Universidade Estadual Paulista "Julio de Mesquita Filho"
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Polimorfismo gênico de receptores TLRs e citocinas: papel na resposta imune em pacientes com tuberculose pulmonar.

Tese apresentada ao Programa de Pós-Graduação em Doenças Tropicais da Faculdade de Medicina de Botucatu

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Mensagem

"(...) O mecanismo filosófico do progresso

são os objetivos em processos consecutivos.

Quando você começar a se deprimir, arranje um objetivo.

De repente, você esbarrará com o grande Objetivo.

Ingressará em um mundo novo!"

Dr. Celso Charuri

Dedicatória

Aos meus pais,

Myiako e Luiz Peresi

Por acreditarem em mim e incentivar a realização deste trabalho.

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Sumário

I - Introdução

A tuberculose (TB) é uma doença infecciosa de evolução crônica, tendo como agente etiológico, a bactéria intracelular *Mycobacterium tuberculosis* (*M. tuberculosis),* que se dissemina pelo ar através de gotículas de saliva expelidas pela tosse, fala ou espirro dos doentes, contaminando as vias respiratórias e se alojando nos pulmões. [(1)] A partir daí, espalha-se pela linfa ou pelo sangue, para outras partes do organismo, principalmente para o ápice pulmonar e os linfonodos regionais, pois, como microrganismo aeróbio estrito, é onde encontra altas concentrações de oxigênio. A tuberculose extrapulmonar ocorre na pleura, sistema linfático, ossos, sistema gênito-urinário, meninges, peritônio ou pele, e corresponde a cerca de 15% dos casos. [(1)] Após a infecção primária, podem ocorrer três desfechos: controle na porta de entrada graças à imunidade inata, doença ativa e tuberculose latente, em que o bacilo permanece por tempo indefinido, contido pelas células de defesa. [(2)]

Durante o estado de latência, há controle, mas não eliminação da infecção. O *M. tuberculosis* fica dormente, replicando intermitentemente e com metabolismo alterado, o que gera um reservatório enorme de micobactérias. A diminuição da imunidade, em situações de desnutrição, subnutrição, estresse ou presença de outras condições mórbidas, como a aids, pode levar à reativação. Quando isso ocorre e a doença se desenvolve, há graves lesões no tecido, ocasionadas pelas próprias defesas do organismo que tentam eliminar o bacilo. [(1, 2)]

De acordo com os dados da Organização Mundial da Saúde (OMS), um terço da população mundial, cerca de dois bilhões de pessoas, está contaminado pelo bacilo. [(3)] Em aproximadamente 5% dos indivíduos imunocompetentes, a infecção progride da forma latente para a forma ativa dentro de dois anos; em outros 5%, a infecção reativa mais tardiamente e de 3,5% a 6,0% destes indivíduos perderão a vida em virtude da doença. [(4,5,6)] Por outro lado, 90% dos imunocompetentes com infecção latente permanecem saudáveis, sem sintomas por toda a vida. [(4)] A cada ano, surgem de 8 a 9,2 milhões de novos casos de tuberculose e 1,2 a 1,5 milhões de pessoas morrem em decorrência da doença. [(3)] No Brasil,

existem mais de 50 milhões de indivíduos infectados e cerca de 90.000 casos novos são notificados por ano, mas calcula-se que este número possa ser de 130.000, em virtude da subnotificação.[5] Nos países desenvolvidos, com a transmissão sob controle desde o advento da terapia específica e a melhora das condições de vida dos seus habitantes, a tuberculose ressurgiu em meados de 1980. Esse ressurgimento, termo que não se aplica aos países subdesenvolvidos, pois estes não chegaram a controlar a disseminação da infecção, tem várias causas. Entre elas, está o advento da aids. O portador do HIV, uma vez infectado pelo bacilo, tem risco estimado de adoecimento de 7% a 10% ao ano, enquanto o não portador, de 10% durante toda a vida (0,3% ao ano). Ao desenvolver a forma ativa da doença, o portador de HIV transmite o bacilo para contactantes, como profissionais da saúde, companheiros de prisão e abrigos, o que aumenta exponencialmente a prevalência da tuberculose. [2]

Outra causa importante do aumento no número de casos da doença é o aumento da resistência do *M. tuberculosis* às drogas antituberculose. Mais de 50 milhões de pessoas no mundo já se encontram infectadas com cepas multirresistentes às drogas (CMR), e 15% dos casos de tuberculose são causados por essas cepas. [7] A mortalidade pelas CMR é de 40% a 60%, que equivale à dos doentes não tratados. [8] Em muitos países, como a Rússia, nos campos de trabalho e nas prisões, os indivíduos infectados pelas CMR praticamente já não alcançam cura, o que pode tornar a tuberculose uma doença incontrolável no futuro. [7,9] No Brasil, a prevalência da tuberculose CMR é menor que 1%.[10]

Além destes, outros fatores que contribuem para o aumento da incidência da doença são a expansão populacional, baixas taxas de diagnóstico de casos e de cura em países mais pobres, transmissão ativa em hospitais, prisões e outros lugares públicos com grande aglomerado de indivíduos, abuso de drogas, decadência social, aumento de pessoas em abrigos e o fluxo migratório a partir de regiões consideradas reservatórios da micobactéria, como África, América do Sul, América Central e Ásia, para os países desenvolvidos. [2, 11]

Todas essas causas caracterizaram a tuberculose como doença em plena expansão, levando a OMS a declará-la, em 1995, emergência sanitária mundial. [2]

O diagnóstico presuntivo da tuberculose pulmonar se faz pelos dados clínicos e radiológicos e a confirmação do diagnóstico é obtida pela baciloscopia e cultura. A baciloscopia identifica os bacilos álcool-ácido resistentes (BAAR), é um método rápido e barato, eleito pelos serviços de saúde pública, mas que apresenta baixa sensibilidade. A cultura tem alta sensibilidade, mas como a reprodução do bacilo é lenta, a definição do diagnóstico é realizada em quatro a oito semanas, tempo muito longo, que pode influenciar o controle da endemia, já que o diagnóstico e o tratamento precoces interrompem o ciclo de transmissão do agente. [12] No Brasil, aproximadamente 26,7% dos pacientes são tratados sem confirmação diagnóstica de tuberculose pulmonar, com base apenas no quadro clínico-radiológico. [13]

A tuberculose é letal sem tratamento que, por ter duração longa, de aproximadamente seis meses, muitas vezes é interrompido pelo paciente, facilitando o desenvolvimento de cepas resistentes às drogas. [4,14] Existem atualmente quatro antimicrobianos de primeira linha, isoniazida, etambutol, pirazinamida e rifampicina, razoavelmente efetivos no tratamento de indivíduos com tuberculose em atividade, porém, ineficientes durante o estágio latente da doença. [15]

