Faculdade de Medicina de São José do Rio Preto

Programa de Pós-graduação em Ciências da Saúde

Christiane Maria Ayo

Genes *KIR*, seus ligantes HLA e polimorfismo do gene *MICA* na toxoplasmose ocular e nas formas clínicas da doença de Chagas crônica

São José do Rio Preto
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Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso de Pós-Graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientador: Prof. Dr. Luiz Carlos de Mattos

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São José do Rio Preto, 30/06/2017.

Ayo, Christiane Maria
Genes *KIR*, seus ligantes HLA e polimorfismo do gene *MICA* na toxoplasmose ocular e nas formas clínicas da doença de Chagas crônica.
São José do Rio Preto, 2017
136 p.

Tese (Doutorado) – Faculdade de Medicina de São José do Rio Preto – FAMERP
Eixo Temático: Medicina e Ciências Correlatas
Orientador: Prof. Dr. Luiz Carlos de Mattos

1. Toxoplasmose; 2. Doença de Chagas; 3. Receptores KIR; 4. Antígenos HLA; 5. Associação genética.

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Dedicatória

Dedico este trabalho aos meus pais Osvanir Ayo e Cidinha Ayo, à minha irmã Jacqueline Ayo, e também ao meu noivo Renan Moreno.

Agradecimentos

A Deus

Pelo amparo, força e inspiração. Por ter me dado discernimento e equilíbrio no decorrer desses anos de doutorado e por ter iluminado meus passos para que eu chegasse até aqui.

Aos meus pais Osvanir e Cidinha

Primeiramente pela educação moral, princípios e valores para que eu possa me tornar uma pessoa melhor a cada dia. Agradeço por investirem na minha educação e por me incentivarem aos estudos. Por depositarem em mim a confiança de que sou capaz e por tanto amor.

À minha querida irmã Jacqueline

Pela companhia e amizade. Por me ensinar a dividir. Pelo apoio incondicional. Por ser tão especial na minha vida.

Ao meu noivo Renan

Pela paciência que tem me dedicado todos esses anos, pela compreensão e companheirismo. Por estar sempre ao meu lado. Por me "colocar pra cima" nos momentos difíceis e me mostrar o lado bom dos acontecimentos. Por me impulsionar a prosseguir.

À toda minha família

Aos meus avôs, tios e primos por compartilharem momentos e conquistas ao meu lado e acreditarem no meu potencial.

À Faculdade de Medicina de São José do Rio Preto - FAMERP

Pela oportunidade e suporte financeiro.

À Diretoria Geral da FAMERP *Prof. Dr. Dulcimar Donizeti de Souza* ***e ao Programa de Pós-Graduação em Ciências da Saúde da FAMERP e seus coordenadores*** *Prof. Dr. Maurício Lacerda Nogueira e Prof. Dr. Mario Abbud Filho.*

Aos funcionários do Programa de Pós-graduação em Ciências da Saúde da FAMERP *José Antônio, Luís Henrique e Fabiana por todos os esclarecimentos.*

Aos colegas e técnicos do Laboratório de Imunogenética da FAMERP

Fernando, Amanda, Fabiana, Geraldo, Ricardo, Cássia, Warlen, Ana Vitória, Vinícius, Alessandro, Marcos, Valquíria, Natália, Mirele, Júlia, Mariana, Marina, Ulysses, Francielly, Cidinha, Márcio e Regina por todo apoio, ajuda, colaboração, motivação, amizade e por todas as vezes que me auxiliaram.

Ao meu orientador Prof. Dr. Luiz Carlos de Mattos

Por ter me dado a honra de sua orientação, pela confiança e estímulo à pesquisa. Pelos valiosos ensinamentos transmitidos ao longo desses anos, e principalmente por acreditar no meu potencial e nas minhas ideias.

À Prof. Dra. Cinara de Cássia Brandão de Mattos

Por ter me dado a oportunidade de estar no Laboratório de Imunogenética. Agradeço pelo carinho e por me auxiliar todas as vezes que precisei.

Aos médicos e demais colaboradores do projeto

Dr. Reinaldo Bulgarelli Bestetti, Dr. Aldenis Albanese Borin, Dr. Luiz Sérgio Ronchi, Dr. Eumildo de Campos Junior, Dr. Rubens Camargo Siqueira, Dr. Fábio Batista Frederico, Dra. Mariana Previato e Dra. Amanda Pires Barbosa por toda colaboração na triagem e seleção dos pacientes para obtenção das amostras. Profa. Dra. Lilian Castiglioni pela atenção e auxílio.

Aos pacientes voluntários

Por aceitarem participar da pesquisa e contribuírem para o desenvolvimento científico da nossa comunidade.

Ao Hemocentro de São José do Rio Preto

Dr. Otávio Ricci Junior por permitir a utilização do espaço físico e equipamentos para realização deste trabalho. Denise, Mirela, Octávia e Camila por dividirem esse espaço comigo, pela paciência e pela ajuda.

À Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP

Pela bolsa de doutorado (processo 2013/06580-9) e apoio financeiro.

Aos membros da banca examinadora

Pela disponibilidade em contribuir na finalização deste trabalho.

Aos meus amigos

Os de perto e os de longe. Agradeço pelos momentos de convivência e pelo privilégio de ter conhecido todos vocês.

Muitas são as pessoas que direta ou indiretamente colaboraram de forma essencial para a realização deste trabalho, por isto, a todos a quem não me referi, o meu muito obrigada.

Epígrafe

"Sonhe com o que você quiser. Vá para onde você queira ir.
Seja o que você quer ser, porque você possui apenas uma vida
e nela só temos uma chance de fazer aquilo que queremos.
Tenha felicidade bastante para fazê-la doce.
Dificuldades para fazê-la forte.
Tristeza para fazê-la humana.
E esperança suficiente para fazê-la feliz!"
(Clarisse Lispector)

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Lista de Abreviaturas e Símbolos

A	Adenina
AME	Ambulatório Médico de Especialidade
C	Citosina
CCC	Cardiopatia Chagásica Crônica
CCHD	*Chronic Chagas Heart Disease*
CD	*Cluster of Differentiation*
CPH	Complexo Principal de Histocompatibilidade
DNA	Ácido Dexoxirribonucléico
DSVE	Disfunção Sistólica Ventricular Esquerda
ECG	Eletrocardiograma
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio imunoenzimático
FAMERP	Faculdade de Medicina de São José do Rio Preto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
Fas-L	Fas-ligante
FcαR	Fc *alfa receptor*
FcεRI-γ	Fc *epsion receptor type γ*
FEVE	Fração de Ejeção do Ventrículo Esquerdo
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
G	Guanina
HAI	Hemaglutinação Indireta
HB	Hospital de Base
HLA	Antígeno leucocitário humano
HLA-A	Antígeno leucocitário humano A
HLA-B	Antígeno leucocitário humano B
HLA-C	Antígeno leucocitário humano C
HLA-E	Antígeno leucocitário humano E

IC (CI)	Intervalo de confiança
IFI	Imunofluorescência Indireta
IFN-γ	Interferon-gama
IgG	Imunoglobulina de classe G
IgM	Imunoglobulina de classe M
IL-2	Interleucina-2
IL-4	Interleucina-4
IL-5	Interleucina-5
IL-6	Interleucina-6
IL-10	Interleucina-10
IL-12	Interleucina-12
IL-13	Interleucina-13
IL-17	Interleucina-17
ITAM	*Immunoreceptor tyrosine-based activation motifs*
ITIM	*Immunoreceptor tyrosine-based inhibition motifs*
KIR	*Killer cell immunoglobulin-like receptors*
LAIR	*Leucocyte associated inhibitory receptor*
LILR	*Leucocyte immunoglobulin-like receptor*
Met	Metionina
MHC	*Major histocompatibility complex*
MIC	*Major histocompatibility complex class I chain-related gene*
MICA	*Major histocompatibility complex class I chain-related gene A*
MICB	*Major histocompatibility complex class I chain-related gene B*
mL	Mililitro
ng/µL	Nanograma/microlitro
NK	*Natural Killer*
NKG2	Receptores semelhantes à lectina
NKp46	*activating NK recptors*
NO	Óxido Nítrico

OCT	Tomografia de coerência óptica
OR	*Odds ratio*
PCR	Reação em cadeia da polimerase
RFLP	Polimorfismo no comprimento de fragmento de restrição
SNP	Polimorfismo de nucleotídeo único
SSO	Oligonucleotídeos específicos de sequência
STR	Sequência repetida em tandem
T	Timina
TAP	Proteínas transportadoras associadas ao processamento de antígenos
TGF-β	Fator de Crescimento Transformador beta
Th1	Linfócitos T auxiliares do tipo 1
Th2	Linfócitos T auxiliares do tipo 2
TLRs	Receptores do tipo toll
TNF-α	Fator de Necrose Tumoral-alfa
TO (OT)	Toxoplasmose ocular
Treg	Células T reguladoras
Tαβ	Linfócitos T alfa-beta
Tγδ	Linfócitos T gama-delta
ULBPs	Proteínas ligantes de UL-16
Val	Valina
3'UTR	Região 3' não traduzida
Δ'	Delta real
µl	Microlitro
$\chi 2$	Qui-quadrado

Resumo

Introdução: A toxoplasmose ocular, caracterizada por uma inflamação intraocular, é a manifestação clínica mais comum da toxoplasmose, doença infecciosa causada pelo protozoário *Toxoplasma gondii.* As lesões podem afetar a mácula, as diversas camadas da retina e a coróide resultando em retinocoroidite com diferentes graus de comprometimento ocular. A doença de Chagas é resultante da infecção pelo protozoário *Trypanosoma cruzi.* Após 20 anos de infecção, cerca de 30% dos pacientes desenvolvem a cardiopatia chagásica crônica, que se manifesta clinicamente por arritimia ventricular malígna, tromboembolismo, morte súbita cardíaca e insuficiência cardíaca crônica. Dez por cento dos pacientes apresentam a forma digestiva, caracterizada principalmente por dilatações do esôfago e/ou cólon devido ao processo de denervação. A progressão de uma infecção, assim como o desenvolvimento de diferentes formas clínicas e diferentes graus de gravidade, podem estar relacionadas com as características genéticas do patógeno e do hospedeiro. Dentre os fatores relacionados ao hospedeiro, a resposta imunológica desperta um interesse especial e os marcadores genéticos exercem importante papel modulador neste contexto, pois podem contribuir para patogênese ou resistência do curso clínico dessas infecções. **Objetivo:** O presente estudo teve como objetivo verificar a hipótese de que os genes *KIR*, seus ligantes HLA e o polimorfismo do gene *MICA* estão associados à toxoplasmose ocular (TO) e às diferentes formas clínicas da doença de Chagas. **Casuística e métodos:** Foram incluídos no estudo 297 pacientes com toxoplasmose, 148 clinicamente classificados com TO e 149 sem TO. Também participaram deste estudo 267 pacientes com doença de Chagas, 78 clinicamente classificados com a forma digestiva da doença, 107 com a forma cardíaca, 82 com a forma mista. O teste de ELISA foi realizado para confirmar as infecções por *T. gondii* e *T. cruzi.* Os polimorfismos destes genes foram identificados por PCR-SSOP e PCR nested. As variáveis contínuas foram comparadas utilizando o teste t não pareado. Para

comparação dos demais resultados foram realizados o Teste Qui-quadrado com correção de Yates ou o Teste Exato de Fisher. **Resultados:** Em relação à toxoplasmose, alelos e genótipos MICA não diferiram entre os pacientes com e sem TO, nem entre os pacientes com a manifestação primária ou recorrente da doença. O gene *KIR3DS1* foi associado positivamente com o desenvolvimento da TO. Genes *KIR* ativadores juntamente com os seus ligantes HLA (KIR3DS1^{+}/Bw4-80Ile^{+} e KIR2DS1^{+}/C2^{+} + KIR3DS1^{+}/Bw4-80Ile^{+}) foram associados com suscetibilidade à TO e às suas manifestações clínicas primária e recorrente. Os pares inibidores - KIR2DL3/2DL3-C1/C1 e KIR2DL3/2DL3-C1 – foram associados com a resistência à TO e suas manifestações clínicas, enquanto que a combinação KIR3DS1^{-}/KIR3DL1^{+}/Bw4-80Ile^{+} foi associada como fator de proteção para o desenvolvimento da TO e, em particular, para a manifestação recorrente. Quanto à doença de Chagas, o alelo MICA-129met foi associado como fator de risco para o desenvolvimento da disfunção sistólica ventricular esquerda (DSVE) em pacientes com cardiopatia chagásica crônica, enquanto que o alelo MICA-129val foi associado com a proteção ao desenvolvimento da DSVE. Em especial o haplótipo homozigoto MICA-129 met/met foi associado com o desenvolvimento da DSVE grave e o genótipo homozigoto MICA-129 val/val foi associado com a proteção desta condição. Também foi possível demonstrar que o haplótipo MICA*008~HLA-C*06 e a combinação entre KIR e seus ligantes HLA - KIR2DS2^{-}/KIR2DL2^{-}/KIR2DL3^{+}/C1^{+} - foram associados como fatores de suscetibilidade à forma clínica digestiva da doença de Chagas. **Conclusões:** Nossos resultados demonstram que os genes *KIR* podem exercer influência tanto na toxoplasmose ocular quanto na forma clínica digestiva da doença de Chagas, enquanto que MICA pode exercer influência nas formas clínicas da doença de Chagas, mas não no desenvolvimento da toxoplasmose ocular.

Palavras-chave: Toxoplasmose; Doença de Chagas; Receptores KIR; Antígenos HLA; Associação genética.

Abstract

Introduction: Ocular toxoplasmosis, characterized by an intraocular inflammation, is the most common clinical manifestation of toxoplasmosis, the infectious disease caused by the protozoan *Toxoplasma gondii*. The lesions can affect the macula and other layers of the retina and the choroid, resulting in retinochoroiditis with different degrees of ocular involvement. Chagas disease is resulting from infection by the protozoan *Trypanosoma cruzi*. After 20 years of infection, about 30% develop chronic Chagas heart disease, which is clinically manifested by malignant ventricular arrhythmia, thromboembolism, sudden cardiac death, and chronic heart failure. Ten per cent of Chagas patients present the digestive form of the disease, characterized mainly by dilatations of the esophagus and/or colon, due to the denervation process. The progression of the infection, as well as the development of the different clinical forms and different degrees of severity may be related to genetic characteristics of the pathogen and the host. Among the factors related to the host, the immunological response is of special interest with genetic markers playing an important modulating role in this context as they may contribute to the pathogenesis or resistance in the clinical course of these infections. **Objective:** The present study aimed to verify the hypothesis that *KIR* genes, their HLA ligands and *MICA* gene polymorphisms are associated with ocular toxoplasmosis (OT) and the different clinical forms of Chagas disease. **Patients and Methods:** This study included 297 patients with toxoplasmosis, 148 clinically diagnosed with OT and 149 without OT. Moreover, 267 patients with Chagas disease were enrolled: 78 clinically diagnosed with the digestive form of the disease, 107 with the cardiac form and 82 with the mixed form. The ELISA technique was used to confirm infection by *T. gondii* and *T. cruzi*. Polymorphisms of the *KIR* and *MICA* genes were identified by PCR-SSOP and nested PCR. Continuous variables were compared using the unpaired t test and the Chi-square test with Yates correction or the Fisher's exact test

were used compare the other results. **Results:** In relation to the toxoplasmosis, MICA genotypes and alleles did not differ between patients with and without OT, or between patients with the primary or recurrent manifestations of the disease. *KIR3DS1* gene was positively associated with the development of OT. *KIR* activating genes along with their HLA ligands ($KIR3DS1^{+}$/$Bw4\text{-}80Ile^{+}$, $KIR2DS1^{+}$/$C2^{+}$ and $KIR3DS1^{+}$/$Bw4\text{-}80Ile^{+}$) were associated with susceptibility to OT and both its primary and recurrent clinical manifestations. The inhibitory pairs - KIR2DL3/2DL3-C1/C1 and KIR2DL3/2DL3-C1 - were associated with resistance to OT and its clinical manifestations, whereas the combination $KIR3DS1^{-}$/$KIR3DL1^{+}$/$Bw4\text{-}80Ile^{+}$ was a protection factor for the development of OT and, in particular, against recurrent manifestations. As for Chagas disease, The MICA-129met allele was associated with the development of left ventricular systolic dysfunction (LVSD) in patients with chronic chagasic cardiomyopathy, while the MICA-129val allele was associated with a protection of developing LVSD. In particular, the MICA-129 met/met homozygous haplotype was associated with the development of severe LVSD and the MICA-129 val/val homozygous genotype protected against this condition. It was also possible to demonstrate that the haplotype MICA*008~HLA-C*06 and the combination between *KIR* genes and their HLA ligands - $KIR2DS2^{-}$/$KIR2DL2^{-}$/$KIR2DL3^{+}$/$C1^{+}$ - were associated with susceptibility for the digestive clinical form of Chagas disease. **Conclusions:** Our results demonstrate that *KIR* genes may influence both OT and the clinical digestive form of Chagas disease, whereas the *MICA* gene may influence the clinical forms of Chagas disease, but not the development of OT.

Key words: Toxoplasmosis; Chagas disease; KIR receptors; HLA antigens; Genetic association

1. Introdução

1. INTRODUÇÃO

1.1 Toxoplasmose

O *Toxoplasma gondii,* agente causador da toxoplasmose, foi descrito oficialmente em 1909 por Nicolle e Manceaux em um pequeno roedor (*Ctenodactylus gundi*) no norte da África.[1] Ao mesmo tempo no Brasil, Splendore[2] observou o mesmo parasito em coelhos. Inicialmente, acreditou-se ser uma forma particular de *Leishmania* sp., no entanto, os primeiros autores constataram que se tratava de um novo parasito.

O *T. gondii* é um protozoário pertencente ao Filo Apicomplexa, Classe Sporozoa, Subclasse Coccídia, Ordem Eucoccidiida, Subordem Eimeriina, Família Sarcocystidae, Subfamília Toxoplasmatinae, Gênero *Toxoplasma* e Espécie *Toxoplasma gondii.*[3] Considerado um parasito intracelular obrigatório, o *T. gondii* consiste em um patógeno de importância médica e veterinária. Este protozoário apresenta três estágios evolutivos como formas infectantes para o homem: oocisto esporulado, taquizoíto e cisto de bradizoíto.[4,5] O ciclo de vida desse parasito é heteroxeno, tendo os felinos como hospedeiros definitivos, e vários mamíferos e aves como hospedeiros intermediários, incluindo o homem.[4] A descrição completa do ciclo de vida do *T. gondii* encontra-se na figura 1.

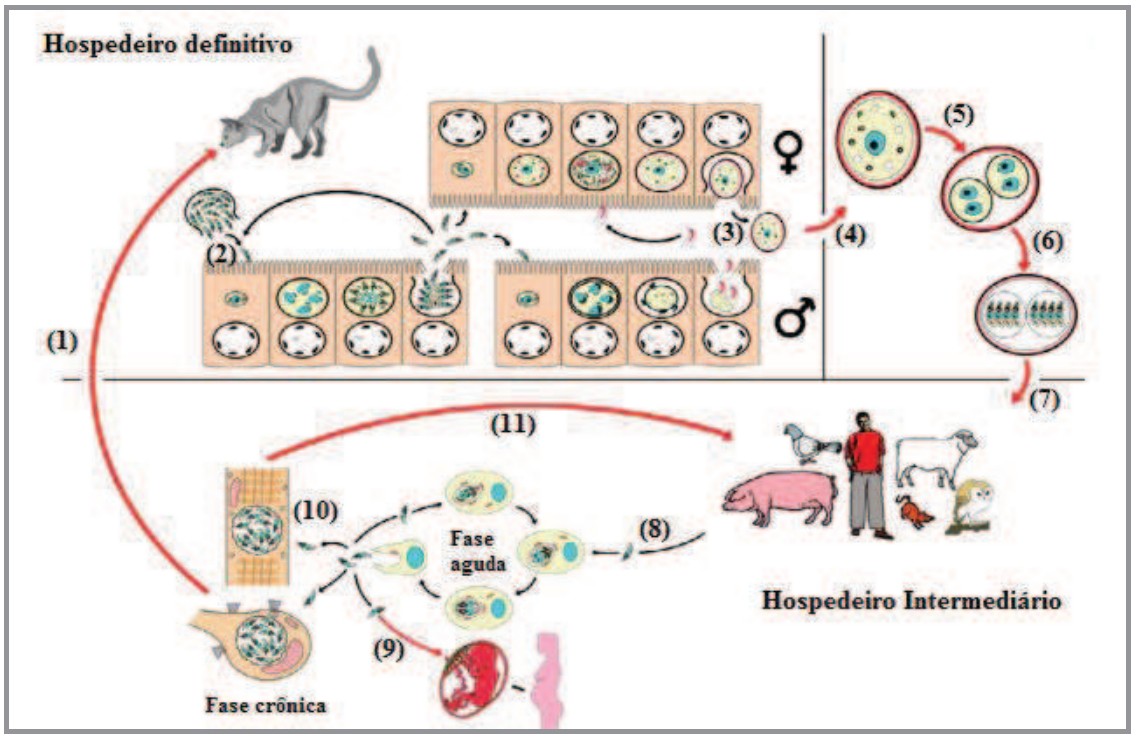

Figura 1. Ciclo de vida do protozoário *T. gondii*. (Adaptado).[6]

(1) Após a ingestão de cistos teciduais de bradizoítos, os quais podem ser encontrados em tecidos de hospedeiros intermediários que servem como presas (ratos, pássaros), (2) os parasitos invadem os enterócitos e se multiplicam de forma assexuada, (3) até a formação do macro e microgametócitos, responsáveis pela reprodução sexuada. (4) Após a fecundação dos gametas, ocorre a formação dos oocistos não esporulados, os quais são liberados juntamente com as fezes do animal. (5) Os oocistos no ambiente sofrem esporulação com formação de dois esporocistos (6) com quatro esporozoítos cada. A forma esporulada do parasito é resistente por anos em ambientes com condições favoráveis. (7) Uma vez ingerido, pelos hospedeiros intermediários, os esporozoítos são liberados dos oocistos e estes penetram nas células epiteliais do intestino, onde sofrem diferenciação em taquizoítos (8), forma extremamente proliferativa, a qual estabelece a infecção aguda. (9) Durante a infecção aguda, os taquizoítos podem ser transmitidos da mãe para o feto, levando a toxoplasmose congênita. (10) Com o desenvolvimento das respostas imunológicas, os taquizoítos diferenciam-se em bradizoítos dentro de cistos teciduais. Neste estágio o parasito pode permanecer latente por toda a vida do hospedeiro intermediário ou (11) pode ser transmitido por carnivorismo para outros hospedeiros, inclusive o gato, reiniciando o ciclo.[4,7]

A infecção pelo *T. gondii* pode ser adquirida pelo homem por vias vertical ou pós-natal. A transmissão pela via vertical ocorre geralmente quando a mãe adquire a infecção primária durante a gravidez com disseminação de taquizoítos ou, menos comumente, quando passa por períodos de reagudização.[8] A transmissão via pós-natal geralmente ocorre por meio da ingestão de alimentos ou água contaminada com os oocistos esporulados, pelo consumo de carne crua ou mal passada de animais com cistos de bradizoítos, ou pelo leite não pasteurizado contaminado com taquizoítos.[5] Além destes meios de infecção há possibilidades de transmissão via transplante de órgãos sólidos [8] e por transfusão de hemocomponentes.[9,10]

Considerada uma zoonose altamente disseminada, estima-se que 25 a 30% da população mundial esteja cronicamente infectada pelo *T. gondii*. Todavia, as taxas de prevalência são variáveis em diversas partes do mundo, variando de 10 a 80% entre diferentes países e dentro do mesmo de acordo com comunidades e regiões devido a fatores climáticos, geográficos e culturais atribuídos principalmente com tipo de alimentação, higiene e exposição ambiental. No Brasil, a prevalência de infecção por *T. gondii* é bastante elevada, estimando atingir cerca de 50% das crianças em idades do ensino fundamental, e entre 50 a 80% das mulheres em idade fértil.[5]

O termo toxoplasmose, na maioria das vezes, é utilizado indistintamente para se referir tanto a infecção como a doença causada pelo agente. Porém, apenas a presença do protozoário caracteriza-se a infecção, enquanto que a doença é acompanhada de sinais e desfechos clínicos. A investigação das consequências da infecção por *T. gondii* em humanos é o principal fator que responde pelo enorme interesse em se estudar a doença atualmente por constituir um problema de saúde pública em todo mundo, e tem

sido direcionada a grupos de risco tais como portadores de imunodeficiências, pacientes transplantados, gestantes e neonatos pelo risco de transmissão congênita e das sequelas resultantes, além de portadores de lesão ocular.[11]

A toxoplasmose ocular (TO) é a manifestação clínica mais comum da toxoplasmose e caracterizada por um processo inflamatório intraocular.[12] As lesões podem ser originadas tanto da infecção congênita, quanto da infecção adquirida após o nascimento[13,14] e pode atingir a mácula, as demais camadas da retina e a coróide, resultando em retinocoroidite, a causa mais frequente de uveíte posterior em pacientes imunocompetentes.[12] As manifestações oculares ocorrem de forma precoce ou tardia, com manifestação clínica primária ou recorrente[15] e apresenta diferentes graus de comprometimento ocular que podem variar de acordo com o estado imunológico do indivíduo e a virulência do parasito.[12,16]

Apesar da soroprevalência da toxoplasmose ser elevada, o percentual de indivíduos com comprometimento ocular é relativamente baixo.[17] Segundo estimativas, a TO ocorre aproximadamente 2 em cada 100 indivíduos com sorologia reagente que adquiriram a infecção após o nascimento.[18] Adicionalmente, a incidência anual global de 19.000 novos casos de infecção congênita traz sequelas oculares significativas,[19] atingindo índices de 20% em crianças até os seis anos de idade.[20] Contudo, o número de casos de TO não deixa de representar um grande fardo para os sistemas de saúde pública em todo mundo, e um bom exemplo disso é que cerca de 250.000 pacientes que procuraram consultas nos oftalmologistas nos EUA em um período de dois anos foi devido à TO.[21] No Brasil, a prevalência de TO é elevada em algumas regiões geográficas e varia de acordo com as regiões do país.[22-27]

A fundocospia e a retinografia são ferramentas utilizadas para o diagnóstico clínico das lesões oculares, que permitem ao especialista visualizar as características da lesão e sua gravidade. As lesões compatíveis com a toxoplasmose apresentam: A- focos de retinocoroidite em atividade caracterizados por lesões brancacentas da coróide e da retina com margens imprecisas, podendo ou não apresentar exudação vítrea e, B- podendo também, estar associada à lesão cicatrizada satélite, indicativa de recorrência (Figura 2). Focos de retinocoroidite em processo de cicatrização ou cicatrizados caracterizados como sendo do tipo: I- cicatrizes atróficas com halo pigmentado; II- cicatrizes pigmentadas com halo atrófico; III- cicatrizes inteiramente pigmentadas ou atróficas (Figura 3).[28] Recentemente, a técnica de Tomografia de Coerência Óptica (OCT) também tem sido utilizada para o diagnóstico e acompanhamento de lesões decorrentes da infecção toxoplásmica.[29,30] A OCT é uma técnica não invasiva que possibilita realizar cortes transversais nas estruturas oculares através da comparação entre diferentes feixes de luz, transmitido e refletido ao olho, os quais possibilitam obter medidas de alta resolução das estruturas oculares.[31] Dessa forma, a imagem da OCT na toxoplasmose ocular representa a refletividade das camadas da retina e dos tecidos subjacentes, o qual permite localizar o infiltrado inflamatório e especificar a camada da retina acometida, sendo útil na avaliação da extensão e gravidade das lesões oculares; além de identificar alterações morfológicas não evidenciadas clinicamente.[29,30] (Figura 4).

