNF-α, granzyme B and perforin.

Frequency of peripheral CD56^{bright} and CD56^{dim} NK cells producing IFN-γ and TNF-α after in vitro stimulation

Regarding IFN-γ, our data showed that in NC and in both SLE groups, 26 to 35% of both NK cell subsets secreted this cytokine, with no significant differences between the three study groups (Table 7.3.). In addition, we observed higher levels of IFN-γ in CD56^{bright} cells from the SLE group compared to NC ($p < 0.05$), without significant differences in CD56^{dim} NK cells between the groups. In contrast, as shown in Table 7.3., 35% of CD56^{bright} NK cells secreted TNF-α in NC, whereas in active disease less than 19% were secreting this proinflammatory cytokine ($p < 0.05$). Likewise, around 40% of CD56^{dim} NK cells produced TNF-α in NC with less than 26 to 22% of those cells producing TNF-α in inactive and active SLE ($p < 0.05$), respectively.

The amount of TNF-α per cell, evaluated through mean fluorescence intensity of positive cells (MFI), was much higher for CD56^{dim} cells than in CD56^{bright} cells in both NC and SLE patients (Table 7.3.). The cytoplasmatic levels of TNF-α on CD56^{bright} cells were similar in the three studied groups of participants, but a significant decrease was noted in CD56^{dim} cells from active SLE (Table 7.3.).

Since only CD56^{dim} NK cells expressed granzyme B and perforin, we focused our analysis in this cell subset, on the three studied groups (as shown in the online resource 7.S1. and Fig. 7.1A.). The proportion of these cells was significantly higher in active SLE compared to NC. A similar trend was observed for these NK specific granules in inactive SLE, but did not reach statistical significance (Fig. 7.1A.). In contrast, analysis of the amount of granzyme B expressed at single cell level demonstrated a significant decrease in inactive and active SLE compared to NC (Fig. 7.1B.). No significant differences were found in the cytoplasmatic expression of perforin on CD56^{dim} NK cell subset among the three groups of participants (Fig. 7.1B.).

Table 7.3. Frequency of NK-cell subsets producing IFN-γ and TNF-α and amount of each cytokine *per* cell, in peripheral blood of healthy individuals and SLE patients.

NK-cell subsets			Normal Controls	Inactive SLE	Active SLE
CD56^{bright}					
	IFN-γ	**%**	26.8 ± 13.6	34.5 ± 19.9	29.1 ± 14.5
		MFI	26.1 ± 13.0	33.3 ± 14.0[b]	37.1 ± 14.0[a]
	TNF-α	**%**	35.0 ± 20.4	28.3 ± 12.1	18.5 ± 12.9[a,c]
		MFI	47.2 ± 19.0	55.1 ± 27.4	43.4 ± 17.1
CD56^{dim}					
	IFN-γ	**%**	29.9 ± 18.3	26.2 ± 18.2	27.6 ± 16.0
		MFI	30.0 ± 13.0	34.1 ± 17.0	38.2 ± 25.1
	TNF-α	**%**	40.4 ± 21.9	25.6 ± 12.6[b]	21.5 ± 16.8[a]
		MFI	94.1 ± 45.2	84 ± 35	65 ± 27[a]

Results are expressed as mean ±standard deviation. % = Percentage of positive cells; MFI = Mean Fluorescence Intensity of positive cells. NC: healthy individuals; ASLE: active disease; ISLE: inactive disease. Statistically significant differences were considered when p□□0.05 (Mann-Whitney U test): [a]ASLE versus NC; [b]ISLE versus NC; [c]ASLE versus ISLE.

Frequency of peripheral CD56^{bright} NK cells and CD56^{dim} NK cells expressing granzyme B and perforin

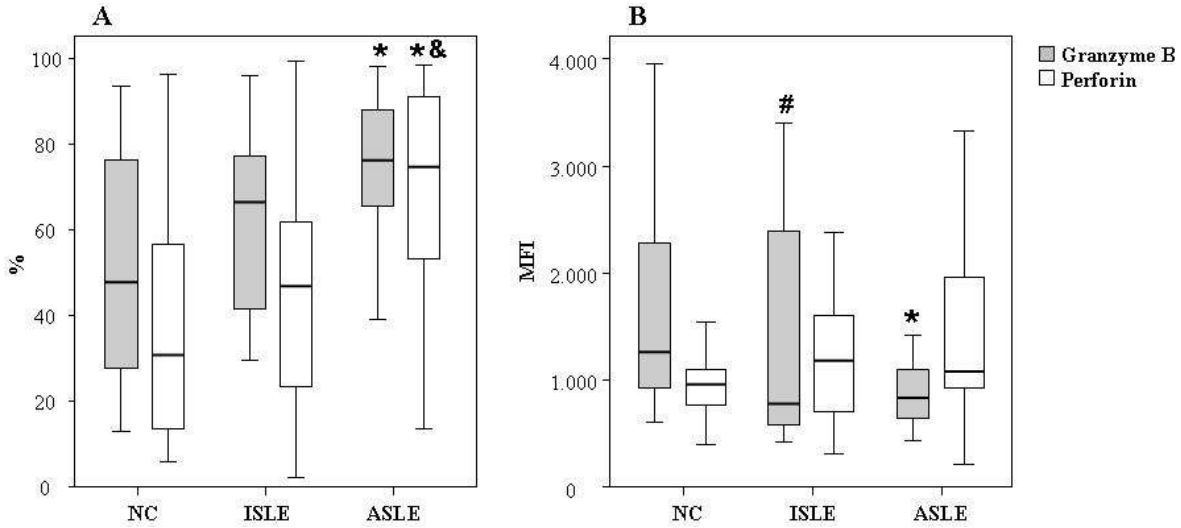


Fig. 7.1. Frequency of peripheral blood CD56^{dim} NK cells expressing granzyme B and perforin and amount of each granule-associated cytolytic effector molecules per cell (MFI) in healthy individuals and SLE patients (a and b, respectively). Statistically significant differences were considered when p <0.05 (Mann–Whitney U test): *ASLE versus NC, #ISLE versus NC, and &ASLE versus ISLE

SLE activity and NK cell numbers and function

The clinical parameters were used to test a possible association with NK cell number and function. We found a negative correlation between the SLEDAI and the absolute numbers of CD56^{bright} NK cells (r =−0.41, p =0.01), while a trend toward a correlation was observed between the SLEDAI and the absolute numbers of CD56^{dim} NK cells (p =0.09 by Spearman's rank correlation). A negative correlation was also observed between SLEDAI and (1) the proportion of TNF-α-producing CD56^{bright} NK cells; (2) the proportion of CXCR3-expressing CD^{56dim} NK cells; and (3) the expression of CXCR3 on both NK cell subsets (Fig. 7.2.). Moreover, the absolute and relative numbers of NK cells and CD56^{dim} NK cell subset were positively correlated with the duration of disease (NK cells, r =0.455, p =0.004 and r =0.514, p =0.001; CD56^{dim} NK cells, r =0.433, p =0.007 and r =0.478, p =0.002), whereas absolute numbers of CD56^{bright} NK cells were negatively correlated with this parameter (r =−0.405, p =0.01).We also noted in active SLE patients a positive correlation between the duration of disease and the levels of IFN-γ expression on CD56^{bright} NK cells (r =0.779, p =0.001).

