UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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A IMPORTÂNCIA DOS FATORES GENÉTICOS DO HOSPEDEIRO NA SUSCETIBILIDADE A DOENÇAS INFECCIOSAS INTRODUZIDAS EM POPULAÇÕES NATIVAS SUL AMERICANAS – A TUBERCULOSE NOS ACHÉ

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Sumário

Lista de abreviaturas, símbolos e unidades

AME: Ameríndios

BCG: *Bacillus Calmette-Guérin*

BCR: receptor de célula B

CCL2 / MCP-1: Quimiocina Ligante 2 (Motivo C-C)

CCL5 / RANTES: Quimiocina Ligante 5 (Motivo C-C)

CD14: aglomerado de diferenciação 14

CD209 / DC-SIGN: aglomerado de diferenciação 209

CEU: residentes de Utah descendentes de Europeus do Norte e do Leste

CHB: chineses de Beijing

CR1: receptor 1 do complemento

EGLN1: Fator 1 indutor de hipóxia da família Egl-9

EPAS1: proteína contendo o domínio endotelial PAS 1

EUA/USA: Estados Unidos da América

F_{ST}: índice de diferenciação populacional

HIV: Vírus da Imunodeficiência Humana

HLA: Antígeno Leucocitário Humano

IgE: Imunoglobulina E

IgG: Imunoglobulina G

IgM: Imunoglobulina M

IL1B: Interleucina 1 Beta

IL2: Interleucina 2

IL4: Interleucina 4

IL4R: Receptor da Interleucina 4

IL6: Interleucina 6

IL8: Interleucina 8

IL10: Interleucina 10

IL12α: Interleucina 12 Alfa

IL12β: Interleucina 12 Beta

IL12Rβ: cadeia beta do receptor da IL12

INFγ: Interferon gama

lNFγR: receptor de interferon gama
iNOS: óxido nítrico-sintase induzida
KIR: receptor das células exterminadoras naturais semelhante à inumoglobulina
MAF: freqüência do alelo menos freqüente
MHC: Complexo Principal de Histocompatibilidade
Mtb: *Mycobacterium tuberculosis*
NK: células exterminadoras naturais
NOD2: domínio de oligomerização contendo 2 ligações para nucleotídeos
NOS2: óxido nítrico-sintase 2
P2X7: receptor purinérgico P2X7
PAMP: Padrão Molecular Associado ao Patógeno
PPD: Derivado de Proteína Purificada
PRR: Receptor de Reconhecimento de Padrão
PTPN22: Proteína Tirosina Fosfatase, não receptor tipo 22
SLC11A1: membro 1 da família 11 de carreadores de soluto
SNP: polimorfismo de nucleotídeo único
SP110: proteína de corpo nuclear
TCR: Receptor de Célula T
TB: Tuberculose
TGF-β: fator de crescimento transformador beta
Th: célula T auxiliar
TLR1: Receptor do tipo Toll 1
TLR2: Receptor do tipo Toll 2
TLR4: Receptor do tipo Toll 4
TLR5: Receptor do tipo Toll 5
TLR6: Receptor do tipo Toll 6
TLR7: Receptor do tipo Toll 7
TLR8: Receptor do tipo Toll 8
TLR9: Receptor do tipo Toll 9
TLR10: Receptor do tipo Toll 10
TLR12: Receptor do tipo Toll 12
TNFα: fator de necrose tumoral alfa

TNFβ: fator de necrose tumoral beta

TNFR1: receptor 1 do fator de necrose tumoral

TPE: derrame pleural tuberculoso

TST: Teste de Sensibilidade à Tuberculina

VDR: receptor de Vitamina D

WHO: Organização Mundial da Saúde

YRI: Yoruba de Ibadan

Resumo

Populações nativas sul americanas apresentam uma suscetibilidade diferenciada a doenças infecciosas introduzidas, como a tuberculose. Diversas teorias procuram justificar essa observação, contudo, ainda não há consenso em relação às bases genéticas que estariam influenciando nessa suscetibilidade diferenciada. Diversos estudos demonstraram que essas populações apresentam uma baixa diversidade genética em diversos marcadores relacionados com a resposta imune, dentre eles, HLA e KIR. Entretanto, sabe-se que as vias de resposta imune inata e adaptativa possuem um papel central no desencadeamento de uma resposta imune adequada frente a um patógeno como o *Mycobacterium tuberculosis*, causador da tuberculose. A presente Tese analisou a variabilidade genética em 32 marcadores relacionados com a resposta adaptativa e 14 marcadores relacionados com resposta inata em diversas populações nativas sul americanas (Aché, Guarani, Kaingang e Xavante), relacionando a variabilidade observada nesses grupos com aquela observada nos três grandes grupos continentais incluídos no HapMap (europeus, africanos e asiáticos). Alem disso, esses marcadores foram relacionados com suscetibilidade a tuberculose e resposta no teste de sensibilidade à tuberculina (TST) na população Aché. Em ambos os sistemas, as populações nativas sul americanas apresentaram baixa diversidade genética em relação às populações do HapMap com alguns marcadores sendo, inclusive monomórficos nesses grupos. Análises de diferenciação populacional (através do F_{ST}) demonstraram uma maior diferenciação entre ameríndios e a população europeia para marcadores do sistema adaptativo (F_{ST} = 0,231), o que provavelmente reflete eventos demográficos associados a eventos de seleção contra alelos inflamatórios que possivelmente tenham ocorrido na Europa em função da adaptação ao clima temperado e a um microbioma diferente. As análises de diferenciação populacional para os marcadores do sistema inato demonstraram uma maior diferenciação dos ameríndios em relação à população africana (F_{ST} = 0,194), provavelmente refletindo os milhares de anos de atuação de eventos demográficos sobre esses grupos desde a saída da África. As análises de

associação com tuberculose na população Aché expuseram uma relação entre marcadores responsáveis pela predominância de um padrão de resposta Th2 (IL-4, IL-10, CR1) e anergia no TST e marcadores relacionados com predominância de resposta Th1 (IL-8 e TLR9) e tuberculose, confirmando o que diversos estudos anteriores haviam inferido analisando somente os níveis de imunoglobulina E. As informações acrescentadas pela presente Tese ajudam a esclarecer as bases moleculares para a suscetibilidade diferenciada a doenças introduzidas em populações nativas sul americanas, além de auxiliar para que, no futuro, políticas de saúde pública específicas sejam direcionadas para esses grupos geneticamente diferenciados.

Abstract

South American Native populations have a differential susceptibility to introduced infectious diseases, such as tuberculosis. There are several theories trying to explain this question, however, at present there is no consensus about the genetic background that could be influencing in this differential susceptibility. Some studies showed a low genetic diversity in several markers related to the immune response, as HLA and KIR, in these populations. However, it is well established that adaptive and innate immune response have a central role in the development of an adequate immune response against pathogens such as *Mycobacterium tuberculosis*, the one responsible for tuberculosis. The current Thesis analyzed the genetic variability in 32 variants related to adaptive immune response and 14 variants related with innate immune response in several South American Native populations (Aché, Guarani, Kaingang and Xavante). The genetic variability observed in these populations was related with those observed in the three major ethnic groups included in the HapMap project (European, African and Asian). Moreover, these markers were related with susceptibility to tuberculosis and tuberculin sensibility test response (TST) in the Aché population. The South American Native populations showed a low genetic diversity in both adaptive and innate systems compared to HapMap populations. Some variants were monomorphic in these Native populations. Population differentiation analysis based on F_{ST} showed a higher differentiation among Amerindians and European populations to adaptive immune response genes (F_{ST} = 0.231). This result probably reflects demographic events associated with selection events against inflammatory alleles in Europe due to the adaptation to temperate climate and a different microbiome. The population differentiation analysis to innate system variants showed a higher differentiation among Amerindians and African populations (F_{ST} = 0.194). This finding probably is the result of thousand years of demographic events action in these groups since the out of Africa event. The tuberculosis association analysis performed in the Aché population showed a relation between variants responsible for Th2 pattern predominance (IL-4, IL-10 and CR1) and anergy in TST. Variants related with Th1 pattern predominance (IL-8 and TLR9) were associated with tuberculosis, confirming the previous studies

findings obtained through immunoglobulin E levels analyze. The knowledge acquired in the present Thesis can help to clarify the molecular basis of the differential susceptibility due to introduced infectious diseases in South American Native populations. Besides that, this knowledge can help to ensure that in the future, specific public health policies will be directed to these genetically differentiated groups.

Capítulo I - Introdução

1.1- Imunogenética em populações nativas sul americanas

Há duas teorias principais que procuram explicar por que os ameríndios são mais suscetíveis aos novos patógenos do que os não nativos americanos: a hipótese da memória imunológica e a hipótese da heterozigosidade do HLA (Black, 2004). Ambas consideram adaptações imunológicas.

A hipótese da memória imunológica argumenta que a falta de exposição aos patógenos na infância levaria a um aumento na suscetibilidade a doenças infecciosas. Sem essa exposição primária, o sistema imune não estaria apto a responder de maneira efetiva quando ocorrer uma exposição subsequente (Neel, 1977). Essa hipótese sugere que após uma exposição apropriada, a capacidade de desenvolver uma resposta imune adequada frente a um patógeno seria equivalente nos nativos americanos e nos não nativos.

Uma segunda hipótese argumenta que uma baixa heterozigosidade relacionada ao HLA entre nativos americanos levaria a uma menor diversidade de fenótipos resistentes a doenças do que aquele observado entre hospedeiros não nativos, uma vez que os primeiros podem reconhecer um menor repertório de proteínas derivadas de patógenos (Black *et al*., 1977). Essa hipótese sugere que mesmo com uma exposição adequada aos patógenos na infância, os nativos americanos nunca teriam uma resposta imunológica equivalente aos não nativos.

Além disso, o balanço entre células Th1, Th2, Th9, Th17 e Th22 no hospedeiro é um ponto crítico para o entendimento da resposta imune. Uma terceira hipótese, mais recente, postula que esse balanço celular seja parcialmente devido à exposição ambiental durante a primeira infância. No entanto, esse mecanismo é muito diferenciado entre nativos americanos e não nativos, podendo ser um fator determinante para as diferenças observadas na suscetibilidade às doenças (Erb, 1999).

Em caucasianos, neonatos têm uma resposta imune predominantemente do tipo Th2 e, por volta dos cinco anos de idade, esse padrão muda para uma resposta predominante do tipo Th1 (Wilson *et al*., 1986). Essa mudança deve-se a capacidade de produzir IFN-γ, que é muito menor em bebês e crianças na primeira infância quando comparado com adultos (Holt *et al*., 1992). Em populações não caucasianas, essa mudança no padrão de dominância parece

não acontecer tão precocemente, e se ocorre, pode ser revertida mais tarde. Africanos (Gold *et al*., 1993; Von Behren *et al*., 1999) e ameríndios tendem a ser mais Th2 dominantes do que Th1 dominantes (Kaplan *et al*., 1980; Sousa *et al*., 1997). As células Th2 estimulam uma produção maior de IgE (imunoglobulina E), que é um anticorpo, do que das demais imunoglobulinas e, através da medida dos níveis desse anticorpo distingue-se uma resposta Th2 dominante de uma Th1 dominante.

1.2- Resposta Imune Inata

A imunidade natural, ou inata, é a linha de defesa inicial contra os micro-organismos, consistindo em mecanismos de defesa celulares e bioquímicos que já existiam antes do estabelecimento de uma infecção e que estão programados para responder rapidamente a esses episódios. Esse tipo de defesa caracteriza-se por uma resposta menos específica e de rápida assimilação, que apresenta uma duração limitada (Saalmüller, 2006). Seus principais componentes são as barreiras físicas e químicas, tais como o epitélio e as substâncias antibacterianas nas superfícies epiteliais; células fagocitárias como os neutrófilos, as células dendríticas e os macrófagos (Steinman & Cohn, 1973; Steinman, 1991) e células NK (natural killer); proteínas do sangue e citocinas (Glimcher *et al.*, 1977). A imunidade inata depende do reconhecimento, através de um repertório bastante limitado de receptores, de componentes microbianos altamente conservados que são compartilhados por grandes grupos de patógenos. Esses receptores são denominados receptores de reconhecimento de padrão (PRRs), enquanto que seus alvos são denominados padrões moleculares associados ao patógeno (PAMPs). A classe de PRRs melhor caracterizada é a dos receptores do tipo Toll-like (TLRs), que são expressos na superfície celular ou em compartimentos intracelulares e detectam micro-organismos através do reconhecimento de vários produtos bacterianos e ácidos nucleicos virais (Akira *et al.*, 2006).

1.3- Resposta Imune Adaptativa

Na imunidade adaptativa ou adquirida há uma especificidade extraordinária para distinguir diferentes moléculas e uma habilidade de lembrar, desencadeando

uma resposta mais intensa quando ocorrerem exposições subseqüentes ao mesmo micro-organismo. Ela necessita de mais tempo para se desenvolver, contudo, é mais específica e duradoura. Além disso, precisa ocorrer uma interação com o sistema imune inato para que a ativação da imunidade adquirida seja efetiva (Saalmüller, 2006). Há dois tipos de resposta adquirida: a humoral e a celular, dependendo do componente do sistema imunológico que está agindo, receptores de células B (receptores imunoglobulínicos) ou receptores de célula T (TCRs) (Cooper & Alder, 2006).

A imunidade humoral é aquela mediada por moléculas presentes no sangue e nas secreções das mucosas, denominadas anticorpos, que são produzidas pelos linfócitos B. Esse é o principal mecanismo de defesa contra micro-organismos extracelulares e suas toxinas, pois os anticorpos se ligam a eles e ajudam na sua eliminação (Lilic, 2009).

A imunidade celular é mediada pelos linfócitos T e promove a destruição de micro-organismos ou a destruição de células infectadas por vírus e bactérias. Uma vez que esses micro-organismos possuem a capacidade de sobreviver e proliferar dentro de células do hospedeiro, onde estão protegidos da ação dos anticorpos, surge a necessidade da ação dos linfócitos T (Lilic, 2009).

Os linfócitos T auxiliares (Th) só conseguem exercer suas funções com a assistência das citocinas, que são pequenas proteínas que atuam na célula que as produzem ou em células próximas. São proteínas mensageiras que regulam o desenvolvimento, o reparo de tecidos e a resposta imune. Essas moléculas ligam-se aos receptores específicos na membrana, ativando uma cascata que leva à expressão de genes que induzem, aumentam ou inibem as vias de sinalização intracelular.

Teoricamente as células Th1 são as responsáveis pela imunidade a patógenos intracelulares; as células Th2 provêm proteção contra parasitas extracelulares; as Th9 estariam envolvidas em doenças autoimunes e alérgicas; as Th17 atuam na resistência a infecções causadas por bactérias e fungos; e as Th22 estariam provavelmente envolvidas na patofisiologia de doenças autoimunes (Zhang *et al*., 2011; Jabeen & Kaplan, 2012). Na prática é bastante complicado determinar essa rígida diferenciação em termos de um conjunto de

células gerando resposta contra uma determinada patologia. A sinalização para diferenciação de determinado conjunto de células Th é dependente da expressão de uma gama de genes. A ação conjunta dos produtos expressos por esses genes acaba por determinar qual o conjunto de células Th que atuará predominantemente naquele momento. No entanto é aceito que determinados produtos gênicos como, por exemplo, as citocinas, estão predominantemente relacionadas com determinado padrão de resposta Th.

1.4- Tuberculose

1.4.1- Características Clínicas

Tuberculose pode ser causada por diversas espécies de micobactérias, como *Mycobacterium bovis* e *Mycobacterium Africanum*, mas *Mycobacterium tuberculosis* (Mtb) é a espécie responsável pela maioria dos casos dessa doença observados em todo o mundo. Uma das principais caracteristicas desse patógeno é sua capacidade de permanecer dormente por longos períodos de tempo, em alguns casos ele pode inclusive não se manifestar durante toda a vida do hospedeiro.

A bactéria geralmente ataca os pulmões, sendo transmitida de uma pessoa infectada para uma sadia através de secreções liberadas durante o espirro, a fala e a tosse. Os sintomas associados com a tuberculose são tosse por três semanas ou mais, produção de catarro, dor no peito, fraqueza ou fadiga, perda de peso, falta de apetite, calafrios, febre e sudorese noturna. Nos casos em que a bactéria se aloja fora dos pulmões (uma condição denominada de tuberculose extra-pulmonar), o quadro clínico varia conforme o tecido afetado (ossos, cérebro, coração, músculos, dentre outros).

Um dos testes que auxilia no diagnóstico de tuberculose é o teste de sensibilidade a tuberculina (TST) utilizando um derivado proteico purificado (PPD), que demonstra se o indivíduo já foi infectado por Mtb. O teste é relativamente simples, consistindo de uma aplicação por via intradérmica do derivado protéico denominado tuberculina. De 72 a 96 horas após a aplicação mede-se o maior diâmetro transverso da área de endurecimento palpável. De

acordo com o resultado, registrado em milimetros, o indivíduo é classificado em termos de reação à tuberculina. Indivíduos com menos de 5mm de reação são ditos não reatores e indivíduos com 5mm ou mais são ditos reatores. Um resultado correspondente à categoria reator demonstra que o indivíduo já foi vacinado com BCG (Bacilo de Calmette e Guérin) ou infectado por Mtb. Já um resultado de não reator pode significar que o indivíduo não foi infectado por Mtb ou que ele apresenta uma hipersensibilidade reduzida, uma condição denominada de anergia.

O tratamento da doença é feito com uma combinação de medicamentos que devem ser administrados por um período de seis meses. Durante os dois primeiros meses, três drogas diferentes são utilizadas: pirazinamida, isoniazida e rifamicina. A partir do terceiro mês, somente isoniazida e rifampicina são administradas. Se o tratamento não for completado, aumenta-se as chances de desenvolvimento de um caso de tuberculose resistente a múltiplas drogas. A vacina BCG é eficiente na proteção contra tuberculose em crianças, por isso deve ser adminstrada em dose única o mais precocemente possível, preferencialmente no nascimento.

1.4.2- Origens nas Américas

Ainda não há consenso em relação à origem da tuberculose humana; contudo, a hipótese mais aceita é a de que há aproximadamente 8000 anos, após a domesticação do auroque (*Bos primigenius*), começou a ocorrer o contato de humanos com o *Mycobacterium bovis* (Nowak, 1991). Esse contato aumentou as chances de transmissão da doença, tanto diretamente, quanto pelo uso de leite e carne contaminados. Quando os grupos urbanos começaram a se formar, surgiu um ambiente favorável para a seleção de formas humanas respiratórias de tuberculose causadas pelo *Mycobacterium tuberculosis* (Mtb) (Cockburn, 1967). Portanto, tradicionalmente se acredita que pequenos grupos nômades não apresentariam condições ideais para manter a tuberculose como uma doença relevante na população, enquanto grandes grupos sedentários seriam ideais para a dispersão da patologia. Contudo, não há como descartar totalmente a possibilidade de que a tuberculose tenha persistido em baixos níveis mesmo em

populações pequenas, já que se trata de uma patologia crônica e que o Mtb pode assumir formas resistentes dentro ou fora do hospedeiro (Johnston, 1995).

O efeito devastador que a tuberculose causou em populações nativas americanas, após o contato com os europeus, lançou a ideia de que esses grupos nunca haviam sido expostos a essa infecção. Contudo, diversas investigações paleontológicas confirmaram a presença dessa patologia em diferentes populações americanas pré-históricas. Portanto, a tuberculose poderia ter surgido nas Américas independentemente do contato com os povos do Velho Mundo que ocorreu a partir do século XV (Gómez & Souza, 2003).

Diversas múmias provenientes de populações pré-colombianas foram diagnosticadas com tuberculose na América do Sul, principalmente no Chile e no Peru, com o caso mais antigo descoberto até agora datando de 160 AD. Considerando que indivíduos de diferentes culturas apresentaram infecção respiratória ou óssea, alguns autores propõem que a tuberculose persistiu como um problema de saúde endêmico na América do Sul por dois milênios, e que já estava presente quando os europeus chegaram à região no século XVI (Gómez & Souza, 2003). Além disso, análises das lesões anatômicas nas múmias revelaram que a patologia existente na época não era diferente daquela observada atualmente.

Baseados na dispersão e persistência da doença, estudos sugerem que o complexo da tuberculose (composto pelas várias espécies de *Mycobacterium* que desencadeiam um quadro clínico similar aquele observado na tuberculose) provavelmente chegou às Américas junto com as primeiras migrações humanas e que, ao menos na América do Sul, a tuberculose foi um importante problema de saúde pública. Apesar disso, muitas das populações nativas podem não ter sido expostas a essa patologia antes do contato com os europeus, devido principalmente ao seu isolamento geográfico em relação às demais populações. Assim, o contato com os povos do Velho Mundo, adicionou novas variedades de micobactérias e novas infecções virais, movimentou grupos de pessoas, impôs estresse a essas populações e, principalmente, levou a patologia até os grupos nativos isolados.

1.4.3- Tuberculose nos dias atuais

Apesar dos programas de controle que são desenvolvidos em todo o mundo, essa doença continua sendo um importante problema de saúde pública. A maioria esmagadora dos casos de tuberculose ocorre em países em desenvolvimento. No último relatório sobre essa doença publicado pela Organização Mundial da Saúde (WHO), dados alarmantes são revelados em relação à dispersão dessa patologia pelo mundo. Dos 216 países ou territórios, 205 relataram algum caso no ano de 2014 (WHO, 2015).

Aproximadamente um terço da população mundial está infectado pelo Mtb. Para o ano de 2014, a estimativa da incidência de casos de tuberculose em todo o mundo foi de 9,6 milhões (133 casos para cada 100 000 indivíduos). Desses, um décimo desenvolverá tuberculose ativa. Apesar dessa minoria de indivíduos infectados que acabará por desenvolver a patologia ativa, a morbidade e mortalidade de humanos ao redor do mundo permanece alta. Cerca de 1,5 milhões de pessoas morreram devido a essa patologia no ano de 2014, sendo 1,1 milhões de mortes entre indivíduos HIV negativos e 0,39 milhões entre indivíduos HIV positivos. Aproximadamente 0,21 milhões de mortes foram de indivíduos com tuberculose resistente a múltiplas drogas (WHO, 2015).

