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Citocinas e a modulação da resposta imune durante a infecção pelo HIV – suscetibilidade à infecção e progressão para a aids

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“A alegria que se tem em pensar e aprender

faz-nos pensar e aprender ainda mais.”

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ABREVIATURAS, SIGLAS E SÍMBOLOS

Aids	*Acquired Immunodeficiency Syndrome*
	(Síndrome da Imunodeficiência Adquirida)
APC	*Antigen Presenting Cell*
	(Célula apresentadora de antígeno)
ARV	*Antiretroviral*
	(Antirretroviral)
AP-1	*Activator protein-1*
	(Proteína ativadora-1)
AZT	*Azidothymidine*
	(Azidotimidina)
CCL5	*Chemokine (C-C motif) ligand 5*
	(Quimiocina CC 5)
CCR4	*C-C chemokine receptor type 4*
	(Receptor de quimiocinas tipo CC 4)
CCR5	*C-C chemokine receptor type 5*
	(Receptor de quimiocinas tipo CC 5)
CCR6	*C-C chemokine receptor type 6*
	(Receptor de quimiocinas tipo CC 6)
CD4	*Cluster of Differentiation 4*
	(Grupo de diferenciação 4)
CD8	*Cluster of Differentiation 8*
	(Grupo de diferenciação 8)
CD25	*Cluster of Differentiation 25*
	(Grupo de diferenciação 25)
CD38	*Cluster of Differentiation 38*
	(Grupo de diferenciação 38)
CDC	*Centers for Disease Control and Prevention*
	(Centro de Prevenção e Controle de Doenças)
cDNA	*complementary Desoxirybonucleic Acid*
	(Ácido desoxirribonucléico complementar)
CXCL10	*C-X-C motif chemokine 10*
	Quimiocina CXC 10
CXCR3	*C-X-C chemokine receptor type 3*
	(Receptor de quimiocinas tipo CXC 3)
CXCR4	*C-X-C chemokine receptor type 4*
	(Receptor de quimiocinas tipo CXC 4)
DC	*Dendrític Cell*
	(Célula dentrítica)
DCSING	*Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin*
	(Molécula de adesão intracelular 3 não-integrina especifica de Célula dendrítica)
DNA	*Desoxirybonucleic Acid*
	(Ácido desoxirribonucléico)
DST	Doença sexualmente transmissível
EC	*Elite controller*

	(Controlador de Elite)
GALT	*Gut-associated lymphoid tissue*
	(Tecido linfóide associado ao intestino)
GWAS	*Genome-wide association study*
	Estudo de Associação Genômica
HAART	*Highly Active Anti-Retroviral Therapy*
	(Tearapia Antrirretroviral de Alta Potência)
HBV	*Hepatitis B Virus*
	(Vírus da hepatite B)
HCV	*Hepatitis C Virus*
	(Vírus da hepatite C)
HIV	*Human Immunodeficiency Vírus*
	(Vírus da Imunodeficiência Humana)
HLA	*Human Leukocyte Antigen*
	(Antígeno Leucocitário Humano)
IFN-γ	*Interferon-γ*
	(Interferon-γ)
IL-1	*Interleukin-1*
	(Interleucina-1)
IL-1ß	*Interleukin-1 Beta*
	(Interleucina-1 Beta)
IL-2	*Interleukin-2*
	(Interleucina-2)
IL-4	*Interleukin-4*
	(Interleucina-4)
IL-5	*Interleukin-5*
	(Interleucina-5)
IL-6	*Interleukin-6*
	(Interleucina-6)
IL-7	*Interleukin-7*
	(Interleucina-7)
IL-10	*Interleukin-10*
	(Interleucina-10)
IL-10R	*Interleukin-10 Receptor*
	(Receptor de Interleucina-10)
IL-12	*Interleukin-12*
	(Interleucina-12)
IL-13	*Interleukin-13*
	(Interleucina-13)
IL-15	*Interleukin-15*
	(Interleucina-15)
IL-17A	*Interleukin-17A*
	(interleucina-17A)
IL-18	*Interleukin-18*
	(Interleucina-18)
IL-21	*Interleukin-21*

	(Interleucina-21)
IL-22	*Interleukin-22*
	(Interleucina-22)
IL-26	*Interleukin-26*
	(Interleucina-26)
IP-10	*Interferon gamma-induced protein 10*
	Proteína induzida por IFN - 10
IRF	*Interferon Regulatory Factor*
	(Fator Regulatorio de Interferon)
LTNPs	*Long-Term NonProgressors*
	(Não progressores de longo prazo)
LTR	*Long Terminal Repeat*
	(Regiões terminais repetidas longas)
NF-κβ	*Nuclear Factor kappa βeta*
	(Fator Nuclear kappa beta)
NK	*Natural killer*
	(Exterminadora natural)
NKT	*Natural killer T*
	(Exterminadora natural T)
NLR	*Nod-Like Receptors*
	(Receptor do tipo Nod)
NRTI	*Nucleoside Reverse Transcriptase Inhibitor*
	(Inibidor Não Análogo de Nucleotídeo da Transcriptase Reversa)
NNRTI	*Non-Nucleoside Reverse Transcriptase Inhibitor*
	(Inibidor Análogo de Nucleotídeo da Transcriptase Reversa)
MDR	*Multifactor dimensionality reduction*
	(Redução Multifatorial de Dimensionalidade)
MHC I	Major Histocompatibility Complex class I
	(Complexo principal de histocompatibilidade classe I)
MHC II	Major Histocompatibility Complex class II
	(Complexo principal de histocompatibilidade classe II)
MS	Ministério da Saúde
PAMPs	*Pathogen-Associated Molecular Patterns*
	(Padrões moleculares associados a patógenos)
PD-1	*Programmed cell death protein 1*
	(Proteína de morte celular programada 1)
PD-L1	*Programmed death-ligand 1*
	Ligante 1 da proteína de morte celular programada 1
PD-L2	*Programmed death-ligand 2*
	Ligante 1 da proteína de morte celular programada 1
PI	*Protease Inhibitor*
	Inibidor de Protease
PRRs	*Pattern recognition receptors*
	(Receptores de reconhecimento de padrões)
RLR	*RIG-1-Like Receptor*
	(Receptor do tipo RIG 1)

RNA	*Rybonucleic Acid*
	(Ácido ribonucléico)
SIV	*Simian Immunodeficiency Virus*
	Vírus da Imunodeficiência Simia
SNP	*Single Nucleotide Polymorphism*
	(Polimorfismo de base única)
SUS	Sistema Único de Saúde
TCR	*T cell receptor*
	(Receptor de Células T)
Tfh	*T follicular helper*
	(T auxiliar folicular)
TGF-β	*Transforming growth factor beta*
	(Fator de Transformação do Crescimento beta)
Th	*T helper*
	(T auxiliar)
TLR	*Toll-Like Receptor*
	(Receptores do tipo Toll)
TNF-α	*Tumor Necrosis Factor-α*
	(Fator de Necrose Tumoral)
Treg	*T regulatory*
	(T regulatória)
Tγδ	*T gamma delta*
	(T gamma delta)
UNAIDS	*United Nations Programme on HIV/AIDS*
	(Programa das Nações Unidas para HIV/AIDS)
WHO	*World Health Organization*
	(Organização Mundial da Saúde)

RESUMO

Décadas se passaram desde que os pesquisadores iniciaram investigações sobre a patogênese da infecção pelo HIV. Atualmente, é possível identificar vários desfechos clínicos após a exposição ao vírus, entre eles estão indivíduos expostos não infectados e indivíduos que após a infecção progridem rápida ou lentamente para a aids. No entanto, a identificação de fatores que permitam diferenciar e monitorar estes desfechos continua um desafio. Neste contexto, citocinas parecem ser marcadores promissores de acompanhamento da progressão para a aids. Neste estudo, avaliamos indivíduos HIV-positivo fenotipicamente (níveis plasmáticos das citocinas IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) e geneticamente (variantes polimórficas) com o objetivo de contribuir para o melhor entendimento da patogênese da infecção e trazer novas perspectivas às questões clínicas e terapêuticas. Nossos resultados sugerem uma associação entre o aumento dos níveis plasmáticos de IL-6 e IL-10 no estágio pré-aids em progressores rápidos ($p<0,05$) e lentos para a aids ($p<0,05$). Assim, o aumento dos níveis de IL-6 e de IL-10 podem indicar um estado inflamatório crítico, podendo ser utilizados como marcadores do curso da infecção em indivíduos HIV-positivo. Os genótipos *IL10*-592 AA+AC (OR 1.89, IC 95% 1,10-3,14, $p<0,01$) e *IL17A*-692 TC+CC (OR 1.61, IC 95% 0,99-2,71, $p<0,05$) foram associados à susceptibilidade a infecção pelo HIV em indivíduos eurodescendentes. Além disso, quando analisados em conjunto, SNPs nos genes *IL4, IL6, IL10* e *TLR9* mostraram efeitos aditivos significativos ($p<0,01$). Já em indivíduos afrodescendentes, observou-se um efeito protetor dos genótipos *TLR9*-1237 TC+CC (OR 0.47, IC 95% 0,23-0,96, $p = 0,038$), e um efeito epistático significativo entre *TLR9* e *IL4* ($p<0,01$). Estes resultados contribuem para a compreensão dos mecanismos envolvidos na resposta imune à infecção pelo HIV. Nossas análises mostram que a origem étnica tem grande influência nas associações entre as variantes genéticas e os desfechos da infecção pelo HIV. Adicionalmente, verificou-se que análises de interação gênica, avaliadas através da redução multifatorial de dimensionalidade (MDR), contribuem para a compreensão dos efeitos dos SNPs e podem ser muito úteis na identificação de redes gênicas que influenciam na susceptibilidade e/ou em diferentes cursos clínicos da infecção pelo HIV.

ABSTRACT

Decades have passed since researchers began investigations on the pathogenesis of HIV infection. Currently, it is possible to identify many clinical outcomes after exposure to the virus, which includes individuals exposed but uninfected, and rapid or slow progressors to AIDS. However, the identification of factors that allows to differentiate and monitor these outcomes remains a challenge. In such context, cytokines seem to be promising markers for monitoring the progression to AIDS. This study evaluated phenotypically (plasmatic levels of cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) and genetically (polymorphic variants) HIV-positive individuals as a contribution to a better understanding of the pathogenesis of infection and bring new perspectives to clinical and therapeutic issues. Our results suggest an association between increased serum levels of IL-6 and IL-10 in the pre-AIDS stage for rapid progressors ($p<0.05$), and for slow progressors to AIDS ($p<0.05$). Thus, the increase of IL-6 and IL-10 levels may indicate a critical inflammatory condition, and they can be used as course markers of infection in HIV-positive individuals. The genotypes *IL10*-592 AA+AC (OR 1.89, 95% CI 1.10-3.14, $p<0.01$) and *IL17A*-692 TC+CC (OR 1.61, 95% CI 0.99-2.71, $p <0.05$) have been associated with susceptibility to HIV infection in European-descendents individuals. Moreover, when analyzed together, SNPs in genes *IL4*, *IL6*, *IL10* and *TLR9* showed significant additive effects ($p <0.01$). Already in African-descendant individuals, a protective effect was observed in genotypes *TLR9*-1237 TC+CC (OR 0.47, 95% CI 0.23-0.96, $p=0.038$) and a significant epistatic effect between *TLR9* and *IL4* ($p<0.01$). These results contribute to the understanding mechanisms involved in the immune response to HIV infection. Furthermore, the analysis show that ethnic origin has a heavy influence on associations between genetic variants and the outcomes of infection by HIV. The interaction through the multifactor dimensionality reduction (MDR) contributed to the understanding the SNP effects and can be very useful to identify genes which has influence on susceptibility networks and / or different clinical courses.

SUMÁRIO

Capítulo 1

1 Introdução

1.1 Aids

1.1.1 Situação epidemiológica

Atualmente a aids (Síndrome da Imunodeficiência Humana) é uma das principais causas de mortalidade por doenças infecciosas em todo o mundo, e caracteriza-se por um conjunto de sintomas e infecções resultantes do dano específico ao sistema imunológico ocasionado pelo HIV (Vírus da Imunodeficiência Humana). Segundo dados da UNAIDS, até o ano de 2014, existiam aproximadamente 36,6 milhões de pessoas infectadas pelo HIV no mundo (*UNAIDS: AIDS by the numbers*, 2015). Campanhas mundiais tentam conscientizar a população que o vírus pode infectar qualquer indivíduo através de diversas vias: uma relação sexual desprotegida, da mãe portadora para o filho, ou ainda, através da exposição a materiais perfuro-cortantes contaminados.

Segundo estimativas do Ministério da Saúde, no Brasil, no ano de 2014, existiam 734 mil pessoas vivendo com HIV/aids, correspondendo a uma prevalência de 0,4% (*Boletim Epidemiologico Hiv aids*, 2014). No Brasil, entre as tendências mais dramáticas da epidemia, destaca-se o aumento do número de casos de infecção em mulheres heterossexuais (casadas ou com parceiros fixos) e, isto, consequentemente, aumenta também o número de crianças infectadas por transmissão vertical (*Boletim Epidemiologico Hiv aids*, 2014). Existe ainda, entre as tendências brasileiras, um aumento do número de casos em populações de baixa renda, afetadas diretamente pela exclusão social, cultural e econômica (*Boletim Epidemiologico Hiv aids*, 2014).

É digno de nota que uma parcela da população possui maior vulnerabilidade à infecção pelo HIV. Por vulnerabilidade entende-se a interação de fatores individuais e

coletivos que fazem com que diferentes pessoas e grupos estejam mais ou menos suscetíveis a infecções e adoecimentos, uma vez que dispõem de maiores ou menores possibilidades de se proteger ou se prevenir (Ayres et al., 2003). Entre esses grupos as taxas de prevalência do vírus encontradas em 2013 foram de 5,9% entre usuários de drogas, 10,5% entre homens que fazem sexo com homens (HSH) e 4,9% entre mulheres profissionais do sexo (*Boletim Epidemiologico Hiv aids*, 2014).

A distribuição proporcional dos casos de aids, estratificada por região, no Brasil mostra uma concentração dos casos nas regiões Sudeste e Sul, correspondendo a 54,4% e 20,0% do total de casos identificados de 1980 até junho de 2014 (*Boletim Epidemiologico Hiv aids*, 2014). A taxa de detecção de aids no Brasil tem apresentado estabilização nos últimos dez anos, com uma média de 20,5 casos para cada 100 mil habitantes (*Boletim Epidemiologico Hiv aids*, 2014). Observa-se também a estabilização da taxa na região Sul, com uma média de 31,1 casos para cada 100 mil habitantes. Contudo, o ranking da taxa de detecção de aids entre as Unidades da Federação indica que, em 2013, o estado do Rio Grande do Sul apresentou a maior do país, com valores de 41,3 casos para cada 100 mil habitantes (*Boletim EPpidemiológico RS HIV/AIDS*, 2015). Além disso, Porto Alegre é a capital com a maior taxa de detecção do país (96,2 casos para cada 100 mil habitantes), mais que o dobro da taxa do estado e quase cinco vezes a taxa do país (*Boletim EPpidemiológico RS HIV/AIDS*, 2015).

Destaca-se ainda, Porto Alegre apresenta o pior cenário nacional no que se refere aos principais indicadores epidemiológicos e operacionais da infecção (taxa de detecção de aids, taxa de mortalidade por aids, taxa de detecção de aids em menores de cinco anos, variações anuais dessas taxas, primeira contagem de células T CD4+ abaixo de 200 cel/mm^3) (*Boletim Epidemiologico Hiv aids*, 2014). Porto Alegre é definida como um *hotspot* da infecção pelo HIV, sendo considerada uma das maiores preocupações do Ministério da Saúde entre as Unidade Federadas (*Boletim Epidemiologico Hiv aids*, 2014).

1.1.2 Antirretrovirais e políticas de saúde públicas no Brasil

Com o agravamento da disseminação da aids na década de 80, muitos estudos abordando questões da infecção pelo HIV foram iniciados. Do ponto de vista de saúde pública duas linhas principais destacaram-se: pesquisas buscando antirretrovirais

(ARVs), visando erradicar o vírus do organismo dos indivíduos infectados; e pesquisas buscando uma vacina eficaz, visando imunizar os indivíduos à infecção pelo vírus.

Em 1986, foi aprovada pelo órgão norte-americano de controle sobre produtos farmacêuticos FDA (*Food and Drug Administration*), a primeira droga antirretroviral, a azidotimidina ou AZT. Contudo, o AZT teve um impacto discreto sobre a mortalidade geral dos infectados pelo HIV (McLeod & Hammer, 1992). Em 1994, uma nova classe de drogas ARVs foi incorporada ao tratamento: os inibidores da protease. A partir do potente efeito da combinação dos inibidores de protease e do AZT, houve a diminuição da mortalidade, melhora dos indicadores da imunidade e recuperação de infecções oportunistas dos indivíduos infectados em aids (Fauci, 2003; Vittinghoff et al., 1999). Entre alguns inconvenientes do tratamento combinado estão o custo elevado, a não eliminação do vírus do organismo e os efeitos colaterais do uso prolongado (Hacker & Bastos, 2007).

A recomendação atual, consenso mundial, para controlar adequadamente a replicação do HIV é utilizar pelo menos três ARVs combinados, sendo no mínimo dois de classes diferentes (Fauci, 2003). No ano de 2010 existiam no mercado aproximadamente 19 ARVs, divididos em cinco classes diferentes: inibidor da transcriptase reversa análogo de nucleosídeo ou nucleotídeo (NRTI); inibidor da transcriptase reversa não análogo de nucleosídeo (NNRTI); inibidor da protease (PI); inibidor da integrase (IN) e inibidor de fusão. Além desses, existiam duas potenciais novas classes em fase de teste pré-clinico (Hughes, 2010).

O Brasil foi um dos primeiros países a garantir o acesso universal e gratuito da população aos ARVs, através do Sistema Único de Saúde (SUS), a partir de 1996 (Reis, 2007). Nos anos seguintes, o país desenvolveu uma Política de Medicamentos através do Programa Nacional de DST/AIDS estabelecendo recomendações técnicas consensuais para a utilização dos ARVs no país. Entre 1999 e 2010, as diretrizes do Ministério da Saúde recomendavam o início do tratamento para os indivíduos HIV-soropositivo com sintomatologia de aids, ou ainda, indivíduos assintomáticos com medidas recorrentes de células T CD4+ abaixo de 350 cel/mm^3; indivíduos assintomáticos com carga viral elevada persistente (>5logs) independentemente dos níveis T CD4+ e indivíduos com hepatite C ou B crônica (*Protocolo clínico*, 2008). No ano de 2010 as recomendações do Ministério da Saúde, para o início do tratamento,

foram flexibilizadas: indivíduos HIV-soropositivo assintomáticos com medidas recorrentes de células T CD4+ abaixo de 500 cel/mm^3 poderiam iniciar o tratamento se o infectologista entendesse ser necessário.

Muitas discussões técnico-científicas foram feitas sobre o estágio clínico ideal para o início do tratamento, uma vez que, sendo uma doença crônica, o indivíduo pode permanecer infectado sem apresentar o quadro de aids por longos períodos. Estudos apontam que o início do tratamento antes de uma queda brusca (valores < 500 cel/mm^3) do número de células T CD4+ reduz o risco de morte (Grant, 2010; Le et al., 2013). Por outro lado, o uso contínuo da medicação aumenta as chances do desenvolvimento de doenças hepáticas, renais e neurológicas, além de estar associado à lipodistrofia e a problemas hematopoiéticos (Hacker & Bastos, 2007). Outra questão preocupante do uso contínuo da medicação é a adesão, pois o uso irregular dos antirretrovirais propicia o surgimento de variantes virais resistentes (Little et al., 2002; Shekelle et al., 2007).

Como citado anteriormente, a partir da caracterização do HIV muitos estudos focaram no desenvolvimento de mecanismos para imunizar a população e evitar novas infecções. Diferentes abordagens vêm sendo avaliadas: vacinas baseadas em anticorpos imunizantes, terapia celular, etc, porém, até o momento sem grande sucesso (Noble et al., 2005; Shattock et al., 2007). Com a falta de um instrumento capaz de imunizar a população e a lenta redução de novos casos de infecção pelo HIV, parte da comunidade científica propôs o uso dos ARVs como forma de redução de novos casos de infecções. O controle da transmissão ocorreria, pois os indivíduos em tratamento tendem a zerar suas cargas virais. Em 2010 a OMS, através da UNAIDS, propôs a meta mundial 90/90/90 até 2020: 90% de pessoas vivendo com HIV/aids com conhecimento de sua sorologia para HIV; 90% das pessoas HIV-positivo em tratamento; 90% das pessoas em tratamento com carga viral indetectável (UNAIDS, 2014).

Seguindo a tendência mundial, o Ministério da Saúde, deu inicio em 2013 a esta nova abordagem para o uso dos antirretrovirais no Brasil (*Protocolo Clínico e Diretrizes Terapêuticas para manejo da infecção pelo HIV em adultos*, 2013). Segundo o protocolo clínico brasileiro, recomenda-se o início da terapia antirretroviral para todos os indivíduos HIV-positivo independentemente de seu estágio clínico, com o objetivo de zerar a carga viral populacional e, assim, controlar o número de novos casos de infecção pelo vírus. Além do compromisso com a meta mundial 90/90/90, outro fator

proposto pelo Ministério da Saúde em 2014 para melhorar o monitoramento da epidemia no país, foi um novo sistema de vigilância epidemiológica (*Boletim Epidemiologico Hiv aids*, 2014). Desde então, a infecção pelo HIV, e não apenas os casos de aids, passou a ser de notificação compulsória. Conforme as novas recomendações técnicas, a vigilância do HIV/aids se dará de forma longitudinal, especificamente em três momentos: na notificação do caso de HIV; na evolução para infecção avançada (caso de aids); e quando do óbito (*Boletim Epidemiológico RS HIV/AIDS*, 2015). Assim, além do uso dos ARVs como forma de reduzir a transmissão, o novo modelo de vigilância epidemiológica de HIV/aids visa uma melhor caracterização e monitoramento de tendências, perfil, riscos e vulnerabilidades na população, com o objetivo de aprimorar a política pública de enfrentamento da epidemia.

1.2 A infecção pelo HIV

1.2.1 Curso clínico da infecção

Clinicamente a infecção pelo HIV pode ser dividida em três fases: infecção aguda, infecção crônica e aids (Figura 1) (Levy, 1993). Nas primeiras semanas de infecção há uma profunda destruição de células T CD4+, células alvo da infecção viral (Brenchley et al., 2004; Veazey et al., 1998). Como consequência da alta taxa de partículas virais presentes no organismo, o sistema imune encontra-se altamente ativo. Os elevados níveis de viremia geralmente diminuem à medida que a infecção aguda é resolvida, quando mecanismos imunes conseguem controlar a taxa de replicação viral e um "set point" da viremia é alcançado. Com a resolução da fase aguda, geralmente 4-8 semanas após a infecção, inicia-se a fase crônica. Na fase crônica, o indivíduo infectado pode permanecer completamente sem sinais ou sintomas clínicos, contudo, na maioria das vezes uma depleção progressiva das células T CD4+ está ocorrendo. No estágio de aids, as células T CD4+ geralmente estão abaixo do nível crítico de 200 células/mm^3, dosagem esta que remete o paciente a uma resposta imune ineficaz contra patógenos invasores, permitindo o estabelecimento de doenças oportunistas (Costin, 2007; Schechter & Rachid, 2005). Apesar de todo indivíduo portador do vírus apresentar as

três fases clínicas, o tempo que transcorre do início da infecção até a aids varia consideravelmente entre os indivíduos (Paroli et al., 2001; Schechter & Rachid, 2005).

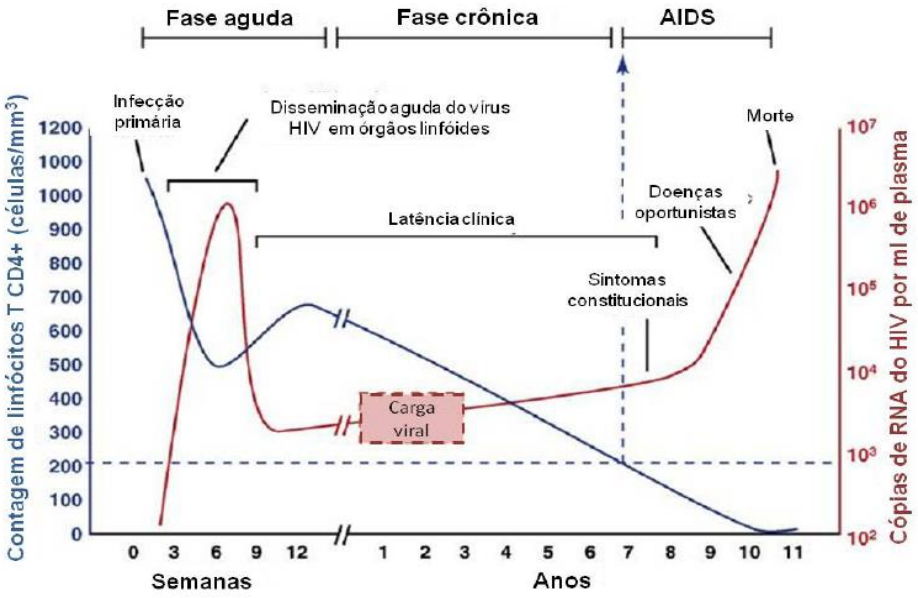

Figura 1 - Fases clínicas da infecção pelo HIV (Adaptado de An e Winkler, 2010).

Os indivíduos infectados pelo HIV que seguem o curso clínico mais comum da infecção são conhecidos como progressores crônicos ou típicos. Esses indivíduos representam entre 75% e 90% da população soropositiva e desenvolvem uma infecção sintomática entre o terceiro e o nono ano após a soroconversão (Schechter & Rachid, 2005). Porém, alguns indivíduos HIV+ podem desenvolver uma infecção sintomática diferenciada, progredindo rapidamente para a aids (em menos de três anos), enquanto outros podem levar mais de dez anos para manifestar os sintomas da síndrome (Okulicz et al., 2009) (Figura 2). Os indivíduos que desenvolvem uma progressão rápida para a aids apresentam duas ou mais medidas consecutivas de células T-CD4+ abaixo de 350 céls/mm^3 em até três anos após a soroconversão, iniciando a terapia antirretroviral por sintomatologia neste período (Casado et al., 2010; Olson et al., 2014). Já os indivíduos conhecidos como progressores lentos, incluídos entre eles os chamados não-progressores de longo prazo (*long-term nonprogressors* LTNPs), parecem controlar por mais tempo o vírus de forma natural (Migueles & Connors, 2010; Paroli et al., 2001). Estes indivíduos possuem diferentes valores de carga viral; porém, apresentam como características comuns a habilidade de manter níveis elevados de células T CD4+ (>500cel/mm^3) e levam mais tempo para apresentar sintomatologia de aids (Deeks &

Walker, 2007; O'Connell et al., 2009; Okulicz et al., 2009). Segundo estimativas, cerca de 5% dos pacientes HIV-positivo são progressores rápidos e 5 a 15% progressores lentos (Schechter & Rachid, 2005). Outro grupo de indivíduos que apresenta características diferenciadas com relação à infecção pelo HIV são os chamados controladores de elite, que possuem a rara capacidade de manter os níveis de vírus no plasma abaixo dos níveis de detecção por ensaios comerciais (<40 cópias por ml).

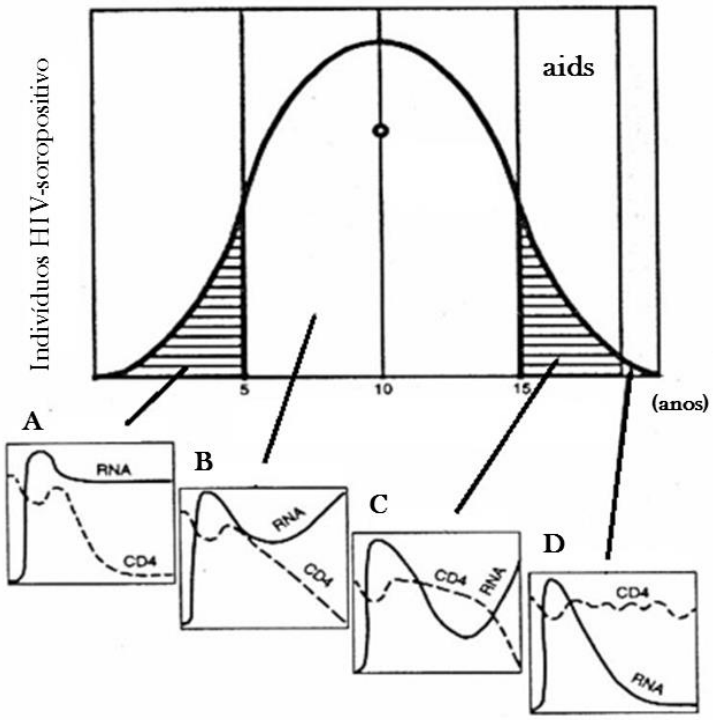

Figura 2 – Representação da distribuição dos indivíduos HIV-soropositivos em função do tempo de progressão para a aids. Perfil clínico (número de cópias de RNA viral e número de células T CD4+) de progressores A - rápidos B - típicos C - lentos D - não-progressores de longo prazo/controlador de elite. (Adaptado de ESCRT - Natural history of infection, www.itg.be/internet/e-learning/written_lecture_eng/5_natural_history_of_infection_cont1).

As complicações clínicas avançadas da infecção pelo HIV, quando não tratadas, compreendem tipicamente complicações infecciosas que refletem a deficiência profunda da imunidade, mediada pelas células T. Com a supressão eficaz da replicação do HIV após a administração de terapias antirretrovirais, a função imune geralmente é recuperada e o risco para estas complicações diminui. Na era atual, doença cardiovascular, doença hepática grave, insuficiência renal, e um espectro de distúrbios malignos são as principais causas de morbidade e mortalidade na infecção pelo HIV (Palella et al 2006; Marin et al., 2009; Hasse et al 2011).

1.2.2 Alvos celulares do vírus e o estabelecimento da infecção

A patogênese do HIV é dinâmica e complexa, com a maior parte dos acontecimentos críticos para o estabelecimento da infecção (por exemplo, a transmissão célula-célula e a destruição de células T CD4+) ocorrendo em tecidos linfóides de mucosa nas primeiras semanas após a entrada do vírus (McMichael et al., 2010). Estes eventos, determinantes para o estabelecimento da infecção, também estão envolvidos na evolução da infecção, ou seja, envolvidos com a progressão para a aids.

Inicialmente, o processo de infecção da célula hospedeira pelo HIV envolve a interação entre a proteína viral gp120 e moléculas CD4 da superfície celular da célula hospedeira (Fanales-Belasio et al., 2010). Após utilizar o CD4 como receptor primário, o HIV interage com os receptores de quimiocinas CCR5 ou CXCR4, que funcionam como co-receptores virais (Dragic et al., 1996). As interações entre o vírus e as moléculas receptoras e co-receptoras permitem a fusão e consequentemente a entrada do capsídeo viral na célula, através de um processo de múltiplas etapas (Fanales-Belasio et al., 2010). A infecção se estabelece a partir da replicação viral dentro das células infectadas, as quais liberam novas partículas virais por brotamento ou por externalização após a lise da célula infectada durante o processo de resposta imune frente à infecção viral (Levy, 1993).

Os receptores e co-receptores utilizados pelo HIV para invadir as células hospedeiras, a molécula de superfície celular CD4 e os receptores de quimiocinas CCR5 e CXCR4, estão presentes nos linfócitos T e nas células da linhagem monócito/macrofágica (Dragic et al., 1996; Levy, 1993). Os linfócitos T CD4+ são os principais alvos celulares do HIV; contudo, o estado de ativação de linfócitos T CD4+ parece ter um impacto significativo sobre a capacidade do vírus de infectar estas células. Linfócitos T CD4+ virgens, que nunca encontraram seu antígeno cognato, estão em um estado de "repouso" relativo e são abundantes no sangue e nos tecidos linfóides (por exemplo, linfonodos e tecido linfóide associado à mucosa gastrointestinal - GALT) (Murphy et al., 2010). De acordo com algumas classificações, os linfócitos T CD4+ já expostos ao seu antígeno são chamados linfócitos T CD4+ de memória, subdivididos em memória efetora (de curta duração), que estão secretando ativamente citocinas, e de memória de longa duração, que estão em repouso, mas podem rapidamente ser ativados a partir de uma exposição ao antígeno (Murphy et al., 2010). Estes estados de ativação

são caracterizados pela expressão de moléculas específicas, além de diferenças no estado de ativação transcricional. Assim, a expressão diferencial, quantitativa e qualitativa, de moléculas na superfície do linfócito T torna alguns estágios menos prováveis de infecção, como é o caso dos linfócitos T CD4+ virgens ou de memória de longa duração que, por expressarem reduzidos níveis de CCR5, seriam mais resistentes à entrada do HIV (Margolis & Shattock, 2006). Além disso, especula-se que o estágio transcricional menos ativo das células T virgens poderia levar o vírus a um estágio de latência e, assim, a infecção destas células estaria relacionada ao estabelecimento de reservatórios virais e não com a replicação ativa do vírus.

Embora os principais alvos do vírus sejam linfócitos T CD4+ CCR5+ de memória efetora, as primeiras células a entrar em contato com o vírus, geralmente, são as células dendríticas (DC) encontradas em vários tecidos humanos, como a mucosa do trato genital e a mucosa anal, principais portas de entrada utilizadas pelo HIV (Cameron et al., 1992; Hladik & McElrath, 2008). As DC são células apresentadoras de antígenos (APCs), fundamentais para a ativação dos linfócitos T imaturos, e podem ser classificadas de acordo com sua origem em DC mielóide e linfóide (Banchereau et al., 2000). DC imaturas encontram-se nos tecidos e expressam receptores fundamentais para a captura de microrganismos, como receptores de manose (DC-SING), pois a principal função das DC imaturas é a captura de antígeno (Banchereau et al., 2000; Rissoan et al., 1999). A captura de antígenos e a concomitante ativação via ligação de moléculas denominadas "padrões moleculares associados a patógenos" (do inglês *Pathogen-associated molecular pattern* ou PAMPs) em receptores específicos, os *toll-like receptors* (TLRs), desencadeia a maturação das DC (Kawai & Akira, 2006). A partir destes eventos as DC ativam a secreção de citocinas específicas, perdem a adesão ao epitélio e via quimiotaxia rumam em direção ao órgão linfóide periférico mais próximo. No órgão linfóide, as DC maduras iniciam a apresentação do antígeno aos linfócitos T e, na presença de um contexto favorável, ocorre à ativação dos mesmos (Hladik & McElrath, 2008). As DCs são capazes de realizar a transfecção do HIV para os linfócitos T: uma vez que o vírus liga-se ao receptor DC-SING mantem-se intacto em invaginações de membrana celular até a passagem para um linfócito T CD4+, processo este chamado "sinapse infecciosa" (Arrighi et al., 2004; Mcdonald et al., 2003; Pope et al., 1994). Embora o HIV possa infectar algumas linhagens de DCs, acredita-se que o principal papel destas células na infecção seja o "carreamento" de vírus até centros de

grande concentração de linfócitos T CD4+ de memória efetora – linfonodos e GALT (Brenchley & Douek, 2008). Uma evidência desta relação é que embora haja um número significativo de linfócitos T CD4+ na lâmina própria da mucosa vaginal, o espalhamento de partículas virais até os linfonodos ocorre antes mesmo de uma replicação local significativa.