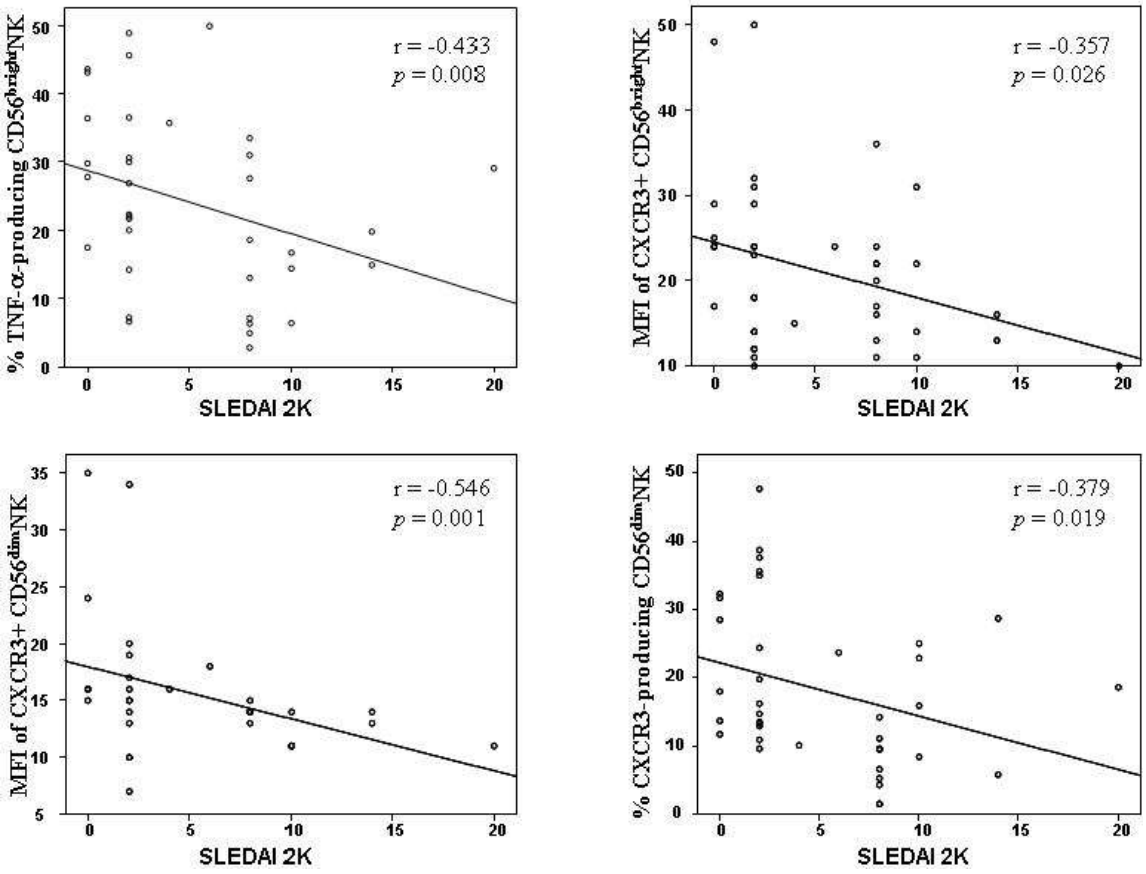


Fig. 7.2. Correlation between SLEDAI-2K and the proportion of TNF-α-producing $CD56^{bright}$ NK cells, the proportion of CXCR3-expressing $CD56^{dim}$ NK cells, and the expression of CXCR3 on both NK cell subsets

As shown in Fig. 7.3., when we grouped SLE patients based on the amount of anti-dsDNA antibodies in negative, low (<20 IU), moderate (20–50 IU), and high positive (>50 IU), we found that absolute number of both NK cell subsets was lower in the all groups with positivity to anti-dsDNA antibodies as compared to those without detectable levels of antibodies and the control group ($p < 0.05$). Another interesting finding was the increase frequency of peripheral blood $CD56^{dim}$ NK cells producing granzyme B and perforin in SLE patients, mainly in those with high expression levels of anti-dsDNA antibodies when compared with control group ($p < 0.05$, Fig. 7.4.).