A rápida emergência de CMR, a falta de uma vacina de efeito universal e o constante aumento de indivíduos infectados com o HIV, em regiões do mundo consideradas desenvolvidas, têm aumentado a dificuldade de um tratamento efetivo dos indivíduos infectados e da eliminação da tuberculose humana. [8] O desenvolvimento de novas terapias antituberculose é de extrema urgência para a interrupção do círculo vicioso de reativação do estado de latência em um futuro próximo. [15]

O *M. tuberculosis* é caracterizado por uma alta taxa de infectividade e um longo período de latência. Em áreas endêmicas, virtualmente todos os adultos susceptíveis se encontram infectados. Entretanto, na maioria dos indivíduos a infecção terá poucos efeitos, já que a bactéria desenvolveu a capacidade de viver em equilíbrio com a resposta imune. Em indivíduos que se encontram com o sistema imune pouco desenvolvido, geneticamente debilitado ou comprometido por outra doença concomitante, subnutrição ou intervenção médica, o equilíbrio pode ser perturbado, levando ao aumento da replicação bacteriana e consequente doença em atividade. A inflamação gerada em resposta ao *M. tuberculosis* pode ser considerada uma "faca de dois gumes". Por um lado, é necessária para o controle inicial da infecção. Falhas no desenvolvimento de uma resposta inflamatória adequada induzem a progressão da infecção para doença. Por outro lado, a bactéria explora esta mesma resposta para se espalhar e infectar indivíduos susceptíveis, completando o seu nicho ecológico. Este delicado equilíbrio requer a atuação dos mecanismos da imunidade inata e adaptativa. [16]

A rota inicial da infecção pelo *M. tuberculosis* ocorre nos pulmões. Gotículas contendo o bacilo se esquivam da defesa dos brônquios, devido ao seu pequeno tamanho, e penetram no alvéolo, onde são fagocitadas pelos macrófagos e células dendríticas (DC). O *M. tuberculosis* também pode infectar células não fagocitárias presentes no espaço alveolar, como células M, células endoteliais alveolares e células epitileiais do tipo I e II (pneumócitos). No início da infecção o bacilo se replica intracelularmente dentro dos fagócitos, podendo estas células atravessar pela barreira alveolar e causar uma disseminação sistêmica. [17,18] A replicação intracelular e a disseminação do patógeno podem ocorrem antes do desenvolvimento de uma resposta imune adaptativa. [11]

A defesa inicial contra o *M. tuberculosis* envolve a interação de várias populações de células imunocompetentes, particularmente de macrófagos e linfócitos T, caracterizando a resposta imune contra a tuberculose como imunidade mediada por células. [19]

Estudos avaliando o efeito da aplicação intradérmica da tuberculina, também conhecido como teste do PPD (*purified protein derivative*), observaram que a maioria dos contactantes de pacientes com tuberculose desenvolve uma reação do tipo hipersensibilidade tardia à tuberculina e são, se ainda sadios, considerados protegidos da tuberculose em atividade. Como a pápula cutânea, indicadora de um PPD positivo, contém linfócitos T reativos, acredita-se que a proteção ocorre devido à imunidade mediada por células T. O PPD negativo pode resultar da falta de uma exposição prévia ou anergia devido a alguma forma de imunossupressão. [20] Além disso, anergia à tuberculina em pacientes com tuberculose pulmonar, durante o curso da doença e a persistência após um efetivo tratamento, tem sido reportada. [21] O PPD negativo também pode indicar uma imunidade inata após exposição, sem a indução dos mecanismos adaptativos.

Desta forma, três desfechos podem ocorrer após a infecção pelo *M. tuberculosis*. Após a fagocitose por macrófagos alveolares o patógeno é destruído pela primeira linha de defesa, sem induzir a resposta imune adaptativa das células T (PPD negativo), ou a infecção estabelece uma resposta imune pelas células T e resulta na contenção da bactéria (PPD positivo), ou 3) tanto a imunidade inata como a adaptativa falham, permitindo o crescimento e disseminação da bactéria (doença). [20]

Na tuberculose, os macrófagos realizam a fagocitose e também regulam a resposta imune contra o agente, auxiliados pelas citocinas. Assim, o *M. tuberculosis* induz o macrófago, as DCs e as células T a secretarem Fator de Necrose Tumoral-α (TNF-α), citocina importante para o controle da infecção ativa, por seu papel na inflamação local e ativação de macrófagos. [22-25] Além do papel na defesa do infectado, o TNF-α pode ser um importante fator na imunopatologia da doença. [26,27] Elevadas quantidades dessa citocina estão presentes no foco da infecção e os monócitos de pacientes com tuberculose produzem mais TNF-α *in vitro* do que aqueles de doadores saudáveis. [28,29]

Após a fagocitose do *M. tuberculosis* e interação macrófago-linfócito é mediada por citocinas, os linfócitos TCD4$^+$ e células *Natural Killer* (NK) secretam Interferon-γ (IFN-γ), que é crítico para o controle da infecção pela micobactéria. Essa citocina ativa os macrófagos alveolares que passam a produzir intermediários reativos de nitrogênio e oxigênio, os quais inibem o crescimento e promovem a morte da micobactéria, além de Interleucina-12 (IL-12), que amplifica essa via. [7,22,23,30-34] No entanto, uma vez dentro do macrófago, o *M. tuberculosis* tem a capacidade de persistir no compartimento fagossômico na forma latente, pela redução da acidificação e inibição da fusão do lisossomo, impedindo a ação das enzimas líticas.[7,15,22,23,34-36] Além das células citadas, linfócitos TCD8$^+$, Tγδ e T reconhecedores de antígenos lipídicos apresentados por moléculas CD1 (TCD1 restritas), também são secretores de IFN-γ e têm propriedades citotóxicas. [37-40]

A IL-12 é produzida principalmente pelas células fagocitárias e a fagocitose do bacilo parece estar diretamente relacionada com a sua produção. [41,42] Esta citocina possui um papel chave na resposta imune ao *M. tuberculosis*, fazendo um link entre a imunidade inata e a adaptativa. Além disso, também induz células T e NK a produzirem citocinas pró-inflamatórias, incluindo IFN-γ e TNF-α e regula a produção da Interleucina- 17 (IL-17). [43-45] Em sinergismo com o TNF-α e o IFN-γ, a IL-12 ativa os macrófagos infectados, estimulando-os a eliminarem o patógeno intracelular, como um dos principais mecanismos efetores da resposta imune celular. [44] A produção desta citocina já foi detectada durante a tuberculose em atividade no infiltrado pulmonar, pleurite, granulomas e linfadenite. [46-50]