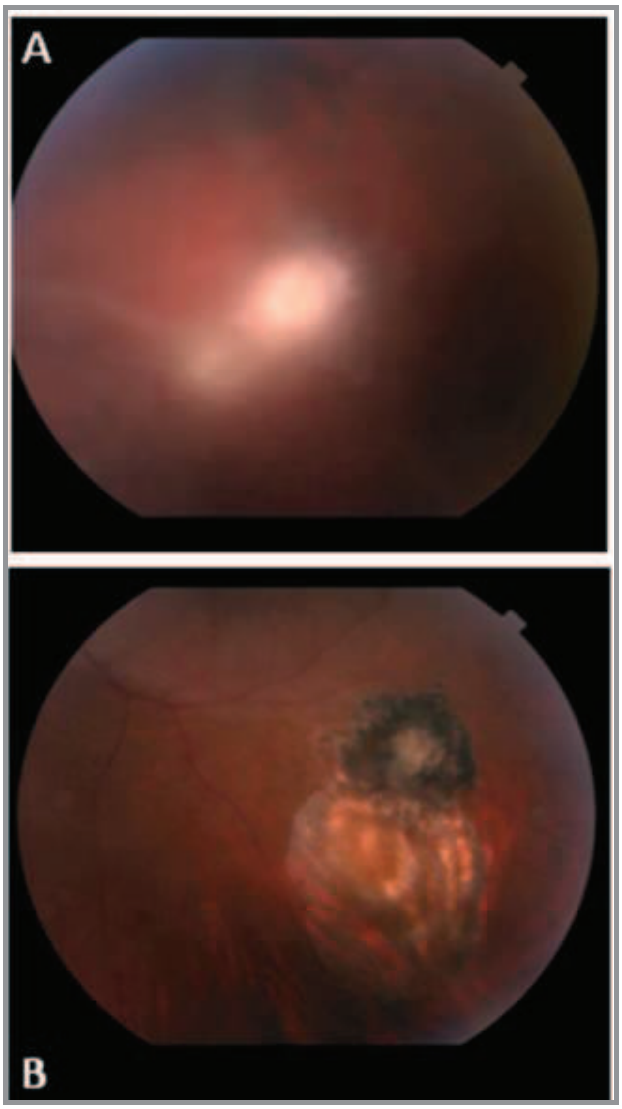

Figura 2. Retinografia colorida mostrando (A) uma lesão coriorretiniana exsudativa aguda e (B) lesão satélite decorrentes da infecção por *T. gondii*. (Adaptado).[32]

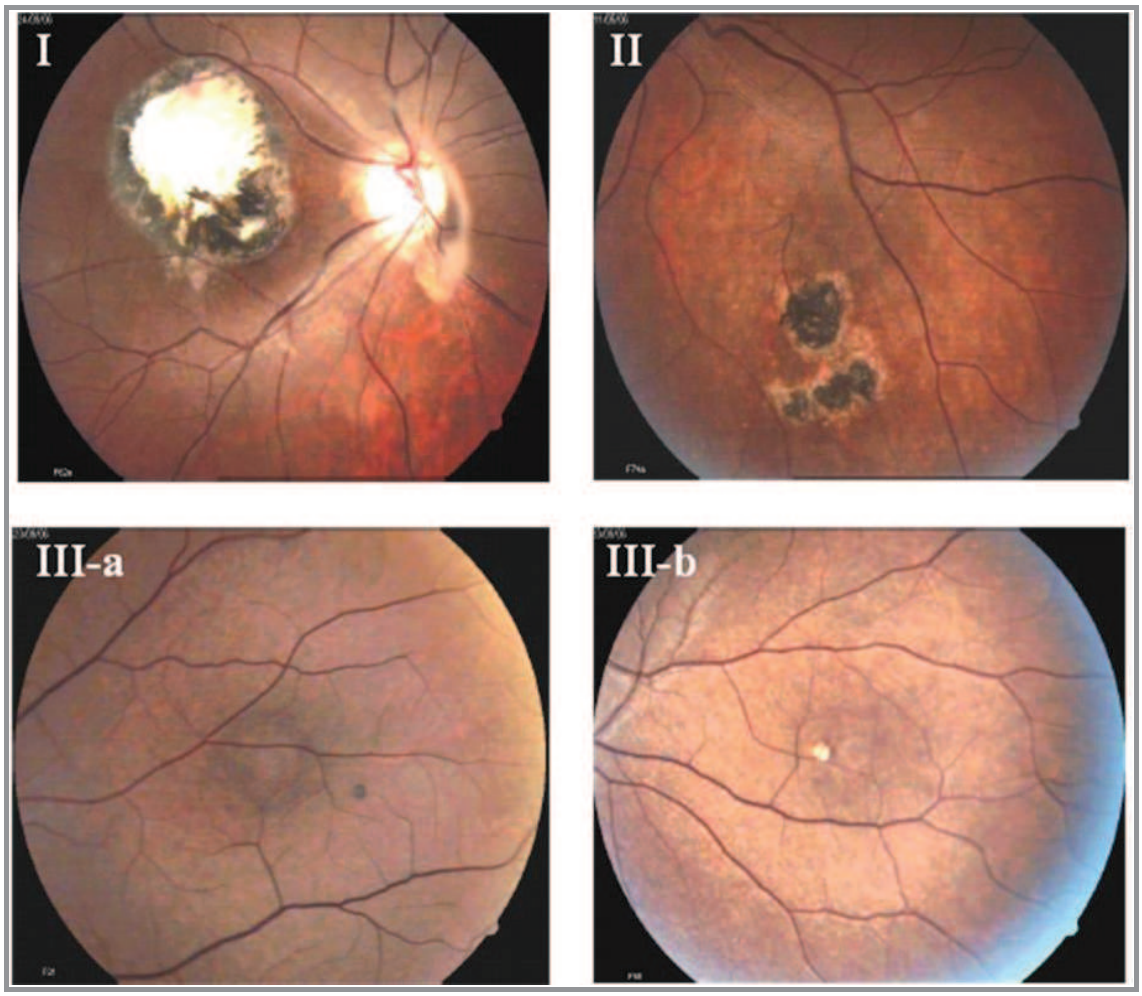

Figura 3. Retinografia colorida mostrando os tipos de cicatrizes oculares decorrentes da infecção por *T. gondii*. (Adaptado).[28]

(I) Área central clara com halo hiperpigmentado, na qual a parte clara representa a esclera, camada mais externa do globo ocular; nesse tipo de lesão o processo inflamatório causou a destruição da retina e da coróide permitindo assim a visualização da esclera. (II) Centro hiperpigmentado com borda clara; nesse tipo de lesão a reação inflamatória é provavelmente de menor intensidade e acarreta em destruição mais moderada do tecido. (III-a) hiperpigmentadas e/ou (III-b) apigmentadas; Nesses tipos de lesões os danos teciduais são menos graves que os causados pelas lesões representadas por (I) e (II), e a etiologia deixa margens de dúvida de ser ou não causada pela infecção por *T. gondii*.[28]

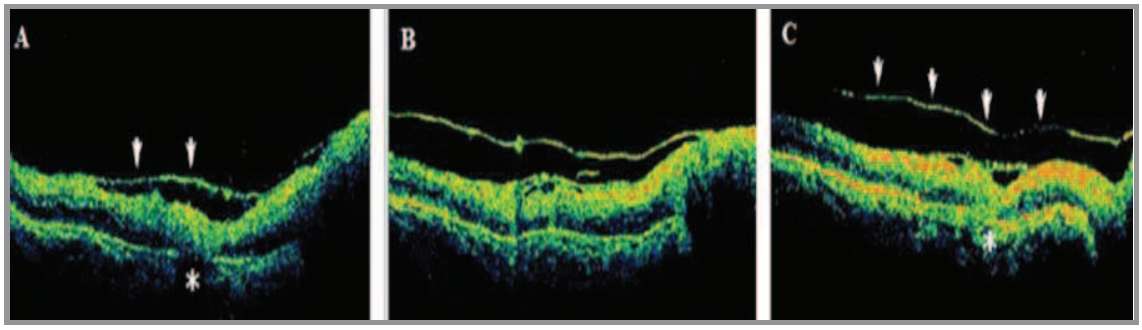

Figura 4. Tomografia de Coerência Óptica (OCT) mostrando diferentes estágios de uma lesão ocular decorrente da infecção por *T. gondii* durante um período de acompanhamento. (Adaptado).[30]

(A) Primeiro exame. Descolamento da membrana hialóide posterior (MHP) (ponta de flechas) e aumento de refletividade das camadas da retina interna com presença de sombreamento óptico posterior localizado na região do complexo estrato pigmentoso da retina coriocapilar-coróide subjacente ao local da lesão ativa (asterisco), caracterizando o processo inflamatório retiniano. (B) Segundo exame, 12 semanas. Aumento da extensão do descolamento da MHP. (C) Terceiro exame, 24 semanas. Aumento na extensão do descolamento da MHP (pontas de flechas), diminuição na espessura retiniana com desorganização completa de suas camadas, e aumento relativo da refletividade da coriocapilar-coróide (asterisco), achados característicos de cicatrização das lesões.[30]

Rotineiramente na prática clínica, emprega-se o uso de testes laboratoriais, os quais atuam de forma complementar para o diagnóstico clínico definitivo.[33] O diagnóstico laboratorial da infecção por *T. gondii* pode ser realizado através de métodos indiretos e diretos de detecção. Os métodos indiretos são baseados em critérios imunológicos, ou seja, na detecção de anticorpos específicos da classe IgM como marcador de infecção recente/aguda e da classe IgG para infecção crônica.[34,35] Os métodos diretos são baseados na identificação do parasito ou de seu material genético através de técnicas moleculares que empregam o uso da Reação em Cadeia da Polimerase (PCR).[36,37]

A terapêutica empregada para o tratamento da TO consiste no uso de pirimetamina, sulfadiazina e ácido folínico. O uso de corticosteróides também é indicado quando há intensa reação inflamatória. No entanto, a administração de corticosteróides durante a parasitemia (sem medicação específica para toxoplasmose) pode causar acentuada destruição da retina e disseminação descontrolada da infecção. Assim, o diagnóstico preciso e o rápido inicio do tratamento são fatores essenciais para a preservação de uma boa acuidade visual final.[38] Também tem sido demonstrado que o tratamento prolongado a base de sulfa reduz as frequências de recidivas.[39]

1.2 Resposta imune contra o *T. gondii* e sua influência na patogênese da toxoplasmose ocular

O controle do *T. gondii* envolve a imunidade inata e adquirida por meio da mobilização de uma variedade de células tais como, neutrófilos, macrófagos, células dendríticas, células *natural killer* (NK) e células T e B.

Neutrófilos, macrófagos e células dendríticas são essenciais na fase inicial da resposta imunológica dirigida ao parasito, pois são células secretoras de Interleucina-12 (IL-12) e de fator de necrose tumoral-alfa (TNF-α), citocinas essenciais para a resistência contra a infecção.[40,41] Essas citocinas estimulam as células NK a produzirem interferon-gama (IFN-γ), e este por sua vez, estimula a atividade microbicida dos macrófagos, mediado por radicais tóxicos de oxigênio, e promove o desenvolvimento de células T helper 1 (Th1), também produtoras de IFN-γ.[42] A ação combinada dessas citocinas, além de outros mecanismos imunológicos, é responsável pela proteção do hospedeiro contra a rápida proliferação de taquizoítos e, subsequente alterações patológicas.[8]

A imunidade celular, mediada pelos linfócitos T, atua como mecanismo de defesa contra os parasitos que sobrevivem dentro de fagócitos ou em células não-fagocíticas infectadas, onde estão protegidas de anticorpos. Os linfócitos helper (Th) são divididos nas subpopulações Th1 e Th2, distinguidas pelo padrão de produção de citocinas. Células Th1 $CD4^+$ produzem citocinas, cuja função é estimular a proliferação e a diferenciação dos linfócitos citotóxicos e macrófagos. Células com perfil Th1 secretam preferencialmente IFN-γ e IL-2, sendo IL-2 responsável pela indução da ativação de células T $CD8^+$, as quais são citotóxica para as células-alvo infectadas com o *T. gondii* devido à produção de IFN-γ, além de estimular a ativação das células NK. No entanto, um efeito efetivo das células T contra o parasito é mediado pelo *Major histocompatibility complex* (MHC).[43,44] Por outro lado, células Th2 produzem IL-4, IL-5, IL-10 e IL-13, citocinas associadas à baixa regulação da resposta imune mediada por células e responsáveis por induzirem a imunidade predominantemente do tipo humoral.[44] Na infecção por *T. gondii*, as citocinas de perfil Th2 também podem ser recrutadas para controlar a resposta imune próinflamatória prejudicial.[43]

Outro conjunto de células Th, denominadas de Th17, também foram descritas como tendo um papel crucial no dano tecidual autoimune, devido à produção de IL-17, potente indutor da inflamação. Além disso, a ausência de IL-17 impede a migração dos neutrófilos para os sítios de infecção o que pode ser prejudicial para o hospedeiro.[17]

Anticorpos também são produzidos em resposta ao *T. gondii*. Em síntese, eles atuam nos taquizoítos extracelulares que são liberados após a lise de uma célula infectada; limitando a multiplicação dos mesmos através da lise do parasito após a ativação da via do complemento, pela opsonização dos parasitos ou aumentando a ação

fagocitária dos macrófagos. Entretanto, esses mecanismos não oferecem proteção contra os parasitos vivos que estão no interior das células.[45]

A resposta imune intraocular é suprimida em circunstâncias normais o que diminui chance de destruição tecidual.[46] Nessas condições, células presentes em vários tecidos do olho expressam constitutivamente ligante de Fas (Fas-L), o qual é capaz de promover a deleção de células T e de células NK no olho. Além disso, também há presença de citocinas como *transforming growth factor beta* (TGF-β) que possui propriedades imunossupressoras e que reduz a expressão de moléculas MHC de classe I,[47,48] na qual pode comprometer as respostas citotóxicas de linfócitos. Contudo, evidências recentes indicam que o olho é um sítio que possui um sistema de imunorregulação bem estabelecido, no qual ocorre apresentação de antígeno, produção de citocinas e de anticorpos.[12] Níveis menores de TGF-β foram encontrados em fluidos oculares de indivíduos portadores de uveítes;[49] além disso, foi demonstrado que o *T. gondii* é capaz de estimular a expressão e modificar a forma ativa do TGF-β favorecendo assim a replicação do parasito.[50]

Os danos aos tecidos oculares levaram à proposição de fenômenos que podem estar relacionados aos mecanismos patogênicos da toxoplasmose ocular, e esses fenômenos podem ser explicados principalmente pela virulência do parasito e pela ação da resposta imunológica dirigida contra o parasito que pode resultar em autoagressão.[16] Em relação a resposta imune, há evidencias de que a infecção por *T. gondii* promove a produção de fatores que anulam o privilégio imune, como IFN-γ, que desenvolve papel crucial na resistência contra a infecção, além de ser um antagonista potente de TGF- β e, hiper-regular a expressão de moléculas MHC.[43,51] Em modelos murinos foi

demonstrado que a resposta imune ocular contra *T. gondii* envolve fatores semelhantes à resposta que ocorre nos demais tecidos podendo levar ao aumento da intensidade das lesões oculares caracterizadas pela necrose ou inflamação acentuada da retina e da coróide.[42,52,53]

A resposta imunológica pode determinar o desenvolvimento das lesões oculares decorrentes da infecção por *T. gondii*, e os mecanismos envolvidos podem estar associados tanto com a patogênese quanto aos efeitos protetores que controlam o dano tecidual. Atualmente sabe-se que uma resposta Th1 exacerbada, conduzida em especial por células Th-17, pode causar danos teciduais e contribuir para a gravidade da toxoplasmose ocular devido à produção de IL-17, um indutor potente da inflamação.[54,55] Entretanto, além das Th-17, existem fontes celulares adicionais de IL-17 que contribuem para o desenvolvimento de condições inflamatórias, incluindo as células NK.[56]

Tem-se demonstrado um aumento do número de monócitos proinflamatórios e células NK $CD56^{dim}$ citotóxicas circulantes e diminuição de células NK $CD56^{bright}$ imunoregulatórias em crianças infectadas por *T. gondii* de forma congênita e que apresentam lesão ocular ativa. Ainda, subpopulações de células NK e T $CD8^{+}$ exercem participação crucial como biomarcadores de lesão ocular cicatricial.[57] Além disso, há evidências de que as células NK apresentam perfil predominantemente pró-inflamatório *in vintro* frente à infecção por *T. gondii*, devido produção aumentada de IFN-γ em pacientes com toxoplasmose ocular congênita.[55]

Os dados encontrados na literatura sugerem que a resposta imunológica mediada por células NK parece ser diretamente influente no combate ao *T. gondii* e no quadro

final da toxoplasmose ocular. Tem sido demonstrado recentemente que as células NK exercem funções biológicas sofisticadas que são atributos da imunidade inata e adaptativa rompendo as fronteiras funcionais entre essas respostas.[58] Assim, interação das células NK com a célula-alvo exerce função relevante na iniciação e regulação da resposta imunológica e, a base molecular dessas interações celulares parece ser crucial para que ocorra uma resposta eficiente e modulada no combate ao parasito evitando danos teciduais. Dessa maneira, polimorfismos em genes que codificam seus receptores e ligantes podem exercer papel relevante nessa patologia.

1.3 Doença de Chagas

A doença de Chagas é uma antropozoonose de larga distribuição no continente americano, especialmente na América Latina, resultante da infecção pelo protozoário flagelado *Trypanosoma cruzi.* A doença foi descrita pela primeira vez em 1909 por Carlos Justiniano Ribeiro das Chagas, médico e cientista brasileiro.[59] Atualmente, as estimativas de prevalência da infecção apontam que aproximadamente 6-7 milhões de indivíduos estão infectados,[60] e cerca de 65 milhões estão sob o risco de contrair a doença, com uma incidência total estimada de 28.000 novos casos por ano.[61] No Brasil, estima-se que pouco mais de 4 milhões de pessoas estariam infectadas pelo *T. cruzi*, e as maiores prevalências foram verificadas em mulheres, com idade superior a 60 anos e residentes nas regiões nordeste e sudeste do país.[62]

A prevalência, a incidência e a taxa de mortalidade associadas à doença de Chagas apresentaram consideráveis variações nas últimas décadas, devido principalmente ao impacto de programas de controle de vetores, migrações de populações rurais e urbanas, além de mudanças sócio-econômicas.[63] No entanto, a taxa da mortalidade relacionada

à doença de Chagas, persiste em níveis elevados.[64-66] Embora conhecida a mais de um século, a doença de Chagas ainda é considerada uma doença negligenciada, que além de afetar áreas rurais e pobres, [67] por causa da crescente urbanização durante as três últimas décadas do século XX também tornou-se uma doença de endemia urbana.[68] Atualmente, sua propagação da América Latina para países não endêmicos, devido à migração internacional, se tornou em um problema de saúde global, atingindo áreas não endêmicas que são destinos dos imigrantes latinos.[69]

A forma clássica e natural de transmissão do *T. cruzi* para o homem e outros mamíferos é através da via vetorial e ocorre pelas fezes contaminadas dos triatomíneos, que possuem o hábito de defecar durante o repasto sanguíneo, e que penetram na pele pelo local da lesão decorrente da picada ou mucosas, sendo considerado o mecanismo de transmissão de maior importância epidemiológica.[70] A infecção também pode ser transmitida por meio de transfusão sanguínea, o segundo mecanismo de maior importância epidemiológica na transmissão da doença de Chagas,[71] de forma congênita e oral, sendo esta última adquirida pela ingestão de alimentos e bebidas contaminados com o *T. cruzi*, no qual os triatomíneos infectados geralmente são triturados acidentalmente junto ao alimento durante o preparo. Existem ainda outros mecanismos de transmissões menos incidentes, tais como transplantes de órgãos e acidentes laboratoriais.[69]

O *T. cruzi* é um protozoário flagelado pertencente à Ordem Kinetoplastida, Família Trypanosomatidae e Gênero *Trypanosoma* [72] e mais de 100 espécies são reconhecidas como reservatórios naturais para esse parasito.[69] Os vetores da doença de Chagas são insetos hematófagos da Ordem Hemíptera, Família Reduviidae e Subfamília

Triatominae.[73] Entre as mais de 150 espécies encontradas como potenciais vetores do *T. cruzi*,[73] as de maior importância epidemiológica para infecção humana são: *Triatoma infestans*, *Triatoma brasiliensis*, *Triatoma dimidiata*, *Rhodnius prolixus*, *Triatoma pseudomaculata*, *Triatoma sórdida* e *Panstrongylus megistus*.[74] Sob condições naturais, o ciclo de vida do *T. cruzi* alterna entre um hospedeiro invertebrado da Família Reduviidae e, um hospedeiro vertebrado, que pode ser qualquer espécie de mamífero de diferentes ordens e envolve várias fases de desenvolvimento. A descrição completa do ciclo do *T. cruzi* encontra-se na figura 5.

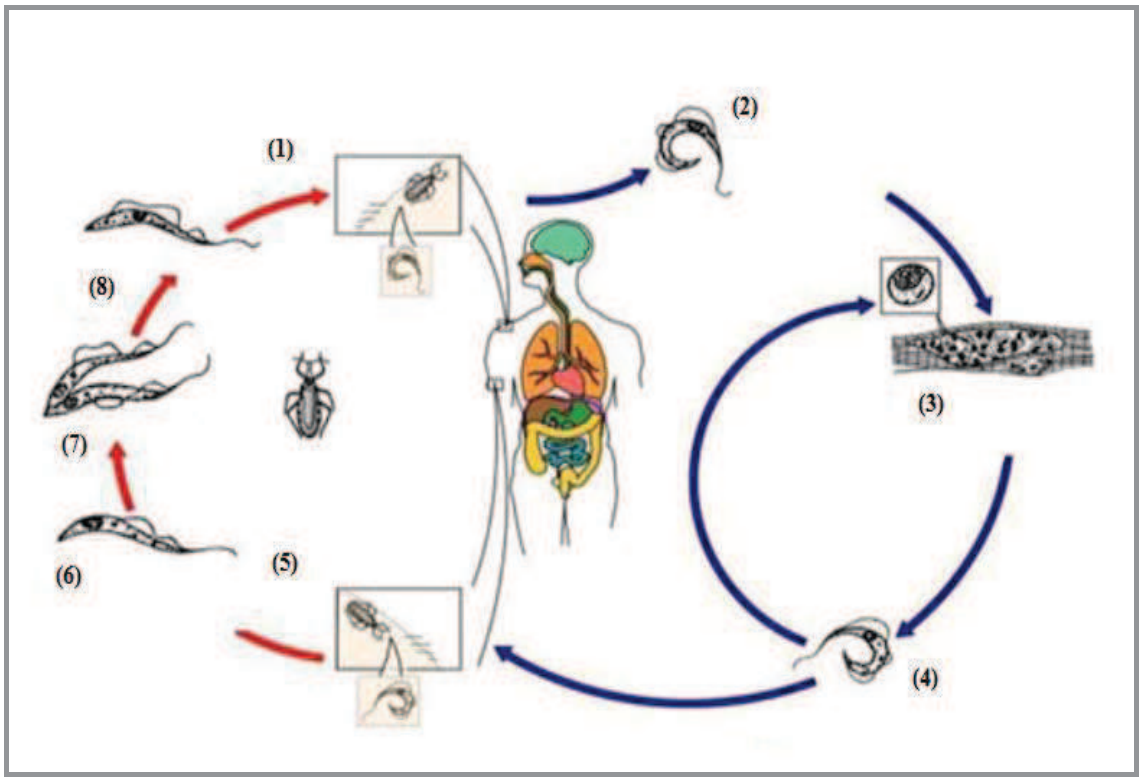

Figura 5. Ciclo de vida do protozoário *T. cruzi*. (Adaptado).[75]
(1) O inseto infectado elimina os tripomastigotas metacíclicos através das excretas durante o hematofagismo. (2) As formas tripomastigotas invadem células do hospedeiro, onde se transformam em amastigotas. (3) Dentro das células, os amastigotas multiplicam-se por divisão binária simples. (4) Amastigotas intracelulares se transformam em tripomastigotas e, com o rompimento das células entram nas correntes sanguínea e linfática, podendo invadir novas células para cumprirem novo ciclo celular no hospedeiro. (5) Os tripomastigotas sanguíneos podem ser ingeridos por novo inseto, continuando o ciclo do parasito. (6) No interior do tubo digestivo do inseto os tripomastigotas sanguíneos se transformam em epimastigotas, (7) os quais se multiplicam por divisão binária simples. (8) Os epimastigotas se diferenciam em tripomastigotas metacíclicos.[75]

O desenvolvimento da doença de Chagas ocorre por fases aguda e crônica e, tanto a variabilidade genética e biológica do parasito quanto do hospedeiro podem influenciar o curso de evolução da doença.[76] A fase aguda pode ser sintomática ou assintomática. Quando sintomática, os sinais clínicos mais relacionados com a infecção

aguda são reação inflamatória local com formação de forte edema na região de entrada do parasito (chagoma de inoculação ou sinal de Romaña, quando a transmissão ocorre de forma natural), febre, mal estar, poliadenia, hepatoesplenomegalia e insuficiência cardíaca.[77]

Após um período de 4-10 semanas os pacientes progridem para uma fase crônica indeterminada que acomete a maioria dos indivíduos (aproximadamente 60%) e podem permanecer assim por toda vida.[78,79] Essa fase é caracterizada por ausência de sintomas, estudos radiológicos e eletrocardiográficos normais e sorologia reagente para o *T. cruzi*.[72]

Os indivíduos infectados cronicamente podem vir a desenvolver manifestações clínicas da doença de Chagas com lesões irreversíveis em alguns órgãos. Cerca de 30% desenvolvem, a cardiopatia chagásica crônica (CCC) de gravidade variável; e 10% apresentam a forma digestiva, caracterizada principalmente por dilatações do esôfago e/ou cólon. Alguns pacientes apresentam também associação das manifestações cardíaca e digestiva, conhecida como forma mista ou cardiodigestiva.[78,80]

A CCC constitui uma das manifestações clínicas graves da doença de Chagas, e em áreas endêmicas, representa a principal causa de incapacidade e mortalidade.[81] A doença manifesta-se por insuficiência cardíaca, transtornos do ritmo dos batimentos e da condução elétrica, fenômenos tromboembólicos, dor no peito precordial e morte súbita. Pacientes portadores desta forma clínica apresentam miocardite usualmente intensa e difusa, sendo acompanhadas de cardiomegalia, lesões vasculares e fibrose.[66,82]

Pacientes portadores da forma digestiva apresentam sintomas decorrentes de comprometimento de órgãos deste sistema devido à dilatação e hipertrofia muscular com formação de megavísceras, principalmente do esôfago (megaesôfago) e do cólon (megacólon). As manifestações clínicas devido ao megaesôfago incluem ptialismo, disfagia, regurgitação, dor epigástrica, podendo levar à desnutrição em alguns casos. O megacólon se manifesta comumente como distensão abdominal, constipação e obstrução intestinal.[83] No entanto, como a doença de Chagas causa comprometimento do sistema nervoso autônomo, podem ocorrer alterações anatomo-funcionais ao longo de todo trato digestivo, até mesmo de órgãos não pertencentes a esse sistema.[66] O desenvolvimento de megaesôfago está associado com redução de aproximadamente 85% do número de neurônios, enquanto no megacólon a redução destas células é cerca de 50%.[84] A figura 6 apresenta as manifestações clínicas mais comuns encontradas na da doença de Chagas crônica.

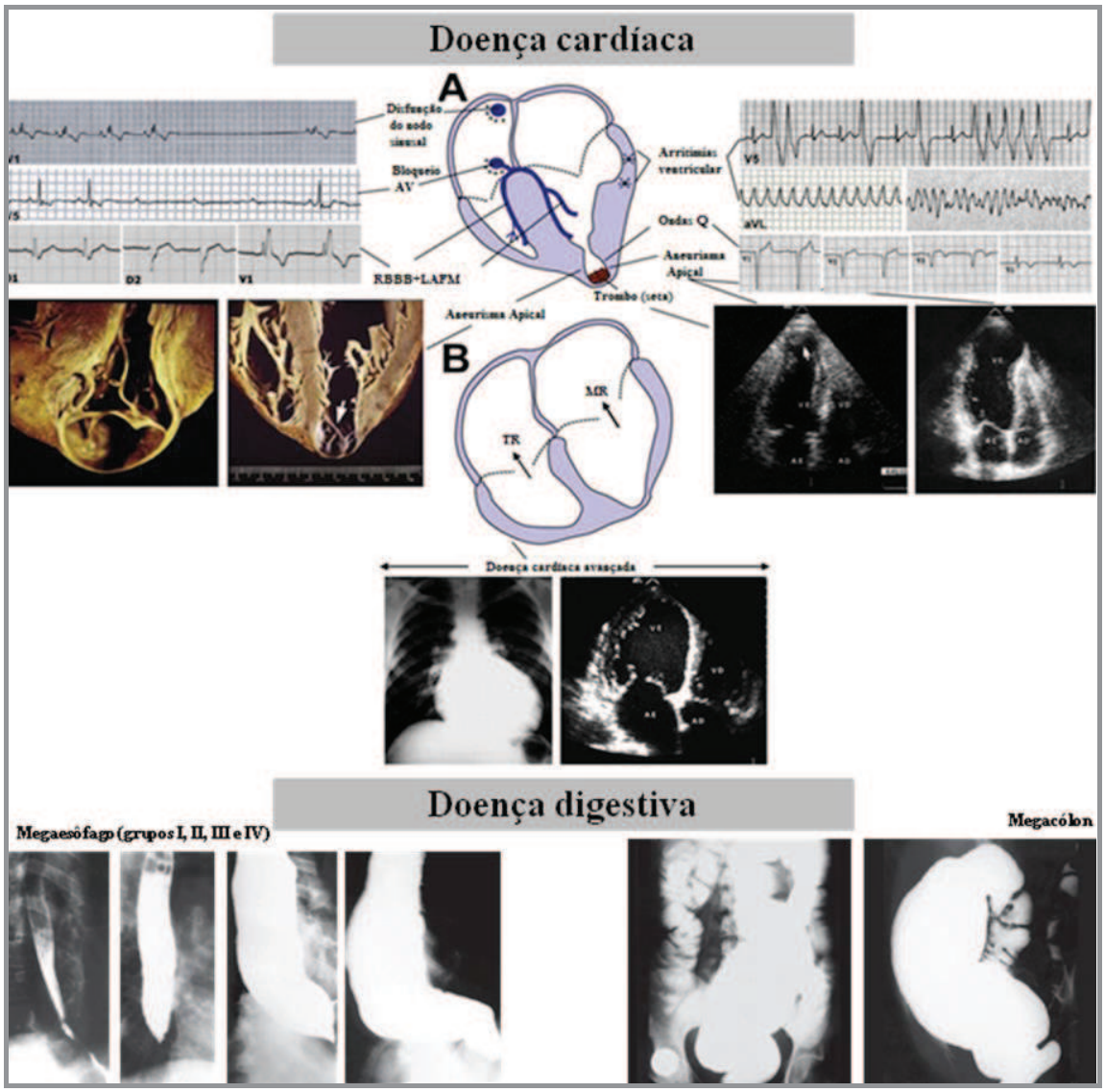

Figura 6. Principais achados na doença de Chagas crônica. (Adaptado).[67]
(A) Forma segmentar cardíaca. (B) Forma cardíaca dilatada. AV, atrioventricular; LAFB, bloqueio fascicular anterior esquerdo; MR, insuficiência mitral; RBBB, bloqueio de ramo direito; TR, insuficiência tricúspide; VE, ventículo esquerdo; VD, ventrículo direito; AE, átrio esquero; AD, átrio direito.