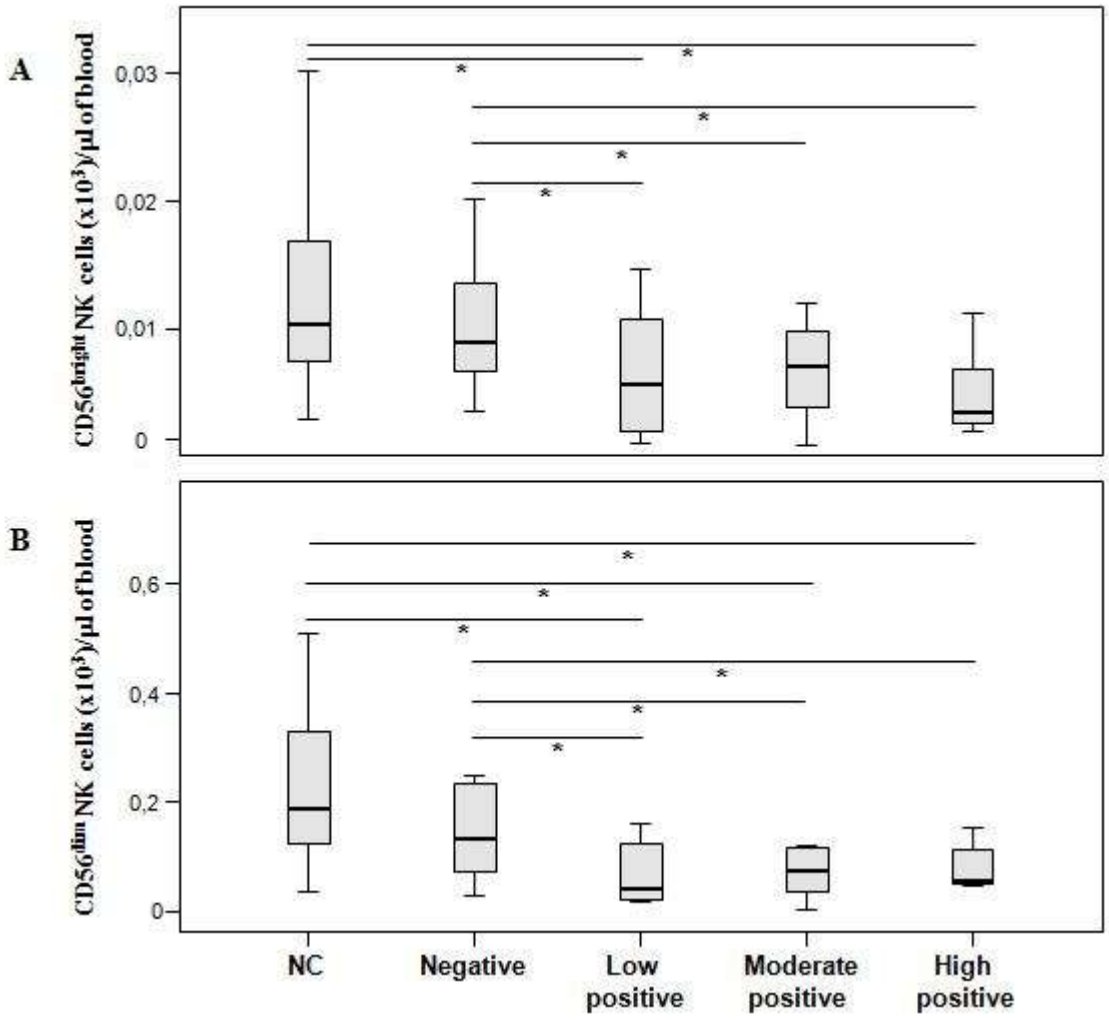


Fig 7.3. Absolute numbers of peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cells according to the expression levels of anti-dsDNA antibodies: negative, low (<20 IU), moderately positive (20-50 IU) and high positive(>50 IU) (**A** and **B**, respectively). * p <0.05.

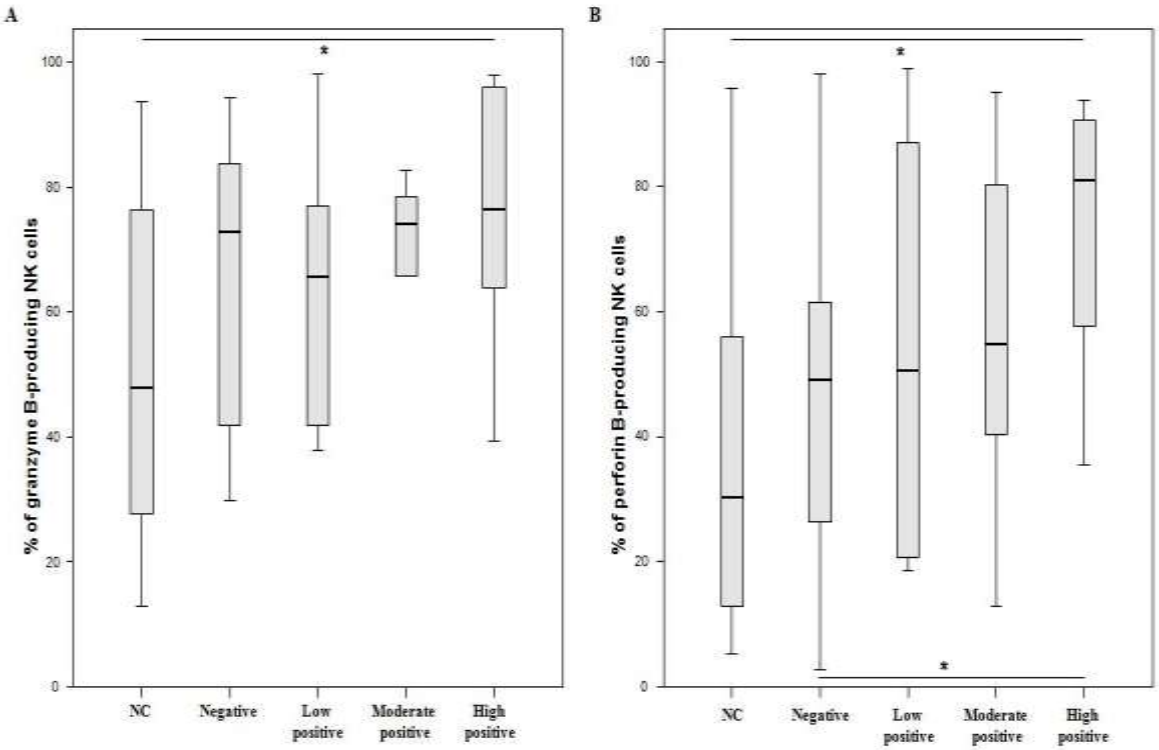


Fig 7.4. Frequency of peripheral blood CD56^{dim} NK cells producing granzyme B and perforin according to the expression levels of anti-dsDNA antibodies: negative, low (<20 IU), moderately positive (20-50 IU) and high positive (>50 IU) (**A** and **B**, respectively). * p <0.05.

Finally, a lower frequency of both TNF-α-producing CD56^{bright} and CD56^{dim} NK cells was observed in SLE patients with high and moderate expression levels of anti-dsDNA antibodies, when compared with those negative to these antibodies and with the control group (p <0.05). A decreased expression of CXCR3 and higher expression of IFN-γ were found on CD56^{bright} NK cells in both groups of patients with and without cutaneous involvement compared to the control group (p <0.05). Conversely, lower frequencies of CD56^{dim} NK cells expressing CXCR3 and producing TNF-α together with an increase frequency of these NK cells producing perforin were observed in both patient groups as compared with the control group (p <0.05). Finally, a decrease frequency of CD56^{dim} NK cells expressing CD57 and increase of those producing granzyme B were also found in patients with cutaneous involvement vs. controls.

Discussion

In the present study, we found that NK cell number, cytotoxic activity, activation, and/or trafficking patterns were altered in patients with SLE. Consistent with previous studies, our findings demonstrated that patients with SLE and more notorious in those with active disease share lower peripheral blood cells counts and low relative and absolute numbers of circulating