A presença do pro-vírus do HIV no genoma é até 10 vezes mais frequente nos linfócitos T presentes nos linfonodos (em sua maioria linfócito T auxiliar folicular - Tfh) do quem em células mononucleares circulantes, tornando-os importantes centros de replicação viral (Brenchley et al., 2004). Contudo, o GALT parece ser o local de maior transmissão do vírus, o principal local para a replicação viral e também de destruição de linfócitos T CD4+ (Brenchley et al., 2004, 2008; Veazey et al., 1998). Mesmo após o uso de ARV, o tecido da mucosa intestinal parece ser o principal local de persistência da replicação do HIV, uma das causas sugeridas é a eficiência com que os ARVs atingem as células infectadas (Macal et al., 2008).

Assim, o HIV infecta principalmente os linfócitos T CD4+ CCR5+ de memória efetora em regiões imunologicamente efetoras como linfonodos e GALT, o que pode ser comprovado pela rápida depleção das células-alvo nesses locais ainda na fase aguda da infecção (Brenchley et al., 2004; Dandekar et al., 2010; Hladik & McElrath, 2008). Nesses locais a replicação do HIV é favorecida pela presença de citocinas pro-inflamatórias, que participam do processo de "amadurecimento" dos linfócitos T CD4+ virgens e participam, também, da ativação da resposta imune em decorrência da presença do vírus.

1.2.3 Resposta imune inata contra o vírus

Como discutido anteriormente, na maioria dos casos de infecção efetiva, a disseminação inicial do HIV ocorre através da rede folicular das DC aos linfonodos, posteriormente alcança o GALT e, então, a infecção torna-se sistêmica. Contudo, como toda invasão viral, a infecção pelo HIV aciona mecanismos imunes para controlar e eliminar o agente patogênico. O sistema imune inato constitui a primeira linha de defesa contra patógenos invasores e se baseia, entre outros mecanismos, no sistema complemento, na ativação de células granulocíticas, células fagocíticas e APCs (Figura 3) (Murphy et al., 2010).

Receptores de reconhecimento de padrões (PRR) possuem um papel central na defesa inata. PRR reconhecem estruturas evolutivamente conservadas de patógenos (PAMPs, padrões moleculares associados a patógenos) (Faure & Rabourdin-Combe, 2011; Kawai & Akira, 2009). Os PRR podem ser encontrados em diferentes populações celulares e estar presentes tanto em membranas plasmáticas ou endossomais, como os receptores TLR (*Toll-Like Receptors*), quanto no citoplasma, como os receptores RLR (*RIG-1-Like Receptors*) e NLR (*Nod-Like Receptors*). Diferentes PRR são expressos em uma mesma célula, permitindo que a célula seja capaz de reconhecer várias classes de microrganismos.

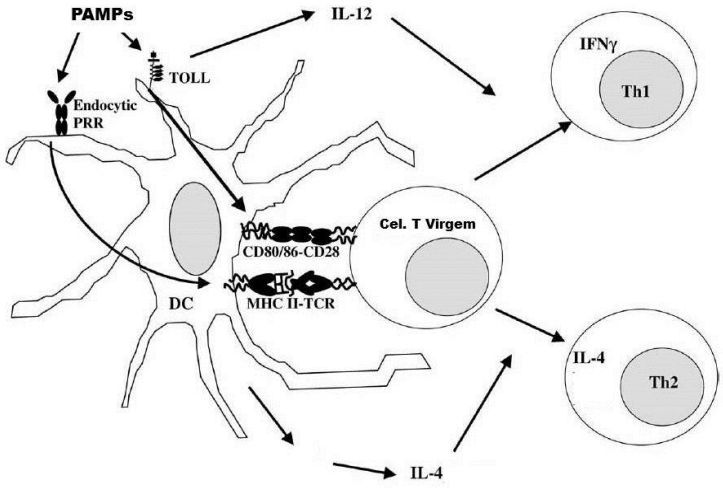

Figura 3 - A apresentação de antígeno pelas DCs é auxiliada pela via dos TLRs na ativação e diferenciação das células T (Adaptado de Werling & Jungi, 2003).

Receptores Toll-like (TLR) são PRRs extensivamente estudados. Nos seres humanos já foram identificados vários TLRs e seus PAMPs, a saber: TLR1, TLR2, TLR6 - lipopeptídeos; TLR4 - lipopolissacarídeos; TLR5 - flagelinas; TLR3 - RNA dupla fita; TLR7, TLR8 - RNA simples fita e TLR9 - DNA exógenos (Kawai & Akira, 2006). Os TLRs 7, 8 e 9 estão localizados em membranas endossomais e são especializados no reconhecimento de microrganismos intracelulares, através do reconhecimento de ácidos nucleicos, como RNA e DNA, presentes no interior da célula (Bauer et al., 2008). Após o reconhecimento PAMP-TLR uma cascata de sinalização ativa vias de transdução de sinal, envolvendo o NF-κB, IRF-3 e IRF-7 (*interferon regulatory factor 3 e 7*), desencadeando a produção de citocinas, quimiocinas e moléculas de adesão celular pró-inflamatórias e antimicrobianas (Kawai & Akira,

2006). Através desse mecanismo uma célula infectada pelo HIV é capaz de reconhecer o RNA viral e o pró-vírus (DNA viral) presentes em seu interior e ativar respostas antivirais, como a expressão do IFN- tipo I.

Contudo, essa mesma via é explorada pelo HIV para facilitar sua proliferação: TLRs 7, 8 e 9 ativam a expressão do IFN tipo-I via NF-kB, o qual também é capaz de ligar-se as regiões LTR do HIV e ativar sua replicação (Brichacek et al., 2010; Báfica et al., 2004). Em cada extremidade do genoma viral existem regiões repetitivas chamadas *long-terminal repeat* (LTR) que contêm promotores, potenciadores, e outras sequências necessárias para a replicação do vírus (Costin, 2007). Diferentes fatores de transcrição celular, como NF-kB (*nuclear factor*-kB), AP-1 (*activator protein-1*) e IRF (*interferon regulatory factor*), são capazes de ligarem-se às LTRs e iniciar a transcrição do genoma viral (Shadrina et al., 2016). Então, ao mesmo tempo em que as células sinalizam uma invasão viral através da produção de citocinas pro-inflamatórias, a replicação do HIV está sendo ativada. Assim, a replicação do HIV é estimulada, quer pela presença do próprio vírus na célula, ou, alternativamente, por vários outros patógenos, acionando respostas imunes via NF-kB. Neste contexto, alguns autores sugerem que a estimulação da via de sinalização dos TLRs desempenha um papel chave na cronificação da ativação do sistema imune e subjacente disfunção do sistema que caracteriza a progressão para a aids (Báfica et al., 2004; Mandl et al., 2008)

O papel da persistência, em curto prazo, dos estímulos gerados pelos TLRs é, de fato, a ativação da resposta imune adaptativa. Nas primeiras semanas após a infecção, a viremia aguda é responsável pela ativação de células T CD8+: o número de células T CD8+ aumenta, enquanto o número de células T CD4+ cai drasticamente (Levy, 1993; Saeidi et al., 2015). A resposta humoral pode ser detectada após 4 a 8 semanas através da identificação de anticorpos anti-HIV (Levy, 1993). Os anticorpos são dirigidos principalmente contra as partículas virais livres, apesar de alguns anticorpos também serem capazes de destruir células infectadas. Estes anticorpos são encontrados no sangue, nas mucosas e em vários fluidos corporais (Pantophlet & Burton, 2006). Contudo, assim como a resposta T citotóxica, anticorpos neutralizantes oferecem pouca proteção, limitando a replicação viral durante as fases iniciais assintomáticas, mas em níveis demasiadamente baixos para promover a eliminação do vírus.

1.2.4 Linfócitos Th1, Th2, Th17, Treg e a infecção pelo HIV

Os linfócitos T CD4+ possuem diversas funções efetoras. O destino da progênie dos linfócitos T CD4+ virgens é decidido durante o período de instrução inicial, regulado por sinais fornecidos pelo ambiente local, originados principalmente das APCs (Murphy et al., 2010). Primeiramente ocorre uma ligação efetiva entre o TCR dos linfócitos T CD4+ virgens e o complexo MHC de classe II + peptídeo da APC (Murphy et al., 2010). Na sequência, os linfócitos T CD4+ necessitam de sinais adicionais para diferenciarem-se, os quais estão presentes no ambiente e/ou são fornecidos pela própria APC (Murphy et al., 2010). Esses sinais adicionais (receptores de superfícies da celular e citocinas) estão envolvidos na sobrevivência ou na expansão dos linfócitos T e na diferenciação dos linfócitos T CD4+ (Murphy et al., 2010). Atualmente as subpopulações de linfócitos T CD4+ podem ser classificadas de maneira simplificada em T *helper* 1 (Th1), T *helper* 2 (Th2), T *helper* 17 (Th17) e T regulatórias (Treg) e outras (Figura 3) (Murphy et al., 2010). Convém, no entanto, salientar que a plasticidade dos linfócitos T está desafiando classificações tradicionais. Estudos estão revelando subpopulações com funções mistas (Geginat et al., 2014).

No fim da década de 80, Mosmann e Coffman (1989) propuseram um modelo em que as respostas dos linfócitos T CD4+, ou *helpers*, se polarizariam em dois grupos distintos de células T: Th1 e Th2 (Mosmann & Coffman, 1989). Os linfócitos Th1 se caracterizam por secretar IL-2 e IFN-γ e conduzir a uma imunidade predominantemente celular, ativando diversos mecanismos para eliminação de microrganismos intracelulares (Mosmann & Coffman, 1989). Além disso, células Th1 auxiliam na ativação da produção de anticorpos pelos linfócitos B virgens após sua ativação pelo antígeno. Já os linfócitos Th2, secretam IL-4, IL-5 e induzem respostas humorais mediadas por anticorpos, sendo essenciais para a mudança de classe de anticorpos (Mosmann & Coffman, 1989). A resposta Th2 é direcionada para combater infecções parasitárias (Mosmann & Coffman, 1989). Atualmente sabe-se que as interações das células T a partir do modelo de Th1/Th2 são mais complexas do que inicialmente proposto. Novas subpopulações de células efetoras foram descritas, as funções de algumas citocinas foram revistas, além, da já mencionada, caracterização da plasticidade das células T.

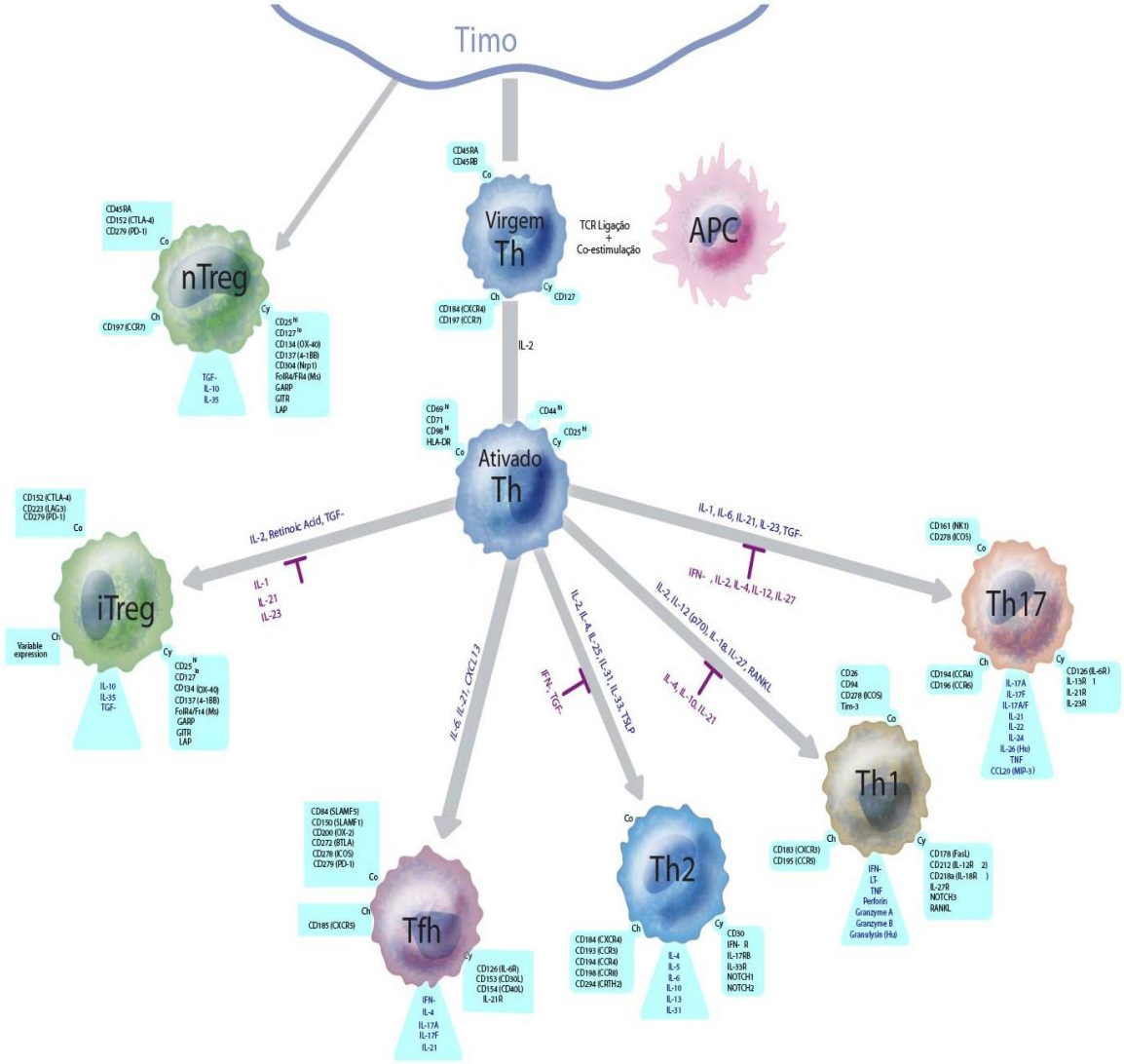

Figura 3 - IL-2 é um fator de sobrevivência para células T ativadas, logo a remoção desta citocina do ambiente pode levar estas células a morte. Além disso, IL-2 promove a diferenciação das células T ativadas em células T efetoras. IFN-γ é a principal citocina Th1; já a IL-4 é a principal citocina Th2. A IL-10, principal citocina Treg, inibe as respostas de células T diretamente pela redução da produção de IL-2, TNF-α, IL-5. As células Th17 produzem citocinas IL-22, IL-26 e as citocinas da família IL-17 (Adaptado de www.bdbiosciences.com/documents/T_Cell_Poster).

No contexto da infecção pelo HIV, Clerici et al. (1990) propuseram que após a infecção pelo vírus, um desequilíbrio entre a resposta Th1 em direção a um aumento da resposta Th2 estaria relacionado ao desenvolvimento da aids (Clerici & Shearer, 1993). A hipótese baseava-se em estudos do grupo que observaram uma redução nos níveis de IL-2 e IFN-γ e aumento nos níveis de IL-4 e IL-10 em indivíduos em aids (Clerici et al., 1989, 1989). Apesar de alguns estudos não apoiarem totalmente a hipótese, ela tornou-se um modelo teórico amplamente aceito para as alterações imunes induzidas pelo HIV (Becker, 2004; Klein et al., 1997). Porém, à medida que outras subpopulações de

linfócitos T CD4+ foram caracterizadas e outras funções foram atribuídas a algumas citocinas, novas relações entre os sinais emitidos pelo sistema imune e a patogênese da infecção pelo HIV foram propostas (Clerici, 2010).

As células Th17 têm importante papel na proteção contra infecção por micro-organismos extracelulares, principalmente, em ambientes de mucosa (Chen & O'Shea, 2008; Smith et al., 2012). As células Th17 produzem citocinas IL-22, IL-26 e citocinas da família IL-17 (Chung et al., 2006; Korn et al., 2009). As citocinas IL-17 são potentes indutoras da inflamação, induzindo a infiltração celular e a produção de outras citocinas pró-inflamatórias, além de ativar mecanismos de apoptose em células potencialmente infectadas (Korn et al., 2009; Park et al., 2005). A via de diferenciação Th17 é antagonizada pelas citocinas dos padrões Th1 e Th2 (Harrington et al., 2005).

Outra importante subpopulação de linfócitos T CD4+ é caracterizada pelas células T reguladoras. Um pré-requisito para o perfeito funcionamento do sistema imune é a sua capacidade de diferenciar moléculas do "próprio" organismo de moléculas "não próprias". Este mecanismo de tolerância central tem como base a seleção negativa de células T auto-reativas no timo. Porém, certa percentagem destas células escapa da tolerância central e para manter as células T auto-reativas sobre controle existem diferentes mecanismos de tolerância e supressão periférica (Murphy et al., 2010). Outro importante papel da supressão imune é evitar danos teciduais irreversíveis gerados por uma resposta imune exacerbada. Assim, células Treg possuem funções opostas às demais células T CD4+ efetoras, uma vez que estão envolvidas na supressão da resposta imune ao invés de sua ativação. As Treg podem ser divididas em Treg naturais, as quais se tornam comprometidas com a função regulatória ainda no timo, e em Treg adaptativas, que se comprometem com a função em órgãos linfóides periféricos após estímulos específicos (Jordan et al., 2001; Roncarolo & Levings, 2000; Weiner, 2001). As principais citocinas imunomoduladoras secretadas por células Treg são a IL-10 e o TGF-β (Annacker et al., 2001; Zheng et al., 2004). A expressão do fator de transcrição *fork-head box P3* (FOXP3) caracteriza a maior parte das células Treg (Walker et al., 2003).

Todas as funções efetoras das células T envolvem sua interação com uma célula-alvo exibindo um antígeno específico. Como já comentado, as ações efetoras dependem de uma gama de interações entre proteínas que as células expressam ou secretam após a

ligação do seu receptor de antígeno. Estas moléculas causam efeitos distintos e apropriados nas células-alvo, tornando a resposta específica. Por sua vez, as subpopulações de células CD4+ efetoras ativadas liberam uma série de citocinas diferentes, que atuam em conjunto, tornando o microambiente propício à resposta necessária.

Em um estudo in vitro, Gosselin *et al*., (2011) descreveram um potencial diferencial entre os subconjuntos de células T CD4+ com relação a permissividade a infecção pelo HIV. Células T de linhagens Th17 ($CCR4^{+}CCR6^{+}$), Th2 ($CCR4^{+}CCR6^{-}$), Th1/Th17 ($CXCR3^{+}CCR6^{+}$) e Th1 ($CXCR3^{+}CCR6^{-}$) foram testadas quanto a sua permissividade em relação à infecção pelo HIV, e os autores observaram que células Th17 e Th1/Th17 foram altamente permissivas e apresentaram elevado potencial para infectar outras células (Gosselin et al., 2010). Curiosamente, em outro estudo, o número de células Th17 mostrou um declínio persistente durante a infecção crônica apesar da terapêutica antirretroviral (Guadalupe et al., 2003). Mais recentemente, Hu et al (2013) observaram que células T CD4+ específicas para o toxóide do tétano (*C. albicans*), com um perfil Th17, eram permissivas à infecção pelo HIV, enquanto que as células T CD4+ específicas para o CMV, com um perfil Th1, eram altamente resistentes (Hu et al., 2013). Estes resultados mostram a ocorrência de uma infecção preferencial a células T CD4+ CCR5+ e CCR6+.

Em parte, esses resultados ajudam a compreender porque o GALT é o maior alvo de depleção de células T CD4+ após a infecção pelo HIV. Além disso, a perda de células Th17 fornece uma ligação direta entre a destruição de células T CD4+ e a disfunção da mucosa intestinal. A disfunção da imunidade da mucosa intestinal pode levar à ruptura da barreira epitelial intestinal causando uma translocação microbiana, ou seja, microrganismos típicos da microbiota intestinal ultrapassam a barreira da mucosa e atingem a corrente sanguínea (Brenchley et al., 2007). A função normal da barreira da mucosa exige um epitélio íntegro e a ação coordenada de vários tipos de células que ocupam posições anatômicas distintas. A destruição súbita e massiva de células Th17 (CD4+ CCR5+ ativadas) interrompe esta rede de comunicação entre as células epiteliais e o sistema imune intestinal (Brenchley & Douek, 2008; Favre et al., 2009; Steinman, 2007). Assim, como resultado da translocação microbiana, ocorre uma "cronificação" da ativação imune.

1.2.5 Respostas Th17 e Treg no contexto da infecção pelo HIV

New Insights about Treg and Th17 Cells in HIV Infection and Disease Progression

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Review Article

New Insights about Treg and Th17 Cells in HIV Infection and Disease Progression

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Treg and Th17 cell subsets are characterized by the expression of specific transcriptional factors and chemokine receptor as well as by secretion of specific cytokine and chemokines. These subsets are important to the differentiation, expansion, homing capacity, and recruitment of several different immune cell populations to the site of infection. Whereas Treg cells maintain self-tolerance and control the activation and expansion of autoreactive $CD4^+$ T effector cells through an anti-inflammatory response, Th17 cells, in an exacerbated unregulated proinflammatory response, can promote autoimmunity. Despite such apparently opposite functions, Th17 and Treg cells share common characteristics, and their differentiation pathways are interconnected. Recent studies have revealed quite intricate relations between Treg and Th17 cells in HIV infection and progression to AIDS. Considering Treg cells, different subsets were already investigated in the context of HIV infection, indicating a fluctuation in the total number and frequency throughout the disease course. This review focuses on the recent findings regarding the role of regulatory T and Th17 cells in the context of HIV infection, highlighting the importance of the balance between these two subsets on disease progression.

1. Introduction

One of the major hallmarks of HIV infection is the immune activation that prompt viral replication and $CD4^+$ T cells loss with disease progression, also leading to an impaired immune competence and consequently to AIDS development. It is still discussed if the loss of immune competence is caused by persistent immune activation, by a suppression of immune cells proliferation or by both phenomena [1].

The $CD4^+$ T cells exert a central role in immune response and represent the preferential target of HIV infection. The most extensive studied $CD4^+$ T cells lineages so far are Th1 and Th2, albeit HIV research now focuses on the immune balance and function of other cellular immune subsets, such as regulatory T cells (Tregs), T helper 17 (Th17), T helper 9 (Th9), and T helper 22 (Th22), where Treg/Th17 cells balance a relevant target of these studies [2, 3]. Treg cells, characterized by Forkhead Box Protein 3 ($FoxP3^+$) expression, represent an important subset that control the proliferation of different immune cell subsets [4]. Meanwhile, T helper 17 most remarkable characteristic is IL-17 production that drives the capacity to these cells to exert an important proinflammatory function against extracellular pathogens [5]. Also, it is known that both subset phenotypes (Treg and Th17) are characterized by specific transcriptional factors and chemokine receptor expressions as well as by secreting specific cytokines and chemokines. Together, all these factors are important to the differentiation, expansion, homing capacity, and immunological cell recruitment into the site of infection or to the injured tissue for restraining the inflammation and dissecting the fine balance between Th17/Treg cells [6, 7].

Natural history of HIV infection involves a variable time of progression to AIDS. HIV long-term nonprogressors (LTNP) are characterized by long periods (>10 years) of AIDS-free symptoms even without antiretroviral treatment and maintain low levels of viremia and elevated $CD4^+$ T cells counts. In contrast, rapid progressor (RP) HIV-1 subjects succumb to AIDS after a few years of infection [8].

Table 1: Immunophenotyping of Treg and Th17 cells and their precursors in different studies.

Subset cells	Markers used	References
Naive Tregs	$CD45RA^+CCR7^+CD25^{high}CD127^-Foxp3^+CD4^+$	DaFonseca et al. [99]
	$CD45RA^+CD45RO^-CCR7^+CD25^+Foxp3^+CD4^+$	Valmori et al. [107]
	$CD45RA^+CD25^+CD127^{low}Foxp3^+CD4^+$	Valmori et al. [108]
	$CD45RA^+CD45RO^-CD25^{high}CD127^{low}Foxp3^+CD4^+$	Duhen et al. [18]
	$CD45RO^-CD25^+CD127^{low}CD4^+$	Tenorio et al. [17]
Memory Tregs	$CD45RA^-CD25^{high}CD127^{low}Foxp3^+CD4^+$	Canavan et al. [133]
	$CD45RA^-CCR7^{+/-}CD25^{high}CD127^-Foxp3^+CD4^+$	DaFonseca et al. [99]
	$CD45RO^+CD25^{high}CD127^{low}Foxp3^+CD4^+$	Duhen et al. [18]
	$CD45RA^-CD25^{high}Foxp3^{high}CD4^+$	Zhou et al. [134]
	$CD45RO^+CD25^+CD127^{low}CD4^+$	Tenorio et al. [17]
Memory Th17	$CD45RA^-CCR6^+CCR4^+CXCR3^-CD4^+$	Gosselin et al. [122]; Becattini et al. [135]; Acosta-Rodriguez et al. [120]
	$CD45RA^-CCR6^+CD26^+CD161$	DaFonseca et al. [99]

Elite controllers (EC) are a particular group of LTNP, because they show persistent undetectable viremia (<50 RNA copies/mL) without treatment, although they represent less than 1% of all HIV-positive population [9]. Recent studies have focused the attention to elucidate the mechanisms involved in the variability of AIDS progression. Several components including viral factors and the host genetic diversity (e.g., the CCR5Δ32 variant and specific HLAs alleles) were already described as important factors that modulate HIV infection [10]. Nevertheless little is known about the cellular immune mechanisms involved in HIV progression and their role in immune molecular signaling, homing regulation, and cell-cell interactions. A better knowledge about these mechanisms could provide additional pieces to the complex puzzle of HIV pathogenesis. This review will focus on the recent findings regarding the role of regulatory T and Th17 cells in the context of HIV infection, highlighting the importance of the balance between these two subsets on disease progression.

2. The Role of Treg Cells on HIV Infection

2.1. Regulatory T Cells: Features and Functions. Regulatory T cells constitute a specialized subpopulation of $CD4^+$ T lymphocytes in the immune system that exerts pivotal roles on establishing and maintaining self-tolerance and immune homeostasis. These specific functions are derived from the regulation of different immune cells proliferation [11]. Based on this, it is expected that Treg cells may participate in the immune regulation in human autoimmune diseases, cancer, allograft rejections, and virus infection [12–15].

As a definition, Treg cells express high amounts of CD4, CD25 (IL-2Rα) and low CD127 (IL-7α) levels on the cell surface, although the Forkhead Box Protein 3 (FoxP3) is characterized as the gold standard marker for natural Treg cells (nTregs or tTregs, from thymic-derived regulatory T cells). IL-2Rα and FoxP3 expression (mediated by STAT5) are critical for Treg cells survival and suppressive function [14, 16]. The limitation to the use of FoxP3 as a marker for Treg is that viable cells cannot be isolated after intracellular staining. In addition, FoxP3 expression is not always indicative of a regulatory status within human $CD4^+$ T cells. A suggested alternative is the combined identification of the cell surface markers CD25 and CD127 ($CD25^{high}$, $CD127^{low/-}$) [17, 18]. In recent years, several studies have proposed a consensus panel of the markers to Treg immunophenotyping (Table 1). Another studied marker, CD39 (an ectonucleotidase involved in the hydrolysis of extracellular ATP into adenosine), identifies a bulk of human T cell regulatory population associated with high FoxP3 expression and inhibits T cell proliferation and cytokine secretion [19–21].

The suppressive capacity of Treg cells is widely dependent and influenced by several factors, such as IL-2, inhibitory cytokines (IL-10, TGF-β, or IL-35), CD152 (CTL-associated antigen 4, CTLA-4), and GITR (glucocorticoid-induced tumor necrosis factor receptor) [21]. IL-2 and, in a lesser degree, IL-7 and IL-15 cytokines are required for the correct differentiation of tTreg cells and the survival of tTreg cells and peripherally Treg cells (pTregs). Also, TGF-β seems to be an important cytokine involved on pTreg cells differentiation and homeostasis, although IL-2 is also required for TGF-β-mediated induction of FoxP3 [22]. Since several cytokines play a pivotal role on Treg cells function and differentiation, recent studies are investigating and suggesting their use on different conditions. The administration of IL-2 has been associated to increase in circulating Treg cells number and activation [23, 24]. IL-7 did not affect Treg cells proliferation but suppresses Treg cells capacity *in vitro* and *in vivo*. Also, IL-7 exerts a synergistic effect through downmodulation of the ectoenzyme CD39, favoring Th17 conversion [25]. In addition, expression of the enzyme indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme, represents another mechanism for immunosuppressive function [26].

2.2. The Role of Treg Cells in HIV Infection and Progression to AIDS: Friend or Foe? Persistent immune activation is considered a reliable predictor for HIV disease progression and may lead to erosion, depletion, and exhaustion of the $CD4^+$ T cell repertoire [27]. One of the immune mechanisms capable of controlling the activation and expansion of immune cells is the suppressive function exerted by Treg cells [28]. The role of Treg cells on HIV infection is still inconclusive since these cells can be involved both in the promotion as well

as in the prevention of disease progression. Some findings point to a beneficial effect through suppression of chronic immune activation and inhibition of activated $CD4^+$ T cells and consequent control of viral replication. On the other hand, a detrimental role is observed since the inhibition of specific HIV immune response through suppressive potential can promote viral persistence at the host [29, 30].

Considering that Treg cells express on their surface the chemokine receptor 4 (CXCR4) and chemokine receptor 5 (CCR5) molecules, these cells can potentially be susceptible to HIV R5-tropic and X4-tropic infection [31]. Some studies reported that HIV-infected Treg cells have its function and phenotype profile altered; however, opposite results have already been described [31–33]. Recently, Angin et al. [34] successfully isolated and *in vitro* expanded CD4 regulatory T cells from (HIV-positive) subjects. Expansion of functional Treg cells from blood and lymphoid tissues of HIV-infected subjects allied with its preserved suppressive capacity possibly indicates that these cells are not intrinsically defective in the context of HIV infection [34].

However, another study demonstrated that HIV-1 infection disrupts Treg cells function and its genes expression [35]. Treg cells infected with HIV-1 seem to be less potent in suppressing autologous $CD8^+$ and $CD4^+$ cell proliferation as compared to uninfected Treg cells. This impairment on Treg cells function can lead to HIV-associated generalized immune activation and inflammation [35]. According to this, infection of Treg cells with HIV X4-tropic strain results in a decrease of FoxP3 expression and decreased suppressive capacity [36]. Also, reduction in the expression of IL-2Rα in Treg cells was observed in HIV-infected subjects with high viral load. This alteration could result in reduced Treg cells capacity function in these individuals, considering that the homeostatic role of this cells depends on IL-2 and the expression of IL-2Rα at the cell surface [37].

Treg cells seems to be a major contributor to the immune activation observed during chronic HIV infection, since a strong relationship between Treg cells depletion and $CD4^+$ T cell activation was observed [38, 39]. It is important to carefully observe that, in chronic HIV infection, a gradual increase of Treg cells (in terms of percentage) and a decrease of its absolute numbers, during progression of the disease, have already been described [39–43]. The opposite results regarding Treg cells relative and absolute frequencies are related to the fact that these cells are preferentially preserved compared to conventional $CD4^+$ T cells [30]. Moreover, it is important to point that the discrepancy observed about Treg cells frequency on HIV infection can be attributed, at least in part, to (i) different surface markers used to characterize/isolate Treg cells; (ii) differential clinical stages of HIV disease, (iii) differences on sample analysed (blood or lymphoid tissues); and (iv) Treg subpopulations.

Human Treg cells have been subdivided according to their activation state: $CD45RA^+$ are defined as naïve Treg cells and $CD45RO^+$ defined as effector/memory Tregs cells in humans. Different cell subsets were already investigated in the context of HIV infection. When approaching the relative frequency (percentage), an increase of memory Treg cells and a decrease of naive Treg cells were observed as $CD4^+$ T cells decline. The level of HIV viremia inversely correlates with memory and naive Treg absolute cell numbers. In addition, immune activation was inversely correlated with lower memory and naive Treg absolute cells numbers [17]. A distinct Treg cells phenotype was already identified on HIV infection. These cells express HLA-G on their surface but do not express FoxP3 or CD25 and are distinct in their profile and function from the classical regulatory T cells. However, these Treg cells ($HLA\text{-}G^+$) seem to be diminished in progressive HIV-1 infection and may contribute to immune overactivation during disease progression [44].

It is noteworthy that Treg cells have an important role in immune homeostasis, and different evidences indicate that these cell repertoires can be disrupted in HIV infection. A better understanding of the Treg cells repertoire frequency and function in HIV-infected subjects with different patterns of progression to AIDS may help to elucidate the mechanisms affected by such cells on HIV pathogenesis and consequently their future therapeutic use.

There is an increasing number of studies approaching the role of Treg cells on different HIV progression groups, although the results are still conflicting. For instance, some of them suggest that low immune activation contributes to a slower disease progression [45]. Chase et al. [46] observed that Treg cells frequency and function were preserved among elite suppressor subjects (elite controllers), which may be a mechanism to limit immune activation. In the same line, Jiao et al. [47] observed a decrease of Treg cells absolute counts during HIV disease progression in the typical progressors group but not in LTNP subjects. One of the main reasons for the differences in Treg cells loss among distinct clinical progression groups would be that Treg cells migrate to lymphoid tissues in the typical progressors, but not in LTNP, which may contribute to Treg cells preservation on this last group and elite controller group [48]. According to this, lower levels of $FOXP3^+$, CTLA-4, and TGF-β, but not IL-10, were observed in the tonsils of HIV-infected subjects classified in the nonprogression group compared to HIV typical progressors [33], indicating that the accumulation of Treg cells within lymphoid tissues is a feature of chronic progression. More recently, it was shown that viremic slow progressors subject has lower Treg cells numbers associated with $CD4^+$ T cell decreased proliferation and surprisingly mucosal T cell activation. In this study, the low Treg cells numbers in the rectal mucosa may contribute to immune activation although they may also support stronger anti-HIV immune responses and a preserved Treg/Th17 cells balance [45].

Although some studies support the evidence of preserved Treg cells frequency and function in slow progressors subjects, there is no consensus in the literature since no differences among Treg cells frequency of slow progressors compared to HIV-infection acute disease and seronegative individuals have been described. Gaardbo et al. [49] showed no alteration on Treg cells numbers among LTNP, EC, viremic controllers, typical progressors, and HIV-seronegative individuals both in blood and in lymphoid tissues. However, activated Treg cells were elevated in LTNP and elite controllers compared to typical progressors and HIV-seronegative

controls, whereas resting Treg cells were diminished, suggesting an important role of different Treg cells subsets on HIV pathogenesis [49]. In this same direction, Brandt et al. [50] observed a lower frequency of Treg cells in EC compared to viremic individuals (HIV-seropositive HAART-naïve), and the frequency was correlated with T cell proliferating and activation.

2.3. Treg Cells in Animal Models: Investigating Treg Cells on SIV Infection. Similar to HIV infection, the exact mechanism of regulatory T cells function as well as its frequency during Simian Immunodeficiency Virus (SIV) infection is unclear. Li et al. [51] observed a higher absolute and relative number of Treg cells in Chinese *Rhesus macaques* in the early stages after SIV infection. No alteration on Treg cells suppressive capacity after infection was described. Estes et al. [52] observed an important regulatory response (mediated by $FoxP3^+$ and TGF-β^+ cells) after SIV exposure that may be involved in immune suppression of antiviral response and favor viral persistence. Although the majority of studies evaluate peripheral blood, Treg cells accumulation in lymphoid tissues was also described [53]. In addition to this, Tregs cells can potentially influence disease progression since lower FoxP3 mRNA levels were observed in an SIV nonprogressors model when compared to SIV progressors [33].

