PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

GUILHERME CERUTTI MÜLLER

ANÁLISE DA PRESENÇA DA INVERSÃO DA RAZÃO CD4:CD8 EM IDOSOS E SEU PERFIL CELULAR E BIOQUÍMICO

Porto Alegre, Setembro de 2015

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ANÁLISE DA PRESENÇA DA INVERSÃO DA RAZÃO CD4:CD8 EM IDOSOS E SEU PERFIL CELULAR E BIOQUÍMICO

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Porto Alegre, Setembro de 2015

"Todo mundo é capaz de envelhecer. Basta viver o suficiente para chegar até lá."

Julius Henry Marx

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1. Lista de abreviaturas

AOPP: Produtos proteicos de oxidação avançada
CI: Cognitive impairment
CMV: Citomegalovírus
DNA: Ácido desoxirribonucleico
EBV: Epstein-Barr virus
FRAP: Atividade antioxidante total pelo método de redução do ferro
HSV: Herpes simplex vírus
IL-1: Interleucina 1
IL-2: Interleucina 2
IL-6: Interleucina 6
IL-7: Interleucina 7
IL-15: Interleucina 15
IRP: Perfil de risco imunológico
KLRG1: killer cell lectin-like receptor G1
Nfr2: NF-E2 related factor 2
NK: Célula Natural Killer
OMS: Organização Mundial da Saúde
OS: Estresse Oxidativo
PCR: Proteína C-reativa
ROS: Espécies reativas de oxigênio
RNS: Espécies reativas de nitrogênio
Tbars: Substâncias reativas ao ácido tiobarbitúrico
Th1: Linfócito T-helper 1
Th2: Linfócito T-helper 2
Th17: Linfócito T-helper 17
TLR: Receptor Toll-like
TNF-α: Fator de necrose tumoral alpha
TREC: T cell receptor excision circles
VZV: Varicella zoster virus

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3. Resumo

O envelhecimento tem sido associado com o aumento da geração de radicais livres, bem como com a imunossenescência. Estudos anteriores já haviam identificado os idosos que apresentavam a inversão da razão CD4:CD8 e a sorologia mais acentuada para citomegalovírus (CMV). Aqui, investigamos marcadores de estresse oxidativo e as defesas antioxidantes em idosos com a relação invertida entre as células T CD4 e CD8. De um total de 421 idosos recrutados na comunidade, 61 sujeitos foram identificados com a inversão da razão CD4:CD8. Os idosos com CD4:CD8<1 tinham níveis aumentados de produtos proteicos de oxidação avançada (AOPP) e atividade antioxidante total pelo método de redução do ferro (FRAP) no plasma, mas níveis reduzidos de substâncias reativas ao ácido tiobarbitúrico (TBARS), em comparação com indivíduos com razão CD4:CD8 normais. A sorologia para CMV (IgG) foi negativamente correlacionada com a razão CD4:CD8. Estes marcadores foram mais evidentes entre os homens do que as mulheres idosas. Nossos dados sugerem uma estreita relação entre a infecção por CMV crônica e perfil oxidativo em indivíduos mais velhos no meio de sua influência sobre os subtipos de células T periféricas.

Palavras-chave: Imunossenescência, Estresse Oxidativo, Citomegalovirus, Perfil de Risco Imunológico.

4. Abstract

Aging has been associated with increased generation of free radicals as well as immunosenescence. Previous studies have identified older individuals with inverted T CD4:CD8 cell ratio and increased immunity to cytomegalovirus (CMV). Here, we investigated markers of oxidative stress and antioxidant defences in older individuals with inverted CD4:CD8 T-cell ratio. From 421 healthy community-dwelling older adults recruited, 61 subjects were identified with inverted CD4:CD8 T-cell ratio. Older individuals with a CD4:CD8 ratio < 1 had increased levels of plasma advanced oxidation protein products (AOPP) and Ferric Reducing Ability of Plasma (FRAP), but reduced levels of thiobarbituric acid reactive substances (TBARS) as compared to subjects with normal CD4:CD8 T-cell ratio. The CMV IgG serology was negatively correlated with CD4:CD8 ratio. These markers were more evident among elderly men than women. Our data suggest a close relationship between chronic CMV infection and oxidative profile in older individuals in the midst of its influence on the peripheral T-cell subsets.

Keywords: Immunosenescence, Oxidative Stress, Cytomegalovirus, Immune Risk Profile.

SUMÁRIO

1 INTRODUÇÃO

O número e a proporção de idosos vêm aumentando rapidamente em países em desenvolvimento, como é o caso do Brasil [1, 2]. Estima-se que ao longo da "*era do envelhecimento*", a população brasileira apresente um crescimento em torno de 88% e que a população idosa, no mesmo período, atingirá cerca de 123% [3]. De acordo com a Organização Mundial de Saúde (OMS), até o ano de 2025, o número de idosos brasileiros será 15 vezes maior, somando perto de 32 milhões de habitantes com idade de 60 anos ou mais (15% de idosos na população), fato que tornará o Brasil um país de idosos [3]. Estes dados revelam que o Brasil vem apresentando um envelhecimento populacional acelerado e que o aumento da população de idosos é três vezes maior que em outros países, como os industrializados [4, 5]. Com este crescimento pode se prever que, haverá muitos desdobramentos relacionados à condição crônica de saúde destes idosos.

O aumento gradativo de doenças infecciosas e tumores, entre outras doenças associadas ao envelhecimento é resultado do aumento da expectativa de vida da população que implica diretamente em mudanças epidemiológicas [6, 7]. O aumento da expectativa de vida somado à precariedade de medidas estratégicas para prevenção e tratamento de doenças crônicas comuns no envelhecimento potencializa este quadro. Desta forma, o aumento de custos relacionados às internações e tratamentos médicos é resultado da falta de estratégias frente a este problema de saúde pública. Por conta desta realidade, um número cada vez maior de estudos está sendo realizado com o objetivo de elucidar os processos relacionados com diferentes sistemas fisiológicos do envelhecimento, em particular, o sistema imune [8].

O estudo da imunossenescência (envelhecimento do sistema imune) é de extrema relevância visto que este processo tem impacto em diferentes patologias ditas comuns no envelhecimento, como exemplo as doenças autoimunes, o câncer, bem como infecções ocasionadas por vírus ou bactérias. Estudos recentes apontam para o aumento da prevalência e da severidade das doenças infecciosas em idosos associadas a inúmeras disfunções na resposta imune. Com base em dados publicados pela OMS, as infecções são uma das maiores *causa-mortis* em idosos (6,4%) no mundo [7, 9]. Nos períodos de epidemias, as infecções causadas pelo vírus *influenza,* representam entre 80% e 90% das mortes em indivíduos acima de 65 anos, demonstrando que indivíduos idosos representam um grupo populacional de alto risco [9].

Uma vez que a expectativa de vida aumenta, o tempo de exposição a fatores ambientais aumenta também, o que pode acarretar em um aumento no número de doenças relacionadas à idade [10]. O envelhecimento está associado com uma reestruturação das funções do sistema imune (**imunossenescência**), tanto na imunidade humoral quanto na imunidade mediada por células, resultando em maior suscetibilidade a infecções, ao câncer, além da diminuição na capacidade de responder a vacinas [11]. Muitas doenças que ocorrem na velhice têm base imunológica e estão associadas com a diminuição das respostas imunes a antígenos exógenos além de maior propensão a reações autoimunes [12].

1.1 Imunossenescência

O envelhecimento é um processo lento e constante que gera alterações fisiológicas que podem ser permanentes ou reversíveis. Durante o processo de

envelhecimento, o funcionamento de muitos órgãos e sistemas passa por mudanças e reestruturação, tanto em termos qualitativos quer em termos quantitativos. A compreensão das alterações imunológicas que ocorrem devido ao envelhecimento é de extrema importância visto que as consequências clínicas da imunossenescência incluem impactos significativos na morbidade e mortalidade do idoso [13].

A imunidade inata apresenta perdas funcionais durante o envelhecimento, em contrapartida com uma estabilidade numérica de seus constituintes celulares. Intrinsicamente ligado ao envelhecimento do sistema imune inato está o fenômeno denominado “inflammaging”, apresentado por um status inflamatório mais acentuado com o passar dos anos. Este perfil inflamatório é caracterizado por níveis plasmáticos elevados de citocinas como: interleucina 6 (IL-6), interleucina 1 (IL-1) e o fator de necrose tumoral alfa (TNF-α); além de proteínas de fase aguda como a proteína C reativa (PCR) e receptores solúveis de citocinas. Paradoxalmente a esse processo, a imunidade inata sofre algumas alterações funcionais, incluindo a redução da função fagocítica e da expressão de receptores Toll-Like (TLRs), sem que a contagem absoluta seja afetada. Processo semelhante ocorre com as células NK, cuja contagem absoluta aumenta com a idade mas sua citotoxicidade (e capacidade de produção de citocinas) decai. Já as células dendríticas sofrem perdas tanto de números absolutos como em termos funcionais na apresentação de antígenos [14-16].

A maioria dos estudos sobre imunossenescência têm focado principalmente o envelhecimento relacionado às funções das células T. Estudos anteriores relatam defeitos nas rotas de sinalização intracelular, diminuição na taxa de proliferação em resposta a antígenos específicos, aumento nos fenótipos de memória, alteração na

produção de citocinas (e.g. aumento de citocinas Th2), diminuição das respostas por células T citotóxicas [17-21].

As células T passam por importantes trocas enumerativas (fenotípicas) durante o envelhecimento. Uma reduzida capacidade hematopoiética, demonstrada pela redução de células-tronco na medula óssea e involução tímica, é observada durante o envelhecimento e está associada com a redução da população de células T naïve, embora o número total de células T se apresente mais ou menos constante. Estas alterações se contrastam com um aumento de células T efetoras diferenciadas e de memória [22-24]. Portanto, é visto que o envelhecimento é caracterizado principalmente pela redução da capacidade do sistema imune adaptativo em responder a antígenos externos, causando um impacto significativo no repertório de células T [25].

A alteração do sistema imune pelo processo do envelhecimento não é uniformemente distribuída e pode ser influenciada por outros fatores imunomoduladores, incluindo fatores genéticos e a exposição ao estresse crônico [26]. Diferentes fatores podem afetar este processo do envelhecimento do sistema imune, como a melatonina [27], e da mesma forma, os fatores nutricionais também têm um impacto significativo [28], assim como a ingestão oral de probióticos específicos [29] e a atividade física [30]. Evidências do nosso laboratório mostraram que intervenções com acupuntura podem também influenciar a qualidade de vida, carga emocional, e também a imunossenescência [31, 32]. Evidências recentes indicam que o estresse psicológico crônico pode causar o envelhecimento prematuro de diversos componentes celulares e moleculares do sistema imune. Este estresse psicológico tem sido amplamente associado com a supressão de algumas funções imunológicas [33].

1.1.1 Alterações fenotípicas em células T

Existem importantes mudanças fenotípicas das células T durante o envelhecimento saudável. Um fenômeno especialmente observado após os 65 anos de idade é a diminuição do repertório das células T. Esse fenômeno foi associado com uma expansão de alguns clones com especificidade restrita, em detrimento da variabilidade clonal que se observa na juventude [34]. A restrição de especificidades de TCR que compõe o repertório T leva à diminuição da capacidade do indivíduo de responder a novas e variadas infecções. A expansão clonal observada no envelhecimento é principalmente de células T CD8+, altamente diferenciadas, e particularmente importantes na imunidade às infecções virais e tumores. O acúmulo dessas células T altamente diferenciadas pode ser explicada pela expansão oligoclonal ao CMV [35, 36]. O fenômeno da expansão clonal de células T CD8+ com a idade já é conhecido em roedores há muitos anos [37]. Ele começa a ser observado em camundongos a partir dos 16 meses de idade, e a existência de populações clonais em camundongos velhos influencia totalmente o perfil da resposta imune obtida em resposta a antígenos nesses animais [38, 39]. Um fato interessante é que essas células clonais não são malignas e não aumentam o número total de células T CD8+ circulantes em um indivíduo, como um tumor de célula CD8+ o faria. Isso indica que há fatores que limitam o crescimento dessas e das demais células T CD8+ em um indivíduo, assim como sugere que vírus como o CMV influenciam o desenvolvimento dessas células agindo sobre um mecanismo específico. Estudos de bloqueio por anticorpos sugerem que a proliferação dessas células está ligada à produção de IL-15 e IL-2 [40].

Com a idade, uma proporção significante de células T perde a expressão de moléculas co-estimulatórias e adquire receptores de inibição [41]. Células T de

idosos demonstram uma resposta reduzida à ativação com estímulo de mitógeno, assim como uma queda da proliferação e da produção de IL-2 [41, 42]. Assim como ocorre um declínio gradativo no número absoluto de células B e T, ocorre também um aumento do número de células NK, de modo que a contagem total de linfócitos acaba não tendo grandes alterações com a idade [15, 43, 44].

As alterações descritas no subtipo de células T CD8+ compreendem boa parte das mudanças fenotípicas nas células T observadas no envelhecimento. As células T CD8+ frequentemente perdem a expressão de CD28 e se acumulam no envelhecimento [45, 46]. A perda da expressão de CD28 é um marco do declínio da função de células T associada à idade, uma vez que CD28 tem um papel vital durante a ativação das células T, como na indução da produção de citocinas (IL-2), e na promoção da proliferação celular, enquanto que a perda deste sinal co-estimulatório durante a ativação resulta em uma ativação parcial, ou até em um estado anérgico das células T [47]. Da mesma forma, o acúmulo de células T que não expressam CD28 ($CD28^{null}$), em geral células T CD8+, está relacionado à menor resposta aos patógenos e às vacinas [48]. Paralelamente à perda de CD28, existe uma redução na expressão de CD27, outra molécula co-estimulatória e expressão aumentada de CD57 e KLRG1 [49]. Estas células fazem parte da subpopulação CD8+CCR7-CD45RA+, onde muitas células ainda não estão totalmente diferenciadas, mas ainda restam algumas células de memória que podem ser reativadas por antígeno. O subtipo com o fenótipo CD8+CCR7-CD45RA+CD27-CD28-CD57+KLRG1+ que pode ser considerado o último estágio de diferenciação celular do linfócito T CD8 é visto principalmente em indivíduos infectados pelo CMV [50, 51].

Moro-Garcia e cols. observou um alto número de células NK e um menor número de células B em indivíduos idosos considerados como tendo um status funcional ruim em comparação com idosos com bom status funcional. Estes mesmo idosos com pior status funcional apresentaram também uma baixa proporção de células CD4+ e uma maior proporção de células CD8+, reduzindo a razão CD4:CD8 [52]. Mais recentemente, novas ferramentas de análise de populações clonais de células T CD8+ em humanos revelou que esses clones possuem um fenótipo alterado, não necessariamente de memória ou naïve. Além disso, foi observado que as células T CD8+ resultantes de infecções repetidas por CMV apresentam um fenótipo semelhante, suscitando a hipótese de que as repetidas infecções virais seriam a força por trás dessas alterações imunes associadas com a idade [53].

Curiosamente, embora as células T CD8+ sejam os principais personagens deste processo do envelhecimento, existe uma perda progressiva de moléculas CD28 em células CD4+ de indivíduos saudáveis com a idade também [45, 54]. Entretanto, as células CD4+CD28^{null} podem representar até cerca de 50% do total de células CD4+ em alguns idosos com mais de 65 anos [55]. Acredita-se que estas células, CD4+CD28^{null}, possam ter um papel importante no controle de infecções virais, como o CMV, onde o tecido endotelial tem uma baixa concentração de células apresentadoras de antígenos em condições normais, mas com as células CD28^{null}, que não requerem um sinal co-estimulatório, a apresentação de antígeno poderia ser efetiva por células não especializadas como as endoteliais [56, 57]. No estudo de Alonso-Arias et al, os autores sugerem que a IL-15 tem um papel fundamental na diferenciação, ativação e proliferação das células CD4+CD28^{null}, e que um tratamento com esta citocina poderia ser efetiva para amenizar os efeitos da queda da efetividade da resposta imune adaptativa decorrente do envelhecimento [57].

O aumento na frequência de células CD27- e CD28- é uma característica marcante das infecções pelo CMV. Analisando a atividade regulatória dessas células, os autores sugerem que elas tenham um papel importante na imunossenescência justamente com a infecção pelo CMV [58]. Outros estudos demonstraram anteriormente que células T específicas para o CMV suprimem a resposta a outros vírus tanto em jovens como em idosos, o que pode ser um fator de aumento no risco de morte em idosos com mais de 70 anos [59, 60].

É conhecido que as células CD8+ são cruciais para a imunidade contra muitas infecções intracelulares, e que após a vacinação mantém diferentes funções como a produção de citocinas e a citotoxicidade. Durante uma infecção crônica as células CD8+ vírus-específicas tornam-se disfuncionais e falham na formação de boas células de memória. Esta exaustão das células CD8 exige a presença do antígeno para continuar sobrevivendo, e não utilizam mais de modo eficiente as citocinas IL-7 e IL-15. Nos casos mais severos de exaustão, quando a carga viral está demasiadamente alta, células T CD8+ vírus-específicas podem ser deletadas [61, 62].

Em paralelo aos efeitos do envelhecimento no sistema imune, também ocorre a redução da diversidade do repertório do TCR (receptor de células T) [54]. Foi visto que a análise de TRECs (alça de excisão do receptor de células T) pode ser um bom método de acompanhamento do processo da imunossenescência, uma vez que eles estão diretamente ligados à variabilidade do TCR [63]. Outro estudo indicou que baixos níveis de TREC estão correlacionados com a baixa frequência de subpopulações naïve de células T (CD45RA+CCR7-) e altas porcentagens de células T efetoras (CD45RA-CCR7-) [52].

Em meio as complexas trocas fenotípicas, a expressão de NKG2D em linfócitos T vem sendo observada durante o envelhecimento. NKG2D um receptor de células NK e de subtipos de células CD8+, no entanto não é comum ser expresso em células CD4 [64]. A infecção pelo CMV pode estar associada à expressão de NKG2D em linfócitos T CD4 [65].

Enquanto as células T CD4 afetadas pelo envelhecimento não tem mais a capacidade de se diferenciar efetivamente em Th1 ou Th2, a capacidade em diferenciar-se em Th17 pode permanecer inalterada [66]. Possívelmente devido ao fato de que acredita-se que as células Th17 sejam o subtipo de células T CD4 mais primitivo, realizando a ponte entre o sistema mune inato e o adaptativo [67].

1.1.2 O perfil de risco imunológico

Vários estudos em imunologia contribuíram para o descobrimento de fatores de risco associados com maior morbi-mortalidade no envelhecimento. Nesse sentido, o perfil de risco imunológico (IRP) parece ser um bom marcador para diferenciar o envelhecimento saudável do patológico. O IRP foi definido a partir de estudos longitudinais com idosos suecos (octa- e nonagenários) com uma razão T CD4/CD8 < 1, baixa proliferação T inespecífica, e elevados níveis de anticorpos IgG para CMV [68]. Os idosos selecionados com este perfil de risco apresentavam uma elevada mortalidade ao longo de quatros anos do estudo, independente do estado de saúde [69]. O perfil de risco foi associado com uma maior atividade inflamatória (e.g. aumento de IL-6 e maior incidência de co-morbidades inflamatórias), aumento de células T CD8 efetoras/memória (CD8+CD28-CCR7-CD27-perforina+) (**Figura 1**), expansão de células CD8+ contra o citomegalovírus (CMV) e aumento de soropositividade ao CMV. A diferenciação de células T CD8+ contra infecções virais

persistentes (especialmente o CMV) parece ser um ponto central da imunossenescência, pois leva ao acúmulo de células senescentes no organismo.