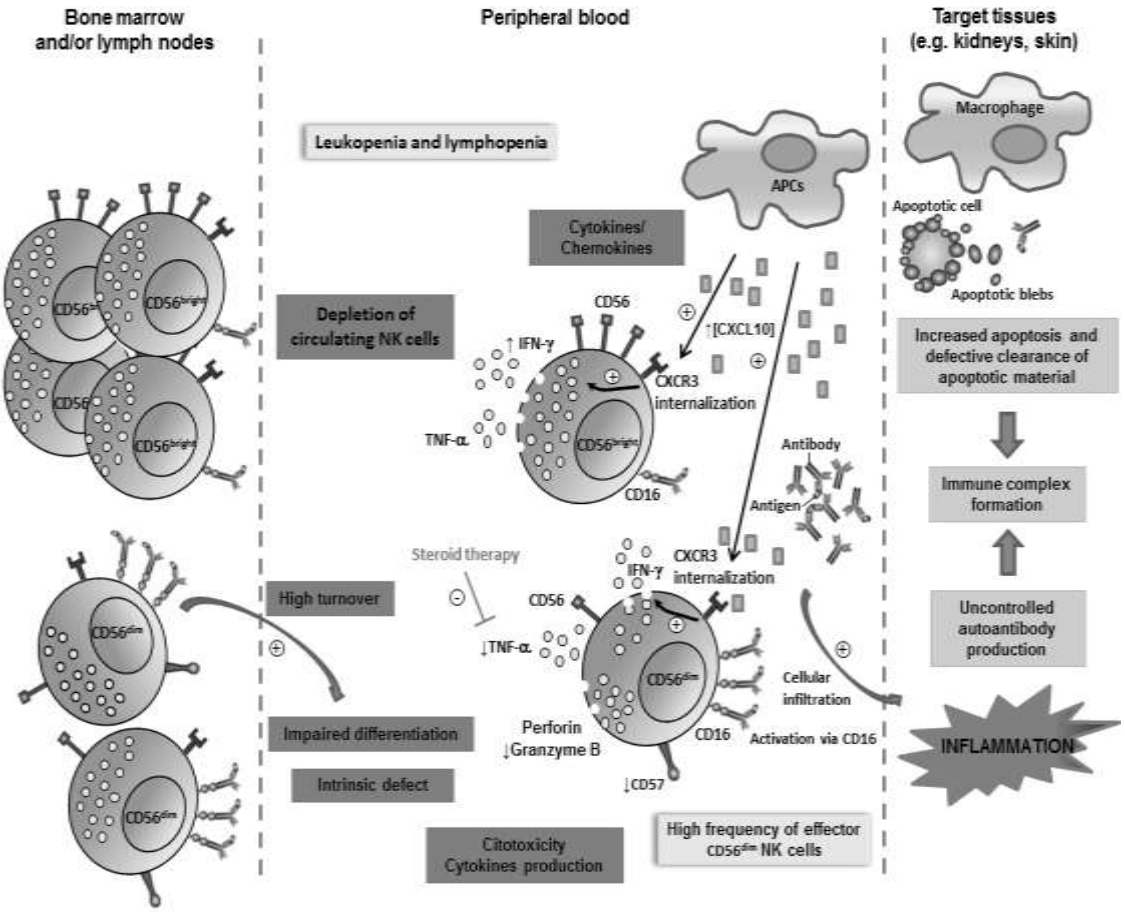
NK cells when compared to healthy subjects.[177, 178] An imbalance between the production and destruction of various types of immune cells can cause cytopenia, which appears associated with autoantibody related apoptosis induction mainly linked to nuclear targeting autoantibodies and associated to disease flare.[179-181] Despite this decreased absolute NK cell count noted in patients with SLE, the proportions of $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were unaffected. Actually, depletion of circulating NK cells in SLE could be due to various factors, such as a reduction in the number of NK cell precursors and their susceptibility to ligand-induced apoptosis via signaling molecules such as CD16 (Fcγ receptor IIIa) related with the formation of immune complexes, as well as influence of serum cytokines, such as enhanced serum IFN-α levels, mainly produced by plasmacytoid dendritic cells that can also mediate the activation-induced cell death of NK cells.[168, 177] Moreover, the NK cell lymphopenia more marked in active disease may arise from NK cell deficiencies and/or due to some of the NK cell subsets being edged out from the blood flow to target tissues. Here, we found that NK cell and their subsets levels in peripheral blood were correlated with parameters of lupus activity, like anti-dsDNA titer and the SLEDAI. Overall, absolute numbers of both $CD56^{bright}$ NK and $CD56^{dim}$ NK cell subsets were significantly decreased in SLE patients with detectable anti-dsDNA antibody levels as compared with those without anti-dsDNA. However, several investigators have described that reduction in NK cell activity was not correlated with serum immune complex levels or the activity of anti-lymphocyte antibodies in SLE.[182, 183] Here, we sought to explore the mechanisms underlying the inappropriate NK cell activation and/or trafficking observed during in SLE through the study of CD57 and CXCR3 expression in patients with active or inactive SLE. We found that CD57 was exclusively expressed by $CD56^{dim}$ NK cells, as previously observed, playing as a cytotoxic marker, while the chemokine receptor CXCR3 assumed to promote cellular infiltration of inflamed tissues was expressed by all $CD56^{bright}$ NK cells and partially by $CD56^{dim}$ NK cells.[184, 185]

According to a recent work, the expression of differentiation markers and NK cell receptors showed that in the mature $CD56^{dim}$ NK population, CD57 defines a subset of highly mature cells representing long-lived cells that have encountered pathogens and represent human "memory" NK cells. Here, we found lower proportions of circulating $CD56^{dim}$ NK cells expressing CD57 and CXCR3 as well as decreased expression levels of CXCR3 per cell in patients with active SLE as compared with those SLE patients with inactive disease and/or normal controls. Thus, these results suggest that the decrease of CXCR3 expression on both CD56 NK cell subsets of SLE patients correlates well with disease activity and could therefore represent a marker of SLE flare. It is tempting to hypothesize that the decrease observed in the proportion of circulating CD57+CXCR3+ $CD56^{dim}$ NK cells together with the decrease of CXCR3 expression per cell observed in active disease may correspond to a sustained CXCR3 internalization resultant from higher serum IP-10 levels described in SLE and from a redistribution and accumulation of these active cells at sites of inflammation (due to high concentrations of ligands for CXCR3 circulating in the serum of patients), in this particular

case, the kidneys and skin, where they will perform cytotoxic functions in the tissues concerned and consequently worsen the disease.[186] In line with this hypothesis, we recently reported higher levels of IP-10 mRNA expression on monocytes and $CD14^{-/low}$ $CD16^{+}$ DC subpopulation in active SLE patients, which could reflect in the peripheral of the tissue injury.[187] Thus, there was a trend toward a negative correlation between the expression of CXCR3 on NK cell subsets, as well as in the proportion of circulating $CXCR3^{+}$ $CD56^{dim}$ NK cells, and the SLEDAI. Interestingly, the changes that we had observed in this chemokine receptor on NK cells were not a result of the SLE treatment regimens since no significant differences in its expression were found in patients with active SLE receiving different doses of steroids and/or immunosuppressants. Considering that activation status enhances NK cell cytotoxicity mediated by several apoptosis-inducing pathways, like perforin/granzyme, and stimulates rapidly cytokine (e.g. IFN-γ, TNF-α) and chemokine production, our results revealed a higher proportion of $CD56^{dim}$ NK cells expressing granzyme B and perforin, but with a decrease of granzyme B expression and no significant differences of perforin expression in SLE patients, especially in active SLE. Interestingly, an increase of $CD56^{dim}$ NK cell proportions expressing granzyme B or perforin was found in those SLE patients with higher serum titers of anti-dsDNA antibodies. This finding may be explained by the activation via CD16 (receptor for constant portion of immunoglobulin G) of peripheral blood $CD56^{dim}$ NK cells, due to the increase of circulating immune complexes, which could contribute for the inflammatory process and maintenance of the disease. Despite the high frequency of $CD56^{dim}$ NK cells expressing granule associated cytolytic effector molecules, mainly in active disease, and highlighting their functional activated phenotype in SLE, the decreased amount of granzyme B per cell may reflect a lower sensitivity to the peripheral inflammatory environment, probably due to an impaired differentiation or an important NK cell lysis defect in patients with SLE.[171] Notably, despite the normal proportion of circulating $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells producing IFN-γ, we found that $CD56^{bright}$ NK cells from patients with inactive and active SLE produced higher levels of this cytokine. It is possible that in a disease context the responses of this latter subset of NK cells may be modulated resulting in the deregulated production of proinflammatory cytokines rather than in immunoregulation reinforcing the potential detrimental role of this cell subset on the inflammatory process and disease pathogenesis. In addition, the proportion of both circulating NK cell subsets producing TNF-α was decreased in SLE and more pronounced for $CD56^{bright}$ NK cells from patients with active disease. In addition, a significant reduction of TNF-α expression per cell was observed on $CD56^{dim}$ NK cells from patients with active disease when compared to control group. In accordance with our recent studies, this finding may be explained once more by the migration of these cells into inflamed tissues as well as by the association of NK cell dysfunction and excessive immune activation, which could contribute, potentially, to the initiation and maintenance of autoimmune responses.[154, 188] Moreover, TNF-α may represent a major therapeutic target in SLE since significant differences in its expression were found in SLE patients receiving different doses of steroids as compared to those without this treatment regimen. Our data showed that NK cell

