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Regis Mariano de Andrade

A Imunologia da infecção pelo HIV em pacientes com idade avançada: caracterização fenotípica e funcional da resposta imune mediada pela célula T $CD4^+$

Rio de Janeiro

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-graduação em Ciências Médicas da Universidade do Estado do Rio de Janeiro.

Orientador: Prof. Dr. Arnaldo Feitosa Braga de Andrade

Coorientadora: Prof^{a}. Dra. Cleonice Alves de Melo Bento

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Rio de Janeiro

2012

DEDICATÓRIA

À minha Família, a quem pertence o mérito por tudo que faço e tudo que sou...

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RESUMO

ANDRADE, Regis Mariano. *A imunologia da infecção pelo HIV em pacientes com idade avançada*: caracterização fenotípica e funcional da resposta imune mediada pela célula T $CD4^+$. 2012. 95 f. Tese (Doutorado em Ciências Médicas) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2012.

A proporção de idosos portadores da síndrome da imunodeficiência adquirida (aids) tem aumentado de maneira importante nos últimos anos e, até a presente data, existem poucos estudos que abordam a infecção nessa população especial. As particularidades imunológicas decorrentes do fenômeno da imunossenescência podem acarretar mudanças significativas na evolução da infecção pelo HIV, bem como na resposta ao tratamento. O objetivo maior desta Tese foi avaliar o impacto da idade na recuperação funcional do sistema imune de pacientes com aids acima de 55 anos, quando tratados adequadamente com terapia anti-retroviral, caracterizando a resultante imunológica da idade avançada e da infecção pelo HIV. Para tanto, foram estudados quatro grupos experimentais: indivíduos jovens saudáveis ou com aids, e indivíduos acima de 55 anos saudáveis ou com aids. Todos os pacientes com aids estavam recebendo terapia anti-retroviral, em sucesso terapêutico. No primeiro artigo apresentado, avaliamos resposta linfoproliferativa e produção de citocinas *in vitro* e resposta humoral *in vivo* mediante desafio antigênico com toxóide tetânico (TT) em indivíduos previamente vacinados contra o tétano. Os resultados mostraram deficiências imunológicas significativas relacionadas à idade avançada no que diz respeito a produção de IgG anti-TT, resposta linfoproliferativa e produção de IFN-γ. Em contrapartida, a produção de IL-10 foi significativamente maior nos indivíduos acima de 55 anos, infectados ou não pelo HIV. No segundo artigo, foram caracterizadas as subpopulações de células T mediante estímulo policlonal ou específico com antígenos do envelope do HIV (Env). Em culturas não-estimuladas de PBMC do grupo com aids e idade avançada, observamos frequência reduzida de células T naive e de memória central, associada a aumento de células T efetoras. Quando estimuladas policlonalmente, essas culturas apresentaram deficiência na produção de IFN-γ e hiperprodução de IL-10, como na resposta ao TT. Mediante estímulo específico com Env, a citometria de fluxo revelou frequência elevada de células T $CD4^+FoxP3^-CD152^+$ com forte marcação intracelular para IL-10, indicando predomínio do fenótipo Tr-1, e não das células Treg clássicas. Interessantemente, em ambos os artigos, a replicação viral *in vitro* foi significativamente menor nos pacientes com aids acima de 55 anos, condizendo com a excelente resposta virológica desses pacientes ao tratamento antirretroviral. A neutralização da IL-10 com anticorpo anti-IL-10 nas culturas ativadas pelos peptídeos Env aumentou de forma significativa a replicação viral no sobrenadante. Tanto na resposta ao TT quanto aos peptídeos Env, o bloqueio da IL-10 aumentou os níveis de citocinas pró-inflamatórias, mas não melhorou a produção de IFN-γ dos pacientes acima de 55 anos com aids. Coletivamente, os achados dessa Tese revelam distúrbios em vários segmentos da resposta imune, particularmente no compartimento Th1, de pacientes acima 55 anos com aids e adequadamente tratados, sugerindo que, para esses pacientes, a reconstituição imune pós-tratamento não ocorre com a mesma eficácia que no jovem. Apesar do aumento da produção de IL-10 provavelmente contribuir, ao menos em parte, para o controle virológico, pode comprometer a resposta tanto ao próprio HIV, quanto a outros desafios antigênicos, a exemplo do toxóide tetânico. Sugere-se, portanto, a necessidade de recomendações específicas de manejo clínico para esse grupo de pacientes.

Palavras-chave: HIV/aids. Idade avançada. Célula $TCD4^+$. Fenótipo Tr-1. Reconstituição imune.

ABSTRACT

The proportion of aged persons living with the acquired immunodeficiency syndrome (aids) has importantly increased in recent years and, up to the present moment, there are few studies that address the infection in this particular population. The immunological nuances resulting from the immunosenescence phenomenon may promote significant alterations in the clinical course of HIV infection, as well as in treatment response. The major purpose of this Thesis was to evaluate the impact of age on the functional immune recovery in aids patients aged more than 55 years, when adequately treated with anti-retroviral therapy, characterizing the immunological result of advanced age and HIV infection. Thus, four experimental groups were enrolled: healthy or HIV-infected young adults, and healthy or HIV-infected adults over 55 years old. All the HIV-infected patients had diagnosis of aids and were under anti-retroviral treatment with therapeutic success. In the first presented article, we evaluated the lymphoproliferative response and cytokine production *in vitro* and humoral response *in vivo*, after antigenic challenge with tetanus toxoid (TT) in previously immunized individuals against tetanus. The results revealed significant age-related immunological impairments concerning anti-TT IgG production, lymphoproliferative response and production of IFN-γ. On the other hand, the production of IL-10 significantly higher in individuals aged more the 55 years, HIV-infected or not. In the second article, T cell subsets were characterized after polyclonal activation or specific stimulus with antigens derived from the HIV envelope (Env). In fresh unstimulated PBMC cultures obtained from the aged aids patients, there was a reduced frequency of naïve and central memory T cells, associated with increased frequency of effector T cells. When polyclonally stimulated, these cultures showed deficient production of IFN-γ and hyperproduction of IL-10, like in response to TT. In Env-stimulated cultures, flow cytometry revealed high frequency of T $CD4^{+}FoxP3^{-}CD152^{+}$ T cells with strong intracellular staining for IL-10, indicating a dominant Tr-1 phenotype, and not the classical Treg cells. Interestingly, in both articles, the viral replication *in vitro* was significantly lower aids patients over 55 years old, which is in consonance with their excellent virological response to anti-retroviral treatment. IL-10 neutralization with anti-IL-10 antibody in Env-activated cultures enhanced the viral replication in culture supernantants. Both in TT and in Env-peptides-stimulated cultures, the IL-10 blockade enhanced the levels of pro-inflammatory cytokines, but it did not improve IFN-γ production from aged aids patients. Altogether, the results reported in this Thesis reveal disturbances in several segments of the immune response, particularly in the Th1 compartment, of anti-retroviral-treated aids patients older than 55 years, suggesting that, for these patients, immune reconstitution after treatment does not occur with the same efficacy as in young patients. And although the enhanced IL-10 production probably contributes, at least in part, to the virological control, it can compromise the immune response both to HIV and to other antigenic challenges, such as tetanus toxoid. It is suggested, therefore, the need for specific recommendations regarding the clinical management of these patients.

Keywords: HIV/aids. Advanced age. $CD4^{+}$ T cells. Tr-1 phenotype. Immune reconstitution.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

ABV	Abacavir
ADCC	Antibody-dependent cell citotoxicity
AICD	Activation-induced cell death
APC	Antigen presenting cell
APV	Amprenavir
ATV	Atazanavir
AZT	Zidovidina ou azidotimidina
BCR	B cell receptor
CD	Cluster of differentiation
CD40L	Ligante do CD40
CD62L	Ligante do CD62
CDC	Centers for Disease Control and Prevention
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen-4
CVP	Carga viral plasmática
D4T	Estavudina
DC	Dentritic cell
DDI	Didanosina
DRV	Darunavir
EFZ	Efavirenz
ELISA	Enzyme-linked immunesorbent assay
ETV	Etravirina
FasL	Liagnte de Fas
fos-APV	Fosamprenavir
FoxP3	Foxhead box P3
FTC	Entricitabina
GITR	Glucocorticoid induced TNF receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HAART	Highly active anti-retroviral therapy
HIV	Vírus da Imunodeficiência Humana
HLA-DR	Human leukocyte antigen-DR

HPA	Hipotálamo-pituitária-adrenal ou Hipotálamo-hipófise-adrenal
IDV	Indinavir
IFN-γ	Interferon-gama
IL-	Interleucina
IL-1β	Interleucina 1 beta
IP	Inibidores de protease
iTreg	Treg induzida
ITRN	Inibidores de transcriptase reversa análogos de nucleosídeos
ITRNN	Inibidores de transcriptase reversa não-análogos de nucleosídeos
LAG-3	Lymphocyte activation gene-3
LPV	Lopinavir
MHC	Major histocompatibility complex
NK	Natural killer
nTreg	T reguladora natural
NVP	Nevirapina
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death-1
PHA	Fitohemaglutina
PMA	Phorbol 12-myristate 13-acetate
ppHIV-1env	Peptídeos do envelope do HIV-1
RNA	Ácido ribonucleico
ROR-γt	Retinoic acid-related orphan receptor γt
RT-PCR	Reverse transcription polymerase chain reaction
RTV	Ritonavir
S1PR1	Receptor 1 de esfingosina-1-fosfato
SIV	Simian immunodeficiency virus
SQV	Saquinavir
TARV	Terapia antirretroviral
3TC	Lamivudina
TCR	T cell receptor
TDF	Tenofovir
Tfh	Follicular helper T
TGF-β	Transforming growth factor-beta

Th	T helper
TNF-α	Tumor necrosis factor-alfa
Tr-1	T reguladora-1
TRECs	T cell receptor excision circles
Treg	T reguladora
TT	Toxóide tetânico
ZDV	Zidovudina ou azidotimidina

SUMÁRIO

INTRODUÇÃO

A Síndrome da Imunodeficiência Adquirida (aids) continua prevalente no mundo inteiro, sem uma cura conhecida. No entanto, a evolução da terapia antirretroviral, capaz de controlar a replicação do vírus, tem aumentado sensivelmente a longevidade da população infectada. Também tem sido observado o aumento da incidência entre idosos, o que contribui para a mudança do perfil epidemiológico da infecção em direção a faixas etárias avançadas, criando a evidente necessidade de estudos envolvendo idosos com aids (Mansky, 2010; Ministério da Saúde, 2012(1)).

A patogênese da infecção pelo HIV é totalmente vinculada à hiperativação crônica do sistema imunitário induzida pelo vírus, levando ao colapso desse sistema devido aos seus próprios mecanismos de auto-regulação (Appay, V. & Sauce, D. 2008). A célula T $CD4^+$, coordenadora da imunidade adaptativa, é a célula mais afetada na infecção pelo HIV e o principal mecanismo responsável pela sua depleção é a apoptose induzida pela hiperativação (AICD – *activation-induced cell death*) (Sodora & Silvestri 2008; Moir, Connors & Fauci 2010).

A terapia antirretroviral é capaz de reverter parcialmente a disfunção imune na aids, na medida em que controla a replicação viral, contendo assim a hiperestimulação imunitária, e, consequentemente, a imunossupressão dela decorrente (Autran, Carcelain & Debre, 2001). No entanto, a reconstituição imune mediante tratamento pode ser influenciada por fatores que alterem de alguma forma a fisiologia imune do indivíduo. Nesse contexto, o fenômeno da imunossenescência tem ganhado crescente importância. O sistema imune senil resulta de uma série de alterações como a involução tímica, a disfunção fenotípica com perda preferencial de Th1 e a redução percentual das células T virgens dando lugar a células em estágios mais terminais de diferenciação (Douek *et al.*, 1998, Gruver *et al.*, 2007; Dorshkind & Swain, 2009). Acredita-se que essas alterações são responsáveis pela progressão mais rápida da infecção nessa população.

No acompanhamento clínico-laboratorial desses pacientes após o início do tratamento, observa-se uma pouca recuperação numérica das células T $CD4^+$ e, paradoxalmente, rápida redução da carga viral plasmática (Grabar *et al.*, 2004; Viard *et al.*, 2001).

Um estudo publicado pelo nosso grupo em 2007 mostrou que indivíduos HIV-positivos de faixa etária avançada adequadamente tratados produzem grandes quantidades de IL-10, e que essa citocina possui clara correlação com o controle virológico *in vitro* (Andrade *et al.*, 2007, anexo 1).

A presente Tese é continuação desses estudos, objetivando a identificação e caracterização fenotípica detalhada da célula responsável por essa superprodução de IL-10 nos pacientes acima de 55 anos com aids adequadamente tratados, em diferentes contextos imunológicos.

1 **HIV E AIDS**

1.1 **Considerações gerais**

A Síndrome da Imunodeficiência Adquirida (aids) continua sendo um grave problema de saúde pública mundial, apesar do grande arsenal terapêutico disponível e da ampla difusão das suas formas de prevenção. A aids tornou-se pandêmica ao longo da década de 80 e assim persiste até os dias atuais, sem uma cura conhecida (Gottlieb *et al.*, 1981; Rozembaum *et al.*, 1982).

O agente etiológico, um retrovírus conhecido hoje como Vírus da Imunodeficiência Humana (HIV), foi isolado em 1983 pelo grupo chefiado por Luc Montagnier, do Instituto Pasteur, na França (Barré-Sinoussi *et al*.,1983). Dentre as peculiaridades do HIV, destacam-se sua capacidade em sofrer mutações constantes e sua predileção por infectar uma célula primordial do sistema imune: o linfócito T $CD4^+$ (Paranjape, 2005).

A morbimortalidade relacionada à infecção pelo HIV, em última instância, resulta da destruição numérica e funcional de componentes-chave do sistema imunitário, tornando o indivíduo suscetível a diversas doenças infecciosas e neoplasias oportunistas (Moir, Connors, & Fauci, 2010).

Os primeiros testes sorológicos para diagnóstico da infecção surgiram em 1985, e a primeira droga eficaz utilizada no tratamento, a zidovidina ou azidotimidina (AZT), teve seu uso aprovado em 1987. A partir de então, iniciou-se uma corrida, que continua até hoje, em busca da descoberta de novas opções terapêuticas e, em 1996, foi difundido o uso da terapia combinada, a que chamamos de terapia anti-retroviral de alta eficácia (HAART – *highly active anti-retroviral therapy*), que ainda é a principal arma no tratamento da doença. A HAART não leva à cura, mas pode prolongar por tempo indefinido a sobrevida do paciente (Graham *et al.*, 1991; Egger *et al.*, 2002).

Atualmente, existem mais de 40 milhões de pessoas vivendo com HIV no mundo, sendo cerca de 2/3 na África sub-saariana. No Brasil há mais de 500.000 casos notificados, sendo 80% nas regiões Sul e Sudeste. Embora a incidência da infecção tenha permanecido estável, a sua prevalência vem aumentando em virtude do aumento da expectativa de vida dos pacientes infectados, decorrente da evolução no arsenal terapêutico (Ministério da Saúde, 2012(1)). Essa melhora no tempo de sobrevida dos pacientes impacta diretamente no aumento da faixa etária da população infectada. A incidência também tem sido crescente em faixas etárias avançadas. O número de casos notificados de aids no Brasil acima de 50 anos passou de 2.345 em 1998 para 3.854 em 2010, ao contrário dos casos abaixo de 50 anos que apresentaram uma redução de cerca

de 30% ao longo desse mesmo período (Nguyen & Holodniy, 2008; Ministério da Saúde, 2012(1)). O gráfico abaixo, construído baseado em dados do boletim epidemiológico de 2012 do Ministério da Saúde, ilustra bem esse panorama no Brasil, mostrando a evolução de 1998 a 2008 no que se refere aos casos novos de aids diagnosticados. Estima-se, com base na população dos Estados Unidos, que em 2015 mais da metade dos pacientes infectados pelo HIV terá mais de 50 anos, denotando a importância epidemiológica crescente desse grupo de pacientes (Rickabaugh, & Jamieson, 2010; Mansky, 2010; Ministério da Saúde, 2012(1)).

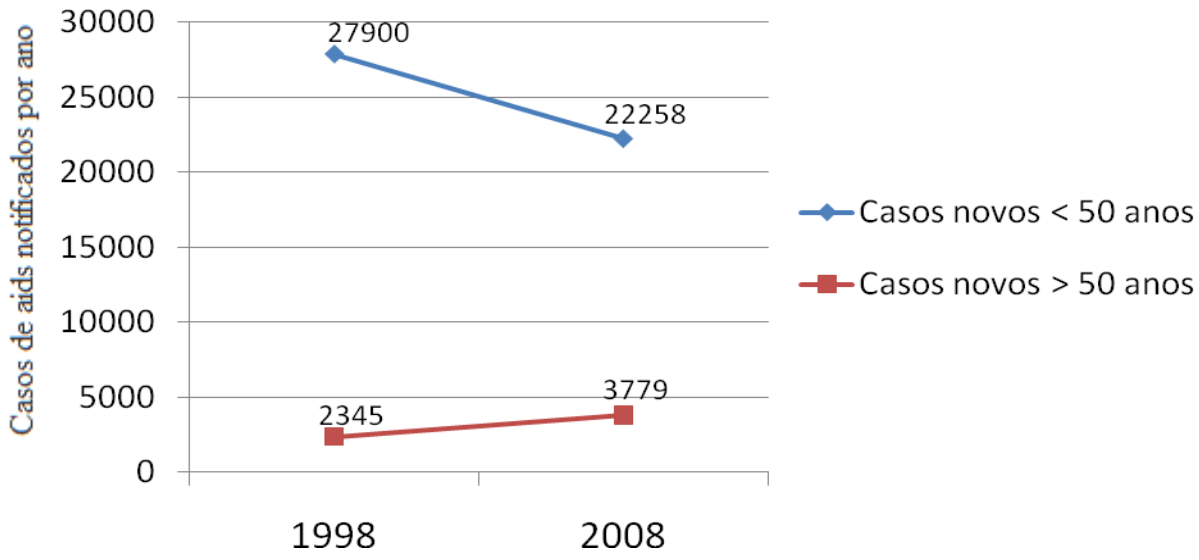

Figura 1. Evolução da incidência de casos de aids no Brasil por faixa etária de 1998 a 2008. A incidência global de aids ao longo de 10 anos reflete uma diminuição evolutiva importante do total de casos novos por ano, porém observa-se um aumento de mais de 40% quando consideramos somente a faixa etária maior de 50 anos.

1.2 **Aspectos clínicos**

A manifestação aguda da infecção pelo HIV, chamada síndrome retroviral aguda ou síndrome de soroconversão, quando clinicamente aparente, ocorre entre uma e seis semanas após o contágio, com pico de incidência na 3ª semana. Caracteriza-se por sinais e sintomas inespecíficos que podem ocorrer em graus variados, como por exemplo: febre baixa, adenomegalias em múltiplas cadeias linfonodais, faringite não-exsudativa, exantema maculopapular, adinamia, anorexia, cefaléia, diarreia e hepatoesplenomegalia. Em metade a 2/3 dos casos, a apresentação é como uma síndrome "mononucleose-like" (poliadenogalia e febre), que se resolve geralmente em cerca de 10 dias. Já foram relatados acometimentos orgânicos graves nessa fase, como encefalite pelo HIV e até doenças definidoras de aids, como pneumocistose, porém são eventos raros (Tindall *et al.*, 1988; Schacker *et al.*, 1996; Vanhems *et al.*, 2002).

Com o desenvolvimento da imunidade adaptativa, os mecanismos efetores celulares e humorais conseguem controlar temporariamente a infecção, sem, no entanto, eliminá-la. Inicia-se o período chamado de latência clínica, em que o paciente permanece assintomático por vários anos. Nesse período, as células T $CD4^+$ estão sendo progressivamente depletadas, tanto numericamente quanto funcionalmente, mas o prejuízo imunológico ainda é relativamente pequeno para provocar expressão clínica (Mellors *et al.*, 1997; Forsman & Weiss, 2008). (Fig. 2)

Após o período de latência, o indivíduo sem tratamento progride para a próxima fase clinicamente definida da infecção, chamada aids, que se caracteriza por uma imunodeficiência avançada em que o paciente fica altamente suscetível a doenças oportunistas potencialmente graves. Essa síndrome é acompanhada laboratorialmente por baixas contagens de células T $CD4^+$ e elevada carga viral plasmática (CVP) (Mellors *et al.*, 1997). Define-se o diagnóstico de aids nos pacientes que atingiram contagem de células T $CD4^+$ abaixo de 200 células/mL ou que apresentaram doenças definidoras da síndrome independente do nível de células $TCD4^+$ (Ministére de la Santé et des Sports, 2010; Ministério da Saúde, 2008). São exemplos de doenças definidoras de aids: pneumocistose, neurotoxoplasmose, criptococose extrapulmonar, tuberculose extrapulmonar, candidíase esofageana, citomegalovirose que não de órgãos linfoides, diarréia por *Critosporidium* sp. ou *Isospora* sp., leucoencefalopatia multifocal progressiva, sarcoma de Kaposi e linfoma da Burkitt (Sterling & Chaisson, 2010).

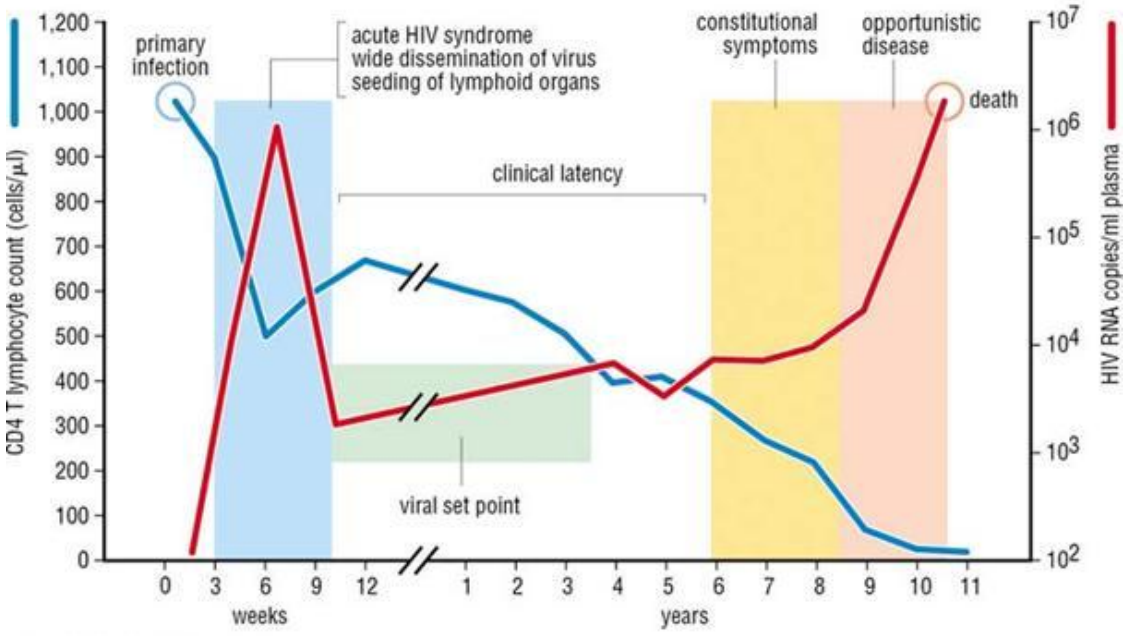

Figura 2. Curso natural da infecção pelo HIV *(Fonte: Forsman & Weiss, 2008)*. Representação gráfica dos 3 grandes estágios evolutivos da infecção pelo HIV. Na fase aguda, observa-se um pico de viremia associado à síndrome de soroconversão; a fase seguinte é o período de latência clínica, que pode durar vários anos, em que as células T $CD4^+$ são lentamente depletadas e o paciente evolui assintomático; por fim, inicia-se a fase caracterizada como aids, onde surgem os sinais e sintomas de imunodeficiência avançada.

1.3 **Diagnóstico e acompanhamento laboratoriais**

O diagnóstico laboratorial da infecção é feito classicamente através da positivação de 2 testes sorológicos de triagem (geralmente ELISA – *enzyme-linked immunesorbent assay*) e 1 confirmatório (*Western-Blot* ou imunofluorescência indireta). A viragem sorológica ocorre geralmente no período compreendido entre a 3ª e a 12ª semana após o contágio, mas considera-se habitualmente até 6 meses o período possível de ocorrer a soroconversão e, em alguns casos raros, até 1 ano (Ministério da Saúde, 2008; Maldarelli, 2005).

O acompanhamento laboratorial é feito, essencialmente, pela contagem das células T $CD4^+$ no sangue periférico do paciente e pela quantificação da CVP. O primeiro é o parâmetro mais importante para definir o momento de início de tratamento e profilaxias para infecções oportunistas, e o segundo assume papel principal no paciente que já está sob tratamento, para monitorar a eficácia da terapia em controlar da replicação viral (Ministério da Saúde, 2008).

1.4 **Terapia anti-retroviral**

Após divulgação, em agosto deste ano, da versão preliminar do consenso brasileiro de terapia antirretroviral (TARV) de 2012, orienta-se agora que o tratamento seja iniciado para todo paciente infectado pelo HIV cujos valores de células T $CD4^+$ no sangue estejam abaixo de 500 células/mL (Ministério da Saúde 2012(2)). Recomendações norte-americanas e francesas já orientavam este início precoce de tratamento desde 2010 (Thompson *et al.*, 2010; Ministére de la Santé et des Sports, 2010). Caso haja manifestações de imunossupressão avançada, também se recomenda seu início, independente da contagem de células T $CD4^+$ (Tsibris & Hirsch, 2010; Ministério da Saúde, 2008).

As três principais classes de anti-retrovirais utilizadas nos esquemas TARV são: 1) os inibidores de transcriptase reversa análogos de nucleosídeos/nucleotídeo (ITRN); 2) os inibidores de transcriptase reversa não-análogos de nucleosídeos (ITRNN) e 3) os inibidores de protease (IP). A combinação classicamente empregada de anti-retrovirais para início de tratamento consiste em dois ITRNs associados a 1 ITRNN ou a 1 IP. Hoje em dia, temos também disponíveis para uso clínico inibidor de co-receptor CCR5, inibidor de fusão, e inibidor de integrase viral (Riddler *et al.*, 2008; Thompson *et al.*, 2010) (Tabela 1).

Tabela 1. Grupos de anti-retrovirais utilizados na terapia anti-HIV atualmente autorizados pelo Ministério da Saúde do Brasil. *(Adaptado de: Ministério da Saúde, 2008*)

Inibidores da Transcriptase Reversa nucleosídicos (ITRN)	**Inibidores da Transcriptase Reversa não-nucleosídios (ITRNN)**	**Inibidores de Protease (IP)**	**Inibidor de Integrase**	**Inibidor de Fusão**	**Inibidores de Co-receptor**
Zidovudina (ZDV, AZT)	Nevirapina (NVP)	Saquinavir (SQV)	Raltegravir	Enfuvirtide	Maraviroc
Didanosina (DDI)	Efavirenz (EFZ)	Ritonavir (RTV)			Vicriviroc
Estavudina (D4T)	Etravirina (ETV)	Indinavir (IDV)			
Lamivudina (3TC)		Amprenavir (APV)			
Entricitabina (FTC)		Lopinavir (LPV)			
Tenofovir (TDF)		Atazanavir (ATV)			
Abacavir (ABV)		Darunavir (DRV)			
		Fosamprenavir (fos-APV)			

Os objetivos da TARV são promover a redução da CVP a níveis indetectáveis (<400 cópias/mL em até 6 meses e <50 cópias/mL em até 1 ano) e, na medida do possível, permitir a

recuperação da competência imunológica do indivíduo. Na prática clínica atual, entretanto, essa reconstituição imune é avaliada unicamente pela contagem de células T $CD4^+$ periféricas, ignorando-se a multiplicidade de fenótipos que esta célula pode apresentar, bem como a modulação funcional desses fenótipos por mecanismos diversos.