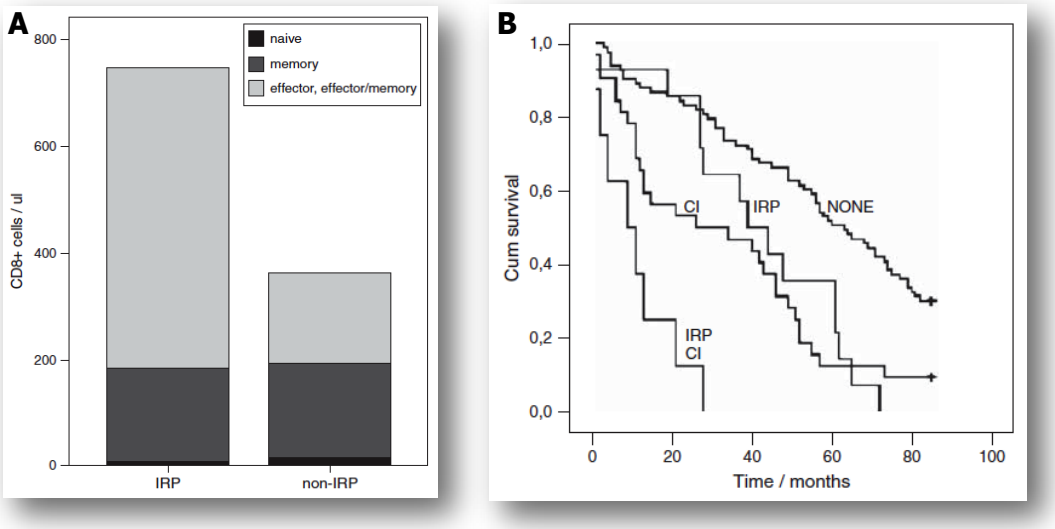

Figura 1. O perfil de risco imunológico (IRP) está associado com um acúmulo de células T CD8+ efetoras/memória **(A)** e menor sobrevivência ao longo de quatro anos **(B)**. Idosos com um perfil de risco e dano cognitivo (IRP + CI) morrem mais cedo do que os sujeitos com perfil de risco apenas. Adaptado de [69].

Alguns estudos já demonstraram uma associação entre as células T CD8+CD28-, a infecção pelo CMV e a baixa resposta imunológica à vacina contra o vírus influenza [70, 71]. Outros estudos também documentaram que em indivíduos idosos que pouco respondem à vacina contra o vírus influenza, as células T CD8+CD28- se acumulam enquanto que as células T CD8 GrB+CD62L+ diminuem [72]. Assim, estes estudos salientam ainda mais a questão já conhecida da maior vulnerabilidade do idoso.

No entanto, em idosos centenários o conceito de IRP não funciona, possivelmente pelo fato de que os idosos que conseguem chegar a essa idade

devem ser justamente aqueles não se encaixavam no perfil de risco nas décadas anteriores de suas vidas [73]. O recente estudo de Wertheimer e cols. jogou uma nova luz no conceito de perfil de risco imunológico, onde eles perceberam uma inversão da razão CD4:CD8 independe da idade, tendo uma relação muito mais forte com os níveis elevados de anticorpos IgG para o CMV [74].

Um estudo recente do nosso grupo demonstrou que a razão CD4:CD8 invertida e o aumento de sorologia para o CMV foram associados com maior fragilidade física e o déficit cognitivo em idosos hexagenários brasileiros [75]. A presente tese visou dar continuidade a esses dados, incluindo informações relacionadas aos estresse oxidativo que surpreendentemente ainda não tinham sido analisadas sob a luz do conceito de vulnerabilidade imunológica.

1.2 Infecções virais

Os fatores e mecanismos por trás do remodelamento do sistema imune com a idade são muitos e abrangem desde uma ação defeituosa da medula óssea, até a involução tímica, passando pela deficiência na geração, maturação, função, homeostase e migração de linfócitos periféricos [76, 77]. Estudos experimentais e clínicos indicam que a população idosa tem uma maior susceptibilidade a infecções microbianas devido ao declínio de sua resposta imune [78].

A maior parte das infecções virais é caracterizada por uma fase aguda onde novas partículas virais são produzidas em células infectadas, e então a resposta imune controla a replicação viral e elimina o patógeno [79]. No entanto, muitos vírus desenvolvem estratégias para resistir aos sistema imune após a infecção primária, sendo assim, o vírus pode persistir no indivíduo hospedeiro apesar da presença de

resposta imune antiviral. Em geral, estas infecções persistentes podem ser divididas em duas categorias: infecções crônicas e infecções latentes [79].

Infecções crônicas não podem ser controladas pelo sistema imune do hospedeiro e são caracterizadas pela contínua replicação do vírus. Alguns vírus como o HIV, o HCV e o HBV têm essa característica, sendo capazes de estabelecer infecções crônicas, podendo permanecer no organismo hospedeiro por longos períodos [80]. No entanto, alguns membros da família *Herpesviridae*, como o vírus Epstein-Barr (EBV), vírus Herpes simplex (HSV) e o vírus Varicella-Zoster (VZV) são bem conhecidos pela sua característica de latência que tornam seus vírus praticamente invisíveis para o sistema imune do hospedeiro. Durante o período de latência há uma produção muito baixa de novas partículas virais, dificultando a pesquisa por antígenos virais de modo sistêmico no organismo durante este período, sendo que a replicação pode ocorrer em um processo denominado reativação. No entanto, este movimento é mais abstrato quando estudamos o citomegalovírus (CMV), um β-herpesvirus que tem complexas implicações na imunossenescência [24, 81-83].

1.2.1 Citomegalovírus

O citomegalovírus (CMV) é um herpesvírus humano com maior genoma, com cerca de 240kb podendo codificar aproximadamente 160 proteínas [84]. O CMV tem alta prevalência em todo o mundo e a sua soro conversão pode ocorrer em qualquer idade, no entanto, o índice de infecção pode aumentar em 70% após os 65 anos de idade, mesmo que na maior parte dos casos a infecção tenha sido adquirida ainda na infância, variando muito em relação a condições socioeconômicas, localização geográfica e [60, 85, 86]. Após a infecção primária, o vírus entra em um estado de

aparente latência no hospedeiro, no entanto o vírus permanece interagindo com o sistema imune do hospedeiro através de complexos mecanismos que desenvolvem um balanço entre a infecção viral e o sistema imune ausente de sinais clínicos. Balanço este que pode ser desfeito caso o sistema imune fique comprometido (**Figura 2**). O vírus é capaz de infectar uma ampla gama de tecidos e fluidos sendo altamente específico para a espécie humana, possuindo uma alta adaptação evolutiva ao organismo humano que pode ser vista pela alta quantidade de genes que não estão diretamente envolvidos com a replicação viral, mas sim com as interações com o hospedeiro humano [87-90].

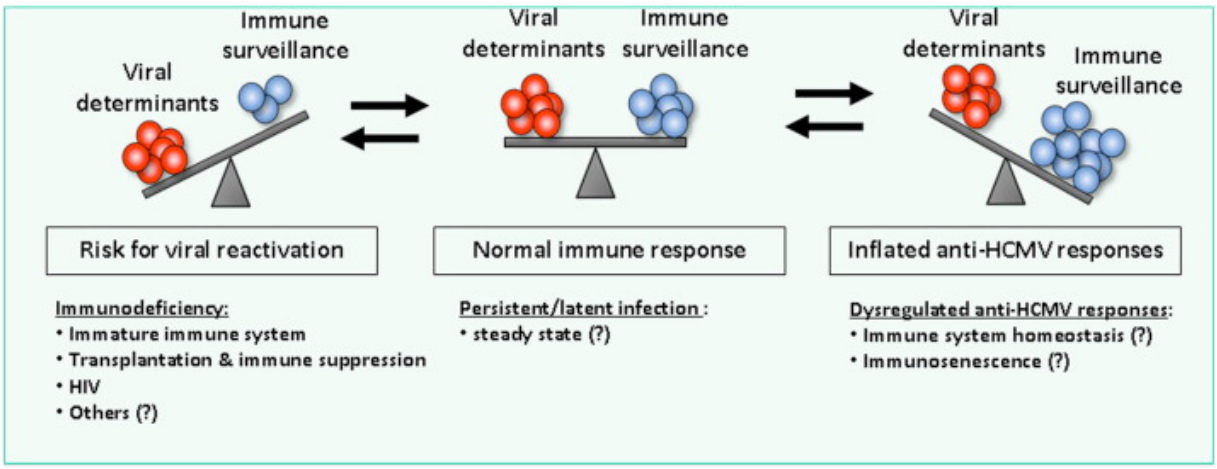

Figura 2. Interação do CMV com o hospedeiro (adaptado de [89]).

Como foi dito anteriormente, existe uma associação entre o IRP e a infecção pelo CMV, que pode ser resumida pela presença da expansão de células CD8+CD28-, e a inversão da razão CD4:CD8 [69]. Nos últimos anos, novos estudos indicam que a maior diferença entre sujeitos idosos do estudo OCTO/NONA no IRP foi no acúmulo de células CD8+ com diferenciação tardia (definidas pela falta dos receptores CD27 e CD28) [91]. Outros estudos recentes indicam uma relação entre

a infecção pelo CMV e um pior status funcional em idosos [52, 75].

Estudos em células T CD8+ reconhecedoras de tetrâmeros (reagentes fluorescentes que coram apenas as células que carregam receptores antigênicos específicos para um epítopo) ligantes de HLA-A2/pp65 (495–503) demonstraram que essas células estão aumentadas em idosos, e que a proporção dessas células que co-expressam CD27 e CD28 (marcadores de ativação funcional e co-estimulação) está muito diminuída nos idosos comparados com jovens [92]. No caso mais dramático, cerca de 30% das células T CD8+ de um indivíduo eram específicas para um epítopo de CMV. Nos jovens, uma proporção significativa de células T CD8+ específicas para CMV é naïve (CCR7+CD45RA+), ou seja, nunca experimentou contato com antígeno. Mas em indivíduos idosos, essas células possuem fenótipo efetor associado com memória (CCR7-CD45RA^{low} ou CCR7-CD45RA+). Existe claramente uma expansão associada com a idade de células de memória que não expressam CD27, CD28, CCR7, ou CD45RA [93]. A ação do CMV afeta inclusive as células T-γδ (gama-delta) no processo da imunossenescência de forma semelhante às células CD8, as células T-γδ contribuem muito no controle da viremia, mas a sua função é severamente afetada com a idade [94, 95].

Em relação à integridade funcional das células anti-CMV expandidas com a idade, foi observado que o numero das células funcionais (respondem ao desafio com peptídeo viral *in vitro* fazendo IFN-γ) é semelhante em idosos e jovens [96]. Contudo, o número dessas células específicas que não são funcionais, ou seja, respondem a desafio com mitógeno *in vitro,* mas não a peptídeos de CMV, é altamente elevado nos idosos [97]. Essas células expressam o receptor *killer cell lectin-like receptor G-1* (KLRG1+), um marcador de diferenciação e resistência à apoptose [98]. Essas células então proliferam em detrimento de células funcionais,

contribuindo para o perfil generalizadamente inflamatório descrito em idosos, resultando na incapacidade do indivíduo de montar respostas policlonais adequadas à medida que envelhece.

O estudo de Elzen et al mostra como o simples acompanhamento clínico e sorológico pode não mostrar com clareza o papel do CMV na imunidade [99], de modo que estudos clínicos combinados à pesquisa a nível celular podem obter resultados mais conclusivos. No entanto, cada vez mais dados demonstram que títulos elevados de anticorpos anti-CMV têm forte relação com a imunosenescência [100, 101].

Estudos recentes indicam que existe uma relação entre o estresse oxidativo e a infecção pelo citomegalovírus. Jaganjac et al. sugere que condições de estresse associadas à peroxidação lipídica podem levar a reativação do CMV [102].

1.3 Estresse Oxidativo

A oxidação é uma parte crítica do metabolismo e os radicais livres são produzidos naturalmente por vários tecidos, incluindo o sistema imune. Eles participam da imunidade celular induzindo inflamação, controlando infecções agudas e crônicas. Os radicais livres incluem espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS). Como podemos observar na figura 3, sob condições fisiológicas normais, existe um equilíbrio entre a geração de radicais de oxigénio livre e defesas antioxidantes. O desequilíbrio entre a produção de ROS e defesas antioxidantes em favor do primeiro é conhecido como estresse oxidativo (OS). Os radicais livres causam efeitos celulares prejudiciais, tais como a oxidação de proteínas, peroxidação lipídica e danos no DNA [103]. Baixa concentração de

ROS leva a sinalização inadequada e, portanto, baixa ativação e proliferação. São necessárias condições ótimas de ROS para que ocorra uma ativação adequada de células T (**Figura 3**). Então um aumento da concentração de ROS leva a uma resposta de células T, incluindo a ativação de TCR e a produção de citocinas. No entanto, um grande aumento da concentração de ROS pode levar ao aumento da apoptose de células T, como resultado de danos no DNA e ativação de p53 induzida por genes e FasL [104].

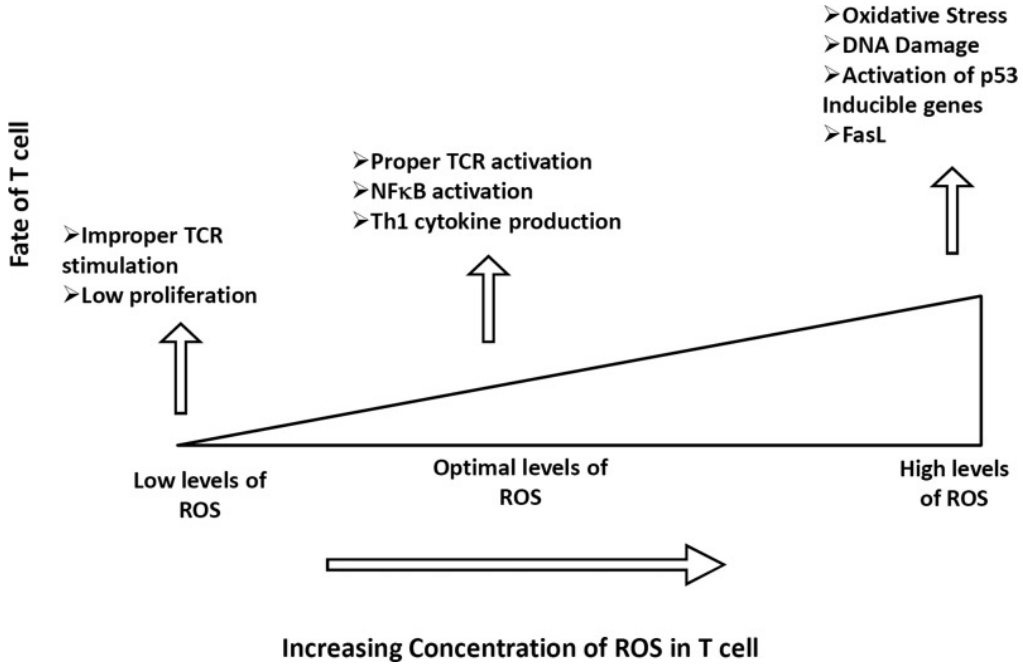

Figura 3. Destino dos Linfócitos T em resposta aos diferentes níveis de ROS (Adaptado de [104]).

O dano oxidativo pode ser estimado pela análise de produtos de oxidação de proteínas e produtos de peroxidação de lípidos circulantes. Estas alterações de proteínas pode resultar em inibição da função de várias proteínas celulares, em alguns casos, de forma permanente. Os produtos proteicos de oxidação avançada (AOPP) são marcadores de dano proteico pertencentes a uma família de compostos oxidados, proteínas reticuladas contendo di-tirosina formados pela reação de

proteínas plasmáticas com oxidantes clorados e são produzidos devido a maior libertação de mieloperoxidase (MPO) de fagócitos ativados, no entanto, as proteínas sensíveis a modificação oxidativa podem também ser adicionadas a este marcador [105-107].

A peroxidação de lípidos pode resultar em danos para a membrana celular, devido à elevada concentração de lípidos presentes. Além disso, os produtos finais da peroxidação lipídica podem ser tanto mutagênicos quanto carcinogênicos, e desempenham um papel importante no envelhecimento e na progressão de diferentes doenças. As substâncias reativas ao ácido tiobarbitúrico (TBARS) são marcadores de peroxidação lipídica, que embora já tenha sido avaliado em relação ao envelhecimento, os resultados são inconclusivos [108]. Diferentes ensaios são usados para determinar TBARS em amostras biológicas, que permite avaliar esta reação de peroxidação lipídica através da análise de alguns produtos como o malondialdeído (MDA). Produtos de peroxidação lipídica podem modificar as propriedades de membranas celulares através da inserção de grupos polares em moléculas de fosfolípido localizados no interior da bicamada lipídica [107, 109].

Para proteger o organismo contra os efeitos deletérios da oxidação, o organismo desenvolveu o mecanismo de defesa antioxidante que mantém o equilíbrio redox, mantendo assim a homeostase celular. São diversas as moléculas antioxidantes já identificadas, e elas desempenham um papel de extrema importância na regulação da função imune e obviamente, se existe algum desequilíbrio deste mecanismo de defesa antioxidante, a função celular será alterada [104]. A análise da atividade antioxidante total pelo método de redução do ferro (FRAP) é um ensaio que permite a avaliação do "poder antioxidante" do

indivíduo, permitindo a investigação de potencial oxidativo e o poder antioxidante [110]. Durante o envelhecimento, no entanto, existe uma clara acumulação de radicais livres e danos associados em vários tecidos [111-113] e isto constitui a base para a teoria dos radicais livres no envelhecimento. Além disso, o aumento do estresse oxidativo contribui para a patogênese de muitas doenças relacionadas à idade [109, 114-116].

As relações entre o estresse oxidativo e fatores de risco imunológicos são em grande parte desconhecidas. Trabalhamos aqui com a hipótese de que o estresse oxidativo pode ter um impacto importante para a imunossenescência, incluindo a inversão da razão CD4:CD8 e o aumento da sorologia para CMV. Esta hipótese é apoiada por estudos anteriores que associaram o estresse oxidativo com o estado inflamatório [117-120] bem como com a reativação do CMV [102]. Além disso, o aumento dos níveis de marcadores de estresse oxidativo foram associadas com a infecção latente por CMV indicando a possibilidade do estresse oxidativo regular a replicação viral [121, 122].

2 OBJETIVOS

2.1 OBJETIVO GERAL

Compreender melhor a relação entre o perfil de risco imunológico, marcadores inflamatórios, metabolismo oxidativo e o perfil antioxidante em idosos

2.2 OBJETIVOS ESPECÍFICOS

1. Identificar sujeitos idosos com um perfil de risco imunológico.
2. Analisar PCR, IL-6, TBARS, AOPP e FRAP plasmáticos;
3. Relacionar a razão CD4:CD8 invertida com PCR e IL-6.
4. Relacionar a razão CD4:CD8 invertida com o metabolismo oxidativo, perfil antioxidante e marcadores bioquímicos.
5. Analisar fatores preditivos para a razão CD4:CD8 invertida.

3 HIPÓTESES

1. Uma pequena parcela dos idosos tem a razão CD4:CD8 invertida.

2. Os idosos com a razão CD4:CD8 invertida apresentam perfis inflamatório e oxidativo acentuados.

3. A oxidação proteica e a peroxidação lipídica são potenciais marcadores do perfil de risco imunológico.

4. O estresse oxidativo está relacionado com os níveis elevados de anticorpos IgG para CMV.

4 CAPÍTULO 4: ARTIGO CIENTÍFICO #1

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The inverted CD4:CD8 ratio is associated with gender-related changes in oxidative stress during aging

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ABSTRACT

Aging has been associated with increased generation of free radicals as well as immunosenescence. Previous studies have identified older individuals with inverted T CD4:CD8 cell ratio and increased immunity to cytomegalovirus (CMV). Here, we investigated markers of oxidative stress and antioxidant defences in older individuals with inverted CD4:CD8 T-cell ratio. Sixty-one subjects were identified with inverted CD4:CD8 ratio. Older individuals with a CD4:CD8 ratio <1 had increased levels of plasma advanced oxidation protein products (AOPP) and ferric reducing ability of plasma (FRAP), but reduced levels of thiobarbituric acid reactive substances (TBARS) as compared to subjects with normal CD4:CD8 ratio. The CMV IgG serology was negatively correlated with CD4:CD8 ratio. These markers were more evident among elderly men than women. Our data suggest a close relationship between chronic CMV infection and oxidative profile in older individuals in the midst of its influence on the peripheral T-cell subsets.