A célula TCD8$^+$ é capaz de secretar citocinas, como o IFN-γ e a IL-4, regulando o equilíbrio entre as células Th1 e Th2 no pulmão dos pacientes com tuberculose. O mecanismo pelo qual as proteínas produzidas pela micobactéria são reconhecias pela molécula de MHC classe I ainda não é completamente compreendido. [1] Estas células possuem a capacidade de lisar macrófagos infectados através da secreção de perforinas e

granzimas. Após a lise, as bactérias que se encontravam em estado latente dentro dos fagossomas são liberadas, deixando-as suscetíveis ao ataque de macrófagos recém-ativados. [51,52] As micobactérias também podem ser liberadas após o macrófago sofrer apoptose, devido à interação deste com a célula T citotóxica, via Fas / FasL. [53]

Células Tγδ reativas ao *M. tuberculosis*, encontradas no sangue periférico de indivíduos saudáveis e reatores ao derivado protéico purificado (PPD) do *M. tuberculosis*, são citotóxicas para monócitos que tiveram contato com antígenos micobacterianos e também secretam citocinas que são importantes para a formação do granuloma. [53] Células Tγδ são mais freqüentes em pacientes imunocompetentes do que em imunossuprimidos. [54,55]

O grupo de células TCD1 restritas, expressando CD8, também lisa macrófagos infectados pelo *M. tuberculosis*, introduzindo grânulos citotóxicos na célula através de poros mediados pela perforina. [56] Por meio da molécula de CD1, lipídios ou glicolipídios são apresentados às células T. [1]

Apesar da ação de todos esses mecanismos de defesa, o bacilo sobrevive e se multiplica no macrófago, fazendo com que células mononucleares, como os linfócitos T, grandes produtores de IFN-γ e TNF-α, sejam recrutadas para a formação do granuloma no foco da infecção. [57-61] Durante este processo, os BAARs resistentes podem ser visualizados no interior dos macrófagos pela coloração de Ziehl-Neelsen. [62] O granuloma aumenta de tamanho, à medida que ocorre o recrutamento de mais células e a área central torna-se necrótica com a morte dos macrófagos infectados, a ativação do sinal de apoptose para as células T e a ação das enzimas lisossômicas que lesam o tecido. [34,62-69] A área de necrose caseosa é circundada por células mielóides, células gigantes multinucleadas e linfócitos $TCD4^+$ e $TCD8^+$. Conforme esta área se solidifica, o bacilo, antes visível, desaparece e a lesão é calcificada e reabsorvida, ou permanece em estado de liquefação. [62] O *M. tuberculosis* se replica exponencialmente nestas áreas de necrose liquefeita. Se ocorrer a

ruptura da lesão para as vias aéreas, os bacilos podem atingir outras áreas do próprio pulmão infectado, ou serem expelidos para o ambiente, infectando novos indivíduos. [34, 65-70]

Estudos têm sugerido, que a imunidade protetora ao *M. tuberculosis* requer além das células Th1, células Th17, produtoras de IL-17. A diferenciação das células Th17é iniciada pela ativação de células T naive, na presença de Interleucina-6 (IL-6) e Fator β de Crescimento e Transformação (TGF-β), e é mantida pela Interleucina- 23 (IL-23) [71]. Estas células apresentam importantes funções pró-inflamatórias, e são definidas pela produção das citocinas IL-17A a IL-17F. Tanto a IL-17A e IL-17F apresentam propriedades próinflamatórias e atuam em vários tipos celulares induzindo a expressão de citocinas (IL-6, IL-8, GM-CSF e G-CSF), quimiocinas (CXCL1, CXCL10) e metaloproteinases, importantes no recrutamento, ativação e migração de neutrófilos [72]. A análise funcional da IL-17 sugere o envolvimento desta citocina na proteção do hospedeiro contra alguns patógenos específicos, incluindo o *M. tuberculosis*. Células dendriticas após a interação com a micobactéria, produzem IL-12 e IL-23, que atuam de forma sinérgica para produção de IL-17 [73-76].

A constante produção de citocinas inflamatórias essenciais, como TNF-α e IFN-γ, assim como outros produtos bactericidas, incluindo reativos do nitrogênio e do oxigênio, gerados pelos macrófagos e células efetoras imunes ao redor, parece ser o fator chave para a latência do *M. tuberculosis* no granuloma. [71,72]

Apesar do efeito protetor da resposta Th1 contra a tuberculose, algumas citocinas, como o TNF-α, estão correlacionadas com a imunopatogenia da doença. [79,80] A destruição tecidual relaciona-se com a elevação da expressão desta citocina e, para limitar esta ação deletéria, a produção sistêmica é suprimida e há elevação de receptores solúveis que bloqueiam sua atividade. Além disso, o excesso de resposta inflamatória, iniciada contra o *M.tuberculosis*, é antagonizada pelas citocinas antiinflamatórias como a IL-4, IL-10, TGF-B e as células T regulatórias. [32,80-84]

Na presença da IL-10, tanto a proliferação das células, quanto a produção de IFN-γ são inibidas, sendo que a IL-10 também compromete os mecanismos microbicidas dos macrófagos e a apresentação de antígenos. [85-91] Como citocina antiinflamatória, a IL-10 relaciona-se ao aumento da gravidade da doença, pela inibição da resposta imune protetora. A desativação dos macrófagos, por efeito de sua ação, faz-se pela inibição de moléculas co-estimuladoras e da síntese de citocinas. [92] Previne danos teciduais, pela regulação da inflamação e da apoptose, tendo efeito contrário ao do TNF-α. Sua produção pelos macrófagos é estimulada por componentes da parede celular micobacteriana. [93-96]

O TGF-β, citocina supressora do perfil Th1, produzida pelos macrófagos, também participa na indução da fibrose em diferentes modelos experimentais de lesão pulmonar, incluindo a inflamação produzida pelo *M. tuberculosis*. [97,98] A ação pró-inflamatória e antiinflamatória do TGF-β depende de sua concentração. Em baixas concentrações, atua como fator quimiotático para monócitos e induz a secreção de Interleucina-1α (IL-1α) e TNF-α. [99] Durante a fase crônica da tuberculose sua produção aumenta tornando-se máxima e inicia processos anti-inflamatório e regenerativo. Essa elevação do TGF-β causa a progressão e cronicidade da doença, ocorrendo diminuição da hipersensibilidade observada em pacientes e animais experimentais com tuberculose avançada. [100,101] O TGF-β, em alta concentração, desativa macrófagos, inibe a expressão e funcionamento de receptores para IFN-γ, IL1-α e IL-2 e diminui a produção do TNF-α, eventos paralelamente relacionados com aumento do crescimento micobacteriano intracelular; ainda, inibe a proliferação de células $TCD4^+$, induz a expressão de receptores CD8 e aumenta a produção de citocinas do perfil Th2, como IL-10. [80,102-107]

A ação anti-inflamatória se dá pela inibição da produção de IFN-γ pelas células $TCD4^+$.