Os exames convencionais no diagnóstico clínico da doença de Chagas na fase crônica incluem: raios X de tórax, eletrocardiograma e ecocardiograma para o diagnóstico cardiológico; manometria anorretal e raios X de enema opaco para o

diagnóstico de megacólon; e manometria esofágica e raios X de seriografia para forma megaesôfago.[85]

O diagnóstico laboratorial da infecção durante a fase aguda da doença de Chagas pode ser realizado por testes diretos de detecção do parasito no sangue periférico por meio de exame de sangue a fresco, gota espessa e esfregaço sanguíneo. O diagnóstico laboratorial durante a fase crônica da doença deve ser realizado, preferencialmente, por métodos sorológicos, os quais detectam anticorpos anti-*T. cruzi* da classe IgG por meio dos testes de imunofluorescência indireta (IFI), ensaio imunoenzimático (ELISA - *Enzyme linked immunosorbent assay*) e hemaglutinação indireta (HAI),[71] e segundo recomendação da Organização Mundial da Saúde dois testes diferentes devem ser empregados para um diagnóstico preciso.[67]

As únicas opções utilizadas no tratamento etiológico da doença de Chagas são os antiparasitários nifurtimox e benzonidazol. No entanto, essas drogas parecem ter maior eficácia terapêutica apenas na fase aguda da infecção. Além disso, podem causar efeitos colaterais graves e induzir resistência no parasito.[86] O nifurtimox, lançado em 1972 com o nome comercial de Lampit®, teve sua comercialização cancelada em diversos países, inclusive no Brasil, por ser considerado tóxico ao homem. O benzonidazol, comercializado desde 1978 sob o nome de Rochagan®, é a única droga comercializada para o tratamento da doença de Chagas no Brasil.[87] Atualmente, um estudo denominado BENEFIT (*Benznidazole Evaluation for Interrupting Trypanosomiasis*) está avaliando a eficácia do tratamento com benzonidazol na doença de Chagas crônica, e os primeiros resultados mostram que o medicamento não conseguiu interromper o agravamento da doença em indivíduos com problemas cardíacos estabelecidos.[88]

1.4 Resposta imune contra o *T. cruzi* e sua influência na patogênese da doença de Chagas

A infecção pelo *T. cruzi* pode ativar múltiplas vias do sistema imune inato e adaptativo do hospedeiro que envolve a participação de um grande número de células, como, células NK, células T $CD4^+$ e $CD8^+$ e células B,[89] as quais favorecem a síntese de moléculas reguladoras e efetoras do sistema imune, tais como citocinas, quimiocinas e óxido nítrico (NO).[90]

Durante a fase aguda da infecção, a presença do parasito induz um rápido aumento na produção, maturação e ativação de monócitos e macrófagos na tentativa de controlar a sua replicação. A interação do *T. cruzi* com essas células envolvidas na resposta imune inata é mediada por receptores específicos de reconhecimento de padrões do patógeno, como os receptores do tipo Toll (TLRs). A infecção dos macrófagos pelo parasito tipicamente resulta na secreção de citocinas pró-inflamatórias, tais como TNF-α e IL-12. Estas citocinas ativam as células NK a produzirem IFN- γ. O IFN- γ exerce *feedback* positivo na ativação dos macrófagos, que destroem os parasitos pela produção NO. Por outro lado, citocinas regulatórias como IL-4, IL-10 e TGF-β inibem a produção de NO e a atividade dos macrófagos,[91] sendo responsáveis pela desativação e pelo controle dos efeitos inflamatórios letais das citocinas tipo 1 produzidas durante a infecção.[92]

A interação de citocinas, macrófagos e outras células do sistema imune inato são essenciais para a evolução da infecção e orienta o organismo para uma resposta imune adaptativa. Os parasitos ao se infiltrarem nas células do hospedeiro vertebrado são processados, e o antígeno é apresentado às células T $CD8^+$ pelas moléculas MHC de classe I. As células T $CD8^+$ exercem efeito citotóxico para as células infectadas,

relacionado principalmente à produção de IFN-γ. Assim, a replicação do parasito é controlada pela capacidade das células T $CD8^+$ destruírem as células infectadas.(93)

Células T $CD4^+$ atuam no reconhecimento de antígenos processados e apresentados pelas moléculas MHC de classe II, que em resposta secretam citocinas, as quais atuam como mediadores da inflamação e, sobretudo são importantes para o controle da parasitemia.(94) Células T $CD4^+$ com fenótipo Th1 estão envolvidas na diferenciação e ativação de células T $CD8^+$ e na produção de IFN-γ, e, por meio da síntese deste, também exerce participação indireta na produção de anticorpos que ativarão o sistema complemanto.(95) Entretanto, a resposta imune não é capaz de eliminar o *T. cruzi*, apenas controla sua replicação.(84)

Os mecanismos patogênicos envolvidos nas formas clínicas da doença de Chagas podem ser explicados pela persistência do parasito como responsável por manter a inflamação, e pela autoagressão decorrente da resposta imune contra antígenos próprios resultando em danos teciduais.(84,96) A evolução clínica da doença, no que diz respeito à resposta imune, depende particularmente da modulação Th1/Th2 e Treg (células T reguladoras). Tanto na forma cardíaca, como na forma digestiva, às manifestações patológicas estão associadas com a ocorrência de uma reação pró-inflamatória do tipo Th1 nos órgãos acometidos, caracterizada pela presença de IFN-γ, TNF-α e IL-6; enquanto que na forma indeterminada da doença a resposta induzida é de caráter predominantemente anti-inflamatório, ou seja, com exacerbação de linfócitos do tipo Th2 com a presença de IL-10 como elemento protetor.(84) No entanto, após a descoberta de uma nova linhagem de linfócitos efetores, chamados de células Th17, o saldo Th1/Th2 passou a ser revisto. Células Th17 produzem IL-17 que tem propriedades

pró-inflamatórias[97] e induz a produção de vários mediadores, levando ao recrutamento de neutrófilos e consequente inflamação,[98] fato que tem associado a resposta Th17 com várias doenças inflamatórias e autoimunes.[99] Além disso, em adição às células Th17, outras células como T $CD8^+$, neutrófilos, monócitos e células NK são produtoras de IL-17.[100]

Assim, o envolvimento da imunidade mediada por células é de suma importância em todas as formas clínicas da doença de Chagas, uma vez que desempenham papel efetor nos danos teciduais de acordo como perfil de moléculas que secretam.[101] Na CCC as principais células que constituem o infiltrado inflamatório do tecido cardíaco são linfócitos T e B, macrófagos e células NK, sendo a população predominante a de células T $CD8^+$.[95,102] Na forma digestiva da doença, da mesma maneira, há associação das lesões encontradas nas células musculares com o infiltrado inflamatório e fibrose, sendo que o infiltrado celular é constituído principalmente por células $TCD4^+$, macrófagos, células $TCD8^+$, B e NK.[84]

Apesar do fato da resposta imune adaptativa ser considerada há anos como o mecanismo protetor mais importante durante a infecção crônica da doença de Chagas, estudos recentes sugerem a importância da resposta imune inata como um mecanismo regulador relevante, uma vez que as citocinas produzidas por células NK e monócitos tem sido relatadas como importantes biomarcadores da morbidade da doença.[103-105]

Em especial, o recrutamento de células NK e monócitos pode ser uma ponte importante entre eventos imunológicos inatos e adaptativos durante infecção por *T. cruzi*. Na doença de Chagas crônica assintomática ou indeterminada, a presença de células NK ($CD3^-$ $CD16^+$ $CD56^+$ e $CD3^-$ $CD16^+$ $CD56^{dim}$) juntamente com a presença de

células imunoreguladoras (Treg^{-} CD4^{+} CD25^{high} e NTK^{-} CD3^{+} CD16^{-} CD56^{+}) e macrófagos circulantes (CD14^{+}CD16^{+}) são responsáveis pelo controle dos mecanismos inflamatórios. No entanto, falha em mecanismos imunorreguladores, com níveis basais das células NK, NKT e CD4^{+} CD25^{high}, associadas com um aumento da expressão de células T CD8^{+} ativadas, estão associados com a doença cardíaca.[106-108] Neste contexto, as citocinas produzidas por estas células podem constituir um mecanismo regulador fundamental.[108] Além disso, a presença de células NK como constituinte do infiltrado inflamatório persistente no miocárdio e no trato digestivo sugere a participação destas na continuidade do processo inflamatório da fase crônica da doença de Chagas.[95,109]

Assim, o claro envolvimento das células NK na infecção pelo *T. cruzi* e nas formas evolutivas da doença de Chagas crônica parece possuir implicações relevantes. Entretanto, tais células precisam ser adequadamente ativadas para tornarem-se competentes na execução de suas atividades efetoras sem causar danos teciduais, sendo importante avaliar polimorfismos em seus receptores e ligantes, os quais podem ter efeitos funcionais e estarem intimamente relacionadas nos processos fisiopatológicos da CCC e dos megacólon e magaesôgfago chagásicos.

1.5 Fatores genéticos do hospedeiro relacionados às manifestações clínicas da toxoplasmose e da doença de Chagas

A interação entre o parasito, o hospedeiro e o vetor (ao se tratar da doença de Chagas), em um ecossistema apropriado e contexto epidemiológico pode determinar a incidência, a prevalência e até mesmo a gravidade das manifestações clínicas resultantes da infecção por *T. gondii* e *T. cruzi*.

Entre os fatores individuais de risco para a ocorrência das formas clínicas da toxoplasmose e da doença de Chagas está o estado imunológico do hospedeiro, uma vez que a maioria dos casos de infecção humana em indivíduos imunocompetentes é assintomática.[4,78]

Assim, fatores genéticos do hospedeiro, em especial a resposta imunológica, parecem ter influência na manifestação dessas doenças, pois apenas parte das pessoas infectadas desenvolve os sintomas clínicos dessas zoonoses. Os mecanismos imunológicos relevantes que contribuem para patogênese ou resistência da toxoplasmose ocular e das formas clínicas da doença de Chagas são numerosos e envolvem vários tipos de células e mediadores do sistema imune inato e adaptativo do hospedeiro, entretanto, tais mecanismos ainda não foram completamente elucidados.

Na fase inicial dessas infecções, a imunidade inata tem papel crucial na resistência ao *T. gondii* e ao *T. cruzi* e as células NK são uma importante subpopulação de linfócitos atuantes nessa fase da resposta imunológica na toxoplasmose e na doença de Chagas. A ativação das células NK limita a replicação dos parasitos além de desencadear a resposta imune adaptativa.[42,110] As funções efetoras das células NK são determinadas tanto pela expressão de moléculas HLA (antígenos leucocitários humanos) de classe I e MICA (*MHC class I chain related*) quanto por distúrbio do equilíbrio mediado por receptores existentes em sua superfície, tais como os receptores KIR (*killer immunoglobulin-like receptor*).[111,112] O extenso polimorfismo genético presente nos genes que codificam essas moléculas, assim como a regulação da expressão das mesmas são fatores importantes no delineamento da resposta imune inata

e adaptativa de cada indivíduo. Nesse sentido é de especial interesse a investigação de genes de resposta imune envolvidos na regulação da atividade das células NK.

1.6 Células *Natural Killer*

As células NK foram descritas inicialmente em 1975 por Herberman et al.[113] e Kiessling et al.[114] e representam uma subpopulação de linfócitos granulosos que exercem função crucial na resposta imune, especialmente contra células infectadas, transformadas ou alogênicas, pela sua capacidade de lise celular sem sensibilização prévia e também pela produção de citocinas e quimiocinas que mediam a resposta inflamatória.[115] Essas células estão presentes em diversos tipos de tecidos e órgãos como medula óssea, baço, linfonodos, fígado, intestino e placenta. Elas também estão presentes na circulação periférica, e possuem a capacidade de migrar para locais específicos de infecção, inflamação e desenvolvimento tumoral.[116]

Em humanos, as células NK são caracterizadas pela expressão do marcador de superfície CD56 e pela ausência de expressão de CD3 e CD19. De acordo com o nível de expressão de CD56 em sua superfície as células NK podem ser classificadas em duas principais populações: as células que expressam abundantemente CD56 são denominadas de células $CD56^{bright}$ e, correspondem cerca de 10% das células NK circulantes, as quais possuem propriedades imunoregulatórias; as células com baixa expressão de CD56 são denominadas de células $CD56^{dim}$ e, apresentam alta capacidade citotóxica correspondendo a 90% de todas as células NK circulantes. Além disso, a maioria das células CD56 também expressa o receptor CD16, que confere a capacidade de mediar a citotoxidade celular dependente de anticorpos.[117]

A função efetora das células NK é regulada por um equilíbrio de sinais gerados por seus receptores de ativação e inibição; e o mecanismo que faz com que as células NK sejam capazes de distinguir as células normais das células infectadas ou transformadas acontece pela interação desses receptores com moléculas específicas expressas na superfície das células-alvo que atuam como ligantes, dentre elas moléculas MHC de classe I clássicas como HLA-A, -B e –C e não clássicas como HLA-E, -G e moléculas MIC.[111]

Moléculas HLA de classe I são normalmente expressas em células saudáveis do organismo, mas podem ter sua expressão alterada em células infectadas ou malignas. A perda ou diminuição da expressão dessas moléculas resulta na ativação das células NK; e a partir dessa característica foi postulada a hipótese *do missing-self* (perda do reconhecimento do próprio), que estabelece que a ausência dessas moléculas remove os ligantes dos receptores inibidores das células NK ativando a função efetora das mesmas.[118] A expressão de moléculas MIC é induzida em resposta ao estresse celular, e ao serem reconhecidas pelo receptor ativador das células NK, desencadeia uma cascata de sinalização que termina com o processo de lise celular da célula-alvo,[119,120] hipótese conhecida como *"induced self"*.[121]

Os receptores das células NK pertencem a duas famílias: a superfamília das imunoglobulinas e a família dos receptores semelhantes à lectina. A superfamília das imunoglobulinas engloba os receptores KIR, LILR (*leucocyte immunoglobulin-like receptor*), LAIR (*leucocyte associated inhibitory receptor*), FcαR (*Fc-alfa receptor*) e NKp46 (*activating NK recptors*). Os membros mais representativos da família dos receptores semelhantes à lectina são as moléculas NKG2 (*C-type lectin receptor*), no

qual as classes NKG2A, B, C, E e H precisam estar associados à molécula CD94 para tornarem receptores funcionais. NKG2D não se liga à CD94. Cada família de receptores contém receptores inibidores e ativadores.[122]

Os receptores KIR e CD94/NKG2A, por exemplo, desempenham importante papel reconhecendo moléculas MHC dos grupos HLA-A, -B e –C e moléculas HLA-E, respectivamente,[123] enquanto que o receptor NKG2D reconhece moléculas MICA e MICB,[112] além várias outras moléculas com expressão celular induzida por estresse, como a família de proteínas ligantes de UL16 (ULBPs).[124] Assim, a interação das células NK com a célula-alvo sugere função relevante na iniciação e regulação da resposta imune inata e adaptativa e a base molecular dessas interações celulares parece ser crucial para que ocorra uma resposta eficiente e modulada.

1.7 Genes *KIR*

Os genes de resposta imune *KIR* (*killer cell immunoglobulin-like receptors genes)* representam uma família de genes polimórficos que estão localizados no cromossomo 19q13.4, em uma região chamada Complexo de Receptores Leucocitários (LRC - *Leukocyte Receptor Complex*).[125] (Figura 7). Esses genes são responsáveis por codificar os receptores KIR que estão envolvidos na resposta imunológica mediada por células NK, sendo encontrados também em algumas subpopulações de células T.[126] Os genes *KIR* são organizados em nove *exons*. Os dois primeiros *exons* codificam as sequências líderes. Os *exons* três, quatro e cinco codificam respectivamente os domínios semelhantes a imunoglobulinas D0, D1 e D2, que são responsáveis pelo reconhecimento dos ligantes. O *exon* seis codifica a região de inserção da molécula na membrana

plasmática e o *exon* sete codifica a região transmembrana. Os *exons* oito e nove codificam domínios citoplasmáticos.[127]

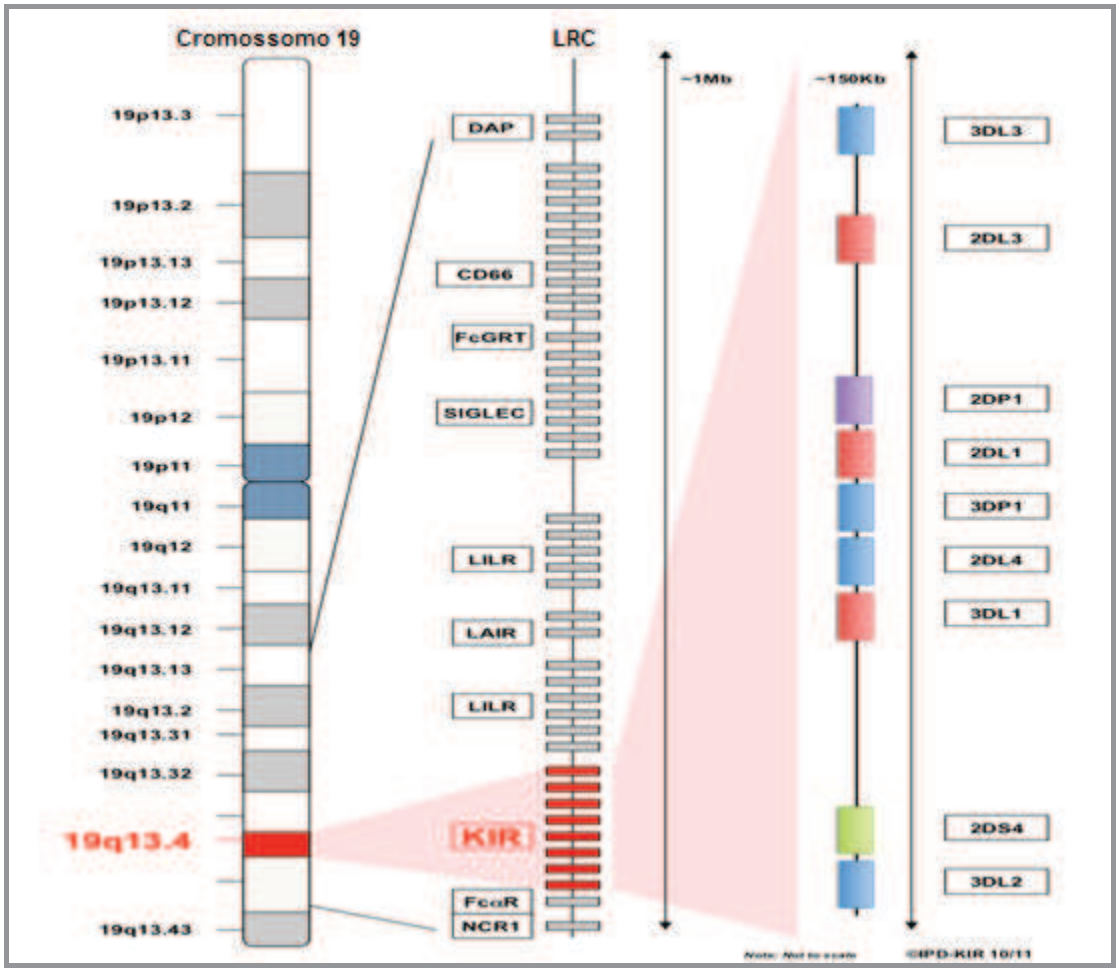

Figura 7: Complexo de Receptores Leucocitários e os genes *KIR*. (Extraído).[128]

Os receptores KIR inibidores e ativadores têm a mesma estrutura quanto aos domínios extracelulares (2D ou 3D), mas em relação à cauda citoplasmática possuem características diferentes: os receptores inibidores possuem cauda longa (L) contendo um ou dois motivos de inibição denominados ITIMs (*Immunoreceptor tyrosine-based inhibition motifs*), enquanto que os receptores ativadores possuem cauda curta (S), ausência de ITIMs, presença de um aminoácido (lisina ou arginina) carregado positivamente na região transmembrana, os quais permitem a associação dos receptores

KIR com a molécula acessória DAP12, e contém motivos de ativação ITAM (*Immunoreceptor tyrosine-based activation motifs*); além dos pseudogenes (P) que não têm função descrita.[129] Uma exceção é o receptor KIR2DL4, pois possui ITIM em sua cauda citoplasmática e o aminoácido arginina na região transmembrana. Desta forma possui dupla função: inibidora e ativadora. Esse receptor ao se associar à molécula FcεRI-γ (*Fc epsion receptor type γ*) envia sinais estimulatórios às células NK via ITAM.[130] (Figura 8).

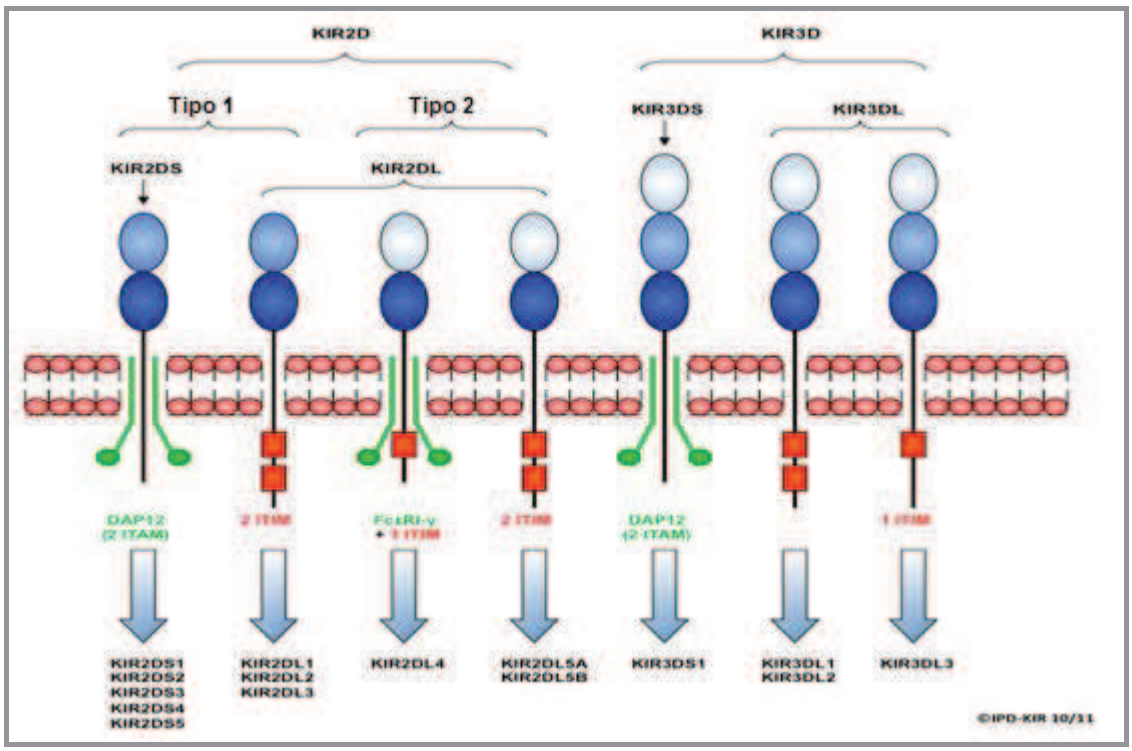

Figura 8: Estrutura molecular dos receptores KIR. (Extraído).[128]
Domínios extracelulares (azul), região transmembrana, molécula acessória DAP-12 (verde) e cauda citoplasmática com presença de ITIMs (vermelho).

Na espécie humana, cada indivíduo expressa em suas células diferentes tipos e números de receptores KIR. Há existência de dois genótipos distintos designados de AA e BX (BB e AB), que são compostos pela combinação de genes inibidores e

ativadores.[129] O genótipo AA é caracterizado pela presença dos genes *KIR2DL1, KIR2DL3, KIR2DL4, KIR2DS4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1* e *KIR3DP1*, e tem como característica a presença de somente um gene que codifica um receptor ativador, o *KIR2DS4*. A presença de um ou mais dos seguintes genes: *KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5* e *KIR3DS1* caracterizam o genótipo BX. Todos os genótipos KIR apresentam os genes *KIR3DL3, KIR3DL2*, *KIR3DP1* e *KIR2DL4*. Esses genes presentes em todos os genótipos são denominados genes de moldura ou genes estruturais (*frameworks genes*).[127]

Os receptores KIR reconhecem moléculas HLA de classe I presente na superfície das células-alvo,[127,131] e ausência ou diminuição da expressão das moléculas HLA faz com que a ação das células NK seja efetiva sobre as células-alvo.[118] KIR2DL1 e KIR2DS1 se ligam a moléculas HLA-C do grupo C2, que incluem as especificidades HLA-C*02, *04, *05, *06, *07,*15, *17, e *18. KIR2DL2, KIR2DL3 e KIR2DS2 interagem com moléculas HLA-C do grupo C1, entre elas: HLA-C*01, *03, *07, *08, *12, *13, *14 e *16. KIR3DL2 liga-se a HLA-A*03 e/ou -A*11 e KIR3DL1 reconhecem epítopos HLA-Bw4 (HLA-A*23, *24, *25, *32; HLA-B*13, *27, *44, *51, *52, *53, *57, *58). Moléculas HLA-Bw4 são divididas em dois grupos baseados na presença de isoleucina ou treonina na posição 80 (Bw4-80Ile e Bw4-80Thr, respectivamente). KIR3DS1 se liga a moléculas Bw4-80Ile e KIR2DL4 se liga ao HLA-G. Ainda permanecem indefinidos os ligantes para KIR2DL5, KIR2DS3, KIR2DS5 e KIR3DL3.[132-134]

Entretanto, a segregação independente dos genes *KIR* e *HLA* possibilita a expressão de moléculas KIR para as quais o ligante HLA não está presente, ou vice-

versa, o que resulta na ausência de funcionalidade das células NK pela falta de sinalização.[132] Além disso, dependendo da combinação entre KIR e ligantes HLA, as células NK podem apresentar vários níveis de resposta, como o excesso de inibição ou ativação ou balanço entre inibição e ativação.[135]

Estudos independentes demonstraram que a presença ou ausência de certos genes e genótipos KIR, bem como diferentes combinações entre os receptores KIR e moléculas HLA de classe I podem estar envolvidos na evolução clínica de várias doenças infecciosas,[136-146] doenças autoimunes ou inflamatórias[147-149] e ainda em muitos tipos de câncer.[150-152] Recentemente foi verificado que a citotoxidade das células NK, mediada pelos receptores KIR e ligantes HLA, pode contribuir no desenvolvimento das lesões teciduais observadas na cardiopatia chagásica crônica.[153] Entretanto, até a realização deste trabalho não havia registros na literatura sobre a influência dos genes *KIR* na toxoplasmose ocular; e não há registros na literatura sobre o papel destes genes na forma digestiva da doença de Chagas crônica.

1.8 Gene *MICA*

O complexo principal de histocompatibilidade (CPH) ou MHC representa um conjunto de locos gênicos situados no braço curto do cromossomo 6 na região p21.31 que são responsáveis pela síntese de moléculas envolvidas na resposta imunológica. Este complexo está dividido em três grandes grupos denominados de classes I, II e III de acordo com a estrutura e função dos produtos gênicos. No entanto, o MHC humano não contém apenas os genes *HLA* clássicos, mas uma grande variedade de genes relevantes, tais como genes *MIC* (*major histocompatibility complex class I chain-related gene*). Os genes *MIC* estão localizados dentro da região de classe I e consiste

uma família de sete genes, *MICA-MICG*, dos quais apenas *MICA* e *MICB* expressam transcritos funcionais; os demais locos (*MICC-MICG*) não codificam nenhum transcrito detectável e são, portanto, pseudogenes.[154-155]

MICA é o mais polimórfico gene de classe I não clássico (até o momento foram identificados 106 alelos)[156] e está situado próximo aos locos *HLA-B* e *HLA-C*. Possui seis *exons*, dentre os quais o primeiro *exon* codifica uma sequência líder, o segundo, terceiro e quarto *exons* codificam as sequências dos domínios externos α-1, α-2 e α-3, respectivamente, o quinto exon codifica o segmento transmembrana da molécula. A cauda citoplasmática e as sequências 3'UTR (região não traduzida) estão fusionadas no *exon* 6.[154] (Figura 9).

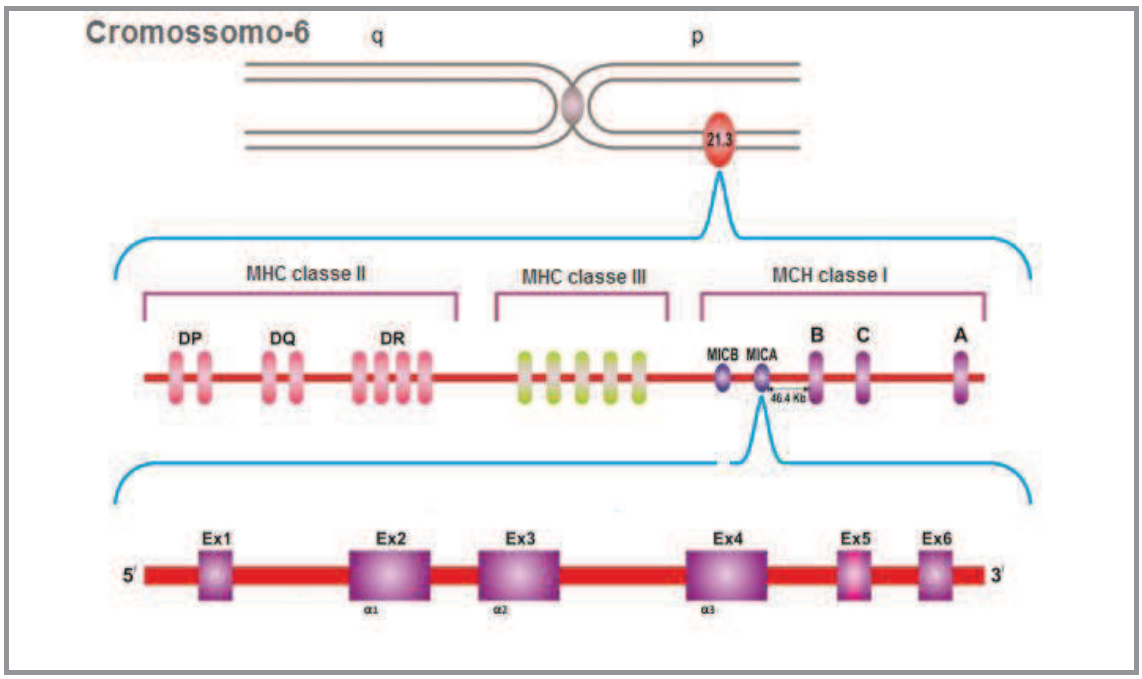

Figura 9: Localização do gene *MICA* no braço curto do cromossomo 6 e representação dos seis *exons* (Ex1-6) que codificam a molécula MICA. (Adaptado).[157]

A expressão de MICA é induzida em resposta ao estresse celular em muitos tipos de células, incluindo epitélio, fibroblastos, queratinócitos, células endoteliais e monócitos.[158,159] As moléculas MICA são reconhecidas pelos linfócitos Tγδ, linfócitos Tαβ ($CD8^+$ e subconjuntos de $CD4^+$) e células NK, através de seus receptores NKG2D presentes em sua superfície em associação com a molécula DAP10, uma proteína adaptadora de membrana. Este complexo NKG2D-MICA ativa a fosforilação dos resíduos de tirosina da molécula DAP10, desencadeando uma cascata de sinalização celular que termina com o processo de lise da célula-alvo.[119,120]

As glicoproteínas MICA apresentam estrutura similar às moléculas HLA de classe I clássicas, incluindo três domínios externos (α1, α2 e α3), um domínio transmembrana e um domínio citoplasmático.[154] Porém, as moléculas MICA apresentam diferenças significantes uma vez que sua estrutura não está associada à molécula β2-microglobulina, são independentes de TAP (moléculas transportadoras associadas ao processamento de antígeno) e não possuem uma região de ligação com peptídeo.[160] (Figura 10).