2 O SISTEMA IMUNE E A INFECÇÃO PELO HIV

2.1 O estabelecimento da imunidade adaptativa e a imunofisiologia da célula T CD4$^+$

Os linfócitos T são classificados sobretudo segundo a expressão de moléculas de superfície. Dessa forma, as duas subpopulações majoritárias são os linfócitos T CD4$^+$ e T CD8$^+$. Os linfócitos T CD4$^+$ são as células mais importantes do sistema imunitário, pois coordenam as ações de todos os demais componentes deste sistema, direta ou indiretamente.

As funções executadas pelos linfócitos T CD4$^+$, embora centrais na resposta imune, dependem de sinais por moléculas de superfície e solúveis liberados por células apresentadoras de antígenos (APC – *antigen presenting cells*), particularmente das células dendríticas (DCs, *dentritic cells*) (Mellman & Steinman, 2001).

As DCs são eficientes em fagocitar, processar e apresentar os antígenos aos linfócitos T. Em áreas ricas em células T de tecidos e órgãos linfóides, ocorre então o contato, chamado sinapse imune, da célula dendrítica com a célula T CD4$^+$ *naïve*, definida fenotipicamente pela positividade para os marcadores de superfície CD45RA, CD127, CD62L e CCR7. O CD62L tem um papel similar ao CCR7, que é direcionar as células T para os órgãos linfóides secundários, onde elas têm maior chance de detectar o antígeno específico apresentado pela APC e iniciar uma resposta adaptativa. A expressão do CD127, ou receptor da IL-7 (IL-7R), é observada em células T virgens ou de memória central, as quais requerem IL-7 para sua sobrevivência (Fukui *et al*., 1997; Boettler *et al*., 2006; Sasson, Zaunders & Kellerher, 2006).

Os principais eventos bioquímicos envolvidos no contato da APC com a célula T CD4$^+$ são a ligação do receptor de célula T (TCR – *T cell receptor*) ao complexo peptídeo-MHC de classe II (*major histocompatibility complex*), a ligação do CD28 com moléculas da família B7 (CD80 e CD86) e a ação autócrina da IL-2 produzida pela célula T CD4$^+$ (Dustin *et al.*, 2006; Henrickson & von Adrian, 2007). O êxito desses eventos determina a ativação eficaz da célula T CD4$^+$, que perde os marcadores citados acima e passa a expressar CD25 (cadeia α do receptor de IL-2), CD45RO (isoforma O do CD45R) e o CD69. Este último marcador é uma das moléculas mais precocemente expressas pela célula T CD4$^+$ ativada, e é perdida também precocemente, logo após a fase de expansão clonal. Sua função é impedir a saída precoce da célula T do órgão linfóide, para que os eventos de ativação/proliferação possam completar-se adequadamente (Nurmi *et al.*, 2007).

Outra molécula induzida precocemente na ativação é o CD40L, cuja expressão já pode se fazer presente ainda durante a sinapse da célula T com a célula apresentadora de antígeno, e

atingirá níveis elevados dentro de 1 a 2 dias, quase concomitante com o pico de expressão do CD25. Sua ligação ao CD40 presente na superfície das APCs amplifica a ativação destas e aumenta a expressão de membros da família B7 de moléculas co-estimuladoras, tornando-as APCs mais eficientes; e quando a APC é uma célula B, essa interação CD40-CD40L também amplifica a produção de anticorpos por essas células (Klaus *et al*, 1994).

Seguindo a expansão clonal mediada pela IL-2, o processo de ativação da célula T $CD4^+$ somente se completa quando esta célula sofre ação das citocinas produzidas principalmente pelas DCs, o que conduz finalmente os linfócitos T ativados à diferenciação em diferentes fenótipos capazes de secretar padrões diversos de citocinas que regulam e coordenam vários ramos da resposta imune (Fig. 3).

Uma vez que as células T $CD4^+$ tenham sido eficazmente ativadas e diferenciadas em diferentes fenótipos que descreveremos a seguir, elas devem sair do tecido linfoide onde ocorreu a ativação e migrar para os sítios onde são requisitadas. Esse evento é possibilitado pela re-expressão de S1PR1 (receptor 1 de esfingosina-1-fosfato) associada à perda das moléculas de membrana CCR7, CD62L e CD127. A meia-vida das células T efetoras, no entanto, é relativamente curta e dependente de citocinas produzidas principalmente nos sítios da agressão (Taylor & Jenkins, 2011).

Apesar da importância dessa expressão seletiva e temporal de diferentes moléculas de superfície e intracelulares no processo de ativação e diferenciação das células T, alguns marcadores, quando expressos em níveis elevados, denunciam um processo crônico e possivelmente patológico de ativação celular. Dentre esses marcadores, podemos exemplificar as moléculas HLA-DR e o CD38, cuja expressão persistente tem sido relacionada à morte celular por exaustão. Assim como ocorre com o linfócito T $CD8^+$, quanto maior a expressão dessas moléculas na superfície da célula T $CD4^+$, mais ativada ela estará, e consequentemente, mais próxima à morte por hiperativação. As células T em estágio terminal de ativação poderão expressar também as moléculas PD-1, Tim-3 e Fas (CD95), além de aumentar a expressão funcional do ligante de Fas (FasL). A ligação do PD-1 a um dos seus ligantes PD-L1 ou PD-L2, presentes nas células dendríticas, exerce um efeito inibitório na proliferação e na produção de citocinas por parte dos linfócitos T e, dependendo da magnitude com que isso ocorra, apoptose. A ligação do Fas, quando funcionalmente expresso na célula $TCD4^+$, ao seu ligante FasL também deflagrará apoptose (Alderson *et al.*, 1995; Day *et al.*, 2006; Jones *et al.*, 2008; Furler & Uittenbogaart, 2010).

Em conclusão, a ativação persistente, associada à produção dominante de perfis pró-inflamatórios de citocinas acarreta distúrbios na fisiologia imune relacionados à hiperativação. Esses distúrbios podem decorrer de falhas nos mecanismos de regulação da resposta imune

(principalmente disfunção das células T reguladoras) ou condições patológicas indutoras e perpetuadoras de ativação (como a infecção pelo HIV).

2.1.1 Os fenótipos da célula T $CD4^+$

Com o desenvolvimento da imunidade adaptativa, os linfócitos T $CD4^+$ ativados passam a coordenar praticamente todas as ações do sistema imune, e para tanto, podem assumir diferentes fenótipos de características funcionais distintas de acordo com o objetivo imunológico na ocasião, seja no combate a desafios antigênicos diversos, seja na manutenção ou recuperação da homeostase. O destino final dessa diferenciação é determinado principalmente pelo perfil de citocinas presente no microambiente da apresentação de antígeno.

A produção de IL-12 pelas DCs durante a apresentação de antígeno induz a diferenciação da célula T $CD4^+$ em linfócito T auxiliar tipo 1 (Th1 – *T helper 1*) (Fazilleau *et al.*, 2007; Henrickson & von Adrian, 2007). Esse fenótipo produz grandes quantidades de IFN-γ e IL-2 e é o grande responsável por coordenar a chamada resposta imune celular, ativando fagócitos, células assassinas naturais (NK – *natural killer*) e os linfócitos T $CD8^+$. Estes últimos, quando adquirem atividade citotóxica após ativação, são denominados linfócitos T citotóxicos (CTL – *cytotoxic T lymphocytes*) e são os principais responsáveis pelo controle das infecções virais, conduzindo as células infectadas à morte por apoptose através da degranulação de produtos citotóxicos (Coquerelle & Moser, 2010). O IFN-γ não apenas aumenta o poder microbicida dos fagócitos (neutrófilos e macrófagos) e a função citolítica das células NK e T $CD8^+$, como também induz linfócitos B humanos a produzirem IgG1 e IgG3 (Cavacini *et al.*, 2003). Ao contrário do que se pensava anteriormente, o fenótipo Th1 é também o maior responsável pela resposta humoral, pois essas classes de anticorpos por ele induzidas são as mais eficazes quanto às funções de opsonização, fixação de complemento e mediação de ADCC (*antibody-dependent cell citotoxicity*) (Cavacini *et al.*, 2003; McKinstry *et al.*, 2010). Portanto, as células Th1 são as coordenadoras da imunidade adaptativa contra a maioria dos patógenos conhecidos.

Na presença de níveis elevados de IL-4, os linfócitos $TCD4^+$ diferenciam-se em Th2, através da indução de um programa de diferenciação envolvendo o fator de transcrição GATA-3. Esses linfócitos produzem IL-4, IL-5, IL-6 e IL-13, promovendo a ativação dos linfócitos B produtores de IgE (Zhu, Yamane & Paul, 2010). A resposta dos linfócitos Th2 está envolvida no combate a infestações por helmintos e têm implicação imunopatológica importante nas reações de hipersensibilidade imediata (Makani *et al*, 2008; Zhu, Yamane & Paul, 2010).

Acreditava-se ser a célula Th2 a responsável por inibir a atividade da célula Th1 e vice-versa. Mas quando as células Th1 e Th2 foram analisadas mais detalhadamente e novas citocinas foram sendo identificadas, viu-se que a função da célula T $CD4^+$ ativada não estava restrita a essas 2 sub-populações, e foram descritos, até o presente momento, vários outros fenótipos.

A produção de IL-1β, IL-6 e a IL-23 pelas DCs têm sido implicadas na indução de Th17 em humanos, através da indução do fator de transcrição ROR-γt (ROR-γt – *retinoic acid-related orphan receptor γt*) (Gutcher & Becher, 2007). O Th-17 é considerado o extremo de fenótipo pró-inflamatório, diretamente envolvido em fenômenos de autoimunidade, como a inflamação sinovial na artrite reumatóide e as lesões cerebrais na esclerose múltipla, mas também possui função protetora importante no sistema imunitário (Kebir *et al*., 2007; Miossec, 2009; Matsuzaki & Umemura, 2007). As células Th17 secretam não apenas IL-17 (também chamada de IL-17A) como também IL-21, IL-22, TNF-α, IL-6 e IL-1β, que dentre suas muitas funções, destaca-se a capacidade de induzir a produção IL-8 em vários tecidos, principal quimiocina responsável pelo recrutamento de neutrófilos para a área de infecção. Ademais, as células Th17 também induzem os linfócitos B a secretarem diferentes isotipos de IgG (Annunziato *et al.*, 2007). A resposta imune mediada por células Th17 vem sendo fortemente associada ao combate a bactérias extracelulares e fungos (Matsuzaki & Umemura, 2007), e também há evidências sólidas de ação sinérgica do fenótipo Th17 com o Th1 na resposta a patógenos intracelulares (Awasthi & Kucho, 2009).

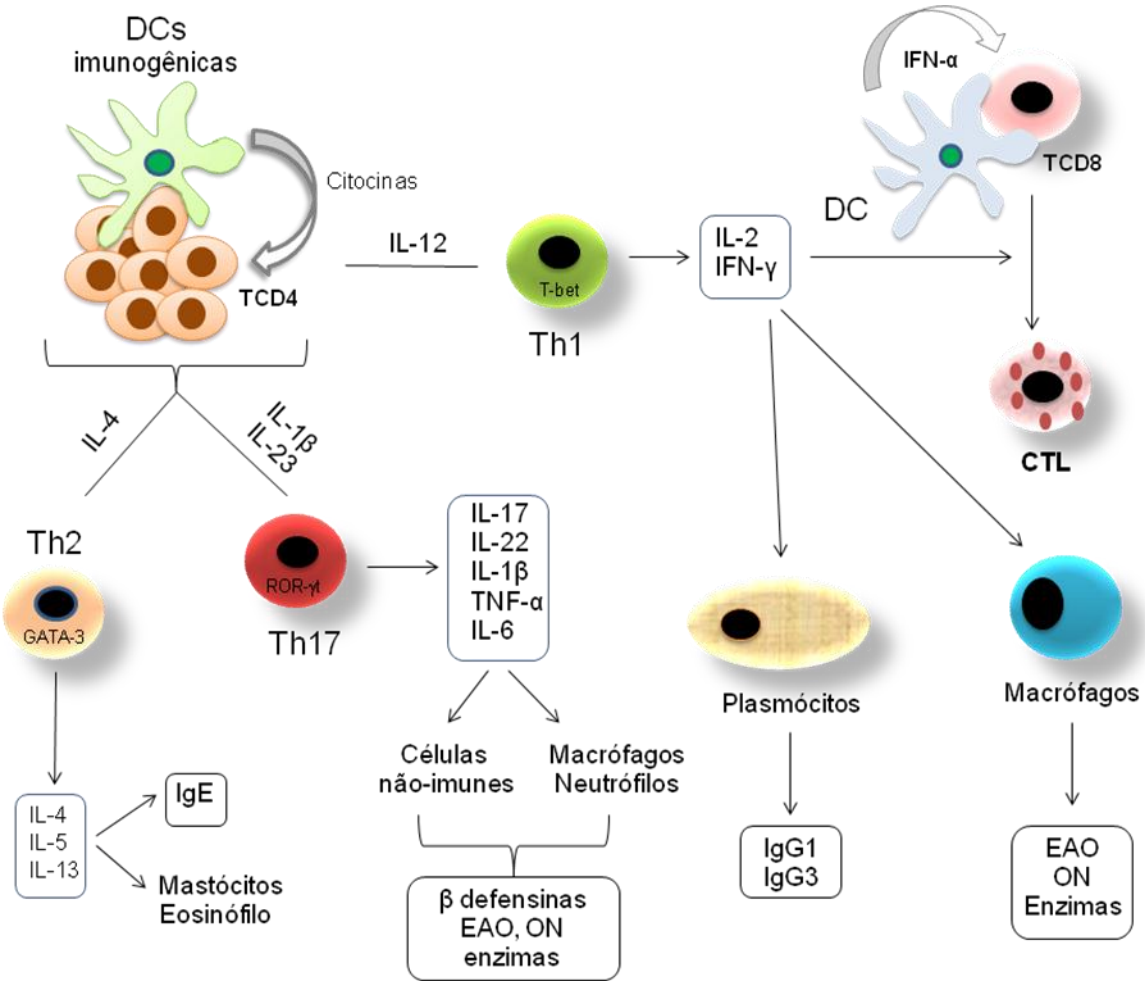

Figura 3. Ativação, diferenciação e função efetora das células T CD4^{+}. Representamos aqui os principais fenótipos efetores que a célula T CD4^{+} *naïve* pode assumir quando ativada por uma célula dendrítica imunogênica. A diferenciação em Th1 , Th2 ou Th17 dependerá do perfil de citocinas presentes no microambiente da ativação. A figura representa também as principais citocinas produzidas por esses fenótipos e seus mecanismos efetores.

No extremo oposto, as células Treg, Tr-1 e Th3 têm função reguladora da resposta imune, por inibirem vários mecanismos efetores e também a ação APC de células dendríticas (Takakubo & Kontinnen, 2012; Vignali *et al.*, 2008). Sua função de frear a resposta imune é fundamental para evitar danos imunomediados aos tecidos do hospedeiro, e até doenças auto-imunes (Vignali *et al.*, 2008; Costantino *et al.*, 2008).

A célula Treg, classicamente identificada pela presença intranuclear do fator de transcrição Foxhead box P3 (FoxP3), além de determinados marcadores de superfície, pode ser classificada em Treg natural (nTreg) ou Treg induzida (iTreg), sendo a primeira gerada no timo pelo contato com antígeno próprio e a segunda gerada perifericamente a partir de uma célula T virgem quando ativada pela DC tolerogênica, na presença de TGF-β (Fantini *et al.*, 2004; Tai *et al.*, 2008). Seu perfil característico de moléculas de superfície é: CD45RO, CD62L, CD25^{high}, GITR (GITR – *glucocorticoid induced TNF receptor*) (Shevach *et al.*, 2006) associada à ausência do receptor para

IL-7 (CD127) (Liu *et al.*, 2006). Células Treg ativadas produzem TGF-β, IL-10 e IL-35 (Dieckmann *et al.*, 2001) e suprimem proliferação e função das células T $CD4^+$ e T $CD8^+$ não só de forma parácrina, através do efeito inibitório dessas citocinas, mas principalmente através de contato, ocasionando: apoptose de célula T efetora (via granzima A), interrupção do ciclo celular (via galectina-1), inibição funcional da apresentação de antígeno (via CTLA-4 e LAG-3) (Piccirillo & Shevach, 2001; Grohmann *et al.*, 2002; Wing *et al.*, 2002; Fallarino *et al.*, 2003; Nakamura *et al.*, 2004; Shevach, 2009; Lee *et al.*, 2009).

O fenótipo Tr1 (célula T reguladora tipo 1), muitas vezes referida como célula reguladora FoxP3-negativa, é induzida por e grande produtora de IL-10 (Barrat *et al.*, 2002; Aluvihare *et al.*, 2004; Carpentier *et al.*, 2009). Esta citocina é uma potente inibidora dos mecanismos efetores da resposta imune celular, por inibir ativação de células do sistema fagocítico mononuclear e células NK (indiretamente), além de inibir os fenótipos Th1 e Th17 descritos anteriormente (Groux *et al.*, 1997; Strobl & Knapp, 1999). Até o momento, não existe um marcador de superfície que identifique a célula Tr-1.

O fenótipo Th3 não é considerado exclusivamente inibitório, pois possui participação na imunidade de mucosas ao induzir localmente a secreção de IgA por células B, importante componente das barreiras mucosas contra microrganismos. Por outro lado, entretanto, a grande quantidade de TGF-β produzida pela célula Th3 inibe ativação de células efetoras da imunidade inata (APCs e NK) e ativação e proliferação de células T efetoras $CD4^+$ e $CD8^+$. Recentemente, alguns trabalhos sugeriram as Treg induzidas podem representar na verdade a população de linfócitos Th3 (Xu *et al.*, 2010).

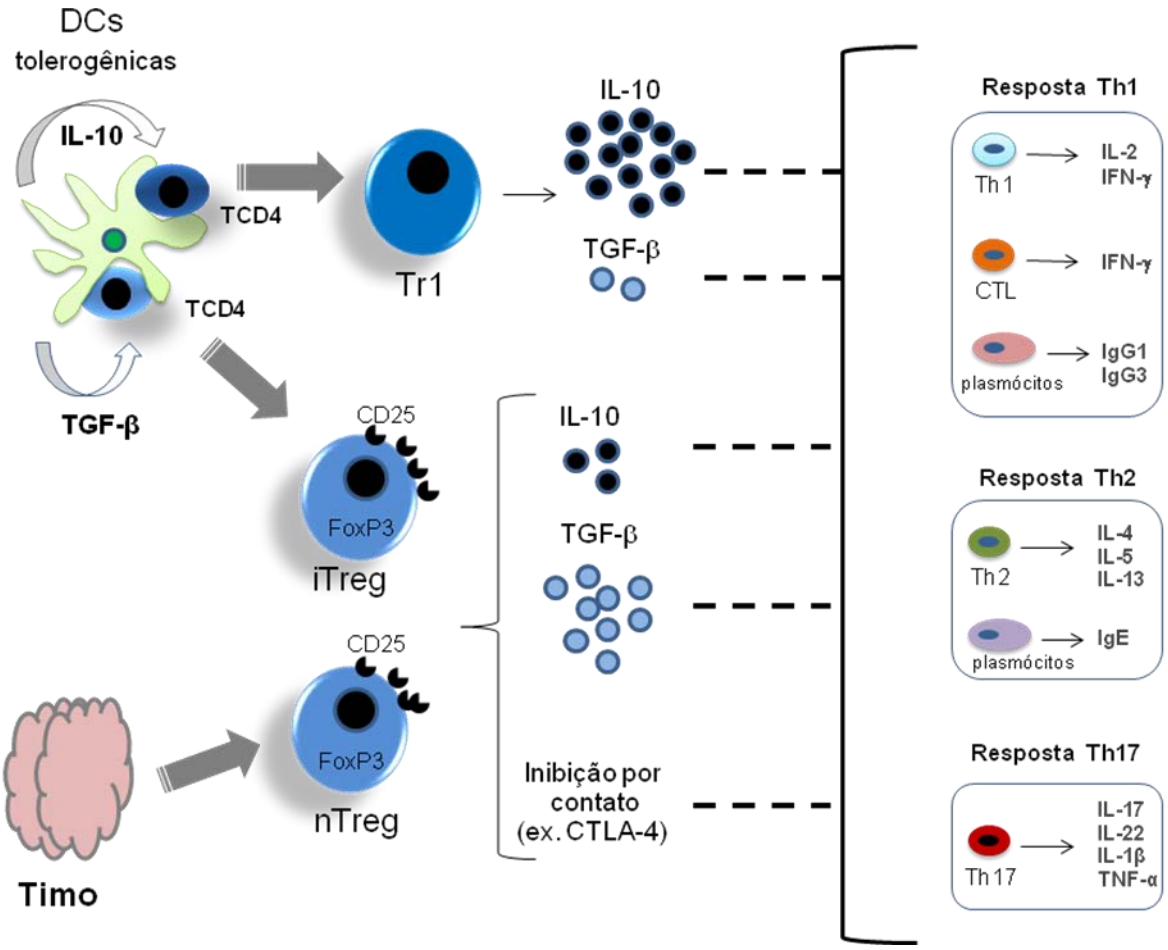

Figura 4. Regulação da resposta imune mediada pelas células T reguladoras. A figura representa as células reguladoras Tr1, Treg induzida (iTreg) e Treg natural (nTreg), sendo a última proveniente do timo e as duas primeiras geradas perifericamente. As citocinas por elas produzidas, bem como moléculas de superfície (no caso das Treg), exercem ação inibitória sobre os fenótipos efetores Th1, Th2 e Th17. A linha tracejada (– – – –) representa inibição.

Mais recentemente, pesquisadores propuseram a classificação de 3 novas subpopulações: Th6, Th9 e Th22, assim nomeadas com base na citocina principal que produzem, além da célula T helper follicular Tfh (*follicular helper T*). Porém, esses fenótipos ainda são relativamente pouco presentes na literatura, havendo muitas indagações sobre sua importância na fisiologia do sistema imune (Veldhoen *et al.*, 2008; Eyrich *et al.*, 2009; Azizi-Semrad *et al.*, 2010).

O padrão de citocinas produzidas pelo hospedeiro, ditada pelo predomínio de um ou outro subtipo de célula T $CD4^+$, constitui fator-chave para determinar o desfecho de qualquer doença infecciosa, na medida em que favorece ou não o tipo de resposta eficaz para o agente infeccioso em questão. E o fenótipo dominante normalmente se auto-amplifica, isto é, as citocinas por ele produzidas retroalimentam positivamente aquele padrão de resposta.

Diante de todos os fenótipos que a célula T $CD4^+$ pode assumir descritos até o momento, alguns inclusive com funções antagônicas entre si, torna-se claro que é uma enorme simplificação

avaliar o sistema imune do paciente HIV-positivo pela simples quantificação das células T $CD4^+$ no sangue periférico, como é feito atualmente no acompanhamento laboratorial desses pacientes (Lane, 2010).

2.2 **Distúrbios imunológicos na infecção pelo HIV: foco na célula T $CD4^+$**

A aids é inegavelmente uma doença do sistema imunitário, e como tal, deve ser estudada, compreendida e tratada à luz de suas bases imunopatológicas.

A infecção pelo HIV acomete vários setores do sistema imunitário, mas o maior impacto recai sobre a célula T $CD4^+$, que é a mais afetada e, ao mesmo tempo, a mais importante desse sistema devido à função de coordenar todos os outros componentes celulares do sistema imune. Dentre eles, citamos aqui em especial o linfócito T $CD8^+$ citotóxico (CTL), principal célula efetora na resposta anti-HIV através da citotoxicidade e produção de IFN-γ, mas que depende da cooperação com a célula T $CD4^+$, particularmente do fenótipo Th1 (Grossman *et al.*, 2006; Appay, 2008).

Ao contrário do que pensa a maioria dos profissionais de saúde que lidam com pacientes infectados, por se tratar de um vírus pouco lítico, a destruição direta das células T $CD4^+$ pelo HIV é um mecanismo relativamente pouco importante para a imunopatogênese da doença. Existem vários outros mecanismos de prejuízo numérico e principalmente funcional dos linfócitos T $CD4^+$ que não estão atrelados à infecção direta (Brenchley *et al*., 2004; Grossman *et al.*, 2006).

Já nas primeiras semanas após a infecção, período correspondente à síndrome retroviral aguda, observam-se não só alterações numéricas da célula T $CD4^+$ (redução do número absoluto), como também distúrbios funcionais identificados pelo aumento na frequência de células PD-1, CTLA-4 e Fas positivas associadas à uma queda na expressão dos níveis de CD127 (Streecka, van Bockelb, & Kelleher, 2008).

No curso da infecção, vários outros mecanismos diminuem a capacidade funcional e a sobrevida das células T $CD4^+$, a saber: 1) Destruição da célula T $CD4^+$ infectada pelo acúmulo de produtos tóxicos virais acumulados no citoplasma ou pela citotoxicidade mediada pelas células NK e T $CD8^+$; 2) Expressão de CD62L na superfície das células T $CD4^+$ ativadas, retendo-as nos órgãos linfoides secundários; 3) Inibição da resposta proliferativa de células T $CD4^+$ por proteínas virais solúveis; 4) Indução de anergia como consequência do sinal sub-ótimo gerado pela ligação de moléculas CD4 à gp120 solúvel; e, principalmente, 6) Hiperativação imune, que será discutida a seguir (Paranjape, 2005; Moir, Connors & Fauci, 2010).

A deterioração imunológica na infecção pelo HIV ocorre, em última instância, como fruto dos mecanismos intrínsecos do sistema imune de auto-regulação. A estimulação antigênica contínua pelo HIV e por antígenos microbianos translocados do trato gastrointestinal conduzem as células T $CD4^+$ a estágios de hiperativação e inflamação crônica que podem levar à exaustão e morte por apoptose (AICD – *activation-induced cell death*) (Hazenberg *et al*., 2003; Appay & Sauce, 2008). As células T $CD4^+$ HIV-específicas são as primeiras acometidas por serem as mais estimuladas antigenicamente. Essas células tendem a aumentar a expressão dos marcadores de superfície citados anteriormente HLA-DR, PD-1, CD38 e Fas. Estudos clínicos, inclusive, já demonstraram de maneira convincente uma relação direta entre os níveis de expressão de CD38 e a progressão da doença (Eggena *et al*., 2005; Sodora & Silvestri, 2008; Moir, Connors & Fauci 2010). A reação fisiológica à depleção numérica desses linfócitos potencializa ainda mais a ativação das células restantes nesse compartimento celular (Lane, 2010). Como resultado, além da redução global da subpopulação $CD4^+$ e da relação CD4/CD8, há um aumento relativo da proporção de células T $CD4^+$ efetoras terminalmente diferenciadas ($CD28^-$) e uma diminuição das subpopulações naïve e de memória central, comprometendo a capacidade de resposta a novos desafios antigênicos, inclusive vacinas (Baarle *et al*., 2005; Sodora & Silvestri, 2008; Lane, 2010).

As citocinas pró-inflamatórias clássicas, TNF-α, IL-1 e IL-6, também estão em alta no paciente com aids, produzidas principalmente por células T $CD8^+$ senescentes (fenotipicamente identificadas pela expressão de níveis elevados de CD57 e CD45RA associada à perda de CD28), como também pelas células da imunidade inata (provavelmente em resposta aos antígenos bacterianos translocados do trato gastintestinal). Essas citocinas potencializam ainda mais a ativação imune, além de tornar a célula T $CD4^+$ mais suscetível a infecção direta pelo HIV com aumento da expressão de CCR5 (Clereci *et al.*, 2002; Effros *et al.*, 2005; Brenchley *et al.*, 2006; Hunt, 2012).