A study performed by Pereira et al. [54] investigated the frequency of Treg cells on two animal models with distinct profiles of SIV progression: African primate *Sooty mangabeys* (SM) (that do not develop immunodeficiency or disease) and Asian *Rhesus macaques* (RM) (a disease progression model). A decrease in Treg cells numbers was observed in chronically SIV-infected RM compared to uninfected animals. In longitudinal analysis, the SIVmac239-infected RM showed a transient increased Treg cells frequency in the acute phase of infection [54]. After the acute phase, a progressive decrease in the frequency and number of Treg cells was observed and correlated with high viral load. Antiretroviral treatment promoted an increase in the frequency and absolute count of Treg cells. None of these differences was observed on the SM model [54]. Another strategy used to investigate the role of Treg cells in HIV infection was to block Treg cells with an anti-CTLA-4 blocking antibody. CTLA-4 blockage in chronically SIV-infected ART-treated macaques was associated with lower IDO and TGF-β levels, as well as decreased viral RNA levels in lymph nodes and an increased immune specific response, suggesting a potentially therapeutic approach on HIV treatment [55].

2.4. The Impact of Highly Active Antiretroviral Therapy (HAART) on Tregs. Highly active antiretroviral therapy can significantly influence Treg cells numbers in HIV-infected subjects, decreasing or even normalizing its frequency at similar numbers to that of healthy controls [56, 57]. Some studies report that lower Treg cells numbers were found in blood and lymphoid tissues of treated compared to untreated subjects [58, 59].

Additionally, it has been hypothesized that Treg cells may contribute to the complete success of the treatment since subjects that do not respond to HAART seem to show higher Treg cells numbers as compared to responders [58, 60, 61]. Gaardbo et al. [62] also demonstrated that subjects with suboptimal immunological recovery had higher percentages of Treg cells and activated Treg cells, as well as lower resting Treg cells frequency in blood. In this same direction, higher levels of Treg cells in blood and lymphoid tissues predict a higher immunological reconstitution in individuals with low $CD4^+$ T cell counts [62]. In a study performed by Jiao et al. [47], HAART increased peripheral Treg cells counts and induced a decrease in the immune activation and $CD8^+$ T cell apoptosis in complete responders but not in nonresponders subjects. In conclusion, considering the important role of Treg cells in the balance between immune activation and/or suppression during HIV progression as well as its influence on HAART response, these cells may be useful as therapeutic targets or for prognostic monitoring in the future.

3. The Role of Th17 on HIV Infection

3.1. Th17 Cells: Features and Functions. Subpopulations of Th17 T helper lymphocytes were recently described and characterized by its involvement in mucosal immune inflammatory response, being its major function to protect the host against extracellular bacterial and fungal infections [5]. Th17 cells can be found under homeostatic conditions, particularly in the lamina propria of the small intestine [63]. However, during infection or under inflammatory conditions, Th17 cells can be induced in other tissues. This cellular lineage is responsible for the release of several cytokines that will act in nearby cells, inducing the production of chemokines able to recruit neutrophils and macrophages to the site of infection [64]. Further, Th17 cells can induce the expression of antimicrobial peptides, as lipocalin-2, Reg3γ, β-defensins, and calprotectin [65].

Th17 human cells are characterized by the expression of the transcription factor RORc and by the surface markers CD161, IL-23R, CCR6, and CCR4 [66, 67]. Moreover, the expression of CCR5 seems to be tissue-specific, with Th17 cells in the peripheral blood being predominantly CCR5-negative although they are CCR5-positive at the gastrointestinal tract [68]. The induction of RORc is dependent on STAT3, preferentially activated by IL-6, IL-21, and IL-23 in the presence of low amounts of TGF-β [69, 70]. Additionally, a balance between IL-6 and TGF-β concentrations has a pivotal role in driving Th17 immune responses, as will be better discussed later [71, 72].

Stimulated Th17 effector cells express several proinflammatory cytokines, such as IL-17, IL-21, IL-22, and IL-26, and chemokines as CXCL-6, CXCL-7, CXCL-8, and CCL20 [73], which contribute to the expansion of the inflammatory response through cells recruitment and activation and induction of antimicrobial peptides production. IL-17 leads to inflammation through NF-$\kappa\beta$ and MAPKs and the induction of genes that code for matrix metalloproteinases, growth factors, other proinflammatory cytokines, and chemokines that attract neutrophils [74].

A balance of proinflammatory and anti-inflammatory or suppressive cytokines in the cellular microenvironment seems to be determinant to the differentiation of the Th17 cells population in specific subsets: Th17 cells expressing both Th17-Th1 and Th17-Th2 surface markers were found in response to the presence of IL-6, IL17, and IL-1β and addition of IL-12 or IL-4, respectively [75]. Another subset, Th17-Treg cells, seems to involve a more complex signalling context [76].

3.2. The Role of Th17 Cells in HIV Infection and Progression to AIDS. Th17 cells are constitutively observed throughout the intestinal lamina propria and in gut-associated lymphoid tissues (GALT). Approximately 80–90% of the $CD4^+$ T cells present in GALT are able to secrete IL-17 [66]. Furthermore, it is recognized that GALT is the main region for HIV replication and massive $CD4^+$ T cells depletion in early infection is observed in this compartment [77]. Indeed, extreme permissiveness of Th17 cells to HIV-1 infection can be explained based on the fact that mucosal $CD4^+$ T cells present a $CD45RO^+$ memory phenotype and express CCR5 and/or CXCR4 [78]. Therefore, the loss of Th17 cells during the HIV infection affects the intestinal mucosal barrier as well as local innate and adaptive immune functions [78].

The presence of HIV-specific Th17 cells in HIV-infected individuals during early infection was already reported; however, this response was not detectable during chronic or nonprogressive stages of the infection disease [79]. Conversely, Brenchley et al. [80] demonstrated that, in HIV-infected and uninfected individuals, Th17 cells respond to bacterial and fungal antigens; nevertheless, Th17 cells response was not specific for viral antigens, including HIV. However many studies found that massive infection of $CD4^+$ T cells in GALT is directly associated with inflammation of the mucosal tissues and a breakdown of the mucosal integrity, resulting in microbial translocation from the lumen of the gut into peripheral blood [81, 82].

As has been suggested by some authors, Th17 cells may have dual impact on HIV infection due to the functional capacity in the mucosal tissue. In the acute phase of infection, in an inflammatory environment, Th17 cells could promote cell migration to the gut and create conditions for viral replication [83–85]. Nevertheless, in the chronic phase of infection, the reduced number of Th17 cells in the gut has been associated with a decrease in mucosal restoration and increase of microbial translocation and immune hyperactivation, which would contribute to exacerbation of the infection [80, 86].

Initial studies evaluating Th17 populations in HIV infected subjects demonstrated that Th17 cells were depleted in the gut-associated lymphoid tissue [39, 87]. In two subsequent studies, Salgado et al. [88] and Ciccone et al. [89] evaluated the numbers of Th17 cells in LTNP and typical progressor subjects. They reported similar results, suggesting that the number of Th17 cells in LTNP is greater than in typical progressor subjects. Furthermore, Salgado et al. [88] also observed a negative correlation between plasma HIV-RNA levels and Th17 cell number and with $CD4^+$ $IL7R^+$ cell number: HIV infected with higher of viral load showed the lowest numbers of Th17 cells and $IL7R^+CD4^+$ cells. These authors suggest that increased numbers of Th17 cells in LTNP subjects could better preserve the immune response against bacterial infections. Thus, low microbial translocation could explain the reduced activation and slower progression of the disease in LTNP subjects. Supporting these results, Singh et al. [90] showed that extensive elimination of $CD4^+$ T lymphocytes in the GALT in the early stages of HIV-1 infection affects the intestinal homeostasis and significantly decreases the effector and regulatory functions of Th17 cells.

3.3. Th17 Cells in Animal Models: Investigating Th17 Cells on SIV Infection. Since Th17 cells of the SIV host have the same phenotype and general functions of the human Th17 cells, these cellular lineages have been investigated in different animal models, as *Sooty mangabeys* (SM) (that do not develop immunodeficiency or disease) and Asian *Rhesus macaques* (RM) (a disease progression model). The Th17 cells studies in SM can be highlighted because, in spite of severe depletion of $CD4^+$ T cells in the mucosal tissues during acute SIV infection, even in the face of high viral replication similar to infections by HIV-human and SIV-RM, they do not progress to AIDS [91].

Raffatellu et al. [92] showed the inability of SIV-infected macaques to assemble an inflammatory GALT response against *S. typhimurium* due to an overall $CD4^+$ T cells depletion in this tissue. Also, a significant systemic spread *S. typhimurium* after the loss of Th17 cells was observed. Another important study, by Paiardini et al. [93], revealed that, after nonpathogenic SIV infection, SM are able to maintain or increase the levels of Th17 cytokines due to the recovery of $CD4^+$ T cells supported by the bone marrow and that this recovery contributes to the resistance against progression to AIDS. Other studies identified significant differences in the mucosal barrier integrity in models of HIV and SIV infection [91]. According to Brenchley et al. [94], Th17 cells are preferentially depleted in the mucosa of HIV^+ humans and SIV^+ *Rhesus macaque* pathogenic infections, but these cells were preserved in SM-SIV infections.

Recent studies have correlated the expression of CCR6 in Th17 cells and preservation of the gut mucosal barrier. This fact can be highlighted by the maintenance of Th17 cells in the gut and the reduced microbial translocation in SIV-infected RM treated with IL-21, a key cytokine in the activation of Th17 response [95]. Also, there are in the human Th17 repertoire, especially prevalent in the GALT, cells expressing high levels of CCR5, which would be a target of a preferential and rapid depletion [96].

SIV replication in the infected RM is restricted by the size of the preexisting Th17 cells compartment: animals with a high representation of such cells in blood and in the intestinal tissue previously to infection experienced peak and set-point viral loads about one log unit lower than those with a lower representation of Th17 cells [97]. Reciprocally, treatment of macaques with IL-2 and G-CSF before infection led to the depletion of Th17 cells, reduction of the ratio between Th17 and Treg cells, and higher viral loads for 6 months after infection [97]. These results suggest that the host immune

system pool previous to the infection has an influence on the disease course after infection and provides a new framework for understanding interindividual variation in response to HIV-infection.

3.4. The Impact of Highly Active Antiretroviral Therapy on Th17 Cells. In the HIV infection, Th17 cells seem to be preferentially depleted in the intestinal mucosa and to a lesser extent in peripheral blood [77]. In the acute phase, the low levels of $CD4^+$ T cells can be restored with the viral load reduction mediated by HAART. Macal et al. [98] showed that the highest level of $CD4^+$ T cells restoration during HAART correlates with a substantial increase in mucosal Th17 cells and a decrease in inflammation markers. However, it is unclear why HAART cannot restore Th17 cells in the intestinal mucosa of some individuals: this same study observed that in some HIV-infected subjects a low level of immune activation persists in GALT despite long-term therapy. A possible explanation is that as Th17 cells are highly susceptible to HIV infection, this subset would be depleted early in HIV infection, leading to nonrestoration of the Th17 cells in spite of HAART. On the other hand, there are evidences showing that the paucity of the Th17-lineage committed precursor cells coincides with the Th17 polarization deficit in HIV chronically infected on HAART individuals versus HIV-negative controls [99]. Therefore, it can be suggested that the initial exhaustion of the precursor Th17 cell subsets in early stages, in some HIV-infected individuals, could be correlated with the Th17 restoration deficit despite an undetectable viral load. These studies are discussed in more detail further in this review (see What about the Balance between Th17 and Treg Cells in HIV Infection?).

Ndhlovu et al. [100] reported that healthy children exhibit a higher frequency of Th17 cells in the peripheral blood than HIV-infected children. Also, infected children with viral load greater than 50 copies/mL had a greater decrease in the frequency of these cells compared to children with undetectable viral load, suggesting that a preservation of Th17 cells depends on viral suppression [100]. Recently Pilakka-Kanthikeel et al. [101] comparing virologic responders and virologic failures HIV-infected children to uninfected pediatric subjects showed that microbial translocation persisted after 44 weeks in both responders and failures HIV-infected groups. A study by Alvarez et al. [102] performed *in vitro* demonstrated that virus replication can be suppressed by 3TC therapy, but the restoration of Th17 response observed in non-infected controllers was only achieved with the combination of 3TC and a "cocktail" of Th17 cytokines (IL-6, IL-1β, TGF-β, and IL-23). Taking into consideration that it was possible to restore Th17 response, it will be interesting to conduct more studies with such potential therapy.

4. Th17 and Treg Balance

The Th17/Treg balance is defined as "a state of equilibrium of the immune system that permits accurate and rapid protective responses against pathogens but curtails potential for causing harm to the host through targeting of 'self' and provoking overexuberant inflammatory processes" [6]. It is known that Th17 and Treg cells have opposite roles in the development and outcomes of autoimmune/inflammatory diseases. Whereas Th17 cells can promote autoimmunity due to a proinflammatory response, Treg cells maintain self-tolerance and controls activation and expansion of autoreactive $CD4^+$ T effector cells through an anti-inflammatory response [7]. However, Th17 and Treg cells share common characteristics, and their differentiation pathways are interconnected.

Recent reports demonstrated that Treg and Th17 cells have a high grade of plasticity due the fact that their initial differentiation is not an endpoint of T cell development [75, 76]. This plasticity allows a functional adaptation to various physiological situations during an immune response and might also be a critical disturbing factor for the Th17/Treg balance, leading to the immunopathogenesis of autoimmune/inflammatory diseases [75].

The maintenance of a Th17/Treg balance mainly depends on environmental factors and genetic predisposition. Besides, the plasticity of both cell subsets is highly dependent on the cytokine milieu and in the inflammatory context. Importantly, the commensal microbiota composition has a particularly significant influence in the immune system regulation and an imbalance in the gut microbiome could lead to alterations of immune responses in both GALT and periphery [6]. Of note, there are mechanisms of peripheral tolerance, achieved in large part through the action of Treg cells.

TGF-β is a critical factor for both Th17 and Treg cells, essential for inducing both RORc and FoxP3 [72, 103]. $CD4^+FoxP3^+RORc^+$ cells represent a transient population, able to give rising to either Th17 or Treg cells depending on the local conditions. If sensing a proinflammatory environment, TGF-β induces RORc expression and Th17 cells differentiation [6, 104]. In the absence of an inflammation, TGF-β promotes FoxP3 expression and in combination with IL-2 promotes differentiation, expansion, and survival of Treg cells that maintain immune tolerance. This fact is due to a FoxP3-mediated inhibition of the activity of RORc and RORα, resulting in abrogation of IL-17 and IL-23 expression [105].

It was observed that Treg cells can acquire a Th17-like phenotype. They are able to release IL-17 and express RORc and high levels of CCR6 but can retain a suppressive capacity (although this capacity is rapidly lost upon strong activation in the presence of IL-1β and IL-6) and FoxP3 expression (Th17Treg profile) [75]. Other studies focused on naïve cells as precursor population of Tregs and Th17 cells and observed that both subsets have a common precursor. It was observed that natural Tregs differentiate from $CD25^+$ naïve T cells (NTregs) [106, 107]. Valmori et al. [108] reported that polarization of human Th17 cells preferentially occurs from $FoxP3^+$ naïve Treg cells in the presence of IL-2 and IL-1β and is increased by IL-23 and TGF-β. Recently, Mercer et al. [109] named these Th17-like phenotype Treg cells as IL-17^+ Tregs cells, due to the fact that this subset produces IL-17, and observed that naïve Treg cells (TNreg) expressing CCR6 have

a predetermined capacity to differentiate into IL-17$^+$ Treg cells with suppressive activity *in vitro*. They also observed that a small portion of naïve Treg cells expressing CCR6 have the propensity to polarize into Th17 cells. CCR6 is expressed by both Treg and Th17 cells and plays a significant role in Treg-mediated suppression and in the migration of Th17 cells to inflammatory sites [110].

Other important factors can influence the Th17/Treg balance. The fine-tuning of Treg cells upregulate chemokine and cytokine receptors in a pattern matching that of the immune T effector cells, whereas chemokine receptors such as CCR6 and CXCR3 facilitate the spatial proximity of suppressive Treg and inflammatory effector cells and cytokine receptors (e.g., IL-1R, IL-6R) that may compete for important factors, thus, limiting the activation or differentiation of T effector cells [6, 104].

Also, the stability of Treg cells has been questioned. It was observed that adoptive transfer of FoxP3$^+$Treg cells into lymphopenic hosts leads to loss of FoxP3 expression in these cells and their differentiation into follicular T helper cells (Tfh) in Peyer's patches [111]. In contrast, in another study, CD25$^+$CD4$^+$ T cells were stable and did not lose FoxP3 upon adoptive transfer into lymphopenic hosts, whereas a relatively minor fraction of CD25$^-$ or CD25^{low} FoxP3$^+$ cells can lose FoxP3 expression and divert into effector T cell lineages [112].

Studies observed a reduction in Treg cell numbers and/or a loss of Treg function in animal models and human autoimmune diseases. Nevertheless, it is important to highlight that an increased number of Treg cells in autoimmune disease do not necessarily mean that these cells are able to control the immune response. As aforementioned, Treg cells have a certain degree of plasticity and can lose their suppressive function, especially under inflammatory conditions. Furthermore, data on peripheral Treg cell numbers and function in human autoimmune/inflammatory diseases are contradictory and remain subject to debate.

4.1. What about the Balance between Th17 and Treg Cells in HIV Infection? Several studies were carried out to investigate the Th17/Treg balance state in typical progressors treated or untreated, EC, slow progressors, HIV-infected subjects, and SIV infection model [113]. Since Treg cells are developmentally linked to Th17 cells, the ratio of Th17 to Treg cells is used as an index of the relative balance between these two cell subsets. An impaired Th17/Treg balance in HIV-1 infection has a deleterious effect on gut mucosal immunity and fuels immune activation by enhancing microbial translocation [3, 80].

The importance of the Th17/Treg balance maintenance was highlighted by experiments performed in animal models using SIV infection. For instance, a loss of the Th17/Treg balance was observed in pathogenic SIV infection in Pig-tailed Macaques (PTs) when compared with nonpathogenic infection in African Green Monkeys (AGMs). SIV-infected PTs, but not SIV-infected AGMs, rapidly developed systemic immune activation and a selective depletion of Th17 cells, suggesting that loss of the Th17/Treg balance is related to SIV disease progression [113].

Li et al. [114] observed a continuous loss of Th17 cells which was accompanied by a concomitant rise in the frequency of Treg cells, resulting in a Th17/Treg cells imbalance during the HIV-1 progression disease in untreated chronic HIV-1 infected followed up for more than 1 year. This study included a small group of EC and remarkably, Th17/Treg cells ratios in those elite controllers remained comparable with ratios observed for HIV-seronegative controls. Complementary to these data, in an Indian HIV-infected cohort, Th17 cells from peripheral blood were significantly more depleted in late stage infected as compared to early stage infected and slow progressor subjects. In this same study, Treg cells frequencies in the subjects with slow progression HIV-1 infection were comparable to the HIV-seronegative controls [115]. Another study performed by Brandt et al. [50] also observed that Th17/Treg cells ratio was similar in EC and HIV-seronegative controls. Taken together, these results suggest that the maintenance of the equilibrium between Th17 and Treg cells would correlate with a "better prognosis" in terms of disease course. In addition, in this study, in untreated viremic and treated HIV-infected subjects, the Th17/Treg cells ratio was lower compared with HIV-seronegative controls. Interestingly, a study followed up HIV/AIDS subjects before and after HAART and observed that the Th17/Treg cells ratio was significantly decreased before treatment, while HAART partially normalized the Th17/Treg cells ratio [116], suggesting that the HAART treatment can restore the Th17/Treg cells balance.

It has been recently shown that IDO induced tryptophan (Trp) catabolism promotes T cell differentiation into Treg cells through FoxP3 overexpression and suppresses the expression of RORc and the generation of Th17 cells [26]. Also, enhanced IDO activity was associated with HIV disease progression, and such activity leads to a Th17/Treg imbalance in the peripheral blood [117]. This chronic activation by IDO pathway diminishes the host's capacity to generate Th17 cells affecting the mucosal immune barrier critically dependent upon Th17 cells [3]. However, a recent study observed that IDO induced Trp catabolism into kynurenine that induces a harmful effect on the Th17/Treg cells ratio that may subsequently contribute to enhanced microbial translocation during HIV-1 infection. Importantly, EC compared to ART successfully treated and healthy subjects displayed a distinctive Trp catabolism characterized by similar Kyn/Trp ratios and preserved IDO expression and Th17/Treg cells ratios [118]. Thereby, efforts to prevent an imbalance (or restore a balance) of Th17/Treg in HIV-infected individuals could be envisaged as a potential treatment alternative.

Studies are showing that precursor populations of Treg and Th17 cells are target of HIV infection, and this phenomenon perturbs the Treg and Th17 cell polarization and consequently the balancing of these subsets. Mercer et al. [109] observed that IL17$^+$Treg cells (derived from naïve Treg cells) are selectively reduced in number in HIV-infected subjects with suppressed viral loads through HAART. Then, DaFonseca et al. [99] showed that a Th17 polarization is impaired and this deficit coincided with the paucity of CD25^{high}CD127$^-$FoxP3$^+$ (naïve Tregs or nTregs) and CD25^{high}CD127$^+$FoxP3$^-$ (called double positive) subset

cells in chronically HIV-infected aviremic subjects under HAART. In this study, the nTreg cells from recently infected untreated viremic subjects harbored higher levels of integrated/unintegrated HIV-DNA when compared with the same cells from chronically HIV-infected aviremic subjects under HAART. Finally, both recent studies suggest the requirement for new therapeutic strategies designed to the preservation of IL17$^+$Tregs- and Th17-lineage committed naïve precursors.

5. Role of Chemokine Receptors on Th17 and Treg Cells: Implications for HIV Pathogenesis

5.1. Characterization of Th17 Cells by Chemokine Receptors and HIV Infection. Chemokine receptors have an important role in the phenotypic characterization of memory T cell subsets with distinct migration capacities and effector functions. The ligands for these receptors are inflammatory chemokines and chemoattractants, which are expressed in inflamed tissues and mediate the selective recruitment of different types of effector cells [119].

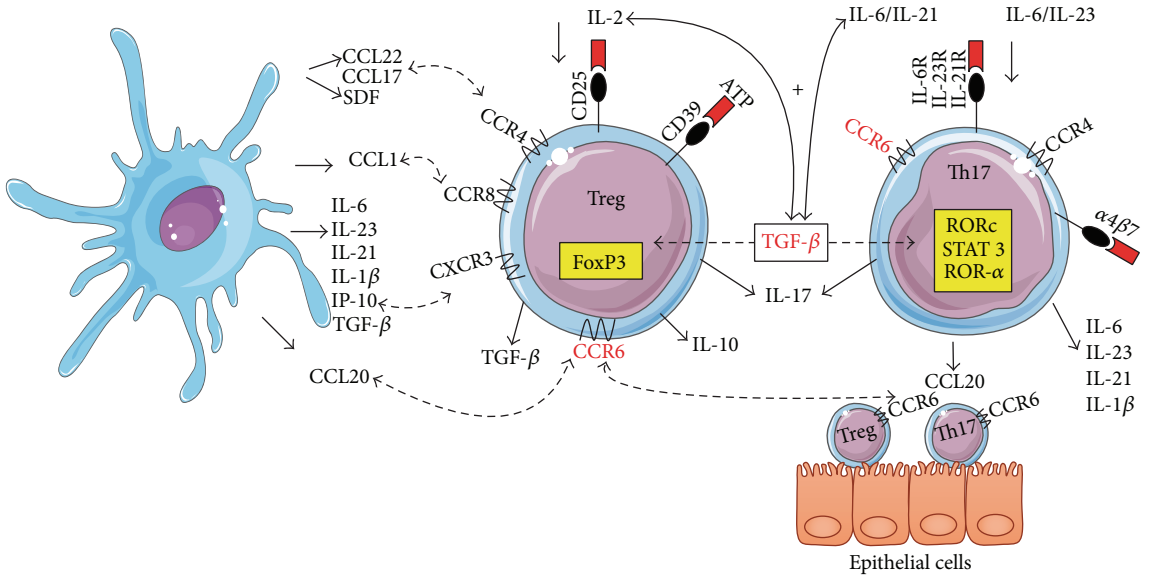
Memory CD4$^+$ T cells are highly heterogeneous in its potential homing and effector functions against a specific pathogen. Studies associate the commitment of lineage and antigenic specificity of CD4$^+$ T memory subsets with chemokine receptors expression [120–122]. It was observed that CCR4$^+$CCR6$^+$CD4$^+$ T cells subsets produce IL-17 and express the transcription factor ROR$\gamma\tau$ (Th17 profile) and were specific for *Candida albicans*, whilst CXCR3$^+$CCR6$^+$CD4$^+$ T cells subsets produce IL-17 and IFN-γ and express the transcription factors ROR$\gamma\tau$ and T-bet (Th1Th17 profile) and were specific for *Mycobacterium tuberculosis* [120].

There is emerging interest in the knowledge of the phenotype of HIV-infected CD4$^+$ T cells, with several studies demonstrating that HIV is very selective in choosing its cellular targets. It is well established that memory CD4$^+$ T cells are more permissive to HIV compared with naive T cells [77]. Thereby, it was observed that CCR4$^+$CCR6$^+$ CD4$^+$ T and CXCR3$^+$CCR6$^+$CD4$^+$ T cell subsets in peripheral blood were highly permissive to replication of both R5 and X4 HIV strains. Interestingly, these CD4$^+$ T cell subsets showed a persistent decline during chronic infection despite antiretroviral therapy [84, 122]. More recently, it was observed that *Tetanus toxoid* and *C. albicans* specific CD4$^+$ T cells with a Th17 profile (and high expression of CCR6 and its CCL20 ligand) were permissive to HIV infection, whereas CMV-specific CD4$^+$ T cells with a Th17 profile were highly resistant to both R5 and X4 HIV strains [121]. These results show a preferential infection of peripheral CCR6$^+$CD4$^+$ T cells by HIV and the importance of different CD4$^+$ T cell subsets against specific opportunistic pathogens that are depleted at different rates [123].

Studies observed that memory and effector Th17 cells are present in a subset of CCR6$^+$ cells in both peripheral blood and inflamed tissues and are preferential target to HIV-1 infection [124]. Even though Th17 cells express more than one trafficking receptor in a tissue-specific manner, CCR6 is the unique receptor that is uniformly expressed by all subsets of Th17 cells [125]. CCR6 is a gut homing chemokine receptor and has a critical role in cell migration into Peyer's patches of the distal small intestine where CCL20 (MIP-3α) is expressed [125]. During normal development and immune homeostasis, CCL20 selectively attract CCR6-expressing lymphocytes and DCs to the mucosal surfaces, organizing lymphoid tissues, such as Peyer's patches, mesenteric lymph nodes, and GALT [126]. Th17 cells subsets express CCR6 and produce CCL20. The production of CCL20 from Th17 cells is regulated similarly as IL-17 (induced by TGF-β along with IL-6) [126]. In contrast, an *in vitro* study observed that CCR6 expression on Th17 cells is coordinately regulated by TGF-β and IL-2. TGF-β, but not IL-6, was able to induce CCR6 on T cells; conversely, IL-2 effectively suppressed the expression of CCR6 on Th17 cells [72, 125]. Th17 cells, by producing CCL20, could also attract other Th17 cells via CCR6, meaning that the production of CCL20 can lead to further recruitment of other CCR6-expressing Th17 cells and sustained chronic inflammation [126].

The high susceptibility of Th17 cells to HIV *in vitro* is reflected by their *in vivo* depletion in the peripheral blood of HIV-infected individuals receiving treatment, compared with HIV-uninfected subjects [83]. It was suggested that CCR4$^+$CCR6$^+$ CD4$^+$ and CXCR3$^+$CCR6$^+$ CD4$^+$ cell subsets could have the potential to be recruited to the intestinal and vaginal mucosa through a CCR6$^-$CCL20 dependent mechanism significantly contributing to HIV dissemination and persistence in cells, also attracting other CCR6$^+$ CD4$^+$ T cells to viral replication sites, *in vivo* [84, 122, 124]. Also, the $\alpha4\beta7$ integrin identifies a subset of Th17 cells that is preferentially infected and depleted during acute SIV infection [125]. Accordingly, a study observed that the loss of peripheral $\alpha4^+\beta7^+$ memory CD4$^+$ T cells correlates with the loss of CD4$^+$ T cells in GALT during pathogenic SIV/HIV infection [127]. Taken together, these studies indicates that the ability of Th17 cells subsets to migrate into the GALT and other infection sites (e.g., periphery) depends on the imprinting for homing which is mediated by a combination of adhesion molecules and chemokine receptors (Figure 1).

5.2. Characterization of Tregs by Chemokine Receptors and HIV Infection. As aforementioned, homing and trafficking of effector cells are mainly facilitated by chemokines and expression of their chemokine receptors on distinct T cell subsets, and Treg cells are no exception. It was observed that CD45RA$^-$ FoxP3$^+$ T cells from peripheral blood express the CCR4, CCR5, CCR6, CXCR3, and CXCR6, chemokine receptors, which are commonly expressed by memory/effector T cells [128]. CCR4 and, even more, CCR8 have already been reported to be important for regulatory human CD25$^+$CD4$^+$ T cells [129]. Of note, mature dendritic cells preferentially attract Treg cells that express CCR4 and CCR8 through CCL17, CCL22, and CCL1 chemokine secretion (Figure 1). Thereby, it was suggested that CCR4 and/or CCR8 may guide Treg cells to inflamed areas and sites of antigen presentation in secondary lymphoid tissues in order to attenuate T cell



IL-2 induces Treg cells proliferation and inhibits CCR6 expression on Th17 cells.
IL-6 or IL-21 induces Th17 cells differentiation and inhibits Treg cells.
IL-23 implicated in the Th17 phenotype stabilization and expansion but is not a differentiation factor.
IL-β and IL-6 amplify Th17 cells differentiation.

Figure 1: The interaction network between transcriptional factors, cytokines, chemokines, and their receptors in Th17 and Treg cells. The fine-tuning of Th17/Treg balance is regulated by expression of transcription factors that are activated by cytokines milieu and their receptors. TGF-β along with mainly IL-6 induces RORc, ROR-α, or STAT3 expression to differentiate Th17 cells while that in combination with IL-2 induces FoxP3 expression to differentiate Treg cells, while homing and immunological cells recruitment of both cell subsets are powerful mechanism mediated by chemokines and their chemokine receptors such as CCR6, CCR4, or CXCR3 which facilitates the recruitment of suppressive Treg and inflammatory effector Th17 cells (e.g., by means of CCR6-CCL20) into the site infection or injured tissue. Of note, other immunological cells, as dendritic cells, influence this balance because they produce cytokines, chemokines, and other molecules that participate in this interaction network.

activation or inhibit APC function [129]. This scenario suggests that chemokines secreted by APCs and chemokine receptors expressed on T cell subsets regulate the competition of T cells for access to antigen-bearing APCs.

Regarding CCR6, an important receptor expressed on Th17 cells, CCR6$^+$ Treg cells exhibit a phenotype of activation, memory, and expansion that are typical for an effector memory function [110]. Unlike Th17, Treg cells do not produce CCL20 [126]. However, it was observed that Treg cells migrate towards to CCL20-producing Th17 cells *in vitro* in a completely CCR6 dependent manner (migratory response was completely abolished in CCR6-deficient Th17 and Treg cells) [126]. In this study, it is proposed that Th17 cells produce CCL20 that attract other CCR6$^+$ Th17 cells as well as CCR6$^+$ Tregs through CCR6.

In the context of viral infections, Qin et al. [130] observed that a simultaneous antagonism of CCR4 by increased CXCR3 ligand expression (CXCL11) and loss of CCR4 ligand expression contributed to reducing homing of FoxP3$^+$ Treg cells to lymph node and intestinal tissues during SIV infection. In this study, the increment of IFN-γ as an upstream regulator of CXCR3 ligand expression and the decrease in TGF-β as an upstream regulator of IFN-γ expression revealed a complex set of interrelationship that control multiple positive and negative feedback system [130]. In the early stage of HIV infection, plasma IP-10 (CXCR3 ligand) levels were predictive of rapid progression than viremia or CD4$^+$ T cells levels [131]. Regarding CCL20 (CCR6 ligand), saliva was shown to increase significantly CCL20 secretion. Thus, it suggests that saliva could facilitate HIV entry and other pathogens through the genital mucosa during sexual intercourse [132].

Nevertheless, little is currently known about how chemokines and chemokine receptors regulate the homing and trafficking of Treg cells in HIV infection. Differential profiles of Treg homing receptors could be critical in the control of the inflammatory response against HIV. Also, interactions between chemokines and their receptors, such as CCL20/CCR6-mediated signals, can be strongly induced by proinflammatory stimuli. Future studies approaching how Treg cell subsets interact with each other and with the remaining cells by means of their chemokine receptors would certainly help in the understanding of the HIV infection pathogenesis.

6. Conclusions

HIV-1 infection is characterized by a gradual decrease of the immunological competence and a massive depletion of

$CD4^+$ T cells, particularly in GALT, which leads to microbial translocation, contributing to immune hyperactivation, an important pathogenic mechanism HIV-1 infection. Th17 cells are proinflammatory $CD4^+$ T cell subsets and play a pivotal role in host defense, mainly in the gastrointestinal tissue. Currently, most evidence suggests that Th17 cells have a beneficial role in HIV infection by promoting gut mucosa recovery, preventing microbial translocation and decreasing immune hyperactivation. However, a pathogenic role of these cells, particularly the induction of an increase in viral replication through the production of inflammatory cytokines, should not be ruled out. The role of Treg cells in regulating T cell activation during immune responses to pathogens such as HIV-1 is a subject of great interest. Their effects can be beneficial or detrimental depending on the balance between attenuating HIV-induced immune hyperactivation and mounting an immune response to HIV-1 and opportunistic pathogens.

The interaction between the cytokines milieu, chemokines, and chemokine receptors and the acquisition of tissue-specific homing form a complex network that is influenced mainly by the plasticity of T cells, genetic host, and environmental factors. Recent studies prompted that this network can disturb the Th17/Treg balance during HIV-1 infection. However, the mechanisms underlying this interaction are still not completely understood, and more studies need to be carried out in that direction. Finally, new findings about Th17/Treg outcomes and the understanding of interindividual variability in HIV infection will be crucial to the development of new treatment strategies and vaccines.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this review.

Authors' Contribution

Jacqueline María Valverde-Villegas, Maria Cristina Cotta Matte, and Rúbia Marília de Medeiros contributed equally to this paper. José Artur Bogo Chies reviewed the final paper.

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1.2.6 Ativação e exaustão do sistema imune durante a infecção

Estudos mostraram que a ativação imune ininterrupta é um dos principais problemas causados pela infecção pelo HIV (Dandekar et al., 2010). Desde o estágio inicial da infecção, APCs, linfócitos T e linfócitos B mostram evidências fenotípicas e funcionais de ativação (Hazenberg et al., 2003). No estágio clínico de aids os linfócitos T CD8+ expressam níveis aumentados de CD38, HLA-DR (marcadores de ativação celular) e PD-1 (marcador de exaustão imune), indicando desregulações funcionais nesta subpopulação celular com o avanço da infecção (Day et al., 2006; Giorgi et al., 1993). O macaco rhesus (*Macaca mullata*), que progride para imunodeficiência severa após a infecção por SIV, mostra sinais de ativação imune e inflamação exacerbada quando comparado com *Sooty mangabeys*, um primata que é naturalmente infectado com SIV e permanece saudável apesar de apresentar carga viral detectável (Chakrabarti et al., 2000; Durudas et al., 2009; Giorgi et al., 1993). Além disso, estudos já sugeriram que o aumento dos níveis de algumas proteínas, consideradas marcadores de ativação imune, como neopterina e β2-microglobulina, estão relacionados a estágios avançados infecção (Fahey et al., 1998; Giorgi et al., 1993; Hazenberg et al., 2003). Do mesmo modo, níveis plasmáticos aumentados de proteína C reativa, marcadores da coagulação (d-dímero), IL-6 e TNF-α estão associados a um maior risco de mortalidade na infecção pelo HIV (Boulware et al., 2011; Kalayjian et al., 2010; Nixon & Landay, 2010).