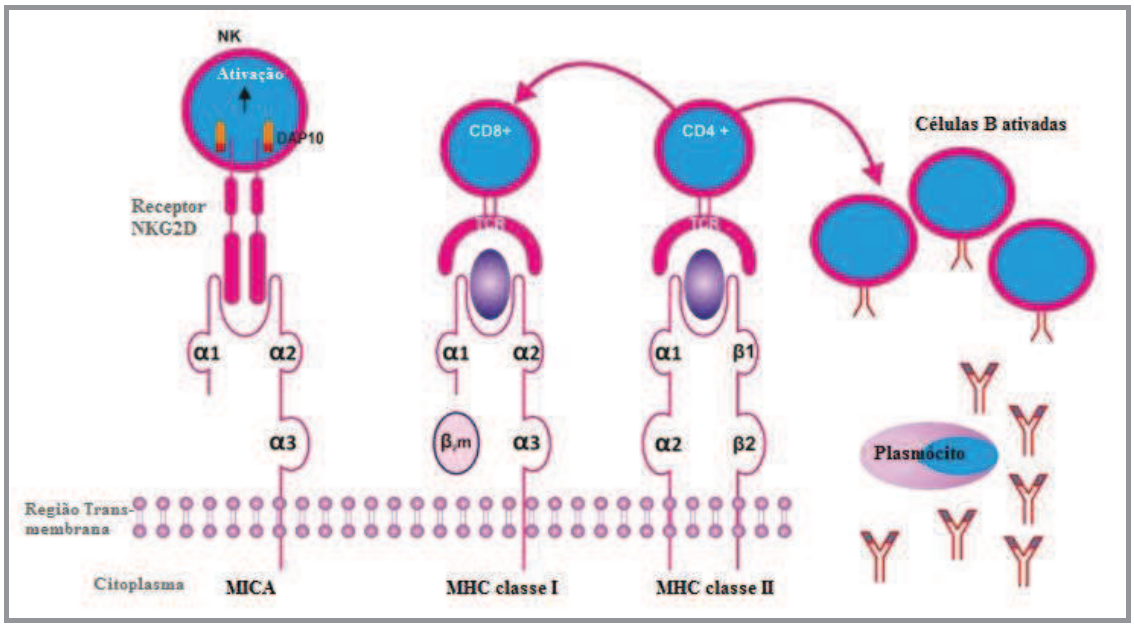

Figura 10: Semelhanças estruturais entre o MHC de classe I e II e moléculas MICA. (Adaptado).[157]

Conforme os genes *HLA* de classe I clássicos, o gene *MICA* possui uma grande variedade alélica devido polimorfismos de nucleotídeo único (SNP) encontrados principalmente nos *exons* 2, 3 e 4, correspondentes aos domínios extracelulares da molécula, dentre os quais um deles apresenta implicação funcional: A presença do aminoácido metionina na posição 454 que corresponde ao aminoácido 129 do domínio α2 da proteína (MICA-129met) tem maior afinidade pelo receptor NKG2D do que as proteínas com o aminoácido valina (MICA-129val) nessa mesma posição, comprometendo a ativação das células NK e a coestimulação dos linfócitos T CD8.[161] Além disso, os alelos MICA*010 e MICA*025, apresentam uma substituição de nucleotídeo que causa uma troca de prolina para arginina na posição 6 do domínio α1, afetando a dobra da molécula e eliminando sua expressão na superfície celular.[162] Ainda, em determinadas condições de estresse, as moléculas MICA podem formar complexos com outras moléculas reguladas positivamente sob tais condições e sofrer

uma clivagem proteolítica devido a uma mudança conformacional de sua estrutura e, dessa maneira serem secretadas na forma solúvel.[157]

Outra característica do gene *MICA* é a presença de um polimorfismo de curta sequência repetida em tandem (STR) localizado no exon 5, que codifica a região transmembrana da molécula. Os STR consistem em repetições (GCT)n. Oito alelos foram descritos, A4, A5, A5.1, A6, A7, A8, A9 e A10, com 4-10 repetições GCT. No caso da variante A5.1, além das 5 repetições, ocorre a inserção de uma guanina após a segunda repetição GCT, esta inserção cria um códon de parada prematuro, deixando a proteína sem cauda citoplasmática e que aparentemente não consegue alcançar a superfície celular não sendo, portanto, uma molécula secretada.[163]

Moléculas MICA expressas na superfície celular parecem ativar células NK e promover a atividade citotóxica das mesmas; por outro lado, estas moléculas na forma solúvel podem bloquear a ativação do receptor NKG2D inibindo a citotoxicidade das NK, situação que pode ser deletéria nos processos antitumorais, autoimunes ou até mesmo em situações de infecção. Mesmo as moléculas ancoradas na superfície celular exibem variabilidade em sua estrutura ou posicionamento na membrana e desta forma provavelmente não interagem com a mesma intensidade com o receptor, principalmente aquelas cujos alelos apresentam polimorfismo funcional parecem trazer consequências para o sistema de defesa do organismo.[164]

Neste sentido, vários estudos de associação têm analisado a diversidade *MICA* em doenças infecciosas[165-169] e em muitos tipos de câncer,[170-172] além do seu papel na rejeição de transplantes.[173] Em relação à toxoplasmose ocular, não havia registros na literatura sobre a influência do gene *MICA* nessa patologia até o memento da realização

deste trabalho. No que diz respeito à doença de Chagas, um estudo realizado na Bolívia mostrou que o haplótipo HLA-DRB1*01~B*14~MICA*01 estava associado com a proteção contra a doença crônica.[174] Entretanto, o papel de MICA no desenvolvimento e gravidade da DSVE em pacientes com CCC também não havia sido abordado.

1.9 Objetivos

O presente estudo teve como objetivo geral verificar a hipótese de que os genes *KIR*, seus ligantes HLA e o polimorfismo do gene *MICA* estão associados à toxoplasmose ocular e às diferentes formas clínicas da doença de Chagas. Os objetivos específicos foram:

1. Identificar os genes *KIR*, os grupos de alelos HLA-A, HLA-B, HLA-C (ligantes de KIR) e MICA em indivíduos infectados pelo *T. gondii*, com e sem toxoplasmose ocular e, dentre aqueles com toxoplasmose ocular, com manifestação ocular primária e recorrente.

2. Identificar os genes *KIR*, os grupos de alelos HLA-A, HLA-B, HLA-C (ligantes de KIR) e MICA em indivíduos com doença de Chagas Crônica, com as formas clínicas cardíaca e digestiva da doença e, dentre aqueles com cardiopatia chagásica crônica, com e sem disfunção sistólica ventricular esquerda.

2. Artigos

2. ARTIGOS

Os resultados deste trabalho estão apresentados na forma de 4 artigos, sendo três artigos publicados e um submetido à publicação.

Em relação à toxoplasmose ocular:

Artigo 1

Título: MHC Class I Chain-Related Gene A Polymorphisms and Linkage Disequilibrium with HLA-B and HLA-C Alleles in Ocular Toxoplasmosis.

Autores: Christiane Maria Ayo, Ana Vitória da Silveira Camargo, Fábio Batista Frederico, Rubens Camargo Siqueira, Mariana Previato, Fernando Henrique Antunes Murata, Aparecida Perpétuo Silveira-Carvalho, Amanda Pires Barbosa, Cinara de Cássia Brandão de Mattos, Luiz Carlos de Mattos.

Periódico: Plos One, 2015; 10: e0144534. IF: 3.05.

Artigo 2

Título: Ocular toxoplasmosis: susceptibility in respect to the genes encoding the KIR receptors and their HLA class I ligands.

Autores: Christiane Maria Ayo, Fábio Batista Frederico, Rubens Camargo Siqueira, Cinara de Cássia Brandão de Mattos, Mariana Previato, Amanda Pires Barbosa, Fernando Henrique Antunes Murata, Aparecida Perpétuo Silveira-Carvalho, Luiz Carlos de Mattos.

Periódico: Scientific Reports 2016; 6: 36632. IF: 5.22.

Em relação à doença de Chagas:

Artigo 3

Título: Association of the Functional MICA-129 Polymorphism With the Severity of Chronic Chagas Heart Disease.

Autores: Christiane Maria Ayo, Amanda Priscila de Oliveira, Ana Vitória da Silveira Camargo, Cinara de Cássia Brandão de Mattos, Reinaldo Bulgarelli Bestetti, Luiz Carlos de Mattos.

Periódico: Clinical Infectious Disease 2015; 61:1310-1313. IF: 8.73.

Artigo 4

Título: *MICA* and *KIR*: Immunogenetic factors influencing the cardiac and digestive clinical forms of chronic Chagas disease.

Autores: Christiane Maria Ayo, Reinaldo Bulgarelli Bestetti, Eumildo de Campos Junior, Luiz Sérgio Ronchi, Aldenis Albaneze Borim, Cinara Cássia Brandão de Mattos, Luiz Carlos de Matttos.

Periódico: Cellular & Molecular Immunology (Submetido para publicação). IF: 5.19.

Artigo 1

PLOS | ONE

RESEARCH ARTICLE

MHC Class I Chain-Related Gene A Polymorphisms and Linkage Disequilibrium with HLA-B and HLA-C Alleles in Ocular Toxoplasmosis

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OPEN ACCESS

Citation: Ayo CM, Camargo AVdS, Frederico FB, Siqueira RC, Previato M, Murata FHA, et al. (2015) MHC Class I Chain-Related Gene A Polymorphisms and Linkage Disequilibrium with HLA-B and HLA-C Alleles in Ocular Toxoplasmosis. PLoS ONE 10(12): e0144534. doi:10.1371/journal.pone.0144534

Editor: Gordon Langsley, Institut national de la santé et de la recherche médicale—Institut Cochin, FRANCE

Received: June 30, 2015

Accepted: November 19, 2015

Published: December 16, 2015

Data Availability Statement: All data files are available from the Figshare database: http://dx.doi.org/10.6084/m9.figshare.1597742; http://dx.doi.org/10.6084/m9.figshare.1597743; http://dx.doi.org/10.6084/m9.figshare.1608313.

Funding: This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grants: 2013/06580-9 to CMA; 2013/10050-5 to MP; 2013/15879-8 to FHAM; 2015/13723-6 to CCBM; 2013/25650-8 and 2009/17540-2 to LCM); by Fundação de Apoio à Pesquisa e Extensão

Abstract

This study investigated whether polymorphisms of the *MICA* (major histocompatibility complex class I chain-related gene A) gene are associated with eye lesions due to *Toxoplasma gondii* infection in a group of immunocompetent patients from southeastern Brazil. The study enrolled 297 patients with serological diagnosis of toxoplasmosis. Participants were classified into two distinct groups after conducting fundoscopic exams according to the presence (n = 148) or absence (n = 149) of ocular scars/lesions due to toxoplasmosis. The group of patients with scars/lesions was further subdivided into two groups according to the type of the ocular manifestation observed: primary (n = 120) or recurrent (n = 28). Genotyping of the MICA and HLA alleles was performed by the polymerase chain reaction-sequence specific oligonucleotide technique (PCR-SSO; One Lambda ®) and the MICA-129 polymorphism (rs1051792) was identified by nested polymerase chain reaction (PCR-RFLP). Significant associations involving MICA polymorphisms were not found. Although the MICA*002~HLA-B*35 haplotype was associated with increased risk of developing ocular toxoplasmosis (*P*-value = 0.04; OR = 2.20; 95% CI = 1.05–4.60), and the MICA*008~HLA-C*07 haplotype was associated with protection against the development of manifestations of ocular toxoplasmosis (*P*-value = 0.009; OR: 0.44; 95% CI: 0.22–0.76), these associations were not statistically significant after adjusting for multiple comparisons. MICA polymorphisms do not appear to influence the development of ocular lesions in patients diagnosed with toxoplasmosis in this study population.

Introduction

Ocular toxoplasmosis, characterized by intraocular inflammation, is the most common clinical manifestation of toxoplasmosis, the infectious disease caused by *Toxoplasma gondii* [1]. Lesions originate both from congenital infection and from infections acquired after birth [2,3].

de São José do Rio Preto (FAPERP) to FHAM. AVSC was supported by scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). CCBM was supported by grant from Faculdade de Medicina de São José do Rio Preto (BAP-FAMERP). LCM was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant: 473579/2009-0). FBF RCS APB APSC were not supported by grants. The opinions, assumptions, and conclusions or recommendations expressed in this material are the responsibility of the authors and do not necessarily reflect the views of FAPESP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

The lesions can affect the macula and other layers of the retina and the choroid, resulting in retinochoroiditis, the most frequent cause of posterior uveitis in immunocompetent patients [1]. Ocular manifestations can have an early or late onset, with primary or recurrent clinical manifestations [4] and present different degrees of ocular involvement that vary according to the immune status of the individual [1,5] and different *T. gondii* strains [6–8]. Whether the ocular manifestation resulting from infection by *T. gondii* is attributable to host or parasite genetic factors or differences in exposure rate remains uncertain [9].

The *MICA* gene is located on chromosome 6 in the region of the class I major histocompatibility complex gene (*MHC*) close to the *HLA-B* and *HLA-C* gene loci [10]. Under stress the *MICA* gene encodes a cell surface protein that is a ligand for NKG2D, an activating receptor of Tγδ lymphocytes, $CD8^+$ Tαβ lymphocytes and natural killer cells (NK) [11,12]. The MICA alleles can be categorized as strong (MICA-129 met) or weak ligands (MICA-129 val) of the NKG2D receptor based on the MICA 129 polymorphism (rs1051792). Corresponding to the 129 amino acid of the protein, this polymorphism alters a single amino acid with the substitution of methionine to valine (A>G) at position 454 of the third exon of the *MICA* gene and most likely alters the activation of these cells [13].

Besides the involvement of both $CD8^+$ T lymphocytes and NK cells, the immune response to *T. gondii* infection is also characterised by a strong T helper-1 (Th-1) response orchestrated by $CD4^+$ T cells and dominated by the production of proinflammatory mediators. However, while the Th-1 response prevents parasite replication, the strong Th-1 response may also cause immune-mediated tissue damage contributing to the severity of ocular toxoplasmosis. More recently, Th-17 cells, characterised by the production of interleukin-17 (IL-17), a potent inducer of inflammation, have been identified as key contributors to immunopathological responses in ocular toxoplasmosis [14–16].

MICA polymorphisms are possibly associated with the susceptibility or progression of several infectious diseases such as dengue fever [17], leprosy [18], tuberculosis [19], schistosomiasis [20], and Chagas disease [21,22], among others. Furthermore, the expression of MICA in inflamed tissues or in autoimmune diseases, in particular the MICA-129 polymorphism, would contribute to the immunopathology of these diseases [22–27]. However, the role of the MICA alleles and the effect of the MICA-129 functional polymorphism in ocular toxoplasmosis remain unknown and there is no data on the expression of MICA in ocular tissue affected by *T. gondii*.

This study investigated whether the MICA alleles and the 129 polymorphism in exon 3 of the *MICA* gene are associated with the development of eye lesions resulting from *T. gondii* infection in a group of immunocompetent patients from southeastern Brazil.

Materials and Methods

Ethics information

This study was approved by the Research Ethics Committee of the School of Medicine in São José do Rio Preto (#1980/2009) and all individuals who agreed to participate in this research were informed about the nature of the study and signed informed consent forms.

Sample selection

A total of 297 unrelated patients were selected from those seeking ophthalmological treatment in the Retinopathy Outpatient Service of Hospital de Base of the School of Medicine in São José do Rio Preto and Medical Outpatient Clinic (AME) in São José do Rio Preto. All patients selected for this study had positive serology for *T. gondii*. Anti-*T. gondii* antibodies were detected by immunosorbent assay (ELISA) according to the manufacturer's instructions (ETI-TOXOK-M reverse PLUS; DiaSorin S.p.A. Italy and ETI-TOXO-G PLUS; DiaSorin S.p.A.

Italy). Of all the patients included in this study, only five of those with ocular scars/lesions due to toxoplasmosis showed positive serology for both IgM and IgG anti-*T. gondii* antibodies. The remaining patients only had positive serology for IgG anti-*T. gondii* antibodies.

The clinical evaluation of patients was conducted by two experienced physicians using an indirect binocular ophthalmoscope (Binocular Ophthalmoscope ID10, Topcon Corporation, USA) as previously described [28]. Subsequently, patients were classified into two distinct groups according to the presence of ocular scars/lesions due to toxoplasmosis (n = 148; 79 men and 69 women; mean age: 42.3 ± 20.6 years) or to the presence of ocular diseases other than toxoplasmosis (n = 149; 73 men and 76 women; mean age: 57.7 ± 16.9 years) such as cataracts (17.5%), pterygium (4.0%), age-related macular degeneration (23.0%), glaucoma (6.7%), retinal detachment (16.1%), optic neuropathy (3.4%), macular edema (4.6%), macular atrophy (2.6%), diabetic retinopathy (8.7%) and other ocular diseases (13.4%). The group of patients with scars/lesions due to toxoplasmosis was further subdivided into two groups according to the type of ocular manifestation observed during a follow up period of at least two years: primary manifestations (n = 120; 65 men and 55 women; mean age: 44.9 ± 20.9 years) and recurrent manifestations characterised by the presence of satellite lesions (n = 28; 14 men and 14 women; mean age: 31.8 ± 30.5 years) (Table 1) [29].

All patients underwent detailed eye examinations including visual acuity (logMAR Early Treatment Diabetic Retinopathy Study [ETDRS] chart) with best correction according to the ETDRS standards [30], measurement of intraocular pressure by Goldmann applanation tonometry, biomicroscopy using a slit lamp, and stereoscopic biomicroscopy performed using a 78-diopter lens (Volk) and classified according to the ETDRS criteria.

Colour fundus photographs and fluorescent photographs were taken using a digital retinal camera (TRC-50DX, Topcon Medical Systems) to document the macula region and optic nerve. Areas of progressive hyperfluorescence (leakage), staining and transmitted hyperfluorescence (window effect) were investigated by fluorescein angiography. Progressive hyperfluorescence with late leakage was considered a sign of lesion activity. Fig 1 shows the different stages of eye lesions caused by *T. gondii* infection by colour retinography.

Although the patients in this study were of European descent, mixed African and European descent, and African descent, they were grouped as a population of mixed ethnicity due to high miscegenation of the Brazilian population [31]. The risk of population stratification bias between patients with scars/lesions and patients without ocular manifestations was minimized by matching the ethnic background, gender and geographic area of residence. These data were carefully checked to select groups.

Table 1. General characteristics of patients with and without ocular toxoplasmosis and its manifestation as primary or recurrent

Characteristic	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
Age (Mean ± SD)	57.7±16.9[a,b,c]	42.3±20.6[a]	44.9±20.9[b,d]	31.8±30.5[c,d]
Median	60	37	46	30
Gender (%)				
Female	76 (51.0%)	69 (46.6%)	55 (45.8%)	14 (50.0%)
Male	73 (49.0%)	79 (53.4%)	65 (54.2%)	14 (50.0%)

t = Student t test.

[a] *P-value*<0.0001 t = 7.00 (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis)

[b] *P-value*<0.0001 t = 5.48 (Patients without ocular toxoplasmosis vs. Patients with primary manifestation)

[c] *P-value*<0.0001 t = 7.51 (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation)

[d] *P-value* = 0.002 t = 3.12 (Patients with primary manifestation vs. Patients with recurrent manifestation)

doi:10.1371/journal.pone.0144534.t001

DNA extraction and MICA, HLA-B, and HLA-C genotyping

Genomic DNA was attained from peripheral blood using a commercial kit for silica column extraction (QIAamp® DNA Blood Mini Kit, QIAGEN, the Netherlands) following the manufacturer's instructions. The genotyping of the MICA, HLA-B and HLA-C alleles was performed using the polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) technique with the rSSO Luminex® genotyping kit (One Lambda, Canoga Park, CA, USA). This technique first targets PCR-amplified DNA using specific biotinylated primers and subsequently the amplified product is hybridized by complementary DNA probes conjugated to fluorescently coded microspheres, with detection using R-Phycoerythrin-conjugated Streptavidin (SAPE). Hybridization was verified by flow cytometry (LABScanTM 100 flowanalyzer) and data were interpreted using computer software (HLA Fusion, version 3.4, One Lambda®).

The 129 A>G (rs1051792) polymorphism of the *MICA* gene was identified by nested polymerase chain reaction (PCR-RFLP) using a technique adapted from Amroun et al. [23]. In this technique, the *MICA* gene-specific amplicon was used as a template in a second round amplification of its exon 3. The primer pair used in the first reaction was 5' CGT TCT TGT CCC TTT GCC CGT GTG C 3' and 5' GAT GCT GCC CCC ATT CCC TTC CCA AA 3' with an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 45 s and a final extension at 72°C for 5 min. The primer pair used in the second reaction was 5' GGG TCT GTG AGA TCC ATG A 3' and 5' TGA GCT CTG GAG GAC TGG GGT A 3' with an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 45 s, 61°C for 45 s, 72°C for 45 s and a final extension at 72°C for 5 min. The MICA-129 val allele was identified by the presence of a restriction site for the RsaI enzyme (FastDigest ™, Thermo Scientific, USA) created by a

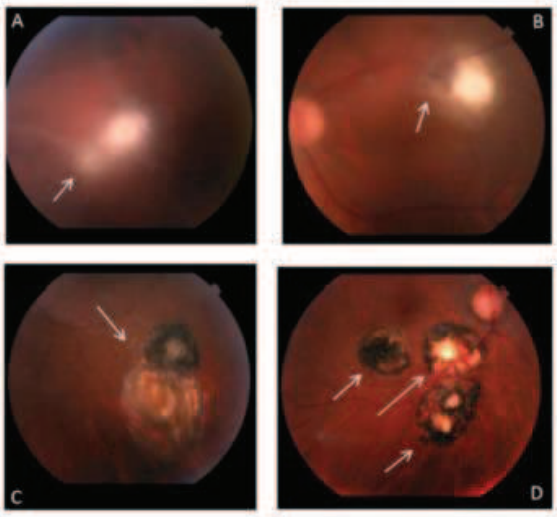

Fig 1. Colour retinography showing the various stages of eye lesions caused by *Toxoplasma gondii* infection in Brazilian patients. In (A) the arrow indicates the region with an acute exudative chorioretinal lesion ("lighthouse in the fog") and cloudy vitreous. In (B) the arrow indicates a chorioretinal lesions in the healing process—the patient had good clinical response to treatment and scar edges in definition. In (C) the arrow indicates presentation of an old chorioretinal scar and an old chorioretinal satellite lesion with pigment mobilization. In (D) chorioretinal scaring with well-defined edges indicated by the arrows with visualization of the sclera.

doi:10.1371/journal.pone.0144534.g001

mismatch introduced into the nonsense primer. For reasons of clarity and to follow the published nomenclature, the alleles will be designated here as MICA-129 met and MICA-129 val.

Statistical analysis

Genotype frequencies were obtained by direct counting, while the ARLEQUIN software (version 3.11; http://cmpg.unibe.ch/software/arlequin3) was used to calculate the allele and haplotype frequencies. The haplotype frequency was estimated by the expectation-maximization algorithm method [32], which allows an estimation of random haplotype frequencies based on the allele frequencies of the sample. Relative linkage disequilibrium (Δ') was calculated according to the Imanishi method [33], and the Hardy-Weinberg equilibrium was verified according to the method described by Guo & Thompson [34].

Comparisons of allele, haplotype and genotype frequencies between groups of patients were attained using the chi-square test with Yates' correction or Fisher's exact test. Odds ratio (OR) with a 95% confidence interval (95% CI) was also calculated to evaluate the risk association. The mean ages were compared using the t-test. Differences, considered statistically significant for *P*-values ≤ 0.05, were corrected by the Bonferroni inequality method for multiple comparisons (*Pc*). Statistical analyses were performed using the GraphPad Instat software (version 3.06).

Results

General characteristics of patients with and without ocular manifestations of toxoplasmosis

The general characteristics of the study participants are shown in Table 1. The group of patients without ocular toxoplasmosis presented a significantly higher mean age compared with the group of patients with ocular toxoplasmosis (*P*-value <0.0001; $t = 7.00$), with the subgroup of patients with primary manifestations of ocular toxoplasmosis (*P*-value <0.0001; $t = 5.48$) and with the subgroup of patients with the recurrent form of the disease (*P*-value <0.0001; $t = 7.51$). Differences in age were also observed between the subgroups of patients: those with the primary manifestation of the disease had a higher mean age than those who had recurrent manifestations (*P*-value = 0.002; $t = 3.12$).

Frequency of MICA alleles in patients with and without ocular manifestations of toxoplasmosis

Table 2 shows the distribution of the MICA alleles. Sixteen alleles were identified in the sample of patients without ocular toxoplasmosis and 20 in patients with ocular toxoplasmosis. The most common alleles in both groups were MICA*008, MICA*002, MICA*004 and MICA*009 totalling 63.5% and 64.8% of the possible alleles in patients with and without ocular toxoplasmosis, respectively. The MICA*030, MICA*041, MICA*044 and MICA*068 alleles were present only in patients with ocular manifestations of toxoplasmosis, with only one individual having each allele (0.3%). No significant differences were found in the distribution of MICA alleles between the groups of patients with and without ocular toxoplasmosis or between those with primary or recurrent clinical manifestations of the disease, so that the distribution of these alleles is in Hardy-Weinberg equilibrium in the study population.

Frequency of MICA-129 genotypes and alleles in patients with and without ocular manifestations of toxoplasmosis

There were no associations of genotypes or alleles of the MICA-129 polymorphism between the groups of patients with and without ocular toxoplasmosis or between the subgroups of patients

Table 2. Distribution of MICA alleles in patients with and without ocular toxoplasmosis and its manifestation as primary or recurrent

MICA alleles	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
	N (%)	N (%)	N (%)	N (%)
*001	7 (4.7)	6 (4.1)	6 (2.5)	0 (0.0)
*002	50 (33.6)	51 (34.5)	37 (15.5)	14 (25.0)
*004	40 (26.8)	37 (12.5)	32 (13.3)	5 (8.9)
*006	1 (0.7)	1 (0.7)	1 (0.4)	0 (0.0)
*007	11 (7.4)	14 (9.5)	11 (4.6)	3 (5.4)
*008	65 (43.6)	59 (39.9)	48 (20.0)	11 (19.6)
*009	38 (25.5)	41 (27.7)	37 (15.4)	4 (7.1)
*010	15 (10.1)	15 (10.1)	11 (4.6)	4 (7.1)
*011	11 (7.4)	17 (11.5)	15 (6.3)	2 (3.6)
*012	4 (2.7)	3 (2.0)	3 (1.3)	0 (0.0)
*015	5 (3.4)	4 (2.7)	3 (1.3)	1 (1.8)
*016	16 (10.7)	10 (6.8)	8 (3.3)	2 (3.6)
*017	5 (3.4)	9 (6.1)	7 (2.9)	2 (3.6)
*018	16 (10.7)	10 (6.8)	8 (3.3)	2 (3.6)
*019	7 (4.7)	6 (4.1)	3 (1.3)	3 (5.4)
*027	8 (5.4)	9 (6.1)	5 (2.1)	3 (5.4)
*030	0 (0.0)	1 (0.7)	1 (0.4)	0 (0.0)
*041	0 (0.0)	1 (0.7)	1 (0.4)	0 (0.0)
*044	0 (0.0)	1 (0.7)	1 (0.4)	0 (0.0)
*068	0 (0.0)	1 (0.7)	1 (0.4)	0 (0.0)

N: number of alleles

doi:10.1371/journal.pone.0144534.t002

with primary or recurrent ocular manifestations of the disease. The MICA-129 val allele and the heterozygous MICA-129 met/val genotypes were the most common in all groups (Table 3).