Com relação ao impacto da infecção pelo HIV na rede fenotípica das células T $CD4^+$, estudos demonstraram que a progressão clínica para aids é estreitamente relacionada à perda funcional das células Th1 e Th17, que são alvos preferenciais tanto da infecção direta pelo vírus como também da exaustão clonal, por serem mais requisitados nesse contexto (Rodriguez *et al.*, 1997; Douek *et al.*, 2002; Lane, 2010; Hunt, 2010). O Th1 é o fenótipo responsável por coordenar a resposta protetora à grande maioria dos patógenos, inclusive ao próprio HIV. A deficiência em Th17, células presentes em grande quantidade em nível de mucosa intestinal e componentes importantes dessa barreira, acelera indiretamente os distúrbios imunitários por facilitar a translocação microbiana, proporcionando mais estímulos antigênicos (Hunt, 2010).

Com a evolução da infecção, portanto, passam a predominar fenótipos menos afetados (principalmente Th2, Tr-1 e Treg), inibidores da imunidade celular; e o paciente torna-se cada vez mais susceptível a infecções e neoplasias oportunistas. Vemos então que o perfil de citocinas ao longo da infecção pelo HIV vai sendo trocado de Th1/Th17 para fenótipos reguladores (Tenorio *et al.*, 2009; Klein *et al.*, 1997; Hunt, 2010, Chehimi *et al*., 1994).

Ironicamente, quanto mais ativada estiver a célula T $CD4^+$, melhor hospedeira será para o HIV, pois terá maior capacidade de produzir novos vírus, devido sua alta atividade metabólica (Giorgi *et al.*, 1999). Portanto, eis o resumo do cenário imunológico: É necessário ativação de fenótipos protetores para o combate eficaz ao HIV e às infecções oportunistas, porém a ativação desses linfócitos pode levá-los à morte e/ou a uma infecção altamente eficiente pelo HIV.

Quando um paciente recebe terapia anti-retroviral (TARV), a replicação viral é freada e observa-se redução da CVP (Li *et al*., 1998). Dessa forma, os anti-retrovirais exercem seu papel principalmente diminuindo a estimulação antigênica pelo HIV e consequentemente reduzindo o grau de hiperativação em que se encontra o sistema imunitário (Andersson *et al*., 1998; Behbahani *et al.*, 2000). A recuperação parcial das barreiras mucosas também ajuda a controlar a hiperativação e inflamação crônica na medida em que reduz a exposição a antígenos bacterianos translocados do trato grastointestinal (Stein & Hsue, 2012). Concomitante com a redução da CVP, a TARV aumenta a contagem de células T $CD4^+$ periféricas. Esse aumento segue um padrão bifásico. Num primeiro momento, o aumento se deve a uma redistribuição rápida de células T $CD4^+$ a partir dos órgãos linfóides secundários e da medula-óssea, com fenótipo ativado/memória efetora ($CD45RO^+CD127^-$). Numa segunda fase, onde a recuperação é mais sutil, ocorre o aumento de células T $CD4^+$ naïve provenientes do timo (Weiss *et al.*, 2002; Behbahani *et al*., 2000; Baker *et al.*, 2008). Portanto, a capacidade do indivíduo manter níveis estáveis de células T $CD4^+$ depende principalmente do sucesso terapêutico. Um rebote na carga viral quase sempre acarreta redução da razão CD4/CD8, tanto por diminuir o número de células T $CD4^+$ circulantes quanto por aumentar o número de células T $CD8^+$ periféricas (Scott-Algara *et al.*, 2001). Para muitos autores, a recuperação de células T $CD4^+$ na segunda fase, reflete o grau de aquisição de imunocompetência do portador do HIV. Nessa fase, que depende diretamente da reserva funcional do timo, observa-se idealmente uma recuperação de linfócitos T $CD4^+$ com repertório TCRvβ diverso, com níveis elevados de TRECs (*T cell receptor excision circles*), CD45RA, CD127 e CD62L (Kolte *et al.*, 2002). Como efeito final, dentro do compartimento T $CD4^+$, eleva-se a proporção células T *naïve* ($CD4^+CD127^+CD45RA^+$) e de memória central ($CD4^+CD127^+CD45RO^+$), diminuindo a de células efetoras ($CD4^+CD127^-CCR7\text{-}CD45RO^+$),

conforme diagrama abaixo adaptado a partir dos resultados de Wilkinson e colaboradores (Fig. 5) (Dunham *et al.*, 2008; Wilkinson *et al.*, 2009).

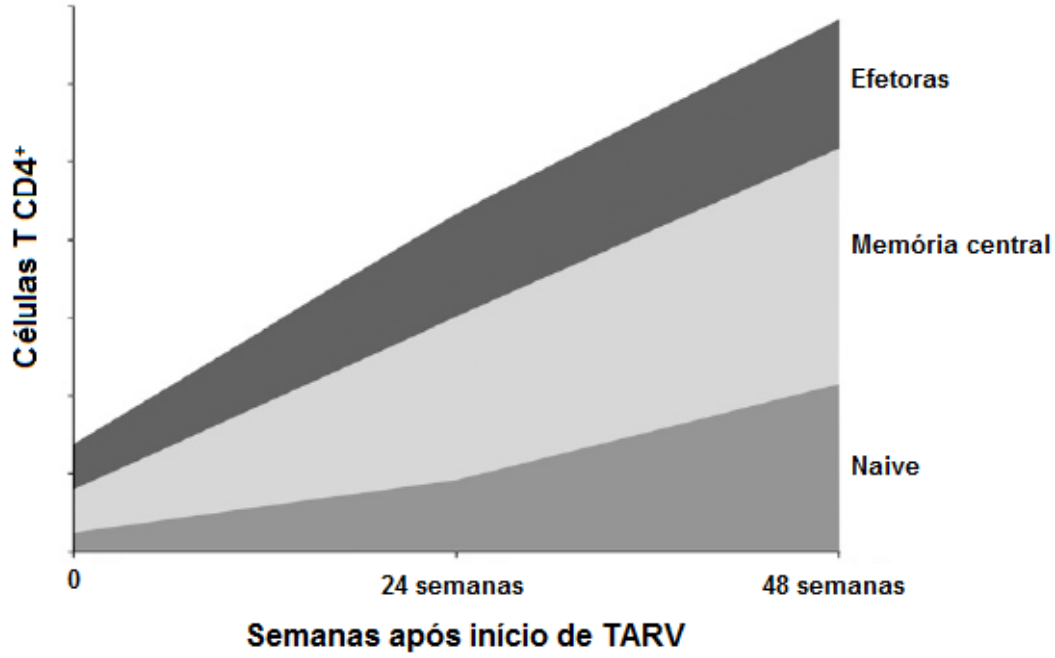

Figura 5. Reconstituição do compartimento T $CD4^+$ após início de TARV *(fonte: Wilkinson et al., 2009).* O gráfico representa a frequência de células T $CD4^+$ *naïve*, memória central e efetora, num paciente com aids, ao longo das primeiras 48 semanas após início de terapia anti-retroviral.

Finalmente, quanto ao perfil de citocinas, a TARV geralmente promove uma recuperação parcial do fenótipo Th1 (evidenciada pela capacidade de produção de IFN-γ) e, conforme recentemente demonstrado, também do Th17, os quais estão habitualmente escassos no paciente em fase avançada de doença. Essa é a definição mais completa de reconstituição imune pós-TARV (Autran, Carcelain & Debre, 2001; Hunt, 2010).

Sabe-se, no entanto, que o grau dessa reconstituição imune pela TARV depende não apenas da adesão do paciente ao esquema terapêutico como também de algumas características dos hospedeiros, tais como a idade do paciente. A idade avançada pode interferir significativamente nessa dinâmica, alterando quantitativa e qualitativamente a reconstituição imune pós-TARV (Viard *et al.*, 2001).

3 **O ENVELHECIMENTO E A INFECÇÃO PELO HIV**

3.1**Os idosos: um grupo especial de hospedeiros?**

Como já discutido anteriormente, o percentual de indivíduos com faixa etária avançada tem aumentado consideravelmente entre as pessoas infectadas pelo HIV (Ministério da Saúde, 2012(1)), e suas particularidades imunológicas não devem ser negligenciadas, pois interferem tanto na fisiopatologia da doença, quanto na sua evolução clínica e resposta à TARV (Adler *et al.*, 1997; Hasse *et al.*, 2011).

À medida que o sistema imune de uma pessoa saudável envelhece, são observadas diversas mudanças anatomo-funcionais, denominadas coletivamente de imunossenescência (Gruver *et al.*, 2007; Jiang *et al.*, 2007). Ocorre uma diminuição da produção de hormônios e citocinas estimuladores da hematopoiese (ex: IL-7, GM-CSF e o hormônio de crescimento), tanto em nível sistêmico quanto no tecido hematopoiético, comprometendo em diferentes níveis a eficácia desse fenômeno. A involução tímica, uma das principais características da imunossenescência, parece começar a ocorrer já no primeiro ano de vida e acarreta numa diminuição progressiva da capacidade de produção de linfócitos T naïve (Douek *et al.*, 1998, Gruver *et al.*, 2007). Como consequência, observamos no idoso fenômeno semelhante ao que ocorre na infecção pelo HIV, isto é, aumento relativo de subpopulações de células T efetoras em detrimento de células naïve e de memória central (Dorshkind & Swain, 2009; Mitchell, Lang & Aspinall, 2010).

Em resumo, a involução morfofuncional do timo associada às múltiplas exposições antigênicas ao longo da vida resultam em alterações no compartimento da célula T. Essas alterações tornam a resposta T-dependente no idoso cada vez mais específica para um número limitado de antígenos e cada vez menos abrangente perante novos desafios antigênicos.

Outro fenômeno característico do envelhecimento do compartimento T, que também é semelhante ao que ocorre nos pacientes infectados pelo HIV, é a perda mais acentuada do fenótipo Th1, cuja importância já foi discutida anteriormente. À semelhança do que ocorre na infecção pelo HIV, o fenótipo Th1 é o mais afetado no idoso, provavelmente por ser o mais requisitado ao longo da vida (Rink *et al.*, 1998; Deng *et al.*, 2004).

Alterações no sistema endócrino senil também repercutem no sistema imune. A queda na produção de hormônios estimulantes da imunidade celular, nomeadamente prolactina, hormônio do crescimento e diidroepiandrostenediona, assim como a hiperativação do eixo hipotálamo-hipófise-adrenal com aumento da produção de cortisol, acarretam num prejuízo ainda maior da

imunidade celular, especialmente do fenótipo Th1 (Deuschle *et al.*, 1997; Ferrari, *et al.*, 2000; Bauer, 2005; Ziemssen & Kern, 2007).

Além da perda preferencial do fenótipo Th1, foi demonstrado que, no idoso, há um aumento de células T reguladoras $FoxP3^+$ em relação a pacientes mais jovens, porém também há controvérsias quanto à integridade funcional dessas células. Devido a hipofuncionalidade tímica, o acúmulo dessas células parece representar majoritariamente as células Treg induzidas $FoxP3^+$, chamadas de iTregs (Lages *et al.*, 2008; Tenorio *et al.*, 2009; Gregg *et al.*, 2005).

A maioria dos autores também concorda que, assim como na aids, a idade avançada está relacionada a um aumento de citocinas pró-inflamatórias da imunidade inata (IL-1, IL-6 e TNF-α) (Roubenoff *et al.*, 2008). Não está claro o motivo desse aumento, mas ele pode estar contribuindo também para o aumento das células T reguladoras no idoso (De Gonzalo-Calvo *et al.*, 2010).

É fundamental que se entenda de que maneira essas alterações imunológicas próprias da senilidade podem afetar a patogênese de doenças incidentes nessa faixa etária e a resposta à terapia, particularmente aos anti-retrovirias seguindo infecção pelo HIV.

3.2 **A infecção pelo HIV em pacientes acima de 50 anos**

As implicações dos fenômenos de imunossenescência no contexto da infecção pelo HIV têm se tornado foco de atenção nos últimos anos, mas ainda há muito mais perguntas do que respostas nesse campo.

Vários distúrbios no sistema imunitário induzidos pelo HIV mimetizam os fenômenos da imunossenescência. A diminuição relativa da capacidade de montar respostas Th1, e o aumento da proporção de células T efetoras e de memória efetora em relação a células T naïve e de memória central são distúrbios inerentes tanto à senilidade quanto à infecção pelo HIV. Esses distúrbios podem somar-se, portanto, no indivíduo portador de ambas as condições acima, causando distúrbio ainda maior e comprometendo a eficácia da reconstituição imune pela TARV (Rickabaugh *et al.*, 2011).

Ademais, a hiperativação e inflamação crônica induzidas pelo HIV podem exacerbar o risco ou até agravar co-morbidades não-infecciosas presentes no indivíduo senil, como coronariopatias, acidente vascular cerebral, osteoporose, demência, diabetes e disfunção renal, aumentando a morbimortalidade do paciente soropositivo de idade avançada (Mocroft *et al.*, 2010; Lane, 2010; Hasse *et al.*, 2011; Stein & Hsue, 2012).

Está também demonstrado que a infecção pelo HIV, em pacientes acima de 50 anos, progride mais rapidamente em direção à aids, e quando esse grupo de pacientes inicia a TARV,

observa-se uma pobre elevação na contagem de células T $CD4^+$ comparada a pacientes mais jovens (Operskalski *et al.*, 1995). Por outro lado, vários ensaios clínicos revelaram fato intrigante: após início da TARV, pacientes HIV-positivos com mais de 50 anos apresentam uma excelente resposta virológica ao tratamento, melhor que nos pacientes jovens (Grabar *et al.*, 2004; Viard *et al.*, 2001; Li *et al.*, 2011). Um estudo feito pelo nosso grupo publicado em 2007 começou a esclarecer, ao menos em parte, essa aparente contradição. Mostramos que indivíduos HIV-positivos tratados de faixa etária avançada produzem grandes quantidades de IL-10, uma das principais citocinas inibidoras da imunidade celular, e que os níveis desta citocina nos sobrenadantes de culturas de células mononucleares do sangue periférico (PBMC, *peripheral blood mononuclear cells*) guarda relação clara e direta com a capacidade de controle virológico *in vitro* (Andrade *et al.*, 2007, anexo 1). De fato, células T $CD4^+$ de fenótipos protetores cuja ativação esteja inibida pela IL-10 são péssimos microambientes para a replicação do HIV, porém são menos capazes de coordenar respostas protetoras ao HIV e outros patógenos (McGowan *et al.*, 2004; Andrade *et al.*, 2007; Darrah *et al.*, 2010).

Esses achados sugerem que, em pacientes HIV-positivos acima de 50 anos, tratados com sucesso, a recuperação funcional do sistema imune pode ser diferente da observada em pacientes jovens, e portanto, é preciso conhecer o comportamento imunológico dessa população particular mediante diferentes desafios antigênicos, inclusive na resposta a vacinas. Cabe ressaltar que as condutas terapêuticas e profiláticas atualmente preconizadas para pacientes com aids baseiam-se em estudos que avaliaram essencialmente populações jovens, e talvez não sejam totalmente adequadas ao manejo da infecção em pacientes idosos.

Na presente Tese, nosso objetivo foi caracterizar fenotipicamente a resposta imune de pacientes acima de 55 anos com aids, adequadamente tratados, identificando e caracterizando funcionalmente as células T $CD4^+$ desses pacientes em diferentes contextos imunológicos: estímulo policlonal, resposta específica anti-HIV e resposta a antígeno de memória (toxóide tetânico).

4 **OBJETIVOS**

4.1 **Objetivo geral**

Avaliar o impacto da idade avançada sobre a resposta imune T-dependente em indivíduos infectados pelo HIV e tratados com sucesso com terapia anti-retroviral.

4.2 **Objetivos específicos**

- Avaliar a resposta linfoproliferativa a antígeno de memória (toxóide tetânico) em culturas de PBMC de pacientes com aids acima de 55 anos vacinados contra o tétano, e estudar o perfil de citocinas no sobrenadannte dessas culturas, comparando-o com pacientes jovens.

- Avaliar a resposta humoral ao toxóide tetânico no soro desses pacientes após dose de reforço da vacina.

- Estudar a linfoproliferação e a produção de citocinas em resposta a ativador policlonal e a antígenos específicos do envelope viral do HIV, em culturas de PBMC provenientes de pacientes com aids com idade avançada.

- Analisar marcadores intra- e extra-celulares nas células T para identificar os fenótipos envolvidos na resposta HIV-específica.

- Avaliar a correlação entre os fenótipos dominantes de célula T $CD4^+$ e a replicação viral *in vitro*.

5 **ARTIGOS CIENTÍFICOS PUBLICADOS**

5.1 **Artigo científico** 1 - Failure of highly active antiretroviral therapy in reconstituting immune response to *Clostridium tetani* vaccine in aged AIDS patients

BASIC AND TRANSLATIONAL SCIENCE

Failure of Highly Active Antiretroviral Therapy in Reconstituting Immune Response to *Clostridium tetani* Vaccine in Aged AIDS Patients

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Abstract: The purpose of this study was to evaluate the impact of age on tetanus-specific immune response in successfully highly active antiretroviral therapy treated AIDS patients, using healthy age-matched individuals as controls. Whole Peripheral blood mononuclear cells or $CD8^+$ cell-depleted peripheral blood mononuclear cells from previously tetanus toxoid (TT) immunized individuals were activated with TT plus IL-2, and cell proliferation, cytokine production, and in vitro HIV-1 replication were measured. The in vivo magnitude of the humoral immune response was also assessed by antibody measurements. Our results showed that, compared with other groups, both in vitro TT-specific lymphoproliferation and serum antibody concentration were lower in older AIDS patients. Although the IL-1β and tumour necrosis factor alpha (TNF-α) production were higher in cultures from aged HIV-1 infected patients, a dramatic damage on the interferon gamma (IFN-γ) release was observed, when compared with younger patients. $CD8^+$ T lymphocytes depletion reduced IL-1β and TNF-α release in the older groups, however, it did not significantly alter their IFN-γ production. Furthermore, the neutralization of endogenous IL-10 did not change the IFN-γ deficiency in older AIDS patients. Finally, the lower cellular immune response in this patient group was not related to in vitro HIV-1 replication. The results suggest that successfully highly active antiretroviral therapy treated aged AIDS patients do not reconstitute the immune response to TT, making them probably more susceptible to tetanus even after vaccination.

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Key Words: aged AIDS patient, cytokines and AIDS, HIV-1, HAART and humoral immune response, tetanus toxoid

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INTRODUCTION

Since the beginning of AIDS epidemic, caused by HIV, the proportion of AIDS cases in adults aged more than 50 years has been increasing fast around the world,[1] which confirms the need for more studies on this particular population.

Despite the frequencies of HIV-1–specific $CD4^+$ and $CD8^+$ T cells that secrete IL-2 and IFN-γ are good predictors of delayed disease progression and long lasting stable peripheral $CD4^+$ T cells counts, in the great majority of patients viral replication proceeds, and disease progression toward AIDS.[2,3] This lack of viral control is mainly accelerated by an early HIV-specific $CD4^+$ T-cell loss and escape mutations in cytolytic T lymphocytes epitopes that commonly develop during infection with HIV-1.[4,5]

As infection proceeds, the main damage on the immune system is the quantitative and qualitative loss of Th1-like lymphocytes by multiple virus-induced events.[6 8] HIV-1 is little cytopathic, and death of target cells by continuous viral budding is not a predominant mechanism of $CD4^+$ T-cell loss.[6] Moreover, the destruction of these target cells by HIV-1–specific cytolytic T lymphocytes seems not to contribute significantly to quantitative immune decline[9,10] as well. Nowadays, there is a consensus that persistent immune activation after HIV-1 infection is the main cause of immunodeficiency.[9,11,12] HIV-1–induced activation is generalized, embracing not only T cells but all components of the immune system, leading to serious disturbances of immune homeostasis.[9,11,12]

The chronic systemic production of pro-inflammatory cytokines favors cell death by inducing the expression of pro-apoptotic proteins (Bim and Bax), whereas downregulates the levels of the antiapoptotic proteins, such as Bcl-2.[10] Furthermore, dysfunctional PD-1^+ and Tim-3^+ T cells accumulate during the course of infection.[13,14] In association with high levels of pro-inflammatory cytokines, impaired IL-2 production and disturbances on its signal transduction pathway have been described as the major deleterious effect on immune

homeostasis.[15,16] As a consequence of these events, there is an increased T-cell turnover.

Additionally, immune activation directly influences the viral replication process. Many authors have proved the existence of a very close relationship between the synthesis of pro-inflammatory cytokines, such as TNF-α and IL-6, and viral replication.[6 8,17 19] Activation of the respective signal transduction pathways by these cytokines induces nuclear factor kB (NF-kB) and nuclear factor of activated T cells (NF-AT), both of them are able to accelerate viral life cycle from HIV-1 proviral DNA.

In chronically HIV-1–infected patients, the production of high levels of pro-inflammatory cytokines can be supported by different immune cells.[6 8,20] Some works have shown that macrophages and HIV-specific and nonspecific activated $CD8^+$ T lymphocytes from infected patients are important sources of IL-1β, IL-12, and TNF-α, with impact on clinical disease progression.[11,21]

As infection proceeds, a preferential loss of the T-helper 1 (Th1)-like responses gradually renders the patients highly susceptible to opportunistic infections and less able to mount protective cellular and humoral immune response after immunizations.[22 24] The successful highly active antiretroviral therapy (HAART) helps these patients to restore the immune response by attenuating these immune dysregulation.[15,23 27]

The marked decrease in the incidence of AIDS-related diseases after the introduction of HAART correlates with the control of plasma viral load (VL) and an increase in peripheral $CD4^+$ cell counts.[23] Many studies with HAART-treated HIV-infected young adults have shown that the risk of some opportunistic infections is reduced when patients achieve $CD4^+$ T-cell counts above a certain value.[23] However, in addition to increasing $CD4^+$ cell count, the impact of HAART on the degree of functional immune recovery depends on the reduction of the hyperactivation state discussed above.[25 29] Normalization of pro-inflammatory cytokines levels and recovery of IFN-γ production have been observed in some patients after successful HAART.[15,30 32]

Besides the reduction of the degree of immune activation, some host variables, particularly age, have been shown to correlate with the extent of immune restoration after HAART initiation.[33 35] Some studies have demonstrated a negative influence of age on $CD4^+$ T-cell numeric recovery after HAART in naive AIDS patients.[1,33 35] Nevertheless, older AIDS patients, in general, have good virological response to HAART.[35] To our knowledge, no work has evaluated the immune function status in this group of AIDS patients in response to recall antigens after immunization.

The present study aimed to evaluate the impact of age in reconstituting the humoral and cellular immune response to tetanus vaccination in young and aged AIDS patients with virological and immunological success after HAART, using healthy age-matched groups as controls.

METHODOLOGY

Patients and Tetanus Immunization

The study examined a group of 20 HIV-1–infected individuals more than 55 years old (mean: 58.9 years; range: 55–65) who were successfully treated with antiretroviral therapy. A second group included 20 young HIV-1–infected patients (mean: 31.1 years; range: 22–38), with similar infection characteristics. All individuals were recruited from the Centre of Epidemiology at the University Hospital of the State University of Rio de Janeiro, Brazil. As controls, gender matched healthy young (n = 20; mean: 27.6 years; range: 20–40, 50% male) and healthy aged individuals (n = 20; mean: 61 years; range: 60–65, 45% male) were enrolled in the study. The written consent for participation was obtained from all, and the study was approved by the Ethics Committee of the same University Hospital.

All characteristics of the HIV-infected individuals, obtained from medical records, are presented in Table 1, including the HAART scheme and the $CD4^+$ T-cell count at baseline and 24 months after the beginning of antiretroviral therapy. The $CD4^+$ cell counts are expressed as absolute values, and the VL was transformed to $\log_{10}$-scale to normalize distribution. As shown in the Table 1, despite the difference in baseline VL, all patients were successfully treated with a 3-drug combination. All of them had achieved undetectable plasma HIV-1 RNA levels, defined here as less than 80 copies per milliliter, within 3 months after therapy, and have remained below this level for at least 2 years (data not shown). Importantly, the patients started therapy with similar baseline $CD4^+$ T-cell counts and responded immunologically by increasing these counts to comparable values of $CD4^+$ cells after therapy. To avoid problems concerning multidrug failure, all the AIDS patients were in their first antiretroviral scheme.

TABLE 1. Characteristics of Patients With Undetectable Plasma VL*

	>50 Years Old (n = 20)	<40 Years Old (n = 20)
Mean age (SD)	58.9 (4.2)	31.1 (5.5)
% Male	60	50
HIV-1 diagnosis (SD)†	7.1 (1.4)	6.8 (2.2)
CDC classification (%)‡		
A3	20	20
B3	10	20
C3	70	60
Mean VL at baseline (log copies/mL) (SD)	4.4 (0.4)	4.8 (0.46)
Mean CD4 cell count (SD)		
At baseline	152.4 (25.2)	144.3 (33.7)
At end point	455 (145)	501 (127)
HAART scheme§		
AZT/3TC/EFZ	4	7
AZT/DDI/EFZ	4	4
AZT/3TC/NFV	4	2
AZT/DDI/NFV	2	1
AZT/3TC/SQV/r	2	2

*Limit of detection: 80 copies per milliliter.
†Time, in years, that the HIV-1 infection was laboratory diagnosticated.
‡Clinical classification from CDC guideline, 1987.
§Twenty-four months after beginning antiretroviral therapy. The HAART basic treatment was with 2 NRTI [AZT (Zidovudine), 3TC (Lamovudine), DDI (Dadanosine)] with 1 NNTRI (Efavirenz) or with 1 PI [NFV (neufinavir), SQV/r (saquinqvir/ritonavir)].

As all these individuals had received tetanus toxoid (TT) vaccine during their childhood, and not during adolescence or adult life, a single boost of TT (Sanofi Pasteur, SA, Lyon, France) in the deltoid muscle was given to all individuals, and the blood samples were collected by venipuncture on day 30 after vaccination for all experiments. Specifically for the evaluation of humoral response, blood samples were also collected immediately before TT immunization. Among the HIV-1–infected patients, the clinical and virological status remained stable 2 months after TT immunization.

Quantification of TT-Specific Immunoglobulin G in the Serum

Immediately before and 30 days after TT immunization, the blood samples were collected and the serum was obtained for TT-specific immunoglobulin G (IgG) quantification by SERION ELISA Classic kit (Immunomat TWIN System, Würzburg, Germany). Values lower than 0.01 IU/mL were considered not protectors, values between 0.11 and 0.5 IU/mL indicated sufficient protection, and values >0.5 IU/mL indicated long lasting protection.

Cell Culture and Stimulation

Thirty days after TT immunization, blood samples were collected from both control individuals and successfully HAART-treated HIV-1–infected patients. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on Ficoll–Hypaque gradients as previously described.[18] For some experiments, $CD8^+$ cell-depleted PBMC were obtained by negative selection using magnetic beads coated with anti-CD8 monoclonal antibody (Dynal Biotech, Great Neck, NY). The efficacy of this procedure was approximately 96% as evaluated by flow cytometry (data not shown). The number of viable cells of each preparation was measured by Trypan blue exclusion in a hemocytometer. Viable cells were adjusted to a concentration of 1×10^5 cells per well and cultured in a 96-well round bottom microtitre plates with 200 μL RPMI 1640 added with 2 μmol/L L-glutamine (GIBCO, Carlsbad, CA), 10% fetal calf serum, 20 U/mL penicillin, 20 μg/mL streptomycin, and 20 μmol/l HEPES buffer. The cultures were stimulated with TT at 1 μg/mL (SBL Vaccin, Stockholm, Sweden) in the presence or absence of recombinant human IL-2 (rhIL-2) at 20U/mL (BD Systems, Minneapolis, MN). In some experiments for evaluation of TT-induced cytokine production, saturating doses of anti-IL-10 mAb (22 μg/mL; B&D System) or isotype control (IgG2a) were added at the beginning of the cultures and 3 days later. The cells were maintained for 7 days at 37° C in a humidified 5% CO_2 incubator for proliferation and cytokine assays.