Muitos dos casos estimados de tuberculose ocorreram na Ásia (58%) e África (28%), com menores proporções de casos na região do leste do Mediterrâneo (8%), na Europa (3%) e nas Américas (3%) (WHO, 2015). Os países com as maiores taxas de incidência dessa patologia são Índia, Indonésia, China, Nigéria, Paquistão e África do Sul. Dentre esses, Índia, China e Indonésia correspondem a 43% dos casos de tuberculose diagnosticados em 2014. Apesar do declínio que se tem observado nas taxas de incidência dessa patologia para as Américas com o passar dos anos, devido principalmente aos programas de controle estabelecidos pelos governos, muitas pessoas ainda morrem de tuberculose nessa região. A taxa de mortalidade associada à tuberculose para a população brasileira HIV negativa foi de 2,6 para 100 000 indivíduos, enquanto a incidência ficou em 44 para cada 100 000. A região das Américas, de maneira geral, atingiu as metas impostas pela Organização Mundial da Saúde para o ano de 2015, que consistiam em decaimento da taxa de incidência para a patologia e

redução de 50% nas taxas de prevalência e de mortalidade em relação ao ano de 1990. Em toda essa região, 77% dos casos de tuberculose foram detectados e 76% foram tratados com sucesso. Os dados são similares para a população brasileira, com 82% dos casos detectados e 72% dos casos tratados com sucesso. Essa taxa de sucesso no tratamento observada para o Brasil está entre as menores observadas para os países que compõem o grupo de risco, e tem se mantido aproximadamente a mesma desde o ano 2000 (WHO, 2015).

Contudo, claramente os grupos que mais sofrem com a tuberculose nas Américas são as populações indígenas. Essas populações nativas apresentam uma suscetibilidade diferenciada a doenças infecciosas introduzidas e algumas etnias já tiveram suas populações drasticamente reduzidas devido à mortalidade por tuberculose (Sousa *et al*., 1997; Hurtado *et al*., 2003; Escobar *et al*., 2004).

1.5 - Resposta imune à tuberculose

A infecção por Mtb necessita de respostas imunes do tipo humoral e celular, além da imunidade mediada pelas células T através das células $CD4^+$ e $CD8^+$, para um adequado combate ao desenvolvimento da doença após a infecção (Flynn & Chan, 2001). A resposta imune específica inicia-se com células T que migram para o foco da infecção, guiadas pelas citocinas produzidas pelas células infectadas. O acúmulo de macrófagos, células T e outras células do hospedeiro (como as dendríticas e os fibroblastos) levam a formação do granuloma no sítio de infecção (Gonzalez-Juarrero *et al*., 2001). A formação do granuloma impede que o bacilo se disperse para o restante do tecido pulmonar e provém um microambiente para interações entre macrófagos e outras células do sistema imune e para as citocinas produzidas por essas células. As células T CD4+ que produzem interferon gama (IFN-γ) reconhecem os macrófagos infectados que estão apresentando antígenos de Mtb e os matam (Ahmad, 2011). Contudo, esse processo raramente erradica o Mtb. Alguns bacilos resistentes são capazes de escapar da morte pelas células T e entrar em um estado de dormência, fugindo da eliminação pelo sistema imune do hospedeiro (Harding & Boom, 2010). Aproximadamente 90% dos indivíduos infectados com Mtb

desenvolvem uma infecção latente sem sinais clínicos aparentes, os 10% restantes desenvolvem tuberculose ativa (Figura 1).

Em aproximadamente 15% dos pacientes com doença pulmonar ativa, ocorre um processo associado com ausência de formação do granuloma e outras manifestações de hipersensibilidade celular, denominado anergia. A anergia, no contexto da tuberculose, é justamente a ausência de reatividade ao PPD em indivíduos infectados por Mtb. Imunologicamente a anergia envolve uma inabilidade das células T de produzir interleucina 2 (IL-2), a qual ocorre junto com um decréscimo na produção de IFNγ, principalmente em casos de doença grave (Delgado *et al*., 2002; Goldfeld, 2004). Anergia também tem sido correlacionada com a expansão de células T produtoras de IL-10 (Goldfeld, 2004).

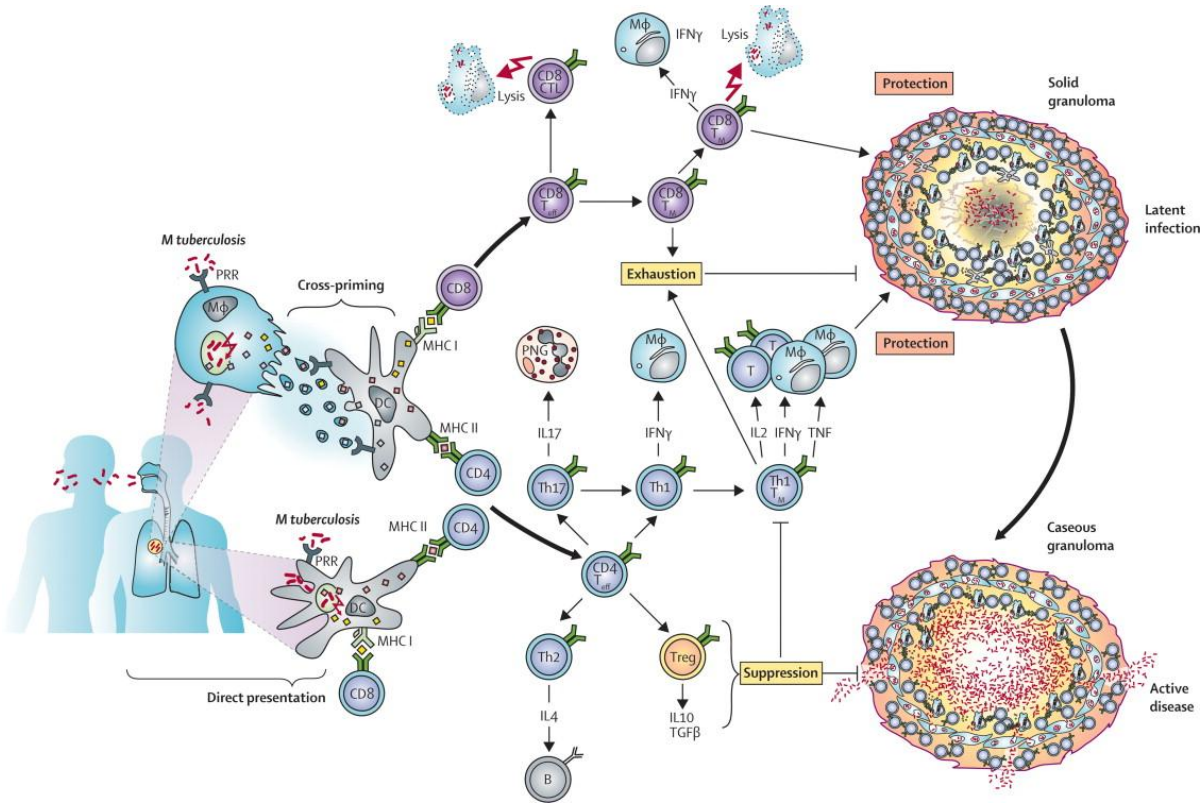

Figura 1- Esquema demonstrando as diferentes interações entre moléculas do sistema imune para desenvolvimento do granuloma, tanto nas infecções latentes quanto na doença ativa. A predominância da resposta Th1 desencadeada durante esse processo é demonstrada como protetora, uma vez que leva à infecção

latente. Quando ocorre supressão dessa resposta, a doença se torna ativa (imagem disponível em Kaufmann *et al*., 2010).

1.5.1- Resposta Inata na tuberculose

Acredita-se que os TLRs são os responsáveis pelo reconhecimento de antígenos derivados de Mtb, levando, portanto, à ativação de macrófagos e células dendríticas, assim como outras células do sistema imune inato. Essa classe de receptores poderia também facilitar o início da expressão de diversos genes de citocinas, regulando a resposta imune do tipo adaptativa (Turvey & Broide, 2010) (Figura 2).

Esses receptores são expressos tanto na superfície celular (como TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 e TLR12) como intracelularmente (como TLR7, TLR8 e TLR9). Vários desses receptores são capazes de reconhecer Mtb e seus componentes da parede celular, dentre eles podemos citar TLR1, TLR2, TLR4, TLR6 e TLR9 (Saraav *et al*., 2014).

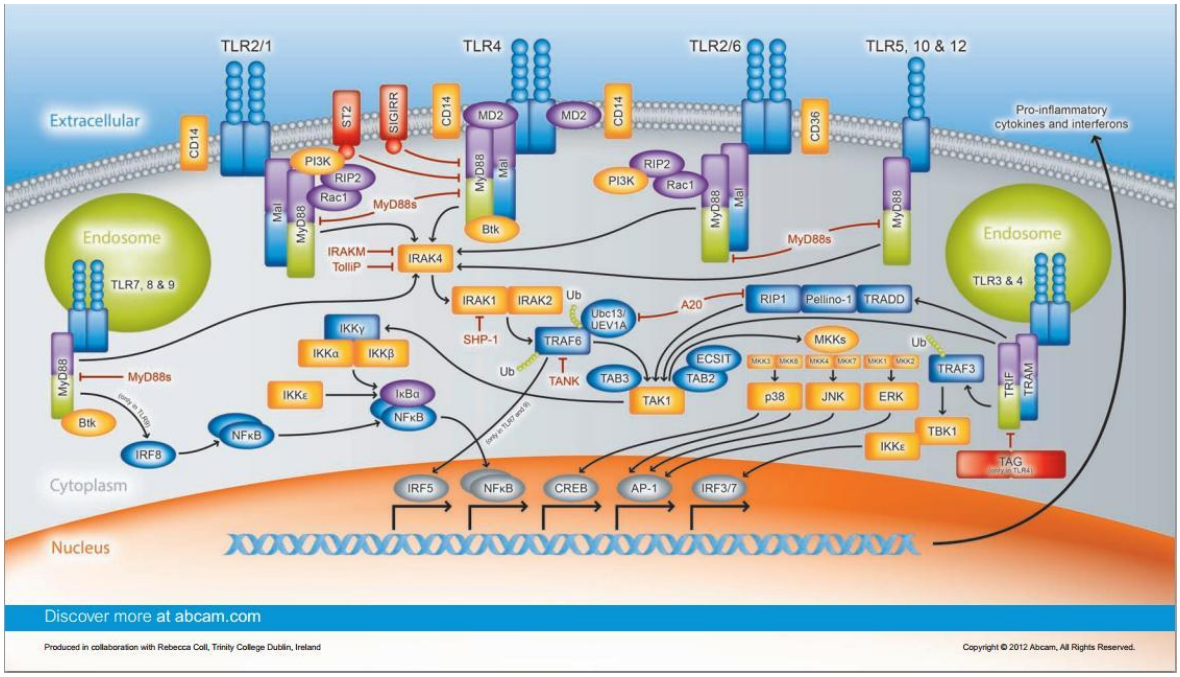

Figura 2- Esquema demonstrando as vias que são ativadas por TLRs. Conforme podemos perceber, esses receptores são responsáveis tanto pela sinalização para outras moléculas do sistema imune inato, quanto pela ativação da expressão

de citocinas pró-inflamatórias, que vão regular a resposta imune do tipo adaptativa (imagem disponível em www.abcam.com).

Diversos estudos tem demonstrado que Mtb é capaz de modular a resposta imune de maneira a desenvolver um ambiente favorável para sua proliferação. Essa modulação pode ser ao nível de imunosupressão de células T, regulação da expressão de TLRs ou alteração na expressão de citocinas. Devido a seu importante papel no reconhecimento da micobactéria e início de uma adequada resposta imune, as estratégias de evasão adotadas pelo Mtb acabam por afetar as vias de sinalização dos TLRs. Dentre essas podemos citar (a) manutenção da fusão fagolisossomal, (b) inibição de intermediários reativos de oxigênio e nitrogênio, (c) inibição da apoptose de macrófagos infectados e (d) interferência na apresentação de antígenos (Saraav *et al*., 2014). Portanto, a ação dos TLRs é diretamente relacionada com o tempo de ativação de suas vias de sinalização. Se essa ativação for demasiadamente prolongada, os mecanismos de evasão do Mtb poderão desenvolver-se; contudo, se essa ativação for demasiadamente curta, não teremos uma resposta subsequente adequada. Dessa maneira, os TLRs precisam manter o sinal de ativação por um tempo ótimo, ou seja, até que as moléculas presentes nas vias de sinalização subsequentes sejam ativadas e desencadeiem uma adequada resposta celular, sem que o Mtb consiga ativar seus mecanismos de evasão. Para tanto, não somente os TLRs são importantes para um adequado combate ao Mtb, mas também as moléculas presentes nas vias de ativação subsequentes, conforme demonstrado na figura 3.

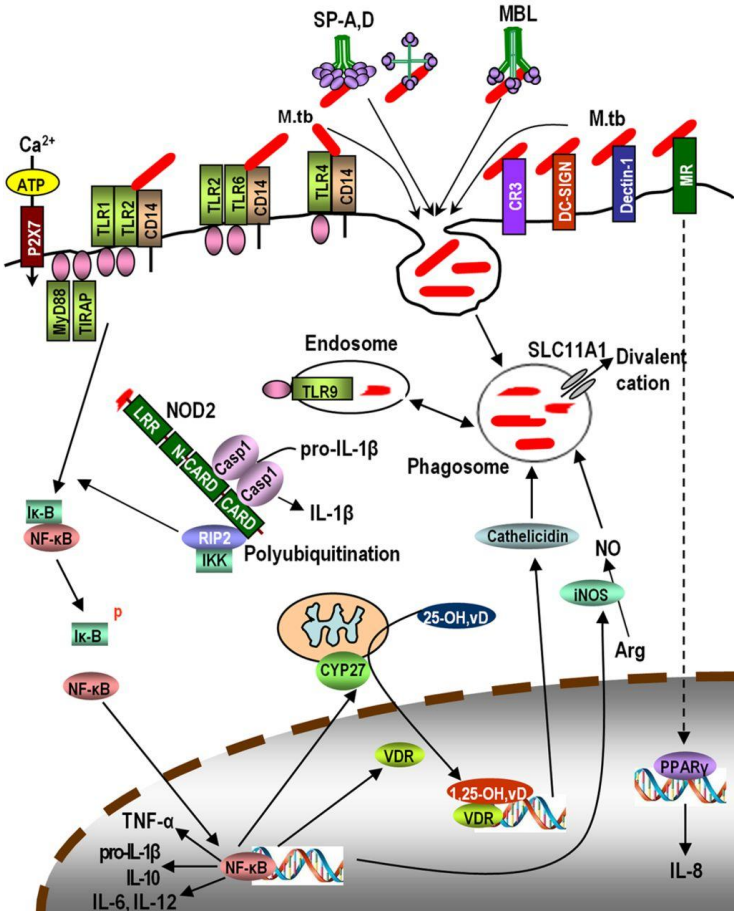

Figura 3- Esquema representando as vias de sinalização utilizadas pelo sistema imune inato nos macrófagos para combater adequadamente o Mtb (imagem disponível em Azad *et al*., 2012).

1.5.2- Resposta Adaptativa na Tuberculose

Está bem estabelecido que a imunidade mediada por células é requerida para uma resposta efetiva contra infecção por Mtb, enquanto que uma resposta do tipo Th1 parece ser essencial para um adequado combate ao patógeno (Figura 1). Portanto, indivíduos capazes de estabelecer uma adequada resposta imune do tipo Th1 são considerados mais protegidos para determinadas doenças infecciosas, dentre elas a tuberculose, em relação àqueles que não conseguem desenvolver adequadamente esse tipo de resposta (Salgame, 2005). As células Th1 secretam citocinas como interferon gama e ativam vias inflamatórias principalmente através da ativação de macrófagos. As células Th2 secretam

citocinas como as interleucinas 4 e 5, que regulam a formação de anticorpos através das células B, eosinófilos e outras vias (Kidd, 2003).

Um recente estudo demonstrou que células Th1 específicas estão presentes no sítio de infecção de pacientes com tuberculose. Além disso, observou-se a presença de células Th17 e Th22. Isso demonstra que essas células também podem ter um importante papel na imunidade contra Mtb (Ye *et al*., 2012). Em humanos as células Th17 originam-se em resposta à combinação de fator de transformação do crescimento beta (TGF-β) e citocinas pró-inflamatórias (IL1-β, lL-6, IL-21, IL-23) (Beriou *et al*., 2010). As células Th22 são aquelas que produzem citocinas como a IL-22, IL-26 e IL-13, das quais a IL-22 é a mais importante do ponto de vista funcional (Zhang *et al*., 2011).

As células Th9 caracterizam-se por produzir IL-9 e se desenvolvem a partir de precursores $CD4^+$ através do efeito combinado de TGF-β e lL-4. Essas células são capazes de induzir inflamação tecidual e contribuir para o desenvolvimento de doenças alérgicas. Ainda não está bem estabelecido se esse conjunto celular tem algum papel na imunidade infecciosa. A “tuberculous pleural effusion” (TPE) é uma reação de hipersensibilidade do tipo tardia grave em resposta à ruptura de um foco subpleural de infecção por Mtb. Já foi relatado um acúmulo de linfócitos, especialmente células T $CD4^+$ em TPE. Recentemente foi demonstrada também a presença de células Th9 nas TPE de pacientes chineses. As células Th9 observadas nas TPE apresentaram-se em um número muito maior àquelas observadas nas amostras de sangue dos indivíduos. A maioria dessas células estava organizada como células de memória (Ye *et al*., 2012). Esses achados, acrescentados às informações de estudos *in vitro,* sugerem que a expressão aumentada de IL-9 pode contribuir para o desenvolvimento da tuberculose (Wu *et al*., 2008).

Apesar de todo o conhecimento que vem sendo adquirido sobre o papel dos diferentes subconjuntos de células Th na resposta à tuberculose, o desequilíbrio entre as respostas desencadeadas pelos subconjuntos Th1 e Th2 ainda permanece como o principal ponto a ser discutido na imunologia da tuberculose. A diferenciação das células Th0 em Th1 ou Th2 é direcionada a partir da expressão de um conjunto de citocinas (figura 4). Nesse contexto, a

resposta Th1 é responsável pela imunidade mediada pelas células e a resposta Th2 é responsável pela imunidade mediada por anticorpos. Como já mencionado anteriormente, o adequado combate ao Mtb ocorre na presença de predominância de resposta do tipo Th1. Dentre as citocinas relacionadas com a predominância desta resposta podemos destacar a IL-2, IL-8, IL-12, IFN-γ e TNF-β. Enquanto que IL1-β, IL-4, IL-6 e IL-10 estão relacionadas com a predominância de um padrão Th2 de resposta imune.

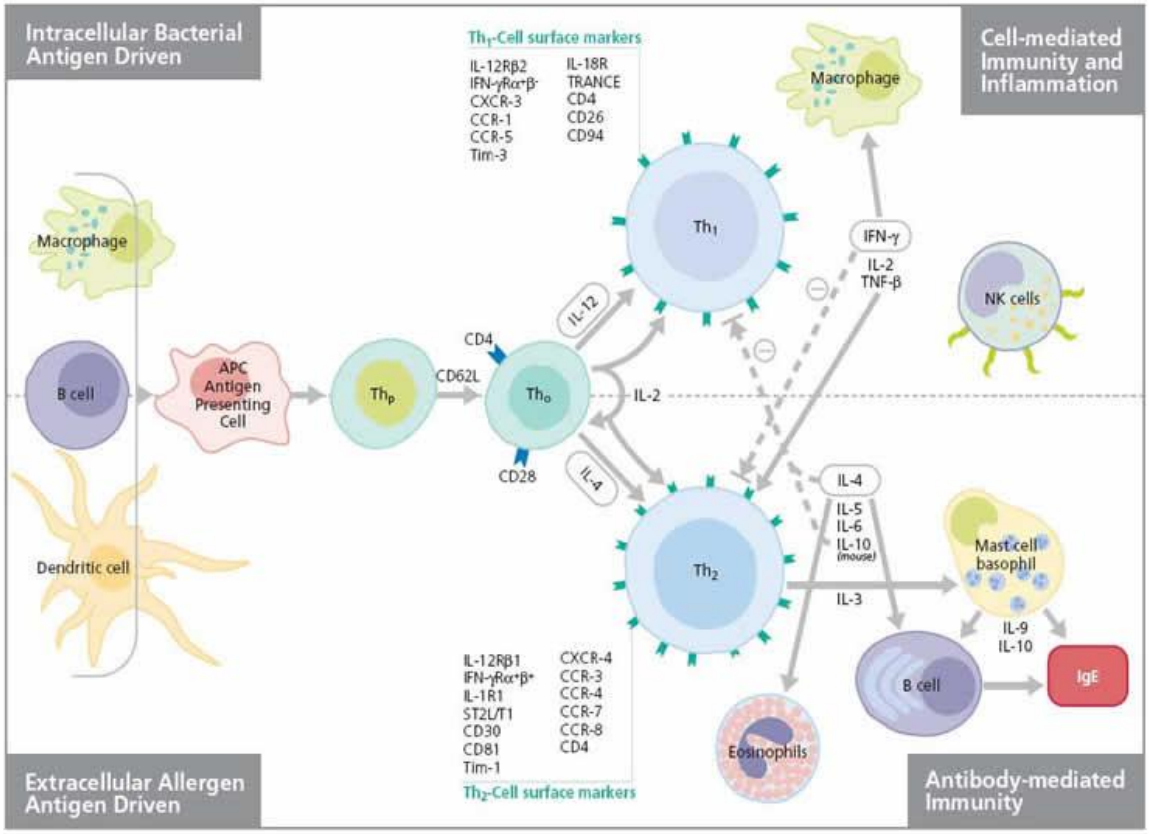

Figura 4- Diferenciação das células Th0 em Th1 ou Th2 através da sinalização de citocinas diante de exposição a antígenos. Antígenos intracelulares, como o Mtb, ativam predominantemente a diferenciação do subconjunto de células Th1, responsáveis pela imunidade mediada por células. A sinalização de citocinas como o IFN-γ e a IL-12 é crucial para a diferenciação nesse subconjunto. Antígenos extracelulares ativam predominantemente a diferenciação do subconjunto de células Th2, responsáveis pela imunidade mediada por

anticorpos. Citocinas como IL-4 e IL-10 são essenciais nesse processo (imagem disponível em www.bd.com).

1.6- Influência dos fatores genéticos na tuberculose

Do ponto de vista genético a tuberculose é uma doença complexa que resulta da interação entre o hospedeiro, o patógeno e o ambiente. Há evidências que fatores genéticos do hospedeiro são importantes na determinação da suscetibilidade ao Mtb. Os trabalhos relacionando resposta imune e tuberculose baseiam-se principalmente em duas frentes de estudo: variabilidade em HLA e em citocinas. As classes de HLA já foram associadas com suscetibilidade a doenças infecciosas (hepatite B, hepatite C, hanseníase, malária, leishmaniose e tuberculose) e apresentam resultados contrastantes dependendo da patologia e população analisada (Blackwell *et al*., 2009).

A família de HLA classe I humano compreende membros clássicos (classe Ia) e não clássicos (classe Ib). As moléculas que formam a classe Ia (HLA -A, -B e -C) são altamente polimórficas e compostas por 506, 872 e 274 diferentes variantes proteicas, respectivamente, com variações em potenciais ligantes de peptídeos. Genes de HLA classe Ib, por sua vez, são menos polimórficos : há 3, 4 e 10 variantes proteicas descritas para HLA -E, -F e –G, respectivamente (Joosten *et al*., 2010).