Frequency of MICA~HLA-B and MICA~HLA-C haplotypes in patients with and without ocular manifestations of toxoplasmosis

The most common MICA~HLA haplotypes are shown in Table 4. The MICA*002~HLA-B*35 haplotype was associated with increased risk of developing ocular toxoplasmosis (*P*-value = 0.04; OR: 2.20; 95% CI: 1.08–4.93), while the MICA*008~HLA-C*07 haplotype was associated with

Table 3. Genotype and allele frequencies of the MICA-129 polymorphism (rs1051792) in patients with and without ocular toxoplasmosis and its manifestation as primary or recurrent

MICA-129 polymorphism	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)	*P*-value
Genotypes	n (%)	n (%)	n (%)	n (%)	
met/met	22 (14.8)	22 (14.9)	18 (15.0)	4 (14.3)	ns
met/val	64 (43.0)	73 (49.3)	57 (47.5)	16 (57.1)	ns
val/val	63 (42.3)	53 (35.8)	45 (37.5)	8 (28.6)	ns
Alleles	N (%)	N (%)	N (%)	N (%)	
met	108 (36.2)	117 (39.5)	93 (38.8)	24 (42.9)	ns
val	190 (63.8)	179 (60.5)	142 (59.2)	32 (57.1)	ns

N: number of alleles; ns: non-significant (when *P*-value >0.05)

doi:10.1371/journal.pone.0144534.t003

Table 4. Haplotype frequencies of MICA, HLA-B and HLA-C in patients with and without ocular toxoplasmosis and its manifestation as primary or recurrent

Haplotypes		Patients without ocular toxoplasmosis (n = 149)		Patients with ocular toxoplasmosis (n = 148)		Patients with primary manifestation (n = 120)		Patients with recurrent manifestation (n = 28)		*P*-value	*Pc*	OR	IC (95%)
MICA	HLA	n	(%)	n	(%)	n	(%)	n	(%)				
*002	B*35	11	3.6	23	7.8	17	7.0	6	10.7	0.04[a,b]	ns	2.20	1.05–4.60
*002	B*53	14	4.7	6	2.0	4	1.7	2	3.6	Ns			
*004	B*44	26	8.7	14	4.7	11	4.6	3	5.3	Ns			
*008	B*07	19	6.3	10	3.3	8	3.3	2	3.5	Ns			
*009	B*51	16	5.3	21	7.0	17	7.0	3	5.3	Ns			
*002	C*04	25	8.3	30	9.8	22	9.2	8	14.3	Ns			
*004	C*16	18	6.0	12	4.0	10	4.1	2	3.5	Ns			
*008	C*07	36	12.0	17	5.7	16	6.6	1	1.7	0.008[a,b]	ns	0.44	0.22–0.76
*009	C*06	12	3.9	7	2.4	7	2.9	0	0.0	ns			
*016	C*04	13	4.4	11	3.4	9	3.1	2	3.5	ns			

ns: non-significant (when *P*-value >0.05)

[a]: Patients without ocular toxoplasmosis

[b]: Patients with ocular toxoplasmosis

doi:10.1371/journal.pone.0144534.t004

protection against the development of manifestations of ocular toxoplasmosis (P-value = 0.009; OR: 0:44; 95% CI: 0.22–0.76). However, the significance of these associations was not statistically significant after correcting for multiple comparisons. There was no significant difference on comparing the MICA~HLA-B and MICA~HLA-C haplotypes between patients with primary manifestations and those with recurrent manifestations of ocular toxoplasmosis.

Discussion

Although the importance of NK cells and CD8$^+$ T lymphocytes to the immune response of individuals infected by *T. gondii* [35,36] and the role of MICA alleles in the activation of these cells has been established [8,9], to the best of our knowledge, this is the first study that addresses the role of MICA alleles and the MICA-129 polymorphism (rs1051792) in the immunopathogenesis of toxoplasmosis. Several factors related to both the host and the parasite have been suggested as possible causes of the initial manifestation and the recurrence of ocular toxoplasmosis, but none is widely accepted [5,6,37,38].

The average ages of the group and subgroups of patients with ocular toxoplasmosis are less than the average age of patients without ocular toxoplasmosis. Moreover, patients presenting recurrent ocular manifestations have a lower mean age than those with primary disease. Several studies have reported the importance of age in the clinical course of ocular toxoplasmosis with most of them showing that the disease most often affects patients from the second to fourth decades of life [29,39–41]. Moreover, the risk of recurrence is higher in the year following the first episode than in following years [42]. A common feature of these studies (including this study) is that the patients with ocular toxoplasmosis were relatively young. Indeed, other eye diseases, those without ocular scars/lesions due to toxoplasmosis, are prevalent in older patients [43], although ocular toxoplasmosis may develop at any stage of life [4]. The majority of cases of ocular involvement due to toxoplasmosis are considered postnatally acquired infections [1].

No distinction was made between congenital and acquired disease in the analysis of the characteristics of eye injuries. During infection, a pregnant woman presents a temporary parasitaemia, which, can cause focal lesions in the placenta and infect the fetus, with varying severity of damage, depending on the virulence of the parasite strain, the immune response of the mother and the gestational period [44]. Reactivation of chronic *T. gondii* infection and consequent disease is common in congenitally infected individuals and some studies report that ocular disease is the most common manifestation of congenital toxoplasmosis [45–48]. In addition to the presence of clinically detectable ocular lesions at birth, new lesions typically appear late in children who receive treatment or not [49,50], although the recurrence rates of congenital and acquired ocular toxoplasmosis appear to be similar [29].

It has been postulated that recurrence is associated with reactivation of cysts in the retina attributed to immaturity or alterations in host immunity [51]. Both patients with congenital infection and older patients seem to be at higher risk of developing ocular lesions [47]. However, independent studies show that individuals with primary toxoplasmic retinochoroiditis without a pre-existing retinochoroidal lesion were older than those with recurrent ocular toxoplasmosis [29,52]. Furthermore, it is possible that recurrent ocular manifestations result from repeated infections of more than one strain of the parasite [53,54], or they may also be associated with more virulent parasite strains [6,7,55].

The frequency of the MICA alleles in this study population was similar to that found by Marin *et al.* [56] in a healthy population of the state of São Paulo and Ribas *et al.* [57] in a healthy population from the state of Paraná, with the MICA*008, *002, *004 and *009 alleles being the most common. However, the data of this study do not suggest that the extensive

allelic polymorphism of the *MICA* gene contributes independently from HLA-B and HLA-C to the appearance of ocular lesions resulting from *T. gondii* infection.

Furthermore, comparisons between the MICA alleles revealed differences in their ability to bind to the NKG2D receptor. Sequences whose codon 129 is encoded with methionine express proteins with a 10 to 50 times greater capacity to form a complex with NKG2D than sequences with valine at this position, which possibly affects the activation and modulation of NK and T cells [13]. However, our results showed there were no associations of the genotypes or alleles of the MICA-129 polymorphism between the groups of patients diagnosed with toxoplasmosis (with or without ocular injury) or between the subgroups of patients with manifestations of primary or recurrent disease.

The intraocular immune response is suppressed in normal circumstances thereby decreasing the risk of tissue destruction [58]. Under these conditions, cells in different tissues of the eye constitutively express the Fas ligand (Fas-L), which can promote the deletion of T cells and NK cells in the eye. Moreover, cytokines, such as transforming growth factor-beta (TGF-β), which have immunosuppressive properties, are also present, reducing the expression of MHC class I molecules [59,60], which may affect cytotoxic lymphocyte responses. However, decreased levels of TGF-β were found in ocular fluids of individuals with uveitis [61] and it has been shown that *T. gondii* is capable of stimulating the release and modifying the active form of TGF-β thereby facilitating replication of the parasite [62].

The pathogenesis of inflammation in ocular toxoplasmosis remains unclear, but several theories have been proposed in an attempt to explain this process [5]. There is evidence that *T. gondii* infection promotes the production of factors, such as interferon-gamma (IFN-γ), that suppress immune privilege which has a crucial role in protecting against infection, as well as being a potent TGF-β antagonist and hyper-regulating the expression of MHC molecules [63,64]. Murine models have shown that the ocular immune response against *T. gondii* involves factors similar to responses in other tissues, possibly leading to increased severity of lesions characterized by marked necrosis or inflammation of the retina and the choroid [65–67].

So far it has been shown that MICA alleles participate in the rejection process of solid organ transplants, immune surveillance of tumours and viruses [68] and the progression of several infectious [17–22], inflammatory and autoimmune diseases [23–27]. However, there is no evidence that immunopathogenic mechanisms related to diseases that involve MICA molecules also act on the immunity of ocular tissue affected by *T. gondii*, as the nature of the expression of MICA as a response to this parasite infection has not been demonstrated in these tissues yet. It has only been reported that, *in vitro*, the MICA protein has a reduced expression in normal corneal epithelium and that an increase in the expression of this protein results in cytotoxic activity of NK cells and $CD8^+$ T cells [69].

Lymphocytes expressing the NKG2D receptor are present in the eye during episodes of inflammation [58,70], and there is evidence that both NK and $CD8^+$ T cells are important components of the immune response against *T. gondii*. It is known that the expression of MICA molecules increases in response to infections and can trigger cytotoxicity and IFN-γ secretion by cells expressing the NKG2D receptor [71]. This study investigated whether the MICA allele and MICA-129 polymorphism, which affect binding affinity to the NKG2D receptor, are associated with the onset of ocular lesions in patients who are serologically positive for toxoplasmosis; however, no correlation was found.

Another possibility is that some MICA allotypes are intimately linked to other alleles responsible for this association, such as HLA, due to the relatively close physical proximity between their loci. According to Stephens [72], it is common for MICA alleles to be associated with HLA alleles, principally with HLA-B and thus exert a synergistic effect when combined. The association between MICA and ocular toxoplasmosis was observed only when the linkage

disequilibrium between the HLA-B and HLA-C loci was analysed. The MICA*002~HLA-B*35 haplotype was associated with increased risk of developing ocular toxoplasmosis, while the MICA*008~HLA-C*07 haplotype was associated with protection against the ocular manifestations of toxoplasmosis. A relatively significant linkage disequilibrium value was observed for the MICA*002~HLA-B*35 haplotype in patients who developed ocular symptoms ($\Delta' = 0.4305$; P-value = 0.002), and for the MICA*008~HLA-C*07 haplotype in the group of patients without ocular manifestations of the disease ($\Delta' = 0.3582$; P-value = 0.001).

When the alleles that make up the haplotypes listed above were analysed separately, no association was detected in respect to ocular toxoplasmosis or to the primary or recurrent clinical forms of the disease (data not shown for the HLA-B and HLA-C loci), so the possibility that the HLA alleles are mainly responsible for the association can be excluded. On the other hand, we cannot exclude chance as an explanation for the observed associations, as the statistical significance was no longer statistically significant after correcting for multiple comparisons. Moreover, as the haplotype frequencies were obtained from the allele frequencies, it is important to emphasize that they may not be accurate. To evaluate the true haplotype distribution, it is necessary to know the ancestors of the individuals in order to identify inherited haplotypes; this was not possible in the current study.

In conclusion, in this study population, the MICA alleles and MICA functional polymorphism-129 do not seem to influence the development of ocular lesions in patients diagnosed with toxoplasmosis. For a better understanding of the influence of MICA~HLA haplotypes as risk factors for ocular toxoplasmosis we suggest that additional studies should be conducted, in particular involving families. As allelic diversity of the *MICA* gene can differ between populations, according to regional variations, associations involving MICA polymorphisms could result in different clinical and immune phenotypes in patients with ocular toxoplasmosis from less racially mixed populations [16]. Furthermore, it is important to emphasize that the ocular toxoplasmosis diagnostic criteria used in this study were the same as in the clinical practice, injury identified by ophthalmoscopy associated with positive serology for *T. gondii*. As no invasive test was performed, this is a presumptive diagnosis when antibodies, antigens and protozoas were not detected in the injury. Furthermore, a histological analysis of the ocular tissue affected by *T. gondii* is necessary as well as investigations of the cytotoxicity of NK and $CD8^+$ T cells to clarify the expression of MICA molecules and to gain a better understanding of the role of cells expressing the NKG2D receptor in the immunopathogenesis of ocular toxoplasmosis.

Acknowledgments

The authors are grateful to all of the volunteers who participated in this study, to Laboratory of Immunogenetics at FAMERP and to David Hewitt and James Joseph Hesson for their help with the English version. Many thanks to Professor Stephen Henry from Auckland University of the Technology for providing library access.

Author Contributions

Conceived and designed the experiments: CMA LCM CCBM. Performed the experiments: CMA AVSC MP FHAM APSC. Analyzed the data: CMA LCM. Contributed reagents/materials/analysis tools: CMA AVSC FBF RCS MP APB CCBM LCM. Wrote the paper: CMA LCM. Head of the FAMERP Toxoplasma Research Group: CCBM. Performed the inclusion of patients: FBF APB RCS MP. Sample collection: FBF APB RCS MP. Developed the clinical evaluation and clinical analyses: FBF APB RCS MP.

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Artigo 2

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SCIENTIFIC REPORTS

OPEN

Ocular toxoplasmosis: susceptibility in respect to the genes encoding the KIR receptors and their HLA class I ligands

Received: 03 August 2016
Accepted: 17 October 2016
Published: 09 November 2016

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The objective of this study was to investigate the influence of the genes encoding the KIR receptors and their HLA ligands in the susceptibility of ocular toxoplasmosis. A total of 297 patients serologically-diagnosed with toxoplasmosis were selected and stratified according to the presence (n = 148) or absence (n = 149) of ocular scars/lesions due to toxoplasmosis. The group of patients with scars/lesions was further subdivided into two groups according to the type of ocular manifestation observed: primary (n = 120) or recurrent (n = 28). Genotyping was performed by PCR-SSOP. Statistical analyses were conducted using the Chi-square test, and odds ratio with a 95% confidence interval was also calculated to evaluate the risk association. The activating *KIR3DS1* gene was associated with increased susceptibility for ocular toxoplasmosis. The activating KIR together with their HLA ligands (KIR3DS1-Bw4-80Ile and KIR2DS1^{+}/C2^{++} KIR3DS1^{+}/Bw4-80Ile^{+}) were associated with increased susceptibility for ocular toxoplasmosis and its clinical manifestations. KIR-HLA inhibitory pairs -KIR2DL3/2DL3-C1/C1 and KIR2DL3/2DL3-C1- were associated with decreased susceptibility for ocular toxoplasmosis and its clinical forms, while the KIR3DS1^{-}/KIR3DL1^{+}/Bw4-80Ile^{+} combination was associated as a protective factor against the development of ocular toxoplasmosis and, in particular, against recurrent manifestations. Our data demonstrate that activating and inhibitory *KIR* genes may influence the development of ocular toxoplasmosis.

Ocular Toxoplasmosis, the most common form of posterior uveitis, results from *Toxoplasma gondii* infection[1]. The prevalence varies widely between different countries however, both the frequency and the severity of the resulting ocular manifestations are higher in Brazil than in many other parts of the world[2,3]. Eye injuries affect the retina and the choroid with local inflammatory reactions being observed in ocular tissues infected by *T. gondii*[1].

The damage to ocular tissues led to the proposal of phenomena that may be related to pathogenic mechanisms of ocular toxoplasmosis, including autoimmune mechanisms[1,4]. Now it is known that an exaggerated T-helper 1 (Th-1) response, in particular by Th-17 cells, can cause tissue damage and contribute to the severity of ocular toxoplasmosis due to the production of interleukin-17 (IL-17), a potent inducer of inflammation[5,6]. In addition to Th-17, other sources of IL-17, including natural killer cells (NK), can contribute to the development of inflammatory conditions[7].

Increases in the numbers of circulating proinflammatory monocytes and NK CD56^{dim} cytotoxic cells and a decrease in immunoregulatory NK CD56^{bright} cells have been identified in children congenitally infected with *T. gondii* who have active eye lesions. Furthermore, subsets of NK cells and CD8^{+} T cells play a crucial role as

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Characteristic	Patients without ocular toxoplasmosis* (n = 149)	Patients with ocular toxoplasmosis** (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
Age (Mean ± SD)	57.7 ± 16.9[a,b,c]	42.3 ± 20.6[a]	44.9 ± 20.9[b,d]	31.8 ± 30.5[c,d]
Median	60	37	46	30
Gender N (%)				
Female	76 (51.0)	69 (46.6)	55 (45.8)	14 (50.0)
Male	73 (49.0)	79 (53.4)	65 (54.2)	14 (50.0)
Serological profile N (%)				
IgM−/IgG+	149 (100.0)	143 (96.6)	115 (95.8)	28 (100.0)
IgM+/IgG+	0 (0.0)	5 (3.4)	5 (4.2)	0 (0.0)
IgM+/IgG−	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Table 1. General characteristics and serological profile of patients with and without ocular toxoplasmosis and its primary or recurrent manifestations. Clinical diagnosis: *Presence of ocular diseases other than toxoplasmosis as cataracts (17.5%), pterygium (4.0%), agerelated macular degeneration (23.0%), glaucoma (6.7%), retinal detachment (16.1%), optic neuropathy (3.4%), macular edema (4.6%), macular atrophy (2.6%), diabetic retinopathy (8.7%) and other ocular diseases (13.4%). **Presence of ocular scars/lesions due to toxoplasmosis. t = Student t test. [a]$P < 0.0001$ t = 7.00 (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [b]$P < 0.0001$ t = 5.48 (Patients without ocular toxoplasmosis vs. Patients with primary manifestation). [c]$P < 0.0001$ t = 7.51 (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation). [d]$P = 0.002$ t = 3.12 (Patients with primary manifestation vs. Patients with recurrent manifestation).

biomarkers of cicatricial lesion of the eye[8]. There is also evidence that NK cells have a predominantly proinflammatory profile *in vitro* during *T. gondii* infections, due to an increased production of interferon-gamma (IFN-γ) in patients with congenital ocular toxoplasmosis[9].

The effector function of NK cells is regulated by a set of receptors named killer immunoglobulin-like receptors (KIR) expressed on the cell surface that recognize human leukocyte antigen (HLA) class I molecules of target cells[9,10]. *KIR* genes are responsible for coding the KIR receptors of NK cells. These genes comprise a family of 15 genes located on chromosome 19q13.4 characterized as inhibitors (*KIR2DL1*, *-2DL2*, *-2DL3*, *-2DL5A*,*-2DL5B* *-3DL1*, *-3DL2*, and *-3DL3*) or activators (*KIR2DS1*, *-2DS2*, *-2DS3*, *-2DS4*, *-2DS5*, *-3DS1* and *-2DL4*,), and two pseudogenes (*KIR2DP1* and *-3DP1*). Moreover, based on the content of the genes, KIR genotypes are divided into two groups designated AA and BX (BB and AB) that differ in the number and type of *KIR* genes[11].

KIR genes have been described as risk or protective factors in different types of non-toxoplasmic uveitis and inflammatory ocular diseases. These diseases include Behcet's uveitis[12], uveitis in patients with spondyloarthropathies[13,14] and Vogt-Koyanagi-Harada syndrome[15,16], all of which are triggered by autoimmune processes. *KIR* genes are also associated with many other infectious diseases[17–19]. Additionally, both murine and human studies have shown that major histocompatibility complex (MHC) class I (called HLA class I in human) are associated with Toxoplasma susceptibility[20–26].

NK cells have great importance in the control of *T. gondii* infection[27] however, the role of *KIR* genes that encode the immune receptors of NK cells and can trigger local inflammation in the eye has not been elucidated in ocular toxoplasmosis yet. The objective of this study was to investigate the influence of the genes encoding the KIR receptors and their HLA ligands in the resistance or susceptibility to the development of ocular toxoplasmosis.

Results

General characteristics of patients with and without ocular manifestations of toxoplasmosis. The characteristics of the study population with respect to age, gender, clinical diagnosis and serological profile are shown in Table 1. The median ages were significantly different between the groups: A higher mean age was observed for the group of patients without ocular toxoplasmosis compared to the group of patients with ocular toxoplasmosis ($P < 0.0001$, t = 7.00), with the subgroup of patients with primary manifestations of ocular toxoplasmosis ($P < 0.0001$; t = 5.48) and with the subgroup of patients with the recurrent form of the disease ($P < 0.0001$; t = 7.51). A higher mean age was also observed for the subgroup of patients with primary manifestations than those who had recurrent manifestations ($P = 0.002$; t = 3.12).

Distribution of KIR genes and KIR genotypes in patients with and without ocular toxoplasmosis. The distribution of genotype frequencies of *KIR2DL2/3* and *KIR3DL1/S1* were in Hardy–Weinberg equilibrium ($P > 0.05$) in this study population. However, *KIR3DL1/S1* for the patient group that developed ocular toxoplasmosis was not in Hardy-Weinberg equilibrium ($P = 0.03$). *KIR* framework genes, *KIR2DL4*, *KIR3DL2*, *KIR3DL3* and *KIR3DP1*, as expected were present in all samples, which are important internal controls to check the quality of genotyping.

The distribution of *KIR* gene frequencies and AA and BX genotype frequencies are shown in Table 2. An increased susceptibility for developing ocular toxoplasmosis (OR = 2.15; CI = 1.31–3.50; $P = 0.003$, $Pc = 0.04$) was observed for the *KIR3DS1* activating gene. There was also a positive association between *KIR3DS1* and recurrent manifestations of disease (OR = 3.25; CI = 1.42–7.44, $P = 0.007$; $Pc = 0.1$) when this patient group was compared

Genes	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
	N (%)	N (%)	N (%)	N (%)
KIR2DL1	149 (100)	146 (98.6)	118 (98.3)	28 (100)
KIR2DL2	95 (63.8)	101 (68.2)	75 (62.5)	23 (82.1)
KIR2DL3	131 (87.9)	128 (86.5)	102 (85.0)	26 (92.9)
KIR2DL4	149 (100)	148 (100)	120 (100)	28 (100)
KIR2DL5	85 (57.0)	93 (62.8)	70 (58.3)	23 (82.1)
KIR2DP1	149 (100)	146 (98.6)	118 (98.3)	28 (100)
KIR2DS1	63 (42.3)	66 (44.6)	51 (42.5)	15 (53.6)
KIR2DS2	40 (26.8)[a]	25 (16.9)[a]	20 (16.6)	5 (17.9)
KIR2DS3	54 (36.2)	58 (39.2)	42 (35.0)	16 (57.1)
KIR2DS4	144 (96.6)	141 (95.2)	113 (94.2)	28 (100)
KIR2DS5	56 (37.6)	62 (41.9)	49 (40.8)	13 (46.4)
KIR3DL1	143 (96.0)	145 (98.0)	117 (97.5)	28 (100)
KIR3DL2	149 (100)	148 (100)	120 (100)	28 (100)
KIR3DL3	149 (100)	148 (100)	120 (100)	28 (100)
KIR3DP1	149 (100)	148 (100)	120 (100)	28 (100)
KIR3DS1	39 (26.1)[b,c]	64 (43.2)[b]	49 (40.8)	15 (53.6)[c]
Genotypes				
AA	41 (27.5)	33 (22.3)	27 (22.5)	6 (21.4)
BX	108 (72.4)	115 (77.7)	93 (80.0)	22 (78.5)

Table 2. Distribution of KIR genes and KIR genotypes in patients with and without ocular toxoplasmosis and its primary or recurrent manifestations. [a]OR = 0.55; CI = 0.31–0.97; $P = 0.05$; $Pc = 0.80$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [b]OR = 2.15; CI = 1.31–3.50; $P = 0.003$, $Pc = 0.04$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [c]OR = 3.25; CI = 1.42–7.44, $P = 0.007$; $Pc = 0.1$ (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation).

HLA ligands*	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
	N (%)	N (%)	N (%)	N (%)
A3 and/or A11	51 (20.8)	42 (28.4)	32 (26.7)	10 (35.7)
Bw4	113 (75.8)	104 (70.3)	84 (70.0)	20 (71.4)
Bw4-80Ile	93 (62.4)	87 (58.8)	72 (60.0)	15 (53.6)
Bw4-80Thr	46 (30.9)	39 (26.4)	29 (24.2)	10 (35.7)
C1C2	62 (41.6)	58 (39.2)	46 (38.3)	12 (42.9)
C1C1	26 (17.4)	25 (16.9)	19 (15.8)	6 (21.4)
C2C2	61 (40.9)	65 (43.9)	55 (45.8)	10 (35.7)

Table 3. Distribution of the HLA class I KIR-ligands in in patients with and without ocular toxoplasmosis and its primary or recurrent manifestations. Bw4 = HLA-A*23, *24, *32; HLA-B *13, *27, *44, *51, *52, *53, *57, *58. Bw4-80Ile = HLA-A*23, *24, *32; HLA-B*51, *52, *53, *57, *58. Bw4-80Thr = HLA-B *13, *27, *44. Group C1 = HLA-C*01, *03, *07, *08, *12, *14, *16. Group C2 = HLA-C*02, *04, *05, *06, *07, *15, *17, *18. *The same individual could express more than one pair KIR-HLA ligand.

to patients without ocular toxoplasmosis, although it was lost after applying the Bonferroni correction. On the other hand, the *KIR2DS2* activating gene was associated with decreased susceptibility for ocular toxoplasmosis (OR = 0.55; CI = 0.31–0.97; $P = 0.05$; $Pc = 0.80$), but the significance was also lost after applying the Bonferroni correction. No significant difference was observed in the AA and BX genotype frequencies between all groups investigated in this study.

Distribution of the HLA class I KIR-ligands in in patients with and without ocular toxoplasmosis. Human leucocyte antigen frequencies was in Hardy-Weinberg equilibrium ($P > 0.05$) in all groups studied. The frequencies of the HLA class I ligands of KIR (A3 or A11, Bw4–80Ile and –80Thr, C1 and C2, in homozygosity and heterozygosity) were analyzed and were similar between groups (Table 3).

Distribution of KIR and their respective HLA ligands in patients with and without ocular toxoplasmosis. Data on the distribution of KIR genes with their HLA class I ligands are listed in Table 4. The KIR3DS1-Bw4-80Ile pair was associated to increased susceptibility for developing ocular toxoplasmosis. The frequency was higher for patients with ocular toxoplasmosis (OR = 2.28; CI = 1.24–4.19; $P = 0.007$; $Pc = 0.01$),

KIR - HLA ligands	Patients without ocular toxoplasmosis (n = 149)	Patients with ocular toxoplasmosis (n = 148)	Patients with primary manifestation (n = 120)	Patients with recurrent manifestation (n = 28)
	N (%)	N (%)	N (%)	N (%)
2DL1-C2	123 (82.6)	121 (81.8)	99 (82.5)	22 (78.6)
2DL2-C1	55 (36.9)	57 (38.5)	41 (34.2)	16 (57.1)
2DL3-C1	79 (53.0)	71 (48.0)	55 (45.8)	16 (57.1)
3DL2-A3/A11	31 (20.8)	42 (28.4)	32 (26.7)	10 (35.7)
3DL1-Bw4	109 (71.2)	101 (68.2)	82 (68.3)	19 (67.8)
3DL1-Bw4-80Ile	90 (60.4)	81 (54.7)	68 (56.6)	13 (46.4)
3DL1-Bw4-80Thr	45 (30.2)	38 (25.7)	28 (23.3)	10 (35.7)
2DS1-C2	49 (32.9)	55 (37.2)	44 (36.7)	11 (39.3)
2DS2-C1	22 (14.8)	16 (10.8)	11 (9.2)	5 (17.9)
3DS1-Bw4-80Ile	19 (12.8)[a,b,c]	37 (25.0)[a]	28 (23.3)[b]	9 (32.1)[c]
2DL1-C2C2	61 (40.9)	63 (42.6)	53 (44.2)	10 (35.7)
2DL2-C1C1	19 (12.8)	20 (13.5)	14 (21.4)	6 (21.4)
2DL3-C1C1	23 (15.4)	23 (15.5)	17 (14.2)	6 (21.4)
2DS1-C2C2	18 (12.1)	31 (20.9)	26 (21.7)	5 (17.9)
2DS2-C1C1	10 (6.7)	4 (2.7)	2 (1.7)	2 (7.1)
2DL2/2DL2-C1C2	6 (4.0)	10 (6.8)	8 (6.7)	2 (7.1)
2DL2/2DL3-C1C2	30 (20.1)	27 (18.2)	19 (15.8)	8 (28.6)
2DL3/2DL3-C1C2	56 (37.6)[d,e,f]	21 (14.2)[d]	19 (15.8)[e]	2 (7.1)[f]
2DL2/2DL2-C1C1	3 (2.0)	2 (1.4)	2 (1.7)	0 (0.0)
2DL2/2DL3-C1C1	16 (10.7)	18 (12.2)	12 (10.0)	6 (21.4)
2DL3/2DL3-C1C1	23 (15.4)[g,h,i]	5 (3.4)[g]	5 (4.2)[h]	0 (0.0)[i]

Table 4. Distribution of KIR and their respective HLA ligands in patients with and without ocular toxoplasmosis and its primary or recurrent manifestations. [a]OR = 2.28; CI = 1.24–4.19; $P = 0.007$; $Pc = 0.01$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [b]OR = 2.08; CI = 1.09–3.95; $P = 0.02$; $Pc = 0.04$ (Patients without ocular toxoplasmosis vs. Patients with primary manifestation). [c]OR = 3.24; CI = 1.28–8.89; $P = 0.02$; $Pc = 0.04$ (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation). [d]OR = 0.27; CI = 0.15–0.48; $P = 0.000007$; $Pc = 0.00002$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [e]OR = 0.31; CI = 0.17–0.56; $P = 0.0001$; $Pc = 0.0003$ (Patients without ocular toxoplasmosis vs. Patients with primary manifestation). [f]OR = 0.12; CI = 0.29–0.59; $P = 0.003$; $Pc = 0.009$ (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation). [g]OR = 0.19; CI = 0.07–0.51; $P = 0.0005$; $Pc = 0.001$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis). [h]OR = 0.23; CI = 0.08–0.64; $P = 0.002$; $Pc = 0.006$ (Patients without ocular toxoplasmosis vs. Patients with primary manifestation). [i]OR = 0.09; CI = 0.05–0.91; $P = 0.01$; $Pc = 0.05$ (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestation).

patients with primary manifestations (OR = 2.08; CI = 1.09–3.95; $P = 0.02$; $Pc = 0.04$) and patients with recurrent manifestations (OR = 3.24; CI = 1.28–8.89; $P = 0.02$; $Pc = 0.04$) than patients without ocular toxoplasmosis.