Of note, the TT dose (1 μg/mL) was previously established by our group by evaluating PBMC proliferative response to different TT concentrations (0,1–10 μg/mL) in a group of healthy immunized individuals (median 39.7 years old; range 22–65). Higher doses of TT did not modify the maximal [^{3}H]-thymidine uptake (data not shown). Similarly, the IL-2 dose was also previously established by a dose–response study in continuous T-cell line proliferation by IL-2-dependent cell line (CTLL) assay (data not shown), and IL-10 mAb doses were established in our previous work.[18]

Proliferation Assay

Approximately 1×10^5 cells per well of PBMC were activated or not with TT (1 μg/mL), with or without rhIL-2 (20 U/mL) for 7 days. The cellular proliferation was measured after addition of [3H]-thymidine (0.51 μCi/well) for the last 12 hours of incubation. The cells were harvested in glass fiber filters in an automatic cell harvester, and radioactive incorporation was measured using a liquid scintillation counter.

Cytokine Determination

The supernatants from PBMC or $CD8^+$ cell-depleted PBMC cultures activated or not with TT plus IL-2 were collected after 7 days and cytokines were measured using OptEIA enzyme-linked immunosorbent assays (BD Pharmigen, San Diego, CA), according to manufacturer's protocol. Briefly, each pair of monoclonal antibodies was used to detect human IL-1β, IL-10, IL-4, TNF-α, and IFN-γ. The reaction was revealed with streptavidin–horseradish peroxidase, using 3,30,5,50 tetramethylbenzidine as substrate. Recombinant human IL-1β, IL-10, IL-4, TNF-α, and IFN-γ at concentrations ranging from 10 to 500 pg/mL were used to construct standard curves.

The Effect of TT on In Vitro HIV-1 Replication

To evaluate the impact of TT addition on in vitro viral replication, the supernatants of TT-activated cell cultures were collected 7 days after the beginning of the cultures and stored at −70°C. This time was chosen because, in previous experiments, the peak of in vitro HIV-1 replication occurred at this point.[18] HIV-1 RNA was measured in the supernatants with a commercial reverse transcriptase–polymerase chain reaction kit (Amplicor HIV Monitor Test, Roche Molecular System, Branchburg, NJ), with a detection threshold of 80 copies per milliliter.

Statistical Analysis

The nonparametric Mann–Whitney *U* test was applied to determine whether the groups were statistically different for each given variable. The impact of TT vaccination on both IgG titers and in vitro HIV-1 replication for the young and aged AIDS patients was analyzed using paired Student *t* test. The significance in all experiments was defined as $P < 0.05$.

RESULTS

In Vivo IgG Concentration and In Vitro Lymphoproliferative Response to TT was Lower in Successful HAART-Treated Aged HIV-1–Infected Patients

The first immune event studied was the lymphoproliferative response induced by TT in cell cultures obtained from previously TT-immunized young and aged individuals. As demonstrated in Figure 1, lymphoproliferative response to TT in younger HIV-1–infected patients was similar to age-matched control group (uninfected young) and it was higher compared with aged groups. On the other hand, among the older individuals, the level of counts per minute (cpm) was lower in the HIV-1–infected patients (Fig. 1). As in both aging and AIDS, the reduction in lymphocyte proliferation is related

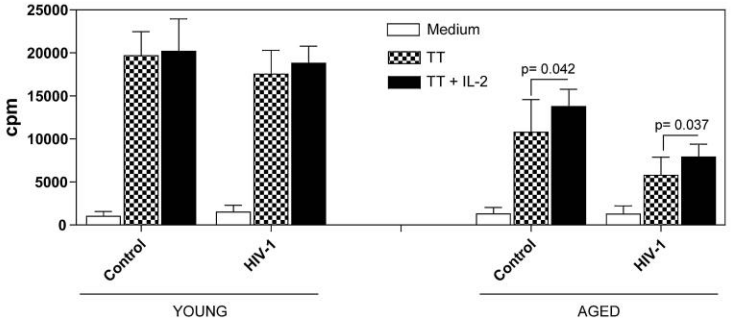

FIGURE 1. Proliferative response to TT in young and aged HAART-treated HIV-1–infected patients. The PBMC (1×10^5 cells per well) proliferative response to TT (1 μg/mL) was evaluated in groups of healthy young and aged individuals or age-matched successfully HAART-treated HIV-1–infected patients. All of them had been submitted to TT immunization 30 days before collecting the blood sample. The level of proliferation was measured as copies per milliliter after the addition of [3H] thymidine. In some experiments, 20 U/mL of hrIL-2 were added at beginning of the culture time. The *P* values are indicated in the figure.

to impairment of IL-2 production,[16,36] we added to each cell cultures optimal doses of human recombinant IL-2. Although the addition of IL-2 had no significant effect on TT-specific cell proliferation among cultures from young individuals, it elevated, but not to normal levels, the response to TT in cell cultures from the aged ones. More important, even in the presence of IL-2, the TT-specific proliferative response remained lower in aged HIV-1–infected group. Of note, without previous TT vaccination, the in vitro lymphoproliferation to TT with or without IL-2 was undetectable in PBMC cultures from young and aged AIDS patients (dada not shown). As IL-2 alone does not induce cell proliferation (data not shown), but it improved the proliferation performance in response to TT, we decided to do the other in vitro immunological assays in presence of TT plus IL-2.

Concerning humoral response, the dosage of TT-specific IgG in the serum revealed lower antibody concentrations in samples from aged patients (Fig. 2). Four weeks after vaccination, all healthy elderly had raised IgG titers, but antibody concentrations were still lower than in young groups. No statistical difference was observed postvaccination in young patient groups. Of note, despite 100% of young AIDS patients had acquired high levels of anti-TT IgG (long lasting protection), only 30% of the aged AIDS patients raised IgG titers higher than 1.1 IU/mL after vaccination.

Higher Pro-Inflammatory Profile Associated With Lower Levels of Th1-Cytokines Observed in Aged HIV-1–Infected Patients

Concerning the investigation of cytokine network disturbances, we observed that TT-activated PBMC cultures from HIV-infected patients produce high levels of IL-1β and TNF-α, mainly in aged patients (Fig. 3). Despite their high tendency to produce pro-inflammatory cytokines, the production of IFN-γ was lower in aged HIV-infected patients (Fig. 3). Furthermore, this low in vitro IFN-γ production correlated with low in vivo anti-TT IgG titers (data not shown).

The levels of IL-10 have been described to increase with age, and this cytokine is known to impair Th1-mediated response.[10,37] Among the aged patients, the neutralization of endogenous IL-10 increased IFN-γ production only in TT-activated PBMC from the healthy group (Fig. 4). Finally, concerning IL-4 release, no statistical difference was observed between the groups.

Unlike the healthy young individuals, both aged and AIDS patients have high counts of abnormal peripheral $CD8^+$ T cells,[21,37–39] and to analyze the contribution of this T-cell subset to the deficient Th1 response observed in older group, we cultured $CD8^+$ cell-depleted PBMC on the same previously mentioned conditions. As observed in Figure 5, despite the

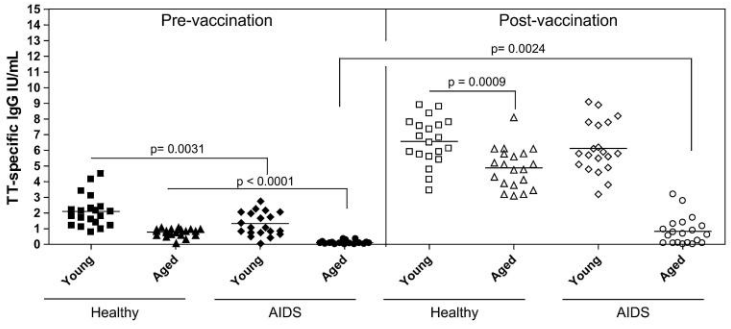

FIGURE 2. Antibody concentrations in young and aged AIDS patients before and after vaccination with TT. Immediately before and 30 days after TT immunization, the blood samples were collected and submitted to enzyme-linked immunosorbent assay. Horizontal bars within boxes correspond to the median, box limits correspond to 25th and 75th percentiles, and vertical lines indicate the range. The *P* values are indicated in the figure.

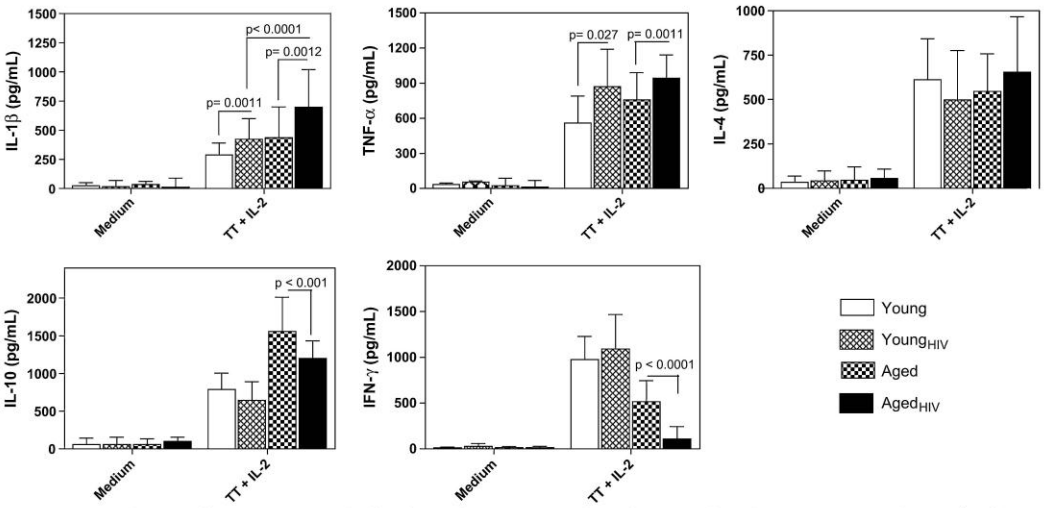

FIGURE 3. Cytokine profile in TT-activated cell cultures from young and aged HIV-1–infected patients. PBMC (1×10^5 cells per well) were obtained from both HAART-treated young and aged HIV-1–infected patients and age-matched healthy individuals. The cell cultures were maintained in the absence (medium) or presence of TT (1 μg/mL) plus rhIL-2 (20 U/mL). The supernatants were collected 7 days later and submitted to sandwich enzyme-linked immunosorbent assay. The results are presented as mean ± SD and *P* values are indicated at the figure.

depletion of $CD8^+$ cells has significantly reduced the IL-1β and, mainly, TNF-α release from aged HIV-infected patients, no modification was observed concerning IFN-γ production. In healthy aged group, the depletion of this T-cell subset reduced all cytokines evaluated but did not change the cytokine profile of the cultures (Fig. 5).

To evaluate whether the lower ability to perform Th1 response to TT in the aged group was related or not to the level of in vitro HIV-1 replication, supernatants from TT-activated cell cultures were collected 7 days after the beginning of the cultures and the concentration of HIV-1 RNA was quantified by reverse transcriptase–polymerase chain reaction. Interestingly, the level of viral replication was significantly lower in the TT-activated cell cultures from successfully HAART-treated aged patients (811.5 ± 1028) when compared with the younger group (2488 ± 2046) ($P = 0.0075$).

DISCUSSION

The pharmacological treatment of AIDS patients by HAART leads to the suppression or reduction in plasma VL and to an increase in $CD4^+$ T-cell count.[15] However, the success of immunological reconstitution after HAART can be less efficient is some patients, particularly in aged ones. Recent studies have described a negative influence of age on $CD4^+$ cell recovery after HAART initiation in HIV-infected patients, despite a better viral control in the older ones.[33–35,40] Nevertheless, in our study, even working with aged HIV-1–infected patients who had augmented the $CD4^+$ T-cell counts to similar levels to those observed in younger patients, the functional humoral and cellular immune recovery to the recall antigen TT was significantly lower. Similar results were obtained by our group when T-cell polyclonal activators were used.[18] Many studies have found a direct correlation between HAART-induced functional immune reconstitution to different antigens and good rise in peripheral $CD4^+$ T-cell count.[25–27] Our results, however, suggest that, in aged patients, who represent an increasing group of HIV-1 victims, the elevation in $CD4^+$ T-cell counts after HAART may be merely a mathematical event, rather than a true functional immune recovery.

In our study, among AIDS patients, the lymphoproliferation to TT was higher in the younger group. Some authors have previously shown that reduction in lymphoproliferation in both young and elderly HIV-1–infected adults is due, at least in part, to their diminished ability to produce IL-2.[16,36] In our system, although exogenous IL-2 had augmented the cell proliferation to TT in the aged group, it remained much lower than in younger patients. The incomplete ability of IL-2 to restore cell proliferation could be related to a damage in CD25 signal transduction pathway described in elderly individuals by some authors.[39,41] The absence of effect of IL-2 on cultures of young patients may represent a saturation of in vitro cellular proliferative potential to TT.

Many immune disturbances described in both healthy elderly individuals and chronically HIV-1–infected patients are related to dysregulation in cytokine network,[38,42] and in AIDS patients, the magnitude of functional immune recovery by HAART is directly proportional to the degree at which

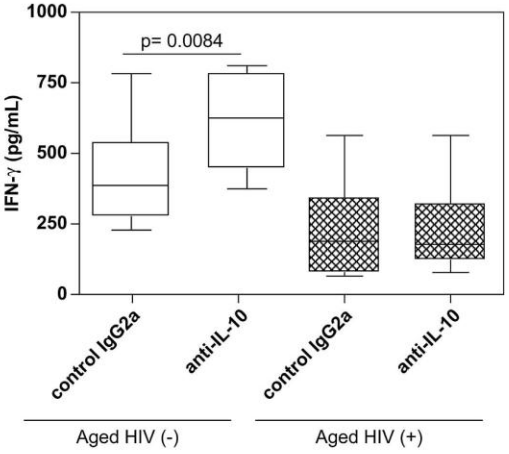

FIGURE 4. The role of IL-10 on the TT-induced cytokine pattern in cultures from healthy and HIV-infected aged individuals. PBMC (1×10^5 cells per well) were obtained from both healthy and HIV-1–infected aged individuals and maintained in the presence TT (1 μg/mL) plus rhIL-2 (20 U/mL) for 7 days. The involvement of IL-10 on the cytokine pattern was evaluated by the addition of neutralizing doses of anti-IL-10 mAb (22 μg/mL) or control isotype (IgG2a). The supernatants were collected and submitted to sandwich enzyme-linked immunosorbent assay. The results are presented as mean ± SD, and the *P* value is indicated at the figure.

immune hyperactivation is controlled.[15,43] Similarly to what happens throughout several decades of a normal person's life, it has been proposed that, in HIV-1 infection, the persistent systemic immune activation by chronic antigen exposure is the main reason of accelerated immunosenescence.[9,11,12,37–39] In our work, IL-1β and TNF-α releases were higher in TT-activated cell cultures from healthy aged individuals and from AIDS patients. Systemic pro-inflammatory cytokines suppress both B-cell formation in bone marrow and thymopoiesis, resulting in progressive reduction of naive B-cell and T-cell counts.[37] In this scenario, an accumulation of abnormal terminally differentiated effector T cells occurs.[38,39] These abnormal T cells have short telomeres, a highly restricted T-cell receptor repertoire, an impaired capacity to migrate to lymph nodes, and a decreased ability to be stimulated by antigen-presenting cells, a result of the loss of the costimulatory molecules CD28 and CD27.[37–39,21] Among these cells, Th1 lymphocytes are the most susceptible to the deleterious effects of oxidative stress related to chronic antigen stimulation and pro-inflammatory cytokines.[44] Furthermore, the defective IL-2 production jeopardizes the long living cells by not inducing anti-apoptotic proteins, like bcl-2.[21,37–39]

IL-2–secreting and IFN-γ–secreting Th1 cells are not only the main coordinators of protective immune response against infectious diseases, but they are pivotal in responding to T-dependent antigens vaccination, such as TT.[45,46] In our study, the stronger pro-inflammatory profile observed in aged HIV-1–infected patients is paradoxically associated with a dramatic damage on IFN-γ production by TT-activated cell cultures, even after IL-2 addition.

Authors have demonstrated that functional damage in Th1 cell subset in healthy elderly individuals impairs Ig isotype switching and somatic mutation, which are essential for the production of high-affinity antibodies and decreases titers of IgG in the serum.[47–49] In these individuals, the antitetanus antibody levels before booster TT dose did not reach protective titers in 50% of them. In our cohort, no aged AIDS patient had protective levels of anti-TT IgG at baseline. These levels efficiently raised in only 30% of them post-vaccination. Altogether, these observations suggest that the prevalence and intensity of the humoral immune response to tetanus decay in aging, and this phenomenon is amplified by HIV-1 infection. Furthermore, our results also demonstrate that HAART does not efficiently restore, in older patients, the ability to adequately mount a humoral and cellular immune response.

In our previous study, the peripheral $CD8^+$ T cells were the main sources of pro-inflammatory cytokines in CD3-activated cell cultures from HIV-1–infected patients.[18] It is

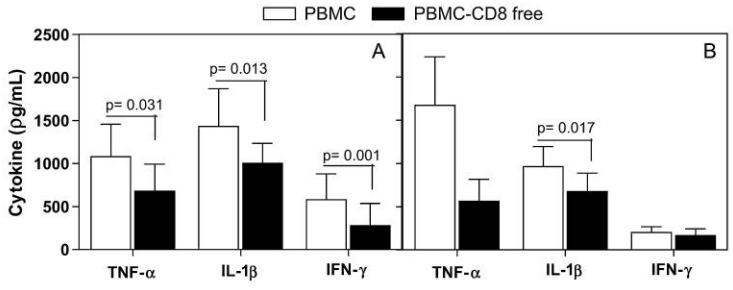

FIGURE 5. The cytokine profile induced by TT in CD8-depleted cultures from healthy and HIV-infected aged individuals. $CD8^+$ T cell-depleted PBMC (1×10^5 cells per well), obtained after negative selection of $CD8^+$ cells, from healthy aged individuals (A) and successfully HAART-treated aged HIV-1–infected patients (B) were maintained in the presence of TT (1 μg/mL) plus rhIL-2 (20 U/mL). The supernatants were collected 7 days later and submitted to sandwich enzyme-linked immunosorbent assay. Horizontal bars within boxes correspond to the median, box limits correspond to 25th and 75th percentiles, and vertical lines indicate the range. The results are presented as mean ± SD, and the *P* values are indicated in the figure.

known that, as disease progresses, specific and nonspecific chronically HIV-activated $CD8^+$ T cells contribute to a generalized state of immune activation by secreting high levels of IL-1β and TNF-α. Successfully HAART-treated patients have a tendency to reduce this immune dysregulation.[21,50] In our study, depletion of $CD8^+$ cells diminished significantly the levels of these pro-inflammatory cytokines, but did not improve the production of IFN-γ by TT-stimulated cultures from aged AIDS patients, indicating that the Th1-mediated response to TT could not be functionally restored in this patient group.

Advanced age has been associated with an expanded peripheral regulatory T-cell pool, such as IL-10–secreting T cells, probably to control the hyperactivated state.[37] In HIV-infected patients, excessive production of IL-10 has been suggested to cause deleterious effects by inhibiting the production of Th1 cytokines that are implicated in promoting resistance against several pathogens.[51] In the present study, although elevated IL-10 production was detected in the TT-activated cell cultures from both HIV-infected and HIV-uninfected aged subjects, the blockade of this cytokine did not improve the IFN-γ production in aged AIDS patients, in contrast to the healthy aged group. This finding is in agreement with the theory that, during the course of HIV infection, IFN-γ-producing cells are the most destroyed cells.[6,24,52]

Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, leads not only to immunologic exhaustion, but also renders $CD4^+$ T cells more susceptible to direct HIV infection by enhancing the expression of the HIV coreceptor CCR5.[53] Despite the pro-inflammatory cytokines detected in cultures from the aged groups, IL-10 was the highest produced cytokine in these cultures in response to TT. In our previous work, the ability of $CD4^+$ T cells from elderly AIDS patients to produce high levels IL-10 was directly associated to low in vitro HIV-1 replication.[18] In aged patients, although anti-IL-10 mAb significantly elevated the number of RNA copies in polyclonaly activated $CD4^+$ T-cell cultures, it did not elevate the IFN-γ release.[18] Therefore, despite the low IFN-γ production, the good virological response to HAART in aged patients observed by physicians and described by some authors[54] could be explained, at least in part, by the higher IL-10 levels in this age group.[18]

In conclusion, our work reveals a complex immune dysfunction in aged HIV-1–infected patients, even successfully treated with HAART. The results reported here indicate a dramatic loss of TT-specific IFN-γ–secreting T cells associated with persistent immune hyperactivation that could possibly be extended to other recall antigens. A better characterization of all immune disorders in aged AIDS patients to recall antigens can provide valuable information that might help to design better immunoprophylatic and perhaps immunotherapeutic strategies for this particular group.

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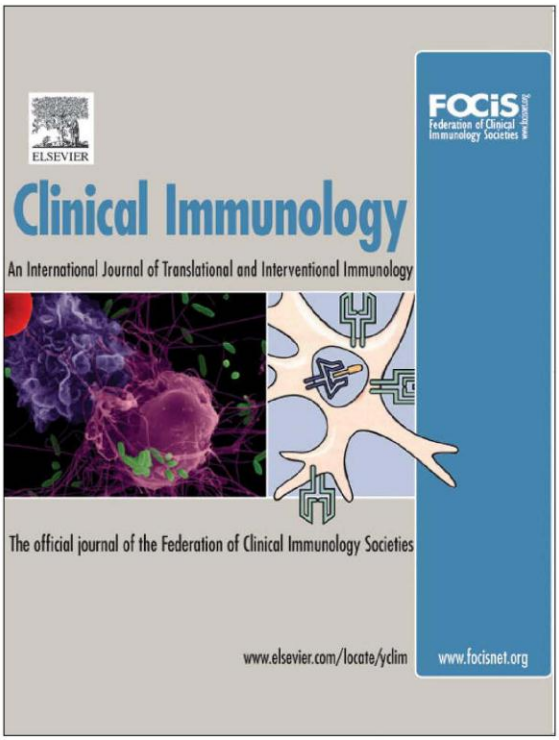

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5.2 **Artigo científico 2-** High IL-10 production by aged AIDS patients is related to high frequency of Tr-1 phenotype and low *in vitro* viral replication

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High IL-10 production by aged AIDS patients is related to high frequency of Tr-1 phenotype and low *in vitro* viral replication☆

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Abstract This work aims to elucidate the effects of age and HIV-1 infection on the frequency and function of T cell subsets in response to HIV-specific and non-specific stimuli. As compared with the younger AIDS group, the frequencies of naive and central memory T cells were significantly lower in aged AIDS patients. Although there was also a dramatic loss of classical $CD4^+FoxP3^+CD25^+$Treg cells in this patient group, high frequencies of IL-10-producing $CD4^+FoxP3^-$ T cells were observed. In our system, the increased production of IL-10 in aged AIDS patients was mainly derived from Env-specific $CD4^+FoxP3^-CD152^+$ T cells. Interestingly, while the blockade of IL-10 activity by monoclonal antibody clearly enhanced the release of IL-6 and IL-1β by Env-stimulated PBMC cultures from aged AIDS patients, this monoclonal antibody enhanced *in vitro* HIV-1-replication. In conclusion, HIV infection and aging undoubtedly

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contribute synergistically to a complex immune dysfunction in T cell compartment of HAART-treated older HIV-infected individuals.

1. Introduction

Aging is now a well recognized characteristic of the HIV-infected population. It is not only a consequence of successful treatment and care of people living with HIV/AIDS, but also an effect of increasing sexual transmission of HIV among older individuals [1]. By 2015, it is predicted that greater than half of all HIV-1-infected individuals will be older than 50 years of age [1]. Therefore, studies are needed to determine the impact of immunosenescence on T cell functional reconstitution in older AIDS patients submitted to highly active anti-retroviral therapy (HAART).

When compared with younger adults, T cells from older individuals (>70 years of age) show qualitative and quantitative deficiencies. Aging is associated with impaired thymic function [2], decreased ratio of naive ($CD45RA^+CD62L^+$) to effector/memory ($CD45RO^+$) T-cells [3,4] and accumulation of exhausted $CD28^-$ T lymphocytes that produce lower levels of IL-2, have shorter telomeres and thus, lower activating, signaling and proliferative potential [4]. Th1 memory cells derived from naive cells from aged individuals produce much less IL-2 than those from young individuals [5]. This damage in the Th1 compartment should have negative impact on the function of cytotoxic $CD8^+$ T cells against viruses. Furthermore, cultures of senescent $CD8^+$ T lymphocytes also produce higher levels of TNFα and IL-6 [6], cytokines that are associated with the frailty characteristic of "normal" aging [7]. Finally, aging is also associated with an increase in frequency and activity of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) [8]. It is believed that chronic quiescent inflammation, a characteristic feature of the elderly, may induce the occurrence of Treg cells [9]. Tregs play a key role in controlling the host immune response to prevent excessive inflammatory damage to host tissues by suppressing both effector T cells and dendritic cells [10]. Nevertheless, when excessive, these regulatory T cell subsets impair the capacity of the host to fight against infections [11].

Interestingly, many of the T cell abnormalities observed in aging are similar to those observed in untreated chronically HIV infected subjects.

In HIV-infected individuals, increased frequency of effector/memory T cells and reduced frequency of naive T cells are associated with $CD4^+$ T cell decline and progression to AIDS [12,13]. Studies have also shown a strict relationship between the frequency of $CD28^-$ T cells and low CD4/CD8 ratios, low naive/memory ratios, reduced T cell repertoire, and reduced responsiveness to vaccines [2,14]. Therefore, the accumulation of senescent immune cells suggests that HIV-1 prematurely ages the T-cell compartment. Many of these immune disturbances in untreated HIV infection are associated with persistently high levels of IL-1β, IL-6, and TNFα [15]. These pro-inflammatory cytokines may also generate more receptive target T cells for viral replication, resulting in a positive feedback loop with further immune activation and $CD4^+$ T cells destruction [16]. Therefore, these immune dysfunctions contribute both to the inability to control HIV-1 replication *in vivo* and to a generalized state of immunodeficiency underlying an increased susceptibility to opportunistic infections and malignancies.

As HIV infection ages the immune system, the 50 years cut-off age is frequently used by the Centers for Disease Control and Prevention (CDC) in HIV/AIDS statistics [17]. Taking into account the immune disturbances of "natural" aging, it is not surprising that HIV-1-infected individuals over the age of 55 exhibit more rapid disease progression with increased likelihood of developing AIDS [17].

The main goal of HAART is to prevent AIDS-related complications by controlling viral replication and elevating peripheral $CD4^+$ T cell counts [18]. When taking HAART, older HIV-infected patients present a lower elevation in $CD4^+$ T cell counts and a reduced reconstitution of naive T lymphocytes [19,20], but, interestingly, a better control of viral load than the younger patients. In this work, we have sought to elucidate the effects of both age and HIV infection on the frequency of different T-cell subsets and the cytokine profile in response to HIV-specific and non-specific stimuli in successfully HAART-treated aged HIV-1-infected patients.

2. Materials and methods

2.1. Patients

The study evaluated a group of 15 HIV-1-infected individuals over 55 years old who were successfully treated with HAART for at least 2 years and were under their first anti-retroviral scheme. A second group included 15 young HIV-1-infected patients (age<40) with similar HIV-infection characteristics. As controls, healthy elderly (age>55, n=10) and young (age<40, n=10) subjects were recruited for this study. All HIV-infected patients were asymptomatic and were recruited from the University Hospitals of the State University of Rio de Janeiro, Federal University of the State of Rio de Janeiro, and Federal University of Rio de Janeiro, Brazil.