1. Introduction

Aging has been associated with increased generation of free radicals and remodeling changes of the immune system (immunosenescence). Of note, previous prospective studies have identified older individuals with compromised immune system and increased morbidity/mortality rates. Indeed, significant higher mortality rates were observed among elderly Swedes with inverted CD4:CD8 ratio and a low nonspecific T proliferation regardless of their health status [1–5]. The CD4:CD8 ratio in healthy adults is about 2:1 and the inversion of this index has been associated with features of premature immunosenescence across different age groups, including children and adults with HIV [6,7], in patients with acute myocardial infarction [6], as well as following physical and psychosocial stressors [8,9]. The prevalence of the inverted CD4:CD8 ratio is around 8% in adults (20–59 years old) and increases to 16% in people aged 60–94 years old [2]. Adults with inverted CD4:CD8 ratio show several remodeling enumerative and functional immune changes resembling premature senescence. For instance, this immune phenotype was associated with fewer B cells (CD19+), expansion of late-differentiated or senescent T cells (CD8 + CD28−), important expansions of CD8 + T cells against cytomegalovirus (CMV), as well as higher CMV seropositivity [10–12]. These results suggest that inverted CD4:CD8 is an immune risk factor in almost any age and could be associated with different pathologies, but was particularly associated with premature immunosenescence and persistent viral infections.

Oxidation is a critical part of the metabolism and the free radicals are naturally produced by several tissues, including the immune system. They participate in the cellular immunity to infectious diseases (e.g. inflammation), controlling both acute and chronic diseases. The free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under normal physiological conditions, there is a balance between the generation of oxygen free radicals and antioxidant defenses. The imbalance between the production of ROS and antioxidant defences in favor of the first is known by oxidative stress (OS). Free radicals cause detrimental cellular effects such as protein oxidation, lipid peroxidation and DNA damage [13]. The oxidative damage may be estimated by the analysis of circulating protein oxidation products and lipid peroxidation products. The analysis of plasma advanced

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oxidation protein products (AOPP) are reliable markers of protein damage and are produced to higher release of myeloperoxidase (MPO) of activated phagocytes. However, proteins susceptible to oxidative modification may also add up to this marker [14]. The thiobarbituric acid reactive substances (TBARS) are standard markers of lipid peroxidation. Previous studies have investigated TBARS during aging, but until now the results are inconclusive [15]. The primary antioxidant system involves coordinated effects induced by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The ferric reducing ability of plasma (FRAP) assay allows the evaluation of the "antioxidant power" of the individual, allowing the investigation of oxidative potential and the antioxidant power [16]. During aging, however, there is a clear accumulation of free radicals and associated damage in various tissues [17–19] – and this constitutes the basis for the free radical theory of aging. In addition, enhanced oxidative stress (OS) contributes to the pathogenesis of many age-related diseases [20–23].

The relationships between OS and immunological risk factors are largely unknown. We hypothesized here that the OS can be particularly associated with immunosenescence phenotype, including the inverted CD4:CD8 ratio and increased CMV serology. This hypothesis is supported by previous studies that have associated OS with the inflammatory status [24–27] as well as with CMV reactivation [28]. Furthermore, the OS can regulate viral replication and increased levels of oxidant markers were associated with latent CMV infection [29,30].

The aim of the present study was to analyze the association between markers of OS and antioxidant defences with CMV serology and CD4:CD8 ratio of community-dwelling healthy older individuals.

2. Materials and methods

2.1. Subjects

We recruited 421 community-dwelling older individuals (>60 years) by random home visits in eight different districts of Porto Alegre, Brazil. Individuals over 60 years old are considered elderly in Brazil. All were non-institutionalized, with cognitive ability. Recruitment was coordinated by the Institute of Geriatrics and Gerontology (São Lucas Hospital), Pontifical Catholic University of Rio Grande do Sul (PUCRS), with the assistance of teams of the Family Health System Health Strategy. Patients with serology positive for HIV and autoimmune diseases were excluded. All subjects signed informed consent and this study protocol, was approved by the institutional review board of the PUCRS (IRB# 10/04967).

2.2. Blood samples and biochemical analyses

Blood samples were collected after a 12 h overnight fasting by venous puncture into tubes without anticoagulant. Specimens were centrifuged for 15 min at 2500×*g*, and aliquots of serum samples were stored at −20 °C. Biochemical examinations of total cholesterol, HDL, LDL, triglycerides and fasting glucose, venous blood samples were examined. The biochemical assays were performed with serum of the elderly patients by spectrophotometry in the semi-automated biochemical analyzer TP Analyzer Basic – Thermo Plate.

The biochemical tests were carried out with Labtest® kits (Lagoa Santa-MG, Brazil). The total cholesterol (kit #4006), triglycerides (kit #3009), and glucose (kit #3004) levels were analyzed by enzymatic systems with endpoint reaction. Cholesterol HDL was analyzed by selective precipitation of the low and very low density (LDL and VLDL) lipoproteins by endpoint reaction – reference values: HDL Low = <40 mg/dl, High (Desirable) = >60. The reference values for total cholesterol are: desirable = <200 mg/dl, high threshold = 200–239 mg/dl, High = >240 mg/dl. The references values for triglycerides are: Desirable = <150 mg/dl, high threshold = 150–199 mg/dl, High = 200–499 mg/dl, Very High = >500 mg/dl. The reference values for glucose levels are: 65–99 mg/dL. LDL was determined by the Friedewald equation for individuals with TG < 400 mg/dL [31].

2.3. Oxidative stress markers

Plasma advanced oxidation protein products (AOPP) levels were measured by Cobas Mira® (Roche Diagnostics, Basel, Switzerland) according method described by Hanasand et al. [32]. Results were calculated by the standard curve as the equivalent of chloramine and are shown as μmol/L. Few studies have standardized this variable, and normal range in adults aged 18–60 years varied from 98.5 ± 38.6 [33] to 82.9 ± 26.7 [34] and 74.6 ± 29.1 for young adults [35].

Thiobarbituric acid reactive substances (TBARS) were measured by a spectrophotometric method described by Janero [36]. The ZENITH study investigated OS markers in non-institutionalized older adults [37]. They reported average TBARS levels of 2.65 ± 0.43 μmol/L (76–95 years) and 2.77 ± 0.38 μmol/L (60–75 years). However, other studies found different levels [38], making it difficult to be compared between different laboratories.

2.4. Antioxidant activity

Ferric reducing ability of plasma (FRAP) levels were measured in the Cobas Mira® (Roche Diagnostics, Basel, Switzerland) according Benzie and Strain [16]. Normal FRAP levels varied from 591 ± 244 μmol/L (76–95 years) and 1274 ± 178 μmol/L (60–75 years)[37]; another study reported an average of 1017 ± 206 μmol/L for Chinese adults aged 21–74 years old [16].

2.5. Peripheral inflammatory markers

IL-6 (Kit 225; reference values: Desirable = <3,4 pg/mL) and ultra sensitivity C-reactive Protein (CRP-us; Kit 313; reference values: low cardiovascular risk = <0.1 mg/dL; average risk = 0.1–0.3 mg/dL; high risk = >0.3 mg/dL) was measured by chemoluminescence immunoassay methods according to the manufacturer's instructions (IMMULITE®/IMMULITE® 1000, Siemens, Eschborn, Germany).

2.6. Immunophenotyping of lymphocyte subpopulations

Ten milliliters of peripheral blood were collected by venipuncture (8–10 A.M.) and stored in EDTA tubes. The CD4 + and CD8 + T cells were identified by four-color flow cytometry (MultiTEST, BD Biosciences, San Jose, CA, USA) as previously described. [3] Briefly, a stain/erythrocyte-lyse/no-wash procedure was used, and this protocol was optimized for use with MultiTEST reagents and with TruCOUNT Tubes to perform absolute counts, as reported by Nemes et al. [39]. Fifty microlitres of anticoagulated whole blood was added into tubes containing absolute counting beads (TruCOUNT tubes, BD Biosciences, San Jose, CA, USA). Twenty microlitres of the BD MultiTEST CD3 FITC (clone SK7)/CD8 PE (clone SK1)/CD45 PerCP (clone 2D1)/CD4 APC (clone SK3) cocktail reagent was added (BD Biosciences, San Jose, CA, USA) and incubated for 15 min in the dark at room temperature (R.T.). Erythrocytes were briefly lysed and samples were immediately acquired in a multi-color flow cytometer (BD FACSCanto II, from BD Biosciences, San Jose, CA, USA). Semi-automatic acquisition and analysis were performed with the BD FACSCanto Clinical

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Software v2.4 (BD Biosciences, San Jose, CA, USA) and collection criteria included 30,000 total events composed of at least 10,000 lymphocytes identified through gating on the CD45 high/SSC low events as recommended by the Centers for Disease Control and Prevention (CDC) guidelines.

2.7. CMV serology

Aliquots of peripheral blood were collected without anticoagulant in order to assess serum CMV-IgM and CMV-IgG using IBL reagents (Cat.# RE57061, International, Hamburg, Germany) by the Basic Radim Immunoassay Operator automated equipment (BRIO, from Radim Diagnostics, Pomezia, RM, Italy). The optical densities (570/620 nm) were estimated in an ELISA plate reader. Samples were considered positive (reactive) for CMV when the values were above the cut off value (>0.4 IU/mL).

2.8. Statistical analysis

All variables were tested for homogeneity of variances and normality of the distribution by means of Levene and Kolmogorov–Smirnov tests, respectively. When data normalization was not possible, the nonparametric tests were performed (Mann–Whitney U test, and Spearman correlation). Statistical interactions between categorical variables and group were compared by means of the χ^2 test. Interrelationships between variables were analyzed by Spearman correlation tests. A logistic regression model (method: enter) was used to analyze AOPP and CMV-IgG levels as predictors of subjects with inverted CD4:CD8 ratio. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) 19.0 software (SPSS Inc., Chicago, Ill., USA). The significance level was set at $\alpha = 0.05$ (two tailed).

3. Results

3.1. Characteristics of the studied population

The subjects were aged between 60–95 years (mean 68.67 ± 6.96 years), 63.9% were female, with low education and low-income, with an average wage of $307.04 monthly. Sixty-one older individuals (38 women and 23 men) were identified with inverted CD4:CD8 ratio with mean age of 68.15 ± 6.57 years (60–87 years) and 360 individuals with normal CD4:CD8 ratio (231 women and 129 men), with mean age of 68.76 ± 7.03 years (60–95 years). Both groups were homogenous regarding socio-demographic and laboratory characteristics, including: age ($p = 0.59$), gender (females in CD4:CD8 > 1 = 64.17%; CD4:CD8 < 1 group = 62.3%), ethnicity (Caucasian represents 50% of CD4:CD8 > group and 52.38% in CD4:CD8 < 1), income, education, smoking and drinking habits (alcohol), dyslipidemic profile, body mass index (BMI in CD4:CD8 > 1 = 28.29 ± 5.14; BMI in CD4:CD8 < 1 = 27.92 ± 5.73), and medication: 34.62% of CD4:CD8 > 1 and 37.5% of CD4:CD8 < 1 used antidiabetic drugs; 50% of CD4:CD8 > 1 and 50% of CD4:CD8 < 1 used anti-inflammatory drugs; 23.08% of CD4:CD8 > 1 and 37.5% of CD4:CD8 < 1 used anti-lipemic agents (e.g. statins). However, both groups had similar clinical conditions in this study, including diabetes mellitus, metabolic syndrome, heart diseases, cerebrovascular diseases, thyroid dysfunction, depression, chronic obstructive lung disease, renal diseases, gastrointestinal diseases, neurodegenerative diseases, infectious diseases, or special needs (all N.S.). These findings are in line with previous published data of this cohort [3].

3.2. CMV serology

As previously reported in a previous study, including the same cohort [3], the older individuals with inverted CD4:CD8 ratio had higher levels CMV-IgG (0.24 to 8.31 IU/mL, mean = 4.18 IU/mL) than subjects with CD4: CD8 ratio > 1 (0.23–9.20 IU/ml, mean = 2.66 IU/ml), $p < 0.001$. These results are in agreement with previous studies that have associated CMV with immune risk profile [4,40,41]. The absence of acute CMV primary infection may be suggested by the absence of reactivity for CMV-IgM.

3.3. Biochemical measures and inflammatory markers

Here, a panel of biochemical and inflammatory markers was analyzed. However, no significant biochemical differences were found between the studied groups (Table 1). Also, CRP-us and IL-6 did not vary between the studied groups (Table 1).

3.4. Lipid and protein oxidation and antioxidant activity

The OS was estimated by subproducts of protein and lipid oxidation (AOPP and TBARS) and the antioxidant activity was analyzed by FRAP. The individuals with CD4:CD8 < 1 showed higher AOPP and FRAP levels than individuals with CD4:CD8 > 1 ($p = 0.029$ and $p = 0.005$) (Table 2). However, subjects with inverted CD4:CD8 ratio had lower levels of TBARS (Table 2).

Interestingly, some gender-related differences were found. Men of the CD4:CD8 < 1 group had higher levels of FRAP ($p = 0.006$) and AOPP ($p = 0.043$) than the CD4:CD8 > 1 group. In contrast, women of the CD4:CD8 < 1 group had lower levels of TBARS ($p = 0.002$) compared to women of the CD4:CD8 > 1 group (Fig. 1).

3.5. Correlations between CMV, redox state and CD4:CD8 ratio

We investigated the relationships between CD4:CD8 ratio, oxidative stress, inflammatory markers and CMV serology in all individuals, without stratification by CD4:CD8 ratio. Only CMV-IgG levels (Spearman correlation (r_s) = −0.26, $p = 0.002$), AOPP levels ($r_s = -0.11$, $p = 0.05$) and LDL levels ($r_s = -0.154$, $p = 0.036$) were found negatively correlated with CD4:CD8 ratio. Triglyceride levels ($r_s = 0.176$, $p = 0.015$) were positively correlated with CD4:CD8 ratio. LDL and Triglycerides were correlated with AOPP ($r_s = 0.288$, $p < 0.001$ and $r_s = 0.161$, $p = 0.005$; respectively).

To further explore the role of independent variables studied, we performed a multivariate logistic regression model entering LDL, Triglycerides, AOPP and CMV serology as predictors of inverted CD4:CD8 ratio. This analysis revealed that biochemical markers had no effects on the inverted CD4:CD8 ratio However, the OR of AOPP levels was 1.01 (95% CI: 1.004 – 1.023, $p = 0.007$) and the OR of CMV serology was 1.31 (95% CI: 1.111 – 1.553, $p = 0.001$).

Table 1
Biochemical measurements, inflammatory markers and T-cell phenotyping.

Biochemical measures	CD4:CD8 < 1		CD4:CD8 > 1		p
	Mean	SE	Mean	SE	
Glucose (mg/dl)	158.38	8.2	144.92	3.26	0.126
Triglycerides (mg/dl)	134.92	9.22	147.09	4.76	0.140
Total cholesterol (mg/dl)	167.35	10.62	168.64	3.99	0.712
HDL (mg/dl)	49.85	2.08	52.21	1.06	0.733
LDL (mg/dl)	115.36	5.86	108.29	2.61	0.115
Insulin (mUI/mL)	15.04	1.71	16.98	0.96	0.616
CRP (mg/dl)	0.38	0.09	0.44	0.03	0.206
IL-6 (pg/ml)	3.14	0.39	3.62	0.2	0.267
CD3 + T cells	2575.33	181.97	2367.78	63.26	0.23
CD3 + CD4 + T cells	1091.57	587.89	1555.00	825.06	<0.0001
CD3 + CD8 + T cells	1480.43	897.98	820.47	474.45	<0.0001

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Table 2
Plasma oxidative stress markers and anti-oxidant defences.

Biomarkers	CD4:CD8 < 1		CD4:CD8 > 1		p
	Mean	SE	Mean	SE	
FRAP	1068.85	89.48	854.18	33.19	0.005
AOPP	110.25	7.34	99.51	3.76	0.029
TBARS	1.51	0.11	1.77	0.08	0.022

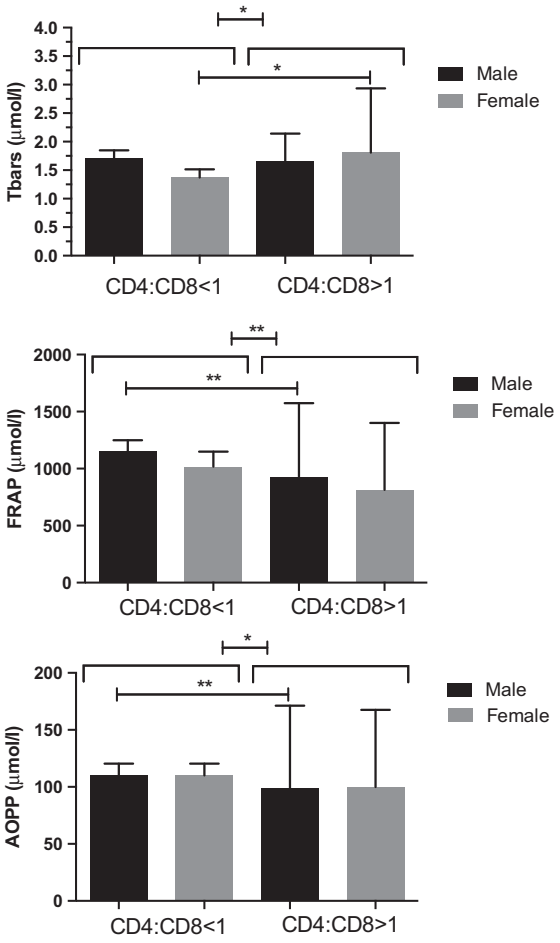

Fig. 1. Circulating oxidant markers (AOPP, TBARS) and antioxidant defences (FRAP) accordingly to gender. Statistically significant differences are indicated: $^{*}p < 0.05$ and $^{**}p < 0.005$.

4. Discussion

To the best of our knowledge, this is the first study that investigated the complex relationships between redox state, CMV serology and CD4:CD8 ratio in older individuals. Two parameters of the immune risk profile (IRP) were incorporated in our study: CD4:CD8 T-cell ratio and CMV serology. However, the IRP definition was not used here because subjects were younger and the cross-sectional design precluded data interpretation of morbidity/mortality status as previously reported in longitudinal studies [4,42]. In addition, our study did not include mortality data. Recent studies estimate that 50% of older individuals with inverted CD4:CD8 ratio had more age-related illnesses [43,44]. In addition, several studies suggested that both the OS and inflammation are underlying the etiology of most age-related diseases [45–47].

The potential effects of OS in peripheral CD4 and CD8 T cells were analyzed. Healthy older individuals with inverted CD4:CD8 ratio showed increased OS, as shown by higher levels of protein oxidation (AOPP). In contrast, FRAP levels were increased in subjects with inverted CD4:CD8 ratio. We believe that increased antioxidant FRAP levels during aging may represent a negative feedback loop promoting the homeostasis between OS/antioxidant defenses. In addition, both groups had similar IL-6 and CRP levels, contrasting to previous studies [48,49]. Previous studies indicate that the CD4:CD8 ratio increased with aging, and was associated with OS and inflammatory markers (e.g. CRP and IL-6) [50–53]. Similarly, previous studies indicate that the CMV seropositivity was strongly correlated to inverted CD4:CD8 ratio, and the amount of IgG antibodies to CMV specifically influenced this inversion [1–4,54].