The *KIR2DL3* inhibitory allele in the homozygous state and presence of its ligands whether homozygous or not (KIR2DL3/2DL3-C1/C1 and KIR2DL3/2DL3-C1) was associated with resistance to ocular toxoplasmosis when patients without ocular toxoplasmosis were compared with those with ocular toxoplasmosis (OR = 0.19; CI = 0.07–0.51; $P = 0.0005$; $Pc = 0.001$ and OR = 0.27; CI = 0.15–0.48; $P = 0.000007$; $Pc = 0.00002$ respectively), primary manifestations (OR = 0.23; CI = 0.08–0.64; $P = 0.002$; $Pc = 0.006$ and OR = 0.31; CI = 0.17–0.56; $P = 0.0001$; $Pc = 0.0003$ respectively) and recurrent manifestations (OR = 0.09; CI = 0.05–0.91; $P = 0.01$; $Pc = 0.05$ and OR = 0.12; CI = 0.29–0.59; $P = 0.003$; $Pc = 0.009$ respectively) (Table 4).

Frequencies of the number of KIR-HLA class I activating and inhibitory ligands in patients with and without ocular toxoplasmosis. Figure 1 shows the influence of the number of KIR-HLA class I activating and inhibitory ligands on the development of ocular toxoplasmosis and its clinical manifestations. There were significant differences between patients with and without ocular toxoplasmosis (OR = 2.29; CI = 1.19–4.39; $P = 0.01$; $Pc = 0.04$) and patients with primary manifestations and without ocular toxoplasmosis (OR = 2.29; CI = 1.16–4.52; $P = 0.01$; $Pc = 0.04$) when only two pairs of activating ligands were present (Fig. 1A). Significant associations were not found in the analysis of pairs of inhibitory KIR-HLA class I ligands (Fig. 1B).

Subsequently, discrimination of the two activator pairs (KIR-HLA class I) for the association mentioned above was investigated. The combination involving KIR2DS1 and KIR3DS1 in the presence of their respective ligands ($KIR2DS1^{+}/C2^{++}$ $KIR3DS1^{+}/Bw4\text{–}80Ile^{+}$) is responsible for increasing the risk of developing ocular toxoplasmosis (OR = 5.56; CI = 2.22–13.88; $P = 0.0001$; $Pc = 0.0004$). The combination is also responsible for its primary (OR = 5.35; CI = 2.09–13.68; $P = 0.0002$; $Pc = 0.0008$) and recurrent clinical forms (OR = 6.50; CI = 1.92–21.96; $P = 0.004$; $Pc = 0.01$) compared to patients without ocular toxoplasmosis (Fig. 2).

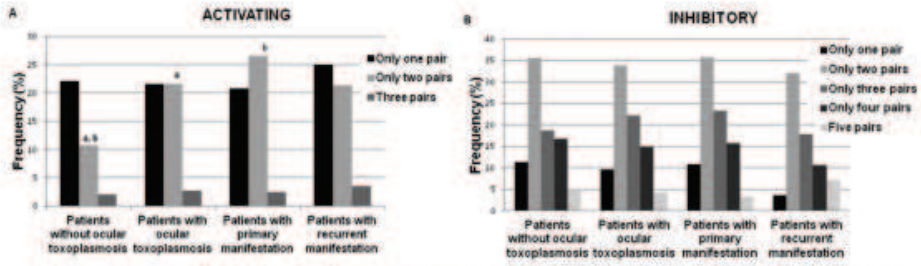

Figure 1. Frequencies of pairs of KIR-HLA class I activating (**A**) and inhibitory (**B**) ligands in patients with and without ocular toxoplasmosis and its manifestation as primary or recurrent. [a]OR = 2.29; CI = 1.19–4.39; $P = 0.01$; $Pc = 0.04$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis); [b]OR = 2.29; CI = 1.16–4.52; $P = 0.01$; $Pc = 0.04$ (Patients without ocular toxoplasmosis vs. Patients with primary manifestation).

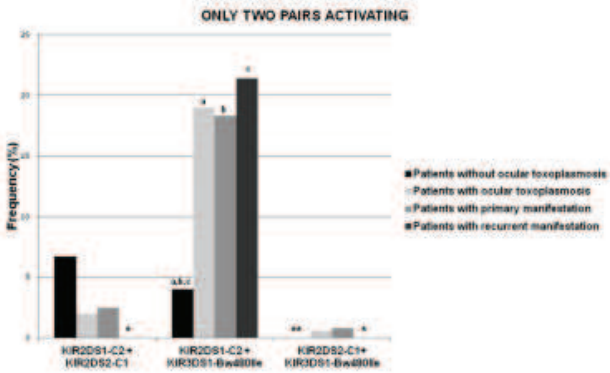

Figure 2. Distribution of the frequencies of the possible combinations of only two KIR activating genes in the presence of their HLA Class I ligands in patients with and without ocular toxoplasmosis and its manifestations as primary or recurrent. * and ** represents respectively that patients with recurrent manifestations and patients without ocular toxoplasmosis presented results of 0% for two activating pairs. [a]OR = 5.56; CI = 2.22–13.88; $P = 0.0001$; $Pc = 0.0004$ (Patients without ocular toxoplasmosis vs. Patients with ocular toxoplasmosis); [b]OR = 5.35; CI = 2.09–13.68; $P = 0.0002$; $Pc = 0.0008$ (Patients without ocular toxoplasmosis vs. Patients with primary manifestations); [c]OR = 6.50; CI = 1.92–21.96; $P = 0.004$; $Pc = 0.01$ (Patients without ocular toxoplasmosis vs. Patients with recurrent manifestations).

Distribution of activating KIR plus inhibitory KIR and their respective ligands in patients with and without ocular toxoplasmosis. The correlation between the distribution of activating and inhibitory KIR and their respective HLA ligands was analyzed (Table 5). A decreased risk of developing ocular toxoplasmosis (OR = 0.52; CI = 0.32–0.84; $P = 0.009$; $Pc = 0.02$) and recurrent manifestations of the disease (OR = 0.13; CI = 0.03–0.45; $P = 0.0003$; $Pc = 0.0009$) was observed for the KIR3DS1$^-$/KIR3DL1$^+$/Bw4–80Ile$^+$ combination (KIR2DL1 and the Bw4-80Ile ligand in the absence of KIR3DS1) when compared to patients without ocular toxoplasmosis. This correlation was also observed when patients with recurrent manifestations were compared to patients with primary manifestations (OR = 0.20; CI = 0.05–0.70; $P = 0.006$; $Pc = 0.01$).

Discussion

Possible causes of ocular manifestations of toxoplasmosis have not been fully elucidated, but it is believed that factors related both to the parasite and the host contribute to the development of this disease[4,28]. Therefore, the study of the impact of genes on infections, such as toxoplasmosis, is extremely important because it provides information about the contribution of the host's genetic factors on the development of this type of disease. We have previously shown that MICA polymorphisms are not associated with the development of ocular toxoplasmosis[29]. Using the same samples, the current study found that KIR receptors genes and their HLA ligands are associated

KIR - HLA ligands	Patients without toxoplasmic retinochoroiditis (n = 149) N (%)	Patients with toxoplasmic retinochoroiditis (n = 148) N (%)	Patients with primary manifestation (n = 120) N (%)	Patients with recurrent manifestation (n = 28) N (%)
KIR-C1				
2DS2+/2DL2−/C1+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2DS2−/2DL2+/C1+	33 (22.1)	41 (27.7)	30 (25.0)	11 (39.3)
2DS2+/2DL3−/C1+	9 (6.0)	12 (8.1)	10 (8.3)	2 (7.1)
2DS2−/2DL3+/C1+	66 (44.3)	67 (45.3)	54 (54.0)	13 (46.4)
2DS2+/2DL2−/2DL3−/C1+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2DS2−/2DL2−/2DL3+/C1+	33 (22.1)	26 (17.6)	24 (20.0)	2 (7.1)
2DS2+/2DL2−/2DL3+/C1+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2DS2−/2DL2+/2DL3+/C1+	33 (22.1)	41 (27.1)	30 (25.0)	11 (39.3)
2DS2+/2DL2+/2DL3-/C1+	9 (6.0)	12 (8.1)	10 (8.3)	2 (7.1)
2DS2+/2DL2+/2DL3+/C1+	13 (8.7)	4 (2.7)	4 (3.3)	0 (0.0)
KIR-C2				
2DS1+/2DL1−/C2+	0 (0.0)	2 (1.4)	2 (1.7)	0 (0.0)
2DS1−/2DL1+/C2+	74 (49.7)	67 (45.3)	56 (46.7)	11 (39.3)
2DS1+/2DL1+/C2+	49 (32.9)	54 (36.5)	43 (35.8)	11 (39.3)
KIR-BW4-80Ile				
3DS1+/3DL1+/BW4−80Ile+	32 (21.5)	53 (35.8)	42 (35.0)	11 (39.3)
3DS1+/3DL1−/BW4−80Ile+	1 (0.7)	3 (2.0)	3 (2.5)	0 (0.0)
3DS1−/3DL1+/BW4−80Ile+	71 (47.7)[a,b]	48 (32.4)[a]	45 (37.5)[c]	3 (10.7)[b,c]

Table 5. Distribution of activating KIR plus inhibitory KIR and their respective ligands in patients with and without ocular toxoplasmosis and its primary or recurrent manifestations. [a]OR = 0.52; CI = 0.32–0.84; $P = 0.009$; $Pc = 0.02$ (Patients without ocular toxoplasmosis vs. Patientes with ocular toxoplasmosis). [b]OR = 0.13; CI = 0.03–0.45; $P = 0.0003$; $Pc = 0.0009$ (Patients without ocular toxoplasmosis vs. Patientes with recurrent manifestation). [c]OR = 0.20; CI = 0.05–0.70; $P = 0.006$; $Pc = 0.01$ (Patients with primary manifestation vs. Patients with recurrent manifestation).

with the development of ocular lesions resulting from *T. gondii* infection. To the best of our knowledge, this is the first study of *KIR* genes and HLA ligands in the immunopathology of ocular toxoplasmosis.

The difference in the mean ages of the patient groups of this study was carefully discussed previously[29]. Briefly, *T. gondii* infection can occur at any time of life, and although most cases of ocular toxoplasmosis occur due to infections acquired after birth, a significant number of patients can acquire the disease congenitally and the resulting scars trend to be persistent[1]. It has also been demonstrated that the risk of recurrence is higher in the year following the first infection than in future years[30,31]. As no distinction was made between congenital and acquired disease in the analysis of the characteristics of eye injuries, this may be one of the possible explanations for the lower mean age observed for patients who developed ocular toxoplasmosis, including those who present with recurrent signs of the disease. Furthermore, other eye diseases, those without scars/lesions due to toxoplasmosis, are prevalent in older patients[32].

Regarding the distribution of *KIR* genes, after Bonferroni correction only the *KIR3DS1* activating gene was associated with increased risk of developing ocular toxoplasmosis with the other associations being lost. The Bonferroni correction decreases the chance of a significant difference by chance alone, making the data more robust[33]. The association observed for the *KIR3DS1* with ocular toxoplasmosis can be explained by the absence of *KIR3DL1*, because *3DS1* and *3DL1* segregate as alleles of a single locus. Thus, the presence of *KIR3DS1*, and consequently the absence of *KIR3DL1*, can create an increased potential for the activation of NK cells owing to decreases in the ratio of inhibitory/activating receptors. Previous studies have shown involvement of the *KIR3DS1/L1* alleles in various types of non-toxoplasmic uveitis and inflammatory eye diseases triggered by autoimmune factors. Levinson *et al.*[15] observed that individuals with the *KIR3DS1* gene in their haplotype have an increased risk of developing Vogt-Koyanagi-Harada syndrome, while the presence of *KIR3DL1* was associated to protection against the development of the disease. A similar result was observed by Moon *et al.*[14] for the development of uveitis related to ankylosing spondylitis: *KIR3DS1* was associated as a risk factor and *KIR3DL1* was associated as a protective factor.

In this study, *KIR3DL1/S1* for the patient group that developed the ocular toxoplasmosis were not in Hardy-Weinberg equilibrium. However, some authors claim that this equilibrium should only be investigated in the control group, because it represents the general population[34,35]. Thus, the high frequency of *KIR3DS1* might be changing the distribution of these alleles in individuals with ocular toxoplasmosis, resulting in a deviation from the Hardy-Weinberg equilibrium. Considering that numerous precautions were adopted to prevent bias in this study, we can safely say that the *3DS1* gene exerts a real influence on the development of ocular toxoplasmosis, even though *KIR3DL1/S1* were not in Hardy-Weinberg equilibrium.

The function of *KIR* genes in immune response is highly dependent on the HLA molecules expressed on the target cell surface. Therefore, KIR receptors influence susceptibility for or protection against certain illnesses by

means of a balance in activation and inhibition signals that regulate the NK cell effector function[9,10]. The recognition of specific HLA ligands by inhibiting KIR is well established[36]. There is clear evidence that HLA-Bw4-80Ile is powerfully recognized by KIR3DL1, but there is still controversy as to whether its homologue, KIR3DS1, interacts with the same ligand, as only indirect evidence was found[37,38]. There is also a hierarchy of inhibition related to KIR2DL receptors in which KIR2DL3-C1 has lower inhibitory potential than KIR2DL2-C1 and KIR2DL1-C2[39].

In this study, where the *KIR* genes were analyzed in the presence of their respective ligands (KIR-HLA), the KIR3DS1-Bw4-80Ile pair was associated with the development of ocular toxoplasmosis irrespective of the type of clinical manifestation (primary or recurrent); while KIR2DL3/2DL3-C1/C1 and KIR2DL3/2DL3-C1 were associated with protection against the development of ocular toxoplasmosis and its clinical manifestations. In accord with our results, other studies found an association involving the KIR3DS1-Bw4-80Ile pair in other human diseases[40–43], suggested an interaction between these molecules. *KIR2DL3* and *KIR2DL2* are considered alleles, as are *KIR3DL1* and *KIR3DS1*. Although the KIR2DL2-C1 interaction is stronger than the KIR2DL3-C1 interaction, the inhibitory signal generated by the absence of KIR2DL2 (KIR2DL3/2DL3-C1/C1 and KIR2DL3/2DL3-C1) appears to be sufficient to inhibit the effector function of NK cells and protect against elevated inflammation and tissue damage. It has been shown that abnormal expressions of inhibitory receptors of NK cells, including the KIR2DL3 receptor, may be associated with the development of Behcet's disease[44].

It is important to highlight the results observed for the number of pairs of KIR-HLA ligands and the correlation between the distribution of activating KIR and their respective HLA ligands. A higher frequency of only two pairs of ligands was observed in patients with ocular toxoplasmosis and in patients with primary manifestations compared to patients without ocular toxoplasmosis. The combination responsible for this association was KIR2DS1^{+}/C2^{++} KIR3DS1^{+}/Bw4-80Ile^{+}, although the *KIR2DS1* and *KIR3DS1* genes are not in linkage disequilibrium as observed among patients with ocular toxoplasmosis ($\Delta' = 0.06$; $P = 0.68$) and patients without ocular toxoplasmosis ($\Delta' = 0.15$; $P = 0.45$). The combination of one pair (inhibitory) in the absence of the other pair (activating) was analyzed. It was possible to observe that the KIR3DS1^{-}/KIR3DL1^{+}/Bw4-80Ile^{+} combination decreases the risk of developing ocular toxoplasmosis and its recurrent clinical forms.

These results may suggest that the activating function mediated by KIR2DS1 plus KIR3DS1 and their respective HLA ligands is, in fact, an important factor interfering in ocular toxoplasmosis, since such a combination may affect the balance of inhibiting/activating signals. NK cells are activated when there is an increase of activation signals, even if there is a combination of strong or weak inhibitory signals[45]. Yet, the absence of KIR3DS1 in the presence of its inhibitor homologue, KIR3DL1, was indicative of the inhibitory or protective role played by NK cells, which perhaps avoids subsequent immune responses that may trigger inflammation and autoimmunity. In support of our findings, Levinson *et al.*[46] demonstrated both negative and positive associations mediated by KIR-HLA pairs in another ocular inflammatory disease, birdshot chorioretinopathy.

NK, CD4 and CD8 T cells, and type 1 cytokines, such as IFN-γ and IL-2, play a protective role in *T. gondii* infection. IL-12 stimulates NK cells to produce IFN-γ and to promote the development of Th-1 cells that produce IFN-γ, a cytokine involved in the activation of macrophages. the main phagocytes in chronic inflammation[47,48]. In the eye, although the immune response is usually suppressed to prevent tissue damage, there is experimental evidence that *T. gondii* infection promotes the production of factors such as IFN-γ that suppress the immune privilege of this organ[49]. This possibly leads to increased severity of lesions with marked necrosis or inflammation of the retina and the choroid[50,51].

The immune response can determine the development of eye injuries resulting from *T. gondii* infection, and the mechanisms involved may be associated with both the pathogenesis and protective effects that control tissue damage. It has been shown that increases in the frequency of circulating NK cells and proinflammatory monocytes in children infected by *T. gondii*, particularly in those with active ocular lesions, are indicative of a strong and persistent proinflammatory response. Moreover, subsets of NK cells and CD8^{+} T cells act as biomarkers for cicatricial lesions of the eye[8]. It has also been demonstrated that NK cells show increased production of IFN-γ in patients with congenital ocular toxoplasmosis[6]. Furthermore, the high imunopathogenicity responsible for tissue damage and deterioration of ocular toxoplasmosis may be due to the presence of a potent inducer of inflammation, IL-17[5,6], which is also produced by NK cells[7]. On the other hand, ocular toxoplasmosis may be linked to autoimmunity[1,4].

The current study investigated the KIR-HLA ligand as a risk factor in ocular toxoplasmosis and these results may improve to understanding of the immunopathogenic mechanism involving NK cells in ocular manifestations related to toxoplasmosis. However, others studies should be performed such as histological analyses of the ocular tissue affected by *T. gondii* and NK cytotoxicity assays to better understanding the role of NK cells and the expression of KIR in the immunopathogenesis of ocular toxoplasmosis.

In conclusion, the results of this study show that activating and inhibitory KIR in the presence of their respective HLA ligands may have influence on the development of ocular toxoplasmosis and its clinical form in this population. In particular this is seen with the strong presence of activating signals as risk factors (*KIR3DS1*, KIR3DS1-Bw4-80Ile and KIR3DS1^{+}/Bw4-80Ile^{++} KIR2DS1^{+}/C2^{+}) and inhibitory signals as protective factors (KIR2DL3/2DL3-C1, KIR2DL3/2DL3-C1/C1 and KIR3DS1^{-}/KIR3DL1^{+}/Bw4-80Ile^{+}).

Methods

The design of this study aimed to follow, as close as possible, the criteria recommended by STrengthening the REporting of Genetic Association Studies (STREGA)[52].

Ethics Information. This study was approved by the Research Ethics Committee of the Medicine School in São José do Rio Preto (#1980/2009) and all individuals who agreed to participate signed informed consent forms. The experiments were carried out in accordance with the approved relevant guidelines and regulations.

Patient selection. A total of 297 unrelated patients from the Retinopathy Outpatient Service of Hospital de Base of the Medicine School in São José do Rio Preto (HB-FUNFARME) and Medical Outpatient Clinic (AME) in São José do Rio Preto participated in this study.

The study subjects have been described previously[29]. Patients were grouped according to the presence of ocular scars/lesions due to toxoplasmosis (n = 148; 79 men and 69 women; mean age: 42.3 ± 20.6 years) or to the presence of ocular diseases not related to toxoplasmosis (n = 149; 73 men and 76 women; mean age: 57.7 ± 16.9 years). The group of patients with scars/lesions due to toxoplasmosis was further subdivided into two groups according to the type of ocular manifestation observed during a follow up period of at least two years: primary manifestations (n = 120; 65 men and 55 women; mean age: 44.9 ± 20.9 years) and recurrent manifestations characterized by the presence of satellite lesions[55] (n = 28; 14 men and 14 women; mean age: 31.8 ± 30.5 years) (Table 1).

All individuals who participated in this study were monitored and evaluated in respect to clinical symptoms, serology for *T. gondii* and epidemiological data. Besides, although patients self-reported themselves as European descent, mixed African and European descent, and African descent, due to high miscegenation of the Brazilian population they were defined as a population of mixed ethnicity[54].

In this study, in order to avoid bias in the results, all patients were selected after clinical examination using the same criteria. Additionally, the probability of variations in the allele frequencies due to ethnic background was minimized by matching patients with ocular toxoplasmosis and patients without ocular toxoplasmosis from similar ethnic backgrounds. Furthermore, gender and residence in the same geographical areas were carefully matched during group selection.

The number of patients enrolled is sufficient to demonstrate whether there is an association between ocular toxoplasmosis and *KIR* genes with statistical power of more than 90% and it was chosen according to frequency of *KIR* genes recorded in Allele*Frequencies database (http://www.allelefrequencies.net) observed in a population located in the southeast region of Brazil and defined as a population of mixed ethnicity.

Inclusion/exclusion criteria. The inclusion criteria of patients with ocular toxoplasmosis were positive laboratory diagnosis of toxoplasmosis, the presence of ocular scars/lesions due to toxoplasmosis and live in municipalities in the northwest region of the State of Sao Paulo (located in the southeast region of Brazil, between 20°49′13″S and 49°22′47″W). The inclusion criteria of patients without ocular toxoplasmosis were positive laboratory diagnosis of toxoplasmosis but without ocular scars/lesions due to toxoplasmosis and living in the same geographical region as the patients with ocular toxoplasmosis. All patients were clinically evaluated by two experienced physicians.

The exclusion criteria were: patients with other infectious and parasitic diseases, patients with any type of mental disability, patients with blood dyscrasia and using oral anticoagulants and related patients.

Laboratory diagnosis. Blood samples were collected into tubes without anticoagulant to obtain serum. Anti-*T. gondii* antibodies were detected by immunosorbent assay (ELISA) according to the manufacturer's instructions (ETI-TOXOK-M reverse PLUS; DiaSorin S.p.A. Italy and ETI-TOXO-G PLUS; DiaSorin S.p.A. Italy). The microplates were read using Epoch™ equipment using the Gen5™ 2.0 software (BioTek, Winooski, Vermont, USA). The samples were tested in duplicate and in cases of indeterminate results, the samples were retested in duplicate. Positive and negative controls were included in all reactions.

Clinical diagnosis. All patients were clinically assessed using an indirect binocular ophthalmoscope (Binocular Ophthalmoscope ID10, Topcon Corporation, USA). The evaluation of visual acuity followed the logMAR Early Treatment Diabetic Retinopathy Study chart (ETDRS) criteria[55]. Intraocular pressure was measured by Goldmann applanation tonometry, and stereoscopic biomicroscopy was performed using a 78-diopter lens (Volk) and a slit lamp and all they were classified according to the ETDRS criteria[55].

As no invasive test was performed, the ocular toxoplasmosis diagnostic criteria used were the same as in the clinical practice: injury identified by ophthalmoscopy associated with positive serology for *T. gondii*. This is therefore a presumptive diagnosis.

KIR and HLA genotyping. Blood samples were also collected into tubes containing EDTA anticoagulant for DNA extraction. DNA of all patients was extracted using the commercial kit for silica column extraction (QIAamp® DNA Blood Mini Kit, QIAGEN, the Netherlands) following the manufacturer's instructions. All DNA samples were subjected to an evaluation of concentration and purity using the ratio of the absorbance at optical densities (OD) of 260 and 280 nm with Epoch™ equipment (BioTek, Winooski, Vermont, USA). KIR and HLA-A, -B and -C were genotyped according to manufacturer's instructions by Polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP) protocols with Luminex® technology (One Lambda Inc., Canoga Park, CA, USA). This technique uses PCR-amplified DNA with specific biotinylated primers. The amplified product is hybridized by complementary DNA probes conjugated to fluorescently coded microspheres, with detection using R-Phycoerythrin-conjugated Streptavidin (SAPE). The data were interpreted using a computer program (HLA Fusion, 2.0 Research, One Lambda).

KIR2DL1 and KIR2DS1 bind to HLA molecules from the C2 group, which include the HLA-C*02, *04, *05, *06, *07,*15, *17, and *18 specificities. KIR2DL2, KIR2DL3 and KIR2DS2 interact with HLA molecules from the C1 group, among them: HLA-C*01, *03, *07, *08, *12, *13, *14 and *16. KIR3DL2 binds to HLA-A*03 or -A*11 specificities and KIR3DL1 recognizes HLA-Bw4 epitopes (HLA-A*23, *24, *25, *32; HLA-B*13, *27, *44, *51, *52, *53, *57, *58). HLA-Bw4 molecules were divided into two groups based on whether isoleucine or threonine was present at position 80 (Bw4-80Ile and Bw4-80Thr). KIR3DS1 binds to Bw4-80Ile molecules. HLA-KIR ligand specificities were considered according to Carr *et al.*[37], Thananchai *et al.*[56] and Kulkarni *et al.*[57].

Two types of KIR genotypes have been described based on the content of the genes: AA and BX (BB and AB) (defined according to http://www.allelefrequencies.net). Individual genotypes were determined to be AA when the genes *KIR2DL1, KIR2DL3, KIR2DL4, KIR2DS4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1* and *KIR3DP1* were present. The presence of one or more of the following genes: *KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5* and *KIR3DS1* characterized the BX genotype.

Statistical analysis. KIR, HLA and KIR-HLA frequencies were obtained by direct counting. The comparisons of the frequencies of HLA ligands, KIR genes, KIR AA and BX genotypes and KIR with or without ligands between groups of patients were performed with the Chi-square test with Yates' correction or, when necessary, Fisher's exact test using the program Graph Pad Instat (http://www.graphpad.com/quickcalcs/contingency1.cfm). Odds ratio (OR) with a 95% confidence interval (95% CI) was also calculated to evaluate the risk association. The mean ages were compared using the t-test. Differences with P-values < 0.05 corrected by the Bonferroni inequality method for multiple comparisons (Pc) were considered statistically significant. A Hardy-Weinberg equilibrium fit was performed by calculating expected genotype frequencies and comparing that with the observed values for KIR2DL2/3, KIR3DL1/S1, and the HLA alleles using ARLEQUIN software, version 3.1. (http://cmpg.unibe.ch/software/arlequin3).

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Acknowledgements

The authors are grateful to all of the volunteers who participated in this study and to Regional Blood Bank of Sao Jose do Rio Preto for their assistance (Denise, Mirela, Otávia and Dr. Octávio). Thanks to David Hewitt for his help with the English version and to Professor Stephen Henry from Auckland University of the Technology for providing library access. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant numbers: 2013/06580-9, 2013/10050-5, 2013/25650-8, 2009/17540-2, 2013/15879-8] and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number: 473579/2009-0]. The opinions, assumptions, and conclusions or recommendations expressed in this material are the responsibility of the authors and do not necessarily reflect the views of FAPESP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: C.M.A., L.C.M. and C.C.B.M. Head of the FAMERP Toxoplasma Research Group: C.C.B.M. Performed the experiments: C.M.A., F.H.M. and A.P.S.C. Performed the inclusion of patients, sample collection, and developed the clinical evaluation and clinical analyses: F.B.F., A.P.B. and R.C.S., M.P. Analysed the data: C.M.A. and L.C.M. Wrote the paper: C.M.A. and L.C.M.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ayo, C. M. *et al.* Ocular toxoplasmosis: susceptibility in respect to the genes encoding the KIR receptors and their HLA class I ligands. *Sci. Rep.* **6**, 36632; doi: 10.1038/srep36632 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Artigo 3

BRIEF REPORT

Association of the Functional MICA-129 Polymorphism With the Severity of Chronic Chagas Heart Disease

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MICA-129 polymorphism affects the binding affinity of MICA molecules with the NKG2D receptor and influences effector cell function. The genotype met/met was associated with the severity of left ventricular systolic dysfunction (LVSD) in patients with chronic Chagas heart disease, while the val/val genotype was associated with the absence of LVSD.

Keywords. MICA polymorphism; Chagas disease; chronic Chagas heart disease; left ventricular systolic dysfunction.

Chronic Chagas heart disease (CCHD) is one of the severe clinical manifestations of Chagas disease. The condition manifests as heart failure, heart rhythm and electrical conduction disorders, thromboembolic events, precordial chest pain, and sudden death [1]. Patients with CCHD usually have a mononuclear cell infiltrate and interstitial and confluent myocardial fibrosis throughout the myocardium, accompanied by microvascular lesions [2], thus provoking left ventricular remodeling. However, the precise pathogenic mechanism of Chagas heart disease is not completely elucidated [3].

Received 18 April 2015; accepted 25 June 2015; electronically published 30 June 2015.

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Clinical Infectious Diseases® 2015;61(8):1310–3

DOI: 10.1093/cid/civ540

The MICA (major histocompatibility complex class I–related chain A gene) molecules are recognized by Tγδ and Tαβ lymphocytes, $CD8^+$ cells, and natural killer (NK) cells by the NKG2D receptors on their surfaces [4]. But, changing a single amino acid, a methionine (met) for a valine (val) at position 454 of exon 3, which corresponds to the amino acid 129 of the protein, changes MICA alleles from strong (MICA-129 met) to weak (MICA-129 val) binders of the NKG2D receptor; this very likely affects the activation of NK cells and the modulation of T cells [5].

The relationship between the MICA-129 polymorphism (rs1051792) and severity of CCHD remains unknown. Accordingly, we investigated the relationship between the MICA-129 met > val polymorphism (A > G) in exon 3 of the *MICA* gene and the severity of left ventricular systolic dysfunction (LVSD) in patients with CCHD.

METHODS

The Research Ethics Committee of the Medicine School in Sao Jose do Rio Preto (FAMERP - # 009/2011) approved this study. An informed consent form was signed by all participants.