To avoid problems concerning multidrug resistance and differences in viral fitness, none of the patients had history of therapeutic failure in their medical records. Furthermore, individuals who were taking any immunosuppressive drug or who had any medical condition potentially affecting the immune system, such as autoimmune diseases, other infectious diseases, malignancies, diabetes, and allergic manifestation were not included.

The consent for participation was obtained from all subjects, and the study was approved by the ethical committee from the University Hospital of the State University of Rio de Janeiro, Brazil.

2.2. Cell cultures and stimuli

Blood samples were collected and the peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll–Hypaque gradients. The viable cells, measured by Trypan blue exclusion, were adjusted to a concentration of 1×10^6 cells/mL and cultured in a 96-well flat bottom microtiter plates in 200 μL RPMI 1640 added with 2 mM L-glutamine, 10% fetal calf serum, 20 U/mL penicillin, 20 μg/mL streptomycin and 20 mM HEPES buffer (GIBCO, Carlsbad, California, USA).

In order to measure HIV-1 specific response, PBMCs (1×10^6/mL) were kept for 5 days in the presence of p24 protein (p24HIV-1_{Gag}, Sigma Co.) at 1 μg/mL or a cocktail of immunodominant synthetic envelope peptides (ppHIV-1_{Env}) at 1 μM each peptide, with recombinant human IL-2 (rhIL-2) at 20 U/mL (BD Systems, Minneapolis, MN). This ppHIV-1_{Env} pool consists of peptides from constant regions [T1 (KQIINMWQEVGKAMYA, aa 428–4430), T2 (HEDIISLWDQSLK, aa 112–124), and TH4 (DRVIEVVQGAYAIR, aa 834–848)] and from hypervariable loop [P18 MN (RIHIGPGRAFYTTKN, aa 315–329) and P18 IIIB (RIQRGPGRAFVTIGK, aa 315–329)]. The concentrations of p24 protein (p24HIV-1_{Gag}) and Env-derived peptides were established in our laboratory as the dose that induces $CD8^+$ T cell proliferation in acutely HIV-1 infected patients. In some experiments, saturating doses of anti-IL-10 (22 μg/mL; BD Systems) were added, at beginning and 3 days after, to the Env-stimulated PBMC cultures. In order to induce polyclonal activation, PBMC cultures (1×10^6/mL) were maintained with phorbol-12-myristate-13-acetate (PMA, 20 ng/mL) plus ionomycin (600 ng/mL) for 24 h or with phytohemagglutinin (PHA) at 5 μg/mL for 7 days. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator.

2.3. Flow cytometry analysis

The mouse anti-human monoclonal antibodies (mAbs) to CD3-PE, CD8-FITC, CD4-FITC, CD45RA-PE, CD45RO-PE, CD69-PE-Cy5, CD152-PECy5, CD62L-APC, CD25-PC, CD127-AlexaFluor647, FoxP3-PE, IL-10-APC, IFN-γ-AlexaFluor®488, and all isotype-control antibodies were purchased from BD Bioscience (San Diego, CA, USA), and were used to characterize the phenotypes of T cells: naive ($CD45RA^+CD62L^+$), effector ($CD45RO^+CD62L^-$), central memory ($CD45RO^+CD127^+$), regulatory ($FoxP3^+CD25^+$) and recently activated T cells ($CD69^+$). Briefly, freshly purified PBMCs (2×10^5/tube) were incubated with various combinations of the aforementioned mAbs for 30 min at room temperature in the dark according to the manufacturer's instructions. After washing with phosphate-buffered saline (PBS), permeabilization was performed by incubating cells with Cytofix/Cytoperm (BD Pharmigen, San Diegio, CA) at 4 °C for 20 min. After washing, the antibodies for intracellular staining (anti-FoxP3 and anti-CD152) or the corresponding isotype control anti-IgG1 were added in various combinations and incubated for 30 min at 4 °C.

For analysis of the frequency IL-10-producing or IFN-γ-producing HIV-1-specific T cells, freshly purified PBMCs were initially kept for 5 days in the presence of p24 protein or envelope peptides with recombinant human IL-2 (rhIL-2) at 20 U/mL (BD systems, Minneapolis, MN) and then submitted to both surface (anti-CD4-FITC, anti-CD45RO-PE, anti-CD45RA-PE, anti-CD8-FITC, and anti-CD69PE-Cy5) and intracellular (anti-FoxP3, anti-CD152, anti-IL-10 and anti-IFN-γ) staining. To optimize cytokine staining, brefeldin A was added 8 h (10 μg/mL; Sigma-Aldrich) before the end of incubation time.

After washing with PBS, the analysis was then performed using either FACSCalibur and CELLQuest software (Becton

Table 1 Characteristics of patients with undetectable plasma viral load. [1]

	Younger group (<40 years)	Aged group (>55 years)
No. in group	15	15
Mean age years (SD)	35.6 (2.1)	60.3 (1.7)
Male (%)	75	83
Time since HIV-1 diagnosis [years (SD)] [2]	6.9 (3.8)	8.8 (3.1)
Mean viral load at baseline [log copies/mL (SD)]	4.2 (0.6)	4.7 (0.8)
Mean CD4 cell count [cells/μL (range)]		
Baseline	161 (51–209)	154.3 (84–270)
End point [2]	614 (401–1007)	603.8 (371–976)
CDC class (%)		
A3	–	08
C3	100	92
Schedule HAART (%) [3]		
AZT/3TC/EFZ	100	92
AZT/3TC/SQV$_r$	–	08

CDC, US left for Disease Control; AZT, zidovudine; 3TC, lamovudine; EFZ, efavirenz; SQV/r, ritonavir-boosted saquinqvir.
[1] Limit of detection: 50 copies/mL.
[2] Diagnosis by laboratory tests.
[3] At least 24 months later at beginning antiretroviral therapy.

Dickinson, San Jose, CA) or Accuri C6 (Accuri™, Ann Arbor, MI, USA) and Cflow software (Accuri™, Ann Arbor, MI, USA). Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 20,000 or 30,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells and doublets.

2.4. Cytokine determination in the supernatants of PBMC cultures

The supernatants collected from PBMC cell cultures activated with PMA/ionomicin or Env peptides were submitted to cytokine measurement by OptEIA ELISA kits (BD, Pharmigen, San Diego), according to the manufacturer's protocol. Briefly, each ELISA was performed using pairs of mAbs directed to human IL-1β, IL-6, IL-10, TGF-β, IL-4, IL-5, TNF-α, IFN-γ, IL-21 and IL-17A. The reaction was revealed with streptavidin–horseradish peroxidase, using 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate. Recombinant human cytokines ranging from 10 to 500 pg/mL were used to construct standard curves.

2.5. Quantification of *in vitro* HIV-1 replication

In experiments to evaluate the impact of endogenous production of IL-10 on *in vitro* HIV-1 replication, saturating

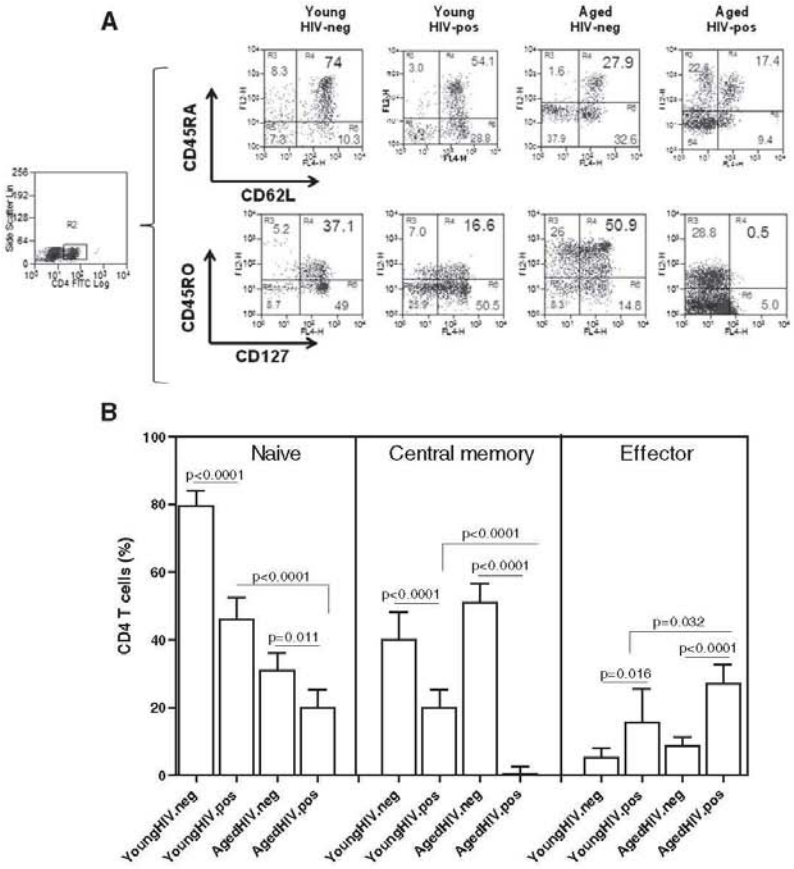


Figure 1 Comparison of the frequency of naive ($CD45RA^+CD62L^+$) and central memory ($CD45RO^+CD127^+$) in $CD4^+$ and $CD8^+$ T cells between young and elderly HIV-infected subjects. Fresh PBMCs from young (n=15) and elderly (n=15) individuals, infected or not with HIV-1, were stained with mAbs to CD4 or CD8 associated with the combination of mAb to CD45RA/CD62L or CD45RO/CD127. (A), (C): Representative dot plots showing identification of naive and central memory $CD4^+$ and $CD8^+$ T cells, respectively. Panels (B) and (C) are the frequency (mean±SD) of naive and central memory $CD4^+$ T cells and $CD8^+$ T cells, respectively. The *p* values were obtained by the Mann–Whitney *U* test.

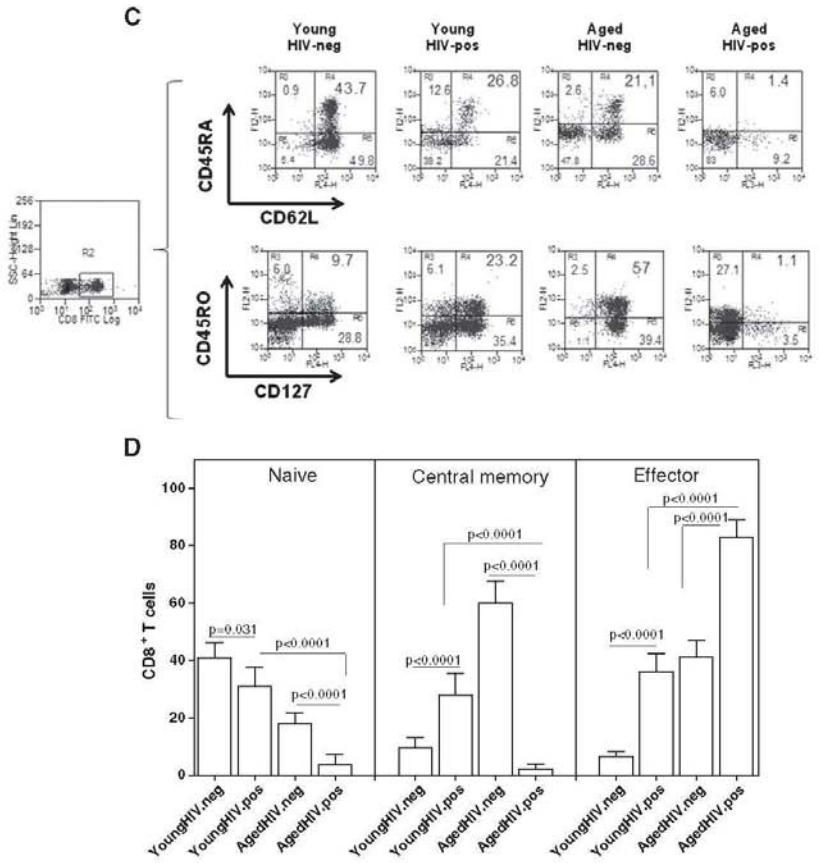


Figure 1 (*continued*).

doses of anti-IL-10 (22 μg/mL; BD Systems) were added in some wells at the time of stimulation of PBMC cultures with PHA (5 μg/mL), and the supernatants were collected 7 days later. This time was chosen because in the previous experiments performed in our laboratory the peak of *in vitro* HIV-1 replication occurred at this point (data not shown). As controls, some wells were incubated with control isotype (IgG2a). Supernatants were stored at −70 °C for posterior HIV RNA quantification by a commercial HIV-1 RNA quantitative reverse transcriptase polymerase chain reaction (Amplicor HIV Monitor Test, Roche Molecular System, Branchburg, New Jersey, USA), with a detection threshold of 50 copies/mL.

2.6. Statistical analysis

Statistical analysis was performed using Prism (GraphPad software). Statistical significance within groups was analyzed with Kruskal–Wallis test. The nonparametric Mann–Whitney U test was applied to determine whether the two groups were statistically different for each given variable. The Student's t-test was applied to verify if a determined variable was statistically different among subjects from the same group. The significance in all experiments was defined as $p<0.05$.

3. Results

3.1. Virological and immunological characteristics of the HIV-infected patients

Two groups of asymptomatic patients, younger (mean age, 35.6 years; range, 31.4–37.8), and older (mean age, 60.3 years; range, 58–64) HIV-infected patients, were enrolled in our study (Table 1). The patients had a similar

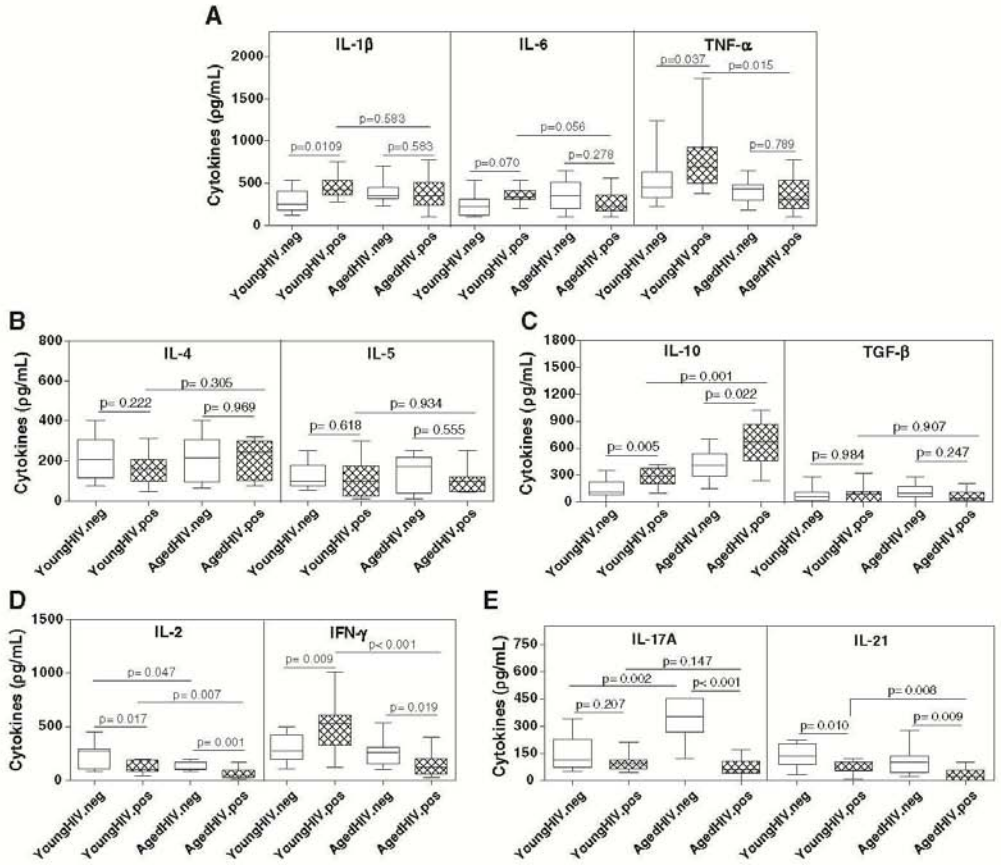


Figure 2 Cytokine profile in the polyclonally-activated mononuclear cells obtained from young and elderly HIV-1-infected individuals. PBMC cultures (1×10^6/mL), obtained from young (HIV-1-negative or HIV-1-positive) and elderly (HIV-1-negative or HIV-1-positive) patients were stimulated with PMA (20 ng/mL) plus ionomicin (600 ng/mL). After 24 h, the supernatants were collected and the cytokine content quantified by ELISA. The horizontal bars within boxes correspond to the median, box limits correspond to 25th and 75th percentiles, and vertical lines indicate the range. In (A), we express the results for the cytokines IL-1β, IL-6 and TNF-α; in (B) the Th2-related cytokines (IL-4 and IL-5); in (C) anti-inflammatory cytokines (Treg-related ones) IL-10 and TGF-β; in (D) Th1-related cytokines (IL-2 and IFN-γ); and in (E) cytokines produced by Th17 phenotype (IL-17 and IL-21). All the *p* values are indicated at the figure.

time in years of HIV-1 diagnosis (young 6.9±3.8×older 8.4±3.1). All of them were successfully treated with a three-drug combination and achieved undetectable plasma HIV-1 RNA levels, defined as <50 copies/mL, within 4 months after initiating therapy and remained below this level for at least 2 years and until the moment of blood sampling. Importantly, the two groups had a similar mean of CD4⁺ cell counts at baseline [young, 154.3 cells/μL (range 51–209)×older, 161 cells/μL (range 84–270), $p=0.557$] and responded immunologically by increasing these counts to similar extension [young, 614 cells/μL (range 401–1007)×older, 603.8 cells/μL (range 387–976), $p=0.488$].

3.2. Severe disturbances in T cell subsets were observed in successfully HAART-treated HIV-infected aged patients

We first performed an analysis of the frequency of T cell subsets in the peripheral blood from older HIV-infected patients. As control for age, the same analysis was performed in HAART-treated younger AIDS patients. In order to compare with the general population, peripheral blood from healthy age-matched individuals (HIV non-infected) was also collected and submitted to the same analysis. As shown in Figs. 1A and B, as compared with all other

groups, the frequency of naive ($CD45RA^+CD62L^+$) and central memory ($CD45RO^+CD127^+$) $CD4^+$ T cells was significantly lower than that in aged HIV-1 infected patients. The same findings were observed in the $CD8^+$ T cell compartment (Figs. 1C and D). On the other hand, the frequency of effector T cells ($CD45RO^+CD127^-$), in both $CD4^+$ and $CD8^+$ subsets, was significantly higher in the aged infected group as compared with all other groups studied (Figs. 1B and D). Importantly, dramatic loss of $CD127^+$ T cells was also identified in this particular group.

3.3. Reduced Th1/Th17-related cytokines and elevated IL-10 production was observed in HAART-treated aged-HIV-infected patients

As we have observed a higher frequency of effector T cells in the peripheral blood of older AIDS patients, we investigated the cytokine profile of PBMC cultures activated with a combination of PMA/ionomycin for 24 h. As shown in Figs. 2A and B, the release of pro-inflammatory cytokines TNF-α and IFN-γ was significantly lower in older AIDS patients, as compared with younger HIV-1-infected ones. Among the HIV-infected patients, the age had great impact on IFN-γ production. Its production was significantly decreased in older AIDS patients. With regard to IL-2, an age-related deficiency in its production was amplified by HIV-1 infection (Fig. 2B). Of note, the release of Th2-related cytokines, IL-4 and IL-5, was not different in all groups studied (Fig. 2C). The release of Th17-related cytokines, IL-17A and IL-21, was significantly damaged in AIDS patients (Fig. 2D). In contrast, when we performed the quantification of anti-inflammatory cytokines, the production of IL-10 was higher in the elderly, and its production was strongly amplified by HIV-1 infection. No difference in the level of TGF-β production was observed in all studied groups (Fig. 2E).

3.4. The higher production of IL-10 in aged AIDS patients was related to the frequency of Env-specific $CD4^+FoxP3^-CD152^+$T cells

The previous results revealed a high production of IL-10 by older AIDS patients associated with impaired production of IFN-γ. Therefore, our next objective was to quantify the frequency of IL-10^+- and IFN-γ^+-T cells targeted to HIV-1 antigens. PBMCs were stimulated with envelope peptides (Env) with recombinant human IL-2 (rhIL-2). Of note, IL-2 alone did not induce any additional cytokine production by T cells as compared with PBMC cultures maintained with only medium (unstimulated) (data not shown). The frequency of effector Env-specific IL-10-producing $CD4^+$T cells was significantly higher in HAART-treated older AIDS patients as compared with younger ones (Figs. 3A and C). The same behavior was observed with $CD8^+$ T cells (Figs. 3B and C). On the other hand, the frequency of effector $CD4^+$ and $CD8^+$ IFN-γ-producing T cells specific to Env antigen was significantly higher in younger AIDS patients (Figs. 3C and F). The same experiments were performed using P24-stimulated PBMC, nevertheless, the frequency of P24-specific T responses was highly variable among younger AIDS patients (data not shown) and some older AIDS patients were unable to respond to it, indicating, probably, a functional loss of this P24-specific T cell subset over time of infection.

As we observed a high level of IL-10 production in response to Env antigen, we sought to evaluate if this event would be related to the frequency of $CD4^+CD25^+FoxP3^+$ regulatory $CD4^+$ T cells (Tregs) in the blood of elderly AIDS patients. Although it has been recently described that these Tregs express low levels of the IL-7 receptor (CD127) [21], we did not use this marker to differentiate non-Tregs from Tregs because, as previously shown (Fig. 1), aged AIDS patients have a severe depletion of CD127 expression on T cell compartment. As demonstrated in Fig. 4B, although we found a clear tendency of normal aging to expand the Treg pool ($p=0.0528$), the frequency of this $CD4^+$ T cell subset was dramatically reduced in HIV-1 infected aged patients. Therefore, in these AIDS patients, Env-specific IL-10-secreting T cells, either $CD4^+$ or $CD8^+$, did not express the classical regulatory T-cell marker, the FoxP3 protein (Fig. 4E). In contrast, the great majority of them were CD152 positive (Fig. 4G). Additionally, approximately 80% (81 ± 6.3) of $CD4^+CD152^+IL\text{-}10^+$ T cells from aged patients were $CD25^-$ following Env-stimulation of PBMCs (Fig. 4F).

3.5. Increased production of IL-10 correlates with *in vitro* control of HIV-1 replication

Previous studies published by us [22] and others [23] have demonstrated that IL-10 down-regulates HIV-1 replication by diminishing the release of pro-inflammatory cytokines. As demonstrated in Fig. 5A, the mean viral load in culture supernatants was significantly lower in the PHA-activated PBMC cultures from aged AIDS patients, and the blockade of IL-10 activity with specific antibodies enhanced the ability of HIV-1 to replicate. Interestingly, IL-10 neutralization up-regulated IL-6 and IL-1β, but not IFN-γ production by Env-stimulated PBMC cultures from aged AIDS patients (Fig. 5B). The same phenomenon was observed in polyclonally-activated PBMCs (data not shown).

4. Discussion

Despite a better HIV control by HAART, older AIDS patients present a lower $CD4^+$ T cell count recovery and a faster disease progression as compared with the younger ones [17,19]. In this study, we investigated the impact of anti-retroviral therapy on the function of T cell subsets in a group of aged AIDS patients who had a good response to therapy by increasing the peripheral $CD4^+$ T cell counts.

The frequency of naive and central memory phenotypes in both $CD4^+$ and $CD8^+$ T cell compartments was significantly lower than that in successfully HAART-treated aged HIV-1 infected patients. On the other hand, we observed an expansion of effector T cells in this population. A severe depletion of $CD127^+$ T cells was also found. In fact, decreased expression of IL-7Rα on T cells has been previously observed in chronic HIV-1 infection [24]. Due to the importance of IL-7 cytokine for the survival of naive and central memory T cells [25], the loss of IL-7Rα expression certainly contributes to the depletion of these cells.

A net damage in Th1/Th17 phenotype is a characteristic of untreated HIV-1-infected patients [26,27]. Here, although elevated IFN-γ production was detected in younger polyclonally-activated PBMC cultures, a dramatic reduction

in the production of Th1/Th17-related cytokines associated with elevated IL-10 release was observed in HAART-treated aged patients. These results suggest that satisfactory CD4⁺ T cell recovery by HAART does not revert the severe disturbances in the cytokine network in older AIDS patients.

Some studies have demonstrated that the pool of classical regulatory T cells, $CD4^+CD25^+FoxP^+CD127^{low/-}$, increases as an individual ages [28], and in our study we observed a clear tendency that healthy elderly patients have more of this T cell subset in the peripheral blood. Although no difference was observed in the frequency of these Treg cells in young individuals, infected or not, a dramatic loss of these cells associated with elevated frequency of Env-specific IL-10-producing $CD4^+FoxP3^-CD152^+$T cells was observed in the peripheral blood of aged AIDS patients. Some studies have demonstrated that Tregs decreased in patients with chronic HIV infection [29–32] and in rhesus macaques experimentally infected with SIV [33]. Direct infection and killing have been proposed as possible causes of the decrease in number of these cells [27]. Therefore, the data presented here suggest that aging elevates the frequency of an HIV-1-specific regulatory T cells that produce IL-10, which are phenotypically compatible with type 1 regulatory T (Tr1) cells [34].

A similar HIV-1-specifc Tr1 cells was described by Torheim et al. [35] in untreated chronically HIV-infected patients. In this population, HIV-1-specific IL-10-producing T cells were either $CD4^+FoxP3^-$ or $CD8^+FoxP3^-$ and were able to potently suppress polyclonal T-cell proliferation, suggesting an involvement of these regulatory T cells in generalized immunodeficiency associated with chronic HIV-1

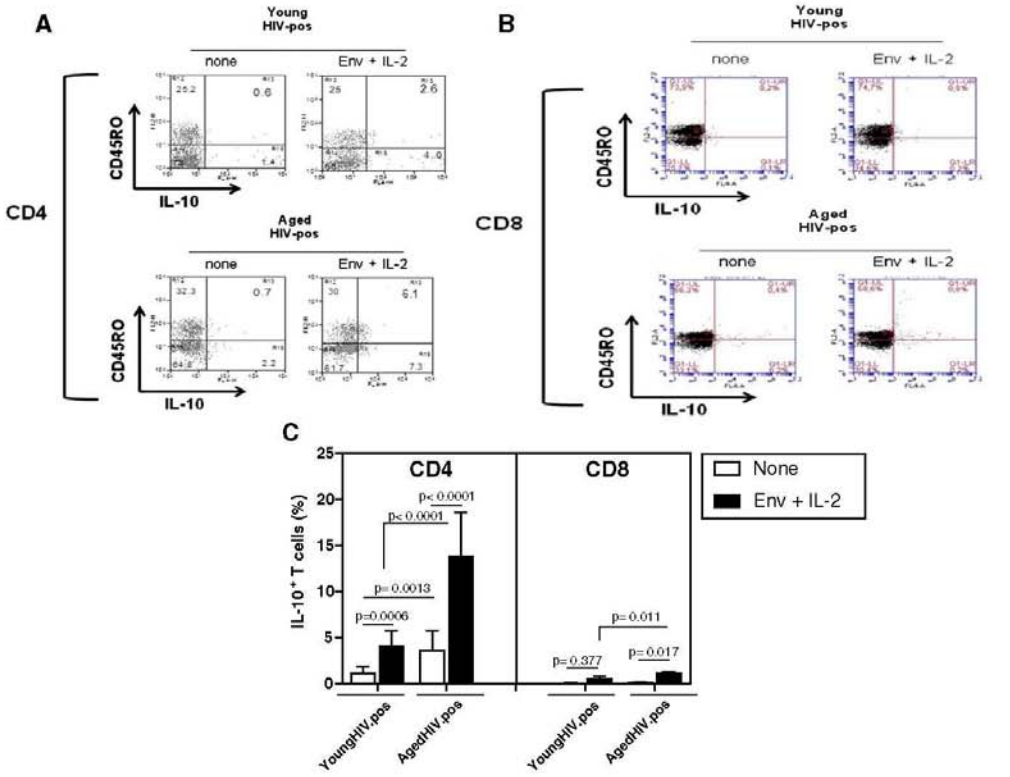


Figure 3 The frequency of HIV-1-specific IL-10- and IFN-γ-producing T cells in young and elderly HIV-1 infected individuals. Purified PBMCs from young (n=15) and elderly (n=15) HIV-1-infected individuals were initially maintained in the presence of a cocktail of immunodominant synthetic envelope peptides (Env) at 1 μM each peptide with recombinant human IL-2 (rhIL-2) at 20 U/mL. After 5 days, the cells were stained with mAbs to CD4 or CD8 associated with CD45RO, then permeabilized and stained with mAb to IL-10 or IFN-γ. Representative dot plots showing CD4CD45RO/IL-10 (A) and CD4CD45RO/FN-γ (D) and CD8CD45RO/IL-10 (B) and CD8CD45RO/FN-γ (E) T cell subsets expressing in the two groups. In (C), the frequency (mean ± SD) of IL-10-producing $CD4^+$ and $CD8^+$ T cell subsets. In (F), the frequency (mean ± SD) of IFN-γ-producing $CD4^+$ and $CD8^+$ T cell subsets in young and elderly HIV-1-infected individuals. The *p* values were obtained by the Mann–Whitney *U* test.