A ativação imune crônica desencadeia a apoptose precoce de células B e T, pelo aumento dos níveis de TNF-α circulantes que induzem a apoptose pelas vias TRAIL e Fas (Ahr et al., 2004; Huang et al., 2006). A inflamação leva à fibrose do tecido linfóide, danos na arquitetura do linfonodo e provoca um desequilíbrio da homeostase das células T (Schacker et al., 2002, 2005). Como resultado da ativação imune sistêmica e estimulação prolongada, pela não eliminação do antígeno, as células T perdem gradualmente a polifuncionalidade e sua capacidade proliferativa, um processo referido como exaustão de células T. A regulação positiva de PD-1 está associada com a exaustão de células T durante a infecção pelo HIV (Day et al., 2006). PD-1 é expressa em células T ativadas, e os seus ligantes (PDL-1 e PDL-2) são amplamente expressos por APCs (Ishida et al., 1992). A ligação de PD-1 aos seus ligantes ativa uma via imunossupressora, atenuando a proliferação de células T CD4+ e T CD8+ e a produção de citocinas, como IFN-γ, IL-10 e IL-2 (Brown et al., 2003). PD-1 é expressa em níveis mais elevados em monócitos infectados pelo HIV, sendo regulada positivamente pelos

produtos microbianos circulantes após translocação microbiana a partir do dano à mucosa do intestino (Said et al., 2010). A expressão de PD-1 pelos monócitos também é capaz de aumentar a expressão de IL-10 que inibe a resposta proliferativa das células T CD4+ (Said et al., 2010). O bloqueio *in vitro* da PD-1 aumentou a produção de IL-2, IFN-γ, IL-13 e IL-21 por células T CD4+ específicas para o HIV e aumentou a produção de IFN-γ por células T CD8+ (Trautmann et al., 2006).

As causas da ativação imune sistêmica associada à infecção pelo HIV não estão claramente definidas, mas certamente alguns mecanismos contribuem para essa ativação: a infecção propriamente dita das células do sistema imune, a contínua produção de citocinas pró-inflamatórias por células do sistema imune inato e a translocação de produtos microbianos para o sangue através do tecido intestinal danificado.

1.2.7 Citocinas e a infecção pelo HIV

A interação entre as proteínas virais ou os produtos intermediários da replicação viral com receptores de padrões parece ser o evento inicial e determinante na qualidade e quantidade da resposta imune que será orquestrada contra o vírus. Tais interações conduzem à síntese e à liberação de uma grande variedade de mediadores imunes, os quais incluem citocinas e quimiocinas, e à modulação de uma ampla variedade de moléculas de superfície celular expressas pelas células que interagem. Como o esperado, algumas horas após a infecção pelo HIV uma "tempestade de citocinas" já pode ser detectada no plasma do indivíduo infectado (Figura 4). Individualmente cada molécula é capaz de ativar ou bloquear vias específicas. Contudo, esta rede de sinalização é extremamente complexa, uma vez que, é necessária a atuação conjunta dos sinais para desencadear uma resposta específica. Um desequilíbrio nesta rede de sinalização pode resultar em desfechos favoráveis ou desfavoráveis para o indivíduo, no contexto da infecção.

A IL-6 é uma citocina pleiotrópica de grande relevância no sistema imune; foi caracterizada inicialmente pela sua capacidade de promover a expansão e a ativação de células T, a diferenciação das células B, e a regulação da resposta aguda a infecções (Hirano, 2014; Yasukawa et al., 1987). Diversas células do estroma e do sistema imune são capazes de produzir IL-6. Entre as principais vias ativadoras de expressão de IL6 estão a IL-1β e o TNF-α (Hunter & Jones, 2015). Concentrações fisiológicas normais de

IL-6 no soro humano são relativamente baixas (1-5 pg/ml) e a sua expressão é sujeita à regulação homeostática basal, porém no contexto de uma infecção ocorre a rápida elevação dos níveis circulantes (Panichi et al., 2004).

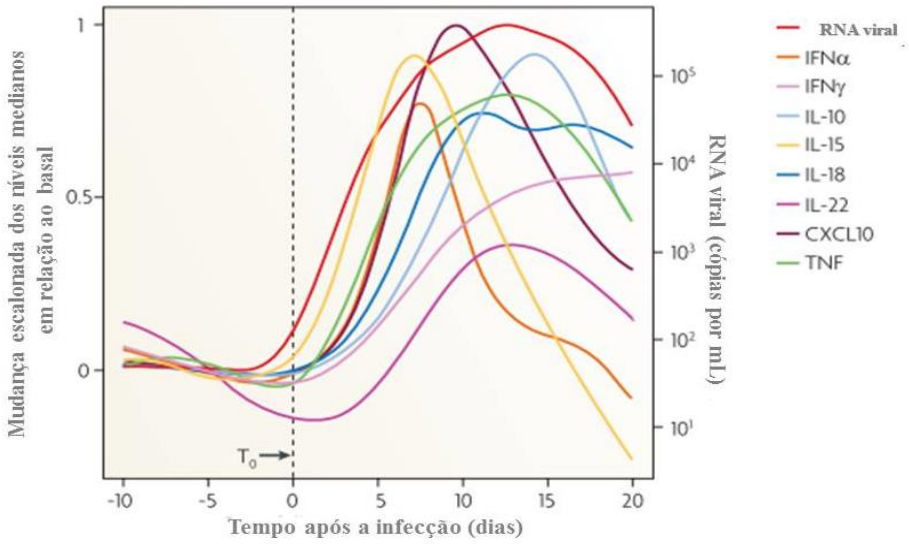

Figura 4 – Poucas horas apos a infecção efetiva pelo vírus HIV já é possivel observar uma "tempestade de citocinas" no plasma dos indivíduos infectados. A cinética observada na fase aguda demonstra à elevação dos níveis de proteínas pro-infmatórias, citocinas e quimiocinas, responsáveis pela ativação de respostas imunes específicas. Existem duas ondas iniciais de citocinas: IL-15, TNF-α e IFN-α, mais tardiamente IL-18, IL-10 e IL-22. Adaptado de (Stacey et al., 2009).

No curso da infecção pelo HIV não é diferente, níveis aumentados de IL-6, assim como de outros marcadores inflamatórios, estão relacionados com um risco aumentado de morte por complicações hepáticas e cardiovasculares, com a severidade de sintomas, desenvolvimento de sarcoma de Kaposi, inflamação e lesões em órgãos (Boulware et al., 2011; Foster et al., 2000; Rose-John, 2012). Estudos mostraram que, em indivíduos HIV-positivo, os níveis de IL-6 podem estar positivamente correlacionados com os níveis de RNA viral (Baker et al., 2012; Kuller et al., 2008).

TNF-α é uma citocina inflamatória capaz de ajudar a controlar infecções virais, induzindo apoptose em células infectadas através da ativação de diferentes vias (Flier et al., 1996). Altos níveis de TNF-α são detectados no plasma e tecidos de indivíduos infectados com HIV e associados com a severidade dos sintomas em indivíduos com aids (Lahdevirta et al., 1988; Rochford & Coffey, 1999). Altos níveis de TNF-α também

já foram associados à maior depleção de células T CD4+, em parte devido à morte direta de células T CD4+ infectadas, mas também, pela produção exacerbada de citocinas pró-inflamatórias capazes de induzir a morte de células T CD4+ não infectadas próximas (Huang et al., 2006).

O IFN-γ é fundamental na regulação da resposta imune contra patógenos intracelulares, virais e bacterianos, através da indução e ativação das células T. Além disso, IFN-γ é capaz de induzir as células infectadas à morte celular programada, utilizando vias de sinalização mediada por Fas e aumentando a sensibilidade das células ao TNF-α (Xu et al., 1998). Uma ampla gama de células do sistema imune secreta IFN-γ: linfócitos T CD4+ e T CD8+, linfócitos B, células NK e NKT, APCs (Gessani & Belardelli, 1998; Harris et al., 2000; Young, 1996). O IFN-γ produzido pelas APCs e células NK atua localmente e é importante na resposta imune inata, já os linfócitos T secretam IFN-γ no contexto da resposta imune adaptativa (Frucht et al., 2001). A produção de IFN-γ é controlada por citocinas secretadas pelas APCs, principalmente IL-12 e IL-18. Após o reconhecimento do patógeno os macrófagos induzem a secreção de IL-12 e outras quimiocinas, as quais atraem as células NK para o local da inflamação, e a IL-12, por sua vez, promove a expressão de IFN-γ (Salazar-Mather et al., 2000). IL-4 e IL-10 regulam negativamente a expressão de IFN-γ (Harris et al., 2000). IFN-γ não tem atividade antiviral direta contra o HIV em culturas, e esse dado é apoiado pelo baixo desempenho do uso de IFN-γ terapêutico em indivíduos infectados (Cao et al., 2003; Dalod et al., 1999). Por outro lado, IFN-γ parece ser fundamental para a ativação da resposta citotóxica e das células NK. Linfócitos T CD8+ de não-progressores de longo prazo apresentam secreção aumentada de INF-γ após serem estimulados por peptídeos virais (Harrer et al., 1996; Rinaldo et al., 1995).

Secretadas para ativar a resposta imune contra o vírus e eliminar a infecção primária, IL-6, TNF-α e IFN-γ, são citocinas pró-inflamatórias envolvidas no estabelecimento de uma ativação imune crônica (Roff et al., 2014). Além disso, algumas citocinas pró-inflamatórias podem induzir diretamente a replicação do HIV devido ao uso do fator de transcrição NF-κβ. Diversos mecanismos são ativados pelo próprio vírus visando estimular a expressão destas citocinas e consequentemente ativar a via do NF-κβ (Haij et al., 2013; Gangwani & Kumar, 2015; Shah et al., 2011).

IL-2 é um fator de sobrevivência para células T e promove a diferenciação das células T ativadas em células T efetoras. IL-2 inicia respostas de células Th1, possibilitando a produção de citocinas como IFN-γ e TNF-α (Catalfamo et al., 2012). Estudos sugerem que células T CD8+ capazes de expressar altos níveis de IL-2 se correlacionam com maior proteção durante a infecção pelo HIV (Akinsiku et al., 2012). Ensaios que testaram o uso de IL-2 como tratamento na infecção pelo HIV demonstraram que esta citocina é capaz de aumentar os níveis de células T CD4+ virgens e de memória, assim como foi capaz de estimular respostas T citotóxicas efetivas. Contudo, o tratamento também induziu aumento de viremia em pacientes sem HAART (Kovacs et al., 1995, 1996). Além disso, ensaios de fase III com IL-2 não demonstraram qualquer benefício clínico devido ao maior número de células T CD4+ (Abrams et al., 2010). A razão para este paradoxo ainda é incerta, mas as células induzidas pelo uso de IL-2 exógena exibiram expressão aumentada de CD25 e FoxP3, perfil semelhante às células Treg (Sereti et al., 2005).

IL-4 é uma citocina pleiotrópica caracterizada em meados de 1980, sendo produzida principalmente por células T ativadas, mas também por mastócitos, basófilos e eosinófilos (Nelms et al., 1999). Funcionalmente, IL-4 é conhecida por ativar a resposta Th2, que inclui indução da produção de imunoglobulinas E (IgE) pelas células B, regulação negativa do receptor CCR5 e regulação positiva do receptor CXCR4 (Jourdan et al., 1998; Nelms et al., 1999; Seder & Paul, 1994). Alguns estudos sugeriram que o aumento de IL-4 estaria associado ao aumento da propagação de sincício induzidos pelo HIV (Valentin et al., 1998; Wichukchinda et al., 2006). A combinação dos efeitos: aumento de células CXCR4 e redução de células CCR5, resultaria em aumento da propagação de variantes virais que utilizam o CXCR4 como co-receptor de entrada, as quais geralmente estão associadas com quadros mais severos de aids. Atualmente as pesquisas em torno da IL-4 tem tentado descrever as vias de sinalização intracelular funcionalmente diferentes que podem ser ativadas após sua ligação a receptores da superfície alternativos (Luzina et al., 2012). Além de definir caminhos distintos para os linfócitos, IL-4 pode conduzir a uma chamada "ativação alternativa de macrófagos"(Luzina et al., 2012). A discussão sobre o papel destas ramificações alternativas centra-se na contribuição à regulação da homeostase do sistema imune e inflamação e não somente na ativação da resposta Th2.

IL-10 foi caracterizada como uma citocina reguladora em infecções virais crônicas em estudos de Brooks e colegas (Brooks et al., 2006, 2010). A IL-10 é produzida por monócitos e células Tregs e, em geral, tem um efeito de "desativar" as respostas do tipo Th1 e reduzir a apresentação de antígeno por regulação negativa do MHC de classe II em APCs (Kwon et al., 2012). Além disso, bloqueio de IL-10R resulta na proliferação de células T, na secreção de IFN-γ e na secreção de IL-2 por células T antígeno-específicas (Kwon et al., 2012). Durante a infecção crónica pelo HIV, os níveis de IL-10 no plasma são significativamente mais elevados em comparação com indivíduos não infectados e indivíduos HIV-positivo controladores de elite (Angin et al., 2012), reduzindo a capacidade do sistema imunitário de eliminar o vírus. Adicionalmente, a perda de Tregs pode levar a uma diminuição da secreção de IL-10 no intestino e desequilíbrio da resposta imune neste local inicial da replicação viral, o que contribui para a ativação imunitária e a destruição da barreira intestinal.

IL-17 é a principal citocina da resposta Th17, responsável por induzir o processo inflamatório e evitar processos autoimunes (Korn et al., 2009; Park et al., 2005). Outros subgrupos de células T, tais como células Th2, células NKT, células Tγδ, além de neutrófilos e eosinófilos também secretam IL-17 em resposta a estímulos da resposta imune inata (Fort et al., 2001; Liu et al., 2007; Lockhart et al., 2006). IL-17 é capaz de induzir células mielóides e mesenquimais a liberar citocinas e quimiocinas pró-inflamatórias, além de, metaloproteinases e peptídeos antimicrobianos com atuação direta no sítio da infecção. Este conjunto de moléculas conduz à expansão e acúmulo de neutrófilos no local (Ferretti et al., 2003; Jovanovic et al., 1998; Fossiez, 1996). Todas estas evidências mostram o papel de IL-17 em intermediar imunidade inata e adaptativa. No contexto da infecção pelo HIV, estudos indicam que menores níveis desta citocina podem estar relacionados ao controle da viremia em modelos animais (macacos rhesus infectados com SIV) e à proteção ao HIV em indivíduos expostos não infectados (Chege et al., 2012; Khowawisetsut et al., 2013).

As citocinas desempenham um papel importante durante a patogênese do HIV, seja através da regulação da replicação viral pelo compartilhamento dos fatores de transcrição, seja pelo seu papel na modulação das respostas imunes inata e adaptativa. Embora a fase aguda da infecção esteja associada com o aumento da produção de citocinas pró-inflamatórias, a relação entre as concentrações de citocinas e a patogênese do HIV ainda não é totalmente compreendida. Rober et al., (2010) avaliaram o nível

plasmático de 30 citocinas e quimiocinas durante a infecção aguda pelo HIV. O estudo incluiu 40 mulheres infectadas com o HIV-1 e que foram acompanhadas longitudinalmente a partir do momento da infecção (12 a 66 meses). Entre as moléculas analisadas, IL-12p40, IL-12p70, IFN-γ, IL-7 e IL-15 mensurados 12 meses após a infecção previram 66% da variação na carga viral na infecção aguda. IL-12p40, IL-12p70 e IFN-γ foram associados com menor carga viral, enquanto que IL-7 e IL-15 foram associadas com maior carga viral. Liovat et al. (2012) analisaram os níveis plasmáticos de 28 citocinas e quimiocinas, também durante a fase aguda da infecção pelo HIV, tentando avaliar seu poder preditivo nos set-points virais e imunológicos e na progressão para aids (Liovat et al., 2012). As moléculas foram quantificadas durante a infecção aguda e pós-aguda em 46 indivíduos não tratados, categorizados em progressores rápidos, progressores típicos e progressores lentos de acordo com seu perfil da evolução espontânea ao longo de 42 meses. Os níveis plasmáticos de CXCL10 (também conhecida como IP-10) foram positivamente associados com a ativação das células T e negativamente associados com a contagem de células T CD4+. Além disso, os níveis de CXCL10, durante a infecção primária, foram preditivos de progressão rápida. Em conjunto, os estudos mostram que citocinas e quimiocinas, as moléculas de sinalização do sistema imune, estão correlacionadas com a patogênese da infecção e podem ter valor preditivo para a progressão da infecção.

1.2.8 Citocinas, polimorfismos genéticos e progressão para a aids

A associação da deleção CCR5Δ32 com a resistência à infecção pelo HIV forneceu a primeira evidência conclusiva sobre a relevância da diversidade genética do hospedeiro na patogênese desta infecção. A deleção de 32 pares de base no gene *CCR5* é responsável pela síntese de uma proteína truncada que não é transportada para a superfície celular (Benkirane et al., 1997). Indivíduos homozigotos para a mutação são resistentes à infecção viral, pois o vírus não consegue invadir a célula. Já indivíduos heterozigotos possuem maiores chances de apresentar uma progressão lenta à aids, uma vez que a menor quantidade de moléculas CCR5 na superfície reduz a possibilidade de o vírus encontrar o co-receptor de entrada.

Após esta descoberta, diversos autores começaram a avaliar a contribuição de variações genéticas na suscetibilidade à infecção e, também, na progressão para a aids (An & Winkler, 2010; Lama & Planelles, 2007). A importância da diversidade das

moléculas de reconhecimento de antígenos das células T no controle da infecção foi demonstrada pela observação de que a homozigose para moléculas HLA está associada com a progressão acelerada para a aids (Carrington et al., 1999; Tang et al., 1999). Ainda, certos tipos de HLA de classe I estão associados com progressão lenta (HLA-B*57, HLA-B*5801 ou HLA-B*27), enquanto outros com progressão rápida (HLA-B+35) (Carrington & O'Brien, 2003; Goulder et al., 1996; Kaslow et al., 1990). Mais recentemente, os resultados de estudos de associação de varredura genômica, ou GWAS, corroboraram o importante papel dos HLAs no controle da infecção. A região codificante do HLA foi associada, além da variante del32 do *CCR5*, com o controle da replicação viral e com progressão lenta para a aids (Fellay et al., 2007, 2009). No entanto, um estudo de varredura direcionado para avaliar o papel da diversidade genética na suscetibilidade ao HIV mostrou apenas um papel protetor da variante del32 do *CCR5* (Mclaren et al., 2013). Contudo, esses estudos são projetados para detectar polimorfismos comuns (geralmente com uma frequência > 5% para o alelo raro) com efeitos biológicos suficientes para criar associações significativas após os testes de múltiplas correções, avançando pouco em análises de interações entre as variantes.

Os estudos genéticos têm auxiliado na compreensão do papel de citocinas em respostas imune frente ao vírus e diferentes variantes em genes de citocinas foram propostas como marcadores de suscetibilidade e progressão da infecção (Tabela1). Em relação a IL-10, por exemplo, trabalhos associaram o genótipo -592AA *IL10*, responsável por uma menor expressão de IL-10, com maior susceptibilidade à infecção pelo HIV (Naicker et al., 2009; Shin et al., 2000). Por outro lado, a variante -1082G da *IL10*, responsável por um aumento na produção de IL-10, foi associada com a progressão lenta da infecção (Erikstrup et al., 2007). Neste sentido, Neiker et al., (2012) sugeriram que o efeito dos níveis desta citocina são dependentes da fase da infecção, de modo que altos níveis de IL-10 durante a fase aguda seriam prejudiciais possivelmente por reduzir a capacidade da resposta imune ao vírus e, durante a fase crônica, altos níveis de IL-10 seriam benéficos por reduzir os efeitos da exacerbação da ativação imune (Naicker et al., 2012). Três estudos, em diferentes coortes (francesa, tailandesa e norte-americana), associaram a variante -589T *IL4* com menor carga viral plasmática e progressão lenta para a aids (Nakayama et al., 2000; Nakayama et al., 2002; Wang et al., 2004; Wichukchinda et al., 2006). Com relação ao *TNF-α*, os SNPs -308G/A e -238G/A já foram associados a diferentes desfechos na infecção pelo HIV. Singh et al.

(2014) associaram a presença da variante -308A, relacionada a uma alta produção de TNF-α, com maiores níveis de apoptose de linfócitos T infectados pelo HIV e progressão rápida para a aids (Singh et al., 2014). Por outro lado, Veloso et al (2010) associaram a variante -208A, associada a um aumento da expressão de TNF-α, com o controle da carga viral em uma coorte espanhola de indivíduos HIV-positivo controladores de elite (Veloso et al., 2010).

A complexidade observada nos estudos de associação se deve em parte às diferenças no desenho do estudo (coortes de soroconversão, corte transversal, estudos longitudinais, etc) e, em parte, a diferenças na estruturação genética populacional. Contudo, é muito importante considerarmos que os marcadores genéticos e imunológicos atuam em conjunto para definir a resposta imune frente à infecção. Deve-se considerar que a maioria das doenças humanas apresenta componentes genéticos de etiologias complexas. Portanto, ao nível genético vários fatores podem atuar simultaneamente para o direcionamento rumo a um determinado desfecho, como a heterogeneidade alélica, heterogeneidade de locus, variabilidade fenotípica, interações gene-gene e gene-ambiente (Thornton-wells et al., 2004). Assim, é crucial que as análises genéticas sejam ampliadas a fim de detectar estes efeitos.

Tabela 1. Presença de polimorfismos em genes de citocinas e associações encontradas csuas possíveis influências na progressão para aids.

Citocina	Polimorfismos	Associação	Refs
IL-2	-330G (↑IL2)	suscetibilidade	(Shrestha et al., 2006)
TNF-α	-308A (↑TNFα)	progressão rápida	(Singh et al., 2014)
	-238A (↑TNFα)	controle da viremia	(Veloso et al., 2010)
IL-4	-589T (↓IL4)	progressão lenta	(Mahajan et al., 2010)
IL-6	-174C (↓IL6)	severidade de sintomas	(Foster et al., 2000)
IL-10	-1082G (↑IL10)	progressão lenta	(Naicker et al., 2009; Vasilescu et al., 2003)
	-592A (↓IL10)	suscetibilidade	(Shin et al., 2000)

Entre as interações gene-gene, está a epistasia que foi reconhecida por Fisher como desvios de aditividade em um modelo estatístico linear (Fischer, 1918). A epistasia estatística trata de efeitos de variações genéticas interindividuais a nível populacional, os quais são difíceis de detectar e caracterizar devido à sua inerente não-

linearidade e elevada dimensionalidade (Jones et al., 2014). Moore et al., (2003) e Templeton et al., (2000) têm proposto que epistasia é um componente ubíquo na arquitetura genética das doenças humanas (Templeton, 2000; Moore et al., 2003; Hahn et al., 2004). Entre as evidências para tal afirmação estão: i) a regulação gênica de sistemas bioquímicos e metabólicos ocorre através de interações moleculares, sugerindo que a relação entre variações no DNA e desfechos biológicos envolva uma ampla gama de variantes; ii) resultados de estudos de associações entre SNPs e desfechos biológicos normalmente não são replicados em todas as amostragens testadas; iii) efeitos epistáticos são comumente encontrados, quando investigados. Deve-se considerar também que o papel da epistasia na estruturação genética de populações segue o principio teórico da seleção estabilizadora, o qual pressupõe que a evolução de redes de genes redundantes permite que populações tornem-se resistentes a perturbações genéticas e ambientais (Gibson, 2009; Waddington, 1942). Assim, desfechos biológicos desfavoráveis são resultados da acumulação de múltiplas variantes em diferentes partes de uma rede de genes. Os efeitos epistáticos podem explicar, por exemplo, por que a maioria das variantes, individualmente, explica muito pouco o risco para qualquer desfecho comum. Neste sentido, os autores têm sugerido novas abordagens estatísticas em estudos de associação genética que incluam inferências sobre efeitos epistáticos.

1.3 Justificativa

O potencial uso prognóstico de moléculas do sistema imune na patogênese da infecção já foi sugerido a partir de estudos epidemiológicos. Como apresentado anteriormente, diferenças, qualitativas e quantitativas, nos níveis plasmáticos de citocinas nos estágios clínicos da infecção já foram descritas, sugerindo que estas moléculas podem estar associadas com diferenças individuais de resposta ao vírus. Assim, caracterizar a modulação das citocinas em indivíduos infectados pelo HIV, do estabelecimento da infecção até o desenvolvimento da infecção crônica é de grande relevância para a compreensão do papel dessas moléculas durante a infecção. E ainda, a avaliação do perfil de citocinas em indivíduos HIV-positivo em diferentes estágios clínicos, quando adequadamente concebida, pode complementar os parâmetros clínicos

atualmente utilizados, como a contagem de células T CD4+ e a carga viral, para uma análise ampla do estado da infecção e auxiliar possíveis intervenções terapêuticas.

Além disso, é preciso ampliar os métodos analíticos utilizados nos estudos genéticos de associações nos desfechos da infecção pelo HIV. A complexidade observada nos estudos de associação se deve em parte às diferenças no desenho amostral e na estruturação genética populacional, mas também a efeitos genéticos não investigados. Análises de interações epistáticas podem auxiliar na compreensão dos efeitos dos SNPs no contexto da infecção pelo HIV. A investigação de redes de genes altamente interativos, como é o caso de genes de citocinas, pode colaborar para o entendimento destes efeitos genéticos.

1.4 Objetivos

Este estudo tem como objetivo geral colaborar para a compreensão do papel da diversidade genética humana nos genes *IL2, IL4, IL6, IL10, IL17A, IFNγ*, *TNFα* e *TLR9* na modulação da resposta imune durante a infecção pelo HIV. As análises apresentadas aqui avaliaram possíveis associações entre a presença de polimorfismos nos genes citados e a suscetibilidade à infecção pelo vírus e a progressão diferencial para a aids. Além disso, as observações descritas ampliam a discussão sobre a mudança de perfil Th1→Th2 como fator determinante para aids, incluindo nesta discussão o perfil Th17 e Treg.

Objetivos específicos:

* Caracterizar diferentes desfechos clínicos (progressão da infecção) de indivíduos HIV-soropositivos regularmente atendidos em um grande centro médico de Porto Alegre, Rio Grande do Sul – Brasil.

* Avaliar alterações nos níveis plasmáticos das principais citocinas Th1, Th2, Th17 e Treg (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ e TNF-α) em indivíduos HIV-soropositivos progressores rápidos e lentos em diferentes estágios clínicos da infecção.

* Avaliar possíveis associações entre a presença de polimorfismos nos genes *IL2, IL4, IL6, IL10, IL17A, TNFα e TLR9* em indivíduos HIV-soronegativos com HIV-soropositivos.

* Avaliar possíveis associações entre a presença de polimorfismos nos genes *IL2, IL4, IL6, IL10, IL17A, TNFα e TLR9* em indivíduos HIV-soropositivos com progressão rápida e lenta para a aids.

* Avaliar interações gênicas entre polimorfismos nos genes *IL2, IL4, IL6, IL10, IL17A, TNFα e TLR9* em indivíduos HIV-soronegativos e em indivíduos HIV-soropositivos.

* Avaliar interações gênicas entre polimorfismos nos genes *IL2, IL4, IL6, IL10, IL17A, TNFα e TLR9* em indivíduos HIV-soropositivos com progressão rápida e lenta para a aids.

* Aplicar testes estatísticos de redução multifatorial de dimensionalidade (MDR) em abordagens genética, auxiliando na elaboração de modelos de interações gênicas mais complexas no contexto da infecção pelo HIV.

* Auxiliar na compreensão do potencial uso de citocinas como marcadores sorológicos e/ou genéticos de suscetibilidade à infecção pelo HIV.

* Auxiliar na compreensão do potencial uso de citocinas como marcadores sorológicos e/ou genéticos da progressão diferencial para a aids.

Capítulo 2

2.1 Definição e obtenção do grupo amostral

Os estudos de coortes em que indivíduos são monitorados do momento da soroconversão ao desfecho aids têm contribuído para a ampliação do conhecimento a respeito da história natural da infecção pelo HIV (An & Winkler, 2010; Casado et al., 2010; Okulicz et al., 2009; Olson et al., 2014). Contudo, estudos deste tipo são raros em países em desenvolvimento como o Brasil. Além disso, segundo o Ministério da Saúde, no país, a grande maioria dos indivíduos infectados pelo HIV chega aos centros de sáude já apresentando sintomas de aids (Boletim epdidemiológico, 2010). Logo, como grande parte da população só tem conhecimento do seu status sorológico no estágio clínico de aids, são escassos os dados quanto à progressão para a aids no Brasil.

A amostra para o estudo foi obtida a partir da análise retrospectiva de >3.500 registros médicos (prontuários médicos contendo testes imunológicos, receituários, encaminhamentos e internações, etc.) de indivíduos atendidos regularmente no Serviço de Infectologia do Hospital Nossa Senhora da Conceição. Aproximadamente 2.000 registros médicos foram analisados no ano de 2011. Posteriormente, no ano de 2013, foram analisados 1.500 registros médicos adicionais. Além disso, nos anos 2014 e 2015 todos os prontuários dos indivíduos incluídos no estudo foram novamente analisados e os dados desse período foram incluídos.

A seleção dos indivíduos HIV-positivo objeto do estudo assumiu os critérios abaixo, propostos a partir de ampla revisão da literatura relacionada à progressão rápida e lenta à aids.

Critérios de Inclusão:

1. Indivíduo com registro de sorologia negativa (HIV-NR) e registro de sorologia positiva (HIV-R) com intervalo de tempo médio de dois anos, além de diagnóstico de aids (notificação de aids/início de tratamento antirretroviral) em até três anos após o diagnóstico;

2. Indivíduo com registro de soroconversão, ou seja, registro de sorologia indeterminada (HIV-inconclusivo) + registro de sorologia positiva (HIV-R) e crise aguda (CD4 abaixo de 500 cel/mm^3 e sintomatologia de infecção viral durante o ano de HIV-R) em até três anos após o diagnóstico;
3. Indivíduo com registro de sorologia positiva (HIV-R) há mais de 10 anos, que ainda esteja sem terapia antirretroviral (*virgem*) ou tenha iniciado o tratamento após 9 anos do diagnóstico da infecção;
4. Indivíduo com registro de sorologia positiva (HIV-R) que apresente carga viral indetectável na ausência do uso de antirretrovirais;

Critérios de Exclusão:

1. Indivíduos com falhas nos registros de contagem de células T CD4+ e carga viral. Cinco anos de registros, para os progressores lentos, foram considerados como tempo mínimo de registro, com isso buscou-se garantir um acompanhamento adequado da evolução da infecção;
2. Indivíduos com indicação de início de tratamento antirretroviral anterior ao início do tratamento antirretroviral efetivo ignorado pelo paciente com intervalo superior a 1 ano;

A partir disto, o grupo amostral de estudo obtido:

108 pacientes foram incluídos no estudo: 60 progressores lentos, 40 progressores rápidos e 8 controladores de elite.
i) progressores rápidos: tempo entre sorologia HIV-, sorologia HIV+ e diagnóstico de aids inferior a 3 anos, além de, no mínimo, 2 contagens consecutivas de linfócitos T CD4+ <350 cél/mm^3 anteriores a coleta;
ii) progressores lentos: tempo após sorologia HIV+ superior a 9 anos sem diagnóstico de aids, além de no mínimo 2 contagens consecutivas de linfócitos T CD4+ >500 cél/mm^3 anteriores a coleta.

A fase clínica aids foi definida como início de terapia antirretroviral segundo os critérios do Ministério da Saúde/BR em vigor até o ano de 2013, ou seja, o início de tratamento antirretroviral era recomendado para pacientes HIV+ sintomáticos; HIV+ assintomáticos com medidas de T CD4+ abaixo de 350 cél/mm3; ou ainda, carga viral elevada persistente, independentemente dos níveis T CD4+.

2.2 Tratamento e regularização dos dados clínicos

Uma medida convencionalmente utilizada para caracterizar a progressão é a inclinação (*slope*) da reta gerada a partir da queda do número de células CD4+ do indivíduo HIV-positivo. Como o seguimento clínico dos pacientes HIV-positivo envolve medidas dinâmicas, que variam durante o curso da infecção, os dados clínicos retrospectivos foram primeiramente regularizados para, então, construirmos a reta e estimarmos o *slope* das células T CD4+.

Ao todo obtivemos 790 registros de dados clínicos (contagem de células T CD4+) pré-HAART, sendo 634 registros referentes a 57 progressores lentos e 156 registros referentes a 28 progressores rápidos. É importante desatacar que no grupo de progressores rápidos do estudo não foi possível calcular o slope das células CD4+, pois eles apresentaram apenas duas medidas de células CD4+ registradas antes do início do tratamento (neste caso abaixo de 350 células CD4+).

O primeiro registro de dados de cada indivíduo foi considerado como o tempo 0 (meses) e o tempo transcorrido do primeiro registro ao último (ΔT) foi estimado. O tempo médio de acompanhamento foi de 103 meses para os progressores lentos e 13 meses para os progressores rápidos. Os intervalos de tempo decorrido entre os registros de dados (ΔT relativo) foram calculados. Observou-se que 85% dos ΔTr dos progressores lentos correspondiam aproximadamente a 6 meses; já para os progressores rápidos, correspondiam aproximadamente a 3 meses. Assim, definido um intervalo de tempo médio para cada grupo de progressão, os dados foram regularizados segundo a fórmula abaixo.

$$\boldsymbol{V_{reg}} = \frac{\sum_{i=1}^{n}(\boldsymbol{V_i} * \boldsymbol{\Delta Tr_i})}{\sum_{i=1}^{n} \boldsymbol{\Delta Tr_i}}, \text{ onde:}$$

V_{reg}: Variável regularizada

V_i: valor original da variável

ΔTr_i: tempo decorrido entre dois registros da variável V, no intervalo de 6 (ou 3) meses

n : número de registros da variável V ao longo do intervalo de 6 (ou 3) meses

Os dados clínicos regularizados para os progressores rápidos (Figura 6) e para os progressores lentos (Figura 7) são apresentados nas figuras abaixo.

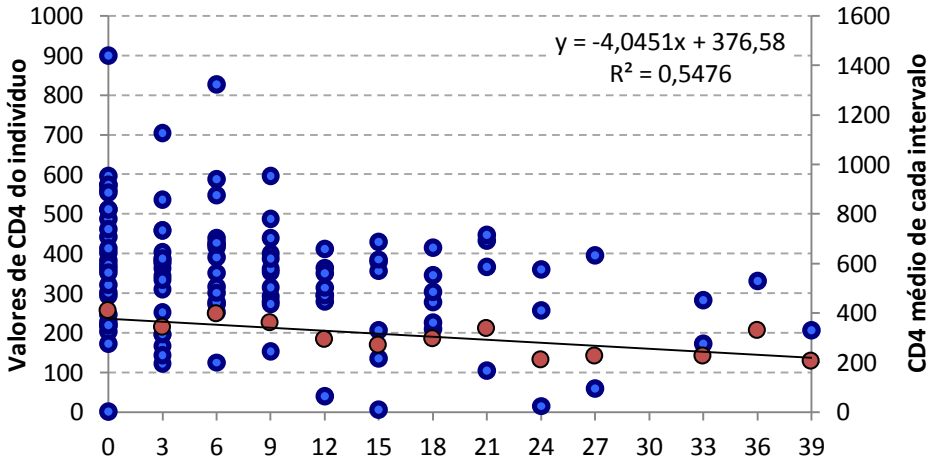

Figura 6 - Em azul o registro do número de células T CD4+ regularizado de cada progressor rápido. Em vermelho o valor médio de células T CD4+ utilizado para o cálculo do slope médio dos progressores rápidos (progressores rápidos *slope* = -4,05).