A total of 189 consecutive unrelated male and female patients with CCHD, treated in the Cardiomyopathy Clinic of Hospital de Base of Fundação Faculdade de Medicina de São José do Rio Preto participated in this study. All patients underwent 2-dimensional echocardiogram. The severity of LVSD was graded according to left ventricular ejection fraction (LVEF) values measured with the Teichholz method according to the Brazilian guidelines of severe chronic heart disease [6]. Patients were classified into the following 3 groups according to LVEF: LVEF > 60% (patients without LVSD), LVEF between 60% and 40% (patients with mild to moderate LVSD), and LVEF < 40% (patients with severe LVSD) (Supplementary Table 1).

The laboratory diagnosis of Chagas disease was made by enzyme-linked immunosorbent assay (ELISA) of serum or plasma using the ELISAcruzi immunoassay (bioMerieux SA Brazil) and following the manufacturer's instructions.

Genomic DNA was extracted from peripheral blood using a silica-membrane column (PureLink, Genomic DNA Mini Kit, Invitrogen, Carlsbad, California) following the manufacturer's instructions. Verification of the MICA-129 polymorphism (A > G, rs1051792) in exon 3 was performed using nested polymerase chain reaction. The val-129 MICA allele was identified by the presence of a restriction site for the RsaI enzyme (FastDigest,

Table 1. MICA-129 Polymorphism in Patients With Chronic Chagas Heart Disease From Southeastern Brazil Using Recessive, Dominant, Additive, and Codominant Inheritance Models

MICA-129 Polymorphism	Severe LVSD n = 48 n (%)	Mild/Moderate LVSD n = 48 n (%)	Without LVSD (Normal) n = 93 n (%)	χ^2	*P* Value
Recessive inheritance model					
met/met	12 (25.0)	5 (10.4)	7 (7.5)	7.19	.007
met/val + val/val	36 (75.0)	43 (89.6)	86 (92.5)		
Dominant inheritance model					
met/met + met/val	36 (75.0)	28 (58.3)	48 (51.6)	9.02	.01
val/val	12 (25.0)	20 (41.7)	45 (48.4)		
Additive inheritance model					
met/met	12 (25.0)	5 (10.4)	7 (7.5)	12.36	.002
val/val	12 (25.0)	20 (41.7)	45 (48.4)		
Codominant inheritance model					
met/val	24 (50.0)	23 (48.0)	41 (44.1)	0.49	.78
met/met + val/val	24 (50.0)	25 (52.0)	52 (44.1)		
Genotype comparisons					
Recessive inheritance model					
Severe LVSD vs without LVSD (normal)				8.29	.004
Severe LVSD vs mild/moderate LVSD				3.50	.06
Mild/moderate LVSD vs without LVSD (normal)				0.33	.56
Dominant inheritance model					
Severe LVSD vs without LVSD (normal)				7.19	.007
Severe LVSD vs mild/moderate LVSD				3.00	.08
Mild/moderate LVSD vs without LVSD (normal)				0.57	.44
Additive inheritance model					
Severe LVSD vs without LVSD (normal)				9.82	.001
Severe LVSD vs mild/moderate LVSD				3.63	.06
Mild/moderate LVSD vs without LVSD (normal)				0.16	.68
Codominant inheritance model					
Severe LVSD vs without LVSD (normal)				0.23	.62
Severe LVSD vs mild/moderate LVSD				0.04	.83
Mild/moderate LVSD vs without LVSD (normal)				0.06	.79

Abbreviation: LVSD, left ventricular systolic dysfunction.

Thermo Scientific) created by a mismatch deliberately introduced into the nonsense primer [7] (Supplementary Table 2).

Groups were compared using the χ^2 test with Yates correction or Fisher exact test using the statistics program GraphPad Instat version 6.3 (http://www.graphpad.com/scientific-software/instat/). The genotype frequencies were also evaluated using the dominant (met/met + met/val vs val/val), recessive (met/met vs met/val + val/val), additive (met/met vs val/val), and codominant (met/val vs met/met + val/val) inheritance models. The odds ratio (OR) and the 95% confidence interval (CI) were calculated to determine the risk of developing LVSD. The Hardy–Weinberg equilibrium was verified using the ARLEQUIN program version 3.11 (http://cmpg.unibe.ch/software/arlequin3/). Differences in *P* values ≤.05 were considered statistically significant.

RESULTS

In this study population, the distributions of alleles associated with the MICA-129 polymorphism were in Hardy–Weinberg equilibrium ($P > .05$).

The data on genotype and allele frequencies are shown in Supplementary Table 3. The genotype met/met ($P = .007$; OR = 4.09; 95% CI, 1.49–11.24) and met allele ($P = .001$; OR = 2.38; 95% CI, 1.43–3.96) ($P = .04$; OR = 1.90; 95% CI, 1.06–3.41) were significantly associated with increased risk of

developing severe LVSD, while the MICA-129 val/val genotype ($P = .01$; OR = 0.35; 95% CI, .16–.76) and val allele ($P = .001$; OR = 0.41; 95% CI, .25–.69) ($P = .04$; OR = 0.52; 95% CI, .29–.93) were associated with a lower risk of developing severe LVSD.

The recessive and dominant inheritance models showed significant association between patients with severe LVSD and patients without LVSD ($P = .007$; $\chi^2 = 7.19$ and $P = .004$; $\chi^2 = 8.20$, respectively). The additive model also produced a significant association ($P = .001$; $\chi^2 = 9.82$), while the codominant model was nonsignificant ($P = .78$; $\chi^2 = 0.49$) as expected. Thus, the met > val polymorphism contributes to CCHD susceptibility under an additive model; individuals who are homozygous for the met allele have the highest risk of developing severe LVSD; those homozygous for the val allele seem to modify CCHD severity (Table 1).

DISCUSSION

To our knowledge, we are the first to address the importance of the functional MICA-129 polymorphism in the severity of LVEF in CCHD. LVEF is the powerful independent predictor of mortality for patients with chronic Chagas disease, in general [8], and one of the most important predictors of all-cause mortality in Chagas disease patients with chronic systolic heart failure in particular [9]. Therefore, the identification of *MICA* as a candidate gene of susceptibility in patients with these conditions has important implications for a better understanding of the immunopathogenesis of this disease.

Differences in the sequences of MICA alleles may influence the interaction of the MICA molecules with the NKG2D receptor. A study of the polymorphism at codon 129 showed that the alleles encoding methionine in this position have a considerably greater capacity to bind to the NKG2D receptor than alleles coding for valine in the same position [5]. Moreover, the interaction between NKG2D and the MICA molecules may potentially increase antitumor immune responses and participate in inflammatory processes with increases in the production of cytokines by NK cells. This interaction may also promote costimulatory signaling to specific $CD8^+$ T-cell self-antigens in autoimmunity [4].

Autoimmunity is accepted as one of the main pathogenic mechanisms responsible for CCHD [10]. Therefore, strong affinity formed by the NKG2D-MICA-129 met/met complex may be related to the tissue damage observed in CCHD due to a high activation of NK cells and $CD8^+$ T cells. The greater damage caused to the cardiac tissue can therefore develop into severe organ dysfunction. On the other hand, the homozygous presence of valine amino acid may generate a less pronounced immune response in cells, so that patients with this polymorphism are protected against the development of severe CCHD due to less affinity to the NKG2D immunoreceptor with consequent less tissue injury. Similar to the results found in this study, the MICA-129 polymorphism has been reported as a risk factor for the development of other autoimmune diseases [7, 11, 12].

Inflammatory infiltration in the chronic phase of Chagas disease presents with signs of cellular activity, with the main cells found being $CD4^+$ and $CD8^+$ T cells. However, NK cells, macrophages, and B lymphocytes are also present [13]. The function of NK cells and $CD8^+$ T cells in the elimination of transformed or stressed infected cells occurs together with self-tolerance, a property that is essential to prevent autoimmunity. Inappropriate expression of ligands for NK cell receptors leads to the activation of autoreactive effector cells and therefore can cause or exacerbate the autoimmunity [4]. It has been demonstrated that baseline levels of NK, NKT, and $CD4^+$ $CD25^{HIGH}$ cells with failed immunoregulation mechanisms associated with an increased expression of activated $CD8^+$ T cells are associated with cardiac events [14, 15].

The data obtained in this study indicated a possible susceptibility related to the homozygous genotype met/met with the severity of LVSD in the setting of CCHD, whereas the homozygous val/val seem to modify CCHD severity. Several studies have shown that uncontrolled activation of autoreactive effector cells may lead to tissue damage that, in turn, leads to the development of the clinical manifestations of severe CCHD [14, 15]. However, to better understand the role of cells expressing the NKG2D receptor as well to clarify the expression of MICA molecules in the immunopathogenesis of Chagas disease, others studies such as histopathological analysis in CCHD and cytotoxicity assays should be done.

CONCLUSION

Our data suggest that the homozygous met/met genotype may be related to a risk factor in the severity of LVSD observed in the setting of CCHD, while the homozygous val/val genotype was associated with protection against this condition in this study population.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. The authors are grateful to all of the volunteers who participated in this study. Thanks to David Hewitt for his help with the English version.

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and do not necessarily reflect the views of the São Paulo Research Foundation (FAPESP).

Financial support. C. M. A. was supported by a grant from FAPESP (2013/06580-9); A. P. d. O. and A. V. d. S. C. were supported by a grant from the Brazilian Ministry of Education—Coordination of Improvement of Higher Education Personnel; L. C. d. M. was supported by grants from FAPESP (2013/25650-8, 2011/08075-4) and Faculdade de Medicina de São José do Rio Preto.

Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Supplementary Table 1

General and clinicopathological characteristics of patients with chronic Chagas heart disease from southeastern Brazil.

Characteristic	Total[a]	Severe LVSD	Mild/moderate LVSD	Without LVSD (normal)	P-value
	n=189	n=48	n=48	n=93	
Age (Mean ± SD)	63.8±10.6	63.4±11.4	63.7±11.3	64.2±10.1	NS
Medin	65	63	66	65	
Gender (%)					
Female	102 (53,4%)	20 (41.7%)	27 (56.3%)	54 (58.1%)	NS
Male	89 (46.6%)	28 (58.3%)	21 (43.8%)	39 (41.9%)	

LVSD: left ventricular systolic dysfunction; SD: Standard deviation. [a]Patients classified as a population of mixed ethnical background

Supplementary Table 2

Methodology: Sequence of primers and their position, size of the amplified products, and enzyme used for genotyping the 129 polymorphism in exon 3 of the gene *MICA*.

PCR	Primers	Site (position nt)[a]	Size of fragment	Restriction enzyme	Size of fragment after digestion by enzyme
PCR-specific MICA[d]					
MICA-FG	5'-CGTTCTTGTCCCTTTGCCCGTGTGC-3'	Intron 1: (6823–6847)	2201 bp		
MICA-RG	5'-GATGCTGCCCCCATTCCCTTCCCAAA-3'	Intron 5: (8999–9023)			
Nested PCR MICA[e]					
MICA-FM	5'-GGGTCTGTGAGATCCATGA-3'	Exon 3: (350–368)	127 bp	RsaI	(A) 127 bp
MICA-RM[b]	5'-TGAGCTCTGGAGGACTGGGGTA-3'	Exon 3: (455–476)			(G)[c] 104 +23 bp

[a]Based on GenBank accession number X92841; nt: nucleotide; [b]The exon 3 *nonsense* primer has a deliberately introduced mismatch to create an RsaI restriction site; bp: base pairs; [c] Single nucleotide polymorphism (SNP)- 129 A>G (rs1051792). [d]cycles: 95°C, 5'; 40 cycles (95°C, 45''; 65°C, 45''; 72°C, 45'') and 72°C , 5'. [e]cycles: 95°C, 5'; 25 cycles (95°C, 45''; 61°C, 45''; 72°C, 45'') and 72°C , 5'.

Supplementary Table 3

Frequency of MICA-129 genotypes and alleles in patients with chronic Chagas heart disease from southeastern Brazil

MICA-129	Total n = 189	Severe LVSD n = 48	Mild/moderate LVSD n = 48	Without LVSD (normal) n = 93	Severe LVSD vs. without LVSD (Normal)		Severe LVSD vs. mild/moderate LVSD	
Genotypes	n (%)	n (%)	n (%)	n (%)	P-value	OR (IC 95%)	P-value	OR (95% CI)
met/met	24 (12.7)	12 (25.0)	5 (10.4)	7 (7.5)	0.007	4.09 (1.49-11.24)	NS	
met/val	87 (46.0)	24 (50.0)	23 (47.9)	41 (44.1)	NS		NS	
val/val	79 (41.3)	12 (25.0)	20 (41.7)	45 (48.4)	0.01	0.35 (0.16-0.76)	NS	
Alleles	n (%)	n (%)	n (%)	n (%)				
129met	135 (35.7)	48 (50.0)	33 (34.4)	55 (29.6)	0.001	2.38 (1.43-3.96)	0.04	1.90 (1.06-3.41)
129val	243 (64.3)	48 (50.0)	63 (65.6)	131 (70.4)	0.001	0.41 (0.25-0.69)	0.04	0.52 (0.29-0.93)

LVSD: left ventricular systolic dysfunction; NS: not significant; OR: Odds ratio; 95% CI: 95% confidence interval.

Artigo 4

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Cellular & Molecular Immunology

Manuscript #	CMI-2017-0200
Current Revision #	0
Submission Date	29th May 17
Current Stage	Manuscript Received
Title	MICA and KIR: Immunogenetic factors influencing cardiac and digestive clinical forms of chronic Chagas disease
Running Title	MICA and KIR in chronic Chagas disease
Manuscript Type	Research Article
Corresponding Author	Prof. Luiz de Mattos (Faculdade de Medicina de Sao Jose do Rio Preto)
Contributing Authors	Mrs. Christiane Ayo , Dr. Reinaldo Bestetti , Dr. Eumildo de Campos Junior , Dr. Luiz Ronchi , Dr. Aldenis Borim , Dr. Cinara Brandão de Mattos
Abstract	Tissue damage observed in the clinical forms of chronic symptomatic Chagas disease seems to have a close relationship with the intensity of the inflammatory process. The objective of this study was to investigate whether the MICA and KIR genes are associated with the cardiac and digestive clinical forms of chronic Chagas disease. Possible influence of these genes on the development of left ventricular systolic dysfunction (LVSD) in patients with chronic chagasic heart disease was also evaluated. This study enrolled 185 patients with positive serology for Trypanosoma cruzi classified according to the clinical form of the disease: cardiac (n=107) and digestive (n=78). Subsequently, patients with the cardiac form of the disease were sub-classified as with LVSD (n=52) and without LVSD (n=55). Genotyping was performed by polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSOP). Statistical analyzes were carried out using the Chi-square test and odds ratio with 95% confidence interval was also calculated to evaluate the risk association. MICA-129 alleles with high affinity for the NKG2D receptor showed a positive association with LVSD (OR=1.90; 95% CI:1.09-3.33; p-value=0.03), while MICA-129 alleles with low affinity for the NKG2D receptor had a negative association with LVSD (OR=0.52; 95% CI:0.30-0.92; p-value=0.03). It was also possible to demonstrate that the haplotype MICA*008~HLA-C*06 (OR = 13.82; 95% CI:1.71-111.63; p-value=0.004; Pc=0.04) and the KIR2DS2-/KIR2DL2-/KIR2DL3+/C1+ combination (OR: 5.83; 95% CI:1.20-4.25; p-value=0.01; Pc=0.04) were factors of susceptibility for the digestive form of the disease. Our data demonstrate that the MICA and KIR genes influence the development of clinical forms of Chagas disease.
Editor	
Keywords	MHC class I, KIR receptors, Chagas disease
Applicable Funding Source	Fundação de Amparo à Pesquisa do Estado de São Paulo [grant number: 2011/08075-4]. [de Mattos] Fundação de Amparo à Pesquisa do Estado de São Paulo [grant number: 2013/06580-9]. [Ayo]

MICA and _KIR_: Immunogenetic factors influencing cardiac and digestive clinical forms of chronic Chagas disease

MICA and _KIR_ in chronic Chagas disease

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Abstract

Tissue damage observed in the clinical forms of chronic symptomatic Chagas disease seems to have a close relationship with the intensity of the inflammatory process. The objective of this study was to investigate whether the *MICA* and *KIR* genes are associated with the cardiac and digestive clinical forms of chronic Chagas disease. Possible influence of these genes on the development of left ventricular systolic dysfunction (LVSD) in patients with chronic chagasic heart disease was also evaluated. This study enrolled 185 patients with positive serology for *Trypanosoma cruzi* classified according to the clinical form of the disease: cardiac (n = 107) and digestive (n = 78). Subsequently, patients with the cardiac form of the disease were sub-classified as with LVSD (n = 52) and without LVSD (n = 55). Genotyping was performed by polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSOP). Statistical analyzes were carried out using the Chi-square test and odds ratio with 95% confidence interval was also calculated to evaluate the risk association. MICA-129 alleles with high affinity for the NKG2D receptor showed a positive association with LVSD (OR = 1.90; 95% CI: 1.09-3.33; p-value = 0.03), while MICA-129 alleles with low affinity for the NKG2D receptor had a negative association with LVSD (OR = 0.52; 95% CI: 0.30-0.92; p-value = 0.03). It was also possible to demonstrate that the haplotype MICA*008~HLA-C*06 (OR = 13.82; 95% CI: 1.71-111.63; p-value = 0.004; Pc = 0.04) and the KIR2DS2^{-}/KIR2DL2^{-}/KIR2DL3^{+}/C1^{+} combination (OR: 2.40; 95% CI: 1.28-4.51; p-value = 0.009; Pc = 0.18) were factors of susceptibility for the digestive form of the disease. Our data demonstrate that the *MICA* and *KIR* genes influence the development of clinical forms of Chagas disease.

Keywords. MHC class I; KIR receptors; Chagas disease.

Introduction

Chagas disease, resulting from infection by the protozoan *Trypanosoma cruzi*, is a neglected disease that affects about 6 million people worldwide, especially in Latin America.[1,2] Chronically infected individuals may develop clinical manifestations of this disease with irreversible lesions of some organs. After 20 years of infection, about 30% develop chronic Chagas heart disease (CCHD), which is clinically manifested by malignant ventricular arrhythmia,[3] thromboembolism,[4] sudden cardiac death,[5] and chronic heart failure.[4] Ten per cent of Chagas patients present the digestive form of the disease, characterized mainly by dilatations of the esophagus and/or colon.[4,6]

In CCHD, an important feature is the interplay of autonomic dysfunction, microvascular abnormalities, and autoimmunity,[7] which leads to chronic inflammation and reparative fibrosis throughout the myocardium. This can result in the remodeling of the left ventricle, which ultimate in left ventricular systolic dysfunction (LVSD). In the digestive form of the disease, the main characteristic is denervation.[8]

The pathogenic mechanisms involved in the clinical forms of Chagas disease can be explained, at least in part, by the persistence of the parasite maintaining the inflammation, and by autoimmunity resulting from the immune response against its own antigens resulting in tissue damage.[8,9] Natural killer (NK) and T cells play a key role in responding to *T. cruzi* infection and the development of clinical forms of Chagas disease.[10] These cells are present in focal inflammatory infiltrations observed in both the heart and the digestive tract.[11-14] However, the inappropriate expression of NK cell and T cell ligands or receptors induces the autoreactive effector function of these cells, and may trigger autoimmune mechanisms.[15,16]

The *MICA* gene (*MHC class I-related chain A*), located on chromosome 6p21.33, is responsible for encoding MICA molecules, which are expressed on the cell surface under stress conditions. These molecules are recognized by Tαβ, Tγδ and NK cells by the NKG2D receptor.[15] The formation of the MICA-NKG2D complex results in a cell-signaling cascade that ends with the target cell lysis process.[17] In addition, *KIR* (*killer cell immunoglobulin-like receptors genes*) are responsible for encoding KIR receptors, which are involved in the regulation of the effector response of NK cells by the recognition of HLA class I molecules of target cells.[18] They comprise a family of 15 genes located on chromosome 19q13.4, classified as activators (KIR2DS and -3DS) and inhibitors (KIR2DL and -3DL) of NK cells and two pseudogenes (KIR2DP1 and -3DP1).[19]

Studies have shown that MICA alleles and certain *KIR* genes and their HLA ligands may influence the clinical course of Chagas disease.[20-22] The objective of this study was to investigate whether *MICA* and *KIR* genes are associated with the cardiac and digestive clinical forms of chronic Chagas disease. Moreover, the possible influence of these genes in the development of LVSD in patients with CCHD was evaluated.

Materials and Methods

As far as possible, the STrengthening the REporting Genetic Association Studies (STREGA)[23] criteria were adopted in the design of this study.

Ethical considerations and clinical characterization of the patients

This study was approved by the Research Ethics Committee of the Medicine School in São Jose do Rio Preto (FAMERP - # 009/2011) and the experiments were

carried out in accordance with the approved regulations. An informed consent form was signed by all participants.

One hundred eighty-five consecutive and unrelated male and female patients serologically-diagnosed with chronic Chagas disease, seen in the Cardiomyopathy Outpatient and General Surgery Services of Hospital de Base of the Fundação Faculdade Regional de Medicina (HB-FUNFARME), São José do Rio Preto, SP, Brazil, participated in this study. All patients underwent 12-lead electrocardiogram, 2-dimensional echocardiogram and chest X-rays. Patients suspected of having the digestive form of the disease were submitted to anorectal manometry, X-rays of the opaque enema, esophageal manometry and X-rays of the esophagus. After clinical evaluation, patients were stratified according to the presence of electrocardiographic or echocardiographic abnormalities consistent with CCHD (cardiac form)[24] and the presence of abnormalities observed in the digestive tract (digestive form).[4] Patients with a mixed form of the disease (cardiac and digestive) were excluded.

Moreover, CCHD patients were subdivided into two groups according to their left ventricular ejection fraction (LVEF) values measured using the Teichholz method: "Patients without LVSD" for those diagnosed with LVEF ≥60% and "patients with LVSD" for those with LVEF <60%.[25]

Patients were classified as being from a mixed ethnic population due to high miscegenation of the Brazilian population,[26] although patients self-reported themselves as European descent, mixed African together with European descent, and African descent. In order to avoid bias in the results, the probability of variations in the allele frequencies due to ethnic background was minimized by matching patients from similar

ethnic backgrounds. Furthermore, gender, mean age and residence in the same geographical areas were matched during group selection.

The following inclusion and exclusion criteria were adopted: Inclusion - Positive laboratory diagnosis of Chagas disease in the chronic phase of the disease at the time of the study and resident in a municipality in the northwestern region of the state of Sao Paulo. Exclusion - Under 18 years of age, suffering other infectious or parasitic diseases, with any concomitant disease that may induce the onset of chronic heart disease and patients with any type of mental deficiency.

Biological samples

Blood samples were collected into tubes containing EDTA anticoagulant for genomic DNA extraction, that it was made using the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The evaluation of concentration and purity of DNA samples was made using the ratio of the absorbance at optical densities (OD) of 260 and 280 nm with Epoch™ equipment (BioTek, Winooski, Vermont, USA).

Blood samples were also collected into tubes without anticoagulant to obtain serum for laboratory diagnosis. The laboratory diagnosis of Chagas disease was made by enzyme-linked immunosorbent assay (ELISA) using the ELISAcruzi immunoassay (bioMerieux SA Brazil) following the manufacturer's instructions. The microplates were read using Epoch™ equipment (BioTek, Winooski, Vermont, USA); test results ≥ 1.0 were considered reactant and test results <0.8 were considered negative. The indeterminate range was defined by values from ≥ 0.8 to < 1.0. Positive and negative controls were included in all reactions and the samples were tested in duplicate.

MICA, KIR and HLA genotyping

MICA, *KIR* and *HLA* class I (HLA-A, -B and -C) were genotyped by Polymerase Chain Reaction-Sequence Specific Oligonucleotide Probes protocols with Luminex technology (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions. Hybridization was detected by flow cytometry (LABScanTM 100 flow analyzer) and the data was interpreted using a computer program (HLA Fusion 2.0 Research, One Lambda).

HLA-KIR ligand specificities belonging to the groups C1, C2, Bw4 (Bw4-80Ile and Bw4-80Thr) and HLA-A*03/-A*11 were considered according to Carr et al.,[27] Thananchai et al.,[28] and Kulkarni et al.[29] KIR genotypes AA and BX (BB and AB) were defined based on the number of the genes encoding activating receptors (according to http://www.allelefrequencies.net). Binding affinities to NKG2D, attributed to the amino acid position at 129 in the *MICA* gene, were determined by Steinle et al.[30] and Karacki et al.,[31] based on amino acid composition (methionine, high; valine, low – in respect to the rs1051792 polymorphism).

Statistical analysis

KIR and MICA-129 frequencies were obtained by direct counting. The ARLEQUIN software version 3.11 (http://cmpg.unibe.ch/software/arlequin3) was used to calculate the allele groups (HLA and MICA) and haplotype frequencies. The Hardy-Weinberg equilibrium was verified according to the method described by Guo & Thompson[32] for KIR2DL2/3 and KIR3DL1/S1, and for MICA and HLA alleles. The relative linkage disequilibrium (Δ') was calculated according to the Imanishi[33] method. Comparisons between groups of patients were attained using the chi-square test with

Yates' correction or Fisher's exact test using the statistics program OpenEpi version 3.01 (http://www.openepi.com/Menu/OE_Menu.htm). The genetic associations were measured by OR (Odds Ratio) and the 95% confidence interval (95% CI). The unpaired t-test was used to compare the mean ages. P-values ≤ 0.05, corrected by the Bonferroni inequality method for multiple comparisons (Pc), were considered statistically significant.

Results

Of the 185 patients with positive serology for *T. cruzi*, 78 (42.2%) (35 men and 43 women; mean age of 65.6 ± 10.4 years) were considered to have digestive form of Chagas disease and 107 (57.8%) (53 men and 54 women; mean age of 64.7 ± 9.8 years) were considered to have cardiac form of Chagas disease. Of the 107 patients with CCHD (cardiac form), 52 (48.6%) (30 men and 22 women; mean age of 64.7 ± 10.0 years) had LVSD, and the remaining 55 (51.4%) (23 men and 32 women; mean age of 64.6 ± 9.7 years) did not have LVSD.

MICA, KIR (*KIR2DL2/3* and *KIR3DL1/S1*) and *HLA* class I frequencies of the studied populations were in Hardy-Weinberg equilibrium (p-value > 0.05).

No significant differences were found in the distribution of MICA alleles between the groups. Moreover, statistically significant differences were not found for both the MICA-129 alleles with high affinity and MICA-129 alleles with low affinity for the NKG2D receptor between patients with the cardiac and digestive forms of Chagas disease. However, MICA-129 alleles with high affinity showed a positive association to LVSD (OR = 1.90; CI = 1.09-3.33; p-value = 0.03), while MICA-129 alleles with low affinity had a negative association with LVSD (OR = 0.52; CI = 0.30-0.92; p-value = 0.03) (Table 1).

Table 2 shows the frequency of the MICA-HLA-B and MICA-HLA-C haplotypes that presented a statistically significant difference between patients with the digestive and cardiac forms of the disease. The haplotypes MICA*002~HLA-B*39 (OR: 3.86; 95% CI: 1.09-8.55; p-value = 0.04; Pc = 0.48), MICA*008~HLA-B*07 (OR: 3.84; 95% CI: 1.06-5.28; p-value = 0.05; Pc = 0.95), MICA*002~HLA-C*07 (OR: 4.52; 95% CI: 1.18-17.30; p-value = 0.03; Pc = 0.99) and MICA*008~HLA-C*06 (OR: 13.82; 95% CI: 1.71-111.63; p-value = 0.004; Pc = 0.04) were associated with an increased risk of developing the clinical digestive form of Chagas disease. Moreover, the haplotype MICA*008~HLA-B*44 (OR: 4.22; 95% CI: 1.13-14.64; p-value 0.03; Pc = 0.48) was associated with an increased risk of developing the cardiac form of the disease. However, the significance of these associations was not statistically significant after correcting for multiple comparisons, except for the haplotype MICA*008~HLA-C*06. There was no significant difference on comparing the MICA~HLA-B and MICA~HLA-C haplotypes between patients with LVSD and those without LVSD. The frequencies of HLA-A, -B and –C alleles also were analyzed and no differences were observed between the groups (data not shown).

Table 3 shows the distribution of *KIR* gene frequencies. Susceptibility for developing CCHD was observed for the *KIR2DS2* activating gene (OR = 1.89; CI = 1.05-3.43; p-value = 0.04; Pc = 0.64), but the significance was lost after applying the Bonferroni correction.

No significant differences were found in the distribution of KIR genotypes (AA and BX) and in the distribution of KIR-HLA receptor ligand pairs between all groups investigated in this study (Figure 1 and Table 4 respectively). The frequencies of HLA class I ligands of KIR (A3 or A11, Bw4 and Bw4-80Ile, C1 and C2, in homozygosity

and heterozygosity) also were analyzed separately and no differences were observed (data not shown).

The combination between the distribution of activating and inhibitory KIR and their respective HLA ligands is shown in Table 5. Both $2DS2^{-}/2DL2^{-}/2DL3^{+}/C1^{+}$ and $2DS2^{-}/2DL3^{+}/C1^{+}$ combinations were associated with increased susceptibility for the digestive form (OR = 5.83; CI = 1.20-4.25; p-value = 0.01; *Pc* = 0.04 and OR = 1.95; CI = 1.07-3.54; p-value = 0.03; Pc = 0.09 respectively), but after Bonferroni correction the significance was lost for the 2DS2-/2DL3+/C1+ combination. The difference between patients with the digestive form and patients without LVSD was also observed for the $2DS2^{-}/2DL2^{-}/2DL3^{+}/C1^{+}$ combination, although it was lost after Bonferroni correction (OR = 2.70; IC = 0.24-5.90; p-value = 0.02; Pc = 0.08). A significant difference was found for $2DS1^{+}/2DL1^{+}/C2^{+}$ combination between patients with LVSD and those without LVSD, but only before p-value correction (OR = 0.35; IC = 0.14-0.85; p-value = 0.03; Pc = 0.09). Furthermore, the $2DS2^{+}/2DL2^{-}/C1^{+}$ combination showed a tendency of a positive association for the cardiac form (OR = 4.41; CI = 0.94-20.50; p-value = 0.07).