We have recently reported the same cohort with the inverted phenotype had similar proportions of major lymphocyte subpopulations (T, B, NK, naïve and memory senescent cells) as compared to older individuals with CD4:CD8 ratio > 1 [3]. These are positive results for the Brazilian older individuals with inverted CD4:CD8 ratio and contrast to previous studies that defined "IRP" in octa- and nonagenarians. Indeed, previous older adults with "IRP" were characterized with increased proportions of effector/memory T CD8 + cells, low B cells (CD19+), increased NK cells and T CD8 + CD28-cells, and higher plasma IL-6 levels during aging [10,55–57]. In accordance to previous work [58], most older individuals (~90%) were seropositive for CMV and subjects with inverted CD4:CD8 ratio had the highest IgG levels to CMV. In addition, the CMV-IgG levels were inversely correlated to CD4:CD8 ratio. Taken together these data support the current knowledge that CMV may drive expansions of T CD8 + cells [59], leading to the inversion of the CD4:CD8 ratio, and would thus be involved with age-related immune alterations [12,60,61]. However, the current study design (cross-sectional) precludes data interpretation concerning the impact of CMV on age-related diseases or mortality of this population. Strindhall et al. have recently shown that hexagenarians had similar CD4:CD8 ratios (and prevalence of inverted phenotype) than individuals with 70 or 80 years old [2]. Future longitudinal studies are necessary to explore the impact of CMV on age-related morbidity development of hexagenarians.

Here, both AOPP and IgG-CMV serology were found negatively correlated with the CD4:CD8 ratio. These findings are in partially agreement with previous studies in adults [29,30,62], showing that increased levels of OS were associated with CMV infection [29]. These studies have also indicated that OS may importantly drive the CMV reactivation and replication [28,30]. This is a very important observation associated with the process of immunosenescence, since the inversion of the CD4:CD8 ratio is much more related to the immune response to the virus rather than aging per se. One mechanism by which the virus may be reactivated and survive the OS is the virus-induced upregulation of Nrf2 (NF-E2 related factor 2), a transcription factor involved in cellular defense protecting the cell against OS [62]. It is very important to remember that the positive IgG CMV serology is a key marker of the theory of the immune risk profile, and more recent studies not only demonstrate the importance of increased CMV serology, but also increase its importance as the main factor for the inversion of the CD4: CD8 ratio [63].

In addition, some gender-related differences were found in this study. Although FRAP and AOPP were found higher only in men of CD4:CD8 < 1 group, females of the inverted phenotype had lower levels of TBARS. Different studies have indicated gender differences in OS profile, including higher antioxidant defences in men, but there is no consensus in this field [64,65]. Age-related increase in production of ROS has been related to the loss of muscle tissue that occurs mainly among men (sarcopenia) and has also been

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associated with increased IL-6 and CRP [66–68]. This loss of muscle tissue is directly related to protein oxidation, both of which are linked with increased physical frailty of the elderly. The protein oxidation occur in both sexes, however, it is not statistically significant among women; unlike the lipid peroxidation that strongly increases among women in the postmenopausal period, but has no relationship to the loss of function [69,70]. Previous work from our group reported the relationship between physical frailty, the inverted CD4:CD8 profile and serology for CMV [3]. Higher levels of AOPP in males further highlight these data, considering AOPP a marker of protein oxidation associated to physical activity. The antioxidant activity may be also activated to restrain (i.e. counter regulate) the production of ROS, explaining the increase of AOPP and FRAP in men. However, data on diet and physical activity were not available here and future studies should include them.

5. Conclusion

The inverted CD4:CD8 ratio phenotype was identified in 14.5% of community-dwelling older individuals in a developing country. This immune phenotype was not associated with increased morbidity. Our data further confirm the impact of CMV on circulating T cells and indicate novel gender-related changes in OS profile during aging.

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5 CONCLUSÕES E CONSIDERAÇÕES FINAIS

As pesquisas na área de imunossenescência são de extrema importância para o melhor entendimento dos mecanismos de adoecimento do idoso, já que as alterações imunes impactam diretamente nas diferentes possibilidades de prevenção e tratamento das patologias mais recorrentes no envelhecimento. Estudos realizados principalmente na última década apontam para o aumento da prevalência e da severidade das doenças infecciosas em idosos associadas a inúmeras disfunções na resposta imune.

Este foi o primeiro estudo que investigou as relações complexas entre o estado redox, a sorologia para o CMV e a razão CD4:CD8 em idosos não institucionalizados. Alguns estudos prévios analisaram o envolvimento do estresse oxidativo com a infecção do CMV [122, 123]. Mas neste estudo, buscamos compreender um pouco mais sobre como estas variáveis tão diferentes podem estar relacionadas. O trabalho de 2011 de Guo e cols.[124] foi pioneiro na compreensão da complexa interação entre inflamação, estresse oxidativo e a razão CD4:CD8, mas apenas dentro do contexto da asma sob o viés de compreender a influência de minerais como zinco, cobre e selênio.

Dois parâmetros do perfil de risco imunológico (IRP) foram incorporadas em nosso estudo: a razão de células T CD4:CD8 e a sorologia para CMV. No entanto, a definição IRP não foi usada no artigo porque a média de idade do nosso estudo esteve abaixo dos 70 anos (os estudos que padronizaram este conceito de IRP estudaram idosos com mais de 80 anos) e porque o delineamento transversal do nosso estudo impede a interpretação do estado de morbidade/mortalidade, essencial para o estudo de IRP [125, 126]. Estudos recentes estimam que 50% dos idosos

com a inversão da razão CD4:CD8 teve mais doenças relacionadas com a idade [127, 128]. Além disso, vários estudos sugeriram que tanto o estresse oxidativo como a inflamação estão relacionados a etiologia da maioria das doenças relacionadas com a idade [129-131].

O processo oxidativo faz parte do metabolismo e o estresse oxidativo ocorre quando existe um desbalanço entre a produção de espécies reativas de oxigênio (ROS) e o sistema antioxidante. Não apenas ROS, mas também as espécies reativas de nitrogênio (RNS) são produzidas naturalmente pelo sistema imune, participando da imunidade celular induzindo inflamação, controlando infecções agudas e crônicas[132]. Foram analisados os efeitos potenciais do estresse oxidativo em células T CD4 e CD8 periféricas. Idosos com a razão CD4:CD8 invertida apresentaram aumento do estresse oxidativo, mostrado por níveis mais elevados de oxidação de proteínas (AOPP). Em contraste, encontramos os níveis de FRAP aumentados em indivíduos com razão de células T CD4:CD8 igualmente invertida. Acreditamos que a variável antioxidante (FRAP) acompanha a AOPP na sua elevação, mantendo o equilíbrio redox dos indivíduos envolvidos no estudo, e sob esta ótica, este equilíbrio a ausência de significância quando analisamos o marcador de lipoperoxidação TBARS quando comparamos os grupos com razão CD4:CD8 maior e menor que 1.

Além disso, em ambos os grupos (razão CD4:CD8 > 1 e razão CD4:CD8 < 1) encontramos níveis semelhantes de IL-6 e PCR, contrastando com estudos anteriores que indicaram uma relação direta entre o aumento do perfil inflamatório com o envelhecimento dos indivíduos [42, 133, 134]. Houve inclusive uma relação direta entre a queda da função tímica e o aumento dos níveis de PCR com a mortalidade [134], a relação equilíbrio/desequilíbrio redox poderia explicar esta

diferença. Outros estudos mostraram que a razão CD4:CD8 aumenta com o envelhecimento, e foi associada com marcadores inflamatórios (por exemplo, PCR e IL-6) e com o estresse oxidativo; embora esta relação com o estresse oxidativo tenha sido encontrada em um contexto completamente diferente do relatado no nosso estudo. Isso evidencia a necessidade de estudar mais o estresse oxidativo sob a luz da imunossenescência [124, 135-137]. Do mesmo modo, estudos prévios indicam que a soropositividade para CMV foi fortemente correlacionado com a inversão da razão CD4:CD8, e a quantidade de anticorpos IgG para o CMV pode influenciar esta inversão [73, 75, 125, 138, 139].

De acordo com trabalho anterior [140], a maioria dos idosos (~ 90%) eram soropositivos para CMV e indivíduos com a inversão da razão CD4:CD8 apresentaram os maiores níveis de IgG para CMV. Além disso, os níveis de CMV-IgG estavam inversamente correlacionados com CD4:CD8. Tomados em conjunto, estes dados apoiam o conhecimento atual que CMV pode conduzir expansões de células T CD8+ [141], que conduz à inversão do CD4:CD8, estando então envolvido com alterações imunológicas relacionadas com a idade [34, 142, 143]. No entanto, o desenho do estudo atual (transversal) dificulta a interpretação dos dados sobre o impacto da CMV sobre as doenças relacionadas com a idade ou a mortalidade dessa população, apenas um estudo longitudinal seria capaz de analisar os dados sob esta ótica.

Strindhall et al demonstraram recentemente que hexagenários possuem uma razão CD4:CD8 semelhante aos indivíduos com 70 ou 80 anos [138]. Futuros estudos longitudinais são necessários para explorar o impacto da infecção pelo CMV sobre o estresse oxidativo e como esta relação está relacionada com a idade de desenvolvimento de morbidade em hexagenários. No nosso estudo, a razão

CD4:CD8 se mostrou inversamente correlacionada tanto com AOPP quanto com IgG-CMV. Esses achados estão parcialmente de acordo com estudos anteriores em adultos [121-123], mostrando que o aumento dos níveis de estresse oxidativo foram associadas com a infecção por CMV [121]. Esses estudos também indicaram que o estresse oxidativo pode conduzir de forma importante a reativação CMV e replicação [102, 122]. Esta é uma observação importante associado com o processo de imunossenescência, uma vez que a inversão da razão CD4:CD8 é muito mais relacionado com a resposta imune ao vírus, em vez de o envelhecimento. Um mecanismo pelo qual o vírus pode ser reativado e sobreviver ao estresse oxidativo é a supra-regulação de Nrf2 (NF-E2 fator relacionado 2) induzida pelo vírus, um fator de transcrição envolvido na defesa celular contra o estresse oxidativo [123]. Dessa forma, o estresse oxidativo faz parte do processo fisiológico de combate a infecções, ao ponto que o aumento da atividade de ROS afeta negativamente a replicação viral entre elas o CMV, no entanto a modulação da expressão de Nrf2 pelo vírus pode auxiliar a sua reativação, assim como o aumento da peroxidação lipídica [102, 122, 123].

É muito importante lembrar que a presença de anticorpos IgG CMV é um marcador chave da teoria do perfil de risco imunológico, e estudos mais recentes não só demonstraram a importância do aumento da sorologia CMV, mas também a sua importância como o principal fator para o inversão da razão CD4:CD8 [74]. Marttila e cols. sugerem que a presença de altos títulos de anticorpos anti-CMV IgG em idosos está associada ao aumento da fragilidade e da mortalidade [101]. Já o estudo de Vasson e cols.[135], que avaliou populações na França, na Espanha e na Austria, não avaliou a sorologia para CMV mas percebeu que a razão CD4/CD8 esteve positivamente correlacionada com a idade apenas na população austríaca,

enquanto que não houve uma relação direta entre idade e perfil CD4/CD8 entre espanhóis e franceses.

Além disso, algumas diferenças ligadas ao sexo foram encontrados neste estudo. Embora FRAP e AOPP tenham sido encontrados mais elevados apenas em homens do grupo CD4:CD8 < 1, as mulheres do fenótipo invertido tinham menores níveis de TBARS. Diferentes estudos têm indicado diferenças de gênero no perfil de estresse oxidativo, incluindo maiores defesas antioxidantes em homens, mas não há consenso neste assunto [132, 144]. O aumento relacionado com a idade na produção de ROS tem sido relacionado com a perda de tecido muscular que ocorre principalmente entre os homens (sarcopenia) e também tem sido associado com o aumento de IL-6 e PCR [145-147]. Esta perda de tecido muscular está diretamente relacionada com a oxidação proteica, a qual está ligada ao aumento da fragilidade física dos idosos. A oxidação de proteínas ocorre em ambos os sexos, no entanto, não se mostrou estatisticamente significativa entre as mulheres; ao contrário da peroxidação lipídica que aumenta fortemente entre as mulheres no período pós-menopausa, mas não tem nenhuma relação com a perda da função [148, 149]. O nosso grupo apresentou a relação entre a fragilidade física, a razão CD4:CD8 invertida e a sorologia para CMV [75] em um estudo anterior (em anexo).

Níveis mais elevados de AOPP em homens realça ainda mais esses dados, considerando AOPP um marcador de oxidação proteica associado à atividade física. A atividade antioxidante também pode ser ativada para conter a produção de ROS, explicando o aumento da AOPP e FRAP em homens. É interessante relacionar estes dados com trabalhos anteriores que verificaram fatores relacionados, como por exemplo o trabalho de 2004 de Drela e cols.[30] onde consideraram que o exercício moderado pode atenuar alguns aspectos da imunosenescência em

mulheres idosas através do aumento da expressão de IL-2. Já em outra estudo mais recente, Derbré e cols. [148] relata que a inatividade física poderia induzir o estresse oxidativo, sendo um importante viés para o desenvolvimento da sarcopenia. Associado aos dois trabalhos, encontramos também a pesquisa de Rosado-Pérez e cols. [150] sugere que a prática diária de Tai chi diminui o estresse oxidativo em idosos. Dessa forma acreditamos que exista de fato uma relação direta entre a razão CD4:CD8 invertida e o aumento da sorologia para CMV com a fragilidade encontrada nestes idosos associada com a inatividade física e o aumento do estresse oxidativo. No entanto, os dados sobre dieta e atividade física não estavam disponíveis neste estudo, e estudos futuros devem incluí-los para que tenhamos um mapa mais completo sobre estes diferentes fatores.

Verdecia e cols. [151] sugerem que haja uma diferença nos subtipos linfocitários entre homens e mulheres devido ao envelhecimento, com a queda nos números de linfócitos B e T (CD4 e CD8) nas mulheres e o aumento da contagem de células CD8+CD28- nos homens. Já Luz e cols. [75] não observaram diferenças entre homens e mulheres quando foram analisados os subtipos linfocitários. Hirokawa e cols. [152] também estudaram as diferenças entre os gêneros em relação ao envelhecimento do sistema imune, percebendo uma queda no número de células B e T CD4 mais acentuada nos homens, embora também ocorresse entre as mulheres. A grande maioria dos estudos que buscam compreender os mecanismos de desenvolvimento da imunossenescência analisa idosos de ambos os sexos, por vezes sem analisa-los separadamente. Nós acreditamos que seja essencial que existam estudos que analisem o envelhecimento sob a ótica das diferenças entre os sexos masculino e feminino, para que seja possível compara-los e buscar alternativas de prevenção e inclusive tratamentos que amenizem os efeitos da

imunossenescência. O estudo de Tsuboi e cols.[153] é um exemplo, onde eles analisaram apenas mulheres que já passaram pela menopausa, e neste estudo eles perceberam uma relação muito interessante entre a redução da citotoxidade mediada por células NK e sintomas depressivos, além da relação entre o aumento dos números de células NK e o bem-estar. Ao mesmo tempo que Sánchez-Rodríguez e cols.[154] que sugeriram que o estresse oxidativo seja um fator de risco para osteoporose, estando as mulheres mais susceptíveis.

Neste estudo, verificamos diferenças relacionadas ao perfil oxidativo em relação ao gênero. Considero, que todos estes dados devem impulsionar novos estudos longitudinais que busquem compreender como estas diferenças impactam o adoecimento (morbidade) e sobrevivência (mortalidade) do idoso.

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7 Anexo 1: TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TÍTULO DA PESQUISA: ANÁLISE DA PRESENÇA DA INVERSÃO DA RAZÃO CD4:CD8 EM IDOSOS E SEU PERFIL CELULAR E BIOQUÍMICO

Ficha N° _____

JUSTIFICATIVA E OBJETIVOS DA PESQUISA:
Os idosos apresentam grandes chances de desenvolver doenças geralmente comuns do envelhecimento, como o câncer, doenças autoimunes e infecciosas (gripe, pneumonia, infecção urinária) que podem prejudicar o estado de saúde do indivíduo e levar a morte. O objetivo deste estudo é: (1) avaliar o seu estado de saúde geral e (2) Avaliar a resposta imunológica (as defesas do organismo) do indivíduo.

PROCEDIMENTOS A SEREM UTILIZADOS:
O meio que vamos utilizar para realizar este trabalho será através do emprego de entrevistas estruturadas e a coleta de 10 ml de sangue. Não existe nenhum risco de contaminação, pois a seringa e agulha são estéreis e descartáveis. No entanto, você poderá sentir um leve desconforto (enjôo e tontura) devido a coleta de sangue. O material obtido será posteriormente analisado no laboratório.

Eu, ... fui informado dos objetivos das pesquisa acima de maneira clara e detalhada. Recebi informação a respeito da coleta a ser feita e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e modificar minha decisão se assim eu o desejar. O pesquisador responsável certificou-me de que todos os dados desta pesquisa serão confidenciais e terei liberdade de retirar meu consentimento de participação na pesquisa, em face destas informações. Declaro, outrossim, que recebi cópia deste consentimento, de que todos os dados sobre a minha pessoa serão confidenciais e mantidos em sigilo.

_____	_____	_____
Assinatura do voluntário	Nome	Data

_____	_____	_____
Assinatura do pesquisador	Nome	Data

Pesquisador para contato: **Dr. Moisés Evandro Bauer (PUCRS)**

Telefone: 0xx51 33203000 / ramal 2347

8 ANEXO 2: DEMAIS PRODUÇÕES DO DOUTORADO

8.1 Artigo científico #1

NeuroImmunoModulation

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The Inverted CD4:CD8 Ratio Is Associated with Cytomegalovirus, Poor Cognitive and Functional States in Older Adults

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Key Words
Immunosenescence · Cytomegalovirus · Lymphocytes · T cells · CD4:CD8 ratio · Cognitive function

Abstract
Background: Some premature features of immunosenescence have been associated with persistent viral infections and altered populations of T cells. In particular, the inverted T CD4:CD8 ratio has been correlated with increased morbidity and mortality across different age groups. ***Objective:*** Here, we investigated the role of persistent viral infections, cognitive and functional states as predictors of inverted CD4:CD8 ratio of older adults in a developing country. ***Methods:*** Three hundred and sixty community-dwelling older adults (aged 60–103 years) were recruited. Cognitive function was evaluated by the Instrument of Brief Neuropsychological Assessment and Mini-Mental State Examination inventory. Functional Activities Questionnaire was used to determine activities of daily living. Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) serologies were determined by ELISAs. Peripheral blood was assessed for lymphocyte subsets by flow cytometry (CD4+, CD8+, NK, NKT, B and CD8+CD28–). ***Results:*** Fifty-nine individuals were identified with CD4:CD8 ratio <1, and had increased IgG titers to CMV ($p < 0.01$), but not to EBV, compared to subjects with CD4:CD8 ratio >1. The older adults with inverted CD4:CD8 ratio had impairments in some cognitive dimensions and had more functional disability and dependency ($p = 0.01$) than subjects with CD4:CD8 ratio >1. The lymphocyte subsets did not vary between groups. The increased CMV-IgG titers alone contributed to 8× higher chance to invert CD4:CD8 T cell ratio (OR 8.12, 95% CI 1.74–37.88, $p < 0.01$). ***Conclusion:*** Our data further indicate the role of CMV on circulating T cells, poor cognition and functional disability/dependency during aging.

Introduction

Aging of the immune system (immunosenescence) has been characterized by remodeling cellular changes in both innate and adaptive immune responses [1], and associated with increased incidence of upper respiratory in-

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fections, autoimmune disorders, and cancer. Several numerical and functional defects in peripheral T cells have been reported in older adults and importantly associated with increased morbidity/mortality. However, distinct elderly populations can be identified based on their immunological profiles, which can differentially impact the progression of immunosenescence and disease outcomes.

Individuals with inverted CD4:CD8 T cell ratios (i.e. <1) have been identified across different age groups and associated with significant immune impairments. The CD4:CD8 ratio in healthy adults is about 2:1 [2], and the inversion of this index has been associated with early immunosenescence in children and adults with HIV [3]. In addition, a significant drop in CD4:CD8 ratios has also been reported in patients with acute myocardial infarction [2], as well as following physical and psychosocial stressors [4, 5]. Longitudinal studies with very old Swedes defined the immune risk profile (IRP) by inverted CD4:CD8 ratios, which were associated with increased mortality rates [6]. This alteration had been associated with lower counts of B cells (CD19+), increased CD8+CD28– cells [7], and particular expansions of CD8+ T cells against cytomegalovirus (CMV) [8] as well as higher CMV seropositivity [9]. These findings suggest that the inverted CD4:CD8 ratio is a risk factor at any age, being particularly associated with accelerated immunosenescence and persistent viral infections.