Muitos estudos indicaram que linfócitos T citotóxicos reconhecidos estritamente por HLA classe I tem um papel importante na rota de controle da infecção por Mtb (Wang *et al*., 2010). Além disso, macrófagos infectados por Mtb têm decréscimo na expressão de moléculas de HLA classe II e decréscimo na apresentação de antígenos, reduzindo o reconhecimento desses macrófagos infectados pelas células T CD4+. Comparações entre a taxa de reconhecimento pelas células T de macrófagos infectados e não infectados demonstraram que a redução no reconhecimento das células infectadas começa de 12 a 18 horas após a infecção (Harding & Boom, 2010). Em relação ao HLA observou-se que as populações ameríndias apresentam um número restrito de alelos, sendo que esse conjunto difere marcadamente entre as populações (Tsuneto *et al*., 2003). Cada alelo de HLA é capaz de se ligar a um determinado número de sequencias de

aminoácidos e então apresentar os antígenos a que correspondem essas sequencias para os linfócitos T ou B. O espectro de tipos de HLA acaba por limitar o espectro de antígenos reconhecidos.

Considerando o grande número de trabalhos envolvendo variabilidade em genes de citocinas, tanto inflamatórias quanto anti-inflamatórias, os resultados para suscetibilidade a tuberculose também são contrastantes em relação à população analisada. A título de exemplificação, podemos citar estudos com polimorfismos nos genes das citocinas IL-8 e IL-12. Variantes em *IL-8* foram associadas como de risco para tuberculose em uma população dos EUA, enquanto que as mesmas variantes não foram associadas em uma população de Gâmbia (Ma *et al*., 2003; Cooke *et al*., 2004). Para *IL-12* foi observada associação com suscetibilidade à tuberculose em populações de Guiné-Bissau e EUA, enquanto que para as populações chinesa, indiana e iraniana não se observou associação (Amirzargar *et al*., 2006; Prabhu Anand *et al*., 2007; Wang *et al*., 2010b). Até o momento somente o estudo de Zembrzuski *et al*. (2010) com a população Xavante analisou a variabilidade em genes de citocinas em populações nativas americanas. Nessa população, variantes nos genes *IFN-γ*, *IL-10* e *IL-4* foram associadas com reação ao PPD [uma completa discussão sobre a importância desse tipo de resposta imune para o combate ao Mtb pode ser encontrada em Jasenosky *et al*., 2015].

Diferentes marcadores do sistema imune inato apresentam associação com suscetibilidade à tuberculose ou com reação ao PPD. Diversos estudos demonstraram a importância do sistema imune inato para a patologia da tuberculose. Foram observadas associação entre tuberculose e variabilidade genética nos receptores de reconhecimento de padrão, nas colectinas, nas citocinas e quimiocinas, além de outros marcadores como iNOS e SLC11A1. Novamente a título de exemplificação, podemos destacar alguns dos resultados em cada uma dessas classes [uma completa discussão pode ser encontrada em Azad *et al*., 2012].

Conforme discutido anteriormente, os TLRs são moléculas centrais para a progressão da resposta imune inata. Variabilidade genética em TLR1, TLR2, TLR8 e TLR9 já foi associada com suscetibilidade a tuberculose em diferentes

grupos étnicos (Azad *et al*., 2012). TLR1 e TLR9 já foram associados com suscetibilidade à tuberculose em populações afro-americanas dos EUA (Ma *et al*., 2007), enquanto que populações de caucasianos desse mesmo país tiveram esse aumento na suscetibilidade relacionado com TLR2 e TLR9 (Velez *et al*., 2010). No entanto, em uma população de caucasianos da Europa também foi possível observar associação entre TLR1 e tuberculose (Uciechowski *et al*., 2011). Assim como observado para os resultados de associação entre tuberculose e marcadores do sistema imune adaptativo, os marcadores de sistema imune inato apresentam um padrão de alteração na suscetibilidade à patologia contrastante em função das populações analisadas. Isso demonstra a importância de considerarmos a variabilidade genética específica de cada população quando estivermos discutindo suscetibilidade a doenças multifatoriais, como a tuberculose.

Dentre as quimiocinas que já foram relacionadas com suscetibilidade à tuberculose podemos destacar a proteína quimiotática de monócitos-1 (MCP-1, também conhecida como CCL2) e CCL5 (RANTES / regulated upon activation, normal T-cell expressed, and secreted). Os marcadores associados com tuberculose nos genes codificantes dessas quimiocinas encontram-se nas regiões promotoras e possivelmente afetam a taxa de transcrição dos mesmos. Diversos estudos mostraram que CCL2 tem um papel relevante na patologia da tuberculose em populações africanas e asiáticas (Flores-Villanueva *et al*., 2005; Thye *et al*., 2009), enquanto que CCL5 parece ser importante para a suscetibilidade à tuberculose em populações de origem europeia e asiática (Chu *et al*., 2007; Sánchez-Castañón *et al*., 2009; Ben-Selma *et al*., 2011). Até o momento a variabilidade em genes de resposta imune inata não foi investigada em populações nativas sul americanas e sua importância para a suscetibilidade a doenças infecciosas nesses grupos não foi discutida.

1.7- Os Aché e a Tuberculose

Os primeiros antropólogos que estudaram a população Aché descreveram-na com características físicas muito diferentes daquelas observadas nos demais grupos indígenas. Eles apresentavam, por exemplo, pele, olhos e cabelos claros,

barba espessa, braços longos, dentre outras características morfológicas distintivas. Atualmente eles se mantêm morfologicamente diferentes dos demais grupos nativos, mas apresentam uma gama de características que são comuns às populações ameríndias como um todo (Callegari-Jacques *et al*., 2008).

A população Aché vive em uma área de 60 000 km² no sul do Paraguai e compreende quatro grupos principais (Nacunday, Northern, Ypety e Yvytyruzu). Durante os primeiros 400 anos após a chegada dos espanhóis, essa população manteve-se hostil, tanto em relação à população colonizadora quanto às demais populações indígenas que habitavam a região. Eles viviam em pequenos grupos, geralmente composto de 15 a 70 indivíduos, e mudavam de lugar constantemente. Essa população começou a ser reunida em uma reserva durante os anos de 1972 e 1973 e foi a partir desse momento que eles passaram a manter contato com os demais grupos. Atualmente, os Aché vivem em cinco reservas principais com uma população composta por cerca de 1000 indivíduos. Sua economia é mista, com algumas comunidades completamente dependentes da agricultura e criação de animais, enquanto outras ainda permanecem parcialmente dependentes da caça e da coleta (Hurtado *et al*., 2003).

Dados da epidemia de tuberculose nessa população mostram que se não tivesse ocorrido tratamento com agentes antimicrobianos, mais de 18% da população teria morrido devido à tuberculose na primeira década após a exposição. Parte da população já foi vacinada com BCG. Diferentemente do que se esperaria, uma grande fração desses indivíduos imunizados (93,3%) apresentam respostas negativas ao teste do PPD. Entre os indivíduos não imunizados a porcentagem de respostas negativas foi de 68,3%. A taxa de incidência acumulada anual de tuberculose ativa observada para a população Aché, considerando os cinco anos iniciais da epidemia de tuberculose (de 1987 a 1992) foi de 3,7% (3700 para 100 000 indivíduos). Essa taxa foi vinte vezes maior do que aquela obervada para a população do Paraguai e cerca de dez vezes maior do que aquela obervada para áreas indígenas paraguaias onde a tuberculose tem sido um problema de saúde pública por décadas (Hurtado *et al*., 2003).

Capítulo II – Justificativa e Objetivos

Embora o número de indivíduos que morrem ou desenvolvem tuberculose seja alarmante, esses números representam uma pequena proporção dos bilhões de indivíduos estimados de estarem infectados com Mtb, ou seja, a grande maioria desenvolve uma resposta imune apropriada e controla a infecção sem o desenvolvimento de doença clínica.

A tuberculose é altamente endêmica no Brasil, mesmo com todas as medidas de controle que são tomadas pelo Governo. A incidência anual de casos notificados supera 40 casos por 100 000 habitantes. Em alguns estados e municípios da região Amazônica a taxa de incidência supera 70 casos para cada 100 000 habitantes (Kritski & Ruffino-Netto, 2000; Barreto *et al*., 2002). Fatores ambientais como baixa condição econômica, desnutrição e estresse tem um papel muito importante na prevalência dessa patologia nas populações humanas. Contudo, fatores genéticos do Mtb e do hospedeiro também são importantes na determinação de uma adequada resposta imune contra a tuberculose.

Entre as populações indígenas do Brasil, a tuberculose é um fator de morbidade e mortalidade importante. Os estudos mais recentes sobre a imunologia da tuberculose em populações indígenas baseiam-se nos resultados obtidos no teste de reatividade à tuberculina (PPD). Esses estudos relatam uma baixa reatividade ao PPD nesses grupos quando comparadas àquelas observadas em populações de descendência europeia.

Uma prevalência extremamente alta de tuberculose ativa (6,4%) foi observada entre os Yanomami que vivem na Amazônia. Essa é uma taxa 100 vezes maior do que aquela observada para a população não nativa que habita a mesma região. Além disso, as taxas de anergia ao PPD foram muito altas também (76%) (Sousa *et al*., 1997). Essa alta taxa de anergia também pode ser observada nos Pakaanóva que habitam uma área de fronteira com a Bolívia, no estado de Rondônia. Nesse grupo 54,8% da população se encaixou na categoria de não reatores ao PPD (Escobar *et al*., 2004).

Em 2003, a população Surui, que também habita o estado de Rondônia, apresentava uma taxa de prevalência de tuberculose de 815,2 para 100 000 habitantes. Esse resultado é muito maior do que aquele observado para a população de Rondônia como um todo na mesma época, que foi de 37,5 para 100

000 habitantes. Esse grupo também teve uma alta taxa de anergia, excedendo 60% (Basta *et al*., 2006).

Mesmo com todas as medidas de controle da tuberculose que são tomadas nessas populações, os dados de incidência dessa doença mostram que essas ações não estão sendo eficazes para alguns grupos. Um exemplo são os Guarani-Kaiowá, que habitam o Brasil Central. Esse grupo, em 2003, apresentou uma taxa de incidência de 740 para 100 000 habitantes, que se manteve a mesma desde 1967, enquanto que a taxa para o Mato Grosso do Sul foi de 49 para 100 000 habitantes (Marques & da Cunha, 2003).

Se considerarmos as informações de taxa de anergia entre os indivíduos vacinados com BCG para as populações Aché e Xavante (93,3% e 74,4% respectivamente), percebemos que as taxas de anergia são geralmente maiores do que 50% para populações nativas sul americanas, mesmo em populações com uma abrangente vacinação por BCG.

A BCG é a vacina mais amplamente usada no mundo, com estimativas de que mais do que 3 bilhões de pessoas tenham sido vacinadas. Entretanto, BCG só oferece proteção eficiente contra tuberculose em crianças e tem um efeito limitado sobre tuberculose pulmonar em adultos. As estimativas de proteção pela BCG variam de 0 a 80% (Fine, 1995). Portanto, o desenvolvimento de novas vacinas mais eficientes capazes de oferecer proteção contra tuberculose é muito necessário.

A compreensão de fatores genéticos que distinguem indivíduos resistentes daqueles suscetíveis possui importantes implicações para o desenvolvimento de novas vacinas e terapias que considerem a predisposição genética e os fatores ambientais vivenciados pelas populações.

Portanto, este trabalho tem como objetivos específicos:

1- Determinar o padrão de variabilidade observado em genes de resposta imune adaptativa e inata em populações nativas sul americanas, relacionando com aquele observado em outros grupos continentais.

2- Determinar quais dessas variantes estão relacionadas com suscetibilidade a tuberculose e/ou com anergia ao PPD na população Aché.

Capítulo III - Distribution patterns of variability for 18 immune system genes in Amerindians – relationship with history and epidemiology

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Distribution patterns of variability for 18 immune system genes in Amerindians – relationship with history and epidemiology

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Abstract

Native American populations generally have a higher prevalence of infectious diseases than non-Native populations and this fact can induce different pressures in their immune system. We investigated the patterns of population differentiation (F_{ST}) of 32 polymorphisms related to adaptive immune response in four Native American populations (Aché, Guarani-Kaiowá, Guarani-Ñandeva and Kaingang), and the results were compared with the three major world population data [Yoruba of Ibadan, Nigeria (YRI), Utah residents with northern and Western Europe ancestry (CEU) and Han Chinese of Beijing, China (CHB)] available in the HapMap database. The Aché clearly differentiated from the other Amerindians, but when all Native Americans were compared with the samples of other ethnic groups the lowest difference (0.08) was found with CHB (Asians), the second lowest (0.15) with YRI (Africans) and the most marked with CEU (European-derived). The considerable intra and interethnic differences found can be explained both in terms of diverse evolutionary distances and more recent environmental pathogen exposures; and they should be appropriately considered prior to any specific public health action.

Introduction

Populations with frequent epidemics that are characterized by many new cases of illness over a short period of time are considered susceptible to the pathogen responsible for the illness. Epidemiologically, they may be unable to produce the 'appropriate' immunological response when faced with pathogenic disease (1). The lack of resistance is usually measured in terms of clinical symptoms and the presence of antibodies or cells having a response to specific antigens against the pathogenic agent due either to their life history or biological constitution. There is now a large amount of data indicating that Native American populations are immunologically different from non-Native populations (2).

Several pieces of evidence point to a major role of host genetic constitution in explaining inter-individual variation in susceptibility to infectious diseases. Previous studies reported that Native American populations have a lower variability in several immune system genes such as KM, GM, Kell and HLA (3, 4). The human leukocyte antigen (HLA) system reduced diversity seems to be especially important for introducing disease susceptibility in Amerindians (5). However, the immune response is dependent of others factors as well. One of these factors is the T-helper (Th) immune response, which is predominantly composed by the balance between Th1, Th2, Th9 and Th17 cells. The differentiation into a specific Th subset is directed by polarizing cytokines and expression of master transcription factors. Every type of these Th cells fight against a different antigen. Th1 cells are hypothesized to lead the attack against intracellular pathogens such as viruses, raise the classic delayed-type hypersensitivity (DTH) skin response to viral and bacterial antigens, and fight cancer cells (6). Th2 cells are believed to enhance protection against extracellular pathogens such as multicellular parasites (6). Th9 cells are proinflammatory, but appear to function in a broad spectrum of autoimmune diseases and allergic

inflammation (7). Th17 has been shown to be an important CD4 T cell subset in human autoimmune diseases, including rheumatoid arthritis and multiple sclerosis (8, 9).

There are several examples that show the adverse consequences of imbalanced cytokine networks during infection: immunopathological lesions when proinflammatory cytokines are not controlled or regulatory cytokines are absent; and inefficient virus elimination, leading to chronicity or pathogen-induced death when the cytokine response is qualitatively aberrant or too weak (10). Numerous other molecules are also required for an adequate immune response development. These molecules can act on different substrates, functioning as signals for specialization of naive T cells into specific subsets of Th cells, or acting on the recruitment of other immune cells or receptor formation.

Environmental exposure to ectoparasites, helminths and physical injuries remained high during the evolutionary history of Native Americans, but not of non-Native American populations (11–16). We therefore expect a different pattern of variability in genes related to the immune response between these two groups. But knowledge about these genes and their variability is still scarce in Amerindians.

In a previous study with a Xavante population of Central Brazil, we observed that allele frequencies for polymorphisms in immune system genes differed from those reported in other major ethnic groups; for instance, *SP110*, *PTPN22*, *IL12RB1* and *IL6* single-nucleotide polymorphisms (SNPs) were not polymorphic in this community (17). This study describes the frequencies of 32 SNPs in immune system genes in four Native South American populations, discusses their evolutionary relationships and their possible adaptations to different relatively recent pathogen exposure.

Methods

Subjects

The Native American populations considered, and the characteristics of the samples investigated are described in Table 1 and their geographical location is shown in Figure 1. Additional information about them follows.

The Aché (or Guayaki) are Tupi-speakers living in eastern Paraguay. They remained isolated from non-Amerindians, subsisting basically in a hunter–gatherer way of living, until the 1970s, when more permanent contact was established. Hill and Hurtado (18, 19) reported an extensive project of study of this population, that included historical, demographic, social and medical data, and these studies are continuing up to the present. Seventeen genetic studies performed among them were reviewed by Callegari-Jacques et al. (20), and aspects related to their exposure and eventual reaction to *Mycobacterium tuberculosis* was examined by Hurtado et al. (2) and Wilbur et al. (21). Currently there are about 1000 Aché living in several rural settlements. A total of 99 subjects were sampled for this study.

Table 1 Characteristics of the populations investigated

Populations and number of individuals investigated	Aché 99	Guarani Kaiowá 72 Ñandeva 72	Kaingang 72
Localities	Arroyo Bandera Chupa-pou	Amambai Limão Verde Porto Lindo	Nonoai
Geographic location	55°W, 23°S 56°W, 24°S	55°W, 23°S 55°W, 23°S 54°W, 23°S	52°W, 27°S
Country and region	South Paraguay	Central Brazil	South Brazil
Linguistic group	Tupi	Tupi	Jê
Non-Indian admixture (%)[a]	0.0	3.0	6.6

[a] Estimated by Callegari-Jacques et al. (72).

Figure 1 Geographic location of the populations investigated in this study.

Aché's origin has been the subject of much controversy, but basically two hypotheses have recently been considered: (a) they would be remnants of a prehistoric Jê population who adopted the Guarani language and culture; or (b) they would be a Guarani group who migrated to the forest and completely lost agricultural skills. These contrasting views will be considered in this article.

Kaingang's territory has always been southern Brazil, and they presently live in four Brazilian states (São Paulo, Paraná, Santa Catarina and Rio Grande do Sul). They are the third

most frequent Native American population in this country, numbering about 28,000 persons. Their presence in the region was already documented at the end of the 16th century (22–24) and population data from Nonoai, the community from which we obtained 72 samples, are available since 1849 (25). Genetic, medical and demographic information about them have been obtained since the 1960s and a selected bibliography can be found in Marrero et al. (26). Relatively recent information about their health status can be found in Hökerberg et al. (27), Diehl (28) and Souza et al. (29). Updated information about them can be accessed at www.socioambiental.org.

The Guarani are the most populous Native American population in Brazil (about 46,000 subjects). Presently, there are Guarani settlements in seven states of the Brazilian south and southeast. Contact with non-Amerindians date to the beginning of the Spanish and Portuguese Conquests, in the 16th century (30). Three main cultural-linguistic subdivisions can be discerned among them: Ñandeva, M'byá (Kaiwá) and Kaiowá. Schaden (31) has written a classical monograph about them, and Monteiro (32) compiled rich historical information about the Guarani-Kaiowá. Extensive genetic comparisons between Guarani and Kaingang have been undertaken, and a review can be found in Marrero et al. (26). Selected examples about medical aspects considered among them are those of Morgado (33; suicide prevalences); Marques and Cunha (34; tuberculosis incidence and treatment); and Souza et al. (29; seroprevalence of herpersvirus type 8). Additional information can be obtained at www.socioambiental.org. A total of 72 Guarani-Ñandeva and 72 Kaiowá were included in this study.

Selected genes

We choose 18 representative genes involved in different stages of the immune response (Table 2). The 32 polymorphisms analyzed in these genes were selected based on association studies with infectious diseases in different populations. Some of these genes are cytokines acting as differentiation molecules, whereas others act as auxiliary factors in the immune response.

Auxiliary factors to the adequate immune response development are the *SP110*, *P2X7*, *VDR*, *PTPN22* and *IL-8* gene products. Very important for monocyte differentiation, apoptosis and activation, including the response to pathogens, the *SP110* gene encoded a component with the same name that participate of the nuclear body, a multiprotein complex assumed to be involved in the regulation of gene transcription (35). Another gene that encoded a molecule that can mediate cell death, killing of infectious organisms and regulation of inflammatory response is *P2X7*. The P2X receptors open a cation-selective channel within milliseconds of ATP binding (36).

The vitamin D receptor (VDR) is involved in a wide range of biological functions, including mediation of vitamin D3 interactions with the immune system, exerting immuno-modulatory effects, which activate monocytes (37). Other important signaling regulator is lymphoid tyrosine phosphatase (LYP), encoded by the *PTPN22* gene, that is an important negative regulator of signal transduction through the T cell receptor (TCR) (38). Among the chemokines, the most potent is interleukin-8 (IL-8 or CXCL8), that have neutrophil chemotactic properties. It is a very important molecule because during infection the neutrophilic granulocytes are often the first immune cells to invade the inflammatory site (39).

Another immune category is composed by molecules involved in the orchestration and regulation of the immune response, as Interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-12, IL-2, IL-6, IL-10 and IL-4. IFN-γ is produced as a by-product of the activation of immune defense mechanisms early in infection or by antigen-specific T cells following the induction of specific immunity. IFN-γ is secreted by subsets of activated CD4+ and CD8+ T cells, as well as activated natural killer (NK) cells and exerts its enormous variety of effects via a specific IFN-γ receptor (IFN-γR) (40). Another gene encoding a molecule that needs a receptor to function is TNF-α . There are two receptors for TNF-α, coded by TNFR1 (also referred to as TNFRSF1A) and TNFR2, and efficient TNF-α binding depends on the self-assembly of these two receptors. Both receptors have been shown to influence TNF-α level. The *TNF*-α gene resides in the major histocompatibility complex (MHC) class III region on chromosome 6 and is in high linkage disequilibrium with MHC class I and class II genes, which code for the various HLA subtypes (41).