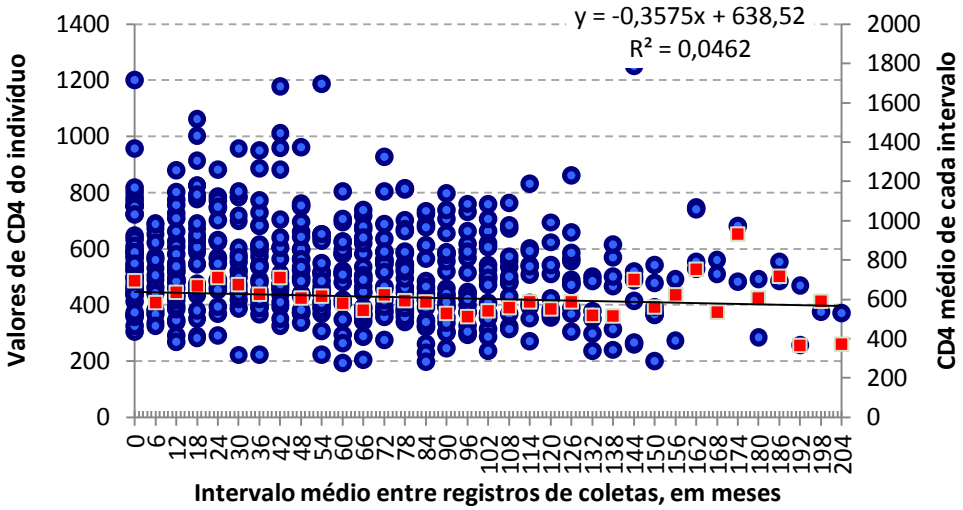

Figura 7 - Em azul o registro do número de células T CD4+ regularizado de cada progressor lento. Em vermelho o valor médio de células T CD4+ utilizado para o cálculo do slope médio dos progressores lentos (progressores lentos *slope* = -0,358).

Capítulo 3

3.1 Níveis circulantes das citocinas IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α e INF-γ em progressores rápidos e lentos para a aids em diferentes estágios clínicos da infecção.

Rapid and Slow progressors show increased IL-6 and IL-10 levels in the pre-AIDS stage of HIV infection

Rúbia M. de Medeiros, Jacqueline M. Valverde-Villegas, Dennis M. Junqueira, Tiago Gräf, Juliana D. Lindenau, Marineide G. de Mello, Priscila Vianna, Sabrina E.M. Almeida, Jose Artur B. Chies.

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RESEARCH ARTICLE

Rapid and Slow Progressors Show Increased IL-6 and IL-10 Levels in the Pre-AIDS Stage of HIV Infection

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Abstract

Cytokines are intrinsically related to disease progression in HIV infection. We evaluated the plasma levels of Th1/Th2/Th17 cytokines in extreme progressors, including slow (SPs) and rapid (RPs) progressors, who were thus classified based on clinical and laboratory follow-up covering a period of time before the initiation of HAART, ranging from 93–136.5 months for SPs and 7.5–16.5 months for RPs. Analyses were also performed based on the different stages of HIV infection (chronic, pre-HAART individuals—subjects sampled before initiating HAART but who initiated therapy from 12 to 24 months—and those receiving HAART). The plasma cytokine levels of 16 HIV-infected rapid progressors and 25 slow progressors were measured using a Human Th1/Th2/Th17 CBA kit. The IL-6 and IL-10 plasma levels differed significantly between the stages of HIV infection. The IL-6 levels were higher in slow progressors pre-HAART than in chronically infected SPs and HIV-seronegative individuals. The IL-10 levels were higher in slow progressors pre-HAART than in slow progressors receiving HAART and HIV-seronegative controls, and in rapid progressors, the IL-10 levels were higher in pre-HAART subjects than in HIV-seronegative controls. The results reflect the changes in the cytokine profile occurring during different clinical stages in HIV+ subjects. Our results suggest an association between increased IL-6 and IL-10 levels and pre-HAART stages independent of the slow or rapid progression status of the subjects. Thus, increased IL-6 and IL-10 levels could indicate a global inflammatory status and could be used as markers of the disease course in HIV-infected individuals.

Introduction

HIV infection progression is commonly defined based on the stability of CD4+ T-cell counts, viral load and the duration of symptom-free HIV infection [1,2]. Subjects with stable CD4+ T-cell counts and other clinical and immunological parameters over a period ranging from 7 to 10 years or more are known as 'slow progressors' (SPs). These individuals represent between 5% and 15% of the HIV-infected population [3,4]. Conversely, approximately 5% of the HIV-infected subjects progress to AIDS within 3 years after viral infection and, based on this time progression, are termed 'rapid progressors' (RPs) [3,5].

Plasma cytokine levels have been postulated to change dramatically over the course of HIV infection [6–8]. This variation involves a change from an environment characterized predominantly by T-helper type 1 cytokines, associated with cell-mediated immune responses, to an environment in which T-helper type 2 cytokines, known to enhance humoral immune responses, are dominant [9–11]. In recent times, however, *cytokinology* has evolved, and multiple T helper populations, such as Th17, and a number of different cytokine functions have been identified and analyzed in the context of HIV infection [12–14]. Although several studies suggest that cytokine levels are different in distinct stages of HIV infection, little is known about this topic in subjects classified according to rapid or slow disease progression.

The understanding of the cytokine profile throughout the course of HIV infection will contribute to the elucidation of the relationships between the immune response and the HIV infection outcome, ultimately improving clinical monitoring. Roberts *et al*. (2010) and Liovat *et al*. (2012) observed a relationship between an increase in plasma viral load, a decline in CD4+ T-cell counts and an increase of certain cytokine levels in HIV-infected subjects and suggested the predictive value of these cytokines for disease progression [15,16]. In the present study, we evaluated the Th1/Th2/Th17 cytokine plasma levels in both the extreme progressor groups (SPs and RPs—thus classified taking into account clinical and laboratory follow-up covering a period of time before the initiation of HAART ranging from 93–136.5 months for SPs and 7.5–16.5 months for RPs). The cytokine evaluation also took into account the different stage of HIV infection in each HIV-seropositive subject. Our results suggest an association between increased IL-6 and IL-10 levels and stages of infection pre-HAART, independent of the slow or rapid progression status of the patient. Thus, increased IL-6 and IL-10 levels could indicate a global inflammatory state and could be used as markers of disease progression in HIV-infected subjects.

Methods

Enrollment of the study population

We reviewed >3,500 medical records of HIV-infected individuals regularly attended in the Infectious Disease Service at Nossa Senhora da Conceição Hospital, Porto Alegre city between 2011 and 2013 to select rapid and slow AIDS progressors. To estimate their AIDS progression profiles (described below), longitudinal clinical and laboratory data were used, including CD4+ T-cell counts, plasma viral loads, stage of HIV infection at the time of sample collection and highly active antiretroviral therapy (HAART) prescriptions. In addition, their demographic data were obtained (Table 1). This study received the ethical approval of the Nossa Senhora da Conceição Hospital Ethical Committee (Project Number 10–123).

Characterization and stage of HIV-infected progressors

Based on the medical records data, the HIV-infected subjects were retrospectively classified into two groups: 16 RPs and 25 SPs (Fig 1). For RPs, the time of HIV seroconversion was

Table 1. Clinical baselines and demographic characteristics of the 41 HIV-infected subjects enrolled in this study as rapid or slow disease progressors.

	No. of subjects	
	RP (n16)	**SP (n25)**
Baselinemeasurements		
Time progression[a]	01 (1–3)	11 (10–13)
First CD4+ T cell count[b]	487 (356–600)	553 (392–712)
First RNA viral load[c]	3.32 (1.84–4.25)	2.2 (1.79–3.96)
Follow-up duration[d,e]	13.5 (7.5–16.5)	111 (93–136.5)
Slope CD4+ T-cell count [e]	-0.66 (-1.28, -0.27)	0.40 (0.22, 0.54)
Median RNA viral load[e]	4.28 (3.8–4.79)	3.73 (3.23–4.10)
Demographiccharacteristics		
Median age [a]	38 (32–50)	42 (35–48)
Sex		
Female	12 (0.75)	22 (0.88)
Male	4 (0.25)	3 (0.12)

RP rapid progressor; SP slow progressor

[a]median (IQ), years;

[b]median (IQ), cells/mm^3;

[c]median (IQ), $\log_{10}$ copies/mL;

[d]median (IQ), months;

[e]estimated for data pre-HAART.

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estimated as the midpoint between the times of the last documented HIV-seronegative test and the first HIV-seropositive test within a maximum interval of 2 years. RPs were defined as subjects who had two or more CD4+ T-cell measurements <350 cells/mm^3 within 3 years of seroconversion and were recommended to initiate HAART. SPs were defined as subjects with asymptomatic HIV infection ≥9 years after diagnosis, with average CD4+ T-cell measurements of ≥500 cells/mm^3 and plasma viral loads<10,000 copies/mL throughout the years. The median follow-up time before the initiation of HAART was 111 months (93–136.5 months, interquartile range) for SPs and 13.5 months (7.5–16.5 months, interquartile range) for RPs. All longitudinal retrospective data were regularized and normalized for statistical analyses, as described in S1 Text.

Samples collected from the RPs and SPswere also classified according to stage of infection (Fig 1). For SPs: 10 subjects were in the chronic stage (stable CD4+ T-cells >500 cells/mm^3 with <10,000 copies/mL plasma viral load), 7 subjects were in the pre-HAART stage [CD4+ T-cells <500 cells/mm^3 in decline, with 10,000 copies/mL plasma viral load; the subjects were sampled before initiating HAART, but they initiated therapy from 12 to 24 months (median 16 months) after sampling] and 8 subjects were in the under-HAART stage (stable CD4+ T-cells >350 cells/mm^3 and undetectable plasma viral loads; the subjects were sampled while under HAART). For the RPs: 8 subjects were in the pre-HAART stage (CD4+ T-cells <350 cells/mm^3, with 10,000 copies/mL plasma viral load; the subjects were sampled before initiating HAART) and 8 subjects were in the under-HAART stage (stable CD4+ T-cells >350 cells/mm^3 and undetectable plasma viral loads; the subjects were sampled while under HAART).

In addition, samples from eight HIV-seronegative voluntary donors, with no known metabolic disorders or other medical conditions at the time of blood collection, were used as controls. The HIV-seronegative samples were matched on the basis of sex and ethnic origin.

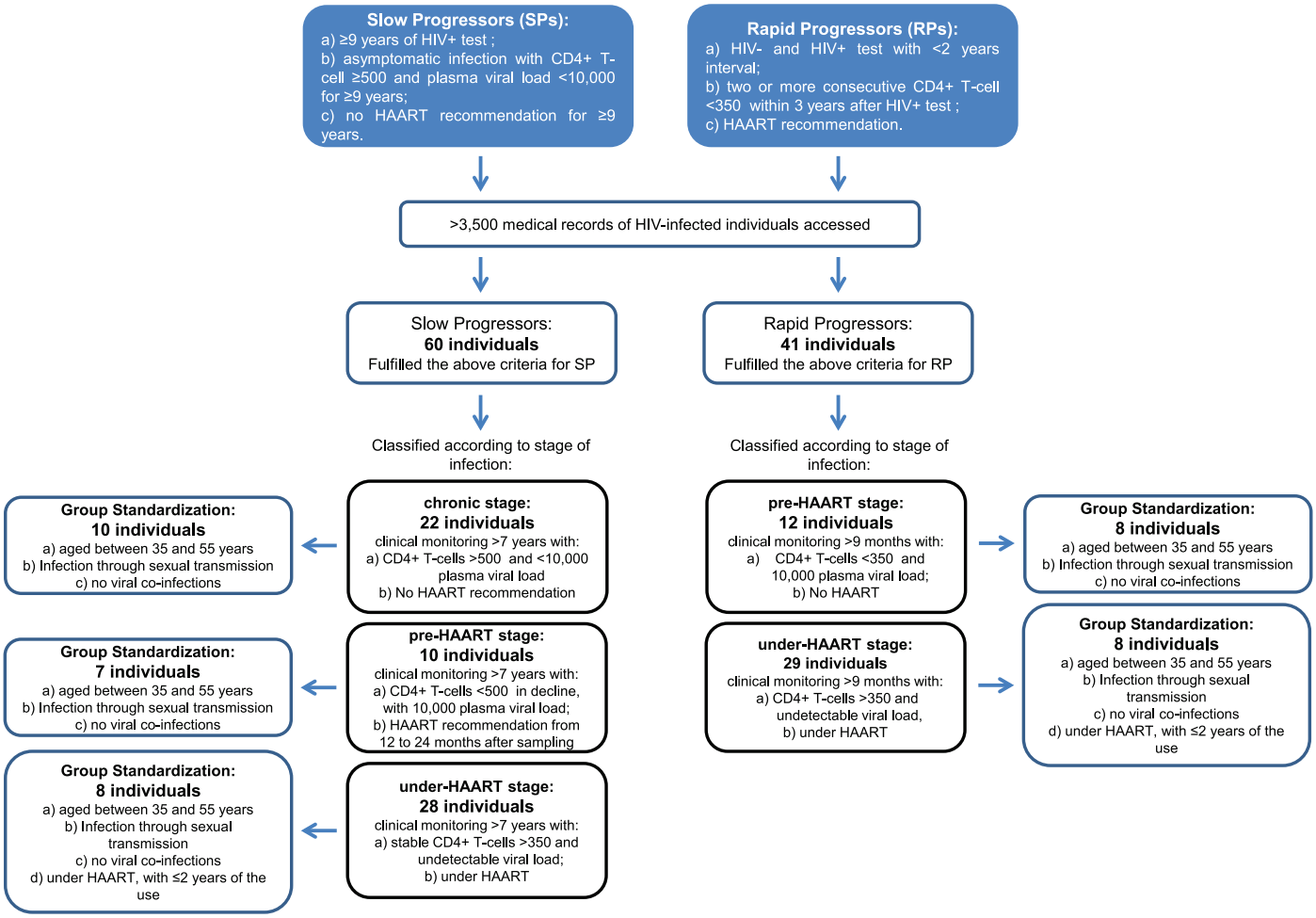

Fig 1. Flowchart of the sampling procedure.

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Th1/Th2/Th17 cytokine profile

Approximately 8 mL blood was collected from each participant after they signed a consent form. The blood was centrifuged, and the plasma was stored at −80°C. Cytokine analysis was performed using the Human Th1/Th2/Th17 Cytometric Bead Array kit (CBA; BD Biosciences, San Jose, CA, USA; Catalog No. 560484), which allowed the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A. Aliquots of plasma were diluted with assay diluent (1:2 v/v), and CBA analysis was performed as per the manufacturer's instructions. Two hundred microliters of each sample was plated on PRO-BIND™ 96-well assay plates and analyzed on the FACS Array Bioanalyzer using FCAP FCS Filter and FCAP Array Software (BD Biosciences). Using these software packages, the debris were filtered from the data, and identification of the bead populations and their mean fluorescence intensities (MFIs) was performed.

Statistical analysis

Global comparisons of the circulating cytokine levels (adjusted by log transformation) were performed for 49 selected individuals using the Kruskal–Wallis test. Two-by-two group comparisons were performed on the basis of Mann–Whitney U tests, and correlations between

cytokine levels and clinical data were calculated using Spearman coefficients. The false discovery rate (FDR) procedure described by Benjamini and Hochberg (1995) was used to account for multiple comparisons.

Results

Description of study participants

Clinical data from infection, specifically the CD4+ T-cell counts and plasma viral loads, were consistent with those previously described for RPs and SPs (Table 1). RPs showed higher plasma viral loads and lower CD4+T-cell counts ($p < 0.001$) than SPs at the first time point. Furthermore, the CD4+ T-cell counts decreased significantly ($p < 0.001$) faster for RPs (−0.66 CD4+ T-cell slope; −1.28, −0.27 IQ) than for SPs (0.40 CD4+ T-cell slope; 0.22, 0.54 IQ). SPs and RPs did not differ significantly in age, sex or ethnicity. No relationships between the exposure category and progression groups were observed, and chronic viral co-infections, such as HCV, HBV and HTLV, diagnosed in the first year of follow-up, did not differ between the extreme phenotypes (data not shown).

Cytokine profiles in rapid or slow progressors in different stages of HIV infection

We assessed the circulating IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ levels in RPs and SPs in different stages of HIV infection and in HIV-seronegative control individuals (Fig 2 and Table 2). Global comparisons revealed that the IL-6 and IL-10 levels differed between the stages of HIV infection ($p = 0.03$ and $p = 0.02$, respectively). Two-by-two evaluations were performed within each group classified according to the stages of HIV infection. Among the SPs, the IL-6 levels were higher in the pre-HAART stage (2.59 pg/mL) than in the chronic stage (2.15 pg/mL, $p = 0.001$), and the IL-10 levels were higher in those in the pre-HAART stage (2.18 pg/mL) than in patients receiving HAART (1.55 pg/mL, $p = 0.04$). Moreover, the IL-6 (2.59 pg/mL) and IL-10 (2.18 pg/mL) levels of the SPs in the pre-HAART stage were significantly higher than the IL-6 (2.13 pg/mL, $p = 0.01$) and IL-10 (1.47 pg/mL, $p = 0.02$) levels in HIV-seronegative controls. In addition, the IL-10 levels were significantly higher in pre-HAART RPs(1.74 pg/mL) than in HIV-seronegative controls (1.47 pg/mL, $p = 0.01$). After the application of the FDR procedure, significant differences revealed higher IL-6 levels in chronically infectedSPs than in pre-HAART SPs ($p = 0.03$) and higher IL-10 levels in pre-HAART RPs than in HIV-seronegative controls ($p = 0.01$). No statistically significant differences were observed between the RP and SP groups. The investigation of the correlation between cytokine levels, the slope of change in CD4+ T-cell numbers and the viral load revealed that an increase in IL-6 levels correlated with an increase in the plasma viral load in SPs with>500 CD4+ T-cells (rho = 0.744, $p = 0.014$). With respect to pre-HAART SPs, a positive correlation between the IL-6 and IL-10 levels was observed (rho = 0.750, $p = 0.02$) (Fig 3). Furthermore, in pre-HAART SPs, a correlation between an increase in the TNF-α levels and a decrease in the CD4+ T-cell slope (rho = −0.686, $p = 0.04$) was observed. In pre-HAART RPs, an increase in the TNF-α levelswas correlated with an increase in the viral load (rho = 0.747, $p = 0.033$).

Discussion

Three decades have elapsed since researchers began to understand HIV infection pathways. Considering the disease course, from the exposure to the virus, through the different clinical symptoms, up to AIDS development, it is now possible to identify several outcomes [1,4,5]. However, the identification of the biological factors modulating the immune system and,

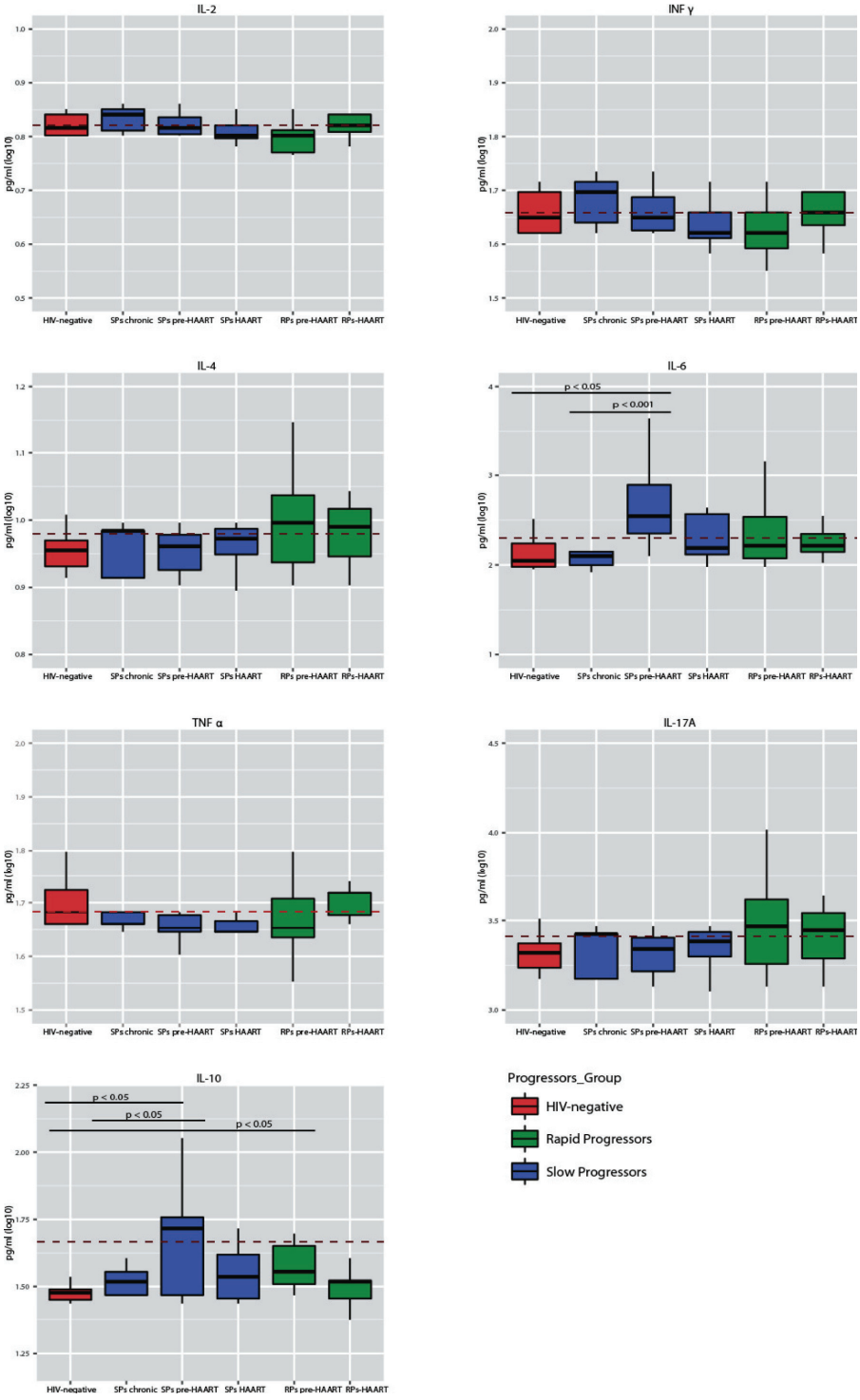

Fig 2. Plasma cytokine levels in rapid and slow progressors subgrouped according to clinical stage. Plasma levels of the cytokines IL-2, IFN-γ, IL-4, IL-6, TNF-α, IL-17A and IL-10 in rapid (green) and slow (blue) progressors grouped by different stages of HIV infection and in HIV-seronegative controls (red). A Kruskal Wallis test showed significant differences between the IL-6 levels for chronically infected*vs* pre-HAART SPs ($p = 0.001$) and pre-HAART SP*vs* HIV-seronegative individuals ($p = 0.01$). In addition, significant differences were observed in the IL-10 levels between pre-HAART SP*vs* SPs receiving HAART ($p = 0.04$); pre-HAART

SPs*vs* HIV-seronegative individuals (p = 0.02); and pre-HAART RPs*vs* HIV-seronegative individuals (p = 0.01). After FDR, significant differences were maintained for the IL-6 levels in chronically infected*vs* pre-HAART SPs (p = 0.03) and the IL-10 levels in pre-HAART RPs*vs* HIV-seronegative individuals (p = 0.01).

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consequently, the immune response that potentially segregates the extreme groups of AIDS progressors (namely, SPs and RPs) remains a challenge.

Our experimental design aimed to quantify the actual Th1/Th2/Th17 cytokine plasma levels in HIV+ individuals. Although at first sight, the sample size seems to be reduced, it was obtained through a methodological approach that ensured the homogeneity of the samples and therefore allowed us to have a high degree of confidence in the results (Fig 1). Stringent sampling was mandatory to avoid the possibility that variable factors masked the effects of the clinical stage of infection. As a result of the stringent inclusion criteria, the sample groups used in this study are homogeneous in size, age, gender, co-infection presence and treatment time. The sample selection method reduced potential bias, focusing on specific groups. In this sense, it was possible to evaluate the cytokine profiles of individuals with extreme rates of progression (i.e., slow and rapid progressors) in well-characterized clinical stages (i.e., in a chronic period and immediately before AIDS development).

Despite the extreme difference in the duration of symptom-free HIV infection, the SP and RP groups presented increased levels of IL-6 and IL-10 during the pre-HAART stage, suggesting a similar pattern in the immunological response in both groups. While the SP subjects in the chronic infection stage maintained relatively constant IL-6 and IL-10 levels, the SP pre-HAART group (composed of subjects who had CD4+ T-cell counts >500 cells/mm^3 at the first collection and then underwent a decrease in CD4+ T-cell counts to <350 cells/mm^3 within 24 months from sampling) revealed a significant increase in IL-6 levels over time. The same trend was observed for the IL-10 levels, although, probably due to a higher variance, the differences did not reach statistical significance. In addition, the IL-6 and IL-10 levels were higher in pre-HAART SPs than in HIV-seronegative controls.

An increase in IL-6 levels has already been associated with the development of opportunistic diseases and mortality in HIV infection [17]. Recently, Williams et al. (2013) showed a significant increase in the plasma IL-6 levels in HIV-seropositive subjects (average of 323.46 CD4 + T-cells/mm^3) compared with HIV-seronegative subjects, demonstrating the important role of IL-6 in the course of the infection [18]. Along the same lines, different studies reported that the transfection of a human astrocyte cell line with a plasmid encoding any of several different HIV proteins (gp120, Nef, Tat or Vpr) resulted in the increased expression of IL-6 through the activation of transcription factors such as NF-κB[19–22]. Therefore, it could be suggested that the increased availability of HIV particles in the pre-HAART stage is involved in the observed increased in the IL-6 level. With respect to IL-10, increased levels of this cytokine are observed with disease progression in chronically HIV-infected subjects, and decreased levels are observed with HAART initiation [23]. Additionally, Roberts et al. (2010) showed that significantly elevated IL-10 levels during acute HIV infection were directly associated with the acute infection viral loads and a high risk of CD4+ T-cell loss [15]. Taken together with our observations, these results suggest a strong association of high IL-6 and IL-10 levels with progression through the stages of HIV infection to AIDS regardless of the patients' progression group.

In addition, our study evaluated the relationship among the classical markers of AIDS progression, i.e., CD4+ T-cell count, median viral load and cytokine levels, and revealed a correlation between increased viral load and increased IL-6 levels (rho = 0.75, p = 0.02). Moreover, in pre-HAART SPs, the IL-6 and IL-10 levels were positively correlated (rho = 0.75, p = 0.01) (Fig 3). The pro-inflammatory role of IL-6 (mononuclear cell recruitment, inhibition of T-cell

Table 2. IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A plasma levels in HIV-seronegative control and HIV-seropositive individuals grouped by different clinical stages of HIV infection.

Group and clinic stage (n)	Female sex	Median age	CD4+ T-cell sampling	VL log sampling	IL2		IL4		IL6		IL10		TNF		INF		IL17	
HIV-seronegative (8)	6	35	—	—	0.82	(0.02)	0.95	(0.03)	**2.13**[c]	(0.19)	**1.47**[d,e]	(0.03)	1.70	(0.05)	1.66	(0.04)	3.32	(0.12)
SPs chronic (10)	9	40	802 (560–1047)	3.49 (2.0–3.9)	0.83	(0.03)	0.96	(0.04)	**2.15**[a]	(0.28)	1.51	(0.05)	1.67	(0.07)	1.68	(0.05)	3.34	(0.13)
SPs pre-HAART (7)	6	41	496 (375–630)	4.25 (3.6–4.5)	0.85	(0.10)	1.01	(0.16)	**2.59**[a,c]	(0.53)	**2.18**[b,d]	(1.12)	1.72	(1.12)	1.71	(0.20)	3.53	(0.59)
SPs HAART (8)	7	42	601 (370–936)	1.69	0.81	(0.04)	0.96	(0.03)	2.30	(0.27)	**1.55**[b]	(0.11)	1.65	(0.05)	1.65	(0.07)	3.35	(0.12)
RPs pre-HAART (8)	6	36	316 (197–387)	4.09 (3.8–4.5)	0.79	(0.05)	1.00	(0.08)	2.35	(0.39)	**1.74**[e]	(0.37)	1.67	(0.08)	1.60	(0.09)	3.48	(0.28)
RPs HAART (8)	6	35	730 (512–858)	1.69	0.82	(0.02)	0.99	(0.07)	2.26	(0.17)	1.50	(0.07)	1.69	(0.05)	1.66	(0.04)	3.46	(0.26)

Mean (Std. Error).

Kruskal Wallis test:

[a] SPs chronic vs SPs pre-HAART (p = 0.001);

[b] SPs pre-HAART vs SPs under-HAART (p = 0.04);

[c] SPs pre-HAART vs HIV-seronegative (p = 0.01);

[d] SPs pre-HAART vs HIV-seronegative (p = 0.02);

[e] RPs pre-HAART vs HIV-seronegative (p = 0.01).

After FDR, significant differences were IL-6 levels in SP chronic vs SP pre-HAART (p = 0.03) and IL-10 levels in RP pre-HAART vs HIV-seronegative (p = 0.01).

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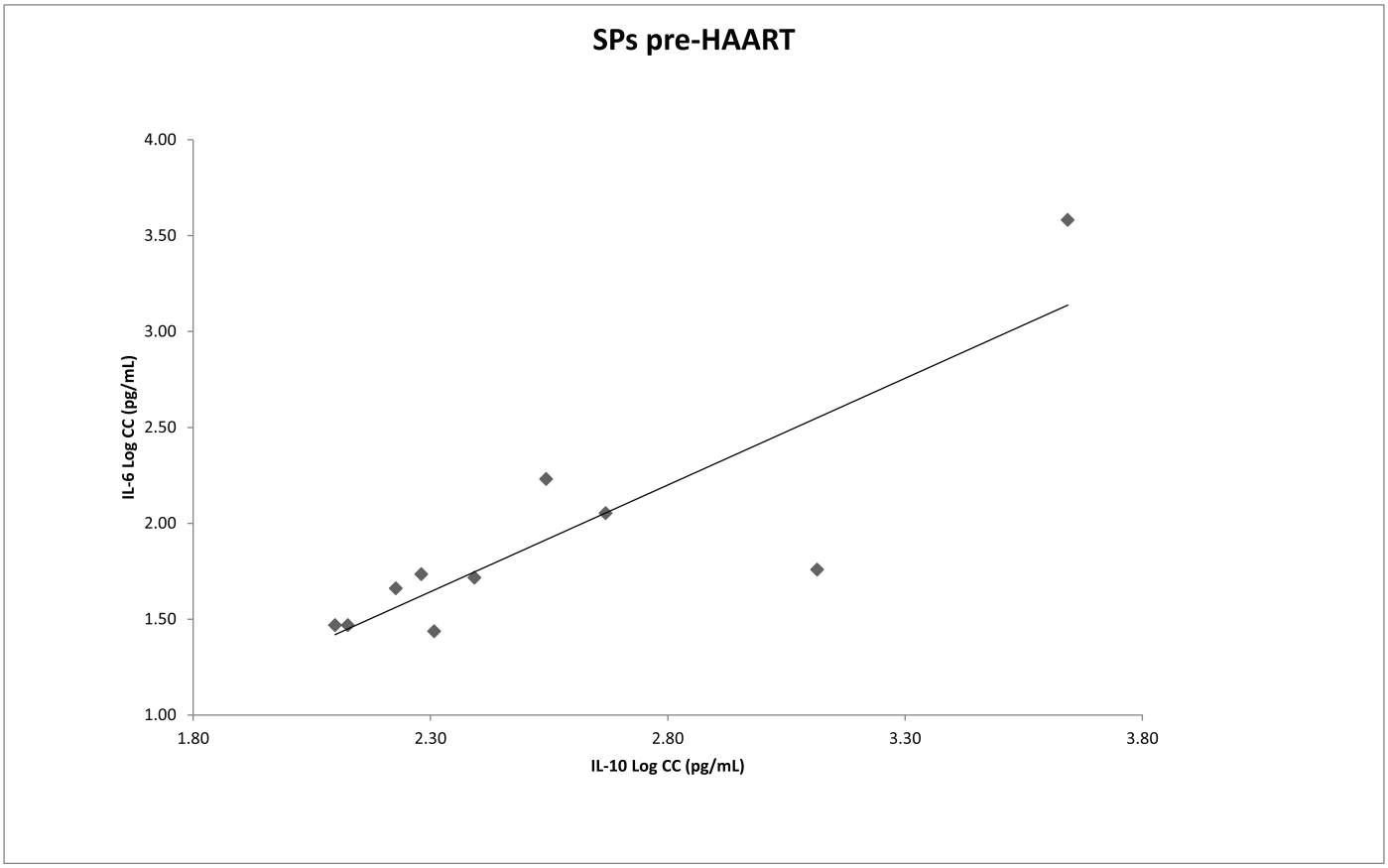

Fig 3. IL-6 and IL-10 plasma levels in slow progressors in a pre-HAART stage. A significant Spearman correlation, rho = 0.750, was observed between theIL-6 and IL-10 plasma levels in pre-HAART SPs (p = 0.02).

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apoptosis, inhibition of T_{reg} cell differentiation and activation of Th17 cell differentiation) has been widely discussed (for a review, see Rose-John, 2012) [24]. Conversely, it has been observed that the expression of IL-10, an anti-inflammatory cytokine, is induced at a later stage of acute HIV infection, after the first burst of pro-inflammatory cytokines [23,25,26]. Liovat et al. (2012) observed that rapid progressors (defined by the accelerated loss of CD4+ T-cells in a follow-up 42 months post-seroconversion) had higher levels of cytokines during acute HIV infection than typical or slow progressors [16]. These authors also suggested that IL-10 is induced as a consequence of the strong inflammatory response, in an attempt to inhibit the exacerbation of immune responses. Therefore, we may speculate that the viral load and IL-6 levels concomitantly increase in the SP group, leading to an increase in the IL-10 levels. However, IL-10 plays a potent anti-inflammatory role, and therefore, it could inadvertently promote viral persistence through the inactivation of effective immune responses. Although it is not possible to conclusively identify increased levels of IL-6 and IL-10 as the direct causes or consequences of progression to AIDS, our results strongly suggest that modifications in the cytokine profile could be used as markers of a global inflammatory state and, consequently, of the disease course, even in HIV infections with extreme phenotype progression.

In the RP group, we observed lower levels of IL-2 and IFN-γ and higher levels of IL-4, IL-6 and IL-17A compared to the SPs or the HIV-seronegative controls, although these differences were not statistically significant. These observations suggest that there is a predominant Th2

(IL-4, IL-6) and Th17 (IL-17A) response during early/acute HIV infection in RPs. To this point, both T cell subsets (Th2 and Th17) were described as susceptible/permissive to HIV replication and are reduced in chronic stages of HIV infection [13,27–29]. Some authors have suggested that Th17 cells have a dual impact on HIV infection. In the acute phase of infection, Th17 cells could promote cell migration to the gut and favor viral replication. Several reports have discussed the Th1-to-Th2 shift in AIDS, and it can be suggested that an increased Th17 response and a possible favoring of HIV replication in the early stages of infection in RPs would accelerate disease progression to AIDS [10,30,31].

In conclusion, slow and rapid progressors show elevated IL-6 and IL-10 levels in the pre-HAART stage. Furthermore, although the relationship between IL-6 and IL-10 is unclear in HIV+ subjects, these cytokines appear to be intrinsically linked to infection progression and AIDS onset. Thus, they could be of great value in the clinical follow-up of HIV+ individuals. On the basis of our findings, we suggest that IL-6 and IL-10 measurement should be incorporated into the clinical management of HIV-infected subjects as a valuable tool in both the decision of HAART initiation and the surveillance of immune activation and inflammation. Finally, the predominant Th2 and Th17 profiles in early HIV infection observed in rapid progressors should be better investigated because if they are corroborated by other studies, this phenotype will define an immune profile in early stages of infection, which will be crucial for decision-making in therapy.