Rojas et al., [83] estudando células TCD4$^+$ ativadas pelo *M. tuberculosis*, não observaram efeito sinérgico entre TGF-β e IL-10. Relatam, no entanto, inibição da resposta das células TCD4$^+$ ao *M. tuberculosis* por ambas citocinas, porém, por diferentes mecanismos de ação. Além disso, Hirsch et al., [104] empregando cultura de células mononucleares do sangue periférico (PBMC) de indivíduos com tuberculose, observaram aumento significativo na produção de IFN-γ, na presença de anticorpos anti-TGF-β, comprovando o efeito imunomodulador dessa citocina. Assim, a tuberculose em atividade associa-se com diminuição de resposta Th1 e aumento de produção e ação de citocinas supressoras de perfil Th2, como TGF-β e IL-10, que agem desativando macrófagos, modulando as citocinas pró-inflamatórias e diminuindo a função apresentadora de antígenos da célula T.

O acúmulo e ativação de células Tregs no foco da infecção, durante a tuberculose, pode inibir o desenvolvimento de uma resposta celular multifuncional. Estas células, de ocorrência natural ou induzida, são uma população heterogenia de células TCD4$^+$ e algumas podem co-expressar marcadores de ativação como o CD25, antígeno-4 de linfócito T citotóxico (CTLA-4) e receptor de fator de necrose tumoral induzido por glicocorticóide (GITR), sendo que o principal deles é o fator de transcrição FoxP3. As células Tregs podem suprimir uma resposta imune excessiva, prevenindo assim o desenvolvimento de uma imunopatologia, incluindo a inibição da produção de IFN- γ por células TCD4$^+$ e a função citolítica dos linfócitos TCD8$^+$, induzidas pelo *M. tuberculosis*, o que poderia resultar em uma infecção crônica ao invés do clerance do patógeno. [108]

O reconhecimento inicial de microrganismos é mediado por receptores celulares expressos em células da imunidade inata. Entre esses receptores destacam-se os receptores semelhantes a toll (TLRs, toll-like receptors), através dos quais as células são capazes de

reconhecer microrganismos com conseqüente estimulação da fagocitose, ativação microbicida e produção de citocinas. [109,110]

Os TLRs são uma família de proteínas transmembrânicas caracterizadas por um domínio extracelular com repetições ricas em leucina (LRR) e um domínio intracelular Toll/IL-1R (TIR). [111] Os TLRs mantiveram-se evolutivamente conservados entre os insetos e mamíferos, podendo também ser encontrados em plantas. [101,111] Em mamíferos, o domínio TIR também está presente em várias proteínas citoplasmáticas, incluindo as moléculas adaptadoras de sinalização MyD88 e TIRAP, ambas com funções na transdução de sinal do receptor. A sinalização do TLR feita através destas proteínas adaptadoras resulta na translocação do fator de transcrição de genes pró-inflamatórios e co-estimulatórios. [112-114]

Todos os TLRs podem utilizar a proteína adaptadora de sinalização MyD88 para propagar o sinal para os genes alvo e gerar uma rápida resposta protetora, tanto pela ativação do Fator de transcrição nuclear (NF)-κB, como por outras vias. O MyD88 também pode ser utilizado em outras vias de sinalização inflamatória, como a da IL-1. Entretanto, existem pelo menos dois receptores, TLRs-3/4, que podem utilizar uma via alternativa de adaptadores, como o domínio TIR-contendo indução adaptativa para o IFN-β (TRIF) e TRIF-com molécula relacionada à adaptação (TRAM), este último utilizado somente pelo TLR-4, podendo assim induzir uma resposta distinta da via fator mielóide de diferenciação 88 (MyD88). Desta forma o TLR-4 pode utilizar tanto o domímio MyD88/TIR, como o TRAM/TIR, sendo este último crítico para a indução da maturação das DCs, promovendo assim resposta distintas. Enquanto a via da MyD88 induz a ativação típica pelo NF-κB, os domínios TRAM e TRIF ativam o fator regulador de IFN -3 (IRF-3) e IRF-7, além da indução tardia da ativação do NF-κB. [115]

Os TLRs reconhecem certos constituintes microbianos denominados padrões moleculares associados ao patógeno (PAMPs). Os PAMPs são produtos produzidos e

conservados pelos microrganismos sendo essenciais para o seu metabolismo e sobrevivência. [116] Os TLRs reconhecem, individualmente, um repertório distinto, mas limitado de PAMPs, tais como, glicolipídeos, LPS de bactérias Gram-negativas, lipopeptídeos e peptídeoglicanos e outros componentes da parede celular microbiana. [117] O TLR-4 e LPS, TLR-5 e flagelina, TLRs-1/2/6 e lipoproteínas e TLRs-3/7/8/9 e diferentes ácidos nucléicos são exemplos de pares ligantes bem caracterizados. [118]

A expressão de TLRs na superfície celular pode ser detectada por anticorpos monoclonais principalmente em macrófagos e DC imaturas. Entretanto, a expressão é observada em outras células, incluindo mastócitos, eosinófilos, neutrófilos, linfócitos T e B, além das células epiteliais, endoteliais, cardiomiócitos e adipócitos. [119-121]

A estimulação dos TLRs, através dos produtos microbianos, ativa a resposta imune inata. Esta ativação resulta na indução da síntese de substâncias microbicidas e citocinas pró-inflamatórias, bem como, a ativação de DCs que regulam positivamente a expressão de moléculas co-estimulatórias e aumentando a expressão de antígenos do MHC, tornando mais efetiva a apresentação de antígenos a linfócitos T e B. Quando a resposta imune inata não é suficiente para eliminar a infecção, ocorre a indução da resposta imune adaptativa. Quando ativadas pelos TLRs, células apresentadoras de antígenos (APCs) liberam níveis elevados de citocinas pró-inflamatórias, como o TNF-α, IL-5, IL-6, IL-8 e IL-12, quimiocinas e óxido nítrico. Ocorre, também, aumento da expressão de moléculas co-estimulatórias como, o CD40, CD80 e CD86. Estas alterações na função das células apresentadoras de antígenos levam a indução da resposta imune adaptativa, determinando a diferenciação em células Th_1 que promovem a imunidade celular ou Th_2, que induz a resposta humoral. [121]

O reconhecimento do *M. tuberculosis* pelos TLRs na superfície celular é um determinante importante da eficácia da resposta imune do hospedeiro. Os produtos micobacterianos podem ser reconhecidos pelos TLR-2 e TLR-4. [122]