Large levels of IL-1β activate platforms, known as inflammasomes, which assemble in response to pathogen-associated molecules. Inflammasome formation and IL-1β activity are required to efficiently control viral, bacterial and fungal pathogen infections (42). IL-12 works as a heterodimeric cytokine composed of p40 and p35 subunits to yield IL-12p70. It is produced primarily by antigen-presenting cells and exerts immunoregulatory effects on T and NK cells (43). IL-2 is important for the proliferation of activated T-lymphocytes and therefore for phagocyte activation involved in the elimination of absorbed material. IL-2 can also enhance activation-induced T-lymphocytes cell death and eliminate self-reactive cells; in addition it participates in the suppression of lymphocyte response by inducing appropriate T cells (44). IL-6 has been shown to stimulate C-reactive protein (CRP) secretion an important biomarker of proinflammatory status in several diseases (45). IL-6 is a proinflammatory cytokine which induces T cell activation and proliferation (46). IL-10 action results in the downregulation of MHC class II proteins and costimulatory molecules on the surfaces of target macrophages. It diminishes the capacity of innate

Table 2 Allele frequencies for 32 single-nucleotide polymorphisms (SNPs) of 18 immune system genes in four Amerindian populations compared to corresponding data of HapMap CEU, YRI and CHB samples

Gene	dbSNP ID	Allele	CEU	YRI	CHB	Aché 99[a]	Kaingang 72[a]	Guarani-Kaiowá 72[a]	Guarani-Ñandeva 72[a]	Amerindians 315[a]
IFN-γ	rs2430561	A	0.433	0.195	0.167	0.005	0.111	0.014	0.028	0.037
IFN-γR1	rs1327474	G	0.398	0.031	0.098	0.347	0.361	0.111	0.153	0.252
	rs2234711	C	0.353	0.480	0.442	0.199	0.347	0.257	0.313	0.272
IL-1β	rs1143629	T	0.633	0.550	0.533	0.036	0.264	0.056	0.125	0.113
	rs1143627	T	0.637	0.354	0.524	0.036	0.257	0.056	0.111	0.108
	rs16944	G	0.642	0.420	0.537	0.036	0.257	0.056	0.111	0.108
IL-2	rs2069762	G	0.232	0.000	0.273	0.031	0.204	0.556	0.694	0.343
IL-4	rs2243250	C	0.863	0.212	0.305	0.207	0.535	0.370	0.274	0.334
IL-4R	rs1801275	G	0.199	0.841	0.195	0.263	0.410	0.444	0.292	0.344
IL-6	rs1800795	C	0.535	0.000	0.000	0.000	0.104	0.014	0.007	0.029
IL-8	rs4073	A	0.400	0.825	0.389	0.327	0.292	0.083	0.215	0.237
IL-10	rs1800872	C	0.788	0.527	0.262	0.071	0.451	0.264	0.493	0.299
	rs1800896	G	0.531	0.274	0.024	0.005	0.194	0.035	0.056	0.067
	rs1800871	C	0.827	0.536	0.312	0.071	0.451	0.250	0.507	0.299
IL-12α	rs568408	A	0.167	0.223	0.062	0.260	0.035	0.000	0.021	0.094
IL-12β	rs3212227	C	0.190	0.327	0.415	0.031	0.354	0.704	0.543	0.375
	rs7709212	T	0.681	0.748	0.598	0.327	0.292	0.285	0.403	0.326
	rs2546890	A	0.562	0.314	0.476	0.321	0.229	0.271	0.403	0.307
IL-12Rβ1	rs375947	C	0.353	0.257	0.195	0.010	0.222	0.028	0.164	0.099
	rs11575934	G	0.375	0.108	0.378	0.010	0.222	0.021	0.136	0.088
PTPN22	rs2476601	T	0.117	0.005	0.012	0.000	0.021	0.000	0.007	0.006
P2X7	rs3751143	C	0.177	0.093	0.256	0.422	0.174	0.222	0.246	0.278
SP110	rs2114592	T	0.094	0.173	0.268	0.000	0.042	0.007	0.000	0.011
	rs3948464	T	0.146	0.124	0.000	0.000	0.035	0.125	0.042	0.046
TNF-α	rs1800629	A	0.173	0.088	0.146	0.000	0.021	0.007	0.007	0.008
	rs1799964	C	0.212	0.124	0.171	0.321	0.194	0.208	0.243	0.248
	rs361525	A	0.074	0.800	0.044	0.313	0.083	0.007	0.021	0.124
	rs1800630	A	0.150	0.097	0.146	0.005	0.164	0.402	0.349	0.204
	rs1799724	T	0.067	0.033	0.178	0.191	0.347	0.472	0.364	0.332
TNF-αR1	rs4149622	G	0.000	0.650	0.125	0.000	0.063	0.049	0.085	0.045
VDR	rs10735810	A	0.412	0.192	0.366	0.253	0.451	0.451	0.349	0.365
	rs1544410	A	0.438	0.279	0.061	0.253	0.139	0.098	0.047	0.147

CEU, Utah residents with northern and Western Europe ancestry; CHB, Han Chinese of Beijing, China; YRI, Yoruba of Ibadan, Nigeria.
[a] Number of individuals investigated.

immune cells to kill pathogens, and reduces their capacity to generate and maintain responsive antigen-specific T cells (47). Finally, IL-4 is derived primarily from CD4 T cells and mast cells, exhibiting a broad spectrum of immune cell biological activities. *IL-4* is essential for total immunoglobulin and IgE production; and it has also been suggested that polymorphisms in this gene could be associated with changes in total serum IgE levels (48).

Laboratory and statistical methods

Genomic DNA was extracted from blood samples and genotyping was carried out by TaqMan® SNP Genotyping Assay methods (Applied Biosystems, Foster City, CA). Allele frequencies were directly obtained by gene counting and compared with those of the Yoruba of Ibadan, Nigeria (YRI), Utah residents with northern and Western Europe ancestry (CEU) and Han Chinese of Beijing, China (CHB) obtained from the HapMap database (www.hapmap.org). Allele frequencies for the non-Native American populations obtained from the database were based in different sample sizes according to the variant. The number of chromosomes considered ranged from 68 to 226 for the CEU population; 100 to 226 for YRI; and 80 to 120 for CHB. Hardy–Weinberg equilibrium (HWE) was tested for each locus within each population using Markov chain as implemented in ARLEQUIN v.3.5. (49). Mean heterozygosities and their standard errors (50) were calculated with the DISPAN software (51). Since these estimates do not follow a normal distribution, they were compared across populations with the Friedman test using the SPSS v.18 software. Interpopulation variability was determined by F_{ST} (52) using the ARLEQUIN v.3.5 program, and their 95% confidence intervals were estimated with the R software using the DIVERSITY package with 3000 bootstraps [http://CRAN.R-project.org/package=diversity]. The D_A genetic distances (53), as well as

neighbor-joining dendrograms (54) were estimated employing the POPTREE software.

Results

Table 2 shows the minor allelic frequencies (MAF) for the polymorphisms in the Native American populations studied here and their frequency range in the HapMap populations. There is considerable variability both within Amerindians and between them and the three other ethnically different samples. Especially noteworthy are the following within Amerindians characteristics: (a) In 15 of the 32 distributions (47%) the Aché present the lowest and the Kaingang the highest frequencies; and (b) In 24 of them (75%) the Guarani-Kaiowá and Guarani-Ñandeva prevalences occupy adjacent positions. As for the interethnic comparisons, the Amerindians show the lowest or second lowest frequencies in 19 of the 32 comparisons (59%). The genotype frequencies for all SNPs evaluated in this study are presented in Table S1. The observed genotype distributions were in agreement with HWE for all SNPs with the exception of rs2243250 in the Guarani-Ñandeva, rs1800630 in Guarani-Ñandeva and Guarani-Kaiowá, and rs1544410 in the Aché. Small sample sizes involved in the comparisons, and eventual deviations from random mating that may occur in these relatively small populations, could account for these findings.

The mean heterozygosity (Table 3) ranged between 0.31 ± 0.03 (Kaingang) and 0.20 ± 0.03 (Aché). Overall heterozygosities varied significantly among groups (Friedman test, $P < 0.001$). After pairwise comparisons two subgroups could be discerned: Kaingang (higher values), and Aché plus Guarani-Kaiowá (lower heterozygosities). Mean heterozygosities were not significantly different when the Amerindian were pooled and compared with HapMap populations (Friedman test, $P = 0.192$; Table S2).

The F_{ST} results obtained for the Native American pairwise comparisons are shown in Table 4. The Guarani subgroups have a low differentiation ($F_{ST} = 0.04$), the values in relation to the Kaingang being similar (0.05–0.07). The Aché, on the other hand, clearly differentiated from the others (Aché *vs* Kaingang: 0.15; *vs* Guarani: 0.15–0.21).

When the four Native American populations were grouped and compared to the non-Native populations (Table 5), the Amerindian/Asian value was of 0.08, almost doubling (0.15) in relation to Africans or tripling when compared to Europeans (0.23). In relation to an Admixed Brazilian population (55), the F_{ST} was almost 0.11.

Essentially the same results were obtained with the genetic distances and dendrograms (data not shown).

Table 3 Mean heterozygosity for 32 single-nucleotide polymorphisms (SNPs) in four Amerindian populations[a]

	Ache	Ñandeva	Kaiowá	Kaingang
rs2243250	0.33	0.40	0.47	0.50
rs4073	0.44	0.34	0.15	0.42
rs361525	0.43	0.04	0.01	0.15
rs1800630	0.01	0.46	0.48	0.28
rs2114592	0.00	0.00	0.01	0.08
rs1800871	0.13	0.50	0.38	0.50
rs2228570	0.38	0.46	0.50	0.50
rs1799724	0.31	0.47	0.50	0.46
rs1801275	0.39	0.42	0.50	0.49
rs1800896	0.01	0.11	0.07	0.31
rs2069762	0.06	0.43	0.50	0.33
rs568408	0.39	0.04	0.00	0.07
rs1800872	0.13	0.50	0.39	0.50
rs3212227	0.06	0.50	0.42	0.46
rs11575934	0.02	0.24	0.04	0.35
rs1800629	0.00	0.01	0.01	0.04
rs7709212	0.44	0.48	0.41	0.42
rs3948464	0.00	0.08	0.22	0.07
rs1143627	0.07	0.20	0.11	0.38
rs1143629	0.07	0.22	0.11	0.39
rs3751143	0.49	0.37	0.35	0.29
rs2476601	0.00	0.01	0.00	0.04
rs4149622	0.00	0.15	0.09	0.12
rs2234711	0.32	0.43	0.38	0.46
rs1544410	0.38	0.09	0.18	0.24
rs1327474	0.46	0.26	0.20	0.46
rs2430561	0.01	0.05	0.03	0.20
rs16944	0.07	0.20	0.11	0.38
rs1799964	0.44	0.37	0.33	0.31
rs1800795	0.00	0.01	0.03	0.19
rs2546890	0.44	0.48	0.40	0.36
rs375947	0.02	0.28	0.05	0.36
Mean ± SD	0.20 ± 0.03^{a}	$0.27 \pm 0.03^{b,c}$	$0.23 \pm 0.03^{a,b,c}$	$0.31 \pm 0.03^{b,c}$

[a] Comparison among heterozygosities: Friedman; $P < 0.001$. Heterozygosities indicated by italics same letter (a, b and c) do not differ significantly by pairwise comparisons.

Discussion

South Native American populations today continue to be vulnerable to all sorts of diseases. The life expectancy at birth in 2000 was frequently 20 years lower among indigenous groups when compared with their non-indigenous counterparts in most countries of South America. The situation is so disappointing that the life expectancy of indigenous peoples in Brazil and Venezuela is lower than that for the United States in 1900 and lower than in Serra Leone in 2000, which has the lowest reported national life expectancy in the world (56). In 1993, the Council of the Pan American Health Organization (PAHO) passed Resolution V – Health of Indigenous Peoples. This document clearly identifies infectious diseases as a major threat to the welfare of indigenous groups (1). Therefore, studies of their immune system can give clues about what happens to populations under heavy epidemiological stress, relating their variability with both their evolutionary history and present health conditions.

The first point to be stressed in our results is the clear differences between the Aché population and the other three

Table 4 Pairwise F_{ST} among Amerindians populations with 95% CI[a]

	Aché	Kaingang	Guarani-Ñandeva	Guarani-Kaiowá
Kaingang	0.146 (0.124–0.177)			
Guarani-Ñandeva	0.209 (0.189–0.238)	0.055 (0.047–0.081)		
Guarani-Kaiowá	0.196 (0.181–0.229)	0.073 (0.065–0.107)	0.036 (0.021–0.062)	

[a] There are significant differences among the pairwise F_{ST} from Aché and Kaingang as well as Aché and Guarani populations since the confidence intervals do not overlap.

Table 5 Pairwise F_{ST} among Amerindians and others populations with 95% CI[a]

	Amerindians	CEU	CHB	YRI	Admixed
CEU	0.231 (0.207–0.257)				
CHB	0.087 (0.065–0.113)	0.179 (0.154–0.203)			
YRI	0.150 (0.133–0.169)	0.221 (0.203–0.243)	0.132 (0.113–0.159)		
Admixed Brazilians	0.107 (0.090–0.124)	0.070 (0.056–0.084)	0.076 (0.060–0.094)	0.119 (0.103–0.135)	

CEU, Utah residents with northern and Western Europe ancestry; CHB, Han Chinese of Beijing, China; YRI, Yoruba of Ibadan, Nigeria.
[a] There are significant differences among the pairwise F_{ST} among Amerindians and HapMap populations since the confidence intervals do not overlap.

Amerindian groups. In terms of history they were the latest to establish permanent contact with non-Natives, and suffered heavily the impact of this encounter. As far as other genetic studies are concerned Aché's origin is still not clear, some data favoring a close relationship with Tupi groups (57–59), while others show a higher genetic similarity with Jê (60, 61); another study concluded that the Aché were distinct from both Amerindian groups (62). After reviewing all these studies Callegari-Jacques et al. (20) suggested a general Tupian background with considerable introgression of Jê genetic material. Our results are in line with this suggestion, since the differentiation estimates observed between Aché and Guarani differs only slightly from that found between Aché and Kaingang (a Jê group) (Table 4). As for the Kaingang/Guarani comparison, the F_{ST} values were low (0.05–0.07), differently from what was observed at the HLA system (58, 63), which clearly differentiated populations of these two Amerindian populations residing in the same region. The low genetic difference observed between the Guarani subgroups was of course expected.

When the Native American populations were grouped and compared with non-Native American populations, the first point to be made is that while all the Amerindian communities are rural, samples from the other ethnic groups were all urban. The CHB sample is composed by Beijing's individuals, from the capital of the People's Republic of China and one of the most populous cities in the world. Beijing is the second largest Chinese city by urban population after Shanghai and is the nation's political, cultural, and educational center (http://www.ebeijing.gov.cn). The YRI sample is composed by individuals who inhabit Ibadan, the capital of Oyo State, the third largest city by population in Nigeria and the largest in geographical area. Ibadan is also the largest Nigerian metropolitan geographical area (http://www.nigeriaembassyusa.org). The CEU sample is formed by descendants from northern and Western Europe who inhabit Utah, one of the 50 US states, located in the Rocky Mountain Region. Utah is one of the fastest-growing states in the United States (http://www.utah.gov/index.html). The burden of infectious diseases has been massive throughout history. Advances in medical science, the use of antibiotics, and vaccines, as well as improved hygienic conditions, have contributed to reduce the burden of infectious diseases and to eradicate some deadly pathogens (16, 64). The development of urban centers, also increased communication between neighboring towns, human settlements became large enough to maintain diseases in an endemic form (16); however, South American Indians continued to live as hunter–gatherers or in small scattered villages until now. Hunter/gatherer communities are thought to have suffered from infections with specific characteristics that favor the maintenance of the agent in a small population. The incomplete immunity, enabling previously infected subjects to remain in the pool of potential victims and a slow or chronic disease course, so that infected members can infect new victims over years are among such characteristics. However, the advent of agriculture about 11,000 years ago led to a set of changes that favored the establishment of the most important epidemic diseases (64). The rural/urban difference pointed between HapMap populations and Amerindians is relatively recent in human populations, but it could also have contributed, at least in part, for the differences found.

How can our F_{ST} results be interpreted? The lowest value (0.08) was obtained between Amerindians and CHB, a result that can reflect the well-known fact that Native Americans originated from Asia. The second lowest (0.15) was found between them and YRI; although in evolutionary terms the two populations are more distant than in the previous comparison, in terms of environmental exposure to pathogens they may be the most similar. Modern man's ancestors lived

in an environment where infectious tropical diseases would have been endemic. Le Souëf et al. (65) postulated that this relatively hostile environment would have caused genetic selection for increased proinflammatory immune responses. On migrating to temperate regions, pronounced proinflammatory responses would have been less important and selected against due to increased mortality from overly vigorous responses to harmless environmental agents. This hypothesis is supported by the observation that proinflammatory alleles in several genes involved in inflammation are more prevalent in populations with long-term tropical ancestry than those with long-term residence in temperate regions. The lower divergence between Africans and Amerindians (0.15) in relation to that observed between Amerindians and Europeans (0.23) might be in part explained by the tropical environment shared by Africans and Amerindians. These observations suggest that there may be general patterns of recent evolutionary adaptation of the human immune system to particular climates, but this suggestion should be tempered by the fact that environmental pressures in Africa and America may be different in these two tropical biomes. Small alterations in the abiotic environment may modify infection outcomes and exaggerate or diminish differences between genotypes. It has been shown that natural selection acts in different ways: negative or balancing selection tends to decrease F_{ST} whereas local positive selection tends to increase F_{ST} (66); therefore, the effect of climate on adaptive immune genes is an open question for further investigation.

Among the different types of adaptation, those affecting immune function are among the most dynamic because the counter-evolution of pathogens drives the need for continuous adaptive change (67). However, immune systems do not evolve one gene at a time; instead, selective pressures are likely to affect multiple interacting genes, resulting in polygenic adaptation (66). If favored mutations increase in frequency at several genes simultaneously, this can reduce the overall strength of selection on each favored mutation, therefore favored mutations may increase in frequency rapidly at first, and then start to drift as the strength of selection becomes weaker (68). Tests based on differences between populations, such as F_{ST} performed in the present study, which examine the variation in allele frequencies between human populations, are powerful for detecting selective differences among populations, especially those that occurred after the out-of-Africa dispersals. These tests can also detect rapid, strong changes in selective pressures between closely related populations but are sensitive to demographic factors (especially bottlenecks) (69). The differentiations among populations observed in the present study were probably shaped by neutral processes such as population history, migration, and drift that can exert powerful influences over the fate and geographic distribution of selected alleles.

The present results must be interpreted in the context of some limitations. In this study, we investigated only genes involved in acquired immune responses but we are aware that to fully disclose the immunogenetic profile of Amerindians and how they differ from other groups, the investigation of innate immune genes should also be performed. Although the coevolution of innate and adaptive immune systems over the last 500 million years has ensured the inextricable linkage of their coordinate roles in mediating host protection (66, 70, 71) much less is known about population genetics and evolution of acquired immune response genes, therefore this study adds to the few available studies.

It is clear that both diverse evolutionary histories and differential more recent exposure to pathogens may be responsible for the observed pattern of relationships. In terms of public health policies the lesson to be learned is that the immunological background may differ in important ways both in populations of the same or of different ethnic groups. Appropriate previous reconnaissance surveys should be performed before any specific action.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Supporting Information

The following supporting information is available for this article:

Table S1. Hardy–Weinberg equilibrium model for 32 SNPs in four South Amerindian populations.

Table S2. Mean heterozygosity for 32 SNPs in Amerindians (AME), CEU, YRI and CHB populations.

Capítulo IV - Cytokine gene polymorphisms are associated with susceptibility to tuberculosis in an Amerindian population

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Cytokine gene polymorphisms are associated with susceptibility to tuberculosis in an Amerindian population

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SUMMARY

SETTING: Cytokines play an important role in anti-tuberculosis immune response, combined with antigen-presenting cells and lymphocytes. Immune response gene polymorphisms have been reported to be associated with tuberculosis (TB) susceptibility in some but not all studies.

OBJECTIVE: To evaluate the association of immune response genes with susceptibility to tuberculin skin test (TST) reactivity and/or TB.

DESIGN: Fourteen single nucleotide polymorphisms were genotyped in 96 individuals of the Aché, a native Paraguayan population, by allelic discrimination using real-time polymerase chain reaction. Univariate and multivariate Poisson regression were employed to assess risk genotypes.

RESULTS: A higher prevalence of purified protein derivative reactivity was associated with the *TNF-α* CCA/TCG haplotype (PR 1.298, 95%CI 1.059–1.589) and with the *IL-10* AT/CC diplotype (PR 1.181, 95% CI 1.024–1.362), and the presence of the IL-8 rs4073 T allele was associated with protection against TB (PR 0.482, 95%CI 0.273–0.851).

CONCLUSIONS: These results suggest that polymorphisms in genes associated with immune response are involved in TST reactivity and susceptibility to TB in the Aché population.

KEY WORDS: infectious diseases; interleukins; Native Americans; anergy

TUBERCULOSIS (TB), an infectious disease caused by *Mycobacterium tuberculosis*, is one of the main causes of morbidity and mortality worldwide. Based on surveillance and survey data, the World Health Organization (www.who.int) estimated that in 2011, 8.7 million people developed TB (125 cases per 100 000 population), and almost 1.4 million died from TB. Despite this high prevalence, only 5–10% of infected people develop clinical TB.

The majority of individuals infected with *M. tuberculosis* develop a delayed-type hypersensitivity response, manifested as a positive response (skin induration) to an intradermal injection with purified protein derivative (PPD) from *M. tuberculosis*. PPD, or the tuberculin skin test (TST), determines if the individual is *M. tuberculosis*-sensitized through vaccination or infection. It is used not only to identify infected persons, but also to assess cell-mediated immune response to *M. tuberculosis,* and to guide decisions about chemoprophylaxis and treatment.[1]

The TST is a measure of adaptive immunity, a response of the immune system that involves B and/or T cells. The immunological response begins with T cells being recruited to the intradermal site where lymphokines are locally secreted. Different cut-offs for a positive TST reaction have been recommended, ranging from 5 to 15 mm induration, depending on the level of exposure to *M. tuberculosis*, on previous contact history and on the immune status of the individual.[2] The lack of skin induration to intradermal PPD injection, defined as anergy, is observed in many individuals. Anergy may occur for two reasons. First, the individual has never been exposed to *M. tuberculosis*. Second, the individual has been exposed but does not have an adequate immune response. Immunologically, anergy involves the inability of T cells to produce interleukin-2 (IL-2),[3] together with decreased production of interferon-gamma (IFN-γ), particularly in severe disease.[1,4] Anergy has also been correlated with the expansion of IL-10-producing T

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cells.[4] Thus a major determinant for the clinical expression of the different forms of TB and their final outcome is the interaction between the pathogen and the host immune system.

Susceptibility to TB is considered to depend on human polygenic variability. Along with antigen-presenting cells and lymphocytes, cytokines play an important role in evoking an anti-tuberculosis immune response. The unique profile of cytokines, determined by the respective functional polymorphisms or haplotypes in cytokine genes or other closely linked genes, might be crucial for inducing protective immune responses. Mutations in these genes may result in altered transcription factor recognition sites, affect transcriptional activation or alter cytokine production levels.[5,6] Many susceptibility studies of different ethnic groups have been conducted, with conflicting results. However, susceptibility studies in Native American populations are scarce, despite the extremely high TB prevalence in these groups.[7]

The Aché is an indigenous population that lives in Eastern Paraguay. There is no evidence that they had regular contact with other ethnic populations in Paraguay or Brazil until the 1960s and 1970s; since that time, they have had periodic contact with outsiders. They currently number approximately 1000, live predominantly in hunting and gathering groups and some agriculture.[7] This population has a high (>30%) prevalence of TB and 7% have developed active pulmonary TB. A complex interplay between host genetic, environmental and cultural factors may contribute to these high rates.[8] This study investigated the association between single nucleotide polymorphisms (SNPs) in genes related to the immune system and TST response or TB disease.