Supporting Information

S1 Text. Regularization and normalization of the longitudinal retrospective clinical data.
(DOC)

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Author Contributions

Conceived and designed the experiments: RM SEA JAC. Performed the experiments: RM JVV DMJ TG JDL MGM PV. Analyzed the data: RM JVV DMJ PV SEA JAC. Contributed reagents/materials/analysis tools: JDL MGM PV SEA JAC. Wrote the paper: RM PV SEA JAC.

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Capítulo 4

4.1 Variações genéticas nos genes que codificam as citocinas IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, no gene do TLR9 e suas relações com a susceptibilidade e/ou a progressão à aids.

Gene-gene interaction among *TLR9* and cytokines polymorphisms in HIV susceptibility and disease progression to AIDS

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Gene-gene interactions among TLR9 and cytokine polymorphisms in HIV susceptibility and disease progression to AIDS

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Abstract

TLR9 and cytokines are candidate molecules to be involved in HIV infection susceptibility and disease progression. In this study, we investigated the relationship between SNPs in *TLR9* [–1237T/C, rs5743836 and +1635G/A, rs352140] and in cytokines genes [*IL2* (-330G/T, rs2069762), *IL4* (-589C/T, rs2243250), *IL6* (-174C/G, rs1800795), *IL10* (-592C/A, rs1800872 and -1082G/A, rs1800896), *IL17A* (-692C/T, rs8193036), *TNFα* (-238G/A, rs361525 and -308 G/A rs1800629) both in susceptibility to HIV-infection and progression to AIDS in a Brazilian population stratified by African and European ancestry. In addition to univariate statistical analysis, interaction analyses were performed by Multifactor dimensionality reduction (MDR). *IL10* -592AA+AC genotypes (OR1.89, CI 95% 1.10-3.14, p=0.009) and *IL17A* -692TC+CC genotypes (OR1.61, CI 95% 0.99-2.71, p=0.037) were related with susceptibility in European-descent individuals. Also, when taken together, *IL4, IL6, IL10 and TLR9* SNPs showed significant additive effects ($p<0.01$). Besides, in African-descent, it was observed a protective effect of *TLR9* -1237TC+CC genotypes (OR0.47, CI 95% 0.23-0.96, p=0.038), and a significant epistatic effect of *TLR9* and *IL4* ($p<0.01$). These results contribute to our understanding of the mechanisms involved in HIV infection immune response, highlighting the influence of the ethnic background. Moreover, genetic interaction analysis help to more precisely define SNP effects and can be very useful in the identification of gene networks that influence susceptibility and/or different clinical courses.

Keywords: HIV susceptibility; AIDS progression; ethnicity; *TLR9; IL10; IL17A.*

1. Introduction

It is well established that there is a considerable variability among individuals concerning their susceptibility to human immunodeficiency virus (HIV) infection and the subsequent disease progression (Casado et al., 2010; Telenti, 2005). Some genetic contributions have been addressed through studies on naturally HIV-resistant individuals, exposed uninfected (EU) subjects and rapid progressors (RPs) or slow progressors (SPs) to AIDS (Olson et al., 2014; Poropatich and Sullivan, 2011). However, GWAS studies have failed to reproduce several SNP associations with susceptibility to HIV infection (Fellay, 2009; Fellay et al., 2007). Recently, McLaren *et. al.* (2013) in a case-control study evaluated more than 6000 individuals, and CCR5del32 was the only polymorphic variant associated to protection against HIV infection. The authors suggested that genetic influences on HIV acquisition are either rare or have effects too small to be detected (Mclaren et al., 2013). Besides, other issues, such as epistatic interactions, should be also considered (Thornton-wells et al., 2004). According to this point of view, gene-gene interaction analysis can contribute to the understanding of the genetic diversity in susceptibility to infection and disease progression (Niel et al., 2015).

Early events during acute HIV infection may determine the pathogenesis of infection, since the immune environment of the initial match antigen seems critical in dictating the long-term equilibrium between host and pathogen (Biasin et al., 2010; Guha and Ayyavoo, 2013). This initial period, is critical to target cells availability, to the seeding of latent reservoirs, and to the initiation and expansion of anti-viral host immune responses (Biasin et al., 2010; Levy, 2001). Genes associated with innate and adaptive immune responses are candidates to be involved in susceptibility and

differential rates of disease progression. Among these genes we should consider those encoding Toll-like receptors (TLRs) and cytokines.

TLRs are innate immune receptors that recognize pathogen associated molecular patterns (PAMPs), such as carbohydrates and nucleic acids from viruses and bacteria. After recognition, TLR triggers the activation of an intracellular signal cascade that induces transcription of inflammatory cytokines, type 1 interferons and chemokines (Blasius and Beutler, 2010; Kawai and Akira, 2009). TLR9 is responsible for the viral DNA recognition inside of infected cells. Some studies reported associations between single-nucleotide polymorphisms (SNPs) in *TLR9* and progression to AIDS. For instance, SNPs in *TLR9*, –1237T/C (rs5743836) and +1635G/A (rs352140), have been linked to viral load, CD4+ T-cell count, and disease progression in HIV-infected individuals (Bochud et al., 2007; Pine et al., 2009; Soriano-Sarabia et al., 2008). Cytokines are key factors involved in antiviral immunological response and recruitment of viral cellular targets (Catalfamo et al., 2012). HIV infection involves disruption of cytokine production and harms responsiveness, particularly related to the T-cell repertoire (Sharma et al., 2011; Vicenzi et al., 1997). Genetic studies have indicated that SNPs in cytokine genes play important roles in HIV infection susceptibility and AIDS progression. In particular, the *IL2* -330G (rs2069762), *IL6* -174C (rs1800795), *IL10* -592A (rs1800872) variants have been reported to be associated with susceptibility, symptom severity and rapid progression to AIDS, while *IL4* -589T (rs2243250), *IL10* -1082G (rs1800896) and *TNFα* -308A (rs361525) have been associated with viral load control and slow disease progression (Foster et al., 2000; Mahajan et al., 2010; Nasi et al., 2013; Shin et al., 2000; Vasilescu et al., 2003; Wichukchinda et al., 2006). Thus, it is possible that these genes, and hence SNPs that functionally modify them, act together altering the susceptibility to HIV infection and/or the clinical outcome.

Moreover, the frequency of immunological response gene variants also fluctuate among populations, probably due to adaptation to local/environmental selective factors (Báfica et al., 2004; Sobieszczyk et al., 2011). The study of populations from different geographic regions and with different ethnic ancestries is important if one wish to understand how the genetic background influences susceptibility to HIV infection and AIDS progression.

Thus, the present study investigates the influence of polymorphisms in *TLR9* and *IL2*, *IL4*, *IL6*, *IL10*, *IL17A*, *TNFα* cytokine genes, concerning to susceptibility to HIV-infection and progression to AIDS in a Brazilian population with European or African ancestry. Also, we investigate the effects of gene-gene interactions in HIV susceptibility and progression to AIDS.

2. Materials and Methods

2.1 Design and setting

The Infectious Disease Service at Nossa Senhora da Conceição Hospital, Porto Alegre city, handles the integral care of almost 5,000 HIV-infected subjects in southernmost Brazil. After the retrospective analysis of about 3,500 medical records, 211 HIV-positive subjects regularly attending this service between 2011 and 2013 were selected to this study. The inclusion criteria were: a) Possibility to estimate the seroconversion by HIV negative test or b) have 5 or more years of regular clinical follow-up after positive diagnosis without HARRT recommendation. For the control group we studied a sample of healthy subjects recruited from voluntary blood donors, whose ethnicity was comparable to the HIV-infected group. Table 1 shows demographic characteristics of the individuals classified according to the study group and clinical features.

This study received the ethical approval of the Nossa Senhora da Conceição Hospital Ethical Committee (Project Number 10-123). All individuals who agreed to participate in the study were at least 18 years old and signed an informed consent form.

2.2 Study population

To estimate the HIV infection disease progression profile, longitudinal clinical and laboratory data were used, including CD4+ T-cell counts, plasma viral loads, clinical stage data at the time of sample collection and highly active antiretroviral therapy (HAART) prescriptions. Following these data, HIV-infected subjects were retrospectively classified into two groups: rapid (RP) and non-rapid (Non-RP) progressors. For RPs, the time of HIV seroconversion was estimated as the midpoint time between the last documented HIV-seronegative test and the first HIV-seropositive test within a maximum interval of 2 years. RPs were defined as subjects who had two or more CD4+ T-cell measurements < 350 cells/mm^3 within 3 years of seroconversion with HAART recommendation. Non-RPs were defined as subjects with asymptomatic HIV infection ≥ 5 years after diagnosis in the absence of any antiretroviral treatment. In addition, within the Non-RPs group a sub-group of elite controllers (EC) was characterized. EC were asymptomatic, with HIV infection of over 8 years duration, in the absence of any antiretroviral treatment and that had undetectable viral load in plasma throughout the years.

Individuals were classified as of European or African ancestry according to the individual phenotypic characteristics self-assertion and as judged by the researcher at the time of data collection, allied to information concerning the ethnicity of parent/grandparents as reported by the participants. The issue concerning the skin-colour-based classification criteria used in southernmost of the Brazil is well

documented and has already been assessed in previous studies of our group (da Silva et al., 2011; dos Santos et al., 2012). According to Pena *et al* (2011), the Brazilian southernmost region has low admixture levels as compared to the rest of the country, probably due to its geographical extent and to cultural and historical differences (Pena et al., 2011).

2.3 SNP selection

The SNPs in innate immune response related genes evaluated in the present work were selected among gene/polymorphisms previously described as involved in susceptibility or outcome of HIV infection (Foster et al., 2000; Mahajan et al., 2010; Nasi et al., 2013; Shin et al., 2000; Vasilescu et al., 2003; Wichukchinda et al., 2006). As criteria for inclusion it was considered: (a) description of the SNP potential relevance for differences on the rate of gene expression and (b) frequency of the rare allele > 5% in European and African-descent populations available in the Entrez SNP Database.

2.4 Genotyping of human genetic polymorphisms

Blood samples from the 516 subjects enrolled were collected through venous puncture, and their genomic DNA was isolated using a salting-out method (Lahiri and Nurnberger, 1991). Eight SNPs in cytokines genes [*IL2* (-330G/T, rs2069762), *IL4* (-589C/T, rs2243250), *IL6* (-174C/G, rs1800795), *IL10* (-592C/A, rs1800872 and -1082G/A, rs1800896), *IL17A* (-692C/T, rs8193036) and *TNFα* (-238G/A, rs361525 and -308 rs1800629)] were genotyped by a minisequencing technique according to the protocol described by Medeiros *et al.,* personal communication (2016). The +1635G/A polymorphism in *TLR9* (rs352140) was amplified according to the protocol described by Cheng et al. (2007). Genotyping analyses were performed using the restriction

endonuclease BstUI, and visualization on a 3% agarose gel (Cheng et al., 2007). The–1237T/C polymorphism in *TLR9* (rs5743836) was genotyped by allele-specific PCR, as described by Carvalho, and was visualized on a 2% agarose gel (Carvalho et al., 2007). The CCR5del32 (rs333) variant was evaluated by conventional PCR and was visualized on a 3% agarose gel (Chies et al., 2003).

2.5 Statistical analysis

Demographic and clinical characteristics were compared among the different groups with a $\chi 2$ or a Fisher's exact test for categorical variables and Mann–Whitney or Kruskal-Wallis non-parametric tests for continuous variables, $p < 0.05$ was considered significant. Allele and genotype frequencies of each polymorphism were determined by direct counting and Hardy-Weinberg equilibrium was tested by $\chi 2$ test. Previous studies, including some of our group, describe significant differences in *TLR9* and cytokines SNPs frequencies among populations with different ancestries, an therefore, the analyses were stratified by ethnicity.

For the genetic comparisons we assumed genetic models based on predicted phenotypic effects due to the presence or absence of the investigated SNPs (Foster et al., 2000; Mahajan et al., 2010; Nasi et al., 2013; Shin et al., 2000; Vasilescu et al., 2003; Wichukchinda et al., 2006), except concerning the *IL17A* -692C/T variant for which three genetic models were tested and an allelic dominant effect was assumed (data no shown). The different genotypic profiles group were evaluated with χ^2 and Fisher's exact tests. Statistical analyses were performed using SPSS for Windows 18.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA).

2.6 Analysis of gene–gene interactions

The gene-gene interactions in HIV infection risk or rapid AIDS progression were evaluated with the Multifactor dimensionality reduction (MDR) method (Moore et al., 2006). The MDR analyses were stratified by ethnicity. Although it leads to a reduction of the sample size, this procedure allows a better understanding of the relationships between SNPs in different ancestry contexts. MDR was developed as a non-parametric data mining and machine learning strategy for the identification of combinations of discrete genetic traits predictive of clinical features. In MDR analysis, cross-validation (CV) consistency and balanced accuracy estimates were calculated for each predictive model (Moore et al., 2006). The model with the highest accuracy and maximal CV is considered to be the best one. In addition, the MDR software determines the best predictive model statistical significance by comparing the accuracy of the observed data with the distribution of accuracy under the null hypothesis of no association, derived empirically from 1000 replicates of permutations.

3. Results

3.1 Description of study participants

HIV-seropositive and control groups did not differ significantly in age and ethnicity, but differed in sex proportion. This difference reflects sex ratios found in the sampling sites. The Nossa Senhora da Conceição Hospital attends a higher proportion of women compared to men and the Blood Donation Center has a greater number of male blood donors. Clinical follow-up of infection, specifically CD4+ T-cell counts and plasma viral loads were consistent with those previously described for RPs and Non-RPs (Table 1). RPs and Non-RPs did not differ significantly in age, sex or ethnicity. No relationship between the exposure category and progression was observed. Chronic viral

co-infections such as HCV, HBV and HTLV, diagnosed in the first year of follow-up, did not differ between groups. The EC group was exclusively composed by European-descent individuals.

3.2 SNP associations with HIV susceptibility

The genotypic profile of the eleven polymorphisms evaluated in HIV-seronegative (HIV-) and HIV-seropositive (HIV+) patients grouped by ethnicity are presented in Table 2 (for the genotypic frequencies see Appendix Table A1). All the SNPs were in HW equilibrium. In comparative analysis between HIV+ and HIV- Brazilian European-descent an increased risk to HIV infection was observed for the *IL10* -592 AA+AC (OR 1.89, CI 95% 1.10-3.14, p-value = 0.009) and *IL17A* -692 TC+CC genotypes (OR 1.61, CI 95% 0.99-2.71, p-value = 0.037). Regarding to HIV+ and HIV- Brazilian African-descent individuals, a protective effect of the *TLR9* -1237 TC+CC genotypes (OR 0.47, CI 95% 0.23-0.96, p-value = 0.038) was observed.

3.3 Gene-gene interactions in susceptibility

Table 3 shows the best models of interactions for 1 to 5 SNPs by MDR analysis. For Brazilian European-descents, a four-locus model including *IL10*_rs1800872, *IL4*_rs2243250, *TLR9*_rs352140 and *IL6*_rs1800795 showed a maximum test balanced accuracy (65.2%) and a maximum CV consistency (9/10), indicating that this was the best model (p-value 0.001-0.002) able to explain HIV+ and HIV- groups. For Brazilian African-descent the best model (p-value = 0.002-0.003) included *IL4*_rs2243250 and *TLR9*_rs5743836 SNPs with the maximum test balanced accuracy (70.5%) and a maximum CV consistency (7/10) of the models. Concerning the entropy algorithm evaluation, the relationships between SNPs in the Brazilian European-descent (*IL10*_rs1800872, *IL4*_rs2243250, *TLR9*_rs352140 and *IL6*_rs1800795) were

predominant synergistic (Figure 1A and 1B), such as additive or correlation effects. As for the African-descent individuals the relationship observed between the SNPs (*IL4*_rs2243250 and *TLR9*_rs5743836) were predominant epistatic (Figure 1A and 1B). Combined effects of the *IL4*_rs2243250 (4.28%) and *TLR9*_rs5743836 (9.48%) beget increased 4.55% in HIV-infection predictive model power. Also, in African-descent group was possible to suppose that genotype combinations that lead to a disbalance between IL-4 and TLR9 levels increase the risk of infection (Appendix Fig A1).

3.4 SNP associations with AIDS progression

Table 4 summarizes SNPs comparisons between RP and Non-RP patients grouped according to their ethnic origin. In RP and Non-RP Brazilian African-descent comparative analyses, the frequency of the *IL17A* -692 TC+CC genotypes were significantly increased in RP (p-value = 0.037). In addition, EC showed an increased frequency of the *TNFα* -238 GA+AA genotypes compared to RPs (p-value = 0.014) and compared to the remaining Non-RP individuals (p=0.037) (data no showed).

3.5 Gene-gene interactions in AIDS progression

Table 5 shows the results from MDR for disease progression. When gene-gene interactions were analysed in RPs and Non-RPs groups with Brazilian European-descent, *IL2_rs2069762 , TLR9_rs5743836, TLR9_rs352140* was the best model with maximum test balanced accuracy (66.1%) and a maximum CV consistency (10/10) for predict rapid progression to AIDS, but this result does not reach significance (p-value 0.089-0.090). For Brazilian African-descent, although not statistically significant, a predominant effect of one SNP as the best predictor of rapid progression to AIDS was observed. *IL17A*_rs8193036 had a maximum test balanced accuracy (69.3%) and a maximum CV consistency (10/10) in rapid AIDS prediction analyses (p-value 0.197-

0.199). From entropy algorithm evaluation, we the relationship between SNPs in the European-descent (*IL2_rs2069762, TLR9_rs5743836, TLR9_rs352140*) and African-descent was predominantly epistatic (data not show).

4. Discussion

In a complex disease, such as the HIV-infection, several loci contribute to distinct outcomes. In this sense, several studies focused the host genetic background and how it would influence HIV infection susceptibility and the subsequent progression to AIDS (Fellay, 2009; Fellay et al., 2007; Mclaren et al., 2013). It is important to identify gene–gene (epistatic) interactions, since they may predict disease risk more accurately than single genes (Nagel, 2005; Thornton-wells et al., 2004). In the present study, we evaluated selected SNPs in *TLR9* and *IL2, IL4, IL6, IL10, IL17A* and *TNFα* cytokine genes, that had been previously associated with susceptibility and outcome of HIV infection in individuals with either European or African ancestry (Foster et al., 2000; Mahajan et al., 2010; Nasi et al., 2013; Shin et al., 2000; Vasilescu et al., 2003; Wichukchinda et al., 2006). Of note, the molecules coded by those genes are highly related to signalling pathways involved in early activation of antiviral immune responses, and therefore represent potential therapeutic targets against HIV infection. Here, MDR analysis was used to predict potential gene–gene interactions that could better explain the complex phenotypes observed in HIV infection.

Concerning the Brazilian European-descent individuals, we observed a significant association of *IL10* -592CA+AA *(*rs1800872) and *IL17A* -692TC+TT (rs8193036) genotypes with an increased HIV infection risk. The *IL10* -592C/A SNP is related to low IL-10 plasma levels, being this a potent anti-inflammatory cytokine,

which plays a key role in regulating the immune response (Sabat et al., 2010; Mahajan et al., 2010). Regarding *IL10* -592CA+AA, our analysis corroborates previous studies performed in South African and North American cohorts, in which the *IL10* -592AA genotype was associated with an increased risk of HIV-infection (Naicker et al., 2009; Shin et al., 2000). Naicker *et al.* (2012) in a more comprehensive study evaluated *IL10* SNPs and IL-10 levels in the context of HIV-infection. These authors hypothesize that high levels of IL-10 could protect against HIV infection possibly by reducing immune activation and counteracting the inflammatory processes that increase the pool of cells susceptible to viral infection (Naicker et al., 2012).

Although this is the first study evaluating *IL17A* -692C/T (rs8193036) on HIV susceptibility, IL-17A is a major player in the regulation of a Th17 immune response, and the potential role of this cytokine in HIV susceptibility has been approached by a few groups (Ancuta et al., 2009). In general, a Th17 immune response was associated with increased immune activation and highly HIV-permissive target cells (Gosselin et al., 2010). Chege *et al.* (2012) demonstrated that IL-17A production was substantially reduced in the genital tract and in the blood obtained from HIV exposed uninfected compared to not exposed individuals. Since the Th17 cell subset is normally enriched at mucosal sites, these authors hypothesized that a reduced Th17 response could be related to a less favourable environment to infection (Chege et al., 2012). Thus, further functional investigation about the *IL17A* -692C/T variant in the context of HIV susceptibility looks promising.

In Brazilian African-descent individuals *TLR9* -1237TC+CC (rs5743836) genotypes presented a protective role in HIV infection. The involvement of TLR9 in the recognition of HIV-1 RNA or DNA leading to NF-kappaB activation and cytokines, chemokines and IFNs production indicates that these markers play a central role in

initiating host immune responses against HIV. Although some studies have described a relationship between the presence of the *TLR9* -1237C/T variant and infection outcomes, few works evaluated its relationship with HIV infection susceptibility (Bochud et al., 2007; Pine et al., 2009; Soriano-Sarabia et al., 2008). Recently, a study of our group (Valverde-Villegas et al., personal communication, 2016) observed a significant association of *TLR9* -1237TC+CC (rs5743836) with protection to HIV-1 infection in another cohort of Brazilian African-descents. In that study, it was concluded that *TLR9* -1237T/C polymorphism has a significant role in HIV-1 infection depending on the ethnic background.

Multifactor dimensionality reduction (MDR) is a data reduction method developed specifically for genotypic data that has been successful employed to the identification of gene–gene interactions in both simulated and actual data (Hahn and Moore, 2004; Ritchie, 2001). The MDR algorithm has enough power to detect epistasis and carries out knowledge discovery in a very specific manner using an exhaustive search and a single classifier to identify the optimal combination of polymorphisms for predicting a discrete disease endpoint (Hahn and Moore, 2004; Ritchie et al, 2003). In Brazilian African-descent group our results show a significant predictive effect for the *IL4*_rs2243250 and *TLR9*_rs5743836 combination in relation to HIV-infection susceptibility. Entropy analyses revealed a strong epistatic effect between these two loci assuming complex relationships between genes. In addition, a significant synergistic relationship among the SNPs *IL10*_rs1800872, *IL4*_rs2243250, *TLR9*_rs352140, *IL6*_rs1800795 in Brazilian European-descent were observed, suggesting that these SNPs can be involved in susceptibility to HIV infection in this ethnic group. Although these results are relevant to understand the combined effect of the SNPs, it is difficult to infer the biological effects of the interactions and their exact influence in susceptibility

to infection (Niel et al., 2015). However, the findings in African and European-descents are consistent with the recently identified links between IL-4 and TLRs in the immune response against HIV-infection. Sriram *et al.* (2014) showed that IL-4 modulates and counteracts the pro-inflammatory stimulation induced by TLR7 and TLR9, and it may negatively affect responses against viruses and intracellular parasites (Sriram et al., 2014). In addition, *TLR9*_rs5743836 and *IL10*_rs1800872 show a protective effect in African and European descent groups on univariate analyses, highlighting coherence between the statistical methods. Further studies are required to determine whether these polymorphisms can account for this epidemiological finding.

In what concerns the progression of HIV infection, the 40 RPs HIV-infected subjects enrolled in this study had significantly low CD4+ T cell counts at diagnosis, and a much more marked slope, representing a significantly decrease in CD4+ T cell numbers throughout the follow-up, when compared to Non-RP. Olson *et al*. (2014), evaluating RPs in the CASCADE cohort (Concerted Action on SeroConversion on AIDS and Death in Europe), associated a low CD4+ T cell count in the first year of infection with a high death risk (Olson et al., 2014). In our study, the plasmatic viral load of RP subjects, in median 4.26 $\log_{10}$ (±0.69, Standard Deviation), was significantly higher as compared to those of the Non-RP subjects, throughout the follow-up periods. This data points out to the inability of RP to recovery lost CD4+ T cells and to control viral replication in the first stage of infection, characterizing a severe acute infection and, consequently, the need of starting an antiretroviral treatment immediately after the positive diagnosis. The *IL17A* TC+CC (rs8193036) genotypes were associated with rapid progression in African-descent individuals by univariate analyses. Also MDR analyses showed a single effect of the *IL17A* rs8193036 on rapid progression in this ethnic group. These results suggest an important effect of the *IL17A* rs8193036 SNP in

HIV infection outcomes. In addition, other studies reported that the number of Th17 cells in slow progressors is higher than in typical progressors suggesting that persistence of Th17 cells can drive a slower progression of the infection (Ciccone et al., 2011; Salgado et al., 2011).

In the present study distinct genetic polymorphisms were associated to HIV infection outcomes in a way strongly affected by the ethnic/genetic background of the subjects. Although it can be argued that this work enrolled a small number of individuals, investigations that include populations of different ancestries and geographic regions are important to understand how the genetic background can influence the immune response and modulate HIV infection progression. Moreover, such results contribute to our understanding of the mechanisms involved in HIV infection immune response, since SNPs in *IL10* and *IL17A* were related to susceptibility in Brazilian European-descent, whereas *IL10* and *IL6, IL4* ,*TLR9* SNPs showed additive effects when considered together. Regarding Brazilian African-descents, besides previous associations of *TLR9* with HIV infection susceptibility, an epistatic effect was detected involving *TLR9* and *IL4* genes. In conclusion, genetic interaction analyses can help to more precisely define SNP effects and can be very useful in the identification of gene networks that may influence susceptibility and/or differential clinical courses. New researches addressing a largest number of SNPs and genes in distinct ethnic populations are mandatory to evaluate these associations. With the identification of the genes that influence clinical course of the infection, it will be possible to implement new epidemic surveillance and prognostic strategies.

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535 **Tables**

536

537 **Table 1 -** General demographic characteristics of the individuals according to the study group
538 and clinical classification.

		HIV- (n=305)	HIV+ (n=211)	RPs (n=40)	Non-RP(162)	ECs (n=9)
Age*		44 ±7	41 ±10	40 ±11	41 ±10	38 ±10
Sex	**Female**	**78 (0.30)**	**148 (0.72)[a]**	26 (0.65)	114 (0.70)	7 (0.77)
	Male	**222 (0.70)**	**63 (0.28)**	14 (0.35)	48 (0.30)	2 (0.23)
Ethnicity	**Euro**	176 (0.58)	125 (0.62)	27 (0.68)	102 (0.63)	9 (1.0)
	Afro	128 (0.42)	73 (0.38)	13 (0.32)	60 (0.37)	0 (0.0)
Plasma HIV-1 RNA viral load **		---	3.99±0.79	**4.26 ±0.69**	**3.80 ±0.72[b]**	<1.59
Slope of CD4+ T-cell count **		---	0.326±0.99	**-1.00 ±1.53**	**0.376 ±0.21[b]**	0.436±0.16
HAART recommendation		---	157 (0.74)	40 (1.0)	117 (0.72)	0 (0.0)
Time between documented infection and the onset of HAART **		---	8.0±4.0	**1.6 ± 0.8**	**9.9±3.2[b]**	10 ±2.5

* mean ± standard deviation; ** median ± standard deviation. RPs = rapid progressors (classified by negative serology test), Non-RPs = non-rapid progressors, EC = elite controllers. [a] Significant difference by χ^2 test (p-value<0.001); [b] Significant difference (p-value<0.001) by Mann-Whitney nonparametric test.

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541 **Table 2** - The genotypic profile of the eleven SNPs evaluated in HIV-seronegative and HIV-seropositive patients, grouped by ethnicity.

Gene	dbSNP ID	SNP	Model	Genotype	Europeandescent, n %				*p*-value	OR	95% C.I		Africandescent, n %				*p*-value	OR	95% C.I	
					HIV+ (n125)		HIV - (n176)						HIV+(n73)		HIV - (n128)					
CCR5	**rs333**	**del32**	co-dominant	**Wt/Wt**	97	0.87	142	0.87	0.520				60	0.91	108	0.93	0.155			
				del32	15	0.13	21	0.13					6	0.09	8	0.07				
IL2	**rs2069762**	**-330G/T**	dominant	**TT**	58	0.53	89	0.53	0.533				35	0.55	44	0.51	0.397			
				TG+GG	52	0.47	79	0.47					29	0.45	42	0.49				
IL4	**rs2243250**	**-589C/T**	recessive	**CC+CT**	104	0.93	166	0.94	0.396				52	0.79	65	0.76	0.395			
				TT	8	0.07	10	0.06					14	0.21	21	0.24				
IL6	**rs1800795**	**-174C/G**	recessive	**GG+GC**	103	0.95	153	0.89	0.061				62	0.98	83	0.98	0.611			
				CC	5	0.05	18	0.11					1	0.02	2	0.02				
IL10	**rs1800872**	**-592C/A**	dominant	**CC**	38	0.34	86	0.49	**0.009**	**Reference**			24	0.36	32	0.37	0.526	Reference		
				CA+AA	73	0.66	89	0.51		**1.89**	**1.10**	**3.14**	42	0.64	54	0.63		1.01	0.79	1.29
	rs1800896	**-1082G/A**	dominant	**AA**	14	0.13	24	0.14	0.464				3	0.05	11	0.13	0.069			
				AG+GG	98	0.88	152	0.86					63	0.95	75	0.87				
IL17A	**rs8193036**	**-692C/T**	recessive	**TT**	58	0.52	102	0.63	**0.037**	**Reference**			33	0.50	33	0.40	0.154	Reference		
				TC+CC	54	0.48	59	0.37		**1.61**	**0.99**	**2.71**	33	0.50	49	0.60		0.84	0.62	1.13
TNFα	**rs361525**	**-238G/A**	dominant	**GG**	95	0.86	149	0.85	0.459				59	0.89	74	0.87	0.430			
				GA+AA	15	0.14	26	0.15					7	0.11	11	0.13				
	rs1800629	**-308G/A**	dominant	**GG**	86	0.79	125	0.71	0.090				55	0.83	65	0.76	0.168			
				GA+AA	23	0.21	51	0.29					11	0.17	21	0.24				
TLR9	**rs5743836**	**–1237T/C**	dominant	**TT**	75	0.68	122	0.70	0.400	Reference			37	0.60	28	0.41	**0.038**	**Reference**		
				TC+CC	36	0.32	53	0.30		1.07	0.75	1.52	25	0.40	40	0.59		**0.47**	**0.23**	**0.96**
	rs352140	**+1635G/A**	dominant	**AA**	37	0.36	50	0.29	0.138				10	0.17	8	0.11	0.250			
				AG+GG	67	0.64	125	0.71					50	0.83	64	0.89				

542

543 **Table 3** Best models of interaction to predict HIV infection susceptibility by MDR analysis.

Number of factors considered	Best Model[a]	Testing balanced accuracy[b]	Cross-validation consistency[c]	*P*-value
Brazilian europeandescent				
1	IL6_rs1800795	0.505	06/10	0.889-0.890
2	IL4_rs2243250, TLR9_rs352140	0.541	04/10	0.578-0.579
3	IL10_rs1800872, IL4_rs2243250, TLR9_rs352140	0.534	04/10	0.652-0.653
4	**IL10_rs1800872, IL4_rs2243250, TLR9_rs352140, IL6_rs1800795**	**0.652**	**09/10**	**0.001-0.002**
5	IL10_rs1800872, IL4_rs2243250, TLR9_rs352140, IL6_rs1800795, IL2_rs2069762	0.599	07/10	0.088-0.089
Brazilian africandescent				
1	TLR9_rs5743836	0.574	9/10	0.332-0.333
2	**IL4_rs2243250, TLR9_rs5743836**	**0.705**	**7/10**	**0.002-0.003**
3	IL2_rs2069762, TLR9_rs5743836, TLR9_rs352140	0.702	5/10	0.002-0.003
4	IL10_rs1800896, IL17A_rs8193036, IL4_rs2243250, TLR9_rs352140	0.579	4/10	0.300-0.301
5	IL10_rs1800872, IL2_rs2069762, IL4_rs2243250, TLR9_rs5743836, TLR9_rs352140	0.602	4/10	0.165-0.166

[a] The best combination of attributes for each order model. [b] Ratio of correct classifications to the total number of instances classified within the training or testing set. This excludes instances that could not be classified. [c] Number of times in a particular cross-validated run that a given attribute combination was selected as the best model.

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545 **Table 4** - Genotypic profile of the eleven SNPs evaluated in rapid and non-rapid progressors grouped by ethnicity.

Gene	dbSNP ID	SNP	Model	Genotype	Eurodescent, n %				*p*-value	Afrodescent, n %				*p*-value
					RPs (n26)		Non-RPs (n89)			RPs (n14)		Non-RPs (n53)		
CCR5	**rs333**	**del32**	co-dominant	**Wt/Wt**	22	91.70	73	84.90	0.315	11	91.70	47	87.00	0.551
				Wt/del32	2	8.30	13	15.10		1	8.30	7	13.00	
IL2	**rs2069762**	**-330G/T**	dominant	**TT**	12	46.20	45	52.30	0.390	6	46.20	29	56.90	0.345
				TG+GG	14	53.80	41	47.70		7	53.80	22	43.10	
IL4	**rs2243250**	**-589C/T**	recessive	**CC+CT**	25	96.20	82	92.10	0.417	11	84.60	41	77.40	0.415
				TT	1	3.80	7	7.90		2	15.40	12	22.60	
IL6	**rs1800795**	**-174C/G**	recessive	**GG+GC**	24	1.00	81	94.20	0.287	13	1.00	49	98.00	0.787
				CC	0	0.00	5	5.80		0	0.00	1	2.00	
IL10	**rs1800872**	**-592C/A**	dominant	**CC**	10	40.9	30	33.70	0.298	4	30.80	20	37.70	0.517
				CA+AA	15	59.1	59	63.30		9	69.20	33	62.20	
	rs1800896	**-1082G/A**	dominant	**AA**	3	13.00	12	13.50	0.602	0	0.00	3	5.70	0.500
				AG+GG	20	87.00	77	86.50		13	1.00	50	94.30	
IL17A	**rs8193036**	**-692C/T**	recessive	**TT**	14	53.00	47	52.80	0.378	**3**	**23.10**	**30**	**56.60**	**0.030**
				TC+CC	12	47.00	42	47.20		**10**	**76.90**	**23**	**43.40**	
TNFα	**rs361525**	**-238G/A**	dominant	**GG**	24	96.00	76	86.40	0.165	11	84.60	48	90.60	0.437
				GA+AA	1	4.00	12	13.60		2	15.40	5	9.40	
	rs1800629	**-308G/A**	dominant	**GG**	20	77.00	67	80.00	0.587	10	76.90	45	84.90	0.327
				GA+AA	5	23.00	20	20.00		3	23.10	8	15.10	
TLR9	**rs5743836**	**–1237T/C**	dominant	**TT**	15	71.40	55	65.50	0.405	10	83.30	27	54.00	0.059
				TC+CC	6	28.60	29	34.50		2	16.70	23	46.00	
	rs352140	**+1635G/A**	dominant	**AA**	9	45.00	27	33.80	0.232	2	16.70	9	17.70	0.409
				AG+GG	11	55.00	53	66.20		10	83.30	42	82.30	

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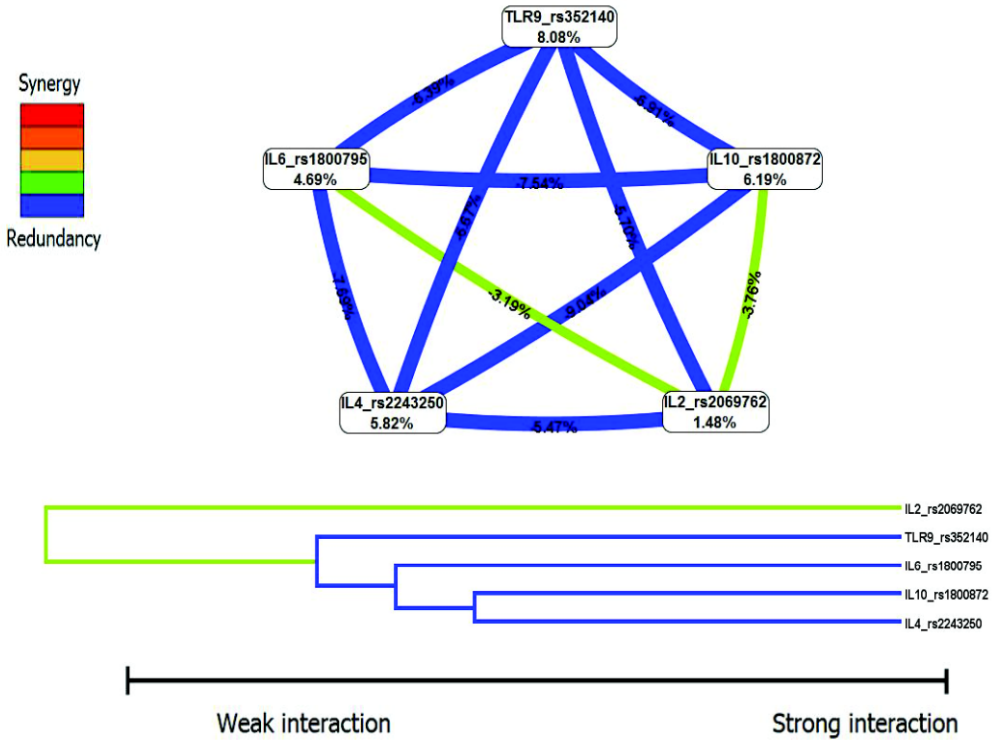
547 **Table 5** - Best interaction models to predict disease progression to AIDS by MDR analysis.