subsets in patients with SLE display unique phenotypic and functional features associated with disease activity that could also reflect their capability for trafficking (as shown in the online resource 7.S2.).

In summary, we suggest that $CD56^{dim}$ NK cells have a high turnover under disease conditions and have to be replaced, and consequently, their precursor cells ($CD56^{bright}$) are released as recent immigrants from the bone marrow and/or the lymph nodes sharing a less differentiated phenotype. In turn, the $CD56^{bright}$ NK cells, a cell type with high cytokine-secreting ability, may contribute to the risk of developing SLE by the potential accumulation of these proinflammatory NK cells in target tissues. The full interpretation of these observations requires further studies on similar markers at the site of tissue inflammation and damage.



Online Resource 7.S2. Leukopenia and lymphopenia are known to occur in SLE and probably related to the destruction of blood cells or to ineffective hematopoiesis. Namely, NK cell apoptosis induced by circulating immune complexes and serum cytokines might contribute to NK cell depletion in SLE, which may be also due to some of the NK-cell subsets being edged out from the blood flow to target tissues, mainly in active disease. The impaired clearance of apoptotic cells by macrophages in patients with SLE may predispose to the development of antibodies which leads to the sustained activation of effector cells. Activation status, in part *via* CD16, enhances NK cell cytotoxicity mediated by several apoptosis-inducing pathways, like perforin/granzyme B, and stimulates cytokine (e.g. IFN-γ, TNF-α) production highlighting the contribution of their activated phenotype for the inflammatory process and maintenance of the disease. Low levels of granzyme B *per* cell together with a decreased CD57 expression on $CD56^{dim}$ NK cells may reflect an impaired differentiation or an important NK cell lysis defect in patients with active SLE. Furthermore, under disease conditions a high turnover of $CD56^{dim}$ NK cells results in the release of cells with a less differentiated phenotype as recent immigrants from the bone marrow and/or the lymph nodes. In turn, $CD56^{bright}$ NK cells, a cell type with high cytokine-secreting ability, may contribute to the risk of developing SLE by the potentially accumulation of these activated proinflammatory NK cells in target tissues. Higher serum CXCL10/IP-10 levels and a redistribution and accumulation of both active CD56 NK-cell subsets at sites of inflammation may explain a sustained CXCR3 internalization and a decreased proportion of circulating CD57+CXCR3+ $CD56^{dim}$ NK cells in active disease. Steroid therapy effect is visible on the lower TNF-α expression levels among $CD56^{dim}$ NK cells.

Chapter 8

Summary and conclusions

Summary and conclusions

The work presented in this thesis aimed at evaluating potential predictors of clinical outcomes of patients with SLE and increasing our knowledge about the relationships between immunological markers and SLE disease activity. Four major contributions have been made towards improving management of SLE patients and understanding the immunological profile of different disease activity states:

1) we contributed to improve the classification of SLE, by clarifying which of the classification criteria currently in use has higher sensitivity, thus being able to identify a higher proportion of the individuals actually suffering from this disease in the general population, especially in earlier phases of the disease course – and this is crucial to improve clinical outcomes by initiating early intervention and appropriate management;

2) we demonstrated that patients satisfying either of the two classification criteria for SLE currently in use, at time of clinical diagnosis, present similar long-term clinical outcomes – this importantly highlights the need to provide to all patients and as early as possible the same standard of treat-to-target management;

3) we have identified clinical predictors of flares of SLE disease activity, that are useful in clinical practice to identify patients that benefit from a tighter monitoring and from interventions designed to prevent flares and maintain a stable state of remission or at least of low disease activity, and hopefully, improving long-term outcomes;

4) we added to the body of knowledge regarding the relationships between immunological markers and SLE disease activity, namely characterizing in patients with and without clinically active disease (as compared to healthy subjects) abnormalities in distinctive immune cell types involved in the innate (NK cells) and adaptive (Th17 and B cells) immune pathways of SLE pathogenesis - the differing profile of immunological abnormalities in clinically active and inactive SLE states may provide clues for better treatment strategies as well as a basis for the biologic validation of clinical definitions of remission. A standardized definition of remission is crucially needed to provide a uniform target for SLE management, in order to optimize long-term clinical outcomes of these patients.

In this final chapter we summarize the main findings of the studies presented in this thesis and will also discuss present and future perspectives.

Performance of the ACR (1997) and the SLICC (2012) classification criteria sets

In the study presented in chapter 2, we compared the sensitivity for SLE clinical diagnosis of the ACR and SLICC classification criteria sets for SLE in a real-life, multicenter, international SLE population. In addition, we tested the sensitivity of each classification criteria set across categories of SLE duration, to determine which set of criteria allows an earlier SLE classification in the disease course. This was a cross-sectional, multicenter, observational study including 2055 SLE patients with a clinical diagnosis of SLE from 17 centers in Portugal and Spain and registered in the Reuma.pt or RELESSER national registries. The sensitivity of the two classification sets was compared using the McNemar's test.

We found that the sensitivity for SLE diagnosis was higher with the SLICC than with the ACR classification criteria (93.2% versus 85.6%, $p<0.0001$). Of the patients not fulfilling the ACR criteria, 62.8% could be classified with the SLICC. Regarding the patients earlier in the disease course, we found that the subgroup of patients within 5 years since disease onset presented the largest difference in sensitivity between the SLICC and the ACR criteria (respectively 89.3% and 76.0%, $p<0.0001$).

In this study we showed that the SLICC classification criteria are more sensitive and may allow a SLE classification earlier in the disease course than the previous ACR criteria, in a large group of patients likely to be representative of the general population of SLE patients. It provided a much needed external validation of the SLICC classification criteria. An important additional advantage of the SLICC criteria demonstrated in this study was the ability to classify as SLE more patients presenting with major organ involvement, namely with lupus nephritis, and with a higher sensitivity for neuropsychiatric lupus. Furthermore, only 1.6% of patients fulfilling the ACR criteria failed to be classified with the SLICC in our study. Thus, the SLICC criteria greatly contributes to reduce the frequent issue of patients with a clinical diagnosis of SLE but not classified as such applying the ACR classification criteria – so called "incomplete lupus".