The CMV is a ubiquitous β-herpes virus infection with increased age-related prevalence, ranging from 40% (18–24 years old) to over 90% (75–80 years old) [10], depending on the socioeconomic status of the population. Although under most circumstances the CMV promotes a latent asymptomatic infection, it has also been associated with chronic conditions including atherosclerosis, autoimmune disorders, periodontitis and inflammatory bowel disease [11–13]. Recent data suggest that CMV is involved with accelerated human immunosenescence [30]. Indeed, CMV has been associated with the accumulation of late-stage differentiated CD8+ T cells (e.g. CD8+CD28– cells) and inflammation in elderly populations [8, 9, 14, 15]. A recent longitudinal study indicated that CMV+ American older adults had a greater incidence of frailty and increased risk of 5-year mortality compared to CMV seronegative subjects [16]. In addition, data from the Women's Health and Aging Studies have shown that CMV serology was associated with higher levels of interleukin (IL)-6 and prevalent frailty in community-dwelling older subjects [17]. These effects of CMV are thought to underlie the age-related increased morbidity/mortality due to infectious disease and premature immunosenescence. However, the association of CMV with early senescence has not been investigated in developing countries.

The inverted CD4:CD8 ratio and persistent infections with neurotropic viruses seem to interact synergistically with poor cognitive function. Previous studies suggested that IRP-related early immunosenescence may augment the mortality associated with cognitive impairment and dementia [18, 19]. Herpes virus infections have been associated with cognitive impairment during and after acute encephalitis. Although chronic latent/persistent infection is considered to be relatively benign, some studies have also documented cognitive impairment in exposed but asymptomatic elderly persons. For instance, higher rates of cognitive decline over four years were observed in older subjects with the highest CMV antibody levels at baseline than in individuals with the lowest levels [20]. More recently, the most heritable component of cognitive performance was found to decline with exposure to CMV or herpes simplex virus type 1 (HSV-1) [21].

Here, we investigated the role of herpes virus infections, cognitive and functional states as predictors of inverted CD4:CD8 ratio of older adults in a developing country.

Methods

Subjects

We recruited 362 community-residing adults aged over 60 years old by random home visits in different districts of Porto Alegre, Brazil. Individuals over 60 years old are considered elderly in Brazil. All were non-institutionalized, with cognitive ability. The recruitment was coordinated by the Institute of Geriatrics and Gerontology (Hospital São Lucas) of Pontifical Catholic University of Rio Grande do Sul (PUCRS) with the assistance of teams from the Family Health Strategy of the National Healthcare System. All subjects signed the informed consent form, and this study protocol was approved by the institutional review board of the PUCRS.

Neuropsychological and Functional Measures

Assessment of cognitive decline used the Instrument of Brief Neuropsychological Assessment (NEUPSILIN) [22] and the Mini-Mental State Examination (MMSE) [23]. Clinical evaluation was performed by trained neurologists. The MMSE scores varied from 0 to 30 points, adjusted for education of the study subjects. The cutoff point for cognitive impairment was ≤13 points for those without schooling and ≤18 points for individuals with less than 8 years of education and ≤26 points for those over 8 years of education. Neuropsychological tests (NEUPSILIN) consisted of a battery of tests with 32 tasks used to assess nine cognitive functions. It was used as a criterion for evaluation of cognitive decline –1.5 standard deviations for expected average of schooling of the population. Older adults were considered cognitively impaired if they had more than two items of the MMSE or NEUPSILIN inventories above the respective cutoff points.

between 0 and 3. The scores higher than 3 are suggestive of functional impairment, greater degree of dependence and fragility of the older adults.

Immunophenotyping

Ten milliliters of peripheral blood were collected by venipuncture (8–10 a.m.) and stored in EDTA tubes. The CD4+ and CD8+ T cells were identified by four-color flow cytometry (MultiTEST, BD Biosciences, San Jose, Calif., USA). Briefly, a stain/erythrocyte-lyse/no-wash procedure was used. Fifty µl of anticoagulated whole blood was added into tubes containing absolute counting beads (TruCOUNT tubes, BD Biosciences). Twenty µl of the BD MultiTEST CD3 FITC (clone SK7)/CD8 PE (clone SK1)/CD45 PerCP (clone 2D1)/CD4 APC (clone SK3) cocktail reagent was added (BD Biosciences) and incubated for 15 min in the dark at room temperature. Erythrocytes were briefly lysed, and samples were immediately acquired in a multi-color flow cytometer (BD FACSCanto II, from BD Biosciences). Semi-automatic acquisition and analysis were performed with the BD FACSCanto Clinical Software v2.4 (BD Biosciences) and collection criteria included 30,000 total events composed of at least 3,000 lymphocytes identified through gating on the CD45 high/SSC low events as recommended by the Centers for Disease Control and Prevention guidelines. The remaining lymphocyte subsets were determined with combinations of the following monoclonal antibodies: anti-CD3 FITC (clone UCHT1), anti-CD3 PECy5 (clone HIT-3a), anti-CD8 PE (clone RPA-T8), anti-CD19 PE (clone HIB19), anti-CD56 FITC (clone NCAM16.2) and anti-CD28 FITC (clone CD28.2; all from BD Biosciences).

CMV and Epstein-Barr Virus Serology

Aliquots of peripheral blood were collected without anticoagulant in order to assess serum CMV-IgM, Epstein-Barr virus (EBV)-IgM (active disease or recent infection) as well as CMV-IgG and EBV-IgG by ELISAs using IBL reagents (IBL International, Hamburg, Germany) by the Basic Radim Immunoassay Operator automated equipment (Radim Diagnostics, Pomezia, Italy). The optical densities (570/620 nm) were estimated in an ELISA plate reader. Samples were considered positive (reactive) for CMV or EBV when the values were above the following cutoff values: CMV-IgG or CMV-IgM (>0.4 IU/ml) and EBV-IgM and EBV-IgG (>20 IU/ml).

Statistical Analysis

All variables were tested for homogeneity of variances and normality of distribution by means of the Levene and Kolmogorov-Smirnov tests, respectively. Main effects between groups were analyzed by Student t test, ANOVA or Mann-Whitney U test, when appropriate. Statistical interactions between categorical variables and group were compared by means of the χ^2 test. Interrelationships between variables were analyzed by Pearson or Spearman correlation tests. A logistic regression model (method: enter) was used to analyze age, CMV-IgG levels and PFAQ scores as predictors of subjects with inverted CD4:CD8 ratio. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) 19.0 software (SPSS Inc., Chicago, Ill., USA). The significance level was set at $\alpha = 0.05$ (two tailed).

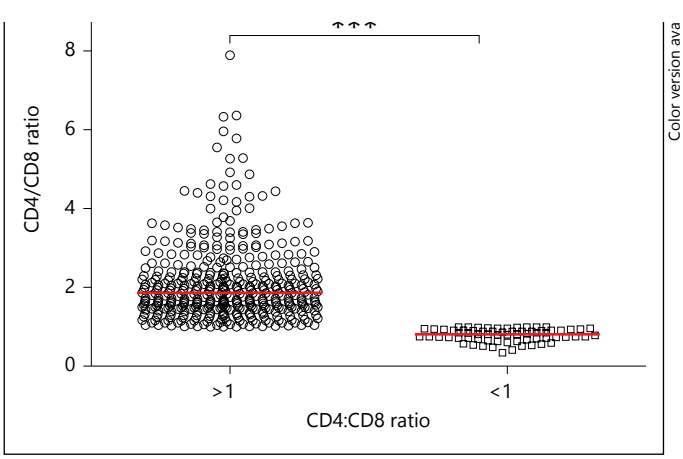

Fig. 1. Sub-grouping accordingly to older adults with CD4:CD8 ratio >1 (n = 303) and <1 (n = 59). Solid lines represent the medians. *** $p < 0.0001$.

Results

Characteristics of the Studied Populations

The subjects were aged between 60 and 103 years (mean 68.86 ± 7.29 years); 50.9% were white, 60.6% were female, with low education and low income, with an average monthly wage of USD 307.04. Fifty-nine older adults (32 women and 27 men) had inverted CD4:CD8 ratio (fig. 1) with mean age of 67.65 ± 5.80 years (60–84 years) and 303 individuals with normal CD4:CD8 ratio (185 women and 118 men), with mean age of 68.97 ± 7.53 years (60–103 years). Both groups were homogenous regarding sociodemographic and laboratory characteristics, including: age, gender, ethnicity, income, education, smoking and drinking habits (alcohol), dyslipidemic profile, and body mass index (all p = not significant, NS). Recent studies estimate that 50% of older adults with inverted CD4:CD8 ratio had more age-related illnesses [25, 26]. However, both groups had similar clinical conditions in this study, including diabetes mellitus, metabolic syndrome, heart diseases, cerebrovascular diseases, thyroid dysfunction, depression, chronic obstructive lung disease, renal diseases, gastrointestinal diseases, neurodegenerative diseases, autoimmune disorders, infectious diseases, or special needs (all NS).

Neuropsychological Assessments of Cognitive Function

A comprehensive analysis of the functional cognitive state was performed in this study. The NEUPSILIN tests revealed that older adults with inverted CD4:CD8 ratio

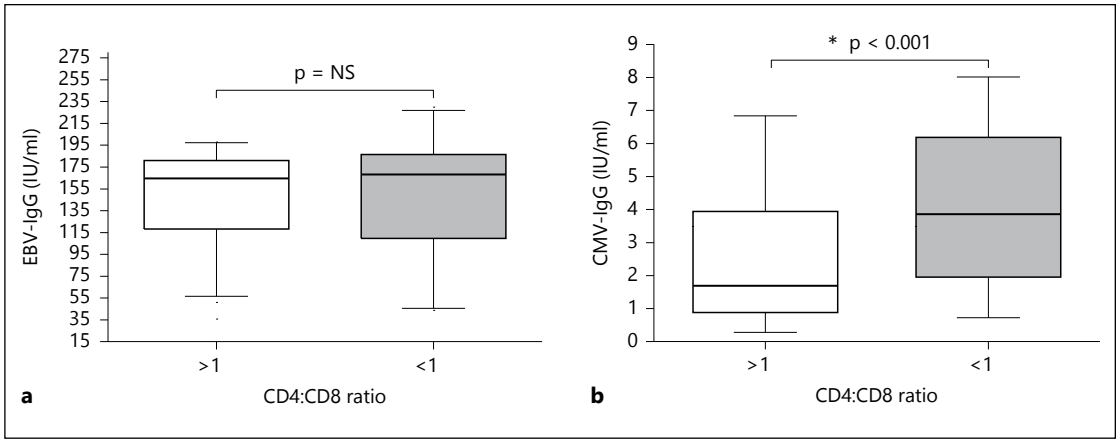

Fig. 2. Serology to CMV and EBV between older adults with CD4:CD8 ratio >1 and <1. Data are shown as box (5–95 percentile) and whiskers (range). Solid lines represent the medians. * p < 0.001.

had cognitive impairments in some dimensions, including reduced capacity to recall a delayed list of words ($p < 0.05$), language and naming ($p < 0.05$; table 1) – adjusting for years of schooling. However, both studied groups had similar MMSE scores (23.60 vs. 23.48), covarying for years of schooling ($p = 0.82$). The PFAQ revealed that older adults with inverted CD4:CD8 ratio had worse functional impairment, greater degree of dependence and fragility compared to non-IRP ($p = 0.01$). We also investigated whether the group with inverted CD4:CD8 ratio had more individuals with cognitive impairment than the comparing group. Subjects were considered cognitively impaired if they had more than two items of the MMSE or NEUPSILIN inventories above the respective cutoff points. However, similar proportions of cognitively impaired older adults were observed between these two groups (MMSE: 8.7 vs. 4.2%; NEUPSILIN: 58.1 vs. 61%), all p = NS.

Immunophenotyping of Peripheral Lymphocytes

We also investigated a panel of major lymphocyte subsets including activated cells, late-differentiated cells and cells previously associated with immunosenescence. The absolute counts of peripheral leukocytes (7,870.75 vs. 7,220.33 cells/mm^3) or lymphocytes (2,223.39 vs. 2,420.71 cells/mm^3) did not vary between subjects with inverted CD4:CD8 ratio and older adults with CD4:CD8 >1, respectively. The proportions and absolute counts of B cells (CD3–CD19+), NK cells (CD3–CD56+), NKT (CD3+CD56+), activated T cells (CD8+CD28+) and differentiated/senescent T cells (CD8+CD28–) did not vary between groups (all p = NS; data not shown) – these data were similarly observed after controlling for age.

Table 1. Cognitive and functional assessments

	CD4:CD8 >1	CD4:CD8 <1	p value
NEUPSILIN			
Immediate memory words	–0.47±0.90	–1.48±5.84	NS
Visuospatial ability	0.14±0.82	0.45±0.79	NS
Delayed memory words	–0.44±0.86	–0.16±0.67	0.03*
Delayed memory drawings	0.07±0.92	0.49±0.79	0.07
Immediate memory	–1.36±0.93	–1.31±1.09	NS
Language and naming	–0.60±1.10	–0.06±0.93	0.03*
Verbal fluency	–0.86±1.42	–0.81±1.00	NS
Verbal fluency animals	–0.58±0.90	–0.44±0.90	0.06
Delayed memory history	–1.46±1.08	–1.37±1.13	NS
PFAQ	1.08±1.79	1.87±2.16	0.01*

Data are shown as mean ± standard deviation. Main effects were assessed by the Mann-Whitney U test. For NEUPSILIN, scoring is shown as standard deviations from the expected average of the population. Statistical significances indicated: * p < 0.05.

Serology for CMV and EBV

The older adults with inverted CD4:CD8 ratio had significantly higher CMV-IgG levels (0.24–8.31 IU/ml; mean = 4.14 IU/ml) than subjects with CD4:CD8 ratio >1 (0.21–9.20 IU/ml; mean = 2.55 IU/ml; $p < 0.001$; fig. 2b). None of the subjects showed IgM reactivity for CMV or EBV, excluding recent acute infections. In this study, the vast majority of the subjects were positive for CMV-IgG and IgG-EBV (data not shown). The EBV-IgG levels between older adults with inverted CD4:CD8 ratio (43.71–250 IU/ml) did not differ significantly from the comparing group (35.82–187 IU/ml; p = NS) (fig. 2a). These data

suggest an impact of CMV in this group and may further indicate the existence of a very specific phenomenon in which CMV may be interfering with the immunological profile of the elderly. Supporting this, we observed a negative correlation between the CD4:CD8 ratio and CMV-IgG levels ($r_s = -0.26$, $p < 0.001$). No correlation was observed for the IgG-EBV levels (NS).

Relationships between CD4:CD8 Ratios, CMV and Cognitive/Functional Measures

First, we sought to explore potential relationships between CD4:CD8 ratio, IgG anti-CMV serology and cognitive/functional measures. Zero-order analyses revealed that only CMV-IgG levels ($r_s = -0.26$, $p = 0.001$) and PFAQ scores ($r_s = -0.28$, $p < 0.0001$) were found negatively correlated with CD4:CD8 ratios of older adults. The remaining sociodemographic, cognitive and immunological variables were neither associated with CD4:CD8 ratio nor with CMV-IgG levels (all p = NS). We then investigated what predictors are more relevant to define older adults with inverted CD4:CD8 ratio. A multivariate model entering CMV-IgG levels, PFAQ scores and age as predictors revealed that only CMV serology had an important impact in defining older adults with inverted CD4:CD8 ratio (OR 8.12, 95% CI 1.74–37.88, p = 0.008). In other words, subjects with elevated CMV-IgG levels had 8× higher chance to invert their CD4:CD8 ratio than people with lower CMV-IgG levels.

Discussion

Here, we investigated the role of persistent viral infections (of note CMV) and cognitive and functional states as predictors of inverted CD4:CD8 ratio of older adults in a developing country. A comprehensive analysis of functional and cognitive states was performed to better discriminate cognitively impaired from normal subjects as well as to address potential effects upon lymphocyte subsets. The NEUPSILIN tests revealed that subjects with inverted CD4:CD8 ratio had significant impairments on some neuropsychological dimensions, including language and naming. Both groups had similar MMSE scores, contrasting with previous studies reporting a worse cognitive status of IRP+ subjects [27, 28]. Although two parameters of the IRP were incorporated in our study (i.e. CD4:CD8 ratio and CMV serology), this definition was not used here because subjects were younger and the study design is cross-sectional unlike previous 'IRP' studies that followed very old elders for morbidity/mortality status. The NEUPSILIN tests are more sensitive than MMSE to detect early and mild changes in cognitive function. These findings are interesting taking into consideration cognitive decline is indeed more clearly associated in the last years of life. Since it is still difficult to examine people with low educational level in order to evaluate cognitive impairment, the decline in activities of daily living may provide a more sensitive and straightforward measure of suspicion of dementia for the elderly. Subjects with inverted CD4:CD8 ratio had worse functional impairment, greater degree of dependence and fragility as compared older adults with normal index. These data are partially in line with a recent study that associated functional impairment with lower proportion of CD4+ T cells, increased CD8+ T cells, and a decreased CD4:CD8 ratio in the elderly [29].

Both groups had similar proportions of major lymphocyte subpopulations. These are positive results for the Brazilian older adults with inverted CD4:CD8 ratio. The aging 'IRP' has previously been associated with increased proportions of effector/memory T CD8+ cells, low B cells (CD19+), increased NK cells and T CD8+CD28– cells, and higher plasma IL-6 levels during aging [9, 31–34]. In accordance to previous work [35], most older adults (~90%) were seropositive for CMV and EBV in this study and subjects had the highest IgG levels to CMV. In addition, the CMV-IgG levels were inversely correlated with the CD4:CD8 ratio. Taken together, these data support the current knowledge that CMV may drive clonal expansions of T CD8+ cells [36] and that repeated viral infections would be involved with age-related immune alterations [8, 37, 38]. In this study, we did not observe synergistic effects of low CD4:CD8 ratio and CMV/EBV serology on cognition or lymphocyte subsets. These results are in partial contrast to previous works reporting increased viral burden (HSV1, HSV2, CMV) or serology associated with cognitive impairment [20, 39].

This study has several strengths. First, a large population-based sample was included to be representative of community-dwelling older adults in Brazil. Second, validated methodology was employed for the first time to better identify peripheral CD4+ and CD8+ T cells. This included semi-automatic multi-color flow cytometry analyses involving four cell-surface markers in peripheral lymphocytes, as recommended by the Centers for Disease Control and Prevention guidelines. Finally, a comprehensive analysis of cognitive and functional states was performed in this study. This adds significantly to previous studies which have only used misleading MMSE

scores to address cognitive function. However, the cross-sectional design should be interpreted as a potential limitation here as it precludes causal relationships.

Concluding, the inverted CD4:CD8 ratio was not associated with increased morbidity in elderly populations in a developing country. However, our data further indicate the role of CMV in circulating T cells, poor cognition and functional disability/dependency during aging.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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8.2 Artigo científico #2

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REVIEW ARTICLE

Psychoneuroendocrine interventions aimed at attenuating immunosenescence: a review

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Abstract There is evidence suggesting that immunosenescence can be accelerated by external factors such as chronic stress. Here we review potential psychoneuroendocrine determinants of premature aging of the immune system and discuss available interventions aimed at attenuating immunosenescence. Chronic stress may accelerate various features of immunosenescence by activating key allostatic systems, notably the hypothalamic–pituitary–adrenal axis. The immunological impact of such neuroendocrine dysregulation may be further amplified by a dramatic decline in dehydroepiandrosterone (DHEA) levels, acting in part as an endogenous glucocorticoid antagonist. Stress-buffering strategies show beneficial effects on various biomarkers in elderly populations. Likewise, supplementation of DHEA, melatonin or growth hormone has yielded significant beneficial effects in a number of studies, including: increased well-being, memory performance, bone mineral density and improved immunocompetence as evidenced by results of in vitro (T cell proliferation, cytotoxicity, cytokine production), and in vivo immune challenges. However, the side-effects of hormonal supplementation are also discussed. Finally, moderate exercise via the promotion of cortisol/DHEA balance or epigenetic modifications, is associated with lower serum pro-inflammatory cytokines, greater lymphoproliferative responses and lower counts of senescent T cells. Taken together, these data suggest that immune system is plastic and immunosenescence can be attenuated psychoneuroendocrine interventions.