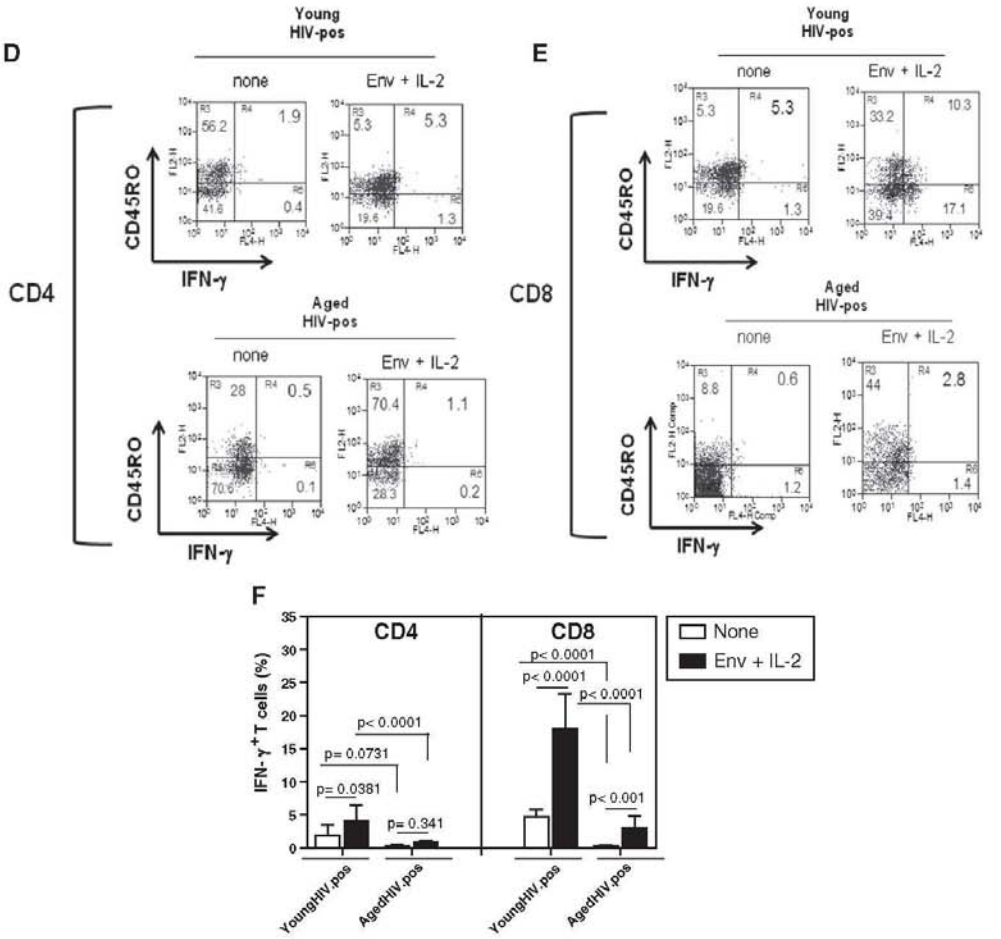
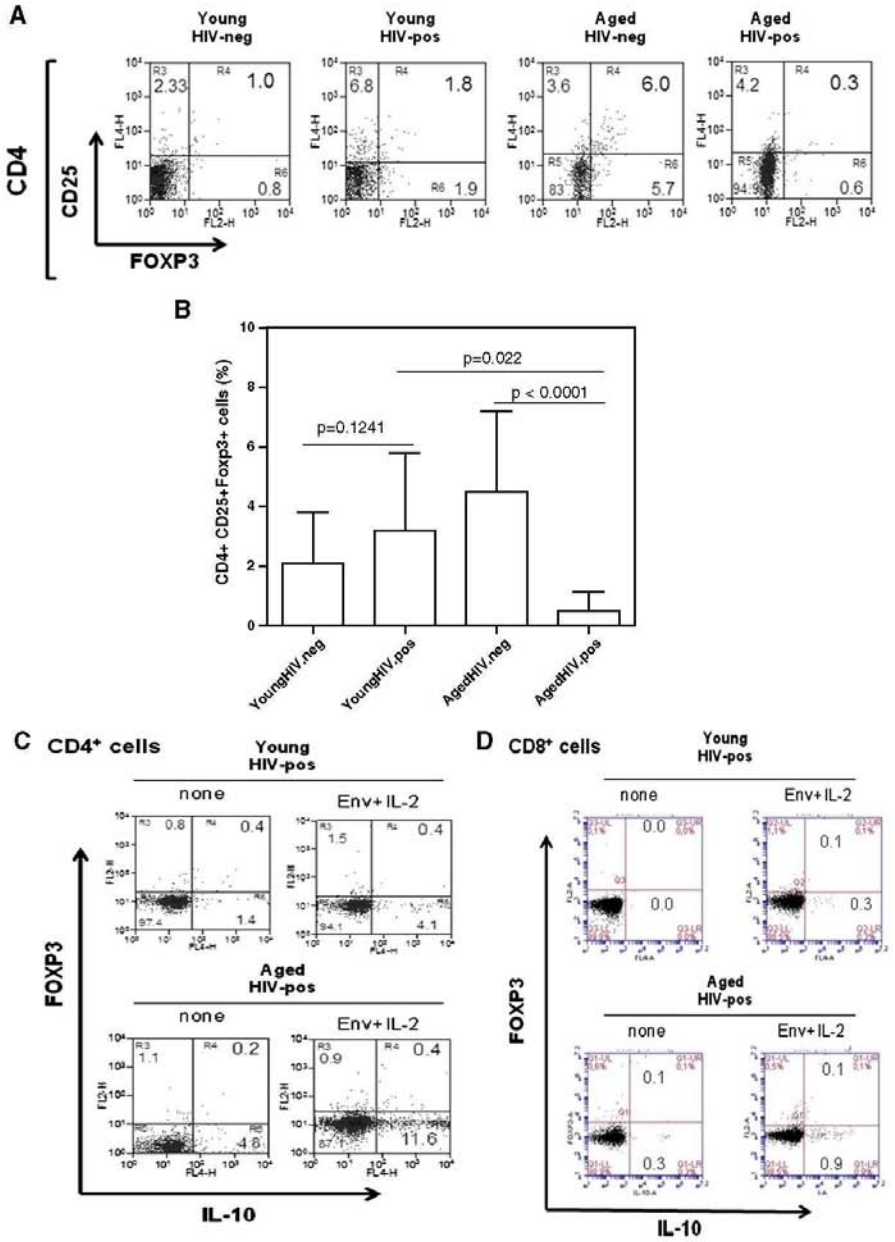


Figure 3 (*continued*)

infection. In their study, however, the frequency of these regulatory T cells was lower than that observed in our present study, and different from our study, the authors did not specify the mean age of those HIV-infected patients. Since lower levels of these Tr1 cells were in fact detected among our younger AIDS patients, we believe that the frequency of this regulatory subset expands as a patient ages. Interestingly, in our system, IL-10 neutralization by anti-IL-10 mAb significantly enhanced IL-1β and IL-6, but not IFN-γ production following activation of PBMCs with Env peptides. These results suggest that Env-specific IFN-γ producing T cells are functionally lost in older AIDS patients and are not recovered by anti-retroviral therapy.

On the other hand, the higher production of IL-10 by aged AIDS patients was related to better *in vitro* control of HIV-1 replication, since the blockade of this cytokine enhanced viral replication, which was related to higher IL-1β and IL-6 production. These present results are in agreement with the findings obtained by us [22] and by other groups [23] that have demonstrated the ability of IL-10 to attenuate HIV-1 replication by diminishing the release of pro-inflammatory cytokines. Thus, while excessive production of IL-10 in HIV-infected patients has been suggested to cause deleterious effects by contributing to down-regulate protective cellular immune response [36], persistently high levels of IL-1β, IL-6, and TNFα are associated with increased viral replication [16]. Therefore, our results suggest that expanded HIV-1-specific IL-10-secreting T cells could help to explain why older AIDS patients have better virological response to anti-retroviral therapy as compared to younger

patients. On the other hand, the net ability of CD152 (CTLA-4) to inhibit accessory cells, mainly dendritic cells through interaction with B7 molecules [37,38], suggests that this elevated frequency of HIV-1-specific CD152$^+$ Tr-1 cells could probably impair any future dentritic cell-based immunotherapeutic approach.



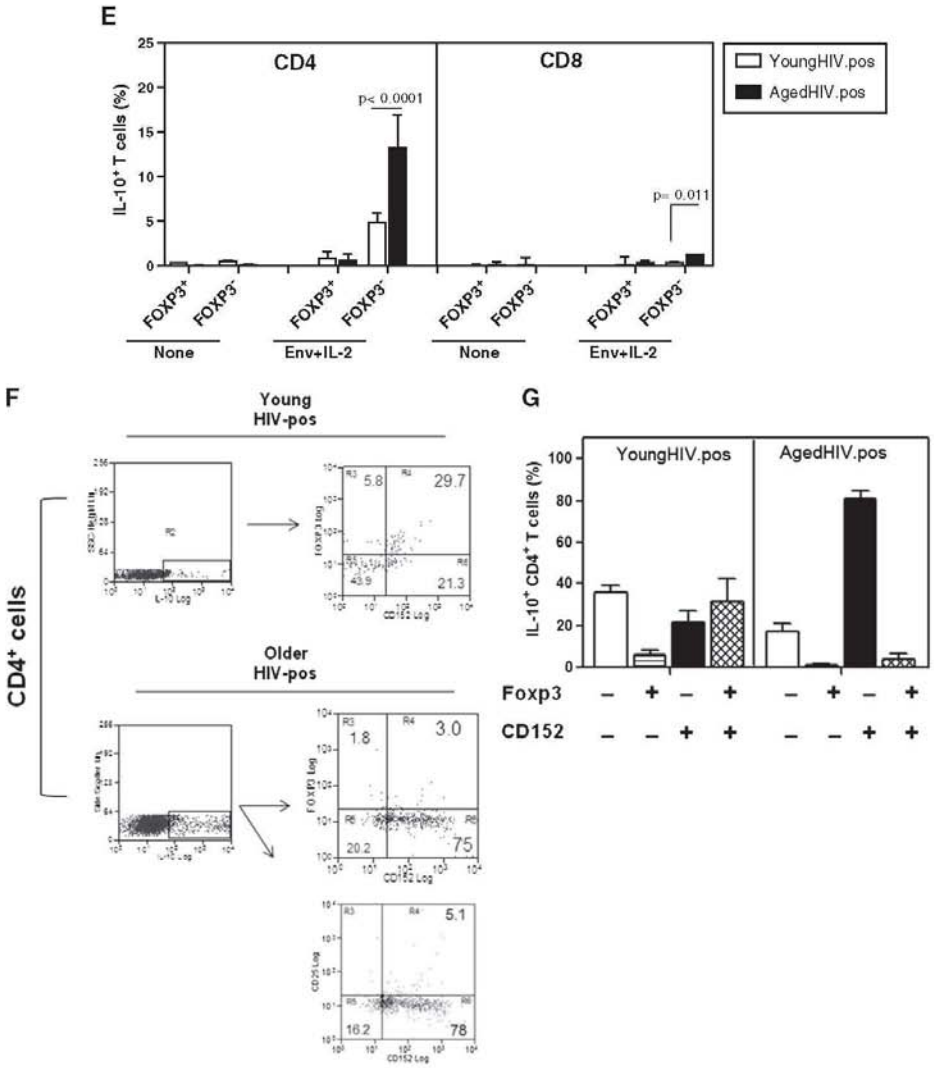


Figure 4 Env-specific IL-10-producing CD4$^+$ T cells are mainly FoxP3$^-$CD152$^+$ in older HIV-1 infected individuals. PBMCs from young and elderly individuals were stained with mAbs to CD4 and CD25, followed by permeabilization and staining with mAb to FoxP3, CD152 and IL-10. In (A), representative dot plots showing CD4$^+$ T cell subsets expressing CD25 and FoxP3 in the different groups. (B) The frequency (mean ± SD) of CD4$^+$ T cell subsets expressing CD25 and FoxP3 in young (HIV-1-negative, n=10; HIV-1-positive, n=15) and elderly (HIV-1-negative, n=10; HIV-1-positive, n=15) individuals. In (C) and (D), representative dot plots showing, respectively, HIV-specific CD4$^+$ (C) and CD8$^+$ (D) T cell subsets expressing IL-10 and FoxP3 after PBMC culture activation with Env peptides plus IL-2 for 5 days, as described in the methodology. In (E), the frequency (mean ± SD) of IL-10-producing $^+$ T cell subsets (CD4 and CD8) co-expressing FoxP3 in younger and older HIV-1-infected individuals. Finally, a better phenotype characterization of Env-specific CD4$^+$ T cells is shown in F and G after staining the cells with anti-CD152. The *p* values were obtained by the Mann–Whitney *U* test.

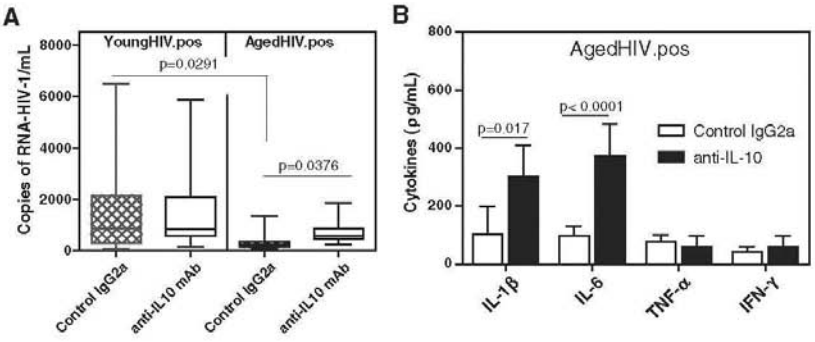


Figure 5 The effect of IL-10 produced on *in vitro* HIV-1 replication and cytokine production. PBMCs (1×10^6/mL) were stimulated in (A) with PHA (5 μg/mL) for 7 days or in (B) with Env peptides (1 μM/peptide) plus IL-2 (20 U/mL) for 5 days in the presence or absence of saturating doses of anti-IL-10 (22 μg/mL). As control, isotype-matched IgG2a was added in some wells. In (A), the horizontal bars within boxes correspond to the median of virus copies in the supernatant, box limits correspond to 25th and 75th percentiles, and vertical lines indicate the range. In (B), the figure shows the mean ± SD of cytokine content in the supernatants quantified by ELISA. The *p* values were obtained by the Student's t test.

4.1. Conclusions

In conclusion, HIV infection and aging undoubtedly contribute synergistically to a complex immune dysfunction in HAART-treated older HIV-infected individuals. Studies like this are needed to provide a better understanding of HIV pathogenesis in the growing older population to provide valuable information for the design of more efficient immunotherapeutic tools, as well as specific treatment guidelines for this aged group of patients with AIDS.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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6 **DISCUSSÃO**

A faixa etária média da população infectada pelo HIV tem se tornado indiscutivelmente maior, devido ao aumento da sobrevida dos pacientes infectados e também à crescente incidência da infecção em indivíduos acima de 50 anos. O envelhecimento, no entanto, traz consigo profundas mudanças sobre o sistema imunitário, conhecidas coletivamente como imunossenescência, potencialmente capazes de modificar o curso da doença e a resposta terapêutica (Deeks, 2011).

Evidências sugerem que a idade avançada pode acelerar a progressão da aids e prejudicar a reconstituição imune nos pacientes tratados com terapia anti-retroviral (TARV). Por outro lado, o eficiente controle virológico desses pacientes pela TARV, aparentemente contraditório, levanta uma série de questões sobre a fisiologia imune desses indivíduos. (Viard *et al.*, 2001; Grabar *et al.*, 2004; Li *et al.*, 2011).

Nossos primeiros resultados, publicados anteriormente ao início deste Doutoramento, demonstraram que culturas de células T periféricas policlonalmente ativadas obtidas de pacientes com mais de 55 anos, com diagnóstico de aids e em sucesso terapêutico, produzem níveis significativamente superiores de IL-10 quando comparadas às culturas de pacientes jovens (Andrade *et al*., 2007, anexo 1). Ademais, documentamos também uma relação inversa desta citocina com a replicação viral *in vitro*. (Andrade *et al*., 2007, anexo 1).

O objetivo maior da presente Tese foi caracterizar fenotipicamente a fonte celular dessa IL-10, inclusive na reposta a antígenos específicos do HIV-1, e avaliar o impacto dessa imunomodulação na resposta a um antígeno de memória, o toxóide tetânico, em pacientes acima de 55 anos com aids e adequadamente tratados com TARV. Dessa forma, procuramos identificar o impacto da idade na reconstituição funcional do sistema imune desses pacientes em comparação a pacientes mais jovens (com menos de 40 anos), também tratados com sucesso. É importante ressaltar que todos os pacientes recrutados apresentavam controle adequado da carga viral plasmática (< 80 cópias de HIV-1/mL) por pelo menos 24 meses e haviam apresentado aumento na contagem de células T $CD4^+$ a níveis semelhantes nos dois grupos amostrais. Indivíduos sadios HIV-negativos, pareados para cada grupo etário, foram também recrutados como controles da infecção pelo HIV-1.

Apesar da Organização Mundial de Saúde (OMS) considerar como idoso o indivíduo acima de 65 anos, o ponto de corte de 50 anos é classicamente utilizado em estudos com pacientes infectados pelo HIV, e é preconizado pelo CDC (*Centers for Disease Control and Prevention*) como referencial de idade avançada a ser considerado para pacientes soropositivos. A justificativa

para isso é a prerrogativa universalmente aceita de que o HIV acelera o envelhecimento sob diversos aspectos, inclusive imunológico (Centers for Disease Control and Prevention, 1992; Nguyen & Holodniy, 2008). Em nossos estudos, usamos um ponto de corte ainda mais alto (55 anos), aumentando a especificidade dos achados.

Estudos publicados por outros autores já tinham demonstrado que, apesar do melhor controle virológico, o ganho numérico de células T $CD4^+$ após introdução da TARV é, em média, inferior em pacientes com idade acima de 50 anos (Grabar *et al.*, 2004). Em vista disso, para que essas diferenças numéricas na recuperação da célula T $CD4^+$ não se tornassem um viés em nossos estudos imunofuncionais, todos os pacientes com aids com mais de 55 anos recrutados apresentavam recuperação numérica semelhante ao grupo jovem de pacientes.

Cabe ressaltar também que todos os pacientes com aids estudados encontravam-se em seu primeiro esquema anti-retroviral e em sucesso terapêutico, ou seja, nunca haviam apresentado falha ao tratamento. Esse dado é importante visto que a aquisição de resistência a drogas anti-retrovirais pode acarretar para o vírus perda da eficiência replicativa ("fitness"), podendo enviesar as análises de replicação viral *in vitro*. (Bleiber *et al.*, 2001).

No primeiro manuscrito desta Tese, avaliamos o impacto da idade na resposta específica T-dependente ao toxóide tetânico (TT). O TT é um clássico modelo experimental de antígeno de memória, já que a vacina é recomendada para todas as crianças e adultos, com reforço a cada 10 anos, e induz resposta T-dependente com grande imunogenicidade. Para esse estudo, todos os indivíduos incluídos receberam uma dose de reforço contra o tétano e a resposta celular e humoral foi comparada entre os indivíduos com menos de 40 ou mais de 55 anos, sadios (grupos controles) ou com aids.

O comportamento da resposta imune TT nos quatro grupos experimentais foi avaliado um mês após a dose de reforço da vacina, e nós observamos prejuízos significativos na eficácia da resposta ao TT no grupo de pacientes com mais de 55 anos, em que os efeitos da idade e da infecção pelo HIV parecem não apenas se somarem, mas atuarem em sinergismo no sentido de amplificar a disfunção imune.

A infecção pelo HIV, nos indivíduos jovens, não acarretou nenhuma diferença significativa na resposta linfoproliferativa ao toxóide tetânico em relação aos jovens saudáveis. Em indivíduos com mais de 55 anos, ao contrário, a infecção causou prejuízo significativo na capacidade linfoproliferativa em resposta ao TT, que a reposição de IL-2 recombinante em cultura não foi capaz de reverter (Artigo 1, Fig. 1). Isso contradiz a premissa defendida por alguns autores de que o déficit de IL-2 na infecção pelo HIV é o grande fator comprometedor da capacidade proliferativa das células T a um desafio antigênico (Schuleck *et al.*, 1993, Schwenerker *et al.*, 2008). Ademais,

vale lembrar que, quando a IL-2 foi usada *in vivo* como imunoterapia em pacientes infectados pelo HIV-1, jamais conseguiu-se mostrar benefício algum na evolução da doença. Demonstrou-se sim elevação da frequência de células T $CD4^{+}$ $CD25^{high}$, sendo provavelmente Tregs induzidas (iTregs) (Sereti *et al.*, 2005).

A dosagem de citocinas produzidas no sobrenadante das culturas de células mononucleares do sangue periférico (PBMC, *peripheral blood mononuclear cells*) dos 4 grupos experimentais em resposta ao TT revelou também achados interessantes. A dosagem de IFN-γ, citocina mais importante da imunidade celular e representativa do fenótipo Th1, não apresentou diferença significativa entre jovens infectados ou não-infectados pelo HIV; mas nos indivíduos acima de 55 anos saudáveis, estava bastante diminuída, e nos pacientes acima de 55 anos com aids estava ainda muito mais baixa. De fato, a célula Th1 é a mais acometida na infecção pelo HIV, tanto diretamente pelo vírus, quanto principalmente, pelas consequências da hiperativação patológica, visto que o Th1 é o fenótipo mais recrutado, e portanto, o mais acometido (Klein *et al.*, 1997; Ledru *et al.*, 1998). Fenômeno semelhante, mas em ritmo mais lento, ocorre ao longo da vida em indivíduos saudáveis (Rink *et al.*, 1998; Deng *et al.*, 2004). Diante disso, a produção deficitária de IFN-γ, tanto devida à infecção pelo HIV quanto devida à idade avançada, justifica a menor capacidade do indivíduo de montar resposta Th1 a antígenos específicos que dependem desse fenótipo, como é o caso do TT, e da maioria dos antígenos existentes. Interessante observar que o efeito da idade e da infecção pelo HIV sobre o fenótipo Th1 na resposta ao TT não foram meramente aditivos, mas sinérgicos.

Ao contrário do IFN-γ, citocinas pró-inflamatórias clássicas da imunidade inata, tais como TNF-α e IL-1, mostraram-se aumentadas tanto na idade avançada quanto na infecção pelo HIV, o que condiz com o observado por outros autores para essas citocinas, e também para IL-6. (Rimaniol *et al.*, 1996; Roubenoff *et al.*, 1998; Franceschi *et al.*, 2000; Breen *et al.*, 2002; Brüünsgaard & Pedersen, 2003). Porém, assim como nos outros distúrbios discutidos até o momento, esse aumento está potencializado no indivíduo que simultaneamente é portador das duas condições: idade avançada e infecção pelo HIV. Estudos conduzidos por Brenchly e colaboradores (2008) demonstraram que a depleção de células Th17 de mucosa intestinal favorece a translocação bacteriana do trato gastrintestinal, a qual tem participação-chave na manutenção do estado de hiperativação crônica na aids, e portanto na patogênese da doença (Brenchly *et al.*, 2008). No idoso, não é clara a origem do aumento das citocinas pro-inflamatórias. Entretanto, apesar da escassez de estudos sobre o assunto, é razoável prever que, na idade avançada, o nível de translocação microbiana também esteja elevado por hipofuncionalidade das barreiras mucosas, induzindo produção inespecífica de citocinas pró-inflamatórias. No contexto da resposta ao TT,

além da contribuição provável da imunidade natural na produção dessas citocinas, células T $CD8^+$ TT-específicas mostraram ser uma fonte adicional importante de IL-1 e TNF-α em todos os indivíduos acima de 55 anos. Interessante notar que dentre esses indivíduos, porém, tais células somente contribuíram com alguma produção de IFN-γ no grupo não-infectado pelo HIV (Artigo 1, Fig. 5)

A produção de níveis elevados de IL-1 e TNF-α pelas células T $CD8^+$ HIV-1-específicas, bem como TT-específicas, podem amplificar os distúrbios celulares e humorais na resposta à vacina contra o tétano. Essas citocinas atuam negativamente na ontogenia das células T e B, inibindo as etapas finais da linfopoiese nos órgãos linfoides primários, o que também terá impacto mais severo nos idosos devido à involução morfo-funcional desses órgãos inerente à idade (Gruver, Hudson & Sempowski, 2007; Aw, Silva & Palmer, 2007). A consequente dificuldade de renovação e manutenção do repertório T e B, associada ao estado de hiperativação crônica e repetitiva, reduzem a frequência de células T e B *naïves* e de memória central com consequente aumento relativo de células terminalmente diferenciadas, identificadas pela não expressão de CD28 e telômeros curtos, denotando senescência replicativa (Franceschi *et al.*, 2000; Franceschi *et al.*, 2007; McElhaney & Effros, 2009). Portanto, o comprometimento da resposta ao TT ocorrerá mediante a redução na frequência de linfócitos *naïve* e de memória central contra o TT, fenômeno esse que está maximizado nos paciente de idade avançada com aids.

Outro interessante efeito adverso induzido pela IL-1, IL-6 e TNF-α no contexto da aids, envolve suas ações em nível de sistema nervoso central. Em elevadas concentrações, essas citocinas estimulam o núcleo paraventricular do hipotálamo, que por sua vez, ativa o eixo hipotálamo-hipófise-adrenal (HPA), assim como eferências simpáticas a partir do tronco encefálico, acarretando, em última instância, no aumento da produção de cortisol e catecolaminas, ambos grandes inibidores da imunidade celular (Ziemssen, & Kern, 2007; Goncharovaa & Tarakanovb, 2007). Esses eventos podem ter mais impacto nos indivíduos com idade avançada, pois eles já possuem tendência à hiperatividade do eixo HPA (Gruver, Hudson, & Sempowski, 2007).

Ainda referente à tríade inflamatória, outro nível de atuação dessas citocinas é estimular a produção de radicais livres e espécimes reativos de oxigênio, principalmente em células do sistema fagocítico mononuclear e polimorfonucleares (Underhill & Ozinsky, 2002). Pessoas de mais idade são também as mais afetadas por potenciais efeitos deletérios do estresse oxidativo, pois esses indivíduos possuem menor capacidade de neutralizá-lo (Cavanagh, Weyand & Goronzy, 2012).