Effect of the classification criteria fulfilled at time of SLE diagnosis and other predictors on long-term outcomes of damage and mortality

The use of the SLICC classification criteria for case definition in clinical trials and observational studies will allow the inclusion of a larger proportion of patients with a clinical diagnosis of SLE, as we concluded in chapter 2. This may lead to enrollment of study populations with a broader spectrum of SLE, with earlier disease as well as those previously named as 'incomplete' lupus or even undifferentiated connective tissue disease. Hence, SLE populations selected with the SLICC instead of the ACR criteria could present differences in disease phenotype, clinical course, management requirements, response to treatment, and

ultimately in long-term outcomes of irreversible damage or survival. In the study presented in chapter 3 we investigated if there are differences in damage and mortality outcomes up to 10 years after diagnosis, between patients satisfying the ACR classification criteria for SLE and those fulfilling only the SLICC criteria at time of diagnosis. In addition, we tested the effect of other patient covariates in determining long-term damage and survival.

This was a prospective, open, inception cohort study at the CHUC Lupus Clinic that included 192 SLE patients followed up foro 10 years from time of diagnosis. Identification of potential predictors for each of the study outcomes, irreversible damage and death, was done through univariate and multivariate survival analysis (applying Kaplan-Meyer curves, log-rank tests and multivariate Cox models with estimation of the adjusted survival curves and hazard ratios with 95% confidence intervals). The putative predictors at inception tested in the models were: the SLE classification status, gender, age at time of SLE diagnosis, lupus nephritis, neuropsychiatric lupus, SLEDAI-2k score, prednisolone daily dosage, antiphospholipid antibodies, lupus anticoagulant, anti-dsDNA antibodies, anti-Sm antibodies, hypertension and smoking.

We found that at inception 30.2% of the 192 subjects fulfilled only the SLICC classification criteria, while the other 69.8% fulfilled the ACR criteria. From the patients fulfilling the ACR criteria, 97.8% also satisfied the SLICC criteria. Compared to patients satisfying only the SLICC criteria at inception, those meeting the ACR criteria presented during follow-up a higher prevalence of lupus nephritis (35.1% versus 13.8%, $p<0.01$), but less thrombotic antiphospholipid syndrome (4.5% versus 17.2%, $p<0.01$). The Cox models showed no significant differences in the risk for damage accrual or death between the two groups. Age at inception was a significant predictor for damage and death, and neuropsychiatric lupus was predictive only for damage. No significant effect was found for the other covariates. Patients fulfilling the ACR criteria required more frequently treatment with prednisolone and immunosuppressants.

In conclusion, in this study we found no differences in major outcomes of organ damage and mortality up to 10 years of follow-up between SLE patients fulfilling, at inception, either the ACR criteria or only the SLICC classification criteria. However, there were differences in the SLE clinical and immunological phenotype captured by the two sets of classification criteria, resulting in heterogeneous management requirements. These differences should be taken in consideration in the design and interpretation of clinical studies as well as in clinical practice.

Taken together, results of the studies presented in chapter 2 and 3 shows that the SLICC criteria provide some added value for SLE classification, as they: (1) allow the inclusion of most cases with a clinical diagnosis; (2) enable classification earlier in the disease course; (3) include a larger proportion of the patients with major organ involvement; (4) extend

classification to a broader spectrum of the disease, namely by including a group of patients with preponderance of secondary antiphospholipid syndrome, which is a potentially severe manifestation. As a result, the cases defined with the SLICC classification criteria are likely to be more representative of SLE in the general population than those identified by the older ACR classification.

Despite the improvements introduced by the SLICC criteria in comparison to the ACR set, as demonstrated in our studies, it still has limitations, particularly in specificity, which impairs its use as diagnostic criteria. Preference for one or another of the classification sets is still controversial, as discussed in a opinion paper in *Nature Reviews Rheumatology* discussing the results of our study presented in chapter 2.[87] Our newly published study, presented in chapter 3, will help settle issues about outcome stratification related to classification criteria. In our view, the prospect of constructing diagnostic criteria or newer classification criteria for SLE with significantly better specificity will require significant progress in the understanding of SLE pathogenesis that may allow identification of sensitive, specific and practical to use SLE biomarkers.[36, 189]

Identification of clinical predictors for SLE flares of disease activity

During the disease course, the level of SLE clinical activity and occurrence of disease activity flares are fundamental outcome measures in monitoring patients with SLE. Flares are known predictors of long-term irreversible organ damage and mortality.[91] Accordingly, prevention of flares and attainment of a stable low disease or remission state is a major objective of the clinical management of SLE patients. The prediction for SLE flares is crucial to optimize monitoring and preventive treatment. However, previous research studies were unable to identify reliable clinical or biomarker predictors of flare.

The study presented in chapter 4 aimed at identifying clinical predictors of SLE flares. This was a prospective cohort study over 24-months of 202 patients followed at the CHUC Lupus Clinic. Flare was defined as an increase in SLEDAI-2k score ≥4 points. As a summary measure of disease activity over time, we calculated the time-adjusted mean SLEDAI-2k (AMS) over follow-up for each patient and compared the AMS between those with and without flares (applying a t-test).[105] Baseline putative clinical predictors of flare (gender, age at SLE diagnosis, severe disease requiring treatment with immunosuppressants, biopsy-proven lupus nephritis, SLEDAI-2k score, disease duration since SLE diagnosis, treatment with hydroxychloroquine, treatment with systemic glucocorticoids) were tested applying survival analysis (univariate analysis with Kaplan-Meyer curves and log-rank tests, followed by multivariate Cox regression models with estimation of adjusted survival curves and hazard ratios with 95% confidence intervals).

We found in this study that 16.8% of 202 SLE patients, followed over 1083 visits for up to 24 months, presented flares. The mean disease activity (AMS) was higher over follow-up in those patients presenting flares compared to those without (6.3 and 3.1, respectively). In the multivariate analysis we identified as clinical predictors of flare: ≤25 years of age at SLE diagnosis, previous lupus nephritis and baseline immunosuppressant treatment/severe SLE. Specifically, at any time-point up to 24-month follow-up, the risk of flare was more than two-fold, four-fold and three-fold higher for patients with SLE diagnosis ≤25 years, previous lupus nephritis or immunosuppressant treatment, respectively. Importantly, we found no evidence for a lower risk of flare associated with baseline low disease activity or longer SLE duration.