Keywords Aging · Immunosenescence · Glucocorticoids · Lymphocytes

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Introduction

Aging is associated with progressive changes in several key physiological systems including the

immune system, which is continuously remodeled over the life course, a process known as immunosenescence. Many age-related diseases are directly associated with immunosenescence such as increased susceptibility to infectious diseases, neoplasias, metabolic diseases, osteoporosis and autoimmune diseases (Castle 2000). Most, if not all, age-related diseases have multifactorial etiology including intrinsic (e.g., genetic background) and extrinsic factors like health-related behaviors and chronic stress exposure (Bauer 2005).

The immunological changes observed during aging are found in similar magnitude following chronic stress or glucocorticoid exposure (reviewed in Bauer et al. 2009). Indeed, the thymic involution and related drop in naïve T cell exports, increased memory and regulatory T cells, a Th1 to Th2 cytokine shift, reduced cell-mediated immunity (e.g., blunted T cell proliferation), restricted TCRαβ repertoire in the CD4+ and CD8+ T cells, increased serum pro-inflammatory markers (inflammaging) and shorter telomere lengths are all similar found during aging, distress conditions and chronic glucocorticoid exposure (Ramirez et al. 1996; Wack et al. 1998; Elenkov and Chrousos 1999; Ashwell et al. 2000; Franceschi et al. 2000; Globerson and Effros 2000; Sapolsky et al. 2000; Kiecolt-Glaser et al. 2003; Effros et al. 2005; Hoglund et al. 2006; Trzonkowski et al. 2006; Damjanovic et al. 2007; Sauce and Appay 2011). Considering these multitude of determinants, efforts have been made to characterize immunosenescence from a multidisciplinary perspective as well as to design broad interventions to attenuate the effects age-related immunological changes. This review will summarizes these approaches, by highlighting several key determinants of aging of the immune system as well as novel interventions aimed to mitigate its effects.

Stress factors as determinants of immunosenescence

Healthy aging has been associated with significant psychological burden (or distress). We have previously reported that strictly healthy elders (SENIEUR) were significantly more stressed, anxious and depressed than young adults (Luz et al. 2003; Collaziol et al. 2004). In accordance with increased psychological morbidity, the healthy elders had higher cortisol (45 %) and lower dehydroepiandrosterone (DHEA, −54 %) levels compared to young adults (Luz et al. 2003), indicating a neuroendocrine imbalance of the hypothalamic–pituitary–adrenal (HPA) axis. Increased cortisol levels were associated with a fall in the numbers of naïve T cells (Collaziol et al. 2004) and reduced T cell proliferation (Luz et al. 2006) during healthy aging. We argued that these neuroendocrine dysregulations may contribute to immunosenescence since all leukocytes exhibit receptors and are thus fully responsive for these neuroendocrine products.

However, healthy aging has been associated with reduced cellular responsiveness to glucocorticoids (GCs). The effects of GCs on the immune system are mediated via both intracellular and membrane GC receptors (GRs) (McEwen et al. 1997). Healthy elders had a reduced (−19 %) in vitro lymphocyte sensitivity to dexamethasone (a synthetic GC) compared to young adults (Luz et al. 2003), suggesting acquired resistance to GCs. We hypothesize that high cortisol levels render lymphocytes resistant to steroids, thus promoting inflammation and "inflammaging". A recent study support this hypothesis, reporting that higher immune glucocorticoid resistance was associated with greater pro-inflammatory cytokine production by cells of young adults infected with common cold virus (Cohen et al. 2012). Taken together, these data show correlations between healthy aging, increased psychological stress, activation of the HPA axis and specific immune changes characteristic of immunosenescence.

Chronic stress may lead to premature immunosenescence

Superimposing chronic stress during aging might thus accelerate features of immunosenescence. It is well known that chronic exposure to psychological stress is correlated with suppressive immune functions (reviewed in Glaser and Kiecolt-Glaser 2005). These associations may be explained by accelerate aging of several lymphoid organs and key immunological functions (Bauer 2008). Stressed elders may thus be at risk for the development of stress-related pathologies because of detrimental additive effects of stress upon the aged immune system.

Is there any elderly population especially at risk for premature immunosenescence? Elderly caregivers of

spouses with dementia represent such model to study the superimposing (and detrimental) effects of chronic psychological stress upon immunosenescence. Caregiving for the first-grade elderly relative with dementia is an exceptionally demanding task associated with increased stress, anxiety, depression and notably suppressed immune functions (Redinbaugh et al. 1995). A longitudinal study has shown that caregivers had increased mortality rate (>63 %) compared to non-stressed controls (Schulz and Beach 1999). Caregiving for a chronically ill partner (stroke or dementia) is associated with increased susceptibility to upper respiratory infections, including influenza (Vedhara et al. 1999), and reduced immune responses to pneumococcal pneumonia vaccines (Glaser et al. 2000). It has been shown that elderly caregivers of Alzheimer patients have impaired T cell proliferation (Bauer et al. 2000), reduced NK cell activity (Esterling et al. 1996), low salivary IgA levels (Gallagher et al. 2008), a reduced IL-2 production (Bauer et al. 2000) in contrast to higher TNF-α, IL-10 (Damjanovic et al. 2007) and IL-6 levels (Kiecolt-Glaser et al. 2003). Stress-related increase in proinflammatory cytokines, such as TNF-α and IL-6, have broad health-related implications, including: several age-related conditions like cardiovascular diseases, osteoporosis, arthritis, type II diabetes, some neoplasias, periodontal disease, frailty and functional decline (Harris et al. 1999). Chronic psychological stress has been correlated with increased oxidative stress, reduced telomerase activity and shorter telomere length, suggesting a state of premature cell senescence, which could implicated with reduced longevity (Epel et al. 2004).

What are the potential mechanisms involved with stress-related premature features of immunosenescence? There is evidence suggesting that neuroendocrine changes are responsible for such changes. Indeed, we have previously observed that elderly caregivers of dementia spouses had significantly higher cortisol levels compared to non-stressed elderly controls (Bauer et al. 2000). Acquired steroid resistance was also demonstrated at the cellular level: caregivers showed increased lymphocyte GC resistance compared to non-stressed elderly controls. Again, we hypothesize that high cortisol levels would render lymphocytes more resistant to steroids, further promoting "inflammaging".

Taking together, chronic stress is involved in the premature dysregulation of key allostatic systems associated with accelerated immunosenescence. It should be considered that other lifestyle factors, coping strategies and temperament may also influence the pace of immunosenescence. It is expected that stress-management interventions would be beneficial for the elderly. The next section will review some interventions designed to reduce the impact of stress on immunosenescence.

Interventions aimed at attenuating immunosenescence

Psychosocial interventions

Psychosocial interventions have been effective in attenuating stress and promoting a better endocrine balance in the elderly. By reducing stress levels and promoting healthy behaviors, psychosocial interventions may also attenuate the rise in cortisol, decline in DHEA, and promote enhanced vagal tone, possibly resulting in better immune responses. For example, following a psychological enrichment program, elderly individuals showed increased levels of DHEA, testosterone, estradiol and growth hormone (GH) (Arnetz et al. 1983). This psychological enrichment program was designed to counteract social isolation and passivity by increasing social activation, competence, and independence. Another study showed that older adults who practiced relaxation techniques, had reduced antibody titers to latent herpes simplex virus-1 (HSV-1). Lower viral antibody titers suggest fewer episodes of viral-reactivation, indicating that a lifestyle intervention was associated with reduced chronic antigenic stimulation (Gouin et al. 2008). These studies suggest that stress-buffering strategies can lead to an improvement in cellular immunity involved in the control of latent viruses (Fig. 1).

Resilience factors may also buffer the impact of stress on key allostatic systems (i.e., neuroendocrine and immune) of the elderly. Recent data produced by our laboratory have suggested that the maintenance of health during aging minimizes the effects of chronic stress exposure (Jeckel et al. 2010). We recruited chronically stressed and non-stressed SENIEUR older adults and investigated neuroendocrine and immunological changes. The stressed group included caregivers of the first-grade elderly relative with dementia. Despite elderly caregivers of dementia patients were

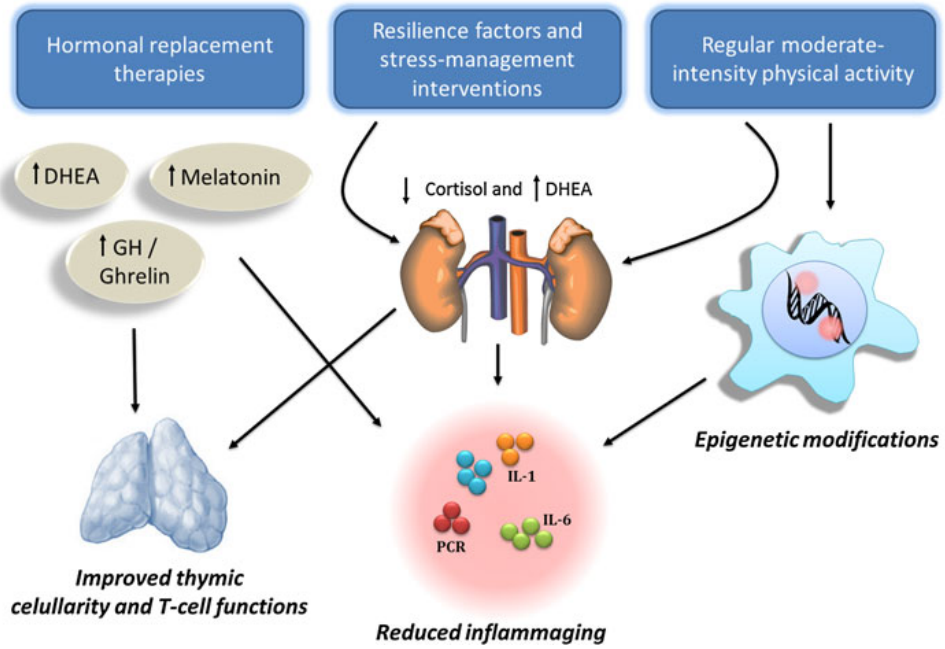

Fig. 1 Some interventions aimed at attenuating immunosenescence. Hormonal replacement therapies have been effective to reduce inflammaging and improve several thymic-related functions such as higher cellularity and T cell proliferation, cytokine production, and T cell repertoire (TCR) diversity. However, the side-effects of long-term hormonal therapies should be considered. Stress-management and regular physical exercise have also been shown to reduce inflammaging and improve T-cell functions most likely via increasing DHEA and reducing cortisol levels as well as via epigenetic modifications

significantly more stressed than elderly controls without a caring burden, no changes in cortisol levels were observed. Positive neuroendocrine-immune interactions (eustress) were also observed in these strictly healthy and stressed elders. Healthy elderly caregivers exhibited increased T cell proliferation and higher cellular sensitivity to GCs compared to non-stressed healthy controls. Elderly individuals engaged in physically active behaviors do not experience enhanced levels of emotional distress (Wrosch et al. 2002) and have attenuated secretion of salivary cortisol (Wrosch et al. 2007). A strong resilience factor for health outcomes may be the induction and maintenance of positive emotion through personality and coping styles. Individuals reporting personality-type of positive affect have lower cortisol levels, reduced inflammatory markers (e.g., IL-6, CRP) and favorable associations with heart rate and blood pressure (Steptoe et al. 2009). In addition, stressed individuals with better social support had stronger immune responses to vaccination (Glaser et al. 1992). Therefore, resilience factors or stress-management interventions appear to be to modulate aspects of immunosenescence most likely by reducing endogenous GC levels.

DHEA supplementation

DHEA and its metabolites have been considered the natural endogenous antagonists of GCs (Hazeldine et al. 2010). Serum DHEA levels decrease significantly after the second decade of life in humans, and the elderly exhibit <5 % of the concentrations seen in younger adults (Hornsby 1995). The impaired DHEA secretion, in parallel with increased cortisol levels, results in an enhanced exposure of leukocytes to deleterious effects of GCs (Butcher et al. 2005; Maninger et al. 2009; Khanfer et al. 2011). DHEA replacement therapy has yielded significant beneficial effects for healthy elders, including increased well-being, memory performance, bone mineral density and altered immune function (Buvat 2003; Nair et al. 2006; Kenny et al. 2010). DHEA replacement can increase glucose tolerance and reduce insulin resistance in elders (Weiss et al. 2011). In addition, flattened DHEA diurnal profile has been associated with impaired physical function during aging (Heaney et al. 2012). However, a recent systematic review indicated no conclusive evidence of benefits of DHEA replacement on muscle strength and physical function

in clinical trials (Baker et al. 2011)—with no recommendation for this use in the elderly. DHEA replacement may also have protective cardiovascular properties, as it can improve arterial stiffness index in elders (Weiss et al. 2012). While the DHEA replacement can induce similar changes in both men and women, in some cases the effects can be genre-specifics, as DHEA replacement can improve the ovarian performance (Gleicher and Barad 2011).

Previous studies have investigated the immunomodulatory effects of DHEA in vitro as well as following in vivo supplementation (Suzuki et al. 1991). DHEA supplementation was associated with increased natural killer (NK) counts and cytotoxic function, as well as decreased IL-6 production and T cell proliferation in vitro, and with increased secretion of IL-2 by T cells (Suzuki et al. 1991; Casson et al. 1993; Di Santo et al. 1996; Straub et al. 1998; Solerte et al. 1999; Hazeldine et al. 2010). Although both T and B cells can be stimulated in vitro, they require different DHEA concentrations (Sakakura et al. 2006). Because of its anti-inflammatory properties, the potential benefits of DHEA have been investigated in autoimmune diseases. However, following encouraging studies demonstrating beneficial effects of DHEA supplementation in murine lupus models, the effect of DHEA on disease activity in lupus patients remains controversial (Sawalha and Kovats 2008). Previous studies have also explored the potential use of DHEA as adjuvant in vaccine preparations. Notwithstanding the clear adjuvant effects of DHEA during immunization to hepatitis B (Araneo et al. 1993) or influenza (Danenberg et al. 1995) in mice, negative effects have been reported following influenza vaccination in older humans (Danenberg et al. 1997; Degelau et al. 1997).

Overall, these data indicate the potential use of DHEA as anti-aging hormone and suggest that DHEA supplementation might attenuate chronic low-grade inflammation and age-related frailty by inhibiting production of pro-inflammatory cytokines. It should be kept in mind that very few data exist regarding the DHEA effects on the immune responses of the elderly and large clinical trials are thus necessary to disentangle conflicting reports. Some studies have found mild adverse effects in the study subjects, who had acne and hirsutism in general. A recent study reported that higher DHEA-S levels were associated with depressive symptoms, but not diagnosis of major depression, during menopausal transition (Morrison et al. 2011). Importantly, pharmacological DHEA levels have been associated with the development of hepatocellular carcinoma (Hazeldine et al. 2010).

Melatonin treatment

Melatonin supplementation could be of potential value for elders due its antagonistic effects on cortisol (Maestroni et al. 1986), as well as potentially enhancing effects of cell-mediated immunity. The production of melatonin by the pineal gland begins as the eyes close and its secretion is associated with serotonin production, which is activated during the dark cycle. Furthermore, melatonin is also secreted by several leukocytes including monocytes, T and NK cells and mast cells (Hardeland et al. 2011). Similar to cortisol, melatonin secretion has a circadian rhythm. The peak secretion occurs at night and very low levels are observed upon waking in the morning. Importantly, melatonin is reduced in elderly individuals, together with a decrease in the amplitude of the circadian rhythm of this hormone (Bastien et al. 2003). Although the age-related reduction of the melatonin levels is considered as a predisposing factor for neurodegenerative diseases such as Alzheimer's, melatonin replacement therapy may have neuroprotective potential (Srinivasan et al. 2006; Cardinali et al. 2008; Markus et al. 2010).

The decline in melatonin levels during aging has been proposed to play an important role in immunosenescence (Cardinali et al. 2008). In addition to its sleep regulatory properties, melatonin is a natural antioxidant with important immunological properties. The immunomodulatory properties of melatonin are related in part to its actions on specific membrane (MT1 and MT2) or nuclear receptors located in leukocytes. The in vitro effects of melatonin include increased T cell proliferation (Konakchieva et al. 1995) enhancement of NK cell cytotoxicity (Currier et al. 2000) and increased production of several cytokines including IL-1, IL-2, IL-12, IL-6, TNF-α and IFN-γ (Garcia-Maurino et al. 1997; Garcia-Maurino et al. 1999; Chen and Wei 2002). Conversely, melatonin has anti-inflammatory properties including inhibition of NO synthases and 5-lipoxygenase (Hardeland et al. 2011). A recent in vitro study with human T cell lines demonstrated that melatonin efficiently downregulates the expression of retinoic

acid-related orphan receptor alpha (RORα), a key transcription factor involved with differentiation of Th17 and regulatory T cells (Tregs) (Lardone et al. 2011). Melatonin also stimulates the production of glutathione, the most abundant antioxidant molecule found in mammalian cells. Aside from detoxifying hydrogen peroxide into water, glutathione is also essential for lymphocyte activation, proliferation and cytotoxicity (Suthanthiran et al. 1990; Liang et al. 1991). Thus, the immune-enhancing properties of melatonin may be in part due to maintenance of intracellular glutathione levels, and the subsequent effects on cell-mediated immunity (Cardinali et al. 2008). In support, melatonin administration increased the total number of thymocytes in old mice (Tian et al. 2003). This protective effect of melatonin on thymocytes was attributed to its anti-apoptotic action, by inhibiting GC-induced thymic apoptosis. Melatonin replacement has been effective in suppressing neoplastic growth in a variety of tumors, including breast and prostate cancer, melanoma, ovarian and colorectal cancer (Srinivasan et al. 2011). Melatonin showed beneficial effects in adjuvant therapy aimed to treat patients suffering from breast cancer, hepatocellular carcinoma or melanoma (Srinivasan et al. 2011). Although there are many studies showing the immunomodulatory properties of melatonin, there is a lack of investigations relevant to immunosenescence. However, the side-effects of melatonin replacement have been reported (e.g., daytime sleepiness, dizziness, headaches and interaction with others medications like anticoagulants and immunosuppressants) and should be considered during long-term treatment.

GH and ghrelin replacement therapies

Aging significantly reduces pituitary GH levels, a phenomenon known as somatosenescence. The absence of appropriate GH-immune signaling may also accelerate immunosenescence. GH-deficient mice exhibit immune dysfunction (including thymic involution), which can be reversed with GH replacement (Kelley 1990). Indeed, GH has many direct (or indirect effects via induction of IGF-1) immunoregulatory effects. For example, GH may enhance lymphocyte cytotoxicity (in both CD8+ T cells and NK cells), increase lymphocyte proliferation, promote the differentiation of neutrophils, as well as increase TNF-α and thymulin production (Welniak et al. 2002). The GH replacement in older subjects may not only modulate the levels of IGFs and IGFBPs but also with little gender-related differences (i.e., increase of IGF-1 and IGFBP-3 in both sexes; IGFBP-2 and IGFBP-5 increase only in men and immunoreactive insulin increase only in women) (Munzer et al. 2006). However, men may have a better response (IGF-1 levels) to GH replacement than women (Gotherstrom et al. 2007). The replacement can also increase the protein synthesis in elderly people, this effect is stronger in men when GH replacement is carried along with testosterone (Huang et al. 2005). In addition, GH is synthesized by leukocytes, mimics the action of IFN-γ, increasing the activity of phagocytes (Kelley 1990). GH replacement therapy may be of clinical value by restoring immune competence in the elderly. However side effects of GH therapy also exist. For instance, GH administration could reduce the levels of sex hormones, promoting secondary changes in the immune system, obesity and even cancer (Lo et al. 2001).