O TLR-2 é o principal receptor para os constituintes micobacterianos, reconhecendo ligantes de superfície, como a lipoarabinomanana (LAM), o seu precursor "phosphatidylinositol mannoside" (PIM) e a lipopotreína de 19-kDa. [123-127] Este receptor está envolvido na resposta à micobactéria íntegra, podendo influenciar também no seu processamento após a fagocitose pelos macrófagos. [128-131] Além disso, a própria micobactéria possui a propriedade de induzir a expressão do TLR-2, através do recrutamento deste receptor para o envelope fagossômico dentro dos macrófagos, apresentando este mesmo receptor como um sensor primário para o reconhecimento do microrganismo invasor. [132,133]

A estimulação dos macrófagos através do TLR-2 inicia a ativação da resposta imune, como produção de TNF-α e IL-1, processamento de antígenos, ativação de linfócitos T e consequente produção de IFN-γ.[129] Evidências recentes sugerem um papel duplo para o TLR-2, que pode atuar tanto na indução de mediadores inflamatórios, como o TNF-α e a IL-12, como interagir com o ESAT-6, proteína secretada pelo *M. Tuberculosis*, fato que induziria uma menor sinalização dos TLRs, por impedir a ligação do MyD88, o que pode levar a inibição da ativação do NF-κB e dos genes envolvidos na ativação da resposta imune inata do hospedeiro. [134,135] O TLR-2 também pode limitar a predisposição dos macrófagos em supra regular a expressão do complexo de histocompatibilidade classe II (MHC II) em resposta ao IFN-γ. [125,136]

O TLR-4 não é um receptor apenas para ligantes exógenos, como o LPS de bactérias gram-negativas, podendo reconhecer ligantes endógenos, como fibronectina, proteínas do choque tóxico e oligossacarídeos hialurônicos. [137] Os ligantes do *M. tuberculosis* para este receptor ainda não são bem conhecidos, mas um estudo recente demonstrou que a via de sinalização da proteína micobacteriana de choque térmico 65 é exclusivamente através do TLR-4. [138] Além disso, foi comprovado que o BCG induziu a transcripção da quimiocina CXCL8 em neutrófilos humanos através dos TLRs-2/4, em conjunto com o MyD88. [139] Em

outro estudo, a glicoproteína de 38-kDa, purificada do *M. tuberculosis*, induziu a ativação de proteínas kinases via TLR-2/4 e a subseqüente produção de citocinas pró-inflamatórias, como TNF-α e IL-6 em monócitos humanos. [140]

Apesar de componentes inativados da micobactéria terem sido demonstrados como agonistas do TLR-4 em estudo *in vitro*, estudos *in vivo* com murinos sobre o papel deste receptor no reconhecimento do *M. tuberculosis* têm demonstrado evidências controversas quanto à sua ativação, mesmo com a utilização da mesma cepa de camundongo. [128,141] Em um estudo, foi demonstrado que camundongos deficientes para TLR-4 apresentavam uma susceptibilidade à infecççao pelo *M. tuberculosis* semelhante ao grupo de animais selvagem. [142] No outro, foi reportado um maior crescimento micobacteriano nos pulmões, baço e fígado e uma diminuição da sobrevivência após a infecção nos animais deficientes para TLR-4 quando comparados ao grupo selvagem. [143]

As moléculas que geram sinais dependentes do TLR-2 são totalmente dependentes da MyD88, mas como já foi citado anteriormente, o TLR-4 possui opções de sinalização independentes da MyD88, podendo promover a ativação do processo inflamatório através de outras vias. [122]

A tuberculose, assim como outras doenças infecciosas, tem caráter complexo uma vez que os diversos aspectos da interação parasito-hospedeiro contribuem para a ocorrência do desfecho. Neste cenário tem importante participação a susceptibilidade genética humana para a doença após a exposição ao *M. tuberculosis*. [144-148] Fatores ambientais, epigenéticos e a deriva genética podem ser responsáveis por diferenças presentes entre diversas populações e possivelmente entre diferentes etnias. [149] Ocorrências familiares têm sido reportadas, por gêmeos monozigóticos terem maiores taxas de concordância para a doença, quando comparados aos dizigóticos. [146,147]

Polimorfismos de base única (SNPs) são variações ou mutações na sequência do DNA que ocorrem quando uma única base [adenina (A), timidina (T), citosina (C) ou guanina (G)] é alterada na sequência do genoma. Muitos SNPs não apresentam efeitos na função celular, mas alguns, principalmente aqueles que afetam a região funcional do gene, podem predispor indivíduos a doenças ou influenciar a resposta perante a um tratamento com drogas. [150] Estudos têm identificado associações entre polimorfismos de diversos genes candidatos e a manifestação clínica da doença ativa, assim como no caso de alelos de genes codificadores do *IFNG* e do receptor para a IL-12, que estão associados com a tuberculose em algumas populações. [149-159]

Diversos genes codificadores de diferentes citocinas podem afetar a susceptibilidade à tuberculose. Polimorfismos podem influenciar diretamente na transcripção dos genes ou, indiretamente, através da um desequilíbrio de ligação com outros polimorfismos que podem ocorre em diferentes regiões do gene de determinada citocina. [160] A natureza polimórfica dos genes das citocinas pode conferir flexibilidade à resposta imune, com alguns alelos promovendo uma produção diferente dessas proteínas, podendo influenciar no desencadeamento de infecções bacterianas ou virais ou aumentar a susceptibilidade/resistência às doenças autoimunes. [160-162]

Dois SNPs para o gene do *IFNG* têm sido bastante estudados. O primeiro se localiza no primeiro intron, a 874 pares de base do sítio onde se incia a translação, local que coincide com a área de ligação para o NF-κB. [163] O outro SNP se localiza no terceiro intron a 2.109 pares de base do sítio onde se incia a translação e tem sido associado com a regulação da transcripção do gene do *IFNG*. [164,165]

Estudos têm reportado que o alelo T do SNP +874 no gene do *IFNG* está correlacionado com uma alta produção desta citocina, enquanto que alelo A correlaciona-se com uma baixa produção. [164] Em estudos realizados na Espanha e no Brasil foi verificada

uma associação entre o genótipo +874TT e uma maior susceptibilidade à tuberculose, devido à baixa produção desta citocina.[166,167] Estudo recente de meta-análise incluindo 11 estudos confirmou o efeito protetor do alelo +874T nesta infecção.[168] Anand et al [169] também verificaram efeito protetor deste alelo, em estudo no qual PBMC de pacientes com tuberculose pulmonar que possuíam o alelo T apresentaram uma maior expressão de IFN-γ, após ativação com *M. tuberculosis*, do que aqueles que apresentavam o alelo A. Alterações de baixa e alta produção de IFN-γ podem estar relacionadas ao fato do alelo +874T apresentar sítio de ligação para a região de acoplamento do NF-kB, fator este que induz a expressão desta citocina. [170]