Finalmente, níveis elevados dessas citocinas pró-inflamatórias amplificam a replicação viral por ativar os fatores de transcrição NF-κB e NF-AT nas células T $CD4^+$, o que aumenta a capacidade da célula fabricar novas partículas virais. (Kedzierska, 2003; McGowan, 2004).

Um aspecto da inflamação sistêmica de extrema importância, e que até recentemente era ignorado, é o aumento do risco de afecções potencialmente graves não-associadas à imunodeficiência. A inflamação crônica está presente na patogênese de várias co-morbidades típicas do envelhecimento, como infarto do miocárdio e outras cardiopatias, acidente vascular cerebral, pneumopatias, disfunção renal, afecções neurológicas como demência, e alterações osteoarticulares degenerativas (osteoartrose e osteoporose). (Mansky, 2010). Esse é um dos argumentos pelos quais tem sido preconizado o início mais precoce de TARV pelas recomendações norte-americanas e francesas, e, desde agosto deste ano, pelas recomendações brasileiras também (Thompson *et al.*, 2010; Ministére de la Santé et des Sports, 2010; Ministério da Saúde 2012(2)). Tem-se considerado mais perigoso o risco cardiovascular da inflamação crônica intensa no paciente não-tratado do que o risco das hiperlipidemias e hiperglicemias induzidas pelos anti-retrovirais, pois estes podem ser controlados farmacologicamente.

Outra citocina de importância central em nossas análises sobre a resposta imune ao TT é a IL-10. Em estudo prévio por nosso grupo, esta citocina apresentou-se significativamente aumentada em pacientes com mais de 55 anos infectados pelo HIV, quando comparados a jovens também infectados, em resposta a estímulos policlonais. Experimentos envolvendo depleção seletiva de subpopulações de células T revelaram que as células T $CD4^+$ policlonalmente ativadas eram as principais responsáveis pela elevada produção de IL-10 nessas culturas. Interessantemente, essa produção elevada de IL-10 mostrou-se capaz de atenuar a replicação *in vitro* do HIV (Andrade *et al.*, 2007, anexo 1), mas pode ter consequências deletérias na resposta imune a diferentes desafios antigênicos, devido à atividade inibitória dessa citocina tanto no território da imunidade inata quanto da imunidade adaptativa. Quando avaliamos a resposta ao TT no presente estudo, observamos que também houve produção exacerbada de IL-10 nos grupos com mais de 55 anos, infectados ou não pelo HIV, concomitante com uma produção deficitária de IFN-γ (Artigo 1, Fig. 3). Interessante observarmos que, apesar do conhecido efeito inibitório exercido pela IL-10 na produção de IFN-γ, a neutralização dessa citocina *in vitro,* através da adição de anticorpo monoclonal anti-IL-10, não restaurou a capacidade das células T TT-específicas de pacientes acima de 55 anos com aids de produzir IFN-γ (Artigo 1, Fig. 4). Esses resultados sugerem que, nesse grupo de pacientes, o prejuízo na produção de IFN-γ em resposta ao TT, estímulo indutor de Th1, não se deve à ação inibitória da IL-10 produzida em excesso, e sim à perda real ou funcional de células secretoras de IFN-γ.

Interessante lembrar que todos esses pacientes estavam adequadamente tratados, apresentando carga viral plasmática indetectável e bons níveis de células T $CD4^+$ totais no sangue periférico, ou seja, aquilo que se classificaria como uma excelente evolução clínico-laboratorial. Portanto, vemos que em pacientes de idade avançada, o emprego com sucesso da terapia anti-retroviral, segundo as recomendações atuais, não restitui a capacidade do indivíduo de montar uma resposta Th1 adequada. De fato, fazendo novamente referência ao nosso estudo prévio, quando as células T $CD4^+$ de pacientes com aids e mais de 55 anos são purificadas e submetidas a ativador policlonal, o bloqueio da IL-10 também não teve nenhum efeito em melhorar a tímida produção de IFN-γ por essas células (Andrade *et al.*, 2007, anexo 1).

Estudos sobre resposta protetora contra o tétano seguindo a vacinação têm inferido um papel crucial dos anticorpos IgG no sentido de neutralizar a toxina do tétano em pacientes infectados pelo *Clostridium tetani*. (Tapia *et al.*, 2006; Gonçalves *et al.*, 2007). Dessa forma, a resposta imune humoral ao TT foi também avaliada em nosso presente estudo e mostrou-se amplamente prejudicada em pacientes acima de 55 anos com aids. Interessante notarmos que, assim como na resposta proliferativa, a idade também parece ter impacto maior do que a infecção pelo HIV sobre a resposta humoral ao TT, pois após reforço de vacinação contra o tétano, o grupo de mais idade, mesmo saudável, produziu títulos consideravelmente mais baixos de IgG anti-TT do que os jovens com ou sem aids. E no grupo acima de 55 anos com aids, a capacidade de produção de IgG anti-TT cai de forma vertiginosa e desproporcional para valores próximos aos encentrados antes da aplicação da vacina, diferindo claramente do prejuízo relativamente pequeno que a infecção pelo HIV causou na resposta humoral nos pacientes jovens. Mais uma vez, os distúrbios da idade avançada e da infecção pelo HIV não apenas se somam no idoso infectado, mas amplificam-se.

Os principais motivos, não necessariamente excludentes, que justifiquem essa deficiência na produção IgG anti-TT podem ser: 1) a depleção do fenótipo Th1, principal colaborador da célula B para a síntese de IgG contra o TT, 2) deficiência na ontogenia, especificamente na diferenciação de pró-B para pré-B (disfunção no rearranjo VDJ, hiporresponsividade a IL-7 e involução morfo-funcional da medula óssea); 4) perda de variabilidade dos BCRs e 5) ativação policlonal nos linfócitos B, levando à senescência replicativa, como ocorre com os linfócitos T. (McElhaney & Effros, 2009; Gruver, Hudson & Sempowski, 2007).

Portanto, definiram-se até o momento várias alterações funcionais celulares e humorais na resposta específica a um clássico antígeno de memória em indivíduos maiores de 55 anos e com aids, tornando esse grupo imunologicamente não-comparável a idosos saudáveis e nem a jovens com aids. Diante desses achados, questiona-se aqui o custo-benefício de submeter o paciente de idade avançada com aids a reforços de vacinação anti-tetânica, que parecem não induzir resposta

protetora nem celular nem humoral. Mas estudos clínicos seriam necessários para alterar as recomendações atuais, que preconizam um reforço a cada 10 anos (Ministério da Saúde, 2008).

Dando continuidade aos nossos estudos, as análises subsequentes foram experimentalmente mais complexas e deram origem ao segundo manuscrito aqui reportado. Evoluímos para uma caracterização fenotípica detalhada da subpopulação T $CD4^+$, HIV-específica ou não, de indivíduos com aids e idade avançada. Caracterizamos os componentes desta população de células T, o perfil de citocinas por eles produzido e sua reação imunofisiológica em resposta a estímulo policlonal potente (PMA+inonomicina) e a antígenos específicos do envelope do HIV-1 (ppHIV-1env).

Para tanto, as células T obtidas do sangue periférico foram marcadas com diferentes combinações de anticorpos monoclonais fluoresceinados e submetidas à citometria de fluxo, objetivando determinar a frequência de linfócitos T virgens ($CD45RA^+CD127^+CD62L^+$), efetores ($CD45RO^+CD127^-$) e memória central ($CD45RO^+CD127^+$) e, em associação com a análise de citocinas, estudar os fenótipos envolvidos na resposta aos estímulos citados. A identificação de fenótipos reguladores envolveu marcação intracelular para IL-10, FoxP3 e CTLA-4, e extracelular para CD25. Finalmente, também foi feita a quantificação do RNA viral, por RT-PCR, no sobrenadante das culturas, para relacionar a capacidade de replicação viral *in vitro* com o(s) fenótipo(s) dominante(s) identificado(s).

A primeira análise fenotípica foi feita em PBMC frescas coletadas dos indivíduos dos 4 grupos experimentais (Artigo 2, Fig. 1). No grupo acima de 55 anos com aids, verificamos, tanto no compartimento T $CD4^+$ quanto no T $CD8^+$, uma grande perda de células T virgens ($CD45RA^+CD62L^+$) e principalmente de memória central ($CD45RO^+CD127^+$), associada a um aumento na frequência de células efetoras ($CD45RO^+CD127^-$). Portanto, o reconhecido fenômeno de inversão da relação *naïve*/efetora, observado tanto no envelhecimento imunológico quanto na infecção pelo HIV, foi amplificado no grupo de mais idade com aids, tornando esses indivíduos ainda menos capazes de reconhecer novos desafios antigênicos.

No passo seguinte, as PBMC foram ativadas por 24 horas com PMA mais ionomicina e a análise do perfil de citocinas foi realizada pela técnica ELISA. Os níveis de citocinas relacionadas ao fenótipo Th1, IFN-γ e IL-2, foram significativamente inferiores nas culturas de pacientes com mais de 55 anos com aids. Esse mesmo fenômeno foi observado nas culturas de PBMC na resposta específica ao estímulo com TT no primeiro artigo dessa Tese. Esse resultado, mais uma vez, confirma a clara deficiência do fenótipo Th1 observada tanto na aids quanto na imunossenescência, por ser o fenótipo mais requisitado e mais susceptível a apoptose (Holzer, D. & Rossol, R. 1997; Deng *et al.*, 2004).

Na avaliação do fenótipo Th17, observou-se fenômeno interessante: nos indivíduos saudáveis, a idade avançada aumentou significativamente a produção de IL-17. Porém, a infecção pelo HIV-1, independente da idade, reduziu de forma significativa à produção não apenas de IL-17, como também de IL-21, outra citocina típica do fenótipo Th17. Esses resultados estão de acordo com dados publicados na literatura demonstrando deficiência no fenótipo Th17 nos pacientes com aids (Hunt, 2010; Brenchley *et al*., 2008).

Quanto à maior tendência das PBMC de indivíduos com mais idade saudáveis em produzir IL-17, resultados similares foram obtidos em outros estudos por outros autores em modelo humano e murino (Stout-Delgado *et al.*, 2009; Tesar *et al.*, 2009; Lee *et al.*, 2011). Uma possível explicação para esse fenômeno pode residir no fato da maior propensão dos idosos em produzir citocinas importantes na indução da célula Th17, tais como IL-6 e IL-1β (Goldstein, 2009). Além disso, com a depleção preferencial do fenótipo Th1 à medida que o sistema imune envelhece, é possível que o Th17 tenha que atuar mais do que o normal no sentido de suprir essa perda durante a resposta a desafios antigênicos diversos.

A infecção pelo HIV, entretanto, anula por completo o aumento do fenótipo Th17 induzido pela idade, trazendo os níveis de IL-17 e IL-21 para valores iguais ou menores aos encontrados em jovens com aids.

Quanto às citocinas pró-inflamatórias IL-1, IL-6 e TNF-α, observou-se um aumento associado à infecção pelo HIV em pacientes jovens, o que condiz com o observado na literatura, e explicado, por alguns autores, pela elevada translocação bacteriana do trato gastrintestinal por disfunção da barreira mucosa (Brenchley *et al*., 2006). Dentre os indivíduos com idade avançada, entretanto, a infecção pelo HIV não elevou os níveis dessas citocinas. Ademais, no caso do TNF-α, sua produção foi significativamente inferior nas culturas de pacientes acima e 55 anos e com aids quando comparados a jovens com aids (Artigo 2, Fig. 2A). Em estudo prévio, demonstramos que a IL-10 produzida por indivíduos de idade avançada com aids é capaz de inibir significativamente a produção de TNF- α nesses indivíduos (Andrade *et al*., 2007, anexo 1).

De fato, quando analisamos a IL-10 no presente estudo, níveis maiores dessa citocina anti-inflamatória foram realmente encontrados nas culturas de células de pacientes acima de 55 anos, quando comparados aos pacientes jovens. Esses resultados, observados nas culturas de PBMC seguindo breve estímulo com mitógenos, são similares ao encontrados nas culturas seguindo ativação com TT por 5 dias, reportados no primeiro manuscrito desta Tese, e também com fito-hemaglutinina por 3 dias em estudo prévio (Andrade *et al*., 2007, anexo 1), o que indica uma tendência de resposta com níveis elevados de IL-10 em ambos os modelos experimentais. Esse

mesmo padrão foi observado também na resposta HIV-1-específica, como demonstrado no segundo manuscrito apresentado nessa Tese.

Neste segundo artigo, uma elevada frequência de células T $CD4^+$ $IL\text{-}10^+$ foi identificada no grupo acima de 55 anos com aids em resposta a peptídeos do envelope viral (ppHIV-1env). O contrário foi observado com IFN-γ, que estava extremamente reduzido nessa subpopulação de células T, mesmo mediante adição de IL-2 exógena. A marcação diferencial CD4/CD8 mostrou que, nos pacientes de mais idade com aids, a tímida produção de IFN-γ detectada é originada da célula T $CD8^+$. Da mesma forma, a produção ainda preservada desta citocina nos jovens infectados é também proveniente em sua maior parte da célula T $CD8^+$. Por outro lado, a IL-10, encontrada em pequena quantidade nos jovens e em grande quantidade nos maiores de 55 anos, provém essencialmente do compartimento T $CD4^+$. Esses resultados sugerem, portanto, que nos pacientes com idade superior a 55 anos, células T $CD4^+$ produtoras de IL-10, específicas ou não para o HIV-1, representam o fenótipo dominante.

Como apresentado na introdução dessa Tese, a IL-10 é uma citocina produzida majoritariamente por um conjunto de células T com função reguladora (Constatino *et al*., 2008; Aluvihare *et al.*, 2004; Shevach *et al.*, 2006). Nosso próximo passo foi identificar qual(is) o(s) provável(eis) subtipo(s) de células T $CD4^+$ reguladora(s) produtora(s) de IL-10 em nosso sistema. Alguns estudos têm demonstrado que o envelhecimento acarreta aumento relativo de células reguladoras T $CD4^+CD25^+FoxP3^+$ (Gregg *et al.*, 2005; Lages *et al.*, 2008; Tenorio *et al.*, 2009). Interessante observarmos que, apesar da aids não ter alterado de forma significativa a frequência de células $FoxP3^+$ em jovens, e da mesma ter-se mostrado aumentada na idade avançada, a frequência desses linfócitos cai de forma muito acentuada no indivíduo mais idoso com aids. Alguns estudos associam a perda de células Treg na aids com a falta de controle virológico adequado e excesso de ativação imune, e essa perda está relacionada a um pior prognóstico (Eggena *et al.*, 2005; Apoil *et al.*, 2005). Estudo mais recente conduzido em primatas não-humanos infectados pelo SIV ("simian immunodeficiency virus") mostrou que a atividade inibitória Treg correlaciona-se diretamente com melhor controle da infecção, ao passo que a perda funcional dessa célula pode favorecer a progressão da doença (Karlsson *et al.*, 2011). Em nosso sistema, observamos uma perda acentuada dessas células, que não pôde ser caracterizada quanto à expressão do CD127, devido a quase completa ausência de expressão desse marcador nos linfócitos T dos pacientes acima de 55 anos com aids, denunciando, na verdade, uma perturbação não apenas no compartimento das células T reguladoras clássicas, como também das células T virgens e de memória central.

A análise fenotípica detalhada, portanto, revelou que as células T $CD4^+$ hiperprodutoras de IL-10 no grupo mais idoso com aids não expressam a proteína FoxP3, porém são positivas para o marcador CD152 (CTLA-4), sugerindo fortemente tratar-se de células compatíveis com o fenótipo Tr-1. (Artigo 2, Fig. 4). Elevada frequência de células semelhantes a Tr-1 foram também identificadas em pacientes sem tratamento anti-retroviral, em resposta a peptídeos estruturais do HIV codificados pelo gene *gag* (Torheim *et al*., 2009).

Nesse nosso último estudo, a hiperprodução de IL-10 pelas células T $CD4^+$ de pacientes mais idosos com aids foi capaz de atenuar a replicação viral *in vitro*, como mostra a quantificação do RNA viral no sobrenadante de PBMCs ativadas com a fitohemaglutina, ou PHA. O bloqueio desta citocina através de IgG murina anti-IL-10 humana favorece a replicação viral, provavelmente, por elevar significativamente a produção das citocinas pró-inflamatórias IL-1 e IL-6 no indivíduo com aids e idade avançada (Artigo 2, Fig. 5), o que está em consonância com achados prévios (Weissman, Poli & Fauci, 1994; Andrade *et al.*, 2007, anexo 1). De fato, IL-1, IL-6 e o TNF-α são reconhecidamente favorecedoras da replicação do HIV (Folks *et al.*, 1989; Granowitz *et al.*, 1995, Furler & Uittenbogaart, 2010; Baker *et al.*, 2012). O bloqueio da IL-10, no entanto, à semelhança dos resultados obtidos através da estimulação das culturas de PBMC com o toxóide tetânico, não conseguiu restaurar a capacidade de produção de IFN-γ, em resposta ao ppHIV-1env, sugerindo que as células produtoras de IFN-γ foram de fato depletadas, pelo menos funcionalmente.

Os achados aqui reportados preenchem algumas lacunas do conhecimento sobre imunologia da aids no paciente de idade avançada, população crescente no cenário epidemiológico atual. Foi demonstrado que, no idoso com aids, tanto na resposta inespecífica como na específica, há um predomínio absoluto de células T $CD4^+$ efetoras sobre células naïve e de memória central, e que essas células majoritárias são fenotipicamente compatíveis com o fenótipo Tr-1, hipersecretor de IL-10.

A importância prática desses achados reside no fato de que os dois parâmetros laboratoriais avaliados no acompanhamento clínico de pacientes com aids, contagem de células T $CD4^+$ e carga viral plasmática, podem passar a ter valor questionável no paciente de idade avançada. A rápida redução da carga viral pode não significar bom prognóstico por ser devida a uma hiperprodução da citocina inibitória IL-10, e o aumento da contagem de células T $CD4^+$ após início de tratamento pode ser apenas matemático e não protetor, uma vez que essas células são, em sua maioria, supressoras da imunidade celular, que é a principal responsável pela resposta a agentes oportunistas.

Erguem-se então questões importantes que requerem ensaios clínicos para respondê-las: 1) O tratamento anti-retroviral no idoso com aids deve ser iniciado nos mesmos níveis de células T $CD4^+$ preconizados para jovens? 2) As profilaxias para infecções oportunistas não deveriam ser iniciadas mais precocemente nos pacientes de mais idade? 3) Podem ser interrompidas nos mesmos níveis de células T $CD4^+$ que o fazemos para jovens com aids? 4) Como adequar as recomendações de imunizações a esse grupo de pacientes?

Parece-nos clara, portanto, a necessidade de serem elaboradas recomendações voltadas especificamente para o acompanhamento clínico e tratamento de pacientes de idade avançada com aids, a exemplo das recomendações específicas que existem para crianças e gestantes, que são também populações imunologicamente diferenciadas. Como demonstrado em vários momentos ao longo desta Tese, o sistema imune de um paciente de idade avançada com aids não reflete simplesmente uma soma da imunossenescência com a imunopatologia da infecção, mas sim um sistema com características absolutamente particulares e ainda pouco conhecido.

7 **CONCLUSÕES**

- Há evidentes particularidades fenotípicas na resposta imune mediada pela célula T a partir da 6ª década de vida de um paciente com aids adequadamente tratado.
- A investigação da imunorreatividade ao toxóide tetânico sugere a ausência de benefício imunológico da vacina contra o tétano para esses pacientes.
- Tanto o toxóide tetânico quanto antígenos específicos do envelope do HIV induziram majoritariamente um fenótipo regulador de resposta T-dependente nos pacientes com idade avançada infectados pelo HIV, diferentemente do ocorrido com os jovens.
- A tendência à produção exacerbada de IL-10 por esses pacientes se deve ao predomínio do fenótipo Tr-1, mas a deficiência na capacidade de produção de IFN-γ não foi reversível pela neutralização da IL-10, sugerindo uma perda celular do fenótipo protetor Th1, e não apenas modulação funcional pelo Tr-1.
- A simples avaliação numérica da célula T CD4 no sangue periférico de indivíduos com idade avançada não é suficiente para o estadiamento imunológico de um paciente de idade avançada com aids, pois a recuperação numérica e a indetectabilidade da carga viral não acompanham uma reconstituição funcional, principalmente com relação ao fenótipo Th1.
- Os achados aqui descritos sugerem a possibilidade de uma maior suscetibilidade desse grupo de pacientes a infecções oportunistas, e uma menor responsividade a imunizações ativas, colocando em questionamento a validade das condutas terapêuticas e profiláticas atualmente preconizadas indistintamente para o paciente jovem e para o idoso com aids.

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APÊNDICE - Artigo científico prévio Interleukin-10-secreting CD4 cells from aged patients with AIDS decrease in-vitro HIV replication and tumour necrosis factor a production.

Interleukin-10-secreting CD4 cells from aged patients with AIDS decrease in-vitro HIV replication and tumour necrosis factor α production

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Objective: To evaluate the impact of age on the proliferative response, cytokine profile and viral kinetics in AIDS patients treated successfully with antiretroviral drugs.

Methods: Peripheral blood mononuclear cells (PBMC), CD4 cell-depleted PBMC or CD4 T cells from young adult and aged HIV-1-infected patients were activated *in vitro* with anti-CD3 with or without interleukin (IL)-2. Lymphoproliferation and cytokines were measured after 3 days and in-vitro HIV-1 replication after 7 days.

Results: Both lymphoproliferation and cytokine [IL-1β, tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ)] secretion were higher in younger than in older AIDS patients. In cultures of cells derived from aged patients and activated by anti-CD3, IFN-γ production was severely damage and IL-10 production was much higher. Although IL-2 addition to activated PBMC elevated IFN-γ secretion, IL-10 production remained elevated in the aged group. The depletion of CD4 T lymphocytes from these cultures dramatically reduced released IL-10 in the older group but did not alter significantly IFN-γ production. Interestingly, higher IL-10 levels produced by CD4 T cells were related to lower in-vitro HIV-1 replication, and the blockade of this cytokine by anti-IL-10 monoclonal antibody enhanced virus replication. This effect may be correlated with elevated TNF-α secretion. Finally, impaired IFN-γ secretion detected in activated CD4 T cells obtained from aged patients was not directly correlated with high IL-10 production.

Conclusions: Elevated IL-10 production by aged AIDS patients contributed considerably to control of HIV replication and to inhibition of TNF-α secretion but not to the reduced IFN-γ production.

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Keywords: aged patient, AIDS, CD4 T cells, cytokines, interleukin-10, HIV-1 replication, interferon-γ, senescence, T-cell responses, tumour necrosis factor α

Introduction

Infection caused by HIV-1 is characterized by a gradual depletion of the CD4 T lymphocytes, which determines disease progression until the development of AIDS and can increase the occurrence of opportunistic infections and malignancies [1]. Besides the decrease in CD4 T cell number, there is also functional damage to these lymphocytes, which is partly related to the virus-induced dysregulation in cytokine network [1–4].

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Macrophages and T lymphocytes from HIV-infected patients are important sources of chronic cytokine production, which has great impact in clinical progression [4–7]. Pro-inflammatory cytokines, for example, can favour intense HIV replication [3–7]. Studies have shown that HIV-derived Nef protein activates macrophages to secrete a variety of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-1α, IL-12, macrophage colony-stimulating factor, granulocyte–macrophage colony-stimulating factor and tumour necrosis factor (TNF)-α, all resulting in attraction of nonspecific CD4 T cells to the area of HIV replication [4–6]. These pro-inflammatory mediators, which are elevated during HIV-1 infection, also diminish the threshold of CD4 T cell activation and, consequently, favour HIV replication as well as activation-induced cell death [2–6].

As infection proceeds, however, many patients show an abnormal cytokine pattern characterized by increased secretion of IL-4 and IL-10 and decreased production of cytokines by T helper 1 cells (Th1), such as IL-2 and interferon γ (IFN-γ) [8,9]. In this phase, the lack of Th1 cytokines makes individuals susceptible to both opportunistic infections and malignancies. Therefore, in the absence of therapy, the HIV-1-related AIDS is uniformly fatal.

The marked decrease in the prevalence of AIDS-related diseases following the introduction of HAART relates to the control of plasma viral load and an increase in peripheral CD4 cell counts [10]. Many studies with HAART-treated HIV-infected young adults have shown that the risk for some opportunistic infection is reduced when patients achieve a certain increased CD4 cell count [11–15]. However, in addition to increasing CD4 cell count, the impact of HAART on the degree of functional immune recovery is also related to reduction of the hyperactivation state that is observed in chronically HIV-1-infected patients [16,17]. Normalization in pro-inflammatory cytokines and recovery of IFN-γ secretion has been detected in some patients following successful HAART [17,18]. However, several host variables, particularly age, have been shown to correlate with the degree of immune restoration during HAART [19,20]. As described in a recent report, the accumulative number of AIDS cases in adults over 50 years in age had a five-fold increase worldwide from 1900 to 2001 [21,22] which confirms the need for more research on this particular population. The influence of age on the numeric CD4 T cell response to HAART was examined in the EuroSIDA study with 1956 patients after 31 months of treatment [22]. The mean increase in CD4 cells during the first 6 months on HAART was lower in patients over 50 years, despite a better virological response in this group. Immune function status in this group of AIDS patients requires further elucidation.

The present study aimed to characterize age-related immune functional events in HIV-infected patients successfully treated with HAART and their impact on viral load. Lymphoproliferation and cytokine production induced by polyclonal activators was examined *in vitro* in cells from adult young and aged AIDS patients and correlated with in-vitro HIV-1 replication.

Methods

Patients

The study examined a small group of 16 individuals over 55 years old (median, 58.9 years; range, 55–65) who had chronic HIV-1 infection and were successfully treated with antiretroviral therapy. A second group included 16 young HIV-1-infected patients (median, 31.1 years; range, 22–38), with similar infection characteristics. All individuals were recruited from the Centre of Epidemiology at the Hospital of the State University of Rio de Janeiro. The consent for participation was obtained from all, and the study was approved by Ethic Committee from University Hospital of the State University of Rio de Janeiro, Brazil.

Characteristics of the HIV-infected individuals were obtained from medical records, including the HAART regimens and CD4 cell count at baseline and 24 months after the beginning of antiretroviral therapy. CD4 cell counts are expressed as absolute values and the viral loads are given as $\log_{10}$ values in order to normalize distribution. To avoid problems concerning multidrug failure, all the participating individuals were previously naive to antiretroviral drugs. Patients with chronic clinical complications other than HIV-1 infection, such as cancer, or those in use of immunomodulating drugs were excluded.

Cell culture and stimulation

Blood samples were collected from the HIV-1-infected patients approximately 2 years after they had started HAART, and the peripheral blood mononuclear cells (PBMC) were collected by centrifugation on Ficoll–Hypaque gradients as previously described [23]. For some experiments, the CD4 cell-depleted PBMC or purified CD4 T cells were obtained by negative selection using magnetic beads coated with anti-CD4 monoclonal antibody or a kit containing antibody mixes to antigens targeted CD8, HLA-DR/DP, CD56, CD14, anti-CD19, CD36 and CDW123 (Dynal Biotech, Great Neck, New York, USA), respectively. The efficacy of this procedure was approximately 97% as evaluated by flow cytometry (data not shown). The number of viable cells for each condition was measured by Trypan blue exclusion in a haemocytometer. The viable cells were adjusted to a concentration of 1×10^5 cells/well and were cultured in a 96-well flat bottom microtitre plates with 200 μl RPMI 1640 added with 2 mmol/l L-glutamine (GIBCO, Carlsbad, California, USA), 10% fetal calf serum, 20 U/ml penicillin, 20 μg/ml streptomycin and 20 mmol/l HEPES

buffer. These cultures were stimulated with plate-bound anti-CD3 (OKT3; 5 μg/ml) in the absence or presence of recombinant human IL-2 (rhIL-2) at 20 U/ml (BD Systems, Minneapolis, Minnesota, USA). The cells were cultured for 3 days at 37°C in a humidified 5% CO_2 incubator for proliferation and cytokine assays.