Number of factors considered	Best Model[a]	Testing balanced accuracy[b]	Cross-validation consistency[c]	*P*-value
Brazilian europeandescent				
1	IL4_rs2243250	0.4439	05/10	0.991-0.992
2	IL2_rs2069762, TLR9_rs5743836	0.5719	09/10	0.544-0.545
3	**IL2_rs2069762 , TLR9_rs5743836, TLR9_rs352140**	**0.6607**	**10/10**	**0.089-0.090**
4	IL10_rs1800896, IL2_rs2069762, TLR9_rs5743836, TLR9_rs352140	0.5784	08/10	0.510-0.511
5	IL17A_rs8193036, IL2_rs2069762, IL4_rs2243250, TLR9_rs5743836, TLR9_rs352140	0.5082	05/10	0.867-0.868
Brazilian africandescent				
1	**IL17A_rs8193036**	**0,6929**	**10/10**	**0.197-0.199**
2	IL17A_rs8193036, TLR9_rs5743836	0,5609	4/10	0.709-0.710
3	IL10_rs1800872, IL4_rs2243250, TLR9_rs352140	0,4474	4/10	0.969-0.970
4	IL10_rs1800896, IL2_rs2069762, IL4_rs2243250, TLR9_rs352140	0,5494	3/10	0.750-0.751
5	CCR5del32, IL10_rs1800872, IL10_rs1800896, IL17A_rs8193036, TLR9_rs352140	0,5462	3/10	0.756-0.757

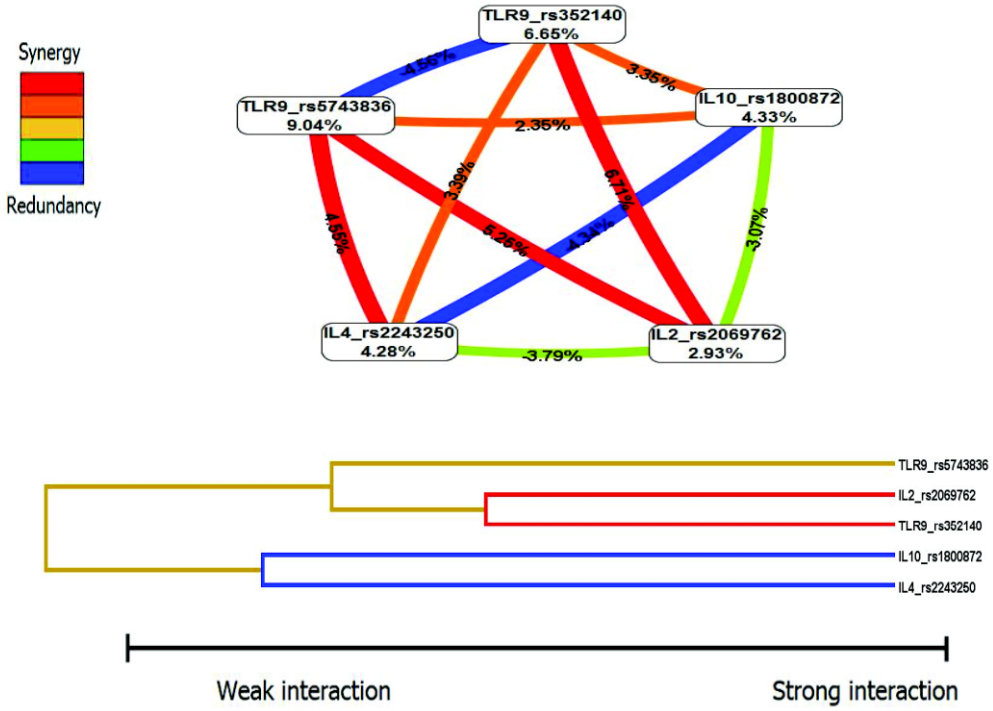
[a] The best combination of attributes for each order model. [b] Ratio of correct classifications to the total number of instances classified within the training or testing set. This excludes instances that could not be classified. [c] Number of times in a particular cross-validated run that a given attribute combination was selected as the best model.

Figure 1

A


Synergy
Redundancy
TLR9_rs352140
8.08%
IL6_rs1800795
4.69%
IL10_rs1800872
6.19%
IL4_rs2243250
5.82%
IL2_rs2069762
1.48%
-6.39%
-6.91%
-7.54%
-4.81%
-5.76%
-7.05%
-3.19%
-9.04%
-3.76%
-5.47%
IL2_rs2069762
TLR9_rs352140
IL6_rs1800795
IL10_rs1800872
IL4_rs2243250
Weak interaction
Strong interaction

B


Synergy
Redundancy
TLR9_rs352140
6.65%
TLR9_rs5743836
9.04%
IL10_rs1800872
4.33%
IL4_rs2243250
4.28%
IL2_rs2069762
2.93%
-4.55%
-3.35%
-2.35%
-3.39%
-6.71%
-4.55%
-5.25%
-4.14%
-3.07%
-3.79%
TLR9_rs5743836
IL2_rs2069762
TLR9_rs352140
IL10_rs1800872
IL4_rs2243250
Weak interaction
Strong interaction

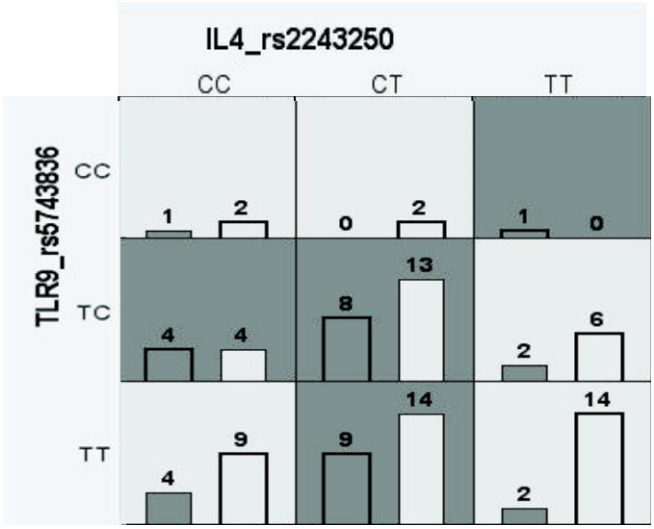
Legend

Fig. 1 - Analysis of SNP interactions in susceptibility to HIV infection stratified by ethnicity. SNPs interaction network and dendogram graphs based in algorithm entropy are presented. Values in nodes represent information gain (IG) of individual SNPs (main effect) while values between nodes are the IG of each pairwise combination (interaction effects). A positive information gain indicates a synergistic or non-additive effect while a negative value indicates redundancy between them. Dendogram graphic showing the level of interaction between attributes. Figure 1A, for Brazilian European-descent, showed the great percentages of the redundancy entropy interaction between the loci analyzed. This indicated a predominant additive effect between *IL10*_rs1800872, *IL4*_rs2243250, *TLR9*_rs352140 and *IL6*_rs1800795 SNPs that were included in best model generated by permutation. Figure 1B, for Brazilian African-descent, showed a great percentages of the interactions between the loci analyzed. In this group, the results shown epistatic effect (4.55%) between *TLR4*_rs2243250 (4.28%) and *TLR9*_rs5743836 (9.04%) SNPs.

Table A1 - Genotypic frequencies of the eleven polymorphisms evaluated in HIV-seronegative (HIV-) and HIV-seropositive (HIV+) individuals grouped by ethnicity.

Gene	dbSNP ID	Genotype	Euro-descent, n %				Afro-descent, n %			
			HIV+ (n125)		HIV- (n176)		HIV+ (n73)		HIV- (n128)	
IL10	rs1800872	CC	38	0.34	24	0.36	86	0.49	32	0.37
		CA	56	0.50	35	0.53	72	0.41	42	0.49
		AA	17	0.15	7	0.11	17	0.10	12	0.14
	rs1800896	GG	46	0.41	68	0.39	29	0.44	41	0.48
		GA	52	0.46	84	0.48	34	0.52	34	0.40
		AA	14	0.13	24	0.14	3	0.05	11	0.13
IL17A	rs8193036	TT	58	0.52	102	0.63	33	0.50	33	0.40
		TC	50	0.45	54	0.34	29	0.44	44	0.54
		CC	4	0.04	5	0.03	4	0.06	5	0.06
IL2	rs2069762	TT	58	0.53	89	0.53	35	0.55	44	0.51
		TG	44	0.40	63	0.38	24	0.38	36	0.42
		GG	8	0.07	16	0.10	5	0.08	6	0.07
IL4	rs2243250	CC	62	0.55	105	0.60	21	0.32	24	0.28
		CT	42	0.38	61	0.35	31	0.47	41	0.48
		TT	8	0.07	10	0.06	14	0.21	21	0.24
IL6	rs1800795	GG	65	0.60	78	0.46	41	0.65	66	0.78
		GC	38	0.35	75	0.44	21	0.33	17	0.20
		CC	5	0.05	18	0.11	1	0.02	2	0.02
TNF	rs361525	GG	95	0.86	149	0.85	59	0.89	74	0.87
		GA	15	0.14	25	0.14	7	0.11	11	0.13
		AA	0	0.00	1	0.01	0	0.00	0	0.00
	rs1800629	GG	86	0.79	125	0.71	55	0.83	65	0.76
		GA	20	0.18	47	0.27	11	0.17	20	0.23
		AA	3	0.03	4	0.02	0	0.00	1	0.01
TLR9	rs5743836	TT	75	0.68	122	0.70	37	0.60	28	0.41
		TC	31	0.28	48	0.27	20	0.32	35	0.51
		CC	5	0.05	5	0.03	5	0.08	5	0.07
	rs352140	AA	37	0.36	50	0.29	10	0.17	8	0.11
		AG	43	0.41	85	0.49	33	0.55	34	0.47
		GG	24	0.23	40	0.23	17	0.28	30	0.42

Figure A1



Legend Figure A1

Fig. A1 - Interaction analysis shows details of epistasis between *TLR9*_rs5743836 and *IL4*_rs2243250 in HIV susceptibility. The genotype combinations IL4_CC+CT + TLR9_CC, IL4_TT + TLR9_TT+TC and IL4_CC + TLR9_TT shows protective effect to infection (grey). Genotype combinations *IL4*_TT + *TLR9*_CC; *IL4*_CC+CT + *TLR9*_TC and *IL4*_CT + *TLR9*_CT increase risk to HIV infection (dark grey).

4.2 Descrição do método desenvolvido e validado para a genotipagem simultânea de 9 polimorfismos nos genes *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-17A*, *TNF-α* utilizados para este estudo.

Development and validation of a method of immunogenic profiling through the evaluation of 23 SNPs of cytokine and chemokine receptor genes using a minisequencing approach

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Genotyping of 23 SNPs of cytokine and chemokine receptor genes trough minisequencing technique: design, validation and standardisation

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Running title: Minisequencing for genotyping 23 immunological SNPS

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Abstract

The minisequencing technique offers accuracy and robustness to genotyping of polymorphic DNA variants, being an excellent option for the identification and analyses of prognostic/susceptibility markers in human diseases. Two multiplex minisequencing assays were designed and standardized to screen 23 candidate SNPs in cytokine, chemokine receptor and ligand genes previously associated with susceptibility to cancer and autoimmune disorders as well as to infectious diseases outcome. The SNPs were displayed in two separate panels (panel 1 - *IL2* rs2069762, *TNFα* rs1800629, rs361525; *IL4* rs2243250; *IL6* rs1800795; *IL10* rs1800896, rs1800872; *IL17A* rs8193036, rs2275913; and panel 2 - *CCR3 rs309125, CCR4* rs6770096, rs2228428; *CCR6* rs968334; *CCR8* rs2853699; *CXCR3* rs34334103, rs2280964*;CXCR6* rs223435, rs2234358; *CCL20* rs13034664, rs6749704; *CCL22* rs4359426; *CXCL10/IP-10* rs3921, rs56061981). A total of 415 DNA samples from healthy individuals were genotyped by minisequencing. To validate the minisequencing technique, and in order to encompass the majority of the potential genotypes for all 23 SNPs, 20 of these samples were genotyped by Sanger sequencing. The results of both techniques were 100% in agreement. The technique of minisequencing showed high accuracy and robustness, avoiding the need for high quantities of DNA template samples. It was easily to be conducted in bulk samples derived from a highly admixed human population, being therefore an excellent option for immunogenetic studies.

Key words: SNPs, cytokine, chemokine, minisequencing, susceptibility markers.

Introduction

The immune system acts in a complex network, involving molecular pathways and cellular components dispersed throughout the body. Health and disease outcome depend on a delicate balance between inhibition and activation, where deficient or uncontrolled responses may result in tissue damage. This complexity requires a fine tuned communication network and cytokines are major signalling molecules in the immune system [1]. Among the main cytokine subfamilies, interleukins are importantly involved in lymphocyte signalling. Specific interleukins play defined roles in pro- and anti-inflammatory responses [2]. Also important to the homeostasis of the immune system, chemokines, small proteins belonging to the chemotactic cytokines subfamily, have pivotal roles for homing and trafficking of immune cell subsets into inflammatory sites [3].

The importance of genetic polymorphisms in immune-related genes, such as those coding for cytokines and chemokines, as well as chemokine receptor genes, to cancer, autoimmune and infectious diseases susceptibility is an important research topic and has already been approached in different studies (see Table 1 for a comprehensive, although not exhaustive, review). Thereby, methodologies for rapid and accurately genotype candidate single nucleotide polymorphisms (SNPs) are avidly sought to investigate and identify prognostic/diagnostic markers in immunological diseases [4]. Of special interest, are methodologies capable to analyse sets of genes involved in specific biochemical pathways or genetic networks. Such approach allows interesting gene-gene interaction studies but, although different methodologies are presently available for genotyping, no technologies for scoring SNPs has become a widely accepted standard [5].

The minisequencing method is based in the use of a probe that targets a sequence immediately upstream to the SNP and extends a single base by incorporation of a fluorescently labelled dideoxy nucleotide. The reactions are runned by capillary electrophoresis and genotypes are determined by the identification of peak position and specific fluorescence emission. Minisequencing analyses give accurate and robust results and require relatively little time in comparison to other genotyping methods [6]. Here we designed and standardized two multiplex assays panels for minisequencing genotyping of 23 SNPs in immune-related genes. The SNPs were displayed in two separate panels: panel 1 focuses on cytokine genes (*IL2* rs2069762; *TNFα* rs1800629, rs361525; *IL4* rs2243250; *IL6* rs1800795; *IL10* rs1800896, rs1800872; *IL17A* rs8193036, rs2275913) and panel 2 focuses on chemokine receptor/ligands genes (*CCR3 rs3091250; CCR4* rs6770096, rs2228428; *CCR6* rs968334; *CCR8* rs2853699; *CXCR3* rs34334103, rs2280964*; CXCR6* rs2234355, rs2234358; *CCL20* rs13034664, rs6749704; *CCL22* rs4359426, *CXCL10/IP-10* rs3921, rs56061981). Both panels were validated through the genotyping of a cohort of 415 healthy individuals. Of note, all evaluated SNPs were selected among candidate genes/polymorphisms previously described as involved in cancer, autoimmunity and/or infectious disease susceptibility and outcome, therefore composing a valuable tool to the evaluation of important potential immunogenetic targets.

Material and Methods

SNPs selection

The SNPs in the immune related genes were selected, as previously stated, among genes/polymorphisms previously described as involved in susceptibility or outcome of autoimmune diseases, cancer, allergy and/or infectious diseases (Table 1). In addition, as criteria for inclusion into the panels it was considered: (a) potential

relevance of the SNP for differences on the rate of gene expression and (b) frequency of the rare allele > 5% in euro and afro-descendent populations available in Entrez SNP Database (www.ncbi.nlm.nih.gov/sites/entrezdb=snp).

Studied population

Samples were obtained of the DNA biorepository from Immunogenetics laboratory of the Federal University of Rio Grande do Sul, Brazil. The group was composed of 415 healthy individuals older than 18 years from Porto Alegre city, 221 (54%) men and 191 (46%) women. Individuals were European or African ancestry according to the individual phenotypic characteristics self-assertion: 310 (74.7%) were European descent and 105 (25.3%) were African descent.

Multiplex PCR-primers and minisequencing probes design

The 23 SNPs selected were combined in two panels. Panel 1 included the SNPs in interleukin and tumor necrosis factor alpha (TNF-α) genes whilst panel 2 included those for chemokines and their receptors (Table 2). Multiplex PCR-primers were designed for each panel using the Primer 3.0 program v.0.2 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) considering two main criteria: a) closely melting temperature among primers; b) all amplicons should range from 150 to 450 bp in order to facilitate visualization of the multiplex reactions simultaneously to minimize the risk of preferential amplification due to reagent competition.

Minisequencing probes for SNP detection were designed with the 3' end base corresponding to the last base before the SNP-position (Table 3). For panel 2, the probes were designed using BatchPrimer3 v1.0 software online adding poly (dCT) 5' tails (http://probes.pw.usda.gov/batchprimer3/). Self- and hetero-dimers annealing between pairs of primers was evaluated using the Multiple Primer Analyzer software tool from Thermo Fisher Scientific (https://www.thermofisher.com/br/en/home/brands/thermo-

scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). This tool was applied for both primer sets (multiplex PCR and minisequencing probes). For minisequencing oligoultramer probes, the recommended scale of synthesis is of 4nmol. All primers were synthetized with purification standards and checked by mass spectrometry.

Multiplex PCR

The panel 1 and panel 2 multiplex PCR were performed separately in 25µL reaction volume with 20ng genomic DNA. In addition, 0.2mM of each primer was used according to the of Qiagen Multiplex PCR Master Mix Kit manufacturer's instructions. All reactions were performed in a Veriti™ 96-Well Thermal Cyclers (Applied Biosystems) with the following settings: pre-denaturation 95°C for 5 min followed by 35 cycles of 30s at 94°C, 90s at 57°C, 90s at 72°C, and a final extension cycle of 10 min at 72°C. Multiplex PCR products were checked in 3% agarose gel.

In order to remove unincorporated primers and dNTPs, 2 µl of Affymetrix™ ExoSAP-IT™ was added in 5µl multiplex PCRs product (for both panels) and reactions were incubated at 37°C for 30 min. The ExoSAP-IT enzyme was inactivated by incubation at 80°C for 30 min.

Minisequencing

Minisequencing reactions were performed in a 10µL final volume using 5µL of SNaPshot™ Kit Reaction Mix, 3µL of the purified multiplex PCR product and 1.5µL of minisequencing probe mix (0.05µM of each primer, Table 3). Reactions were performed in a Veriti™ 96-Well Thermal Cyclers (Applied Biosystems) with the following settings: initialization at 95°C for 5min, followed by 25 cycles of 10s at 96°C, 5s at 50°C and 30s at 60°C.Then, 1.5 µl of Affimetrix SAP® enzyme was added, and the reaction was incubated at 37°C for 30 min to remove the 5’ phosphoryl groups of

the unincorporated fluorescent ddNTPs. SAP® enzyme was inactivated by incubation at 80°C for 30 min.

Capillary electrophoresis

For capillary electrophoresis 1 µL of minisequencing products purified mixed with 8.5µl of HiDi™ formamide and 0.5 µl of GeneScan-120LIZ size standard (ABI - Foster City, USA) was denatured at 95°C for 5 min. Capillary electrophoresis was performed on an ABI 3130xl genetic analyzer (ABI - Foster City, USA) with a 36 cm length capillary and POP-4™ polymer. Data analyses were performed with SNaPshot™ tool in GeneMapper 4.0 software (ABI - Foster City, USA) (see Figure 1A and B for an example).

Sanger Sequencing

To validate the minisequencing results, 20 samples were sequenced for the 23 SNPs by Sanger sequencing. These were selected amongst the previously minisequenced samples, in order to encompass the majority of the potential genotypes for all 23 SNPs evaluated. The amplified fragments were obtained by single PCR using Qiagen Multiplex PCR Master Mix Kit under the same minisequencing conditions described above. The Sanger sequencing reactions were performed using 20-30ng purified single PCR product, primers (both forward and reverse for each SNP in separated reactions) and ABI Big Dye Terminator v.3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. The amplification cycling profile was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) using 35 cycles of 10 sec at 96°C, 10 sec at 54°C; 4 min at 60°C. All reactions were run in an ABI 3130xl genetic analyzer (Applied Biosystems).

Results

Validation of miniseqeuncing approach

Figure 1 shows representative electropherograms obtained from both panels for a control individual. Figure 1A concerns panel 1 (9 different cytokine gene SNPs) and Figure 1B concerns Panel 2 (14 different chemokine receptor or ligand gene SNPs). All SNPs are indicated by their rs nomenclature and genotyping is achieved by reading the peaks according to the size and fluorescent emission defined by each specific probe. As previously stated, Sanger sequencing was performed in 20 samples to encompass the majority of the potential genotypes for all 23 SNPs in order to validate the minisequencing technique. All genotypes obtained by the Sanger sequencing method (considering all the 23 tested SNPs for the 20 individual samples) mirrored the results from the minisequencing (data not show).

Immunogenetic profiling: allelic and genotype frequencies

The Table 4 and 5 summarize allelic and genotype frequencies obtained for the 415 individual considering all 23 evaluated SNPs. In order to compare with a so-called standard human population, allelic and genotype frequencies for the same SNPs were extracted from the HapMap database, considering the 1000 Genomes Project Phase 3 for all populations, and are also presented in Table 4 and 5. As expected taking into account that our control group is an admixed human population, the results as a whole are quite similar.

Discussion

In the development of this experimental strategy to define an immunogenetic profile, several points had to be addressed. The quality and quantity of amplified product obtained after the multiplex PCR is very important for a good performance in minisequencing genotyping. Therefore, the multiplex PCR was standardized using a

commercial master mix kit according to the manufacturer's instructions and all amplicons were obtained with enough quality and quantity for the following steps. Importantly, the multiplex product purification step can significantly affect genotyping accuracy. Failure in removing the unincorporated ddNTPs can yield extraneous fluorescence, and therefore special attention should be given to this step. It is important to mention that it is possible to use the same primary amplicon for the annealing of two different probes in the subsequent minisequencing reaction, consequently genotyping different SNPs on the same gene. This approach was used for the identification of both rs1800629 and rs361525 of the *TNF-α* gene in Panel 1 and for the identification of rs34334103 and rs2280964 of the *CXCR3* gene in Panel 2. However, special attention should be given if these SNPs are very close to each other, due to proximity interference of the fluorescent probes, the signal intensity in the electropherogram could be reduced.

Two different principles were used to design the probes for the different panels used in this minisequencing approach. The panel 1 probes were designed with different lengths and their whole sequence encompassing and pairing to the region adjacent to the target SNP. For panel 2, only ~20 bp of the region adjacent to the SNP was selected to be incorporated in the probe and CT tails with different sizes were added. An increase in the size of the probe results in better efficiency, although simultaneously can favor nonspecifically annealing. The addition of tails can bypass this paradoxical situation. In our panels both principles to design probes worked equally well and we strongly suggest a close investigation of the regions to be genotyped to choose the appropriate criteria to design new probes.

In electropherograms, the electrophoretic migration of the shorter probes assigned by the automated sequencer were slightly different from those expected considering only the size of the synthesized fragments. Quintáns B et al., 2004 also

observed this discrepancy between the expected and the observed distance of migration concluding that the length, sequence, and the dye used to label the extended primer could interfere with its electrophoretic mobility [7]. Thus, this should be taken into consideration in the design of probes, avoiding the use of very small fragments or fragments able to fold in complex structures. Electropherograms were analyzed, it becomes apparent a residual signal accumulation that does not match any SNP at the region around 40 to 45 bp in size (see Figure 1). Based on our experience, we recommend to avoid the use of probes that will fall in this region.

In the present study two separated panels were designed allowing the independent genotyping of two different groups of SNPs. In this sense, researchers can choose the most convenient panel for their specific needs. Also, other SNP targets can be added to enrich the panels (Quintáns et al., 2004; Coutinho et al., 2014; Fanis et al., 2014). Alternatively, the products of the two multiplex panels could be combined and minisequenced in a single reaction. However, in this case, some considerations data would be lost: the allele dyes and bin positions of *CCR8* rs2853699 and *IL10* rs1800872 as well as *CCL20* rs13034664 and *IL17A* rs8193036 are the same. For this reason, it would be necessary or eliminate one marker, or make a new choice of fluorescent dyes.

In order to validate the minisequencing results 20 DNA samples from healthy individuals which included the majority genotype combinations for all 23 SNPs were sequenced by Sanger sequencing. The results were always in agreement with the minisequencing. Regarding immunogenetic profiling from 415 healthly individuals, as expected, fluctuations of allelic frequencies are found in admixed populations, but even this fact, frequencies are quite similar when compared with HapMap database

In conclusion, the technique of minisequencing showed high accuracy and robustness, avoiding the need for high quantities of DNA template samples. It was

238 easily to be conducted in bulk samples derived from a highly admixed human
239 population, being therefore an excellent option for immunogenetic studies.
240 Minisequencing allows the analyses of various SNPs with high specificity at the same
241 reaction conditions. Another advantage of this method is the simultaneous genotyping
242 of several SNPs using small quantities of template DNA (approximately 20ng). Thus,
243 minisequencing is a promising approach for multiplex high-throughput genotyping
244 assays.

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372 **Table 1.** SNPs previously described as involved in susceptibility or outcome of autoimmune diseases, cancer, allergy and/or infectious diseases.

Gene	Chromosome	SNPs	rs	Ancestral allele	MAF	Associations
Panel 1						
IL2	4	-330 G/T	rs2069762	G	0.27 (T)	Cancer, Multiple sclerosis (Wu et al., 2009; Fedetz et al., 2009)
TNFα	6	-308 G/A	rs1800629	G	0.09 (A)	Asthma, Crohn's disease, Liver cancer, Systemic lupus erythematosus
	6	-238 G/A	rs361525	G	0.06 (A)	Psoriasis, Lymphoma (Ferreira et al., 2005; Nasi et al., 2013; Aoki et al., 2006)
IL4	5	-590 C/T	rs2243250	C	0.46 (T)	Type 1 diabetes, Rheumatoid arthritis [15]
IL6	7	-174 C/G	rs1800795	G	0.13 (C)	Kaposi's sarcoma, Hodgkin's lymphoma, Crohn's disease (Foster et al., 2000; Hohaus et al., 2007)
IL10	1	-1082 A/G	rs1800896	A	0.27 (G)	Allergy and asthma exacerbations
	1	-598 C/A	rs1800872	C	0.43 (A)	Lymphoma, Atherosclerosis (Shin et al., 2000; Hunninghake et al., 2008; Kube et al., 2008)
IL17A	6	-737 T/C	rs8193036	T	0.38 (C)	Chronic hepatitis B, Rheumatoid arthritis,
	6	-197 G/A	rs2275913	A	0.30 (A)	Tuberculosis susceptibility, Rheumatoid arthritis, Gastric cancer (Li et al., 2014; Shibata et al., 2009; Shen et al., 2015)
Panel 2						
CCR3	3	-520 G/T	rs3091250	G	0.35 (T)	Kawasaki and macular degeneration diseases (Breunis et al., 2007; Sharma et al., 2013)
CCR4	3	1014 C/T	rs2228428	C	0.15 (T)	Chronic fatigue syndrome [26]
	3	512 C/T	rs6770096	C	0.19 (T)	
CCR6	6	-98+594 A>G	rs968334	G	0.37 (A)	Rheumatic diseases (Kochi et al., 2010; Koumakis et al., 2013)
CCR8	3	80 G/C	rs2853699	G	0.13 (C)	AIDS progression [29]
CXCR6	3	7 G/A	rs2234355	G	0.14 (A)	Long-Term Nonprogression to AIDS

	3	*42 T>G	rs2234358	G	0.45 (T)	HIV infection susceptibility (Duggal et al., 2003; Limou et al., 2010)
CXCR3	X	12+213 G/A	rs34334103	G	0.06 (A)	Systemic lupus erythematosus, Asthma (Cheong et al., 2005; Im et al., 2014)
	X	12+234 C/T	rs2280964	C	0.18 (T)	
CCL20	2	-786 C>T	rs6749704	T	0.23 (C)	Ulcerative colitis, Atopic dermatitis [34]
	2	-1706 C>T	rs13034664	T	0.59 (C)	
IP-10	4	-135 C/T	rs56061981	C	0.11 (T)	Hepatitis B, malaria and tuberculosis infection diseases susceptibility (Tang et al., 2009; Wilson et al., 2013; Sheikh et al., 2015)
	4	+1642 C/G	rs3921	C	0.31 (G)	
CCL22	16	5 A>C	rs4359426	C	0.08 (A)	Atopic dermatitis, Gastric cancer susceptibility (Wang et al., 2009; Hirota et al., 2011)

MAF, minor allele frequency.

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Table 2. Panel 1 and 2 multiplex primer sequences.

Gene	rs	Sequence (5'->3')	Tm	Product size (bp)
Panel 1				
IL2 Fw	rs2069762	CCATTCTGAAACAGGAAACCA	59.96	301pb
IL2 Rev		AAACCCCCAAAGACTGACTG	59.04	
IL4 Fw	rs2243250	ACCCAAACTAGGCCTCACCT	59.99	174pb
IL4 Rev		ACAGGTGGCATCTTGGAAAC	59.97	
IL6 Fw	rs1800795	TCGTGCATGACTTCAGCTTT	59.60	328pb
IL6 Rev		GCCTCAGACATCTCCAGTCC	59.80	
IL10-SNP1 Fw	rs1800896	TTCCCCAGGTAGAGCAACAC	60.11	190pb
IL10-SNP1 Rev		ATGGAGGCTGGATAGGAGGT	59.92	
IL10-SNP2 Fw	rs1800872	GGGGTCATGGTGAGCACTAC	60.29	230pb
IL10-SNP2 Rev		CAAGCAGCCCTTCCATTTTA	60.20	
IL17A-SNP1 Fw	rs2275913	GCCAAGGAATCTGTGAGGAA	60.20	328pb
IL17A-SNP1 Rev		TTCAGGGGTGACACCATTTT	60.21	
IL17A-SNP2 Fw	rs8193036	CCTTCTCTCTTTCCCCCATC	60.01	158pb
IL17A-SNP2 Rev		TGCATGCTACCAAGCAACTT	59.49	
TNFα-SNP1/2 Fw[a]	rs1800629/ rs361525	GCCCCTCCCAGTTCTAGTTC	60.07	244pb
TNFα-SNP1/2 Rev[a]		AAAGTTGGGGACACACAAGC	60.01	
Panel 2				
CCR3 Fw	rs3091250	TGACAGGAGAAATGGACATGG	60.91	282
CCR3 Ver		CTGTCTCTTACGGCATTTTGC	59.90	
CCR4-SNP1 Fw	rs2228428	TGCTGCCTTAATCCCATCAT	60.44	176
CCR4-SNP1 Ver		TCATGATCCATGGTGGACTG	60.34	
CCR4-SNP2 Fw	rs6770096	TCTTGCTTTTGCGGAACAAT	60.75	221
CCR4-SNP2 Ver		GTTTGGAAAGCAGACCTTGG	59.71	
CCR6 Fw	rs968334	TGCATTGCAGCATCAAGAAT	60.37	360
CCR6 Ver		AAATGCAGATCCCACAGACC	59.93	
CCR8 Fw	rs2853699	CAGACCACAAGGACCAGGAT	59.96	198
CCR8 Ver		CGCTGCCTTGATGGATTATAC	59.58	
CXCR3-SNP1/2 Fw[a]	rs34334103/ rs2280964	CTGTGACTGCAGGTTTCCAA	59.87	398
CXCR3-SNP1/2 Rev[a]		AGCACGCCAAGAGTCAAAGT	60.06	
CXCR6-SNP1 Fw	rs2234355	CCCCTAAATGTGGTCAATGG	60.04	240
CXCR6-SNP1 Ver		CCACAGACAAACACCACCAG	60.04	
CXCR6-SNP2 Fw	rs2234358	CCTTACCTTGGGGTCTCACA	59.96	409
CXCR6-SNP2 Ver		TCCCAATCAAGGAGAACCTG	60.04	
CCL20-SNP1 Fw	rs6749704	CTGTTATTTGACATTTGCTGTGCTG	59.0	260
CCL20-SNP1 Rev		CTGTCCGCAGTTAGAGTGGA	59.3	
CCL20-SNP2 Fw	rs13034664	GACATGAGAGAGAGGGAGGAGA	59.8	340
CCL20-SNP2 Ver		AAGGGGATTGGGGAGTGACT	60.9	
CCL22 Fw	rs4359426	AGTGAGGCTTGTGGGTGGA	60.8	130
CCL22 Ver		CCACAGCAAGGAGGACGAG	60.0	
CXCL10-SNP1 Fw	rs56061981	CCCCAACAACTTGTACAGCC	59.05	380

CXCL10-SNP1 Rev	rs3921	TGCAAAAGGAAATGAGAAGGAAATCA	59.65	150
CXCL10-SNP2 Fw		GATGGACCACACAGAGGCTG	60.30	
CXCL10-SNP2 Rev		AACATTAACCTTCCTACAGGAGTAGT	60.30	

[a] The PCR product includes two polymorphisms.
Notes: Primer final concentrations = 0.20µM for 1 Reaction final volume = 25µL.

Table 3. Panel 1 and 2 minisequencing probe sequences.