Estudos funcionais demonstraram que pacientes com tuberculose que possuem o genótipo *IFNG* +874AA produziam um quantidade menor de IFN-γ do que aqueles que apresentavam genótipos +874AT e +874TT. [162,171]

Em pacientes indianos com tuberculose pulmonar foi verificada uma possível associação do SNP *IL4* -590C/T com a susceptibilidade à doença, entretanto este SNP não teve associação com os níveis de IL-4, produzidos por PBMC destes pacientes. Além disto, neste mesmo estudo, os autores não conseguiram associar o SNP +874 T/A do *IFNG* nem com a susceptibilidade à doença, nem com os níveis produzidos desta citocina. [172]

Apesar da associação do SNP +2109 A/G com a regulação da transcrição do gene do *INFG*, suas contribuições ainda não estão bem claras. [164,165] Alguns estudos relataram que este SNP pode estar associado com algumas doenças, como a esquistossomose hepática. [165,173] Outro demonstrou que *IFNG* +874 e +2109 podem estar associados com a tuberculose em atividade, através da correlação destes SNPs com a positividade para o diagnóstico microscópico e para cultura de micobactéria, apesar do estudo não ter demonstrado que estes dois SNPs estejam associados com a susceptibilidade à doença. [174]

Selvaraj et al. [175] sugeriram que o polimorfismo 3´UTR+1188A/C para a *IL12B* está associado com diferentes níveis de IL-12p40, o que poderia influenciar nos níveis de IL-12p70 ou de IL-23, podendo ter um papel na indução e manutenção da resposta imune celular adquirida contra a tuberculose pulmonar. Neste mesmo estudo, também foi avaliada a produção de outras citocinas, como IL-10, IFN-γ, IL-2 e IL-6, na presença de um SNP correspondente, porém houve alteração nestes níveis quando comparados os diversos genótipos e os grupos de doentes e controles.

Estudos com pacientes com hepatite B verificaram que SNPs em regiões específicas dos genes do *IFNG* e da *IL12B* podem influenciar na produção destas citocinas. Foi verificado que o SNP +1188A/C da subunidae p40 do gene da IL-12 está envolvido na regulação da expressão desta citocina. [176] Outro estudo que avaliou a produção de IL-12 através da cultura de PBMCs estimuladas com tuberculina (PPD) verificou níveis mais elevados desta citocinas em indivíduos homozigotos CC, do que aqueles que eram heterozigotos (AC) ou homozigotos AA. Neste mesmo estudo também foi verificado que indivíduos que apresentavam o alelo A produziam níveis inferiores desta citocina após o estímulo com o PPD. [177]

Polimorfismos no gene do *TNF* podem variar entre populações e podem estar associados a algumas doenças autoimunes e alguns estudos demonstraram associações entre estes SNPs e a tuberculose. [178-180] O SNP funcional - 308 A/G na região promotora do gene do TNF-α tem sido descrito como uma região altamente polimórfica, com o alelo A associado à uma alta expressão deste gene. Estudos demonstraram que o alelo A pode estar associado à doenças, como malaria, leishmaniose mucocutânea e a hanseníase. [181-184] Observações com pacientes com tuberculose, de diferentes etnias, demonstraram evidências conflitantes para a associação entre polimorfismos nos genes do *TNF* e da *IL10* (-1082) com a tuberculose,

entretanto não foram avaliadas diferentes formas clínicas da doença. [166] Outros estudos não encontraram associações entre alelos de SNPs para o gene o *TNF* e a tuberculose. [184-192]

A região promotora do gene da *IL17A* possui diversos sítios para fatores de transcrição, incluindo sítios de ligação para o fator de transcrição nuclear de células T ativadas (NFATC), que possui um papel importante na regulação da expressão da IL-17A. [193] Estudos têm associado SNPs no gene da *IL17A*, como aquele na posição -197 G/A, com doenças como a artrite reumatóide, colite ulcerativa e na rejeição de transplantes de medula. [194-197] O alelo A neste SNP está associado a uma alta produção desta citocina, com elevada atividade promotora e uma maior afinidade com o Fator Nuclear de Células Ativadas (NFAT), em comparação com o alelo G. [198] Em outro estudo foi demonstrado que haplótipos constituídos por SNPs no gene da *IL17F*, como 126A/G e 161A/G, têm contribuído para a susceptibilidade à doença de Behçet. [199]

Diferentes níveis na produção de IL-10 diferem bastante entre indivíduos, e isto pode ocorrer devido à presença de SNPs na região promotora do gene da *IL10*. [200,201] Três SNPs, nas posições -1082G/A, -819T/C e -592C/A, estão associados a diferenças na expressão desta citocina. [202] Estudo revelou que indivíduos com o alelo G para o SNP *IL10* -1082G/A apresentam aumento da gravidade à infecção por pneumococos. [203] Em pacientes com aids estudos apresentam divergências quanto à associação do -1082G/A e a elevada produção de IL-10, sendo que em um deles esta elevação foi associada à presença do alelo G e no outro estudo à presença do alelo A[199,204] Estudo que avaliou a produção de IL-10 com SNPs na região promotora do gene da *IL10*, como o -819 C/T, não verificou nenhuma associação em cultura de PBMC estimulada com PPD. [177]

Associações entre SNPs no gene do *TGFB1* e diversas doenças não infecciosas têm sido identificadas, como na asma, câncer, artrite, osteoporose, infarto do miocárdio e lupus eritrematoso. Poucos trabalhos têm avaliado a influência deste gene em doenças infecciosas.

Estudo com brucelose encontrou associação entre os SNPs *TGFB1* +869 T/C e +915G/C e o risco da doença. [205] Evidências indicam que estes SNPs estão envolvidos com uma elevada ou baixa produção desta citocina durante infecção com dengue, tanto em experimentos *in vitro* como *in vivo*. [206] Estudo avaliando pacientes com Doença de Chagas também encontrou associação para o SNP *TGFB1* +869T/C, sugerindo que o alelo C seja um fator de risco para susceptibilidade à doença. [208] Estudo avaliando pacientes com tuberculose não encontrou associação entre a doença e *TGFB1* SNPs. [205]