Proliferation assay

Approximately 1×10^5 cells/well of the cell cultures containing PBMC or CD4 T lymphocytes were activated or not with anti-CD3 (5 μg/ml), with or without rhIL-2 (20 U/ml) for 3 days. The cellular proliferation was measured after addition of [^{3}H]-thymidine (0.5 μCi/well) for the last 8 h of incubation. The cells were harvested in glass fibre filters in an automatic cell harvester and radioactive incorporation was measured using a liquid scintillation counter. In some experiments, the [^{3}H]-thymidine uptake by activated CD4 T cell cultures was evaluated after addition of saturating doses of anti-IL-10 (22 μg/ml; BD Systems). Results for each patient group are shown as means (±SD).

Cytokine determination

The supernatants from the different cell cultures were collected after 3 days and cytokines were measured using OptEIA enzyme-linked immunosorbent assays (ELISA) (BD Pharmigen, San Diego, California, USA), according to manufacturer's protocol. Briefly, each ELISA used pairs of monoclonal antibodies directed against human IL-1β, IL-10, IL-4, TNF, and IFN-γ. The reaction was revealed with streptavidin–horseradish peroxidase, using 3,3′,5,5′-tetramethylbenzidine as substrate. Recombinant human IL-1β, IL-4, IFN-γ, TNF and IL-10 at concentrations ranging from 10 to 500 pg/ml were used to construct standard curves.

The effect of interleukin-10 produced by CD4 T cells on in-vitro HIV-1 replication

In experiments to evaluate the impact of endogenous production of IL-10 on in-vitro HIV-1 replication, saturating doses of anti-IL-10 (22 μg/ml; BD Systems) were added in some wells at the time of stimulation of CD4 T cells cultures with anti-CD3 (OKT3; 5 μg/ml) plus rhIL-2 (20 U/ml), and the supernatants were collected 7 days later. This time was chosen because in previous experiments the peak of in-vitro HIV-1 replication occurred at this point (data not shown). As controls, some wells were incubated with control isotype (IgG_{2a}). HIV RNA was measured in the supernatants stored at −70°C by a commercial HIV-1 RNA quantitative reverse transcriptase polymerase chain reaction (Amplicor HIV Monitor Test, Roche Molecular System, Branchburg, New Jersey, USA), with an average detection threshold of 80 copies/ml.

Statistical analysis

Student's *t* test was used to determine statistically differences between the two groups for a given variable. For correlation of different variables within a group, Spearman's correlation coefficient was calculated and tested for statistical significance. Significance was defined as $P < 0.05$.

Results

Virological and immunological characteristics of the patients

Table 1 shows the characteristics of the two groups of patients, who all had undetectable plasma viral load. Despite the differences in baseline viral load, all patients were successfully treated with a three-drug combination. All achieved undetectable plasma HIV-1 RNA levels, defined as < 80 copies/ml, within 3 months of initiating therapy and remained below this level for at least 2 years (data not shown). Importantly, the patients started therapy with similar baseline CD4 cell counts and responded immunologically by increasing these counts following therapy.

The lymphoproliferative response was lower in aged patients successful treated with HAART than in their counterpart young HIV-1-infected individuals.

The first immune event studied was lymphoproliferative response induced by nonspecific T cell stimuli. Figure 1 shows the lymphoproliferative response to anti-CD3, which was higher in younger HIV-1-infected patients than in aged patients. As reduction in the T cell proliferation in AIDS patients is related to impairment of IL-2 secretion [24], rhIL-2 was added to some cultures containing anti-CD3. As demonstrated in Fig. 1, T cell lymphoproliferative response induced by anti-CD3 was elevated by exogenous IL-2 in both groups, but it remained lower in aged HIV-1-infected group.

Cytokine secretion by HAART-treated aged HIV-1-infected patients

An important immune disturbance in HIV-1-infected patients involves the persistent production of pro-inflammatory cytokines [3–7], and better immune reconstitution following HAART has been linked to normalization of the cytokine network [11,14,16,17]. PBMC from younger HIV-infected individuals spontaneously secreted higher concentrations of IL-1β and TNF-α than those from the older patients (Table 2). When these cultures were polyclonally activated, those from the younger group continued to secrete higher levels of these pro-inflammatory cytokines than did those from the older group (Table 2). While pro-inflammatory cytokines were secreted in lower amounts in cell cultures from aged patients compared with the young group, IL-10 secretion was significantly higher in the older group (Table 2). No statistical difference was observed between the two groups for IL-4 release. The most interesting

Table 1. Characteristics of patients with undetectable plasma viral load[a].

	Aged group (> 50 years)	Younger group (< 40 years)
No. in group	16	16
Mean age [years (SD)]	58.9 (4.2)	31.1 (5.5)
Male (%)	60	50
Time since HIV-1 diagnosis [years (SD)[b]]	7.1 (1.4)	6.8 (2.2)
ADIS		
CDC class (%) [35]		
A3	20	20
B3	10	20
C3	70	60
Mean viral load at baseline [log copies/ml (SD)]	4.4 (0.4)	4.8 (0.46)
Mean CD4 cell count [cells/μl (SD)]		
Baseline	152.4 (25.2)	144.3 (33.7)
End point	455 (145)	501 (127)
Schedule HAART[c]		
AZT/3TC/EFZ	4	7
AZT/ddI/EFZ	4	4
AZT/3TC/NFV	4	2
AZT/ddI/NFV	2	1
AZT/3TC/SQV/r	2	2

CDC, US Center for Disease Control; AZT, zidovudine; 3TC, lamovudine; ddI, didanosine; EFZ, efavirenz; NFV, nefinavir; SQV/r, ritonavir-boosted saquinqvir.
[a]Limit of detection: 80 copies/ml
[b]Diagnosis by laboratory tests.
[c]24 months later at beginning antiretroviral therapy. HAART comprised two nucleoside reverse transcriptase inhibitors, plus one nonnucleoside reverse transcriptase inhibitor, or one protease inhibitor.

result was observed when IFN-γ production was assayed. As demonstrated in Table 2, a dramatic age-related deficiency in the ability to secrete IFN-γ was detected. Although IL-2 addition significantly elevated IFN-γ secretion in anti-CD3-activated PBMC cultures from aged patients ($P < 0.001$), it remained lower than that detected in the younger group. Therefore, critical cytokine dysregulation following HIV-1 infection was more dramatic among the aged patients.

The role of CD4 T cells on cytokine secretion in aged HIV-1-infected patients with immunological response to HAART

To understand the involvement of the rise of peripheral CD4 cell count in the cytokine pattern observed, PBMC were treated with T cell polyclonal activators after removal of the CD4 T cells using anti-CD4-coated immunomagnetic beads. As demonstrated in Fig. 2, IL-1β and TNF-α release, in contrast to IFN-γ secretion, was not statistically modified by depletion of CD4 cells from whole PBMC in the young group. Interestingly, such depletion of PBMC from the aged group significantly elevated IL-1β and TNF-α secretion (Fig. 2). In the absence of the CD4 T cell subset, IL-10 production was reduced in the majority of activated cell cultures, an effect that was stronger in cell cultures purified from the aged individuals (Fig. 2). In this activation model system, the secretion of these cytokines was not significantly changed by depletion of CD19 cells (B lymphocytes) from PBMC cultures from either age group (data not shown). These results suggest that activated CD8 T cells may be the main source of the pro-inflammatory cytokines detected in all cell cultures. In contrast, elevated IL-10 secretion, detected mainly in the activated aged cell cultures, is probably a CD4 cell-dependent event. Finally, these results suggest that recovery of peripheral CD4 T cells following HAART had a different impact on the pattern of cytokine production in younger and older HAART-treated AIDS patients.

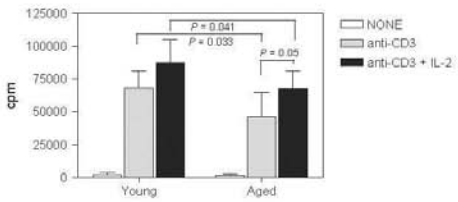

Fig. 1. Proliferative response of peripheral blood mononuclear cells purified from 16 young and 16 aged subjects with chronic HIV-1 infection. Cells (1×10^5/well) were cultured with medium alone or with different stimuli for 3 days. Stimulators were anti-CD3 with or without recombinant human interleukin 2 (IL-2). Means (SD) are given for each group.

The impact of interleukin 10 produced by CD4 T cells on in-vitro HIV-1 replication and on cytokine secretion

As shown in the Table 2, the highest levels of IL-10 were detected in activated cell cultures from elderly patients. Furthermore, the depletion of CD4 cells from PBMC dramatically reduced the secretion of this cytokine, indicating the involvement of this T cell subset in IL-10 production (Fig. 2). Highly purified CD4 T cells were

Table 2. Cytokine production by polyclonally activated mononuclear cells from young and aged HIV-1-infected patients.

Cytokine	None	Anti-CD3	Anti-CD3+IL-2
Mean interleukin-1β [pg/ml (SD)]			
Young	128 (45)*	958 (267)**	1156 (521)***
Aged	82 (32)*	402 (131)**	678 (371)***
Mean tumour necrosis factor-α [pg/ml (SD)]			
Young	280 (112)***	10 715 (3046)***	11 521 (4510)***
Aged	78 (42)***	2050 (1036)***	2155 (1653)***
Mean interferon-γ [pg/ml (SD)]			
Young	29 (27)*	1750 (550)***	2512 (951)***
Aged	8 (11)*	358 (216)***	607 (248)***
Mean interleukin-4 [pg/ml (SD)]			
Young	92 (36)	411 (103)	731 (371)
Aged	77 (350	578 (228)	933 (451)
Mean interleukin-10 [pg/ml (SD)]			
Young	13 (22)	1646 (692)***	2015 (791)***
Aged	21 (17)	6750 (2135)***	8851 (3771)***

Significant differences: $^{*}P<0.05$; $^{**}P<0.001$; $^{***}P<0.0001$.

examined during polyclonal activation (Fig. 3), which induced significantly higher levels of IL-10 in aged patients than in the young group ($P=0031$). The mean IL-10 production in aged and young groups was 5168 pg/ml (SD, 3557) and 1296 pg/ml (SD, 1007), respectively. Lower virus replication was also detected in supernatants from aged activated CD4 T cells cultures (Fig. 3a). Interestingly, when these cultures were sorted by their IL-10 secretion and levels of in-vitro HIV-1 replication, the highest IL-10 secretion clearly correlated with lower in-vitro virus replication in almost all patients (Fig. 3b,c). In this context, 69% (11/16) of the young patients had HIV-1 RNA > 5000 copies/ml against 25% (4/16) in the aged group. To understand the impact of this correlation, saturating doses of anti-IL-10 were added at the beginning of the culture period, and virus replication was evaluated 7 days later. As demonstrated in Fig. 4A, there is a clear correlation between the blocked of IL-10 activity and the ability of HIV to replicate. Anti-IL-10 elevated HIV-1 RNA in 88% (14/16) of the activated CD4 T cell cultures from the aged group, increasing the virus replication up to six times compared with isotype-matched controls. The direct relationship between lower virus replication with elevated IL-10 secretion was only significantly observed in the aged group ($P=0.036$; (Fig. 4b).

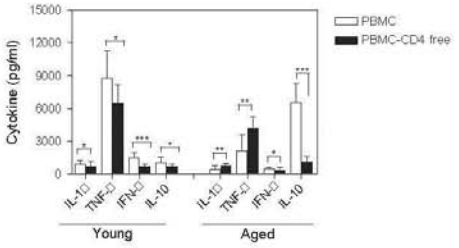

Fig. 2. Involvement of CD4 T cells in cytokine secretion in young and aged HIV-1-infected patients with immunological response following HAART. CD4 cell-depleted peripheral blood mononuclear cells (PBMC; 1×10^{5}/well), obtained as described in the Methods, were stimulated with anti-CD3 (5 μg/ml) plus recombinant human interleukin (IL) 2 (20 U/ml); after 3 days, supernatants were collected and the levels of IL-1β, tumour necrosis factor α (TNF-α), interferon γ (IFN-γ) and IL-10 were measured. Means (SD) are given for each group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$.

To evaluate if the ability of IL-10 to downregulate HIV-1 replication was related to the capacity of this anti-inflammatory cytokine to reduce proliferation of CD4 T lymphocytes, [^{3}H]-thymidine uptake was evaluated in the presence of anti-IL-10 or isotype-matched control. As shown in Fig. 5a, IL-10 neutralization did not significantly modify CD4 T lymphocytes proliferation. Many pro-inflammatory cytokines have been known to support HIV-1 replication *in vitro* [3–7] and so TNF-α and IFN-γ secretion was also examined in these cultures. Blockade of IL-10 significantly augmented TNF-α secretion but did not change that of IFN-γ in activated CD4 cells cultures from aged patients (Fig. 5b). These data suggested that the IL-10 secretion by CD4 T cells was involved in the downregulation of virus replication, probably by inhibiting TNF-α. Additionally, these data suggest that reduced IFN-γ secretion observed in cultures from aged HIV-1-infected patients was not directly related to their elevated IL-10 production.

Discussion

The pharmacological treatment of AIDS by HAART is associated with decreasing plasma viral load and increasing CD4 T cells counts [10]. However, the success of immunological reconstitution following HAART can be less effective in some patients, particularly in older individuals. In our study, functional cellular immune recovery was significantly lower in the older

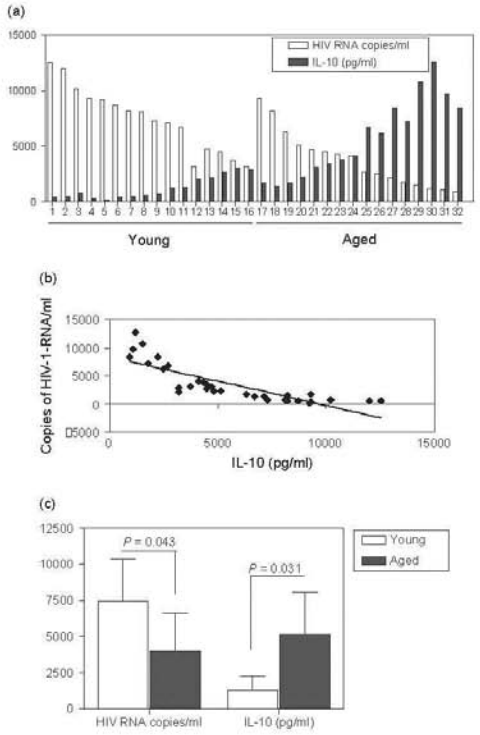

Fig. 3. The inverse correlation between HIV-1 replication and interleukin (IL) 10 production. CD4 T cells (1×10^5/well) from the 16 young and 16 aged patients, obtained as described in the Methods, were stimulated with anti-CD3 (5 μg/ml) plus recombinant human IL-2 (20 U/ml). Supernatants were collected after 3 days for IL-10 measurement or after 7 days for measurement of HIV-1 replication. (a) HIV-1 RNA and IL-10 production for each patient. (b) The inverse correlation between in-vitro HIV-1-RNA measurements and IL-10 secretion. (c) In-vitro HIV-1-RNA measurements and IL-10 secretion presented as mean (SD) for each group.

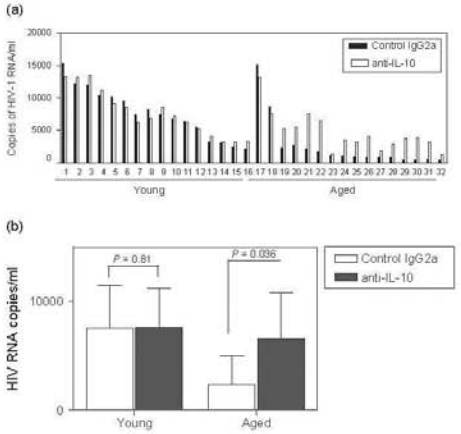

Fig. 4. The effect of interleukin-10 (IL-10) produced by CD4 T cells from 16 young and 16 aged patients on in-vitro HIV-1 replication. CD4 T cells (1×10^5/well), obtained as described in the Methods, were stimulated with anti-CD3 (5 μg/ml) plus recombinant human IL-2 (20 U/ml) in the presence of saturating doses of anti-IL-10 (22 μg/ml) at the beginning of the cultures. As control, isotype-matched IgG_{2a} was added in some wells. Supernatants were collected after 3 days for IL-10 measurement or after 7 days for measurement of HIV-1 replication. (a) Virus replication detected in activated CD4 T cells for each HIV-1-infected patient. (b) Mean (SD) in-vitro HIV-1 replication obtained after IL-10 blockage for the two patient groups.

HIV-1-infected patients even when they had augmented their CD4 cell counts to similar levels to those observed in younger patients. Many studies with HAART-treated young HIV-infected adults have stated that once patients have achieved a certain CD4 cell count, the risk for some opportunistic infections is accepted as low [11–13]. Our results, however, suggest that the interruption of prophylaxis for opportunistic agents following increase in CD4 cell counts after HAART may not be a merely mathematical clinical decision.

In our study, the T cell proliferative response to polyclonal activators was higher in the HAART-treated young HIV-1-infected patients than in the older group. This difference was also observed when we evaluated the specific lymphoproliferative response to tetanic toxoid in those in both groups who had been previously immunized against *Clostridium tetani* (data not shown). Some authors have previously shown that HIV-1-infected young adults treated with IL-2 had a marked increase in proliferative response mediated by CD4 and CD8 T cells to mitogens and recall antigens [24]. However, in other HIV-1-infected patients, particularly older ones, this immune approach could contribute less to improve the immune status.

Many of the immune disturbances described in individuals with chronic HIV-1 infection are related to the dysregulation in cytokine network induced by the virus [25,26], and the magnitude of functional immune recovery is related in part to the degree to which immune hyperactivation is controlled [27]. In this context, spontaneous and activated IL-1β and TNF-α secretions were lower in the PBMC cultures from older patients than from younger ones. The analysis of the cell subsets involved in the cytokine pattern observed in our system suggested that the main source of these pro-inflammatory cytokines in CD3-activated cell cultures from both groups was probably from CD8 T cells. It has

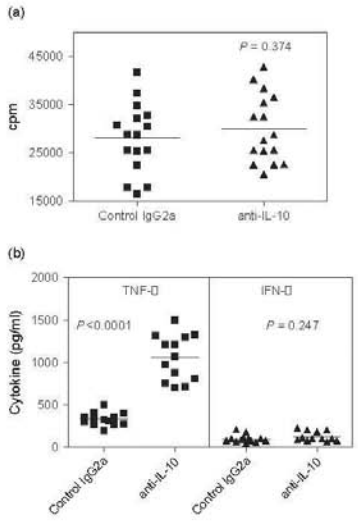

Fig. 5. The role of interleukin 10 (IL-10) produced by CD4 T cells from 16 aged patients on in-vitro proliferation and cytokine production. CD4 T cells (1×10^5/well), obtained as described in the Methods, were stimulated with anti-CD3 (5 μg/ml) plus recombinant human interleukin IL-2 (20 U/ml) in the presence saturating doses of anti-IL-10 (22 μg/ml) at the beginning of culture. As control, isotype-matching IgG_{2a} was added in some wells and the wells were incubated for 3 days. (a) CD4 T cell proliferation, detected by [^{3}H]-thymidine uptake. (b) Tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) secretion.

been known that, as HIV disease progresses, specific and nonspecific chronically activated CD8 T cells contribute to a generalized state of immune activation by secreting high levels of IL-1β and TNF-α; patients successfully treated with HAART tend to reduce this immune dysregulation [28].

The immune hyperresponsiveness observed in individuals with chronic HIV-1 infection is paradoxically associated with a decrease in IFN-γ-producing CD4 T cells upon antigenic stimulation [28], and the ability of HAART to restore IFN-γ production seems to depend on endogenous IL-2 release [18,29,30]. In our study, even after IL-2 addition, severe dysfunction in IFN-γ production by polyclonally activated T cell cultures was observed in the aged AIDS patients, despite their good numerical CD4 T cell recovery following HAART. A similar IFN-γ-release deficiency was also observed in cultures of PBMC from aged AIDS subjects during restimulation with tetanic toxoid compared with PBMC from younger patients (data not shown). These results suggest that, in older patients, HAART does not efficiently restore the ability to mount an adequate cellular immune response. Furthermore, these novel observations raise important issues for immunotherapeutic approaches such as subsubcutaneous IL-2 injection to improve immune function in aged AIDS patients.

In our study, there was a higher tendency of activated CD4 T cells to produce IL-10 in aged AIDS patients. Furthermore, elevated IL-10 production was also detected in the anti-CD3-activated CD4 T cells from healthy aged subjects (data not shown), suggesting that this phenomenon is age related and not AIDS associated. Excessive production of IL-10 in HIV-infected patients has been suggested to cause deleterious effects by contributing to decrease the production of Th1 cytokines, which are implicated in promoting resistance to different pathogens [31]. In our system, the blockade of IL-10 secreted by activated CD4 T lymphocytes increased the TNF-α secretion but did not alter the IFN-γ production in aged subjects, in contrast to young HIV-1-infected patients. In healthy aged individuals, however, blockade of IL-10 did elevate IFN-γ production (data not shown). These findings are in agreement with the theory that IFN-γ-producing cells are the group most prone to destruction in the course of HIV infection [1,5,8].

Our experiments have shown that the ability of CD4 T cells from elderly AIDS patients to secrete higher IL-10 is directly associated with low in-vitro HIV-1 replication. Anti-IL-10 elevated significantly the number of RNA copies in activated CD4 T cell cultures from older AIDS patients. This phenomenon was not related to any change in CD4 T lymphocytes proliferation, because anti-IL-10 did not significantly alter lymphocyte polyclonal expansion. However, blockade of IL-10 in our model enhanced significantly TNF-α release by activated CD4 T cells from the aged AIDS patients. Weissman *et al.* [32] demonstrated that IL-10 blocked HIV-induced TNF-α and IL-6 release and inhibited virus replication in monocyte-derived macrophage cultures. Therefore, the good virological response to HAART in older patients observed by physicians and described by some authors [33,34] could be explained, at least in part, by the IL-10 produced in high levels in this age group, which reduces the TNF-α secreted by some immune cells such as CD8 T lymphocytes.

In conclusion, our results reveal a complex immune dysfunction in aged HIV-1-infected patients, even in those successfully treated with HAART. In our system, the IL-10 produced by CD4 T cells had an antiviral effect by diminishing HIV-1 replication, probably by decreasing TNF-α production. However, the low IFN-γ secretion in older AIDS patients does not appear to be directly related to the high levels of IL-10. A better characterization of this IL-10-secreting CD4 cell group could provide valuable information that might help in the design of better immunotherapeutic tools for this older group of patients with AIDS.

Acknowledgements

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APÊNDICE B – Termo de Consentimento Livre e Esclarecido

"A IMUNOLOGIA DA INFECÇÃO PELO HIV EM PACIENTES COM MAIS DE 55 ANOS: Reconstituição imune e possíveis implicações imunoterapêuticas"

Investigador Principal: Dr. Regis Mariano de Andrade – Médico Infectologista

EXPLICAÇÃO DO PROJETO DE PESQUISA AOS PACIENTES

1. Propósito de estudo

O propósito dessa pesquisa é aprender mais sobre o vírus da AIDS em pacientes acima de 55 anos. Vamos dar continuidade aos nossos estudos sobre a recuperação do sistema imunológico dos pacientes da terceira idade, quando são tratados com os anti-retrovirais. Acreditamos que os resultados obtidos pelo nosso estudo terão grande importância para o acompanhamento clínico desse grupo de pacientes.

2. Procedimentos

Nós precisaremos saber há quanto tempo que você tem ciência de seu estado portador e tempo vem sendo tratado. Vamos avaliar todos os seus resultados de carga viral e a contagem de células T $CD4^+$ e T $CD8^+$ desde que você iniciou o tratamento. Ainda teremos que avaliar a quais esquemas anti-retrovirais você já foi submetidos e os prováveis motivos de troca terapêutica, caso tenha ocorrido.

Precisaremos também colher uma única vez 20 mL de seu sangue, para avaliarmos a sua competência imunológica. Acompanharemos o seu prontuário para obter informações sobre o seu tratamento.

O seu médico será notificado se os testes imunológicos sugerirem que a conduta terapêutica adotada deve ser modificada por outra que possa ser melhor para você.

Esse estudo não afetará o seu tratamento. O médico que lhe assiste continuará recomendando o tratamento de acordo com o Consenso do Ministério da Saúde do Brasil.

A parte do seu sangue que será usado em nosso estudo será enviada para o laboratório de *Imunofisiologia e Imunopatologia dos Linfócitos T* (UNIRIO), que está trabalhando conosco nesse projeto. O seu sangue retirado não será utilizado para nenhum outro fim (não será usado para testes genéticos, não será mandando para outros laboratórios e nem será usado para propósitos comerciais). Caso você decida que quer que o sangue retirado seja jogado fora, fale com o seu médico e nós o jogaremos fora a seguir.

3. Riscos e desconfortos

Nós não iremos acrescentar nenhum risco ou desconforto além da retirada de sangue, que você já faz habitualmente para seus exames de rotina.

4. Benefícios

Os resultados obtidos no laboratório serão cruzados com os dados clínicos para que toda a equipe possa trabalhar junta. A pesquisa ajudará a nós, e a outros, a aprender mais sobre essa doença, nos pacientes de idade mais avançada, que ainda se conhece pouco.

5. Custos e compensações

Você não pagará nada para participar nesse estudo e também não será pago por estar no estudo.

6. Confidencialidade

Seus dados pessoais são informações confidenciais. Essas informações serão mantidas estritamente em sigilo. O seu nome não será dado para ninguém além do grupo clínico que o acompanha. Qualquer publicação científica dos resultados não identificará você.

7. Direito para não participar ou para se retirar da pesquisa

Sua participação nesse estudo é voluntária. Caso você não queira participar, você continuará a receber o melhor tratamento disponível nesse serviço. Você também é livre para retirar o seu consentimento a qualquer hora. Caso você venha a desistir de participar, isso não em nada afetará o tratamento atual.

8. Consentimento

Uma vez que você leu (ou lhe foi explicado) e entendeu o propósito desse estudo, os procedimentos que serão realizados, os riscos e benefícios, e você concorda VOLUNTARIAMENTE em fazer parte desse estudo, favor assinar seu nome abaixo:

Paciente: Prontuário:

Eu expliquei o propósito do estudo para o(a) paciente. Ao meu entender, ele(a) entendeu o propósito, procedimentos, riscos e benefícios desse estudo.

Nome do Investigador:

Assinatura do Investigador:

Testemunha:

Assinatura da Testemunha:

Data:

ANEXO – Aprovação do Comitê de Ética

UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO
HOSPITAL UNIVERSITÁRIO PEDRO ERNESTO
COMITÊ DE ÉTICA EM PESQUISA

Rio de Janeiro, 10 de junho de 2003

Do: Comitê de Ética em Pesquisa
Prof.: Wille Oigman
Para: Drª. Cleonice Alves de M. Bento

O Comitê de Ética em Pesquisa do Hospital Universitário Pedro Ernesto, após avaliação, considerou o projeto (755-CEP/HUPE) "RELAÇÃO ENTRE A CINÉTICA DA REPLICAÇÃO VIRAL E RECOSNTITUIÇÃO EM PACIENTES INFECTADOS PELO HIV-1: IMPACTO DE DIFERENTES ESQUEMAS ANTI-RETROVIRAIS" aprovado, encontrando-se este dentro dos padrões éticos da pesquisa em seres humanos, conforme Resolução n.º 251 sobre pesquisa envolvendo seres humanos de 07 de agosto de 1997, do Conselho Nacional de Saúde, bem como o consentimento livre e esclarecido.

O Comitê de Ética solicita a V. Sª., que ao término da pesquisa encaminhe a esta comissão um sumário dos resultados do projeto.

Prof. Wille Oigman
Presidente do Comitê de Ética em Pesquisa

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