STUDY POPULATION AND METHODS

Population, epidemiological, clinical and laboratory analyses

Of 96 Aché individuals recruited during community meetings in Arroyo Bandera (55°50′W, 23°30′S) and Chupa Pou (56°30′W, 24°10′S), in Paraguay, 52 were males and 44 were females. Tuberculous infection or TB disease status was unknown at the time of the sample collection. Infection was determined using PPD skin tests. Individuals with a wheal size $\geqslant 5$ mm were considered positive. This lower cut-off value reflects the documented tendency of the Aché to be unresponsive to PPD.[7] Unresponsive individuals were considered anergic. PPD information was available for 84 persons. At time of data collection, 30% of the Aché aged <20 years had received bacille Calmette-Guérin (BCG) vaccinations. There was no policy for adult vaccination.[7]

TB was diagnosed with a combination of X-ray, contact history, and clinical symptoms. It was not possible to diagnose TB based on sputum smear analysis because the Aché have difficulty in expectorating, perhaps due to their dehydrated state. Bronchoscopy and gastric aspirations were not viable diagnostic options given the difficult field conditions. Due to these limitations, TB was diagnosed as previously described.[7] Briefly, buccal swab samples were tested three times for the presence of *M. tuberculosis* DNA. The target was insertion sequence (IS) *6110*, which is diagnostic of *M. tuberculosis* complex. PCR status was assigned based on the number of positive tests. Each of the four possible PCR statuses was analyzed separately, as test sensitivity and specificity have still not been determined.

All individuals with a positive diagnosis of TB were first treated with isoprodian; recurrent cases were treated with rifampicin, isoniazid, pyrazinamide, or ethambutol.[7]

Genomic DNA, genotyping procedures, and polymorphism frequencies have been described elsewhere.[9] In that study, 32 variants were investigated, but for analyses of the present association only 14 SNPs were selected based on minor allele frequencies (MAF) $\geqslant 0.05$.

Statistical analysis

Linkage disequilibrium (LD) was measured for all markers in the same gene using the MLocus software. PHASE software was used to infer haplotypes for each individual when the markers were in LD. Due to small numbers, genotypes were grouped according to the following criteria: when functionality data for the allele were reported in the literature, the lower expression allele carriers were grouped (heterozygotes plus homozygotes); when that information was unavailable, a frequency criterion was applied, in which the low frequency categories were grouped. The association between the outcomes (reaction to PPD and TB susceptibility) with genotypes was evaluated using prevalence ratios (PR) estimated by univariate Poisson regression with robust variance. The lowest prevalence category was used as reference. All markers with a $P \geqslant 0.2$ in the univariate analysis were analyzed in a multivariate Poisson regression, with age and sex as covariates. $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS for Windows v18.0 (SPSS Inc., Chicago, IL, USA). The effect size for significant P values was obtained through the transformation of the r correlation in the Cohen effect size measure.[10]

Ethical approval

Our research program was approved by the Brazilian Ethics National Committee (Resolution 123/98). As the Aché did not read or write at the time of data collection, we were unable to obtain written consent. Instead, the aims of the study were translated into the

Aché language in a community meeting, and the community members agreed to participate.

RESULTS

Of 96 individuals investigated, 38 had active TB. The mean age of the affected individuals was 39.4 ± 13.7, which was higher than that observed among the healthy subjects (33.2 ± 13.4). Age had a significant effect in disease status ($P = 0.008$), but no significant effect in PPD reactivity ($P = 0.98$). Among the 84 subjects with known PPD status, anergy was observed in 36 (43%), and 48 (57%) had an induration $\geqslant 5$ mm. About 24% ($n = 9$) of the individuals with active disease were anergic to PPD. Due to limited sample size, it was not possible to estimate genetic associations with these individuals. Sex had no significant effect on disease status or PPD reactivity (data not shown).

Genotype association tests with PPD reactors and non-reactors are shown in Table 1. A higher PPD reactivity prevalence was associated with the tumor necrosis factor-alpha (*TNF-α*) CCA/TCG haplotype (PR 1.298; 95%CI 1.059–1.589) and with the interleukin-10 (*IL-10*) AT/CC diplotype (PR 1.181; 95%CI 1.024–1.362). Although on the threshold of significance ($P = 0.057$), *IL-4R* AA homozygous individuals showed almost 14% higher prevalence of reaction than G carriers.

Table 2 shows the PRs for TB in relation to genotypes. *IL-8* rs4073T allele carriers had a lower prevalence of TB (PR 0.482; 95%CI 0.273–0.851) in comparison to the reference genotype (AA). The effect size obtained for this estimate is a standardized value that can be compared among studies. *r* correlations were 0.23, 0.33, and 0.32 for IL-10, TNF and IL-8, respectively. The corresponding effect sizes were 0.5, 0.7, and 0.7.

DISCUSSION

The association of host genetic factors with TB has been extensively studied, and the results are controversial. In Amazonian Indian populations, where a high BCG vaccine coverage is observed, anergy rates are higher than those observed in the surrounding non-Indian populations.[11–15] It has been suggested that the frequent low reactivity to TST observed in

Table 1 Association analysis of PPD reaction prevalence ratios and immune gene polymorphisms in the Aché population

Gene	dbSNP ID ‡	Genotypes	PPD = 0	PPD >5	Model 1* PR (95%CI)	Model 1* *P* value	Model 2† PR (95%CI)	Model 2† *P* value	Effect size§
IL4	2243250	C carriers	11	18	1.049 (0.913–1.204)	0.501			
		TT	25	30	1				
IL4R	1801275	AA	15	30	1.140 (0.996–1.305)	0.057¶	1.140 (0.996–1.306)	0.058	
		G carriers	21	18	1		1		
IL8	4073	A carriers	15	29	1.125 (0.983–1.287)	0.087¶			
		TT	21	19	1				
IL10	1800872/1800871	AT/AT	34	38	1	0.009¶	1	0.022#	0.5
		AT/CC	2	10	1.200 (1.046–1.377)		1.181 (1.024–1.362)		
IL12A	568408	A carriers	17	21	1	0.752			
		GG	19	27	1.022 (0.892–1.171)				
IL12B	7709212/2546890	CG/TA	11	20	1.161 (0.930–1.450)	0.187¶			
		CG/CG	17	22	1.104 (0.885–1.377)	0.379			
		TA/TA	7	5	1				
		CA/CG	0	1	**	**			
		CA/TG	1	0	**	**			
TNFα	361525/1799724/ 1799964	TCG/TTG	10	5	1		1		0.7
		TCG/TCG	7	12	1.224 (0.979–1.529)	0.076¶	1.218 (0.974–1.522)	0.084	
		CCA/TCG	8	23	1.306 (1.070–1.595)	0.009¶	1.298 (1.059–1.589)	0.012#	
		Others ††	11	8	1.066 (0.840–1.352)	0.599	1.069 (0.844–1.355)	0.580	
IFNGR1	2234711/1327474	TA/TA	8	10	1.026 (0.850–1.238)	0.789			
		TA/TG	15	16	1				
		TG/CA	6	9	1.055 (0.870–1.281)	0.586			
		Others‡‡	7	13	1.088 (0.917–1.292)	0.334			
P2X7	3751143	GG	6	4	1				
		TG	20	31	1.148 (0.910–1.449)	0.243			
		TT	9	12	1.122 (0.870–1.449)	0.375			

* Model 1: univariate analyses.
† Model 2: multivariate Poisson regression with age and sex as covariates for all polymorphisms with $P < 0.2$ in the univariate analysis.
‡ dbSNP is a public database of SNPsfound at http://www.ncbi.nih.gov/projects/SNP/
§ Effect size calculated according to Randolph & Edmondson.[10]
¶ $P < 0.2$.
# *P* values that reached statistical significance (at the 0.05 level).
** Haplotypes not analyzed due to low frequency.
†† Others: TTG/TTG; CCA/TTG; CCA/CCA.
‡‡ Others: CA/CA; TA/CA; TG/TG.
PPD = purified protein derivative; PR = prevalence ratio; CI = confidence interval; SNP = single nucleotide polymorphism.

Table 2 Association analysis of tuberculosis susceptibility prevalence ratios and immune gene polymorphisms in the Aché population

Gene	dbSNP ID ‡	Genotypes	TB negative	TB positive	Model 1*		Model 2†		Effect size§
					PR (95%CI)	*P* value	PR (95%CI)	*P* value	
IL4	2243250	C carriers	21	14	1.017 (0.610–1.696)	0.95			
		TT	37	24	1				
IL4R	1801275	AA	32	20	1				
		G carriers	26	18	1.064 (0.649–1.744)	0.807			
IL8	4073	AA	4	8	1		1		0.7
		AT/ TT	54	30	0.536 (0.327–0.876)	0.013¶	0.482 (0.273–0.851)	0.012#	
IL10	1800872/1800871	AT/AT	51	32	1				
		AT/CC	7	6	1.197 (0.627–2.286)	0.586			
IL12A	568408	A carriers	32	14	1				
		GG	26	24	1.577 (0.934–2.662)	0.088¶			
IL12B	7709212/2546890	CG/TA	20	14	1.153 (0.513–2.590)	0.730			
		CG/CG	29	17	1.035 (0.466–2.298)	0.933			
		TA/TA	9	5	1				
TNFα	361525/1799724/ 1799964	TCG/TTG	14	6	1				
		TCG/TCG	10	9	1.579 (0.695–3.586)	0.275			
		CCA/TCG	22	13	1.238 (0.558–2.745)	0.599			
		Others**	12	10	1.515 (0.673–3.409)	0.315			
IFNGR1	2234711/1327474	TA/TA	11	10	1.515 (0.780–2.943)	0.220			
		TA/TG	24	11	1				
		TG/CA	9	7	1.392 (0.664–2.919)	0.381			
		Others††	14	10	1.383 (0.704–2.719)	0.347			
P2X7	3751143	GG	7	4	1				
		TG	36	23	1.072 (0.461–2.494)	0.872			
		TT	14	10	1.146 (0.459–2.858)	0.770			

* Model 1: PRs for each polymorphism.
† Model 2: multivariate Poisson regression with age and sex as covariates for all the polymorphisms with $P < 0.2$ in the univariate analysis.
‡ dbSNP is a public database of single nucleotide polymorphisms found at http://www.ncbi.nih.gov/projects/SNP/
§ Effect size calculated according to Randolph & Edmondson.[10]
¶ $P < 0.2$.
# P values that reached statistical significance (at the 0.05 level).
** Others: TTG/TTG; CCA/TTG; CCA/CCA.
†† Others: CA/CA; TA/CA; TG/TG.
TB = tuberculosis; PR = prevalence ratio; CI = confidence interval; SNP = single nucleotide polymorphism.

these Amazonian Indian groups may be due to imprecise immunological mechanisms that may cause diminished cell-mediated immune response against *M. tuberculosis*.[7,11,16] In the present study, an association between the *TNF-α* and *IL-10* genes and PPD response was observed, whereas for TB susceptibility a protective effect of *IL-8* genotypes was found.

In their observations of anergic patients, Montiel et al. suggested that a Th2 pattern of immune response was associated with anergy.[17] Our results suggest that polymorphisms in genes related to Th2 immune profile predominance (*IL-4R* and *IL-10*) are involved with anergy to PPD (G carriers in IL-4R and AT/AT in IL-10). On the other hand, polymorphisms in genes related to the Th1 immune profile predominance (*TNF-α*) might be involved in a positive response to PPD. This assumption is supported by the observation that delayed-type hypersensitivity is an important Th1 marker.[18]

The response of *M. tuberculosis* infection is characterized by a strong inflammatory cell-mediated immune response, with elevated expression of both *TNF-α* and *IFN-γ*. These two cytokines are essential for mycobacterial infection control, but in most cases *M. tuberculosis* survives and establishes latent infection that can rapidly reactivate if *TNF-α* production is blocked.[19] *TNF-α* is a key component of the immune response to TB, and is involved in granuloma formation.[20]

An association was observed between TST reactivity and haplotypes derived from *TNF-α* rs361525, rs1799724, and rs1799964. The prevalence of reaction was almost 1.3 for individuals with the CCA/TCG haplotypes. To our knowledge, ours is the first study to observe this association. However, no association between this and active TB was found. Conflicting results on the association between *TNF-α* and TB have been observed. In Iranians, the rs361525 GG genotype was positively associated with pulmonary TB[21] whereas in North Indians the same SNPs investigated in the present study were not associated with TB.[22] Cobat et al. recently identified a region that controlled TNF production in response to mycobacterial stimulation (TNF1 locus).[23] This locus was mapped in the vicinity of the TST1 locus, which controls TST negativity itself; i.e., T-cell-independent resistance to *M. tuberculosis* infection. This finding suggests that there is a connection between TST negativity and TNF production.

IL-10 is an important anti-inflammatory cytokine and is considered one of the macrophage-deactivating

cytokines. It downregulates IFN-γ production and Th1 response to *M. tuberculosis*.[24] In a study of the Xavante of Central Brazil, the rs1800896 GG genotype carriers showed a PPD anergy prevalence of 1.5.[25] The Aché are not polymorphic at this site,[9] but we observed an association between the *IL-10* rs1800872/1800871 haplotype and PPD. The AT/CC carriers have a higher prevalence of reaction than AT/AT individuals. In a Peruvian study, the rs1800872 A allele was associated with protection to TB and was correlated with higher levels of IL-10.[26] Higher IL-10 levels modulate the Th1 response, potentially preventing excessive inflammatory responses.

IL-4 is also a Th2 type cytokine that downregulates the protective Th1 response to TB.[27] The rs1801275 G allele was associated with increased IL-4R levels,[28] a situation that could determine a predominance of the Th2 immune response pattern. Although on the threshold of significance, our study showed that the AA genotype was more prevalent among individuals who developed reactivity to PPD. The predominance of the Th1 immune response was predictable in this case, as the A allele determines lower IL-4R levels.

IL-8 functions as a potent chemo-attractant in leukocyte recruitment to inflammatory sites. It is produced and released by leukocytes in response to *M. tuberculosis* or its components. A case-control study of Whites and African Americans in the United States found that the rs4073A allele was associated with an increased risk of TB.[29] However, in a Gambian population this result was not replicated.[30] Among the Aché the presence of a T allele was associated with disease protection. As the A allele has been associated with increased IL-8 production, a Th1 pattern would explain the greater susceptibility to disease.[31] One explanation for the higher risk of TB in individuals with higher expression of IL-8 (the AA genotype) is that the high secretion of this cytokine may increase inflammation by retarding apoptosis of polymorphonuclear leukocytes.[32] This increased expression of IL-8 attracts an excess of leukocytes to the disease site, resulting in extensive tissue damage by the generation of free radicals, proteases and elastases.[29]

This study has some limitations. First, although the sample is representative of the Aché population, which comprises only approximately 1000 individuals, it is nevertheless small.[7] Although the effect sizes of the significant results obtained reveal the contribution of single genes to TB susceptibility, the effect sizes were nevertheless small, as would be expected for single genes in multifactorial diseases. Secondly, the subjects in our study live in several scattered agricultural villages, which made field work difficult. Opportunities to study genetic susceptibility to infectious disease among Native Americans who still live isolated from their non-native neighbors are rare; this was a unique opportunity to investigate genetic susceptibility to TB in this ethnic group. Finally, we are aware that the gold standard for TB diagnosis is sputum smear analysis; however, due to cultural and health issues, the Aché did not expectorate. Our clinical and laboratory criteria were nevertheless sufficiently stringent to minimize the possibility of a false diagnosis. Our results may therefore be considered reliable.

The identification of essential host genes, their susceptibility-associated alleles and the mechanisms through which they influence disease pathogenesis and host resistance in primary or latent infection will provide key insights into effector mechanisms of protection. More importantly, it will provide new targets for preventive or post-infection intervention.[33]

CONCLUSIONS

We observed an association between IL-10 and anergy to PPD. This finding reinforces the importance of IL-10 polymorphisms in PPD response in native populations, as previously reported in studies of the Xavante.[25] Furthermore, a TNF-α haplotype (CCA/TCG) was associated with greater TST reactivity, and the presence of the IL-8 rs4073 T allele was associated with protection against TB. In a study by Wilbur et al., the VDR rs10735810 C allele was found to be protective against tuberculosis infection and disease among the Aché.[8] The TT genotype determines an increased transcription rate, and this has provided the main explanation for the association between this genotype and the development of infectious diseases.[34] Consequently, alleles that determine greater activation of a Th2 pattern of immune response could be responsible for higher rates of PPD anergy and TB susceptibility; conversely, when they determine a greater activation of the Th1 pattern, they could lead to protection against TB and higher PPD reaction rates.

In South American indigenous groups with a high prevalence of several infections, the Th1/Th2 balance may be disturbed, leading to increased susceptibility to several pathogens.[35] In a previous study, we demonstrated that the immune patterns observed in Native Americans could be different from those observed in non-Native Americans.[9] Therefore, the investigation of immune response polymorphisms in Amerindian communities is essential for understanding the susceptibility to common pathologies in these populations.

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RESUME

CONTEXTE : Les cytokines jouent un rôle important dans la réponse immunitaire à la tuberculose (TB), combinées aux cellules présentant les antigènes et aux lymphocytes. Le polymorphisme génétique de la réponse immunitaire a été déclaré associé à la susceptibilité à la TB dans certaines études, mais pas dans toutes.

OBJECTIF : Evaluer l'association des gènes de la réponse immunitaire avec la réactivité au test à la tuberculine (TST) et/ou à la TB maladie.

SCHÉMA : Quatorze polymorphismes de nucléotide simple ont été génotypés chez 96 individus de l'ethnie Aché, une population indigène du Paraguay, par discrimination allélique avec une PCR en temps réel ; une régression de Poisson univariée et multivariée a été utilisée pour évaluer les génotypes à risque.

RÉSULTATS : Une prévalence de réactivité PPD plus élevée a été associée à l'haplotype *TNF*-α CCA/TCG (PR = 1,298 ; IC95% 1,059–1,589) et au diplotype *IL-10* AT/CC (PR = 1,181 ; IC95% 1,024–1,362) et la présence de l'allèle IL-8 rs4073 T était associée à une protection vis-à-vis de la TB (PR = 0,482 ; IC95% 0,273–0,851).

CONCLUSIONS : Ces résultats suggèrent que le polymorphisme des gènes associés à la réponse immunitaire est impliqué dans la réactivité au TST et à la susceptibilité à la TB dans la population Aché.

RESUMEN

MARCO DE REFERENCIA: Las citocinas cumplen una importante función en la respuesta inmunitaria frente a la tuberculosis (TB), en asociación con los linfocitos y las células que presentan los antígenos. En algunos estudios se comunicado la asociación de polimorfismos génicos que intervienen en la respuesta inmunitaria con la vulnerabilidad a la TB, pero este efecto no lo confirman todos los estudios.

OBJETIVO: Evaluar la asociación entre los genes que regulan la respuesta inmunitaria y la reactividad a la reacción tuberculínica o la susceptibilidad a la enfermedad TB.

MÉTODOS: En 96 personas de la población Aché, un grupo étnico indígena del Paraguay, se practicó la genotipificación de 14 polimorfismos de un solo nucleótido por discriminación alélica mediante la reacción en cadena de la polimerasa en tiempo real; mediante el método de la regresión monofactorial y multifactorial de Poisson se evaluaron los genotipos que conferían un riesgo de padecer TB.

RESULTADOS: Se observó que la mayor prevalencia de reactividad a la tuberculina se asociaba con el haplotipo CCA/TCG del gen del TNF-α (razón de probabilidades [RP] 1,298; IC del 95% 1,059–1,589) y con el diplotipo AT/CC del gen de la IL-10 (RP 1,181; IC95% 1,024–1,362); la presencia del alelo rs4073 T del gen de la IL-8 se asoció con protección contra la TB (RP 0,482; IC95% 0,273–0,851).

CONCLUSIÓN: Estos resultados indican que en la población Aché, los polimorfismos en los genes que intervienen en la respuesta inmunitaria participan en la reactividad a la tuberculina y la susceptibilidad a la TB.

Capítulo V - Association between HLA-DR4 haplotypes and tuberculin skin test response in the Aché population

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BRIEF COMMUNICATION

Association between HLA-DR4 haplotypes and tuberculin skin test response in the Aché population

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Abstract

The human leukocyte antigen (HLA) system has a major role in the regulation of the immune response as it is involved in the defense against pathogens. Evidence for association with tuberculosis (TB) is more consistent for class II than for class I HLA genes. TB is important among indigenous peoples in South America, not only because of its historical role in regional depopulation, but also because it is still widespread. The aim of this study was to evaluate the association of HLA class II alleles, haplotypes and genotypes and tuberculin skin test response (TST) in 76 individuals of the Aché population. Poisson Regression was employed to assess risk genotypes. *DRB1*04*, *DQA1*03* and *DQB1*03:02* were associated with TST response in this population.

The major histocompatibility complex (MHC) is located on the short arm of chromosome 6 and comprises more than 200 genes, many of which are involved in immune response. The classical class I and class II human leukocyte antigens genes (HLA) map to this region and encode molecules that are highly polymorphic cell surface glycoproteins playing a fundamental role in self/non-self-immune recognition. The high variability of these HLA classes I and II molecules is driven and preserved by balancing selection (1, 2).

Short peptides are presented on the cell surface where they are recognized in the context of the class I or class II HLA molecules by CD8+ or CD4+ T cells, respectively. Considering that HLA molecules are one of the major components of the immune system it is not surprising that polymorphisms in these molecules are associated with various immune-mediated diseases such as autoimmune disorders and infections. Evidence for association with tuberculosis (TB) is more consistent for class II than for class I genes (3). The protective response to *Mycobacterium tuberculosis* (Mtb) relies on cell mediated immunity. Yet, both the protective and pathologic responses to Mtb are complex, involving many components of the immune system. An effective T cells response determines whether the infection resolves or develops into clinically evident disease and direct recognition of infected cells by class II MHC-restricted CD4+ T cells is required for control of intracellular Mtb (4, 5).

TB is important among indigenous peoples in South America, not only because of its historical role in regional depopulation, but also because it is still widespread. These groups have TB prevalence higher than those of the general populations of their countries, and seem particularly vulnerable to it, with a greater risk of acquiring and dying from TB than non-Indians (6–10). The majority of individuals infected with Mtb develop a delayed-type hypersensitivity response, which is manifested as a positive response (skin induration) to intradermal injection with purified protein derivative (PPD) from Mtb. PPD or tuberculin skin test (TST) determines if the individual is Mtb sensitized, through vaccination or infection. Intradermal application PPD revealed that the success in infection control is highly dependent on adaptive immune responses. PPD anergy rates are higher in Amerindians than in other major ethnic groups, it

occurs in about 50% of the subjects tested, even in populations with relatively high BCG coverage (11–13).