Polimorfimos no intron 2 do *TLR2*, como os SNPs na região -16943A>T e as repetições curtas tipo GT (short tandem GT repeats), podem estar ligadas à diferenças quanto à susceptibilidade à doença. [208] Estudos reportaram que variações no *TLR2* podem predispor indivíduos à sarcoidose, à infecção com patógenos Gram-positivos e também à tuberculose. [208-210] O alelo T na região promotora do gene do TLR-2 -16943A/T tem sido considerado como protetor em casos de sensibilização atópica, asma alérgica e febre do feno em crianças que vivem em fazendas na Europa. [211] Outros SNPs no *TLR2* (Arg753Gln e R677W) também estão associados com um aumento no risco em desenvolver tuberculose. [212-214] No estudo de Lorenz et al. [215] foi reportado pela primeira vez que um polimorfismo no *TLR2* (Arg753Gln) pode levar a uma diminuição da resposta dos macrófagos aos peptídeos micobacterianos, resultando em uma resposta imune mais atenuada no homem. Baseado nestes dados, Ogus et al. [209] avaliaram este mesmo SNP em pacientes com tuberculose pulmonar e, demonstraram que os pacientes apresentavam uma maior frequência do genótipo AA em relação aos indivíduos controles, sugerindo que os indivíduos com genótipos AA e GA têm maior risco de desenvolver a doença ativa do que aqueles que apresentam genótipo GG. [216] Este polimorfismo pode ser um dos fatores que influenciam na susceptibilidade genética para tuberculose, apesar de outros fatores ou defeitos em diferentes etapas da resposta imune, inclusive diferentes polimorfismos no próprio *TLR2* ou em outros TLRs,

também podem ser responsáveis pela progressão da infecção pelo *M. tuberculosis* em direção à doença. [166]

O gene do *TLR4* apresenta uma maior variação na sequência que codifica o domínio de sinalização extracelular, do que na região codificadora do domínio citoplasmático. Estudos confirmaram que dois SNPs não sinônimos, ou seja, nos quais a mudança de uma base promove mudança no aminoácido, do TLR-4 possuem uma frequência populacional maior do que 5%. Estes consistem na transição A/G que promove a substituição do ácido aspártico pela glicina (Asp299Gly) e na transição a C/T que promove a substituição da treonina pela isoleucina (Thr399Ile). Estes SNPs existem em uma forma co-segregada, resultando na possibilidade de existência de 4 haplótipos na população geral, sendo eles nomeados como wt/wt, Asp299Gly/wt, Thr399Ile/wt e Asp299Gly/Thr399Ile. [217] O *TLR4* Asp299Gly parece prejudicar a resposta imune ao LPS, mesmo quando apresentado na forma heterozigota. [21]

Em trabalho realizado na Gâmbia não foram encontradas associações entre a variação *TLR4* Asp299Gly e a tuberculose pulmonar, sugerindo que este SNP não possui papel na susceptibilidade à tuberculose pulmonar nesta população. Também, não foram encontradas associações entre a presença do *TLR4* Asp299Gly alelo G e uma baixa resposta imune ao LPS, utilizando-se ensaio com sangue total, nesta mesma população. [216] Outro estudo com tuberculose pulmonar, na população mexicana, verificou um aumento na expressão de TLR-4, antes do tratamento antituberculose em relação a um grupo controle, sendo que esta se normalizou ao final do tratamento, entretanto os autores não observaram uma associação destes resultados com os genótipos e frequências de alelos do *TRL4* Asp299Gly. [218]

Todos os TLRs podem ativar rapidamente o NF-kB. Esta ativação induz a produção e secreção de mediadores pró-inflamatórios, como citocinas, incluindo o TNF-α, IL-1, IL-12, IL-18; quimiocinas, que atraem neutrófilos, células NK, células T e outras DC e macrófagos para o foco das infecção; e NO, que irá atuar na eliminação do patógeno. [219,220] Mudanças

qualitativas ou quantitativas deste fator podem gerar alterações na regulação da transcrição dos genes das citocinas inflamatórias, levando a alterações na produção destas. [118, 221] Defeitos no reconhecimento do *M. tuberculosis* e/ou na subseqüente sinalização intracelular do NF-kB, podem estar relacionados à polimorfismos presentes no gene do *TLR2*, levando a uma baixa produção de citocinas e baixa produção de células T $CD4^+$, em pacientes com tuberculose extrapulmonar. [217] Além disso, foi demonstrado que os polimorfismos TLR4 Asp299Gly e Thr399Ile induzem uma diminuição da atividade do NF-kB, quando comparados ao gene normal do TLR-4, sugerindo que possam influenciar no fenótipo, levando a uma baixa produção de citocinas durante a resposta imune. [222]

O desenvolvimento da tuberculose ativa depende de um complexo relacionamento entre o homem e o bacilo. É fundamental ressaltar que 10% dos indivíduos imunocompetentes que entraram em contato com o *M. tuberculosis* irão desenvolver a doença ativa ao longo de suas vidas e, que os mecanismos moleculares responsáveis por essa susceptibilidade ao bacilo ainda não estão bem compreendidos. [223]

Os receptores TLRs e as citocinas possuem um papel chave na defesa contra o bacilo, e seus genes promotores, dependendo do polimorfismo gênico apresentado, podem influenciar na estimulação e manutenção de uma resposta imune ineficiente contra o bacilo, sendo considerados genes candidatos à susceptibilidade do hospedeiro ao aparecimento da tuberculose em atividade.

A associação entre os polimorfismos dos TLRs e das citocinas com a susceptibilidade a agentes infecciosos têm sido reportada, entretanto, poucos estudos têm investigado seus papéis na modulação da resposta imune durante a tuberculose pulmonar.

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III – Objetivo

1. Objetivo geral

Verificar a influência de polimorfismo nos genes dos receptores *TLR2* e *TLR4* e das citocinas *IFNG*, *IL12B*, *TNF*, *IL17A*, *IL10*, *TGFB1* na resposta imune em pacientes com tuberculose pulmonar, durante o tratamento antituberculose.

2. Objetivos específicos:

1 – Avaliar a influência de SNPs nos genes dos receptores *TLR2* e *TLR4* e das citocinas *IFNG*, *IL12B*, *TNF*, *IL17A*, *IL10*, *TGFB1* sobre a expressão destes genes, em pacientes com tuberculose pulmonar.

2 – Avaliar a expressão gênica do mRNA dos receptores TLR-2 e TLR-4 pela PCR em tempo real, em PBMC do sangue periférico de pacientes com tuberculose pulmonar no início, aos três meses e ao final (seis meses) do tratamento antituberculose.

3 – Avaliar a expressão dos receptores TLR-2 e TLR-4 na superfície das células mononucleares do sangue periférico de pacientes com tuberculose pulmonar, através da citometria de fluxo, no início, aos três meses e ao final (seis meses) do tratamento antituberculose.

4 – Avaliar a expressão gênica do mRNA do fator de transcrição NF-kB pela PCR em tempo real, em PBMC do sangue periférico de pacientes com tuberculose pulmonar no início, aos três meses e ao final (seis meses) do tratamento antituberculose.