Discussion

The data obtained in this study provide evidence that broadens and reinforces our knowledge about the influence of the *MICA* and *KIR* genetic variants and their interactions with HLA class I molecules in the different clinical evolutions of chronic Chagas disease. In particular, MICA-129 alleles with high affinity for the NKG2D receptor showed a positive association for LVSD, while there was a negative association between MICA-129 alleles with low affinity for the NKG2D receptor and LVSD. It was also possible to demonstrate that the haplotype MICA*008~HLA-C*06

and the KIR2DS2^{-}/KIR2DL2^{-}/KIR2DL3^{+}/C1^{+} combination were associated as factors of susceptibility to the digestive clinical form of the disease.

MICA and KIR molecules are directly involved in the activation and regulation of NK cell activity. The effector mechanisms of NK cells act on the target cell when there is no recognition of HLA class I molecules by KIR receptors and/or when MICA molecules are recognized by the NKG2D receptor,[16] hypotheses known as "missing self" and "induced self" respectively.[34] Furthermore, MICA molecules act as co-stimulators of Tαβ (CD8^{+} and subsets of CD4^{+}) and Tγδ cells (Lanier, 2015). It is known that NK and T cells are part of the inflammatory infiltrate found in the cardiac and digestive forms of chronic Chagas disease.[8] The interaction of these cells with the target cell exerts a significant role in the initiation and regulation of the innate and adaptive immune responses and the molecular basis of these cellular interactions seem to be crucial for an efficient and modulated response against the parasite to avoid tissue damage.

The met allele (high affinity for the NKG2D receptor) and the homozygous met/met genotype of the MICA-129 polymorphism were previously reported by our group as risk factors for the development of severe LVSD in patients with CCHD. Similarly, the val allele (low affinity for the NKG2D receptor) and the homozygous val/val genotype are associated with a lower risk of developing severe LVSD.[21] Even though the strategies adopted for the composition of the present study were different from those adopted previously,[21] MICA-129met seems to be a susceptibility factor and MICA-129val a protective factor for the development of LVSD in a population of patients with CCHD from the northwestern region of the state of São Paulo (southeastern Brazil).

The amino acid methionine at position 129 of the α2 domain of the MICA protein (MICA-129met) has higher affinity for the NKG2D receptor than proteins with the amino acid valine (MICA-129val) in the same position due to a conformational change of the molecule, which may compromise NK cell activation and the co-stimulation of $CD8^+$ T lymphocytes.[30] The strong affinity formed by the NKG2D-MICA-129met complex may be related to the tissue damage that causes LVSD in patients with CCHD, since the NKG2D-MICA interaction may participate in inflammatory processes with increased cytokine production by NK and $TCD8^+$.[15] The involvement of these cells in the immunopathological mechanisms of the clinical forms of Chagas disease can be attributed to their cytotoxic effects, mainly because of the production of cytokines with a Th1 response profile such as interferon-gamma (IFN-γ).[12,36]

Furthermore, the interaction between NKG2D and the MICA molecules may also favor autoimmune conditions by promoting costimulatory signaling of specific $CD8^+$ T cell self-antigens.[15] Thus, a milieu of potent immune stimuli may overcome the threshold of activation needed to breach self-tolerance. Autoimmunity is accepted as one of the pathogenic mechanisms responsible for the different clinical manifestations of Chagas disease, as the infectious agent can mimic self-antigens, induce autoreactive cell proliferation or increase the expression of major histocompatibility complex (MHC) and costimulatory molecules in infected cells.[37] Associations of $CD8^+$ T cells with degenerated ganglion cells has been reported in patients with megacolon.[38] This cells were also seen to be related to the destruction of myofibers in heart tissue.[9]

This study also demonstrated that the MICA*008~HLA-C*06 haplotype was associated with an increased risk of developing the clinical digestive form of Chagas

disease. The MICA*008 allele carries the microsatellite A5.1 characterized by the insertion of a guanine nucleotide after the second GCT (GGCT) repeat, which generates a stop codon in the exon that encodes the transmembrane domain and results in a truncated form of the protein.[39] Furthermore, the MICA*008 allele has the amino acid valine at position 129 of the protein. Thus, susceptibility to the digestive form of the disease may be related to the expression of the mutated protein, A5.1, which would affect its recognition by NKG2D receptors, leading to lower NK and T cell activity via MICA ligands with greater persistence of the parasite and consequently of the immune response. On the other hand, the result of a weak MICA-129val interaction could lead to an increased expression of NKG2D as in autoimmune diseases,[40,41] and favor interactions with other ligand binding proteins such as UL16-binding proteins (ULBPs).[42] This creates an environment rich in cytokines that enhances the cytotoxic activity of $CD8^+$ T cells and NK cells and therefore the production of IFN-γ, again favoring the autoimmunity condition.[40,41] It is also known that MICA stimulates Tγδ cells in the intestinal mucosa, a phenomenon that could be related to the digestive form of the disease.[43]

Class I HLA molecules play a crucial role in determining the individual immune response through the peptide presentation of pathogens endogenous to $CD8^+$ T cells. Thus, peptide affinity for MHC class I appears to be determinant for an effective antigen-directed response, as this interaction may result in distinct patterns of $CD8^+$ T cell responses.[44] As the linkage disequilibrium between the MICA*008 and HLA-C*06 was strong ($\Delta' = 1.0$; p-value = 0.03) and associations involving MICA and HLA alleles were not separately observed, it is difficult to determine the primary associated locus within the haplotype. It is know that some MICA alleles may be intimately linked to

other alleles also responsible for the association, such as HLA, due to the relatively close physical proximity between these loci and thus, when combined they exert a synergistic effect.[45] Thus, both alleles may be exerting an influence on the development of chagasic megacolon and/or magaesophagus.

To the best of our knowledge, this appears to be the first study to evaluate the influence of *KIR* on the clinical digestive form of chronic Chagas disease and on the development of LVSD in patients with CCHD. The data from this study indicate possible susceptibility related to the inhibitory KIR2DL3 and its C1 ligand in the absence of both KIR2DL2 and KIR2DS2 ($2DS2^{-}/2DL2^{-}/2DL3^{+}/C1^{+}$) in the development of the digestive form of chronic Chagas disease.

The interaction between KIR and HLA class I molecules, KIR ligands, is required for the functional activity of NK cells, so that the presence of one, but not the other, is not sufficient to influence their function.[29] It is known that different KIR receptors (inhibitors and activators) have affinity for the same ligand, however the affinity for the inhibitory receptor seems to be stronger than for its homolog activator.[46] There is also a well-established inhibition affinity scale for the KIR2DL-HLA-C pairs: KIR2DL1-C2 has the greatest inhibitory potential, followed by KIR2DL2-C1 and KIR2DL3-C1.[47]

KIR2DL2 and KIR2DL3 secrete as alleles of the same locus and both recognize C1 group ligands. However, these receptors present qualitative differences in their functional effect and clinical influence.[48] Thus, the weak inhibitory signal generated by the absence of KIR2DL2, that is, the homozygosity of KIR2DL3 in the presence of the C1 ligand found in the $2DS2^{-}/2DL2^{-}/2DL3^{+}/C1^{+}$ combination, appears not to be sufficient to inhibit the effector function of NK cells and protect against the exacerbated

inflammation that results in tissue lesions of the gastrointestinal tract. KIR2DL-HLA-C inhibitor pairs have been shown to exert influence on inflammatory bowel diseases.[49-51]

In addition to the results of this study, the KIR2DS2-C1 activating pair, in the absence of its inhibitory homolog, KIR2DL2, independently of the presence or absence of KIR2DL3, has been shown to constitute a possible risk factor for the development of Chagas disease and CCHD in patients from a population in southern Brazil.[22] In this study, the $2DS2^{+}/2DL2^{-}/C1^{+}$ combination showed only a tendency of a positive association with the cardiac form. However, the possible distinction of results can be explained by the influence of ethnicity on the distribution of *KIR* genes and HLA alleles in patients chronically infected by *T. cruzi* from different Brazilian regions. Other methodological variables may have contributed to the observed differences.

NK cells can increase T cell response activity through the production of IFN-γ and both can migrate to the region of inflamed tissues. In addition, NK cell-mediated target cell death also affects T cell responses, possibly by decreasing parasite burden and/or because target cell debris can promote the cross-presentation of antigens to $CD8^{+}$ T cells.[52] According to Sathler-Avelar et al.[10] strong and uncontrolled activation of NK cells as well as proinflammatory monocytes may result in the tissue damage observed in the clinical manifestations of chronic Chagas disease. Baseline levels of NK, NKT, and $CD4^{+}$ $CD25^{high}$ cells, increased expression of activated $CD8^{+}$ T cells associated with failed immunoregulation mechanisms were associated with cardiac symptoms of chronic Chagas disease.[53] Thus, NK and T cells need to be properly activated to become competent in performing their functional activities without causing tissue damage, and the molecular interactions between receptors and stimulatory and costimulatory molecules and the production of cytokines in the activation process of these cells may

be closely related in the immunophysiological processes of CCHD and chagasic megacolon and magaesophagus. Of course, there are other cells and other immunophysiological mechanisms involved in the pathogenesis of both cardiac and digestive Chagas disease.

The results of this study show that *MICA* and *KIR* may exert an influence on the inflammatory activity carried out by NK and T cells in the clinical manifestations of chronic Chagas disease. In the cardiac form of the disease, a higher activation threshold generated by the MICA-129met allele seems to result in more pronounced inflammatory activity that would lead to the development of LVSD; while in the digestive form of the disease a less marked activation threshold of the T and NK cells generated by the MICA*008~HLA-C*06 haplotype appears to be sufficient to induce their cytotoxic activity. Furthermore, the weak inhibitory signal generated by the $2DS2^{-}/2DL2^{-}/2DL3^{+}/C1^{+}$ combination may not be sufficient to inhibit the effector function of the NK cells present in the gastrointestinal tract. However, to better understand the role of NK and T cells, as well as to clarify the expression of KIR and MICA molecules in the immunopathogenesis of CCHD and chagasic megacolon/megaesophagus, others studies such as histopathological analysis and cytotoxicity assays should be performed.

Acknowledgements

The authors are grateful to all of the volunteers who participated in this study, to Laboratory of Immunogenetics at FAMERP and to David Hewitt for his help with the English version. Thanks to Professor Stephen Henry from Auckland University of the Technology for providing library access. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant numbers: 2013/06580-9 and 2011/08075-4]. The opinions, assumptions, and conclusions or recommendations

expressed in this material are the responsibility of the authors and do not necessarily reflect the views of FAPESP.

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Hapl Group	Genotype ID	Inhibitory KIR								Activanting KIR						Pseudoge		Digestive (n=78)		Cardiac (n=107)		With LVSD (n=52)		Without LVSD (n=55)	
		2DL					3DL			2DS					3DS	2DP	3DP								
		1	2	3	4	5	1	2	3	1	2	3	4	5	1	1	1	n	%	n	%	n	%	n	%
AA	1	■		■	■	■	■	■	■				■			■	■	24	30,8	21	19,6	12	23,1	9	16,4
Bx	2	■		■	■	■	■	■	■	■			■	■	■	■	■	8	10,3	6	5,6	3	5,8	3	5,5
Bx	3	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	5	6,4	7	6,5	3	5,8	4	7,3
Bx	4	■	■	■	■		■	■	■		■		■			■	■	3	3,8	9	8,4	3	5,8	6	10,9
Bx	5	■	■	■	■	■	■	■	■		■	■	■			■	■	3	3,8	8	7,5	4	7,7	4	7,3
Bx	6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	2	2,6	5	4,7	2	3,8	3	5,5
Bx	8	■		■	■	■	■	■	■	■		■	■		■	■	■	2	2,6	1	0,9	0	0,0	1	1,8
Bx	12	■		■	■	■	■	■	■	■	■		■	■	■	■	■	1	1,3	5	4,7	1	1,9	4	7,3
Bx	18	■	■	■	■	■	■	■	■	■			■	■	■	■	■	1	1,3	3	2,8	1	1,9	2	3,6
Bx	19	■	■	■	■		■	■	■				■		■	■	■	3	3,8	5	4,7	3	5,8	2	3,6
Bx	20	■	■	■	■	■	■	■	■				■	■		■	■	1	1,3	3	2,8	1	1,9	2	3,6
Bx	21	■	■	■	■	■	■	■	■		■		■	■		■	■	0	0,0	3	2,8	2	3,8	1	1,8
Bx	25	■		■	■	■	■	■	■		■	■	■	■		■	■	0	0,0	2	1,9	0	0,0	2	3,6
Bx	27	■		■	■	■	■	■	■	■			■		■	■	■	2	2,6	0	0,0	0	0,0	0	0,0
Bx	30	■		■	■	■	■	■	■			■	■			■	■	1	1,3	0	0,0	0	0,0	0	0,0
Bx	36	■		■	■	■	■	■	■		■		■			■	■	0	0,0	3	2,8	2	3,8	1	1,8
Bx	56	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	57	■		■	■		■	■	■	■	■	■	■	■	■	■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	64	■	■	■	■	■	■	■	■	■		■	■		■	■	■	2	2,6	1	0,9	1	1,9	0	0,0
Bx	69	■		■	■	■		■	■	■		■		■	■	■	■	3	3,8	3	2,8	2	3,8	1	1,8
Bx	70	■	■	■	■	■		■	■	■	■	■		■	■	■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	71	■	■		■	■	■	■	■		■	■	■			■	■	2	2,6	1	0,9	0	0,0	1	1,8
Bx	72		■		■		■	■	■		■	■					■	4	5,1	3	2,8	1	1,9	2	3,6
Bx	73	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	1	1,3	0	0,0	0	0,0	0	0,0
Bx	79	■		■	■	■	■	■	■	■		■	■	■	■	■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	88	■		■	■	■	■	■	■	■	■			■	■	■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	89	■	■		■		■	■	■		■		■			■	■	1	1,3	0	0,0	0	0,0	0	0,0
Bx	90	■	■		■	■	■	■	■	■	■	■	■		■	■	■	3	3,8	2	1,9	1	1,9	1	1,8
Bx	92	■	■		■	■	■	■	■	■	■		■	■		■	■	1	1,3	0	0,0	0	0,0	0	0,0
Bx	112	■	■		■	■	■	■	■		■	■	■	■		■	■	0	0,0	2	1,9	2	3,8	0	0,0
Bx	118	■	■		■	■	■	■	■	■	■		■	■	■	■	■	2	2,6	0	0,0	0	0,0	0	0,0
Bx	176	■	■		■	■	■	■	■		■		■	■		■	■	1	1,3	0	0,0	0	0,0	0	0,0
Bx	188	■	■	■	■		■	■	■				■	■	■	■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	192	■	■	■	■		■	■	■	■	■		■			■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	193	■	■	■	■		■	■	■				■			■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	228	■	■		■	■	■	■	■		■	■	■	■		■	■	1	1,3	2	1,9	2	3,8	0	0,0
Bx	271	■	■	■	■	■	■	■	■			■	■	■	■	■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	339	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	0	0,0	1	0,9	1	1,9	0	0,0
Bx	391		■	■	■		■	■	■	■	■	■	■		■	■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	424	■	■		■	■	■	■	■		■	■	■	■	■	■	■	0	0,0	1	0,9	0	0,0	1	1,8
Bx	433	■		■	■	■	■	■	■				■		■	■	■	1	1,3	0	0,0	0	0,0	0	0,0

Figure 1 - Distribution of KIR genotypes in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

ID: Genotype identification (according to http://www.allelefrequencies.net). Shaded rectangles represent the presence of the gene. LVSD: left ventricular systolic dysfunction

Table 1 - Distribution of MICA alleles in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

MICA		Digestive (n = 78)	Cardiac (n = 107)	With LVSD (n = 52)	Without LVSD (n = 55)
		n (%)	n (%)	n (%)	n (%)
Alleles	Position 129				
*001	met	2 (1.3)	6 (2.8)	2 (1.9)	4 (3.6)
*002	met	33 (21.2)	40 (18.7)	23 (22.1)	17 (15.5)
*004	val	26 (16.7)	25 (11.7)	11 (10.5)	14 (12.7)
*006	val	2 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)
*007	met	4 (2.6)	9 (4.2)	6 (5.8)	3 (2.7)
*008	val	41 (26.3)	51 (23.8)	22 (21.2)	29 (26.4)
*009	val	15 (9.6)	27 (12.6)	13 (12.5)	14 (12.7)
*010	val	8 (5.1)	8 (3.7)	3 (2.9)	5 (4.5)
*011	met	4 (2.6)	6 (2.8)	5 (4.8)	1 (0.9)
*012	met	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
*015	met	1 (0.6)	3 (1.4)	2 (1.9)	1 (0.9)
*016	val	3 (1.9)	3 (1.4)	0 (0.0)	3 (2.7)
*017	met	5 (3.2)	5 (2.3)	1 (1.0)	4 (3.6)
*018	met	6 (3.8)	12 (5.6)	7 (6.7)	5 (4.5)
*019	val	1 (0.6)	7 (3.3)	4 (3.8)	3 (2.7)
*024	val	0 (0.0)	1 (0.5)	(0.0)	1 (0.9)
*027	val	3 (1,9)	8 (3.7)	2 (1.9)	6 (5.4)
*029	met	0 (0.0)	2 (0.9)	2 (1.9)	0 (0.0)
*030	met	0 (0.0)	1 (0.5)	1 (1.0)	0 (0.0)
*045	val	1 (0.6)	0 (0.0)	(0.0)	0 (0.0)
MICA-129					
High affinity[§]		56 (35.9)	84 (39.3)	49 (47.1)[a]	35 (31.8)[a]
Low affinity[§]		100 (64.1)	130 (60.7)	55 (52.9)[b]	75 (68.2)[b]

[a]OR = 1.90; CI = 1.09-3.33; p-value = 0.03 (With LVSD vs. Without LVSD)

[b]OR = 0.52; CI = 0.30-0.92; p-value = 0.03 (Without LVSD vs. With LVSD)

LVSD: left ventricular systolic dysfunction; Met: methionine; Val: valine. [§]Based on amino-acid composition (methionine, high; valine, low - related to the rs1051792) in position 129 of the *MICA* gene (Steinle et al., 2001; Karacki et al., 2004).

Table 2 - Haplotype frequencies of MICA, HLA-B and HLA-C in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

Haplotypes	Digestive (n = 78)	Cardiac (n = 107)	With LVSD (n = 52)	Without LVSD (n = 55)
	n (%)	n (%)	n (%)	n (%)
MICA~HLA-B				
*002~*39	12 (15.3)[a]	6 (5.6)[a]	2 (3.8)	4 (7.2)
*008~*07	18 (23.0)[b]	12 (11.2)[b]	7 (13.5)	5 (9.1)
*008~*44	3 (3.8)[c]	15 (14.0)[c]	7 (13.5)	8 (14.5)
MICA~HLA-C				
*002~*07	9 (11.5)[d]	3 (2.8)[d]	1 (1.9)	2 (3.6)
*008~*06	9 (11.5)[e]	1 (0.9)[e]	0 (0.0)	1 (1.8)

[a]OR = 3.86; CI = 1.09-8.55; p-value = 0.04; Pc = 0.48 (Digestive form vs. Cardiac form).

[b]OR = 3.84; CI = 1.06-5.28; p-value = 0.05; Pc = 0.95 (Digestive form vs. Cardiac form).

[c]OR = 4.22; CI = 1.13-14.64; p-value = 0.03; Pc = 0.48 (Digestive form vs. Cardiac form).

[d]OR = 4.52; CI = 1.18-17.30; p-value = 0.03; Pc = 0.99 (Digestive form vs. Cardiac form).

[e]OR = 13.82; CI = 1.71-111.63; p-value = 0.004; Pc = 0.04 (Digestive form vs. Cardiac form).

LVSD: left ventricular systolic dysfunction

Table 3 – Distribution of KIR genes in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

KIR Genes	Digestive (n = 78)	Cardiac (n = 107)	With LVSD (n = 52)	Without LVSD (n = 55)
	n (%)	n (%)	n (%)	n (%)
Inhibitory				
KIR2DL1	74 (94.9)	103 (96.3)	51 (98.0)	52 (94.5)
KIR2DL2	36 (46.2)	64 (59.8)	31 (59.6)	33 (60.0)
KIR2DL3	62 (79.5)	96 (89.7)	46 (88.5)	50 (90.9)
KIR2DL5	43 (55.1)	64 (59.8)	31 (59.6)	33 (60.0)
KIR3DL1	75 (96.2)	103 (96.3)	49 (94.2)	54 (98.2)
Activating				
KIR2DS1	33 (42.3)	42 (39.3)	18 (34.6)	24 (43.6)
KIR2DS2	30 (38.5)[a]	58 (54.2)[a]	26 (50.0)	32 (58.2)
KIR2DS3	17 (21.8)	33 (30.8)	17 (32.7)	16 (29.1)
KIR2DS4	75 (96.2)	101 (94.4)	48 (92.3)	53 (96.4)
KIR2DS5	26 (33.3)	50 (46.7)	23 (44.2)	27 (49.1)
KIR3DS1	33 (42.3)	40 (37.4)	18 (34.6)	20 (36.7)
Framework and Pseudogenes				
KIR2DL4	78 (100.0)	107 (100.0)	52 (100.00)	55 (100.0)
KIR3DL2	78 (100.0)	107 (100.0)	52 (100.00)	55 (100.0)
KIR3DL3	78 (100.0)	107 (100.0)	52 (100.00)	55 (100.0)
KIR3DP1	78 (100.0)	107 (100.0)	52 (100.00)	55 (100.0)
KIR2DP1	74 (94.9)	104 (97.2)	51 (98.1)	53 (96.4)

OR = 1.89; CI = 1.05-3.43; p-value = 0.04; Pc = 0.64 (Cardiac form vs. Digestive form).

Table 4 - Distribution of KIR and their respective HLA ligands in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

KIR - HLA ligands	Digestive (n = 78)	Cardiac (n = 107)	With LVSD (n = 52)	Without LVSD (n = 55)
	n (%)	n (%)	n (%)	n (%)
Inhibitory				
2DL1-C2	63 (80.8)	90 (84.1)	44 (84.6)	46 (83.6)
2DL2-C1	33 (42.3)	50 (46.7)	25 (48.1)	25 (45.5)
2DL3-C1	53 (67.9)	68 (63.6)	34 (65.4)	34 (61.8)
3DL2-A3/A11	19 (24.4)	26 (24.3)	13 (25.0)	13 (23.6)
3DL1-Bw4	41 (52.6)	78 (72.9)	36 (69.2)	42 (76.4)
2DL1-C2C2	10 (12.8)	25 (23.4)	10 (19.2)	15 (27.3)
2DL2-C1C1	3 (3.8)	12 (11.2)	6 (11.5)	6 (10.9)
2DL3-C1C1	10 (12.8)	13 (12.1)	6 (11.5)	7 (12.7)
2DL2/2DL2-C1C2	14 (17.7)	10 (9.3)	5 (9.6)	5 (9.1)
2DL2/2DL3-C1C2	16 (20.5)	28 (26.2)	14 (26.9)	14 (25.5)
2DL3/2DL3-C1C2	27 (34.6)	27 (25.2)	14 (26.9)	13 (23.6)
2DL2/2DL2-C1C1	1 (1.3)	1 (0.9)	1 (1.9)	0 (0.0)
2DL2/2DL3-C1C1	2 (2.6)	11 (10.3)	5 (9.6)	6 (10.9)
2DL3/2DL3-C1C1	8 (10.3)	4 (3.7)	3 (5.8)	1 (1.8)
Activating				
2DS1-C2	27 (34.6)	34 (31.8)	13 (25.0)	21 (38.2)
2DS2-C1	26 (33.3)	45 (42.1)	22 (42.3)	23 (41.8)
2DS1-C2C2	5 (6.4)	10 (9.3)	4 (7.7)	6 (10.9)
2DS2-C1C1	3 (3.8)	8 (7.5)	4 (7.7)	4 (7.3)
3DS1-Bw4-80Ile	16 (20.5)	19 (17.8)	9 (17.3)	10 (18.2)

Bw4 = HLA-A*23, *24, *32; HLA-B *13, *27, *44, *51, *52, *53, *57, *58. Bw4-80Ile = HLA-A*23, *24, *32; HLA-B*51, *52, *53, *57, *58. Group C1 = HLA-C*01, *03, *07, *08, *12, *14, *16. Group C2 = HLA-C*02, *04, *05, *06, *07, *15, *17, *18. HLA-KIR ligands specificities were considered according to Carr et al. (2005),

Thananchai et al. (2007) and Kulkarni et al. (2008). LVSD: left ventricular systolic dysfunction

Table 5 - Distribution of activating KIR plus inhibitory KIR and their respective ligands in patients with the digestive or cardiac forms of chronic Chagas disease and in patients with LVSD and without LVSD.

Activating and/or inhibitory KIR and HLA ligands combinations	Digestive (n = 78)	Cardiac (n = 107)	With LVSD (n = 52)	Without LVSD (n = 55)
	n (%)	n (%)	n (%)	n (%)
KIR-C1				
2DS2+/2DL2-/C1+	2 (2.5)[a]	11 (10.3)[a]	4 (7.7)	7 (12.7)
2DS2-/2DL2+/C1+	7 (9.0)	14 (13.1)	7 (13.5)	7 (12.7)
2DS2+/2DL3-/C1+	15 (19.2)	10 (9.3)	6 (11.5)	4 (7.3)
2DS2-/2DL3+/C1+	41 (52.6)[b]	37 (34.6)[b]	18 (34.6)	19 (34.5)
2DS2+/2DL2-/2DL3-/C1+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2DS2-/2DL2-/2DL3+/C1+	34 (43.6)[c,d]	26 (24.3)[c]	14 (26.9)	12 (21.8)[d]
2DS2+/2DL2-/2DL3+/C1+	1 (1.3)	9 (8.4)	4 (7.7)	5 (9.1)
2DS2-/2DL2+/2DL3+/C1+	7 (9.0)	13 (12.1)	7 (13.5)	6 (10.9)
2DS2+/2DL2+/2DL3-/C1+	14 (17.9)	10 (9.3)	6 (11.5)	4 (7.3)
2DS2+/2DL2+/2DL3+/C1+	11 (14.1)	27 (25.2)	13 (25.0)	14 (25.5)
KIR-C2				
2DS1+/2DL1-/C2+	0 (0.0)	1 (0.9)	0 (0.0)	1 (1.8)
2DS1-/2DL1+/C2+	36 (46.2)	55 (51.4)	30 (57.7)	25 (45.5)
2DS1+/2DL1+/C2+	26 (33.3)	32 (29.9)	10 (19.2)[e]	22 (40.0)[e]
KIR-BW4-80Ile				
3DS1+/3DL1+/BW4-80Ile+	15 (19.2)	18 (16.8)	8 (15.4)	10 (18.2)
3DS1+/3DL1-/BW4-80Ile+	1 (1.3)	1 (0.9)	1 (1.9)	0 (0.0)
3DS1-/3DL1+/BW4-80Ile+	28 (35.9)	38 (35.5)	17 (32.7)	21 (38.2)

[a]OR = 4.41; IC = 0.94-20.50; p-value = 0.07 (Cardiac form vs. Digestive form).

[b]OR = 1.95; IC = 1.07-3.54; p-value = 0.03; Pc = 0.09 (Digestive form vs. Cardiac form).

[c]OR = 5.83; IC = 1.20-4.25; p-value = 0.01; Pc = 0.04 (Digestive form vs. Cardiac form).

[d]OR = 2.70; IC = 1.24-5.90; p-value = 0.02; Pc = 0.08 (Digestive form vs. Without LVSD).

[e]OR = 0.35; IC = 0.14-0.85; p-value = 0.03; Pc = 0.09 (With LVSD vs. Without LVSD)

Bw4 = HLA-A*23, *24, *32; HLA-B *13, *27, *44, *51, *52, *53, *57, *58. Bw4-80Ile = HLA-A*23, *24, *32; HLA-B*51, *52, *53, *57, *58. Group C1 = HLA-C*01, *03, *07, *08, *12, *14, *16. Group C2 = HLA-C*02, *04, *05, *06, *07, *15, *17, *18. HLA-KIR ligands specificities were considered according to Carr et al. (2005), Thananchai et al. (2007) and Kulkarni et al. (2008). LVSD: left ventricular systolic dysfunction

3. Conclusões

3. CONCLUSÕES

Toxoplasmose ocular:

- O polimorfismo do gene *MICA* parece não influenciar o desenvolvimento de lesões oculares em pacientes diagnosticados com toxoplasmose.
- Genes *KIR* na presença de seus respectivos ligantes HLA exercem influência no desenvolvimento da TO e suas manifestações clínicas primária e recorrente: $KIR3DS1^{+}/Bw4\text{-}80Ile^{+}$ e $KIR2DS1^{+}/C2^{+}$ + $KIR3DS1^{+}/Bw4\text{-}80Ile^{+}$ foram associados como fatores de risco e, KIR2DL3/2DL3-C1/C1 e KIR2DL3/2DL3-C1 como fatores de proteção.

Formas clínicas cardíaca e digestiva da doença de Chagas crônica:

- O alelo MICA-129 met foi associado com o desenvolvimento da DSVE, e o alelo MICA-129 val foi associado a um menor risco de desenvolvimento da DSVE. O genótipo homizigoto MICA-129 met/met foi associado com o aumento do risco de pacientes com CCC desenvolverem a forma grave da DSVE, enquanto que o genótipo homozigoto MICA-129 val/val foi associado com a proteção desta condição.
- O haplótipo MICA*008~HLA-C*06 foi associado como fator de risco à forma clínica digestiva da doença de Chagas.
- A combinação $KIR2DS2^{-}/KIR2DL2^{-}/KIR2DL3^{+}/C1^{+}$ foi associada como fator de suscetibilidade à forma clínica digestiva da doença de Chagas.

4. Referências

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