The Aché population lives in the tropical forests of the southwestern part of South America in Paraguay. There is no evidence that they ever experienced amicable relations with other ethnic populations in Paraguay until the 1960s and 1970s, when various groups made contact with outsiders. In fewer than 15 years after first contact, 18% of the population was diagnosed with active TB and the negative tuberculin reaction rate was observed in almost 68% of the population tested (13). In this study, the association between HLA class II alleles and PPD response was investigated in the Aché population.

The study sample for human leukocyte antigen (HLA) analyses comprised 76 subjects, all of whom had been clinically examined and tested with PPD during community meetings in Arroyo Bandera (55°50′W, 23°30′S) and Chupa Pou (56°30′W, 24°10′S), in Paraguay. Thirty-eight were males. Individuals with a wheal size of 5 mm or greater were considered PPD positive. This lower cut-off value reflects the documented tendency of the Aché to be unresponsive to PPD (13). Unresponsive individuals were considered anergic. Genomic DNA, genotyping procedures, and polymorphism frequencies were previously described (14).

HLA-DRB1, -DQA1, and -DQB1 loci genotype and haplotype frequencies were estimated previously (14). Hardy–Weinberg equilibrium was tested for each locus using Markov chain as implemented in Arlequin v.3.5 (15). This test is performed using a modified version of the Markov chain random walk algorithm described by Guo and Thompson (16). The association among the outcome and genotypes or haplotypes was evaluated using prevalence ratios (PR), estimated by univariate Poisson regression with robust variance. All markers were analyzed in a multivariate Poisson regression, with age and gender as covariates. This method provides correct estimates and is a better alternative for cross-sectional studies with binary outcomes with high frequencies (>10%) than logistic regression, because the PR is more interpretable (17, 18). *P*-values <0.05 were considered significant (Table 1). Statistical analyses were performed using SPSS for Windows v18.0 (SPSS Inc., Chicago, IL). The power of tests was calculated with WINPEPI software (19).

Among the 76 individuals investigated in which PPD status was available, anergy was observed in 32 (42%) and 44 had 5 mm or greater wheal size. The age of individuals ranged from 17 to 64 years (36.2 ± 13.8). The observed genotype distributions were in agreement with Hardy–Weinberg equilibrium for all markers (HLA-DRB1 p-value = 0.37; -DQA1 p-value = 0.82; -DQB1 p-value = 0.81). Genotype and haplotype association tests with PPD reactivity are shown in Table 1. Higher PPD reactivity prevalence was associated with *DQA1*03*, *DQB1*03:02* and *DRB1*04:11* homozygous genotypes [PR = 1.709; confidence interval (CI): 1.079–2.707; $p = 0.022$]. When HLA class II haplotypes (composed by DRB1, DQA1 and DQB1) were derived, a significant higher PPD reaction prevalence was observed with 04:11-03-03:02 homozygous in relation to others (PR = 1.709; CI: 1.079–2.707; $p = 0.022$). The power of tests was 80%. We were not able to detect associations between class I HLA

Table 1 Association analysis of purified protein derivative (PPD) reaction prevalence ratios and HLA variants in the Aché population[a]

				Model 1		Model 2	
HLA	Genotypes or haplotypes	PPD = 0	PPD > 5	PR (95% CI)	*P*-value	PR (95% CI)	*P*-value
DRB1	04:11 homozygous	13	31	1.734 (1.094–2.749)	0.019	1.709 (1.079-2.707)	**0.022**
	Others[b]	19	13	1		1	
DQA1	03 homozygous	13	31	1.734 (1.094–2.749)	0.019	1.709 (1.079–2.707)	**0.022**
	Others[c]	19	13	1		1	
DQB1	03:01 carriers	10	7	0.657 (0.360–1.198)	0.170	0.675 (0.370–1.232)	0.200
	Others[d]	22	37	1		1	
DQB1	04:02 carriers	10	6	0.592 (0.306–1.147)	0.120	0.580 (0.300–1.122)	0.106
	Others[e]	22	38	1		1	
DQB1	03:02 homozygous	13	31	1.734 (1.094–2.749)	0.019	1.709 (1.079–2.707)	**0.022**
	Others[f]	19	13	1		1	
DRB1-DQA1-DQB1	04:11-03-03:02 homozygous	13	31	1.734 (1.094–2.749)	0.019	1.709 (1.079–2.707)	**0.022**
	Others[g]	19	13	1		1	

CI, confidence interval; PR, prevalence ratios.

[a]Model 1: univariate analyses. Model 2: multivariate Poisson regression with age and gender as covariates for all polymorphisms of the univariate analysis; *P*-values that reached statistical significance (at the 0.05 level) are in bold.

[b]Others: 04:11/08:07 (9); 04:11/14:02 (6); 04:11/14:13 (8); 04:11/08:02 (4); 04:03/08:07 (1); 14:13/14:02 (1); 08:02/14:02 (1); 08:07/08:07 (1).

[c]Others: 03/05 (15); 04/05 (1); 03/04 (14); 04/04 (1); 05/05 (1).

[d]Others: 03:02/04:02 (14); 03:02/03:02 (44); 04:02/04:02 (1).

[e]Others: 03:02/03:02 (44); 03:02/03:01 (15); 03:01/03:01 (1).

[f]Others: 03:01/03:01 (1); 03:02/03:01 (15); 03:02/04:02 (14); 04:02/04:02 (1); 04:02/03:01 (1).

[g]Others: 04:11-03-03:02/08:02-04:01-04:02 (4); 04:11-03-03:02/08:07-04:01-04:02 (10); 04:11-03-03:02/14:02-05:01-03:01 (8); 04:11-03-03:02/14:13-05:01-03:01 (9); 04:03-03-03:02/08:07-04:01-04:02 (1).

(-A, -B and -C) and PPD reaction due the low degree of polymorphisms observed in the Aché population in these loci (14). These analyses presented a low statistical power (5% to HLA-A; 7% to HLA-B and 9% to HLA-C).

The basis of HLA/disease associations remains unknown. Most hypotheses depart from the antigen-presentation function and the peptide-binding properties of the classical HLA molecules. The products of different HLA alleles bind different though overlapping peptide sets. The HLA/peptide complexes generated select the specific T cell clones via their TCR (T cell receptor). Thus, the HLA genotype of any individual shapes the T cell repertoire during negative and positive selection in the thymus and drives the antigen-specific effector and regulatory responses in the periphery. Furthermore, the HLA/peptide complexes may determine the patterns of cytokines secreted and influence the immune response outcome (20, 21). Apart from their role in antigen presentation, HLA molecules can contribute to differential susceptibility to complex diseases through antigen-presentation independent allele-coded ligands that activate signaling events (22). HLA class I molecules also interact with natural killer (NK) cell receptors modulating their cytotoxic response and the release of cytokines that contribute to inflammation and immunoregulation. T cell-mediated immune mechanisms have a crucial role in the development of resistance to Mtb infection. CD4+ T cells are activated by the recognition of pathogen-derived peptides in the context of HLA class II molecules presented by antigen presenting cells.

The cytokine variability observed in Native American groups could be different from that observed in non-Native Americans populations (23) therefore their interaction with HLA could also differ. Several evidences show that the occurrence of some HLA alleles determine IFN-γ expression modification, altering Th1 immune response. Selvaraj et al. (24) showed that the HLA-*DRB1*03* allele group was associated with an increased production of IFN-γ, while *HLA-DRB1*15* was associated with a decrease of this cytokine production. Furthermore, it has also been suggested that *HLA-DRB1*15* carriers have a higher spontaneous IL-10 production compared to non-carriers of this allele. Considering that IL-10 is a potent inhibitor of HLA class II expression, antigen specific proliferation and IFN-γ synthesis, their disturbed expression can contribute to increased suppression of Th1 response (24). Additionally, *HLA-DRB1*04* carriers have an increased IL-6 response, suggesting that this allele may enhance IL-6 responses which in turn may up regulate the production of Mtb-specific antibodies by B cells (25). These unbalanced cytokines expression determines a suppression of the Th1 response and, consequently, an increase in Th2 response. Th1 response is an important marker for delayed-type hypersensitivity (26); whereas a Th2 pattern is associated with anergy (27). Previous investigations reported different HLA alleles associated with TB with the results varying among populations. The most associated HLA alleles were *DRB1*08*, **12*, **13*, **14* and **15* (28–34) and *DQA1*03* (35, 36). In line with these findings *DQA1*03* and *DRB1*04:11* were associated with higher PPD reactivity in the Native American population investigated herein. These findings show the importance of HLA variants for TB adequate immune response, where these alleles act with cytokines to begin the more appropriate Th response according to the intracellular environment.

Recently, it was reported that Mtb, a highly successful persistent human pathogen, has highly conserved epitope sequences. This apparently conflicts with the general model of a host–pathogen coevolution, and emphasizes that Mtb employs unique approaches to achieve success as a pathogen (37). T-cell subsets at different stages of development may be important for maintaining a strong and long-lived immune response. A pool of 'precursor' T cells, and immediate-acting 'effector' T cells or memory T lymphocytes which are free to leave the bloodstream and to enter the site of infection with different potentials for proliferation or cytokine production may be advantageous to achieve a maximum and long-lived protection. The immunogenic Mtb peptide, ESAT, can bind differently with HLA alleles, altering the T cell effector response. Therefore, individuals homozygous for specific nucleotide changes in the HLA alleles can be more susceptible to TB, due the differences in the binding to ESAT (38).

The frequencies of most HLA alleles and haplotypes vary widely among human populations. This is the principal reason of the apparent inconsistency between the results of studies of the same disease in different populations, along with the statistical power, which in turn depends of the sample size and the relative effect of the different genotypes (39). The Aché have very particular HLA frequencies. The frequency of haplotype *DRB1*04:11-DQA1*03-DQB1*03:02* is close to 78% and only 1 of the 76 individuals here analyzed do not have this haplotype. Such a high frequency of a single HLA-DRB1 allele and class II haplotype is quite unusual in populations worldwide. The other haplotypes seen in that population are *DRB1*08-DQA1*04:01-DQB1*04:02* (10%) and *DRB1*14-DQA1*05:01-DQB1*03:01* (11.2%). Therefore, the Ache did not present *DRB1*15* and DR2 (an antigenic determinant shared by *DRB1*15* and *DRB1*16* allele products) that is associated with susceptibility to TB and whose frequency is highest in Europe, East and South Asia and Oceania (3; allelefrequencies.net). Several other alleles/allele groups associated to increased or decreased susceptibility to the disease in other populations: *DRB1*03*, *DRB1*07*, *DRB1*11*, *DRB1*13*, and most of the HLA-DQ alleles and antigens associated to these HLA-DRB1 alleles were not observed. These alleles could occur in low frequencies or they could be absent in this population. More studies with bigger sample sizes would be needed to disclose the HLA variability in this ethnic group. Moreover, all HLA-DRB1 allele groups seen in the Aché (*DRB1*04*, *DRB1*08*, *DRB1*14*) were associated to increased susceptibility to TB in at least one other population (3). On the same direction, this study showed positive tuberculin skin test response in this Amerindian population

was more frequent in individuals homozygous for haplotype *DRB1*04:11-DQA1*03-DQB1*03:02*.

The results presented here should be viewed in the context of some limitations. The sample is small but it is representative of the Aché population which has approximately 1000 individuals only (13). The *p*-values were not corrected for multiple tests because we consider this study as exploratory. The opportunities to study genetic susceptibility to infectious disease in Native Americans isolated from their non-native neighbors are becoming increasingly rare. In this context this study was an unparalleled opportunity to increase the knowledge about genetic susceptibility to TST response in this ethnic group. In conclusion, this study showed the importance of the HLA alleles on susceptibility to PPD reaction, a test that measure the exposition to one of the most prevalent infectious diseases in the Native Americans populations. More studies are necessary to better understand the basis of HLA and TB association.

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Conflict of interest

The authors have declared no conflicting interests.

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Capítulo VI - Variability of innate immune system genes in Native American populations - relationship with history and epidemiology

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Brief Communication: Variability of Innate Immune System Genes in Native American Populations—Relationship With History and Epidemiology

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KEY WORDS Amerindians; innate immune genes; pathogens; Amerindian demography; natural selection

ABSTRACT

Objectives: The immune system of a host, defending him/her against invading pathogens, has two main subsystems: innate immunity and acquired immunity. There are several evidences showing that Native American populations are immunologically different from non-Native populations. Our aim was to describe the variability of innate immune system genes in Native American populations.

Materials and Methods: We investigated heterozygozities and patterns of population differentiation (F_{ST}) of 14 polymorphisms related to the innate immune response in five Native American populations (Aché, Guarani-Kaiowá, Guarani-Nandeva, Kaingang, and Xavante) and the results were compared with the three major world population data (YRI, CEU, and CHB) available at the 1,000 genomes database.

Results: Mean heterozygosities ranged between 0.241 ± 0.057 (Aché) and 0.343 ± 0.033 (Kaingang), but no significant differences were observed (Friedman test, $P = 0.197$). Mean heterozygosities were also not significantly different when Amerindians were pooled and compared with the 1000 genomes populations (Friedman test, $P = 0.506$). When the Native American populations were grouped as Amerindians, a significantly higher F_{ST} value (0.194) was observed between the Amerindian and African populations. The Ewens-Watterson neutrality test showed that these markers are not under strong selective pressure.

Discussion: Native American populations present similar levels of heterozygosity as those of other continents, but are different from Africans in the frequency of polymorphisms of innate immune genes. This higher differentiation is probably due to demographic processes that occurred during the out-of-Africa event. Am J Phys Anthropol 000:000–000, 2015.

Individuals display variable ability to fight infections, as well as variable susceptibility to inflammatory and auto immune diseases (reviewed in Quintana-Murci and Clark, 2013). Immune function is likely to be a critical determinant of an organism's fitness, yet most natural populations exhibit tremendous genetic variation for immune traits. In vertebrates this system is composed by two main subsystems: innate and acquired immunity (Pancer and Cooper, 2006). The primary characteristic of the innate immune system is speed, since the protective inflammatory response will start immediately after pathogen exposure. After this first response, innate immunity will play a central role in activating the subsequent adaptive immune response.

Population genetics is an approach that can provide invaluable genetic and statistical information about immune targets and involves the analysis of allele distributions in human populations at loci known or presumed to be involved in host defense and/or self-tolerance. Immunological heterogeneity, both among individuals and among populations, can help to understand the way in which natural selection has acted on host genes over time, by determining the current patterns of variability in the general population (Casanova et al., 2013).

South American Indians present a remarkable number of populations spread over a vast territorial area. The long history of genetic isolation and the great interpopulation diversity make Amerindians very unique. The populations are small, they still follow kinship rules, and mortality is caused mostly by infectious diseases. Many studies had been undertaken among them that

Additional Supporting Information may be found in the online version of this article.

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TABLE 1. Characteristics of the populations investigated

Populations and number of individuals investigated	Aché 98	Guarani; Kaiowá 72; Ñandeva 72	Kaingang 72	Xavante 78
Localities	Arroyo Bandera Chupa-pou	Amambai Limão Verde Porto Lindo	Nonoai	Pimentel Barbosa
Geographic location	55°W, 23°S 56°W, 24°S	55°W, 23°S 55°W, 23°S 54°W, 23°S	52°W, 27°S	51°W, 13°S
Country and region	South Paraguay	Central Brazil	South Brazil	Central Brazil
Linguistic group	Tupi	Tupi	Jê	Jê
Non-Indian admixture (%)[a]	0.0	3.0	6.6	0.0
Sampling period	1998	1992–1993	2000	1990

[a] Estimated by Callegari-Jacques and Salzano (1999).

involved not only genetics, but other areas that are essential for evolutionary interpretations, such as demography, epidemiology and social anthropology. Examination of genetically isolated populations permits analysis of the evolutionary basis for immune gene variation, allowing insight into the role of these genes in health and disease.

There are several evidences showing that Native American populations are immunologically different from non-Native populations (Hurtado et al., 2004; Lindenau et al., 2013). Several investigations reported that Native American populations have a lower variability in several immune system genes such as KM, GM, Kell, HLA, and KIR (Bhatia et al., 1995; Black and Pandey, 1997; Black, 2004; Prugnolle et al., 2005; Augusto et al., 2013, 2015). Recently it has been reported that the pattern of adaptive immune system variability in Amerindians populations differs from that observed in the HapMap CEU population as evaluated by F_{ST} analyses (Lindenau et al., 2013). It is well established that environmental exposure to parasites, helminths, and physical injuries were very different during the evolutionary histories of world populations, leading to differences in immune response patterns observed among these groups (Finkelman and Urban, 2001; Hurtado et al., 2003; Lazzaro and Little, 2009; Schulenburg et al., 2009; Cagliani and Sironi, 2013). Nevertheless, knowledge about the Native American gene population variability of the immune system is still scarce, especially in relation to innate immunity. The present study describes the frequencies of 14 SNPs in innate immune system genes of five Native South American populations, discusses their evolutionary relationships, and the possible implications for immune response when pathogen exposure happens.

MATERIALS AND METHODS

Study subjects

A total of 392 individuals from five Amerindian populations were investigated. Their names and the characteristics of the samples investigated are described in Table 1.

The Aché (or Guayaki) are Tupi-speakers that live in eastern Paraguay. They remained isolated from non-Amerindians, subsisting basically in a hunter-gatherer way of living, until the 1970s. After that, more permanent contact was established. Extensive studies of this population including historical, demographic, social, and medical data were reported by Hill and Hurtado (1996, 1999). Currently there are about 1,000 Aché living in several rural settlements. A total of 98 subjects were sampled for this study.

Kaingang's territory has always been southern Brazil, and they presently live in four Brazilian states (São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul). They are the third most frequent Native American population in this country. Genetic, medical, and demographic information about them have been obtained since the 1960s and a selected bibliography can be found in Marrero et al. (2007). Farming is their main subsistence activity, although hunt and gather has also been important (Marrero et al, 2007). We analyzed 72 individuals from their Nonoai settlement.

The Guarani are the Brazilian most populous Native American population. Contact with non-Amerindians date to the beginning of the Spanish and Portuguese Conquests, in the 16th century (Monteiro, 2009). They are agriculturalists and fishermen. Three main cultural-linguistic subdivisions can be discerned among them: Ñandeva, M'byá (Kaiwá), and Kaiowá. Extensive genetic comparisons between Guarani and Kaingang have been undertaken, and a review can be found in Marrero et al. (2007). A total of 72 Guarani-Ñandeva and 72 Guarani-Kaiowá were included in this study. At present both populations (Kaingang and Guarani) are in advanced stage of acculturation.

The Xavante live in over 100 villages in seven reserves in the state of Mato Grosso, Central Brazil. Data collection for this study was conducted at the Pimentel Barbosa village in 1990. It is the largest village in the reserve of the same name. We analyzed 78 individuals from this settlement. Permanent contact of the Xavante with outsiders took place in the late 1940s (Coimbra et al., 2002). Until recently they were predominantly hunters and gatherers with incipient agriculture (Salzano and Callegari-Jacques, 1988).

Selected genes

We have chosen 13 genes involved in different stages of the innate immune response (Table 2). The polymorphisms investigated were selected based on association studies with infectious diseases in different populations, considering diverse pathogen exposition of continental populations, pattern recognition receptor, chemokine, and nitric oxide systems. Brief information about them follows.

Ten of them are pattern recognition receptors: *TLR1*, *TLR2*, *TLR4*, *TLR7*, *TLR8*, *TLR9*, CD209 (*DC-SIGN*), *CR1*, *NOD2*, and *CD14*. Toll like receptors (TLRs) are expressed on many cell types, being immune response

TABLE 2. Minor allele frequencies for 14 SNPs in 13 innate immune system genes in five Amerindian populations, compared with corresponding data of 1,000 genomes CEU, YRI, and CHB samples

						Aché	Kaingang	Guarani Kaiowá	Guarani Ñandeva	Xavante	Amerindians
Gene	dbSNP ID	Allele	CEU 99	YRI 108	CHB 103	98	72	72	72	78	392
TLR2	rs111200466	Del	0.172	0.218	0.383	0.000	0.129	0.024	0.026	[a]	0.040
CD14	rs2569190	C	0.470	0.685	0.383	0.386	0.556	0.639	0.849	[a]	0.604
TLR1	rs4833095	T	0.793	0.093	0.320	0.311	0.465	0.479	0.557	0.662	0.483
NOD2	rs2066842	T	0.318	0.000	0.000	0.005	0.104	0.000	0.000	0.000	0.021
CCL5	rs2107538	T	0.146	0.440	0.379	0.319	0.146	0.127	0.074	0.027	0.149
NOS2	rs8078340	A	0.131	0.231	0.010	0.182	0.174	0.226	0.160	0.128	0.174
CCL2	rs1024611	A	0.677	0.792	0.364	0.005	0.319	0.140	0.134	0.006	0.113
CD209	rs2287886	G	0.667	0.796	0.311	0.057	0.655	0.873	0.678	0.720	0.563
TLR4	rs1927911	G	0.717	0.301	0.583	0.495	0.761	0.543	0.771	0.821	0.670
CR1	rs2274567	A	0.859	0.745	0.825	0.197	0.796	0.536	0.715	0.691	0.563
TLR8	rs3764880	A	0.727	0.736	0.194	0.366	0.606	0.421	0.470	0.704	0.507
TLR7	rs179008	T	0.197	0.157	0.000	0.000	0.194	0.083	0.197	0.333	0.152
TLR9	rs352140	C	0.535	0.722	0.578	0.175	0.764	0.778	0.646	0.526	0.551
	rs352143	C	0.172	0.389	0.034	0.011	0.076	0.021	0.042	0.299	0.088

[a] Not determined due to genotyping problems.

mediators to a variety of pathogens (reviewed in Kawai and Akira, 2010). They are either expressed on cell surface (as TLR1, TLR2, and TLR4) or intracellularly (as TLR7, TLR8, and TLR9). Several common polymorphisms associated with infectious diseases have been reported for different TLRs (Turvey and Broide, 2010). CD209 is a type II transmembrane protein predominantly expressed on dendritic cells (the antigen-presenting cells). Its presence in macrophages depends on tissue type and state of activation (Geijtenbeek et al., 2000). Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), also known as caspase recruitment domain-containing protein 15 (CARD15), is a cytoplasmic sensor protein that is implicated in a variety of inflammatory and infectious diseases (Inohara and Nunez, 2003). Complement receptor (CR1) is a complement regulator that has three binding sites for C4b and two for C3b. It is found on the red cell surface, but mostly on white cells, and on glomerular podocytes (Gelfand et al., 1975; Fearon, 1985). It can also bind C1q and MBL (mannose binding lectin) and, thus, might play a role in the complement-independent removal and phagocytosis of particles coated by these proteins (Ghiran et al., 2000). Cluster of differentiation 14 (CD14) is a monocytic differentiation antigen that regulates innate immune responses to pathogens, acting as a co-receptor for TLR4 (Liu et al., 2012). It is expressed mainly by monocyte/macrophage lineage cells and it is required for the recognition of extracellular lipopolysaccharides (LPS) and lipoteichoic acid (LTA). Recent research findings revealed associations between the CD14 gene promoter polymorphism and infectious diseases (Anas et al., 2010; Areeshi et al., 2013).