This study is important because it demonstrated the feasibility of clinically stratifying SLE patients according to risk of disease flares. In addition to the predictors identified, it is equally important the finding that risk of flares does not subside with longer disease duration or in those with a lower disease activity state at baseline. For clinical trials with occurrence of new flares as endpoint, this set of predictors can be applied to enroll a high risk study population, in order to increase the efficiency of the trial. Patients with predictors of flare should receive a tighter clinical monitoring and efficient preventive management strategies to achieve a stable remission or low disease activity state. Of note, this study also provides evidence that current immunosuppressive strategies are inefficient in providing flare prevention. Development of effective treat-to-target strategies and effective immunosuppressants remains an unmet need in SLE.[11]

Regardless of the definition applied, flares are a transitional measure from a lower to a higher state of disease activity at a subsequent time-point. During a given period of time, patients experiencing flares are likely to present higher mean disease activity levels, as we found in our study. Higher disease activity over time is associated with increased damage accrual.[54] A stable state of remission or low disease activity is associated with a decrease in damage progression.[65, 66, 69] A recent study from Italy found that patients needed to be maintained in a stable clinical remission, without flares, for at least two consecutive years in order to obtain a significant decrease in damage progression.[65] From the 293 Caucasian patients followed in that cohort study, 78.8% maintained a state of clinical remission for at least 2 years, which is consistent with our own findings. On the contrary, a large cohort study from the North-American Hopkins Lupus Clinic found that durable remission was rare, with most patients presenting flares with a median time to flare of 3 months.[63] A more intensive treatment at baseline was the major predictor of clinically active SLE, which is consistent with our results. Differences between studies are likely due to multiple factors, in particular the high proportion of African-Americans in the USA cohort and the different definitions of remission used.[71]

Knowledge about the relationships between immunological markers and SLE disease activity

A broadly accepted definition of remission or low disease activity state to serve as target for SLE management remains elusive, despite recent progress.[64, 65, 69, 70] A major constraint to this objective is the lack of a gold-standard for the derivation and validation of such a definition.

Patients with SLE are carriers of susceptibility genes associated with abnormalities in immune cells and molecules, which develop self-sustained loss of tolerance and chronic maintenance of autoimmunity: resulting markers of immunological dysfunction are likely to be present even in clinically quiescent phases of the disease. In contrast, enhanced autoimmune responses and target organ inflammation lead to clinically active disease: associated immunological abnormalities may be reversible when SLE becomes clinically inactive.

The aim of the studies described in chapters 5-7 is to increase knowledge about the relationships between immunological markers and SLE disease activity – ultimately seeking to provide a basis for the biologic validation of clinical definitions of remission and disease activity states.

B cell lineage

B cells play a crucial role in SLE pathogenesis. Failure in both central B-cell tolerance and peripheral checkpoints at the transitional-näive B-cell stage, lead to expansion of autoreactive B cells.[17] Self-reactive B cells and their products – autoantibodies - are typically found long before clinical manifestations of SLE become recognizable.[117] The B-cell-activating factor (BAFF) is primarily secreted by myeloid cells and through its receptor, BAFFR, plays a key role in stimulating B cell expansion, differentiation, survival and autoantibody production.[190] An anti-BAFF monoclonal antibody, belimumab, has benn proven effective in the treatment of SLE.[191]

In the study presented in chapter 5, we searched for unique phenotype patterns in peripheral blood B cell subsets capable of distinguishing between SLE patients with clinically inactive and with active disease activity, as well as healthy control subjects. This was a cross-sectional study in 41 SLE patients (including 24 with clinically inactive and 17 with active disease) and 28 matched healthy subjects. Briefly, peripheral blood samples were collected and analyzed with a flow cytometry immunophenotyping protocol for identification of B cell subsets.[192, 193] In each subset, the individual expression of CD19, CD20, CD38, CD81 and BAFFR was quantified as mean fluorescence intensity (MFI). Selected clusters derived from principal component analysis according to the pattern of immunophenotypic markers were compared to

search for cellular subsets that would distinguish among groups of subjects. Group comparisons were performed with Kruskal-Wallis, Mann-Whitney-U and Chi-squared tests.

We found that the absolute number and frequency of B cells among lymphocytes in patients with clinically inactive SLE is similar to healthy controls, but show an abnormal distribution among the B cell subsets: the frequency of transitional B cells is higher (10.1% versus 4.1%, $p<0.0001$) and the frequency of memory B cells is lower (22.5% versus 34.4%, $p<0.001$). Patients with clinically active SLE, compared to inactive disease, presented a lower number and frequency of B cells in the peripheral blood (1.1% versus 2.8%, $p<0.01$), together with an abnormal distribution among functional subsets, most notably a higher number and frequency of plasmablasts (3.0% versus 0.9%, $p<0.05$).

Most importantly, applying principal component analysis of the cell surface signal transduction molecules (CD19, CD20, CD38, CD81, and BAFFR) on the B cell functional subsets, we identified two major clusters in transitional B cells, providing good discrimination of the participants' disease status: cluster 1 integrated all healthy subjects and 79% of SLE patients with clinically inactive disease, while cluster 2 included only patients with SLE and 82% of those with clinically active disease. Furthermore, in the 12 months after blood collection, all patients with active disease in cluster 1 improved to inactive disease state, while this was observed in only 21.4% of those in cluster 2. No clusters could be identified in the remaining B cell subsets.

In conclusion, this study provides proof-of-concept that a panel of biomarkers may be used as a basis for the biologic validation and refinement of clinical definitions of disease activity states. In this study, the combined expression of BAFFR, CD81 and CD38 on transitional B cells was able to discriminate groups of patients with active and inactive disease states. Further studies with larger populations are required to confirm these results and eventually to derive and validate an improved biomarker panel with optimized accuracy. Research should also focus on biomarkers from other immune pathways that may need to be integrated in the panel, due to the heterogeneous immunopathogenesis of different SLE clinical manifestations and patient populations.

Th17 cells

Interleukin-17 (IL-17), in particular IL-17A and its major producer, the T helper cell subset (Th17) play critical roles in the pathogenesis of a variety of autoimmune and inflammatory rheumatic diseases, such as psoriasis, psoriatic arthritis and ankylosing spondylitis. This is reflected by the efficacy of anti-IL-17A targeted agents in the treatment of these conditions.[194] There is also evidence suggesting a role for IL-17 and Th17 cells in the pathogenesis of SLE: high levels of IL-17-producing cells and IL-17 were found in target organs

of SLE patients, namely in the kidneys and skin in periods of active organ disease.[143, 145, 195] IL-17A can promote the inflammatory process in tissues by inducing local production of chemokines and cytokines and recruiting other inflammatory cells.[196, 197] Furthermore, IL-17 stimulates B cells, acting in synergy with BAFF.[198] However, studies evaluating IL-17 levels and circulating Th17 cells in peripheral blood of SLE patients reported conflicting results.[196, 199-201]

In the study presented in chapter 6, we compared the frequency and functional characteristics of the interleukin 17-producing subset of $CD4^+$ effector T helper cells (Th17) in the peripheral blood of healthy subjects, SLE patients with clinically inactive and those with clinically active disease. This was a cross-sectional study in 34 SLE patients (including 19 with clinically inactive and 15 with active disease) and 15 matched healthy subjects. The samples were analyzed with flow cytometry immunophenotyping protocols.

We found no significant differences in the frequency of Th17 in the three groups. The amount of IL-17 produced at single cell level was lower in SLE patients compared to healthy individuals, without significant differences according to disease activity status. We demonstrated that in addition to IL-17, most Th17 cells also can express the Th1 proinflammatory cytokines IL-2, TNF-α and/or IFN-γ; however, the pattern of distribution of these functional Th17 subsets was similar in healthy subjects and the SLE patient groups.