Considering patients with GH deficiency, the replacement has shown beneficial effects to the thymus and to the T cell proliferation as well as bone mineral density, which reinforces the concept that GH is capable to attenuate senescence (Morrhaye et al. 2009; Elbornsson et al. 2012). Furthermore, we have previously observed that low pituitary GH levels were not associated with a reciprocal decline in immunoreactive GH produced by lymphocytes during human aging (Luz et al. 2006). The potential side-effects of long-term GH replacement shall be considered, and include carpal tunnel syndrome, arthralgia, increased risk for diabetes (as it increases insulin resistance) and it has been associated with increased risk for Hodkin's lymphoma (Freedman et al. 2005).

Reduced ghrelin levels during aging may also contribute to immunosenescence. Ghrelin binds to a GH secretagogue receptor (GHS-R) expressed on lymphoid cells, having anti-inflammatory effects (lowering TNF-α, IL-1 and IL-6 levels) (Dixit et al. 2004). Ghrelin is produced by stomach cell, which modulates energy balance, stimulating appetite and pituitary GH secretion. Ghrelin may be an important factor for thymopoiesis during aging as mice deficient for ghrelin or GHS-R showed profound age-related thymic involution. In contrast, ghrelin replacement in old mice is associated with increased thymic mass, and a greater production of recent thymic emigrants with

enhanced TCR diversity (Dixit et al. 2007). The ghrelin replacement can increase the GH secretion in elders (Nass et al. 2008), and can also help to increase the bone mineral density in women (Napoli et al. 2011). Cytokine replacement could be also of interest in rejuvenating thymopoiesis and T cell function. Sportés et al. have recently shown that IL-7 can rejuvenate the aging immune system in humans by promoting thymopoiesis (increasing the numbers of naïve T cells) and expanding TCR repertoire diversity (Sportes et al. 2008). In addition, it was shown that treatment with thymosin α1 (Tα1) resulted in increased T cell responses (e.g., cytokine production, proliferation and cytotoxicity). Tα1 is a 28-amino acid biologically active protein that has pharmacologic effects enhancing cellular immunity. Tα1 administration was highly effective in the restoration of cell-mediated functions when used in combination with cytokines (Naylor et al. 2007). However, the long-term beneficial effects of ghrelin replacement therapies should be considered with caution as it can be pro-oncogenic (Akamizu and Kangawa 2012).

Regular moderate-intensity physical activity is associated with better immune responses

Physical activity is associated with many beneficial health effects, and has the advantage of being a non-invasive, low-cost and easy to implement therapy or intervention. Interventions including health behavior changes are likely to be more effective in attenuating aging of the immune system. Indeed, increasing fitness is probably one of the most powerful interventions in restoring cortisol/DHEA balance and can improve psychological and physiological well-being. Long-term moderate-intensity exercise can decrease cortisol and increase DHEA, GH and IGF-1 levels (Cotman and Berchtold 2002), as well as reduced anxiety (Petruzzello et al. 1991) and depression (Barbour et al. 2007).

Moderate-intensity exercise has been associated with anti-inflammatory effects, including lowering serum TNF-α, IL-6 levels and higher IL-10 and Treg counts (Gleeson et al. 2011). These effects might be mediated by the reduction of visceral fat mass and correlated decreased released of adipokines, as well as the induction of the anti-inflammatory environment promoted by the moderate-intensity exercise. In addition, moderate-intensity exercise training in the elderly can also increase T cell proliferation, reduced the frequency of senescent T cells (i.e., CD45RO+, KLRG1+, CD57+, CD28-), enhance IL-2 production and T cell expression of the IL-2 receptor, and is associated with longer leukocyte telomeres and better in vivo immune responses to vaccines and recall antigens (Simpson and Guy 2010). Ogawa and col. compared the Th1/Th2 cytokines in active vs. sedentary elderly, and observed greater numbers of IL-2 secreting CD8+ cells in physically active elderly compared to those who were sedentary (Ogawa et al. 2003). Enhanced T cell responses may be associated with stronger immunity to control viral and bacterial infections: It has been reported that physically active elderly women have a lower risk of community-acquired pneumonia compared to sedentary elderly women (Baik et al. 2000). On the other hand, a lack of regular exercise is associated with an increased the risk of hospitalization due to infection (Leveille et al. 2000). This concept concurs with another study suggesting that physical activity may increase mucosal immune responses in the elderly, promoting resistance to upper respiratory infections (Sakamoto et al. 2009).

Moderate-intensity exercise also appears to buffer the age-related increase in pro-inflammatory cytokines via epigenetic modifications. For instance, the methylation of the pro-inflammatory ASC gene, involved in the secretion of IL-1 and IL-18, reduced significantly with age, suggesting an age-related increase in ASC expression (McGee et al. 2009). Of particular note, the ASC methylation levels were higher in the older exercise group than in the older controls. To what extent these changes are related to cortisol/DHEA balance induced by moderate-intensity exercise is largely unknown.

Conclusions and future perspectives

Normal aging is associated with major changes in key allostatic systems, notably immune and neuroendocrine, that increase the risk for age-related diseases. These effects do not appear driven by reverse causality (i.e., an effect of age-related health impairments on immunity), as healthy aging is also associated with significant psychological distress and HPA axis activation (increased cortisol and reduced DHEA levels). Although the exact mechanisms of immunosenescence

remain to be elucidated, it is becoming apparent that many of the physiological changes associated with aging are characterized by epigenetic modifications (Fraga and Esteller 2007).

We reviewed here that psychological or pharmacological strategies aimed to attenuate or prevent the increase in HPA axis activation with aging may benefit the elderly. Many studies aimed to understand how lifestyle factors promote or maintain good health. In this scenario, for instance, regular moderate-intensity physical activity may help to delay the onset of immunosenescence. Some clinical strategies, such as hormone replacement or cytokine therapy may be useful to attenuate immunosenescence. However, the potential harmful side-effects of prolonged hormonal supplementation shall be also considered.

The concept of hormesis should be discussed here considering the genetic, hormonal and lifestyle factors involved in accelerating or buffering immunosenescence. Aging, senescence and death are the final consequences of impaired homeostasis or failure of homeodynamics (Rattan 2006). The most important component of homeodynamics is represented by the capacity of living systems to deal (cope) with stress. A progressive shrinking of the homeodynamic space (or buffering capacity) is the hallmark of aging and strongly associated with age-related diseases (Rattan 2008). The stress responses in mammals include apoptosis, inflammation, and increased glucocorticoids—that are also associated with healthy aging. The clinical consequences of stress responses can be both harmful and beneficial, depending on characteristics of the stressor (Calabrese 2008). This phenomenon of biphasic dose response was termed hormesis (Southam and Ehrlich 1943) and it has been described across different disciplines including toxicology, pharmacology, medicine, radiation biology and gerontology (Calabrese et al. 2012). A good example of stress-induced hormesis is the beneficial effects of moderate exercise (hormetic agent) to increase immunity and lower levels of oxidative stress (Radak et al. 2008). Physical inactivity or overtraining are associated with damaging oxidative stress and blunted immune responses. Furthermore, acute or mild stress is generally associated with enhanced immune functions (Dhabhar and McEwen 1999), which prepare the organism to better cope with the stressor. In contrast, chronic stress, which is not resolved via coping or adaptation, is considered to be *distress* and it has been associated with suppressed immune functions (Glaser and Kiecolt-Glaser 2005) and inflammation (Kiecolt-Glaser et al. 2003). These effects are related to GC concentration and to duration of tissue exposure to peripheral GCs. For instance, low cortisol levels produce permissive or stimulatory immune changes whereas long-term or high cortisol levels are immunosuppressive (Sapolsky et al. 2000). Hormetic stressors (hormetins) can be applied successfully to interventions in aging and can be categorized as physical, nutritional, or psychological hormentins (Rattan 2008). Moderate exercise, hormonal and nutritional supplementation, and psychosocial interventions are good examples of stress-induced hormesis aimed to improve homeodynamic space in aging. Recent studies suggest that lifestyle factors (such as physical activity) and human health may be linked through epigenetic mechanisms such as DNA methylation, histone modifications and micro-RNAs (Sanchis-Gomar et al. 2012). Therefore, by favoring hormesis, the interventions reviewed here would render epigenetic changes involved with the attenuation of immunosenescence.

In comparison to younger adults, the elderly who are chronically stressed may be at a greater risk of stress-related pathologies. Therefore, stress management and psychosocial support should promote a better quality of life for the elderly as well as reducing hospitalization costs. Preliminary evidence suggests that differences in personality traits (temperament) and coping skills could have protective properties. Finally, the maintenance of regular moderate-intensity physical activity, social support, personality (positive affect) and coping skills may protect the elderly from the detrimental effects of chronic stress exposure. More studies are needed to address the relationships of health-related behaviors on immunity that might promote better resilience to stress exposure.

This review has presented current evidence that it is possible to attenuate and potentially reverse many features of immunosenescence via stress-management therapies, improved health-related behaviors, and hormone replacement therapies. Low-cost practices, such as moderate regular exercise, may also allow us to age healthily by attenuating the effects of immunosenescence.

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8.3 Capítulo de livro

Inmunopatogenia y aspectos genéticos del asma grave

Guilherme Cerutti Müller, María Isidoro García, Ignacio Dávila González

4.1. INTRODUCCIÓN

El asma es una entidad nosológica de notable complejidad y heterogeneidad, susceptible de ser desencadenada por muy diversos factores y que presenta niveles de gravedad variables, tanto entre los distintos pacientes como en su evolución temporal. En líneas generales, en el asma existe una inflamación eosinófila, encuadrada dentro de un perfil linfocitario Th2, asociado a una activación de mastocitos y otras células, capaces de producir obstrucción bronquial y, en consecuencia, limitación del flujo aéreo[1]. Recientemente, se ha intentado uniformar la definición de *asma grave*[2] como "un asma no controlada que puede originar exacerbaciones frecuentes graves (o el fallecimiento) y/o reacciones adversas al tratamiento y/o morbilidad crónica (incluyendo deterioro de la función pulmonar o reducción del crecimiento pulmonar en los niños)". Como sucede con la enfermedad, la definición de *asma grave* muestra una notable heterogeneidad, al incluir, dentro de la misma, distintos aspectos, como las exacerbaciones o las reacciones adversas al tratamiento, que podrían presentar manifestaciones inmunopatológicas diversas. Esta heterogeneidad se pone de manifiesto también en los distintos grupos de asma grave que se definen: no tratada, difícil de tratar y resistente al tratamiento. Nuevamente se observa una gran variabilidad; es decir, puede no ser lo mismo un asma no tratada que una que sólo es controlada con el máximo nivel de tratamiento u otra que, aun así, no se consigue controlar. En los últimos tiempos esta diversidad se ha reflejado en la definición de distintos fenotipos, tanto del asma en general como de la grave en particular. En cualquier caso, resulta evidente que es mejor disponer de una definición ampliamente aceptada que de ninguna. De todos modos, muchos de los estudios sobre asma grave son, lógicamente, anteriores a esta definición, hecho que añade aún más complejidad y que debe ser tenido en cuenta a la hora de interpretar los datos provenientes de los mismos.

No se conoce con exactitud si en el asma grave se producen las mismas alteraciones que en las formas más leves de esta enfermedad, aunque se sabe que la desregula-

ción de la polarización Th1/Th2 y la inmunorregulación se produce de modo distinto en el asma grave. A continuación, se realizará una revisión centrada en los aspectos etiopatológicos e inmunopatogénicos del asma grave, con especial énfasis en las características que la diferencian de las formas leves y moderadas.

4.2. INMUNOPATOGENIA DEL ASMA GRAVE

Aunque pueda observarse una notable variación en cuanto a las manifestaciones clínicas de las distintas formas de asma, el hecho es que la reacción inflamatoria que se produce en las vías respiratorias es, básicamente, muy similar[3]. Normalmente, la respuesta inflamatoria conlleva infiltración eosinófila, desgranulación de mastocitos, lesión de las paredes bronquiales y activación de linfocitos Th2, que se extiende por todo el tracto respiratorio, si bien las alteraciones más intensas se localizan en zonas intermedias del árbol bronquial y son similares entre las diferentes formas clínicas[3]. No obstante, pueden observarse diferencias entre las formas graves y menos graves de asma; así, por ejemplo, la presencia de linfocitos citolíticos naturales (NKT, del inglés *natural killer T*) parece estar, principalmente, relacionada con el asma grave. Además, la infiltración eosinófila, especialmente cuando es intensa, se acompaña de fibrosis subepitelial, incremento de las cifras de mastocitos y aumento de la expresión del factor de crecimiento tumoral beta (TGF-β), lo que es más patente en las formas graves del asma. En el asma infantil, por su parte, la presencia de linfocitos T activados se correlaciona con la eosinofilia y la gravedad clínica.

4.2.1. Células inflamatorias

Los mastocitos secretan diversos mediadores, bien almacenados en sus gránulos (por ejemplo, histamina), bien sintetizados tras la activación celular (por ejemplo, leucotrienos y prostaglandinas)[1]. La activación se puede producir a través de los receptores de alta afinidad para la inmunoglobulina E (IgE) (FcεRI) o por otros mecanismos, y, a largo plazo, origina la síntesis y la liberación de citocinas, entre ellas las interleucinas (IL) IL-4, IL-5 e IL-13. En los últimos años se han asociado los mastocitos con el asma grave. Así, se ha observado una infiltración de mastocitos en el músculo liso de los pacientes con asma grave[4], posiblemente activados y reclutados a través de quimiocinas. Además, la participación de los mastocitos en el asma grave es reforzada por el hecho de que el tratamiento con anti-IgE, que reduce la activación mastocitaria desencadenada por la IgE y el número de receptores FcεRI, disminuya las exacerbaciones agudas en los pacientes con asma grave que reciben esta terapia[5]. Por su parte, los macrófagos pueden ser activados a través de los receptores de baja afinidad para la IgE (FcεRII) y liberar diferentes mediadores capaces de incrementar la respuesta, como el factor de necrosis tumoral alfa (TNF-α) y el óxido nítrico[6].

En la reacción de hipersensibilidad inmediata, tras la liberación y síntesis de mediadores inflamatorios, se produce el reclutamiento de linfocitos T, eosinófilos, neutrófilos

y basófilos. Diversos estudios han demostrado un incremento de linfocitos T CD4+ en las vías respiratorias de los pacientes con asma y han observado, en la mayoría de ellos, un predominio del patrón Th2 sobre el patrón Th1. Este patrón Th2 se relaciona directamente con la hiperrespuesta bronquial y con el incremento en la producción de moco. Se ha comprobado que, en pacientes con asma grave tratados con fármacos antiinflamatorios, la presencia de linfocitos T activados y eosinófilos se puede asociar a una resistencia al tratamiento. Si bien las citocinas Th2 son de gran importancia, no son los únicos factores que influyen en la hiperrespuesta bronquial. Incluso se han encontrado autoanticuerpos en algunos pacientes con asma, en concreto anticuerpos dirigidos contra la enolasa alfa (presente en el epitelio), en el caso del asma grave.

Si bien su papel en la patogenia de la enfermedad no está bien establecido, puede observarse un incremento del número de neutrófilos en muchos de los pacientes con asma grave, hasta el punto de que se ha sugerido que el asma con infiltrado neutrófilo pueda constituir una entidad distinta de aquélla con infiltrado eosinófilo. Este hecho es más propio del asma grave, aunque también pueda observarse una infiltración de neutrófilos en los pacientes con asma leve. En el estudio *European Network for Understanding the Mechanisms of Severe Asthma* (ENFUMOSA)[7] se ha observado un aumento del recuento de neutrófilos y de la liberación de mediadores eosinófilos en los pacientes con asma grave respecto a aquéllos con una forma leve o moderada de la enfermedad. La IL-8 desempeña un importante papel en la acumulación de neutrófilos y se han observado concentraciones aumentadas de esta citocina en las vías respiratorias de los pacientes con asma grave[8]. No se conocen bien los mecanismos por los que se puede producir este incremento de la IL-8, aunque algunos estudios apuntan a una relación entre el infiltrado neutrófilo y los linfocitos Th17.

Los linfocitos Th17, inducidas por la IL-6 y mantenidas por la IL-23, también parecen ser importantes en el asma, encontrándose incrementadas en el esputo de los pacientes asmáticos; además, la presencia de IL-17 se relaciona con una infiltración neutrófila que puede asociarse con el asma grave; también estimulan la secreción de quimiocinas por el epitelio de las vías respiratorias, como CXCL1 y CXCL8 (IL-8), lo que contribuye a la formación del infiltrado neutrófilo. Tras la estimulación con IL-23, los linfocitos Th17 pueden presentar características tanto Th1 como Th2[9]. Se cree que la IL-17 puede estar correlacionada con el subgrupo de pacientes con asma neutrófila. Asimismo, existe la posibilidad de que los recientemente descubiertos linfocitos Th22 puedan también participar en la patogenia del asma, puesto que se están describiendo ya en distintas entidades alérgicas.

Los linfocitos T reguladores (Treg) tienen una gran importancia en todos los procesos inflamatorios; su efecto inmunosupresor (mediante la secreción de IL-10 y TGF-β, entre otros mecanismos) evita que los leucocitos, especialmente los linfocitos T, produzcan una exacerbación del efecto; el desequilibrio de estas células ayuda a explicar el mecanismo mediante el cual se desarrollan las enfermedades inflamatorias[10].

Los linfocitos Treg pueden ser regulados por la citocina epitelial linfopoyetina del estroma tímico (TSLP), que actúa también sobre los mastocitos y las células dendríticas (además de sobre los eosinófilos y los linfocitos Th2), y que se puede encontrar en gran cantidad en el epitelio de los pacientes asmáticos, y se ha observado que sus concentraciones se relacionan con la gravedad de la enfermedad.

Como comentan Poon *et al.*[9], la transmisión de señales en el asma grave es sumamente compleja, y no se limita al modelo de patrón Th2 que caracteriza al asma leve o moderada. Es un subtipo de asma que también implica linfocitos Th1 y Th17, en un modelo de interacción desconocido hasta el momento. Es necesario realizar más estudios para poder desvelar estos mecanismos, que, en un futuro, podrían contribuir al tratamiento del asma grave.

4.2.2. Mediadores inflamatorios

Actualmente se han relacionado más de 100 mediadores distintos con el asma y la compleja respuesta inflamatoria de las vías respiratorias. Entre estos mediadores se encuentran las citocinas (que dirigen la respuesta inflamatoria), las quimiocinas (que reclutan a las células implicadas en el proceso inflamatorio) y las neurotrofinas (cuya importancia en relación con el sistema inmunitario es cada vez más evidente)[11].

4.2.2.1. Citocinas

La diferenciación del linfocito T virgen hacia un linfocito Th1 o Th2 está influida por la secreción de IL-12 o IL-4, respectivamente. Además de los linfocitos T, otras células son capaces de producir citocinas Th1 y Th2[12]. En el asma grave se aprecia un aumento de la eosinofilia junto con la presencia de linfocitos Th2 activados; además, en un estudio se ha observado, respecto a los pacientes con asma moderada, una disminución de los niveles de IL-4 y un incremento de las concentraciones de interferón gamma (IFN-γ) e IL-8, sin que hubiera diferencias en las de óxido nítrico e IL-5[8]. La vía de transmisión de señales a través de IL-4/IL-13 es muy importante en el asma grave, especialmente en el desarrollo de los síntomas relacionados con la alergia[9], ya que se pueden formar los complejos IL-4/IL-4Rα/IL-2Rγ_c e IL-4/IL-4Rα/IL-13/IL-13Rα1, capaces de activar el factor de transcripción STAT6[13], que media las acciones de linfocitos T de perfil Th2 de estas citocinas y que se puede expresar con mayor intensidad en el epitelio bronquial de los pacientes con asma grave, frente a lo que sucede en las muestras obtenidas de sujetos con una forma leve de la enfermedad o de controles. Incluso teniendo un perfil Th2, el asma grave se diferencia por expresar, además, otras citocinas, como IFN-γ, IL-8, IL-18 e IL-17[8].