Another set is composed by molecules that act as chemokines, namely CCL2 and CCL5. Monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) is a β-chemokine produced by monocytes and macrophages (Gu et al., 2000). Regulated on Activation, Normal T-Cell Expressed and Secreted (RANTES, also known as CCL5) is a member of the C-C chemokine subfamily. These chemokines are very important for granuloma formation in tuberculosis. CCL2 and CCL5 gene variants were associated with higher susceptibility to infectious diseases (Azad et al., 2012).

Finally, NOS2 (nitric oxide synthase), also known as iNOS2, does not belong to the two previously described categories, but nitric oxide is a pleiotropic regulator of neurotransmission, inflammation, and autoimmunity (Foster et al., 2013).

Laboratory and statistical methods

Genomic DNA was extracted from blood samples and genotyping was carried out by TaqMan® SNP Genotyping Assay methods (Applied Biosystems, Foster City, USA), except for the TLR2 and CD14 variants. The TLR2 deletion (rs111200466) was detected by 7% polyacrylamide gel electrophoresis after PCR amplification; while the CD14 polymorphism (rs2569190) was genotyped by PCR-RFLP as previously described (Greene et al., 2009; Ayaslioglu et al., 2013). Allele frequencies were directly obtained by gene counting and compared with those of the Yoruba of Ibadan, Nigeria (YRI), Utah residents with northern and Western Europe ancestry (CEU), and Han Chinese of Beijing, China (CHB) obtained from the 1,000 genomes database (McVean, 2012). The number of individuals considered was 99 for the CEU population; 108 for YRI; and 103 for CHB. Hardy-Weinberg equilibrium was tested for each locus within each population using the Markov chain as implemented in Arlequin v.3.5 with Bonferroni correction (Excoffier and Lischer, 2010). Arlequin was also employed to perform the Ewens–Watterson neutrality test (infinite allele model) (Ewens, 1972; Watterson, 1975). Mean heterozygosities and their standard errors (Nei, 1987) were calculated with the DISPAN software (Ota, 1993). Since these estimates do not follow a normal distribution, they were compared across populations with the Friedman test using the SPSS v.18 software. Interpopulation variability was determined by F_{ST} and their 95% confidence intervals were estimated with the R software using the diveRsity package (Keenan et al., 2013).

RESULTS

Table 2 shows minor allele frequencies (MAF) of the investigated polymorphisms in Native Americans, CEU, CHB, and YRI populations. The first point to consider is the difference observed among Amerindians. The Aché showed the lowest allele frequencies in 10 out of the 14 variants investigated (71%). In contrast, the Xavante

TABLE 3. Mean heterozygosities for 12 SNPs in five Amerindian populations[a]

	Aché	Ñandeva	Kaiowá	Kaingang	Xavante
rs4833095	0.431	0.497	0.503	0.501	0.450
rs2066842	0.010	0.000	0.000	0.188	0.000
rs2107538	0.437	0.138	0.223	0.251	0.053
rs352143	0.022	0.081	0.041	0.141	0.422
rs8078340	0.299	0.271	0.352	0.289	0.225
rs1024611	0.010	0.234	0.242	0.437	0.012
rs2287886	0.108	0.439	0.223	0.455	0.406
rs1927911	0.502	0.355	0.499	0.366	0.296
rs2274567	0.318	0.411	0.501	0.327	0.429
rs3764880	0.466	0.502	0.491	0.481	0.419
rs179008	0.000	0.319	0.153	0.315	0.447
rs352140	0.290	0.460	0.348	0.363	0.502
Mean ± SD	0.241 ± 0.057	0.309 ± 0.048	0.298 ± 0.052	0.343 ± 0.033	0.305 ± 0.054

[a] Comparison among heterozygosities: Friedman; $P = 0.197$. Heterozygosities do not differ significantly.

TABLE 4. Mean heterozygosities for 12 SNPs in Amerindian, CEU, CHB and YRI populations[a]

	AME	CEU	CHB	YRI
rs4833095	0.500	0.330	0.437	0.169
rs2066842	0.041	0.436	0.000	0.000
rs2107538	0.254	0.251	0.473	0.495
rs352143	0.161	0.286	0.066	0.477
rs8078340	0.288	0.229	0.019	0.357
rs1024611	0.201	0.439	0.465	0.331
rs2287886	0.493	0.446	0.431	0.326
rs1927911	0.443	0.408	0.488	0.423
rs2274567	0.493	0.243	0.290	0.382
rs3764880	0.501	0.399	0.314	0.390
rs179008	0.258	0.318	0.000	0.266
rs352140	0.495	0.500	0.490	0.403
Mean ± SD	0.344 ± 0.047	0.357 ± 0.027	0.290 ± 0.060	0.335 ± 0.040

[a] Comparison among heterozygosities: Friedman; $P = 0.506$. Heterozygosities do not differ significantly.

TABLE 5. Pairwise F_{ST} among Amerindian populations with 95% CI[a]

	Aché	Guarani-Kaiowá	Guarani-Ñandeva	Kaingang	Xavante
Guarani-Kaiowá	0.263 (0.233–0.292)	–			
Guarani-Ñandeva	0.271 (0.227–0.317)	0.050 (0.034–0.077)	–		
Kaingang	0.264 (0.230–0.299)	0.058 (0.035–0.087)	0.042 (0.022–0.069)	–	
Xavate	0.306 (0.265–0.344)	0.117 (0.089–0.151)	0.049 (0.031–0.071)	0.075 (0.050–0.102)	–

[a] There are no significant differences among the pairwise F_{ST} between Aché and Kaingang, Aché and Xavante, as well as Aché and Guarani, since the confidence intervals overlap.

showed the highest frequency in 5 (42%) of the 12 variants studied among them. These two populations showed very contrasting allele frequencies for nine (75%) of the 12 allele distributions. As for the interethnic comparisons, Amerindians showed the lowest or second lowest frequencies in nine (64%) of the 14 comparisons.

The genotype frequencies for all SNPs tested in this study are presented in Supporting Information Table 1. The observed genotype distributions were in agreement with Hardy-Weinberg equilibrium (HWE) for most SNPs after Bonferroni correction. The exceptions were rs3764880 in Aché, Kaingang and Xavante, and rs179008 in Kaingang and Xavante. The small sample sizes used for these comparisons, and eventual deviations from random mating that may occur in these relatively small populations, could contribute to these findings.

Mean heterozygosities ranged between 0.241 ± 0.057 (Aché) and 0.343 ± 0.033 (Kaingang), but no significant differences were observed (Table 3; Friedman test, $P = 0.197$). Mean heterozygosities were also not significantly different when Amerindians were pooled and compared with the 1,000 genomes populations (Friedman test, $P = 0.506$; Table 4).

Pairwise F_{ST} among Amerindian populations are shown in Table 5. Since the confidence intervals for all comparisons overlap, there are no significant differences among populations. When the Native American populations were grouped as Amerindians, a significantly higher value (0.194) was observed between the Amerindian and YRI populations as compared with the Amerindian versus CEU (0.127) and Amerindian versus CHB (0.113) comparisons (Table 6).

The Ewens-Watterson neutrality test showed that the probability of observing random samples with F values identical or smaller than the original sample could be accepted, suggesting that these markers are not under strong selective pressure (Table 7).

DISCUSSION

Infectious diseases and epidemics have always accompanied and characterized human history, representing one of the main causes of death. Even today, despite progress in sanitation and medical research, infectious diseases still are a major killer. Individuals vary in their resistance to infectious disease. Much of this variation is genetic and, in natural populations, considerable attention has been focused on the potential for pathogens to act as a selective force on genetic diversity (Cagliani and Sironi, 2013).

Modern humans encountered changeable environments during the colonization of the world. A significant phenotype variation, involving different behaviors, lifestyles and cultures, was then generated among modern human populations. Wu and Zhang (2011) analyzed the level of population differentiation among different sets of human genes. They concluded that few genes involved with the immune system showed high levels of population differentiation. Quintana-Murci and Clark (2013) argued that the innate immune system position, as first host defense line against pathogens, makes it an excellent model to evaluate the selective pressures that pathogens have exerted in the host genome. Therefore, innate immunity genes would be perfect targets for natural selection.

TABLE 6. Pairwise F_{ST} among Amerindians and others populations with 95% CI[a]

	AME	CEU	CHB	YRI
AME	–			
CEU	0.127 (0.107–0.147)	–		
CHB	0.113 (0.094–0.133)	0.179 (0.153–0.205)	–	
YRI	0.194 (0.174–0.214)	0.173 (0.150–0.197)	0.205 (0.177–0.234)	–

[a] There are significantly higher differences in the pairwise F_{ST} between Amerindians and YRI populations as compared with CEU and CHB, since the confidence intervals do not overlap.

Several studies have demonstrated that Native American populations have a differentiated pattern of variability in the immune system, either in HLA-KIR diversity or in the adaptive profile (Tsuneto et al., 2003; Augusto et al., 2013, 2015; Lindenau et al., 2013). They showed a reduced number of HLA and KIR alleles in relation to non-Native populations, that was considered as one of the explanations for their differentiated susceptibility to introduced diseases (Augusto et al., 2013, 2015; Lindenau et al., 2014). The pattern of adaptive immune system variability in Amerindian populations also differs from that observed in the HapMap CEU population (Lindenau et al., 2013**).**

This same trend seems not to happen with the innate immune markers studied in the present investigation. Our results show that average heterozygosities do not differ among world populations. On the other hand, Amerindians show, as expected, a higher genetic distance considering these alleles from Africans, as compared with European and East Asian samples. This agreement with historical data, at face value, would indicate the absence of differential selection.

Wang et al. (2007) found that Native Americans are strongly differentiated from the rest of the world. Considering that these populations are the youngest in the world, they discussed that it is difficult to infer selection as the main responsible for this differentiation. The little time allowed for selection to operate and small population sizes raised the possibility that demographic factors would be the better explanation for these results. Hofer et al. (2009) also suggested that demographic factors are probably the best explanation for the differentiation observed between Africa and Americas. Taking into account human evolutionary history, we need to consider, for instance, the spatial and demographic bottlenecks that occurred during the out-of Africa to Eurasia and the Americas. As discussed by Travis et al. (2007), these bottlenecks could be responsible for allelic surfing during subsequent spatial expansions.

CONCLUSION

Our results suggest that Native American populations present similar levels of heterozygosity as those of other continents, but are different from Africans in the frequency of polymorphisms of innate immune genes. This

TABLE 7. Ewens-Watterson neutrality test for 14 SNPs in five Amerindian populations[a]

	Aché	Ñandeva	Kaiowá	Kaingang	Xavante
rs111200466	–	0.691 (>0.999)	0.610 (>0.999)	0.363 (>0.999)	–
rs2569190	0.083 (0.996)	0.294 (>0.999)	0.108 (>0.999)	0.039 (0.546)	–
rs4833095	0.150 (>0.999)	0.044 (0.572)	0.014 (0.182)	0.026 (0.364)	0.119 (>0.999)
rs2066842	>0.999 (>0.999)	–	–	0.373 (>0.999)	–
rs2107538	0.135 (>0.999)	0.500 (>0.999)	0.342 (>0.999)	0.323 (>0.999)	0.679 (>0.999)
rs352143	0.800 (>0.999)	0.565 (>0.999)	0.729 (>0.999)	0.474 (>0.999)	0.156 (>0.999)
rs8078340	0.276 (>0.999)	0.301 (>0.999)	0.212 (>0.999)	0.306 (>0.999)	0.341 (>0.999)
rs1024611	>0.999 (>0.999)	0.368 (>0.999)	0.345 (>0.999)	0.137 (>0.999)	>0.999 (>0.999)
rs2287886	0.473 (>0.999)	0.135 (>0.999)	0.338 (>0.999)	0.128 (>0.999)	0.176 (>0.999)
rs1927911	0.004 (0.048)	0.206 (>0.999)	0.031 (0.403)	0.214 (>0.999)	0.272 (>0.999)
rs2274567	0.249 (>0.999)	0.183 (>0.999)	0.023 (0.299)	0.230 (>0.999)	0.138 (>0.999)
rs3764880	0.093 (>0.999)	0.022 (0.286)	0.048 (0.624)	0.085 (>0.999)	0.169 (>0.999)
rs179008	–	0.250 (>0.999)	0.426 (>0.999)	0.269 (>0.999)	0.134 (>0.999)
rs352140	0.265 (>0.999)	0.102 (>0.999)	0.238 (>0.999)	0.227 (>0.999)	0.030 (0.330)

[a] Values in parentheses are adjusted for multiple comparisons by the Bonferroni test. The dashes indicate the impossibility of performing the test due to either absence of variation in the indicated population, or absence of study for the given polymorphism.

difference is probably due to demographic processes. Native Americans provide good models to elucidate human evolutionary history, but interpretation of the findings are difficult due to decimation of many groups as a consequence of diseases introduced by non-Natives. Further investigations on carefully selected samples using a genomic approach would be welcome.

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Capítulo VII - The role of variants from the innate immune system genes in tuberculosis and skin test response in a Native American population

Em preparação

The role of variants from the innate immune system genes in tuberculosis and skin test response in a Native American population

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Short tittle: Tuberculosis susceptibility in Amerindians

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ABSTRACT

Native American populations show higher tuberculosis (TB) mortality and infectivity rates than non-Native populations. Variants in the innate immune system seem to have an important role on TB susceptibility. The role of some innate immune system variants in TB susceptibility and/or skin test response (PPD) were investigated in the Aché, a Native American population. Complement receptor 1 and toll like receptor 9 variants were associated with anergy to PPD and protection to TB, respectively. These findings demonstrate an important role of the innate immune system variants in TB susceptibility.

Keywords: tuberculosis, Amerindians, skin test response, innate immunity, CR1, TLR9, Aché

Several immune system genes have been involved in tuberculosis (TB) progression or in the *Mycobacterium tuberculosis* (Mtb) control. Several studies reported that variants in the innate immune system seem to have an important role on TB susceptibility. Besides its role in the defense against strange antigens, the innate system is emerging as an important regulator of the adaptive immune response (1).

Tuberculosis is an important health public problem among Native American populations since these populations show higher mortality and infectivity rates due to TB than non-Native populations (2, 3). This differential susceptibility is usually explained by nontraditional variability in the HLA/KIR, adaptive and innate immune systems genes (4-7). The reduced variability in the HLA system was associated with higher purified protein derivative skin test response (PPD) reaction in the Aché population (8). This test is not only used to identify infected persons, but also to assess cell-mediated immune response to Mtb, and to guide decisions about chemoprophylaxis and treatment. The lack of reaction in this test is called anergy and it can be due to two different procedures, non previous pathogen exposure or deficient immune response against the pathogen. Some variants in the adaptive immune system were also associated with higher PPD reaction or with TB susceptibility in the Aché group (9). It is important to understand how the genetic variability in the innate immune system can act in the susceptibility or resistance to Mtb. Here we investigated the role of some variants from this system in TB susceptibility and/or PPD skin test response in the Aché, a Native American population.

The Aché population was recruited in Arroyo Bandera (55°50'W, 23°30'S) and Chupa Pou (56°30'W, 24°10'S) settlements in Paraguay. The sample was composed by 96 individuals, which 52 were men. TB was diagnosed with a combination of X-ray, contact history, and clinical symptoms. Infection was determined by PPD skin tests, where a wheal size of ≥5mm was considered positive. We used this low cut-off value because the Aché population shows a tendency to be unresponsive to PPD (3). All the unresponsive individuals were considered anergic. PPD status is available for 83 individuals. Genomic DNA, genotyping procedures, and polymorphism frequencies have been described

elsewhere (7). In that study 14 variants were investigated, but for association analyses presented here only 8 SNPs were selected based on minor allele frequencies (MAF) >0.15. To avoid categories with few individuals, a frequency criterion was applied for rare genotype grouping.

The association between PPD reaction and/or TB susceptibility was evaluated with prevalence ratios (PR) estimated by univariate Poisson regression with robust variance. This method provides correct estimates and it is a better alternative for cross-sectional studies with binary outcomes with high frequencies (>10%) than logistic regression, since the prevalence ratio (PR) is more interpretable (10). The variants with a p-value <0.1 in the univariate analysis were analyzed in a multivariate Poisson regression, with age as covariate. Sex did not show any effect, so it was not included as covariate in the multivariate analysis. Statistical analyses were performed using SPSS for Windows v18.0 (SPSS Inc., Chicago, IL, USA). P-value <0.05 was considered significant. The effect sizes for significant p-values were obtained through the transformation to the Cohen effect size measure (11).

Among the 83 individuals with PPD status available, 36 (43%) were anergics, while 47 (57%) had 5mm or greater wheal size. Table 1 shows the association analyses of PPD reactions. The A allele carriers from complement receptor 1 (CR1) were more prevalent into those subjects with PPD induration ≥5 mm (PR: 1.165, CI: 1.021-1.329, p-value: 0.023). Among the 96 individuals screened for TB, 39 (41%) have active TB. The Toll like receptor 9 (TLR9) TT genotype was associated with lower prevalence of active TB (Table 2). Therefore, this genotype seems to be protector to TB (PR: 0.607, CI: 0.377-0.977, p-value: 0.040). All other SNPs tested were non-significant.

Several studies postulated that TLR signaling has a major involvement in host resistance to Mtb through the well-characterized TLR ligands found in Mtb (12). These ligands are potent stimuli to a number of proinflammatory cytokines, including TNF-α and IL-12, affecting the innate and adaptive immune response through the activation of a predominantly Th1 pattern. TLR9 recognizes unmethylated Cytosine-phosphate-Guanine (CpG) DNA motif found in DNA viruses and prokaryotic genomes. In humans, this receptor is predominantly

expressed in professional type I IFN-producing dendritic cells and B cells. It was demonstrate that TLR9 acts regulating the Th1 response, either alone or collaborating with TLR2 (13). A predominance of Th1 response can be an ideal scenario to fight against Mtb, leading to TB protection. TLR9 gene functions are affected by several SNPs, including the rs352140 variant investigated herein. It is located in exon 2 and functional studies demonstrated that the TT genotype is associated with enhanced TLR9 expression (14). This higher TLR9 expression could enhance innate immune reactivity functions against Mtb, leading to a better Th1 response and, consequently, to TB protection.

Erythrocyte complement component (3b/4b) receptor 1 (CR1) is a member of the receptors of complement activation family. It encodes a momomeric single-pass type I membrane glycoprotein found on several cells. Its function is to prevent immune complex deposition in the vessel wall by binding complement-tagged inflammatory particles and facilitating their clearance. Elevated levels of circulating immune complexes have been reported in diseases like leprosy and TB, demonstrating the importance of the CRs in the pathology of mycobacterial diseases (15-17). It was reported that human monocyte CR1 and CR3 mediate Mtb phagocytosis and the C component C3 in the serum is the bacterium-bound ligand (18). After CR1 binding, the C3 containing immune complexes are carried to the macrophages where CR1 is cleaved and hence continuously lost. The nonsynonymous variant rs2274567 results in a change from histidine to arginine and it is located in one conserved β sheet (β_2). Kullo et al. (19) predicted this variant as “probably damaging” and showed that this one might alter the secondary structure of the sheets, affecting the binding affinity of CR1 to C3b and C4b. Our results showed an association between this variant and PPD reaction. To our knowledge, this was the first time that this association was reported. A allele carriers showed a higher prevalence of PPD ≥5 mm reaction than individuals with GG genotype. Therefore the GG genotype may be related to higher rates of anergy to PPD. Fitness et al. (20) observed an association between another variant that also affects the binding affinity to CR1 and TB susceptibility. Apparently, a lower affinity between CR1 and C3 confers a poor immune response against Mtb, leading to higher susceptibility to the disease and higher anergy

rates. Senbagavalli et al. (21) observed reduced levels of CR1 in patients with TB, speculating that they were due to the greater bacillary load. This load leads to more immune complexes formation demanding the engagement of CR1 molecules, which are subsequently lost in the process of immune complexes elimination. We hypothesized that a similar procedure happens when the binding affinity is altered, as in the variant analyzed here. The G allele confers lower binding affinity which leads to a high bacillary load free in the system. This can be one reason to the higher anergy rates observed in the studied population.

Our results should be considered in the view of some limitations, among them sample size. Although small, the sample size is representative for the Native American group investigated, since the whole Aché population has almost 1000 individuals (3). The p-values were not corrected for multiple tests because we consider this study as exploratory. The small effect sizes are in line with those expected for multifactorial diseases as TB. The opportunity to study Native populations is became rarer due to population decimation due to introduced diseases as well as government politics. So our results add important knowledge about the TB epidemiology in Native American populations.

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Table 1 Association analyses of PPD reaction prevalence ratios and immune system genes polymorphisms in the Aché population

Gene	dbSNP ID	Genotypes	PPD=0	PPD≥5	Model 1		Model 2		
					PR (95% CI)	P-value	PR (95% CI)	P-value	Effect Size
CD14	rs2569190	CC	5	7	1				
		CT	16	14	0.926 (0.748-1.148)	0.484			
		TT	11	17	1.015 (0.824-1.251)	0.889			
TLR1	rs4833095	CC	14	24	1.080 (0.944-1.236)	0.256			
		T carriers	22	23	1				
CCL5	rs2107538	CC	13	23	1.069 (0.934-1.223)	0.333			
		T carriers	21	24	1				
NOS2	rs8078340	GG	26	28	1				
		A carriers	10	18	1.082 (0.941-1.243)	0.268			
TLR4	rs1927911	AA	7	14	1.000 (0.837-1.195)	1.000			
		AG	23	20	0.879 (0.745-1.037)	0.127			
		GG	6	12	1				
CR1	rs2274567	GG	28	27	1		1		
		A carriers	7	20	1.168 (1.025-1.330)	**0.019**	1.165 (1.021-1.329)	**0.023**	0.084
TLR8	rs3764880	AA	5	12	1.098 (0.939-1.284)	0.240			
		AG	10	8	0.930 (0.774-1.117)	0.438			
		GG	21	26	1				
TLR9	rs352140	TT	24	32	1				
		C carriers	12	15	0.990 (0.855-1.146)	0.892			

dbSNP is a public database of single nucleotide polymorphisms (SNPs) found at http://www.ncbi.nih.gov/projects/SNP/

Model 1: Prevalence ratios (PRs) for each polymorphism. The P-values lower than 0.1 are in bold.

Model 2: multivariate Poisson regression with age as covariate for the polymorphisms with p<0.1 in the univariate analysis, P-values that reached the statistical significance (at the 0.05 level) are in bold.