In conclusion, our study does not support that the frequency or functional status of Th17 cells in peripheral blood could be used as a biomarker of disease activity states in SLE patients. It is possible that the frequency of IL-17 producing cells in target organs may correlate with disease activity, but due to unpractical sample collection that would not be a feasible biomarker.[159, 202-204]

Natural killer cells

Natural killer (NK) cells are a main component of the innate immune system and they may play both regulatory and/or disease-promoting roles in SLE.[205] Gene polymorphisms of the killer cell immunoglobulin-like receptor (KIR), expressed by these cells, were associated with increased susceptibility for SLE.[206] Human peripheral blood NK cells present two functional subsets, based on their cell-surface density of CD56: the $CD56^{dim}$ NK cells are the most frequent, express higher levels of KIR and exert cytotoxicity through perforin and granzyme-containing granules; the $CD56^{bright}$ NK cells produce abundant amounts of proinflammatory cytokines and chemokines, including IFN-γ and TNF-α, and present lower cytotoxic capacity. NK cells' expression of chemokine receptors, such as CXCR3, drive migration to sites of inflammation and may contribute to SLE disease activity.[175] CD57 is a marker of NK cell differentiation and abnormalities of $CD57^+$ cells were associated with systemic autoimmune

diseases.[207] However, the contribution of NK cells to SLE pathogenesis and disease activity is far from clear.

In the study presented in chapter 7, we compared the frequency and functional characteristics of the NK cell subsets in peripheral blood of healthy subjects, SLE patients with clinically inactive and with clinically active disease. This was a cross-sectional study in 44 SLE patients (including 26 with clinically inactive and 18 with active disease) and 30 matched healthy subjects. We analyzed by flow cytometry the frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells, and their expression of CD57, CXCR3, granzyme B, perforin, IFN-γ and TNF-α.

We found that SLE patients, regardless of disease activity status, presented a lower number and frequency of NK cells than healthy subjects, without differences in the distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. A lower frequency of $CD56^{dim}$ NK cells expressing CXCR3 was a marker of clinically active SLE (12.5% versus 24.1% in the active and inactive SLE group, respectively, $p<0.01$), concomitantly with a lower CXCR3 expression level (MFI) in the same cells. The $CD56^{dim}$ NK cells from both groups of SLE patients expressed a lower amount of granzyme B compared to healthy subjects, while patients with clinically active disease presented a higher frequency of $CD56^{dim}$ NK cells expressing perforin, compared to those with inactive SLE. Analysis of the $CD56^{bright}$ NK cell subset showed higher expression (MFI) of IFN-γ in SLE patients, regardless of disease activity status, compared to healthy subjects, while a lower frequency of $CD56^{bright}$ NK cells expressed TNF-α in the patients with clinically active SLE.

In conclusion, this study demonstrated a low number and frequency of NK cells and altered functional markers in both the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets from peripheral blood of SLE patients; we identified expression of CXCR3 in $CD56^{dim}$ NK cells and TNF-α in $CD56^{bright}$ NK cells as candidate markers of disease activity states. Most recently published studies corroborate our findings.[208-210]

Overall, our flow cytometry immunophenotyping studies (chapter 5-7) of peripheral blood immune cell types in SLE and healthy subjects, suggests: an upregulation of B cells, with subset clusters differentiating SLE patients from healthy subjects and clinically active from inactive SLE; a downregulation of NK cells in SLE and less clear changes of Th17 cells.

SLE is a disease with high heterogeneity of the predominant immunopathogenesis among different organ manifestations, patients and populations: this is highlighted by results of clinical trials with belimumab, a monoclonal antibody targeting BAFF, which demonstrated clinical efficacy in less than 60% of SLE patients with clinically active disease.[102, 191] The subgroup of patients with high serum levels of BAFF were more likely to be responders.[211] In

other subgroups of patients, disease activity is likely dependent on immune pathways other than B cell activity, particularly of type I IFN activation. Indeed, expression of type I IFN-inducible genes (IFN-gene signature) is increased in peripheral blood mononuclear cells of many SLE patients.[212] Accordingly, in a clinical trial of sifalimumab, an anti-IFN-α monoclonal antibody, SLE patients with high IFN-gene signature were more likely to be responders.[213] In a most recent cutting edge study, Banchereau *et al* longitudinally profiled the whole blood transcriptome of 158 patients with pediatric SLE and healthy controls.[214] By applying an analysis with a rationale similar to our study on B cells, they aimed at identifying clusters of transcriptomic markers correlating with SLE classification and disease activity states. They found an increased IFN signature to be the best correlated with SLE classification, within a cluster also including a variety of other molecular markers, that was shown to highly correlate with SLE classification criteria (R^2 =0.94). They further identified seven clusters of combined transcriptional immune markers that best identified subsets of patients with clinically active SLE, each displaying a specific combination of immune signatures, including increased plasmablasts and IFN response, while NK cell transcripts were negatively correlated. The plasmablast signature was particularly enriched in African-Americans and was overall the best correlated with disease activity. However, highlighting the broad heterogeneity of immune pathways in SLE, they found that plasmablast or IFN signatures alone failed to identify clinically active SLE in two-thirds of patients. The results of this study are consistent with our own, presented in chapters 5-7, regarding positive (B cell lineage), negative (NK cell lineage) and not clearly related (Th17 lineage) markers of disease activity states. Importantly, it demonstrates that an integrated transcriptional panel can reliably identify SLE patients and disease activity states. In future research, such panels can be applied to compare groups of patients fulfilling different SLE classification criteria sets for potential differences in immunopathogenesis, in order to further validate and optimize classification criteria.[189] A similar approach can be applied to redefine and provide a molecular validation of clinical criteria for SLE remission. This might require integration of additional information, such as epigenetic, proteonomic, immunophenotyping and cytokine analysis. Among serum markers, high BAFF levels were found to be predictive of flares in the following 52 weeks.[61, 211] Accordingly, patients defined as in a clinical remission state who present low BAFF levels have an increased likelihood of maintaining a long-term stable remission, an important predictor of improved outcomes in organ damage accrual and survival.[65, 71] Such complex combined molecular panels are unlikely to be applicable in the clinical setting in the near future; they should instead be used in translational research settings to help identify and validate (by providing a molecular explanation) panels of clinical predictors of outcomes and stratification of patients appropriate for individualized treatment strategies. As an example, we identified lupus nephritis and standard immunosuppressant usage as clinical predictors of flare (chapter 4); these were more recently found to associate with high serum levels of BAFF ≥2 ng/mL, which is a molecular predictor of flares.[211]

A critical strategy for derivation, validation and optimization of predictive models of clinical outcomes in SLE is the standardized acquisition and merging of high-quality data in large multicenter prospective cohorts from several national registries. [215] We applied such a strategy in the study described in chapter 2, by integrating the large SLE datasets from the Reuma.pt and RELESSER, and intend to expand it in future research for modeling SLE clinical outcomes.

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