Gene	Rs	Minisequencing probes	Len (w)	w + t-tail
Panel 1				
IL2	rs2069762	F:TTATTCTTTTCATCTGTTTACTCTTGCTCTTGTC CACCACAATATGCTATTCACATGTTCAGTGTAGT TTTA	72	NA
IL4	rs2243250	F:GATACGACCTGTCCTTCTCAAAACACCTAAACT TGGGAGAACATTGT	47	NA
IL6	rs1800795	F:AAAGAAAGTAAAGGAAGAGTGGTTCTGCTTCT TAGCGCTAGCCTCAATGACGACCTAAGCTGCACT TTTCCCCCTAGTTGTGTCTTGC	88	NA
IL10	rs1800896	F:AAATCCAAGACAACACTACTAAGGCTTCTTTG GGA	35	NA
	rs1800872	F:ATCCTAATGAAATCGGGGTAAAGGAGCCTGGA ACACATCCTGTGACCCCGCCTGT	55	NA
IL17A	rs2275913	F:GCATAGCAGCTCTGCTCAGCTTCTAACAAGTAA GAATGAAAAGAGGACATGGTCTTTAGGAACATG AATTTCTGCCCTTCCCATTTTCCTTCAGAAG	97	NA
	rs8193036	F:CATCACTCTCTACTCCCCCCTGCCCCCCTTTTCT CCATCT	40	NA
TNFα	rs361525	F:AAAAGAAATGGAGGCAATAGGTTTTGAGGGGC ATGGGGACGGGGTTCAGCCTCCAGGGTCCTACA CACAAATCAGTCAGTGGCCCAGAAGACCCCCCT CGGAATC	105	NA
	rs1800629	F:AGGAAACAGACCACAGACCTGGTCCCCAAAAG AAATGGAGGCAATAGGTTTTGAGGGGCATG	62	NA
Panel 2				
CCR3	rs3091250	F:$(CT)^{14}$CTTCAAGGTTCAATTTCCCCATTAACTAT AATGAATG	37	65
CCR4	rs2228428	F:$(CT)^{21}$GCCAATACTGTGGGCTCCTCCAAATTTA	28	70
	rs6770096	F:$(CT)^{4}$cAGCCAGATGTATGAAGAAACAATTAG	26	35
CCR6	rs968334	F:ATGTTAGATCCACCAGCACCCCCC	24	24
CCR8	rs2853699	F:$(CT)^{16}$TGCCATTTGTCTGAATAAGTTCC	23	55
CXCR3	rs2280964	F:AGCCTTCGAGTCTACTTGCCCCCCGCCCCC	20	30
	rs34334103	R:*$(CT)^{14}$cTCTCCTCTCCTCTCTGGCTTC*	21	50
CXCR6	rs2234355	F:$(CT)^{17}$cGTTCATCAGAACAGACACCATGGCA	25	60
	rs2234358	F:$(CT)^{27}$CGAGAAGCTGCTCTGGAATTTGCAAG	26	80
CCL20	rs6749704	F:$(CT)^{22}$cTTCCTCAACAATTCTGAGGCTCTATATTG AGTTATATTAG	40	85
	rs13034664	R:$(CT)^{9}$*cTGTTCATTTCTCCTTCCTCCA*	21	40
CCL22	rs4359426	F:$(CT)^{39}$AGACATACAGGACAGAGCATGG	22	100
IP10	rs56061981	F:$(CT)^{26}$GGGGAAGTCCCATGTTGCAGACT	23	75
	rs3921	F:$(CT)^{30}$AGTTTGCAGTTACACTAAAAGGTGACCA AT	30	90

Notes: Primers F:left side of the SNP and R: right side of the SNP; CT +C tails was used according the size desired (24-100pb); Primer final concentration = 0.05 μM for 1 Reaction final volume = 10 μL. NA, not applicable.

Table 4. Allelic and genotypic frequencies of cytokine genes SNPs from healthy individuals compared with the HapMap database.

Gene	SNPs /genotypes /alleles	Controls (n=415) Frequencies	HapMap database*	Gene	SNPs/alleles/ genotypes	Controls (n=415) Frequencies	HapMap database*
	rs2069762				**rs2275913**		
	TT	0.524	0.553		AA	0.126	0.120
IL2	TG	0.390	0.353		AG	0.395	0.346
	GG	0.087	0.095		GG	0.479	0.534
	T	0.719	0.729		A	0.324	0.293
	G	0.281	0.271	***IL17A***	G	0.676	0.707
	rs2243250				**rs8193036**		
	CC	0.492	0.361		TT	0.556	0.413
IL4	CT	0.389	0.339		TC	0.403	0.413
	TT	0.118	0.300		CC	0.041	0.174
	C	0.687	0.530		T	0.757	0.620
	T	0.313	0.470		C	0.243	0.380
	rs1800795				**rs1800629**		
	GG	0.563	0.766		GG	0.725	0.827
IL6	GC	0.359	0.186		GA	0.256	0.165
	CC	0.078	0.048		AA	0.019	0.008
	G	0.742	0.859		G	0.853	0.910
	C	0.258	0.141	***TNFα***	A	0.147	0.090
	rs1800896				**rs361525**		
	GG	0.114	0.098		GG	0.858	0.883
	GA	0.485	0.348		GA	0.138	0.112
	AA	0.401	0.554		AA	0.004	0.005
	G	0.357	0.272		G	0.927	0.939
	A	0.643	0.728		A	0.073	0.061
IL10	**rs1800872**						
	CC	0.354	0.346				
	CA	0.513	0.438				
	AA	0.132	0.216				
	C	0.611	0.565				
	A	0.389	0.435				

* 1000 Genomes Project Phase 3 for all populations.

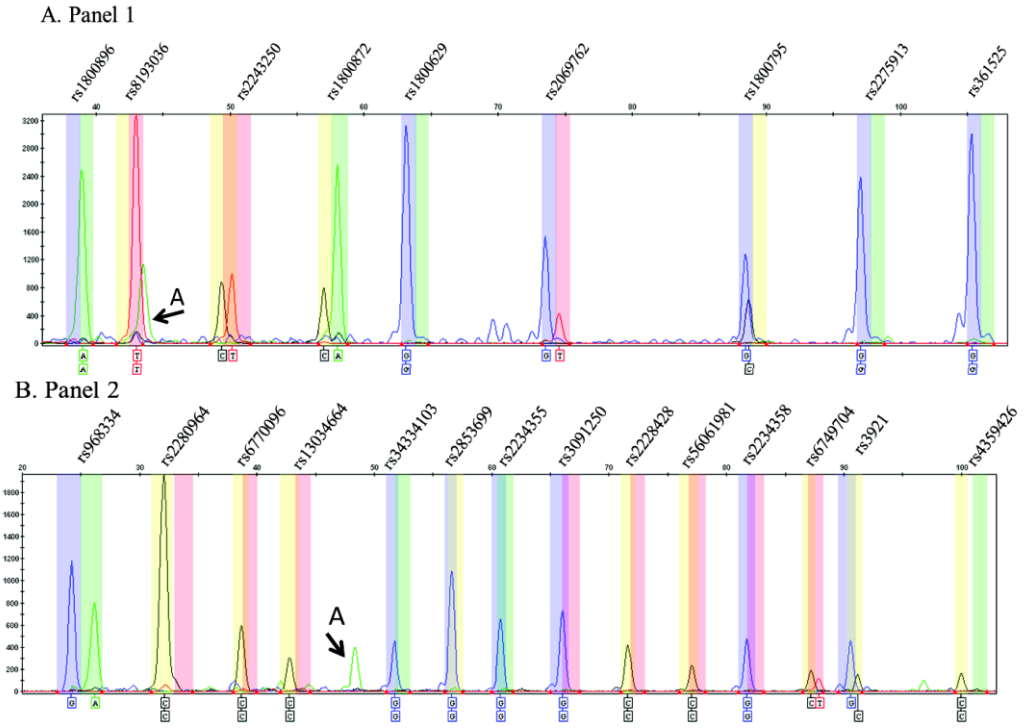
Table 5. Allelic and genotypic frequencies of chemokine receptor/ligand genes SNPs from healthy individuals compared with the HapMap database.

Gene	SNPs/alleles/ genotypes	Controls (n=415) Frequencies	HapMap database*	Gene	SNPs/alleles/ genotypes	Controls (n=415) Frequencies	HapMap database*
	rs3091250				**rs2234355**		
	GG	0.558	0.447		GG	0.847	0.786
CCR3	GT	0.361	0.414		GA	0.129	0.148
	TT	0.082	0.139		AA	0.024	0.066
	G	0.738	0.654		G	0.912	0.860
	T	0.262	0.346	***CXCR6***	A	0.088	0.140
	rs6770096				**rs2234358**		
	CC	0.821	0.675		GG	0.288	0.331
	CT	0.164	0.278		GT	0.476	0.439
	TT	0.015	0.047		TT	0.236	0.229
	C	0.903	0.814		G	0.526	0.551
CCR4	T	0.097	0.186		T	0.474	0.449
	rs2228428				**rs3921**		
	CC	0.582	0.742		CC	0.408	0.509
	CT	0.344	0.223		CG	0.510	0.367
	TT	0.075	0.035		GG	0.082	0.124
	C	0.753	0.853		C	0.663	0.692
	T	0.247	0.147	***CXCL10***	G	0.337	0.308
	rs968334				**rs56061981**		
	GG	0.395	0.404		CC	0.860	0.797
CCR6	GA	0.466	0.446		CT	0.140	0.192
	AA	0.139	0.151		TT	0.000	0.011
	G	0.628	0.627		C	0.930	0.893
	A	0.372	0.373		T	0.070	0.107
	rs2853699				**rs13034664**		
	GG	0.565	0.772		TT	0.204	0.210
CCR8	GC	0.388	0.198		TC	0.541	0.395
	CC	0.048	0.031		CC	0.255	0.395
	G	0.759	0.870		T	0.474	0.407
	C	0.241	0.130		C	0.526	0.593
	rs34334103			***CCL20***	**rs6749704**		
	GG ♀	0.489	0.449		TT	0.561	0.609
	GA ♀	0.011	0.056		TC	0.361	0.330
	AA ♀	0.000	0.003		CC	0.078	0.062
	G [a]	0.973	0.936		T	0.741	0.774
CXCR3	A [b]	0.027	0.064		C	0.259	0.226
	rs2280964				**rs4359426**		
	CC ♀	0.354	0.364		CC	0.888	0.845
	CT ♀	0.146	0.096		CA	0.112	0.144
	TT ♀	0.000	0.040	***CCL22***	AA	0.000	0.012
	C [a]	0.800	0.824		C	0.944	0.917
	T [b]	0.200	0.176		A	0.056	0.083

* 1000 Genomes Project Phase 3 for all populations.

[a,b] frequencies in general population

Figure 1



Legend

Figure. 1. Detection of cytokines and chemokine receptor/ligand gene SNPs by two multiplex minisequencing assays. Panel 1 (A) shows nine cytokine gene SNPs from a healthy individual donor. Peaks correspond to the fluorescence signal detected for each SNP. Panel 2 (B) shows fourteen chemokine receptor/ligand gene SNPs. The A arrow indicates a residual signal that does not match any SNP located among 40 to 45 bp position in both panels (discussed in the text).

Capítulo 5

5.1 Discussão

Em nosso estudo, indivíduos caracterizados com progressão rápida apresentaram contagens de células T CD4+bastante reduzidas já no momento do diagnóstico e, ainda, uma acentuada perda de células T CD4+ ao longo do acompanhamento clínico (mediana de 13 meses). Além disso, níveis elevados de partículas virais circulantes (mediana de 4,28 log; 3,8-4,79 log) foram detectados. Estes dados apontam para a incapacidade dos indivíduos com progressão rápida para aids de recuperar naturalmente as células T CD4+ perdidas na fase aguda e controlar o "*burst*" inicial da replicação viral, caracterizando uma infecção aguda grave e, por conseguinte, a necessidade de começar o tratamento antirretroviral em um curto período de tempo após a infecção.

No extremo oposto, indivíduos com progressão lenta foram capazes de manter um equilíbrio dinâmico entre a contagem de células T CD4+ e carga viral plasmática durante longos períodos. Destes, 17 indivíduos (aproximadamente 28% dos progressores lentos) foram classificados como não-progressores de longo prazo (LTNP) mantendo contagens de células T CD4+ acima de 500 cel/mm^3 por período superior a 15 anos. Contudo, por tratar-se de um estudo retrospectivo-longitudinal, foi possível acompanhar o aumento da carga viral e queda de células T CD4+ em 34 indivíduos caracterizados com progressão lenta (correspondendo a 57% dos progressores lentos). Os progressores lentos incluídos em nosso estudo, ao terem o equilíbrio dinâmico entre vírus e sistema imune interrompido, apresentam uma queda brusca de células T CD4+ acompanhada de um aumento da carga viral.

O papel das células T reguladoras na infecção pelo HIV ainda não está completamente compreendido, uma vez que, trabalhos de diferentes autores afirmam que estas células podem estar envolvidas, tanto na promoção, como na prevenção da progressão da infecção pelo vírus. Alguns autores apontam para um efeito benéfico através da supressão da ativação imune crônica pela inibição de células T CD4+ ativadas e, consequentemente, um maior controle da replicação viral (de StGroth &

Landay, 2008). Por outro lado, um papel prejudicial também já foi proposto, pois a inibição da resposta imune específica ao HIV através do potencial papel supressor das Treg pode beneficiar a multiplicação do vírus no organismo (Kwon et al., 2012; Piconi et al., 2010). Nossos trabalhos tentam auxiliar na compreensão do papel da imunidade das células T regulatórias através de inferências sobre o papel da IL-10, principal citocina supressora, na patogênese da infecção pelo HIV.

Em acordo com estudos de Shin et al. (2000) e Naicker et al, (2009), nossos resultados indicam que um perfil genético associado a níveis reduzidos de IL-10 (variante -592A *IL10*), em um contexto de exposição ao vírus, aumenta o risco de efetividade da infecção (Naicker et al., 2009; Shin et al., 2000). A redução de um efeito supressor provavelmente reflete uma maior disponibilidade de células alvo preferenciais do vírus em um estado transcricional ativo. Contudo, IL-10 tem papel chave na supressão de respostas imunes antivirais. Estudos já mostraram que em contexto de infecções virais o *knockout* do gene da IL-10 resulta em melhores respostas de células T específicas e na depuração viral (Brooks et al., 2006; Ejrnaes et al., 2006). Brockman et al. (2009) também demonstraram *in vitro* que o bloqueio do receptor de IL-10 em PBMCs de indivíduos HIV-positivo resultou no restabelecimento da função das células T CD4+ efetoras (Brockman et al., 2009). Além disso, o controle natural da replicação do HIV, observado em indivíduos controladores de elite, já foi associado à redução do número de células Treg quando comparado com indivíduos com infecção crônica (Brandt et al., 2011). Em adição, demostramos que o nível plasmático de IL-10 está aumentado no estágio clínico pré-aids tanto em progressores rápidos quanto lentos. Nos progressores lentos é possível observar o aumento de IL-10 antes da perda acentuada de células T CD4+, corroborando a hipótese de que o mecanismo de supressão imune está fortemente relacionado à patogênese da infecção pelo HIV. Assim, podemos supor que a supressão do sistema imune, através da IL-10, durante a infecção aguda e/ou estágio clínico pré-aids, estágios que o vírus encontra-se com taxas elevadas de replicação, favorece o espalhamento viral e acentua a depleção de células fundamentais para a orquestração das respostas imunes.

No mesmo sentido, uma resposta Th17 aumentada parece favorecer a efetividade da infecção pelo HIV (Chege et al., 2012). A maior disponibilidade dos alvos celulares favorece a replicação viral, auxiliando o vírus a ultrapassar barreiras iniciais impostas pelo sistema imune. Hartigan-O´Connor et al (2012) demonstraram em modelos animais

(infectados por SIV) que a composição do sistema imune do hospedeiro no momento de exposição ao vírus tem influência sobre a infecção e posterior progressão da doença (Hartigan-O'Connor et al., 2012). Contudo, estudos já associaram respostas Th17 preservadas à progressão lenta em LTNP e EC (Brandt et al., 2011; Khowawisetsut et al., 2013; Nigam et al., 2011). Em nossas análises encontramos o SNP -692C/T *IL17A* (rs8193036) associado com proteção à infecção e, também, com progressão rápida. Embora os efeitos funcionais deste SNP ainda sejam desconhecidos, o papel da resposta imune Th17 na patogênese da infecção pelo HIV, e sua relação com as células Treg, parece ser dependente do estágio clínico da infecção. Em adição, o perfil de citocinas dos progressores rápidos, em nossas análises, sugere que na infecção aguda progressores rápidos apresentam predominantemente um perfil imune Th2 (IL-4, IL-6) e Th17 (IL-17A). Ambas as subpopulações de células T (Th2 e Th17) são descritas como altamente permissivas à infecção pelo HIV, sugerindo que um número elevado de células Th17 na fase aguda pode estar envolvido na aceleração da progressão para a aids (Gosselin et al., 2010).

Em ambos os grupos, progressores rápidos e lentos, o aumento dos níveis de IL-10 foi associado ao aumento dos níveis de IL-6, citocina pró-inflamatória já associada ao quadro clínico de aids. A partir de nossas análises, e dados apresentados em outros estudos, podemos propor marcadores pró-inflamatórios, como IL-6, bem como marcadores de supressão imune, como IL-10, como valiosas ferramentas no monitoramento da infecção (Baker et al., 2012; Boulware et al., 2011; Kuller et al., 2008; Liovat et al., 2012; Roberts et al., 2010). Por estar relacionado com o mecanismo imune propriamente dito, o monitoramento dos níveis plasmáticos dessas citocinas, aliado a outros marcadores clínicos, pode auxiliar em intervenções terapêuticas. IL-6 e IL-10 podem até mesmo auxiliar a monitorar a falha terapêutica, uma vez que já foi descrito que seus níveis basais são reestabelecidos com o tratamento antirretroviral (Baker et al., 2012; Kamat et al., 2012). Já a correlação entre o aumento dos níveis de IL-6 e IL-10 deve ser melhor investigada, uma vez que a literatura aponta para um papel inibitório de IL-6 sobre células secretoras de IL-10. Assim, espera-se que níveis aumentados de IL-6 inibam a produção de IL-10. Por outro lado, sabe-se que mecanismos pró-inflamatórios exacerbados desencadeiam uma resposta imune supressora. Desta forma, é fundamental que estudos investiguem os mecanismos

intermediários entre a correlação encontrada no contexto da infecção pelo vírus HIV e como este quadro colabora para o estabelecimento da aids.

Com relação aos indivíduos controladores de elite, embora nossos resultados não possam ser generalizados devido ao reduzido tamanho amostral, é digno de nota que dos 8 indivíduos pertencentes a esse grupo 7 apresentaram a variante genética -208A *TNF-α*. Outros dois estudos em coortes italiana e espanhola já haviam encontrado resultados semelhantes (Nasi et al., 2013; Veloso et al., 2010). Níveis elevados de TNF-α geralmente estão associados à carga viral elevada e a um mau prognóstico (Singh et al., 2014), contudo, os impactos da presença desta variante genética em indivíduos HIV-soropositivo capazes de controlar naturalmente a infecção devem ser investigados.

Em nossas análises genéticas focamos duas questões principais: a estratificação da amostra de acordo com a origem étnica dos participantes e a aplicação de métodos estatísticos não-lineares desenvolvidos para detectar efeitos epistáticos. Utilizamos o método analítico de dados não paramétricos livre de modelos genéticos *a priori* chamado redução multifatorial de dimensionalidade (MDR) (Ritchie et al., 2003). No MDR, genótipos multilocus são agrupados em grupos de alto e baixo risco, reduzindo efetivamente a dimensionalidade dos genótipos preditores (atributos) de *n* dimensões para uma dimensão, permitindo detectar interações em amostras de tamanho relativamente pequeno. O processo de definição de um novo atributo como uma função de dois ou mais outros atributos é referido como indução construtiva (Michalski, 1983). O novo atributo genótipo multilocus é então avaliado pela sua capacidade de classificar e prever o desfecho (Moore et al., 2006).

Seguindo esta metodologia, foi possível detectar efeitos de interação entre os genes de citocinas e *TLR9* no contexto da infecção pelo HIV. Demostramos também que os resultados obtidos foram fortemente afetadas pela origem étnica dos indivíduos, reforçando que o estudo de populações de diferentes regiões geográficas e com diferentes ascendência étnica é uma importante ferramenta para a compreensão do papel da variabilidade genética na susceptibilidade à infecção pelo HIV e progressão à aids. Neste sentido, podemos argumentar que parte das dificuldades encontradas nos estudos de associação em larga escala (GWAS), que tem falhado em encontrar variantes genéticas significativas no contexto da infecção pelo HIV, se deve à heterogeneidade genética e efeitos epistáticos de difícil caracterização.

Além disso, nossos resultados contribuem para ampliar a compreensão dos mecanismos envolvidos na resposta imune à infecção pelo HIV, uma vez que SNPs em *IL10* e *IL17A* foram associados, através de análise estatística linear clássica, com suscetibilidade em euro-descendentes. Além disso, quando utilizamos o teste não-linear encontramos efeitos de interação significativos entre os SNPs de *IL10, IL6*, *IL4*, *TLR9*. Da mesma forma, com relação aos indivíduos afro-descendentes, encontramos uma associação significativa entre *TLR9* e a susceptibilidade à infecção pelo HIV. Na análise não lineare, detectamos efeitos epistáticos significativos entre *TLR9* e *IL4*. Assim, a análise de interação genética pode ajudar a definir com mais precisão os efeitos de SNPs e pode ser útil na identificação de redes de genes que estejam influenciando a susceptibilidade e os diferentes cursos clínicos da infecção.

5.2 Perspectivas

A fim de ampliar as discussões sobre nossos resultados já está em andamento em nosso laboratório a caracterização imunofenotípica das subpopulações de células T CD4+ efetoras, assim como a análise do perfil de expressão gênica de fatores de transcrição e cofatores envolvidos com a diferenciação e plasticidade de células Th17 e Tregs em indivíduos progressores rápidos e lentos em diferentes estágios clínicos da infecção pelo HIV. Estas novas análises poderão nos trazer informações sobre quais são as células secretoras e possíveis alvos celulares das citocinas com níveis alterados nos diferentes estágios clínicos da infecção em indivíduos com progressão rápida e lenta para a aids.

A respeito das análises genéticas, pretende-se replicar as avaliações dos SNPs em outras populações de estudo, considerando diferentes componentes étnicos, para ampliar o espectro das associações encontradas. Pretende-se avaliar os SNPs no contexto de suscetibilidade e progressão para a aids e, também, avaliar possíveis associações em um grupo HIV-positivo por transmissão vertical. Estas análises serão desenvolvidas através de colaborações já estabelecidas com outros grupos de pesquisa nacionais e internacionais. Análises estatísticas que auxiliem a compreensão dos efeitos epistáticos serão intensificadas, buscando contribuir na compreensão dos mecanismos genéticos que colaboram para os distintos desfechos frente à infecção pelo vírus HIV.

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ANEXO A

Aprovação do projeto no Comitê de Ética do Grupo Hospitalar Conceição, instituição de origem dos indivíduos participantes do estudo.

GHC Grupo Hospitalar Conceição

HOSPITAL N. S. DA CONCEIÇÃO S.A. Av. Francisco Trein, 596 CEP 91350-200 - Porto Alegre - RS Fone: 3357.2000 CNPJ: 92.787.118/0001-20

HOSPITAL DA CRIANÇA CONCEIÇÃO (Unidade Pediátrica do Hospital Nossa Senhora da Conceição S.A.)

HOSPITAL CRISTO REDENTOR S.A. Rua Domingos Rubbo, 20 CEP 91040-000 - Porto Alegre - RS Fone: 3357.4100 CNPJ: 92.787.128/0001-76

HOSPITAL FÊMINA S.A. Rua Mostardeiro, 17 CEP 91430-001 - Porto Alegre - RS Fone: 3314.5200 CNPJ: 92.693.134/0001-53

100% SUS

Vinculados ao Ministério da Saúde - Decreto nº 99.244/90

COMITÊ DE ÉTICA EM PESQUISA - CEP/GHC

O Comitê de Ética em Pesquisa do Grupo Hospitalar Conceição (CEP/GHC), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS desde 31/10/1997, pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB0001105) e pelo FWA - Federalwide Assurance (FWA 00000378), em 30 de novembro de 2010, reavaliou o seguinte projeto de pesquisa:

Projeto: 10-213 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
JOSÉ ARTUR BOGO CHIES
LUIZ FERNANDO JOBIM
MARIA CRISTINA COTTA MATTE
RÚBIA MARÍLIA MEDEIROS
DENNIS MALETICH JUNQUEIRA
LEONARDO AUGUSTO LUVISON ARAÚJO
CYNARA CARVALHO NUNES
MARINEIDE GONÇALVES DE MELO
BRENO RIEGEL SANTOS
MARIA LÚCIA ROSA ROSSETTI
SABRINA ESTEVES DE MATOS ALMEIDA

Título: Avaliação de polimorfismos em genes envolvidos na resposta imunológica de pacientes infectados com HIV-1.

Documentação: Aprovados
Aspectos Metodológicos: Aprovados
Aspectos Éticos: Aprovados

Parecer final: Este projeto, por estar de acordo com as Diretrizes e Normas Internacionais e Nacionais especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde, obteve o parecer de APROVADO.

Considerações Finais: Toda e qualquer alteração do projeto, deverá ser comunicada imediatamente ao CEP/GHC. Lembramos do compromisso de encaminhar dentro dos prazos estipulados, o(s) relatório(s) parcial(ais) e/ou final ao Comitê de Ética em Pesquisa do Grupo Hospitalar Conceição e ao Centro de Resultado onde a pesquisa for desenvolvida.

Porto Alegre, 30 de novembro de 2010.

Daniel Demétrio Faustino da Silva
Coordenador-geral do CEP/GHC

ANEXO B

Termo de Consentimento Livre e Esclarecido do projeto, o qual foi proposto e aceito por todos os indivíduos participantes do estudo.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto de Pesquisa: "Avaliação de polimorfismos em genes envolvidos na resposta imunológica de pacientes infectados com HIV-1"

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Você está sendo convidado a participar de uma pesquisa intitulada: **"Avaliação de polimorfismos em genes envolvidos na resposta imunológica de pacientes infectados com HIV-1"** que tem como objetivo principal avaliar fatores imunológicos que estão envolvidos na progressão da AIDS. O tema escolhido se justifica, pois pode propiciar um maior entendimento sobre os mecanismos envolvidos na infecção pelo HIV e auxiliar em um acompanhamento e tratamento adequado para todos os pacientes soropositivo. Além disso, pode propiciar novos estudos que tenham como objetivo o desenvolvimento de novos medicamentos ou vacinas. O trabalho está sendo realizado sob orientação da pesquisadora Sabrina Esteves de Matos Almeida. Para alcançar os objetivos do estudo será realizada uma entrevista individual, na qual você irá responder 22 perguntas pré-estabelecidas. Os dados de identificação serão confidenciais e os nomes reservados. Os dados obtidos serão utilizados somente para este estudo, sendo os mesmos armazenados pelo(a) pesquisador(a) principal durante 5 (cinco) anos e após totalmente destruídos (conforme preconiza a Resolução 196/96).

Como são feitas as análises? As análises do DNA dos genes do sistema imune serão realizadas a partir de coleta de sangue, como uma coleta normal para hemograma. Com o uso de agulhas e seringas descartáveis será coletada de você uma amostra de sangue (quantidade aproximada de uma colher de sopa). Esta coleta será feita por um indivíduo treinado. Após, o sangue será examinado para determinar variações genéticas referentes ao sistema imune. As amostras serão identificadas por números. Todos os dados que vinculem sua identidade com os dados obtidos a partir de sua amostra de sangue serão mantidos em um banco de dados sigiloso, ao qual só terão acesso os pesquisadores acima citados.

Quais os riscos em participar? Não há riscos em participar do projeto. Poderá, no entanto, haver formação de um hematoma no braço em função da coleta de sangue.

O que o paciente ganha com este estudo? Embora este trabalho não possa gerar nenhum benefício imediato aos participantes, este estudo poderá trazer vários benefícios em longo prazo (conhecimento das características genéticas presentes na nossa população) podendo assim, auxiliar em novas diretrizes do tratamento e acompanhamento futuro dos pacientes que vivem com HIV/AIDS. Este estudo não fornecerá nenhum auxílio financeiro aos participantes.

Quais são os seus direitos? Os seus registros médicos serão sempre tratados confidencialmente. Os resultados deste estudo só poderão ser usados para fins científicos, e você não será identificado por nome. Sua participação no estudo é voluntária, caso você decida não participar, isto não afetará no tratamento normal que você tem direito. Além disso, você terá a liberdade de retirar seu consentimento a qualquer momento, caso desejar. Você poderá procurar qualquer pesquisador envolvido para responder a qualquer pergunta ou obter esclarecimentos acerca dos assuntos relacionados a esta pesquisa. Caso você queira esclarecer alguma dúvida sobre as questões éticas deste projeto você poderá entrar em contato Daniel Demétrio Faustino da Silva, Coordenador-geral do Comitê de Ética em Pesquisa do GHC pelo telefone 3357-2407.

EU_____, recebi as informações sobre os objetivos e a importância desta pesquisa de forma clara e concordo em participar do estudo.
Declaro que também fui informado:
* Da garantia de receber resposta a qualquer pergunta ou esclarecimento acerca dos assuntos relacionados a esta pesquisa.

Versão Aprovada em

30 NOV. 2010

Daniel Demétrio Faustino da Silva
Coordenador-geral do CEP-GHC

. * De que minha participação é voluntária e terei a liberdade de retirar o meu consentimento, a qualquer momento e deixar de participar do estudo, sem que isto traga prejuízo para a minha vida pessoal e nem para o atendimento prestado a mim.
. * Da garantia que não serei identificado quando da divulgação dos resultados e que as informações serão utilizadas somente para fins científicos do presente projeto de pesquisa.

* Sobre o projeto de pesquisa e a forma como será conduzido e que em caso de dúvida ou novas perguntas poderei entrar em contato com a pesquisadora Sabrina Esteves de Matos Almeida no telefone 3352-0336, email: sabrinamatos.almeida@gmail.com e endereço: Av. Ipiranga, 5400. 3° andar. Bairro Jardim Botânico – Porto Alegre.
* Também que, se houverem dúvidas quanto a questões éticas, poderei entrar em contato com Daniel Demétrio Faustino da Silva, Coordenador-geral do Comitê de Ética em Pesquisa do GHC pelo telefone 3357-2407.

* Se houverem dúvidas quanto a questões éticas, poderei entrar em contato com qualquer um dos pesquisadores envolvidos.

Declaro que recebi cópia deste Termo de Consentimento Livre e Esclarecido, ficando outra via com a pesquisadora.

Nome do entrevistado: _____**Assinatura do entrevistado** _____

Nome do Pesquisador: _____**Assinatura do Pesquisador:** _____

Este formulário foi lido para _____(nome do paciente) em / / (data) pelo _____(nome do pesquisador) enquanto eu estava presente.

Versão Aprovada em

30 NOV. 2010

Daniel Demétrio Faustino da Silva
Coordenador-geral do CEP-GHC

ANEXO C

Questionário sócio demográfico aplicado a todos os indivíduos participantes do estudo.

1. Identificação do participante: _____
2. Data de nascimento: _____
3. Sexo: ( )Masculino ( )Feminino
4. Município de Residência:_____
5. Estado Civil: ( )Solteiro ( )Casado ( )Acompanhado
6. Etnia (auto-declaração): ( ) Branco ( ) Não-branco (Pesquisador:_____)
7. Profissão: _____ Em atividade: ( )Sim ( )Não
8. Escolaridade:_____
9. Data da última sorologia negativa para HIV: _____ / _____ / _____
10. Data da primeira sorologia positiva para HIV: _____ / _____ / _____
11. Possível forma de Transmissão: ( )Heterossexual ( )HSH ( )UDI ( )Transfusão sanguínea ou Transplante ( )Transmissão vertical (Materno fetal) ( )Outro. Qual?_____
12. Gestante (atualmente): ( )Sim ( )Não
13. Fumo: ( )Sim ( )Não
14. Uso de Álcool (+ de 3 vezes por semana): ( )Sim ( )Não
15. Uso de drogas: ( )Sim ( )Não Se sim, qual? _____
16. Comorbidades e Coinfecções: ( )Diabetes ( )Cardiopatia ( ) Hemodiálise ( ) Tuberculose ( )HTLV ( )Hepatite B ( )Hepatite C ( )Outras, qual? _____
17. Viajou para o exterior: ( )Sim ( )Não - Continentes: ( )África ( )Europa ( )Ásia ( )América do Sul ( )América do Norte ( ) Oceania
18. Teve contato sexual/drogas injetáveis no exterior: ( )Sim ( )Não Onde?_____
19. Teve contato sexual/drogas injetáveis com estrangeiros no Brasil: ( )Sim ( )Não
20. Tem (já teve) alguma doença crônica ou histórico familiar deste tipo de doença?Se sim, qual?
    _____
21. Tem (já teve) alguma doença autoimune ou histórico familiar deste tipo de doença?Se sim, qual?
    _____

ANEXO D

Ficha de revisão de prontuário médico realizada para todos os indivíduos participantes do estudo.

FICHA DE REVISÃO DE PRONTUÁRIO

Data desta revisão	**Data da coleta**	**Código no projeto**

Nome

Registro de prontuário	**Data de Nasc.**

Ultimo teste HIV -	**Primeiro teste HIV+**	**Diagnóstico AIDS**

Relações sexuais	**Uso de drogas**

Provável transmissão

Gestantes

Gestações após HIV+:	
Data ARV nas gestações:	
Uso de ARV pós-gestação:	

Co-infecções:

HCV		**HTLV**	
HBV		**CMV**	
Toxoplas.		**Cripto.**	
Outras			

Observações:

Esquema terapêutico

Data	CD4	CD8	rel	CV	CV log

ANEXO E

Co-orientação de trabalho relacionado ao tema da tese: "Fatores que influenciam o diagnóstico tardio do HIV/Aids: um olhar sociológico de casos em porto Alegre" apresentado em janeiro de 2015 no Instituto de Filosofia e Ciências Humanas da UFRGS para Conclusão do Curso de Ciências Sociais pela aluna Bruna Gre Marques.

Resumo

Ainda que o curso clínico da infecção pelo vírus da imunodeficiência humana (HIV) seja variável, a maioria dos indivíduos infectados desenvolve a Síndrome da Imunodeficiência Adquirida (AIDS) de quatro a nove anos após a infecção pelo vírus. Contudo, mesmo após 30 anos de estudos relacionados ao HIV/aids, cerca de 30% dos brasileiros HIV+ descobriram-se portadores do vírus já com um quadro clínico considerado grave. Na maioria dos casos, portanto, trata-se de um diagnóstico tardio para HIV/aids. O presente trabalho teve como principal objetivo examinar fatores sociais relacionados ao diagnóstico tardio em pacientes atendidos no Hospital Nossa Senhora da Conceição em Porto Alegre (RS) no ano de 2013. Os dados foram obtidos através da revisão de prontuários médicos dos indivíduos em atendimento regular no Serviço de Infectologia do Hospital Conceição e analisados com base nos conceitos de determinantes sociais da saúde e de vulnerabilidade. As análises dos resultados obtidos indicam que fatores como o sexo do indivíduo, a sua escolaridade, o bairro em que reside e a presença de coinfecções está associado ao estagio clínico da infecção no momento do diagnóstico. A discussão das dimensões de vulnerabilidade e as determinações sociais da doença contribui para a melhor compreensão do diagnóstico tardio e pode apontar estratégias de ações em saúde visando a diminuição destes casos.

Abstract

Even though the clinical course of the infection by the Human Immunodeficiency Virus (HIV) varies widely, infected people usually develop the Acquired Immunodeficiency Syndrome (AIDS) between four and nine years after the HIV virus infection. However, even after 30 years of studies related to HIV/aids, around 30% of HIV+ Brazilians found out the infection among a serious clinical. In most cases, therefore, it is a late diagnosis for HIV/AIDS. This paper aimed to look over the social factors associated to a late diagnosis of the patients treated at the Hospital Nossa Senhora da Conceição, in Porto Alegre (RS) in 2013. The data were obtained by review of medical records of individuals in regular attendance at Infectious Disease Department of Hospital Conceição e analyzed based on the concepts of social determinants of health and vulnerability. The results analysis indicates that factors as patient sex, education, the neighborhood that lives and the presence of coinfections may have strong association with the infection"s advanced stages. The discussion about the vulnerability dimensions and disease"s social determinations contributes to a better epidemiological phenomenon"s comprehension and can point to health"s strategies and actions aiming to late diagnosis reduction.