Table 2 Association analyses of the tuberculosis susceptibility prevalence ratios and immune system gene polymorphisms in the Aché population

Gene	dbSNP ID	Genotypes	TB negative	TB positive	Model 1		Model 2		
					PR (95% CI)	P-value	PR (95% CI)	P-value	Effect Size
CD14	rs2569190	CC	8	5	1.084 (0.470-2.500)	0.850			
		CT	22	11	0.939 (0.477-1.848)	0.856			
		TT	20	11	1				
TLR1	rs4833095	CC	26	17	0.952 (0.584-1.553)	0.845			
		T carriers	31	22	1				
CCL5	rs2107538	CC	23	16	1.035 (0.627-1.711)	0.892			
		T carriers	32	21	1				
NOS2	rs8078340	GG	40	22	0.757 (0.460-1.246)	0.274			
		A carriers	17	15	1				
TLR4	rs1927911	AA	11	11	1.000 (0.546-1.832)	1.000			
		AG	34	17	0.667 (0.371-1.197)	0.175			
		GG	10	10	1				
CR1	rs2274567	GG	39	24	0.850 (0.509-1.418)	0.533			
		A carriers	16	13	1				
TLR8	rs3764880	AA	15	10	1.020 (0.566-1.839)	0.947			
		AG	11	7	0.992 (0.506-1.943)	0.981			
		GG	31	20	1				
TLR9	rs352140	TT	44	21	0.570 (0.356-0.913)	**0.019**	0.607 (0.377-0.977)	**0.040**	0.276
		C carriers	13	17	1		1		

dbSNP is a public database of single nucleotide polymorphisms (SNPs) found at http://www.ncbi.nih.gov/projects/SNP/

Model 1: Prevalence ratios (PRs) for each polymorphism. The P-values lower than 0.1 are in bold.

Model 2: multivariate Poisson regression with age as covariate for the polymorphisms with p<0.1 in the univariate analysis, P-values that reached the statistical significance (at the 0.05 level) are in bold.

Capítulo VIII - Discussão

As discussões específicas dos resultados obtidos nesta Tese encontram-se nos capítulos III a VII. A discussão apresentada aqui tem como propósito ser um texto mais abrangente cujo objetivo é integrar as ideias abordadas nos capítulos anteriores, dentro do contexto de resposta imune em populações nativas sul americanas, demonstrando as inovações e as perspectivas advindas dos resultados obtidos nesta Tese.

VIII. 1 - Genética de populações de genes associados com a resposta imune

O conhecimento sobre a variabilidade em marcadores do sistema imune em populações nativas sul americanas é ainda escasso. Alguns estudos descreveram a variabilidade genética nos sistemas HLA/KIR (Bathia *et al*., 1995; Tsuneto *et al*., 2003; Black, 2004; Augusto *et al*., 2013; Augusto *et al*., 2015) e adaptativo (Zembrzuski *et al*., 2010) em algumas dessas populações, enquanto que informações sobre a variabilidade genética no sistema inato são inexistentes na literatura. De maneira geral, os resultados obtidos no presente trabalho, reunidos com as informações anteriormente disponíveis, nos permitiram concluir que os Ameríndios possuem baixa variabilidade genética para os três sistemas citados anteriormente.

As populações Aché, Guarani e Kaingang investigadas no presente trabalho já apresentaram uma reduzida diversidade alélica e haplotípica no sistema HLA classe II em relação às demais populações continentais (Tsuneto *et al*., 2003). Essa baixa diversidade também pôde ser constatada no sistema KIR, onde somente três ou quatro perfis explicam cerca de 70% da variabilidade genética observada nessas populações (Augusto *et al*., 2015). Esse é um achado especialmente interessante, uma vez que nas demais populações mundiais pode-se observar uma tendência contrária àquela que foi observada nos ameríndios. Enquanto as populações nativas sul americanas apresentam poucos perfis de HLA/KIR, mas cada um deles possui alta frequência, as demais populações mundiais apresentam muitos perfis, mas cada um deles apresenta baixa frequência. Ou seja, as demais populações mundiais não apresentam nenhum

perfil de HLA/KIR predominante, diferentemente das populações nativas sul americanas. Outro ponto divergente no sistema KIR entre ameríndios e as demais populações relaciona-se com a presença do haplogrupo A e B. Enquanto o haplogrupo mais prevalente nas populações mundiais é o A, as populações nativas sul americanas apresentam frequências elevadas do haplogrupo B, variando de 36% a 58% (Augusto *et al*., 2015).

Essa baixa variabilidade genética observada no sistema imune das populações nativas sul americanas pode também ser observada nos sistemas adaptativo e inato. Informações sobre a variabilidade genética em marcadores do sistema adaptativo para a população Xavante descritas anteriormente (Zembrzuski *et al*., 2010) revelaram que dos 19 marcadores analisados nessa população, nove (correspondendo a 47% do total) apresentaram a prevalência do alelo menos frequente (MAF) menor do que 10%. Desses nove marcadores, três foram monomórficos nessa população (correspondendo a 16% do total de marcadores analisados). Esses achados foram corroborados pelas informações acrescentadas pela presente Tese, onde a variabilidade genética dos 32 marcadores do sistema adaptativo analisados nas populações Aché, Guarani e Kaingang foi comparada com aquela observada nos três grandes grupos populacionais disponíveis no HapMap (europeus, africanos e asiáticos). As populações nativas sul americanas apresentaram a menor ou a segunda menor MAF para 19 dos 32 marcadores analisados (correspondendo a 59% do total). Se considerarmos as frequências observadas para a população Aché (que assim como os Xavantes, é uma população não miscigenada), o padrão apresenta-se bastante similar àquele observado por Zembrzuski *et al*. (2010). Dos 32 marcadores aqui investigados, 18 apresentaram MAF menor do que 10% (correspondendo a 56% do total), enquanto que seis marcadores foram monomórficos nessa população (correspondendo a 19% dos marcadores analisados). O mesmo padrão pôde ser visualizado quando analisamos marcadores do sistema imune inato, onde os ameríndios apresentaram a menor ou segunda menor MAF em nove dos 14 marcadores analisados (correspondendo a 64% do total). Considerando os resultados específicos para a população Aché, seis dos 14 marcadores apresentaram MAF menor do que 10% (correspondendo

a 43% do total) e dois marcadores foram monomórficos (correspondendo a 14% dos marcadores analisados).

Considerando a variabilidade em marcadores de resposta imune nesses grupos, diferenças significativas entre as populações nativas sul americanas e as populações representativas das populações continentais (europeus (CEU), africanos (YRI) e asiáticos (CHB)) foram demonstradas no presente trabalho. A maior semelhança observada entre as populações nativas e a população CHB, independentemente do conjunto de marcadores analisados, deve-se, sobretudo, à dinâmica de dispersão da população humana após a saída da África. Considerando que as populações asiáticas são os ancestrais mais próximos dos grupos nativos sul americanos, uma maior semelhança seria esperada devido aos efeitos estocásticos observados durante esse processo. No entanto, uma grande diferenciação entre as populações ameríndias e a população europeia foi estimada com os marcadores do sistema imune adaptativo (FST = 0,231), enquanto que os marcadores do sistema imune inato mostraram-se mais diferenciados quando consideramos as populações nativas e a população africana (FST = 0,194).

O sistema inato é a primeira linha de defesa contra qualquer patógeno invasor; portanto, esse sistema precisa estar apto a desenvolver a resposta imune inicial a qualquer momento em que houver contato com o antígeno estranho. Nesse contexto, espera-se uma menor pressão seletiva para mudanças no mesmo, o que justifica o fato desse sistema ser altamente conservado entre primatas, uma vez que qualquer variação pode acarretar mudanças significativas na resposta imune desencadeada (Barreiro *et al*., 2010). Entretanto, não podemos excluir a possibilidade de que os patógenos exerçam algum tipo de pressão seletiva sobre os genes que compõem esse sistema, o que os tornariam um alvo perfeito para a seleção natural (Quintana-Murci & Clark, 2013). Nesse sentido, por exemplo, TLRs parecem ter evoluído sobre forte seleção purificadora, não tolerando mutações sem sentido ou mutações de perda de sentido. Contudo, TLRs de superfície celular apresentam uma diversidade genética e funcional muito maior (Ferwerda *et al*., 2007; Barreiro *et al*., 2009). Alguns estudos demonstraram que a diversidade genética observada no gene NOD2 parece ser

consistente com a neutralidade, enquanto alguns alelos desse gene foram possivelmente alvos de seleção positiva recente ou seleção balanceadora (Nakagome *et al*., 2012; Vasseur *et al*., 2012). Entretanto, existe muita dificuldade em distinguir os efeitos de seleção positiva dos efeitos da demografia, principalmente devido aos eventos demográficos do passado. Gargalos de garrafa populacionais ou grandes expansões podem mimetizar as assinaturas genéticas de uma varredura seletiva (Hofer *et al*., 2009). Se considerarmos novamente a dispersão da população humana, uma maior diferenciação em relação aos marcadores do sistema imune inato entre as populações ameríndias e a população africana poderia ser explicada em função dos milhares de anos em que os processos demográficos têm atuado nessas populações desde a saída do *Homo sapiens* da África. Esses processos demográficos parecem ser a explicação mais parcimoniosa nesse caso em função dos resultados obtidos para os testes de neutralidade nesses marcadores. Não foi possível observar nenhum desvio da neutralidade, sugerindo que esses marcadores não estão sobre nenhum evento de seleção nessas populações.

O cenário é distinto em relação aos marcadores do sistema adaptativo, uma vez que observamos uma maior diferenciação dos ameríndios em relação à população europeia (FST = 0,231) e uma diferenciação mediana entre ameríndios e a população africana (FST = 0,150). Esses resultados sugerem que ambientes tropicais (como aqueles que populações nativas sul americanas e africanas compartilham) levariam a uma maior prevalência de marcadores pró-inflamatórios, que desencadeariam a predominância de uma resposta imune do tipo Th2, a resposta adequada para combater helmintos e parasitas em geral (Gold *et al*., 1993; Von Behren *et al*., 1999). Dessa maneira, a maior semelhança em termos de variantes do sistema adaptativo observada entre nativos sul americanos e africanos pode ser o resultado de uma origem comum aliada a um ambiente tropical compartilhado, que apresenta níveis similares de seleção. Podemos considerar um cenário diferente quando as populações humanas saíram da África em direção aos ambientes temperados, uma vez que os patógenos teriam uma prevalência diferenciada nesses ambientes em relação àquela observada em ambientes tropicais. Em um ambiente consideravelmente mais frio, indivíduos

com forte resposta inflamatória poderiam ter problemas ao desencadear uma resposta imune robusta diante de antígenos inofensivos. Eventos demográficos, como a deriva genética, aliados a milhares de anos de seleção contra essas fortes respostas inflamatórias, podem ter levado a uma mudança no perfil imune adaptativo, tornando-o menos inflamatório (Le Souëf *et al*., 2000). Nesse contexto, justifica-se a predominância de um padrão de resposta Th1 em populações de origem europeia (Wilson *et al*., 1986) e a maior diferenciação observada entre populações nativas sul americanas e europeia observada na presente Tese.

Poucas informações relacionadas ao balanço entre respostas Th1 e Th2 em populações nativas sul americanas estão disponíveis na literatura para confirmação dessas hipóteses. Estudos medindo os níveis de IgE determinaram que africanos e ameríndios tendem a ser mais Th2 dominantes do que Th1 dominantes, enquanto europeus tendem a ter um padrão contrário, com predominância de Th1 (Kaplan *et al*., 1980; Wilson *et al*., 1986, Sousa *et al*., 1997). Os resultados obtidos na presente Tese ajudam a elucidar ao nível molecular essas observações, uma vez que, em relação aos marcadores do sistema adaptativo, foi observada uma menor diferenciação genética entre as populações nativas sul americanas e a população africana do que aquela observada com a população europeia. Essa maior diferenciação reforça as informações fenotípicas de uma troca no padrão de resposta Th que predomina nessas populações e corrobora a hipótese de que a predominância do padrão Th1 nas populações europeias foi uma adaptação ao clima.

A presente Tese aumentou significativamente o conhecimento em relação à variabilidade genética em marcadores do sistema imune em populações nativas sul americanas, contudo, não podemos considerar esse assunto como esgotado. Diversas vias atuam para o desenvolvimento de uma resposta imune adequada e, consequentemente, uma série de genes apresentam funções essenciais para o desencadeamento dessa resposta. Assim, determinar a variabilidade genética em genes que compõe essas diversas vias será extremamente importante para traçar o perfil imunogenético das populações nativas sul americanas.

A via do complemento, composta por cerca de 50 proteínas encontradas no plasma e nas células sob a forma de proteínas de controle ou receptores, seria um alvo interessante de estudo. Sua atuação pode ser através da lise direta do corpo estranho ao organismo ou pela opsonização e recrutamento de leucócitos que promovem fagocitose. Essa via foi parcialmente abordada na presente Tese, uma vez que tem relação direta com o sistema inato, mas um aprofundamento em relação aos seus diversos marcadores seria importante para uma melhor compreensão do mecanismo de desencadeamento da resposta imune adequada. Além disso, existe uma série de evidências demonstrando um importante papel das plaquetas na imunidade inata através de interação com moléculas do sistema complemento (Speth *et al*., 2013; Speth *et al*., 2015). Essa ativação plaquetária estaria associada com a liberação de quimiocinas e citocinas derivadas do megacariócito e de lipídios pró-inflamatórios, que podem induzir efeitos pleiotrópicos em diversos tipos de células e tecidos, inclusive nos leucócitos. Esse recrutamento leucocitário é considerado um passo importante para a formação dos trombos, ligando a trombose à resposta inflamatória (Cerletti *et al*., 2012). Nesse contexto, um maior conhecimento em relação aos marcadores relacionados com a variabilidade plaquetária pode contribuir para uma melhor compreensão da resposta imune.

Entretanto, o perfil imunogenético de uma população não estaria completo sem a análise da diversidade dos receptores de célula B e T. Cada receptor de célula B (BCR) é composto por duas cadeias pesadas e duas cadeias leves de imunoglobulina e uma subunidade de sinalização de imunoglobulina alfa e beta. Cada receptor apresenta uma especificidade única e é justamente essa diversidade de especificidades de BCRs que é o ponto central para a imunidade adaptativa. Os receptores de célula T são também importantes para a imunidade adaptativa, uma vez que são as moléculas capazes de reconhecer o MHC ligado ao peptídeo. Cada TCR é um heterodímero de uma cadeia alfa e uma cadeia beta, ou uma cadeia gama e uma cadeia delta, sendo cada cadeia composta de um domínio constante e um domínio variável. Entretanto, devido à alta variabilidade observada em ambos os tipos de receptores, é extremamente difícil quantificar a diversidade de BCRs e TCRs de um individuo. Atualmente métodos

como o sequenciamento de alta resolução são os que apresentam resultados mais acurados em relação à diversidade desses receptores, contudo, ainda apresentam o viés da PCR e os erros de sequenciamento, que podem levar a identificação errônea de um receptor e inflar a diversidade (Bolotin *et al*., 2012). O aprimoramento em técnicas laboratoriais e nas análises de bioinformática e bioestatística é constante no ambiente de pesquisa e diagnóstico e, provavelmente, nos próximos anos será possível medir adequadamente a diversidade desses receptores, o que contribuirá significativamente para a elucidação dos mecanismos que determinam uma resposta imune efetiva.

Um ponto importante observado nos resultados obtidos que deveria ser mais investigado é a diferença de heterozigosidade observada entre as populações nativas sul americanas. De maneira geral, observou-se uma menor heterozigosidade nas populações Aché e Xavante e uma maior heterozigosidade na população Kaingang. As populações Guarani apresentaram valores intermediários. Se considerarmos as informações em relação ao nível de miscigenação dessas populações, os Aché e Xavante não apresentam evidencias de miscigenação com suas populações vizinhas não ameríndias. Os Guaranis possuem cerca de 3% de mistura enquanto os Kaingang apresentam os maiores níveis de mistura interétnica (cerca de 7%; Callegari-Jacques & Salzano, 1999). Dessa forma, não podemos extrapolar qual a real redução de variabilidade que ocorre nesse grupo étnico. A investigação de mais populações com pouca ou nenhuma evidência de miscigenação seria fundamental para responder essa questão.

Ségurel & Quintana-Murci (2014) propuseram que a diversidade imune pode ter sido adquirida através da miscigenação com espécies de hominídeos que se encontram extintas atualmente (como Neanderthal e Denisova) através de introgressão adaptativa. Esse processo poderia ter permitido que variantes funcionalmente vantajosas relacionadas com as respostas imunes fossem adquiridas mais rapidamente. Recentemente, Jeong *et al*. (2014) demonstraram um caso de adaptação a altas altitudes facilitada pelo fluxo gênico entre populações humanas modernas nos genes *EGLN1* e *EPAS1*, que são associados com menor concentração de hemoglobinas. Contudo, não há nenhum estudo que

demonstre se poderia ser possível a introgressão adaptativa entre populações humanas modernas que sofrem pressões patogênicas diferenciadas. A introgressão adaptativa em genes relacionados com melhor resposta a patógenos poderia ser um evento plausível em populações miscigenadas modernas. Nesse contexto, as populações e informações adicionadas pela presente Tese apresentam-se como uma opção adequada para testar essa teoria, uma vez que as populações analisadas aqui apresentam diferentes níveis de miscigenação com populações não nativas.

VIII. 2 - Suscetibilidade Genética para a Tuberculose

A importância de marcadores do sistema imune para a suscetibilidade a doenças infecciosas introduzidas em populações nativas sul americanas ainda não foi totalmente explorada. De maneira geral, os estudos realizados com essas populações focavam somente em suas respostas imune ao nível fenotípico, através da análise de anticorpos reconhecidamente relacionados com predominância de resposta Th1 ou Th2. Esse tipo de abordagem não permitia conclusões em relação à dinâmica da resposta imune nesses grupos, assim como diretrizes mais enfáticas em relação a prováveis mudanças na política de saúde pública aplicada atualmente a esses grupos. Além disso, poucos estudos haviam analisado como o sistema imune reagia diante do teste de sensibilidade à tuberculina (TST) e quais marcadores poderiam estar relacionados com o processo de anergia nessas populações, apesar da alta taxa de anergia comumente observada nesses grupos.

O TST apresenta-se como um importante teste para tuberculose (TB), uma vez que seu resultado serve como informação relevante para a utilização de quimioprofilaxia e diagnóstico, principalmente em crianças, em áreas endêmicas de TB. Contudo, poucas informações relacionando os resultados obtidos nesse teste e a resposta imune que provavelmente estava sendo desencadeada estavam disponíveis. De maneira geral, podemos considerar o estudo de Zembrzuski *et al*. (2010) com a população Xavante como pioneiro nesse tópico. As informações obtidas com a análise dos resultados no teste de sensibilidade a

tuberculina na população Aché demonstraram um importante papel de marcadores dos sistemas imune adaptativo e inato na reação ao TST. Níveis elevados de reação ao TST estariam relacionados com uma resposta predominantemente Th1, enquanto que a anergia estaria relacionada com predominância de um padrão Th2 de resposta imune. Em suas observações de pacientes anérgicos, Montiel *et al*. (2002) já haviam sugerido que esse fenótipo estava relacionado com predominância de um padrão Th2 de resposta adaptativa. O presente trabalho acrescenta uma confirmação ao nível molecular desses resultados. Essas informações são importantes auxiliares nas políticas públicas de combate ao *Mycobacterium tuberculosis* (Mtb), visto que permitem uma determinação rápida (uma vez que o resultado do teste está disponível em até 72 horas após a aplicação) do possível panorama imune da população exposta ao Mtb ou vacinada com BCG.

Os resultados aqui obtidos demonstram a importância de considerarmos as diferenças entre as populações em relação ao *background* genético disponível para desencadear uma resposta imune adequada, quando ocorrer confronto com patógenos. Isso parece particularmente importante no contexto da tuberculose, uma vez que as populações nativas sul americanas apresentam uma maior suscetibilidade a essa patologia em relação a outros grupos étnicos. Mesmo com altas taxas de vacinação com BCG, essas populações não conseguem desenvolver uma resposta imune adequada frente ao Mtb. Atualmente a BCG é a única vacina licenciada contra Mtb, mas sua eficácia na prevenção de tuberculose pulmonar pode variar de 0 a 80%. Diversos estudos demonstraram que aproximadamente metade da variabilidade na resposta a vacinação pode ser explicada em função das diferenças geográficas entre os estudos, principalmente na latitude (Fine, 1995). Fatores ambientais também mostraram ser essenciais para uma adequada eficácia da BCG. Dentre esses fatores podemos considerar como principais o índice de massa corporal da mãe durante a gestação e o peso do recém-nascido, além das exposições prévias a micobactéria (revisão em Kollmann, 2013).

Além disso, diferenças no tipo de resposta Th predominante na população (no caso Th1 em europeus e Th2 em africanos e nativos americanos) também

parecem ter uma importante contribuição na resposta desencadeada no processo de imunização, sugerindo que crianças de diferentes regiões do mundo possam responder diferentemente à vacinação. Estudos demonstraram que genes do sistema imune inato, como os TLRs, estão associados com o desenvolvimento da resposta imune efetiva após a vacinação (Randhawa, 2011). Isso implica que a variação genética entre as populações tenha um importante papel na resposta à vacinação por BCG. Nesse contexto, as informações adicionadas pela presente Tese em relação à variabilidade em marcadores do sistema imune em populações nativas sul americanas, assim como a importância desses marcadores para a patologia da TB nesses grupos, podem colaborar para uma futura elaboração de políticas de saúde mais eficazes para grupos humanos isolados.

O presente estudo deve ser, no entanto, visto no contexto de algumas limitações. A ausência de estudos de expressão dos marcadores do sistema imune aqui investigados não permite que análises de causa e efeito possam ser abordadas. No entanto, esse tipo de abordagem seria de difícil execução em populações isoladas. Inicialmente devemos considerar o tempo que transcorreu entre a coleta das amostras e a execução do projeto. Quando essas amostras foram coletadas há algumas décadas, esse tipo de abordagem não estava entre os objetivos da coleta, consequentemente não houve preservação adequada para estudos funcionais. Além disso, para obtermos um verdadeiro panorama de como o sistema imune atua diante de um antígeno estranho, os estudos de expressão deveriam ser conduzidos durante um episódio de infecção. Sabe-se que há diferenças consideráveis entre a expressão de marcadores durante o estágio de não infecção e o estágio de infecção (além de variações nos diferentes estágios clínicos da doença), o que acaba por limitar ainda mais esse tipo de abordagem (Singer & Ouburg, 2016).

Apesar das questões ainda não completamente elucidadas, a presente Tese acrescenta importantes informações em relação aos padrões de resposta imune que podem ser comumente observados em populações nativas sul americanas e sua relação com a patologia de doenças infecciosas introduzidas. Os dados apresentados aqui poderão auxiliar, no futuro, na escolha de políticas de saúde pública mais apropriadas para essa etnia.

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