Se sabe que un déficit de IL-4 e IL-13 disminuye la respuesta Th2 y favorece la respuesta Th1[12]. Esta acción, compartida por la IL-4 y la IL-13, podría estar también relacionada con un efecto inductor de la producción de quimiocinas que participan en el reclutamiento de eosinófilos. La IL-13, además, produce una reducción de la expresión de TNF-α, IL-1β, IL-12

y CCL5 (RANTES)[14]. En líneas generales, se cree que la IL-13 se relaciona más con las manifestaciones clínicas del asma, mientras que la IL-4 está más implicada en la diferenciación y estimulación de los linfocitos Th2, la síntesis de IgE y la activación de los macrófagos.

Aunque el infiltrado eosinófilo que se observa en la mucosa puede ser similar tanto en los pacientes sensibles como en los resistentes al tratamiento con corticoesteroides, en los últimos no se produce una reducción de la eosinofilia, ni de la secreción de IL-4 e IL-5 ni de la proliferación de células mononucleares de sangre periférica *in vitro*. Del mismo modo, en adultos con asma grave se ha observado una menor sensibilidad a los corticoesteroides, respecto a los pacientes con asma leve[15]. Sin embargo, no se observa lo mismo cuando se estudian niños en edad escolar, lo que sugiere que esta menor sensibilidad puede aparecer con la evolución de la enfermedad y la exposición a los corticoesteroides[16].

Al igual que sucede en otros tipos de asma, existen pacientes con asma grave que no presentan datos de alergia, que pueden sufrir exacerbaciones graves, con presencia de un infiltrado eosinófilo y resistencia a los corticoesteroides inhalados, aunque respondan a los corticoesteroides orales y a la anti-IL-5. Sobre esta base, Poon *et al.*[9] han sugerido una posible nueva vía de transmisión de señales en el asma grave, independiente de la IL-4, con la participación de IL-5 e IL-33, ya que esta última parece ser capaz de inducir la diferenciación de linfocitos T vírgenes a linfocitos Th2, productores, principalmente, de IL-5. Se ha especulado que esta vía podría estar más relacionada con el asma eosinófila grave no atópica de inicio tardío.

En los pacientes con asma grave se han encontrado concentraciones elevadas de IFN-γ durante las exacerbaciones; además, las células subepiteliales de los pacientes con asma grave pueden expresar más cantidad de IFN-g que las de los que presentan asma moderada. Asimismo, se ha observado que el porcentaje de linfocitos CD8 productores de INF-g en sangre periférica se correlaciona con la gravedad del asma, el recuento de eosinófilos y la hiperrespuesta bronquial. Otra citocina implicada es la IL-18, también denominada *factor inductor de INF-*γ*.* Se ha observado que la IL-18 es capaz de inducir la producción de citocinas Th1 y Th2 por los linfocitos Th1[9]. Se ha considerado que IL-18/INF-γ se relacionan más con la gravedad del asma en sí misma que con formas concretas de asma grave.

4.2.2.2. Quimiocinas

Las quimiocinas actúan directamente en el proceso de extravasación y migración de los leucocitos. La quimiocina CCL5 es la principal quimiocina con efecto quimiotáctico de los eosinófilos encontrada en el lavado broncoalveolar (LBA) de los pacientes asmáticos expuestos a los alérgenos. Junto con CCL3 (MIP-1α) o CCL2 (MCP-1), tras una sensibilización con un alérgeno, las concentraciones de CCL5 se mantienen elevadas durante 4 h en el LBA y vuelven a los valores basales en unas 24 h. No se han

observado diferencias significativas respecto a la producción de CCL5 en pacientes con asma grave ni leve, incluso tras la estimulación *in vitro* con un lipopolisacárido (LPS) e inhibición con dexametasona[15]. No obstante, en un estudio más reciente, se sugiere que la CCL5 puede ser un potencial biomarcador para monitorizar la gravedad del asma, así como un biomarcador no invasivo de obstrucción de las vías respiratorias[17].

Una de las primeras quimiocinas que se relacionaron con el asma fue la eotaxina, de la que se ha observado un incremento de su expresión en el asma atópica. Esta quimiocina induce desgranulación de los eosinófilos y de los basófilos independiente de la IgE. Existen tres variantes, CCL11 (eotaxina-1), CCL24 (eotaxina-2) y CCL26 (eotaxina-3), que, aunque estructuralmente diferentes, actúan específicamente como una quimiotaxina de eosinófilos, que lo hace junto a la IL-5. Sin embargo, hasta el momento no se ha encontrado una asociación específica con el asma grave[8,18]. Algo parecido sucede con las quimiocinas de los monocitos (MCP), que son capaces de inducir la migración de los monocitos, aunque también ejercen su efecto sobre los eosinófilos y basófilos. Dentro de un modelo de asma alérgica, junto con las citocinas Th2, los homólogos múridos de la IL-8 (KC e MIP-2) modulan la respuesta inflamatoria pulmonar y la producción de IgE, con lo que actúan sobre el desarrollo de la hiperrespuesta bronquial. De este modo, la IL-8 parece tener importancia en la fisiopatología del asma; así, *in vitro,* se ha observado que los pacientes con asma grave producen más IL-8 que aquéllos con asma leve en respuesta a la dexametasona[15]. Asimismo, se ha evidenciado un incremento de las concentraciones de IL-8 en el esputo de los pacientes con asma grave[19].

Por tanto, parece existir un patrón de producción de citocinas y quimiocinas en los pacientes con asma, como se refleja en la figura 4-1, si bien aún faltan datos que permitan establecer claramente las diferencias entre asma leve y grave.

4.2.2.3. Neurotrofinas

Las neurotrofinas (también denominadas *factores neurotróficos*) son péptidos identificados como factores de crecimiento esenciales para la diferenciación, supervivencia y plasticidad neuronales. Cada vez son más los datos que indican que estas moléculas ejercen sus efectos en diversos tipos de tejidos[11]. Se ha demostrado que no sólo el factor de crecimiento nervioso (NGF, del inglés *nerve growth factor*) sino también el factor neurotrófico derivado del cerebro (BDNF, del inglés *brain-derived neurotrophic factor*) tienen importancia en el asma, aunque, hasta ahora, sólo se ha relacionado el BDNF directamente con el asma grave, más concretamente, con la forma grave en niños[18]. Aún no están bien definidos los mecanismos responsables de la interacción entre el sistema inmunitario y el sistema nervioso periférico en la patogenia del asma, pero hay indicios rigurosos que señalan que las neurotrofinas pueden desempeñar un papel importante en la regulación de este proceso[11]. Las neurotrofinas están presentes de forma natural tanto en el epitelio de las vías respiratorias como en los macrófagos alveolares e intersticiales[20], en los monocitos, los eosinófilos, y los linfocitos B y T.

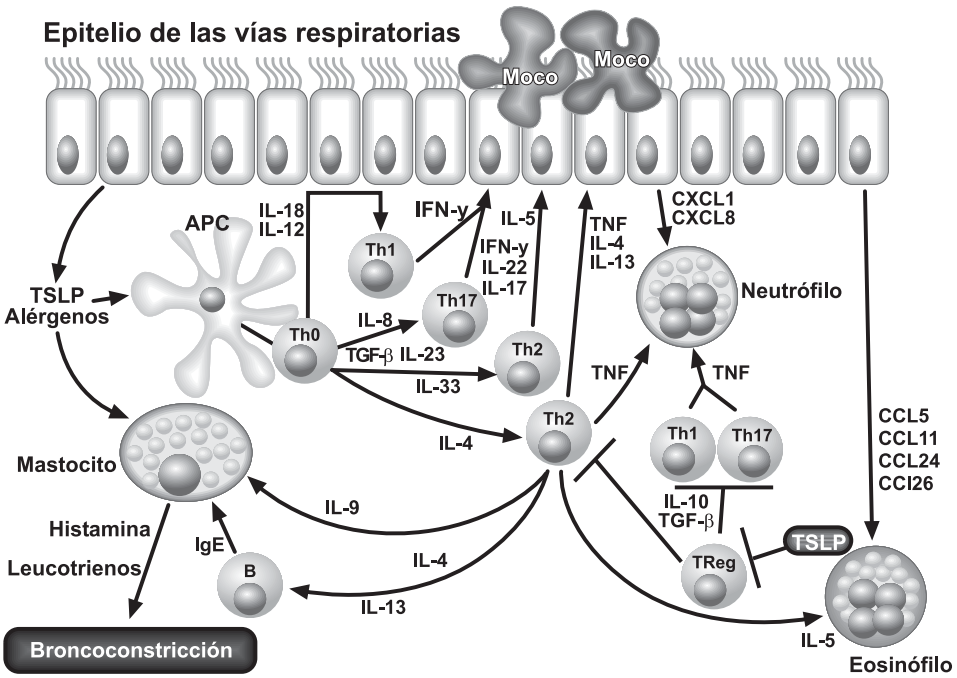

Figura 4-1. Principales citocinas y quimiocinas implicadas en el asma grave. Los linfocitos Th2 tienen una gran importancia en la regulación de la respuesta inflamatoria e incluso, según datos recientes, pueden actuar sin la influencia de la interleucina (IL) 4. Además, en el asma grave se activan los linfocitos Th1 y Th17 y puede aparecer un infiltrado neutrófilo, además del eosinófilo. Las células epiteliales no son agentes pasivos en el proceso, sino que producen quimiocinas y citocinas propias, como la linfopoyetina del estroma tímico (TSLP), contribuyendo así al desarrollo de la enfermedad. CPA, celulas presentadoras de antígenos; IFN-γ, interferón gamma; TGF-β, factor de crecimiento tumoral beta; TNF-α, factor de necrosis tumoral alfa; Treg, linfocitos T reguladores.

Estos factores neurotróficos pueden influir sobre la inflamación alérgica de diferentes formas, como a través del reclutamiento local de células efectoras (por ejemplo, eosinófilos y mastocitos) o de la activación y supervivencia de estas células en las vías respiratorias[20]. Además, también pueden influir mediante la inflamación neurogénica que se produce durante la que acompaña a la alergia[20]. Se ha sugerido que el BDNF actúa como mediador en la patogenia de la hiperrespuesta de las vías respiratorias y, consecuentemente, en la limitación del flujo aéreo, y se han observado concentraciones elevadas del mismo en los pacientes asmáticos, tanto en el LBA como en suero o plasma. Otros estudios resaltan la importancia del BDNF y del receptor específico del BDNF (TrkB) en el aparato respiratorio[21]; además, los corticoesteroides pueden regular la activación de TrkB y reducir significativamente las concentraciones de BDNF.

4.3. ASPECTOS GENÉTICOS DEL ASMA GRAVE

Aunque se conoce la importancia de los factores genéticos en el asma, en la actualidad no se sabe bien cómo influyen éstos en el desarrollo de la enfermedad y en su gravedad.

4.3.1. Genes asociados al asma grave

Como ya se ha comentado, el asma es una enfermedad multifactorial y su manifestación depende de la expresión de múltiples genes y de sus interacciones con los factores medioambientales, lo que complica aún más la comprensión de los diferentes mecanismos implicados. Se ha identificado la asociación de múltiples polimorfismos con la susceptibilidad al asma[22-24]; sin embargo, son escasos los estudios que muestran una asociación con su gravedad. Diversos ensayos han tratado de identificar polimorfismos en los genes que codifican citocinas; entre ellos, se ha detectado la asociación con polimorfismos en el gen *IL1RA,* tanto en población pediátrica como en adultos, en los genes que codifican IL4 y su receptor, y en los genes *IL12* e *IL18*[25]; sin embargo, estos resultados son controvertidos.

La vía de los leucotrienos también ha sido vinculada con la gravedad de asma, en concreto en relación con los genes *LTC4S* y *CYSLTR1* en población pediátrica[26]. Estudios más recientes han identificado otros genes asociados con la gravedad asma, como es el caso del gen de la fibrosis quística *CFTR* (del inglés *cystic fibrosis transmembrane conductance regulator,* 'regulador de la conductancia transmembranosa de la fibrosis quística'), que se ha relacionado con el asma grave en niños de la India, o el del gen *ADAM33* (del inglés *A disintegrin and metalloproteinase domain 33,* 'metaloproteinasa y desintegrina 33'), cuyas variantes se han asociado tanto con la gravedad como con la susceptibilidad al asma.

Considerando la importancia de las citocinas Th2 en el asma alérgica, diversos estudios han mostrado una relación de ésta con genes implicados en la respuesta Th2 y los niveles de IgE[27]. Koster *et al.*[27] observaron que la variante T2206C de *FCER2*, de la que ya se había establecido su asociación con un incremento en los niveles de IgE, se relacionaba también con una menor eficacia del tratamiento esteroideo y con las exacerbaciones. Sin embargo, algunos autores sugieren que la asociación con la gravedad del asma es independiente de la relación con la susceptibilidad a padecer asma. Es el caso de Zhang *et al.*[28], quienes describieron, en una población china, que los polimorfismos de *TLR4* estarían relacionados exclusivamente con la gravedad del asma pero no con la susceptibilidad al asma, o el del SNP-675 4G/5G del gen *SERPINE1,* que no se asociaría a la susceptibilidad a desarrollar asma pero sí con la gravedad y con la progresión de la respuesta a los corticoesteroides inhalados a largo plazo.

Estudios realizados en linfocitos CD4 han relacionado la vitamina D con reversibilidad de la resistencia al tratamiento con corticoesteroides[29]. Sobre esta premisa, se

ha observado una relación entre los polimorfismos del gen del receptor de la vitamina D y el asma, así como con el gen *CRTAM* (del inglés *class I MHC-restricted T cell-associated molecule gene,* 'linfocitos T restringidos por la molécula del complejo principal de histocompatibilidad de clase I'), durante las exacerbaciones del asma en niños. Otros genes que se han asociado con la gravedad de asma en distintas poblaciones son *MCP-1, PDGFRA, CTTN, MYLK, PHF11, RANTES* o *CRTH2;* sin embargo, muchas de estas asociaciones aún no han sido confirmadas en otras poblaciones.

En relación con las neurotrofinas, cada vez se está relacionando más más la neurotrofina BDNF con la patogenia del asma y, consecuentemente, se ha intentado identificar la base genética de esta asociación. En un reciente estudio realizado en niños germanos, se sugirió que los polimorfismos del gen *BDNF* se asociarían con la gravedad del asma[30]. No obstante, existe cierta controversia[31], lo que implica la necesidad de realizar más ensayos para establecer el peso del factor genético en la relación entre el BDNF y el asma.

La reciente aplicación de las técnicas de análisis genético de alto rendimiento, como los estudios de asociación pangenómica (GWAS) (del inglés *genome wide association study*), no ha permitido detectar nuevos polimorfismos específicos relacionados con la gravedad de asma, aunque sí confirmar asociaciones descritas previamente para la susceptibilidad de presentar asma, como los *loci* ORMDL3/GSDMB e IL1RL1/IL18R1[32]. Aunque, como se ha indicado antes, no se dispone de muchos estudios de polimorfismos relacionados exclusivamente con el asma grave, los estudios de polimorfismos asociados con el asma son susceptibles de poder ser utilizados como base para futuros estudios sobre la gravedad del asma, como puede ser el caso de los genes relacionados con la respuesta Th2, con la inmunidad de mucosas o con la función pulmonar (fig. 4-2).

4.3.2. Farmacogenómica

El asma es una patología de base poligénica, por lo que la farmacogenómica puede tener mucho que decir en relación tanto con formas más eficaces de tratamiento como con nuevos abordajes terapéuticos, al buscar terapias cada vez más específicas, individualizadas y precisas, así como con menos efectos secundarios.

En este sentido, el efecto antiinflamatorio de los corticoesteroides constituye uno de los principales métodos para tratar y controlar el asma. La respuesta a estos fármacos es variable, lo cual puede deberse a múltiples causas[15,16]; en concreto, los genes relacionados con los receptores glucocorticoideos (RG) son dianas farmacogenéticas muy interesantes. Así, en un reciente estudio se ha detectado que el polimorfismo N363S del *NR3C1* (gen del RG) se asociaba tanto con la susceptibilidad de presentar asma como con el desarrollo de formas moderadas, graves y no controladas de la enfermedad[33]. También se ha identificado una variante del gen *GLCC1* (del inglés *glucocorticoid-induced transcript 1*) asociada a la disminución de la respuesta a los corticoesteroides inhalados. Del mis-

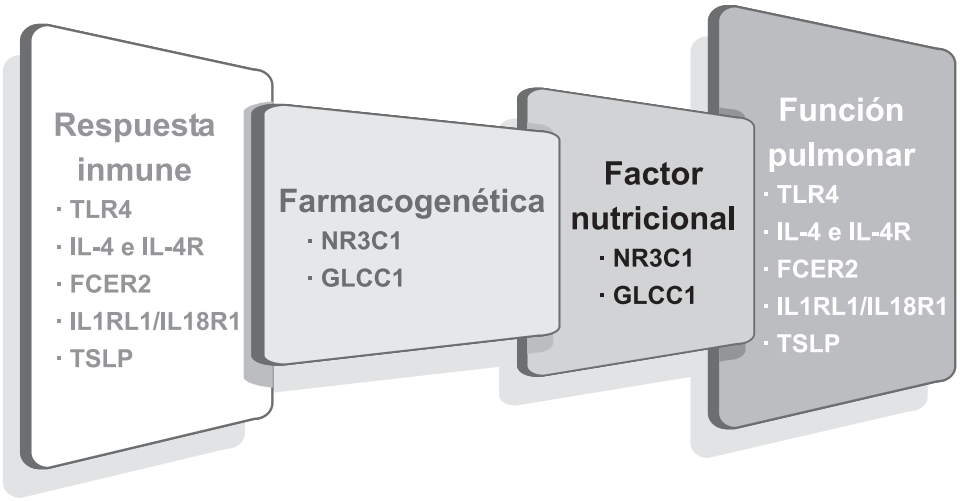

Figura 4-2. Posibles genes implicados en el asma grave[9,25-30,32-36].

mo modo, se ha demostrado la importancia del gen *FCER2* en relación con la respuesta a los corticoesteroides, especialmente en lo que respecta a las exacerbaciones graves[9].

Otra de las asociaciones farmacogenéticas significativas es la relacionada con el receptor de IL-4; las variantes génicas de *IL4RA* han permitido identificar a un grupo de pacientes con asma de moderada a grave con mejor respuesta al tratamiento con antagonistas de IL4RA. Especial consideración merece el gen que codifica TNF-α, ya que en individuos con asma grave se han detectado concentraciones de este factor significativamente superiores que explicarían la mejor respuesta al tratamiento con antagonistas del TNF-α. Se ha propuesto que podría deberse a la coexistencia del asma con otras enfermedades inflamatorias que incrementan la actividad del TNF-α o a variaciones en el gen que codifica esta citocina o alguna de las moléculas que participan en su procesamiento y liberación[34]. Por último, estudios realizados en biopsia bronquial de pacientes con distintos grados de gravedad de asma han permitido identificar una asociación de los grados de expresión de los genes *ADRB2, p-CREB, TSLP, ADAM 33* y *ADAM8* con la gravedad de asma[35,36].

Como se ha comentado previamente no son muchos los estudios centrados en las bases genéticas de la gravedad del asma y, con frecuencia, se caracterizan por presentar discrepancias en las asociaciones, lo que dificulta la identificación de nuevas dianas terapéuticas. Estas controversias pueden ser debidas tanto a aspectos étnicos como a otros relacionados con la calidad de los análisis de genotipificación, con los criterios estadísticos aplicados y, muy especialmente, con los criterios de inclusión. En definitiva, se precisa una exhaustiva caracterización fenotípica tanto celular como molecular basada en protocolos estandarizados que permita abordar con éxito los aspectos genéticos que subyacen en el asma grave.

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