5 – Avaliar a expressão gênica do mRNA das citocinas TNF-α, IL-12, IFN-γ, IL-10, TGF-β e IL-17 pela PCR em tempo real, em PBMC do sangue periférico de pacientes com tuberculose pulmonar no início, aos três meses e ao final (seis meses) do tratamento antituberculose.

6 – Avaliar a produção de citocinas TNF-α, IL-12, IFN-γ, IL-10, TGF-β e IL-17 no soro de pacientes com tuberculose pulmonar, através do método de ELISA, no início, aos três meses e ao final (seis meses) do tratamento antituberculose.

IV- Capítulo 1

Influence of *TLR-2* -16934 and GT repeats polymorphisms in immune profile of pulmonary tuberculosis patients undergoing anti-tuberculosis treatment.

Abstract

It is estimated that one-third of the total world population is latently infected with *M. tuberculosis* and only 5-10% of the infected individuals will develop active TB disease during their life-time. The reason why some infected individuals develop active disease, while others do not is not yet entirely understood. Given the central role of TLR-2 in the incitement of inflammation, polymorphisms in its gene might be involved in both infectious and inflammatory diseases. The aim of this study was to evaluate the influence of *TLR2* -16934A/T and GT repeat polymorphisms on the immune response of PTB patients undergoing anti-TB treatment at different time points of anti-tuberculosis treatment: T1 (beginning), T2 (3 months) and T3 (end). For this we genotyped TLR2 -16934 and $(GT)_n$ repeats polymorphisms and evaluated the immune response of pulmonary tuberculosis patients during the time of anti-tuberculosis treatment. The present study suggests that *TLR2* -16934A/T and GT repeats polymorphisms can influence differential TLR-2, NF-κB and cytokine levels during anti-TB treatment. We also suggest that PTB patients with *TLR2* -16934 AA genotype may have a worst outcome of the disease, since they have a lower IFN-γ, cytokine essential to initiate the protective immunity to active TB. This association could not be made in our study due to the low number of patients evaluated. Since TLR-2 play a major role in initiating immune response against *M. tuberculosis* other polymorphisms in *TLR2* would be crucial to better understand protective immune responses and may serve as biomarkers of protection or susceptibility to TB.

Key words: Pulmonary tuberculosis, polymorphisms, toll-like receptor 2, cytokines

1. Introduction

Toll-like receptors (TLR) are proteins associated with the human bodies response to *Mycobacterium tuberculosis* (*M. tuberculosis*) and when stimulated can quickly activate NF-κB pathway which results in production and secretion of pro-inflammatory mediators including cytokines TNF-α, IL-1, IL-12, IL-18, chemokines and nitric oxide (Korbel et al 2008; Liu and Modlin 2008). Qualitative or quantitative changes of this nuclear transcription factor may cause changes in the regulation of gene transcription of inflammatory cytokines, leading to changes in production of these mediators (Motsinger-Reif1 et al. 2010; Sutmuller et al. 2006).

TLR-2-deficient mice are highly susceptible to M. tuberculosis infection, suggesting mutations that affects TLR2 expression my impair host response to this pathogen (Drennan et al. 2004; Reiling et al 2002). The G to A (Arg753Gln) polymorphism at position 2258 in exon 3 and the guanine-tymine (GT) microsatellite repeat polymorphism (100bp upstream of the translational star site), have been associated with susceptibility to clinical TB disease (Ogus et al. 2004; Yim et al. 2006). Defects in the recognition of MTB and/or subsequent intracellular signaling of NF-κB may be related to polymorphisms in *TLR-2* gene, leading to low production of cytokines and low expression of $CD4^+$ T cells in patients with extra pulmonary TB (Zhang et al 1994).

It is estimated that one-third of the total world population is latently infected with *M. tuberculosis* and only 5-10% of the infected individuals will develop active TB disease during their life-time. The reasons why only some of the individuals exposed to *M. tuberculosis* develop active disease while others eradicate or limit the disease remains unknown. Also little is known about the mechanisms that influence the rate of progression from infection to disease. Evidence suggests that genetic factors may be important

determinants of increased susceptibility to progressive TB disease development (Kaufmann 2002).

Since TLRs have an essential role in initiating protective immune response to TB, SNPs in its genes could interfere in this response, leading to active TB disease. Many studies have reported association of *TLR* SNPs with TB, but few have demonstrated its role to modulate the immune response during active TB disease. Thus the aim of this study was to verify the influence of *TLR-2* -16934A/T and GT repeats polymorphisms on the immune profile of Brazilian patients with pulmonary TB (PTB) under anti-TB treatment.

2. Material and Methods

2.1. Study population

The study group enrolled 31 Brazilian patients attending the Infectious and Parasitic Diseases Services at Botucatu Mecdical School University Hospital – UNESP, Botucatu Teaching Health Centre and Primary Healthcare units of Botucatu and surrounding region with pulmonary TB diagnose confirmed by sputum smear or culture positive for *M. tuberculosis*, or else by clinical-epidemiologic data, laboratory and image exams compatible with active tuberculosis. Patients with pulmonary tuberculosis concurrent with other active granulomatous disease or HIV positive were excluded. All patients diagnosed with pulmonary TB received treatment for six months, using different combinations of the four first-line drugs: isoniazid, ethambutol, pyrazinamide and rifampicin. For the evaluation of immunological function, patients samples were collected based on the anti-tuberculosis treatment time line, defined as T1: after diagnosis and with no more than one month of treatment; T2: with three months of treatment; and T3: with six months of treatment. As

normal controls (C), we studied 20 health care workers from Botucatu Medical School (Botucatu, São Paulo, Brazil), 9 males (mean age 40.4 years) and 11 females (mean age 34.1 years), without clinical complaints and with no history of TB disease, autoimmune disease and other infectious disease. All controls were tuberculin test (PPD) positive (hardering ≥ 5mm). All patients and controls agreed to participate in the study, after due clarification and signing of the written informed consent.

2.2. Genotyping

Two *TLR2* polymorphisms were studied: *TLR2* -16934A/T and *TLR2* GT repeats.

For this proposal peripheral blood (5 mL - per standard procedure) was drawn in EDTA, from patients (*n*=31) with pulmonary tuberculosis and controls (*n*=20) groups at base line T0, and genomic DNA was extracted from leukocytes employing DNAzol commercial reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantification and purity determination of the extracted DNA was determined on a spectrophotometer (Nanodrop 2000 ThermAmplification of the genomic regions of interest was made of 50 ng /μL of DNA performed by PCR using 3μL of DNA, Master Mix (Invitrogen) and primers (20μM). Specific primers for each of the *TLR-2* polymorphisms investigated are described in Table 1. Conditions of PCR comprised 10 min of initial denaturation at 95°C, then repeated cycles of 94°C for 30s, annealing for 30s (see Table 1 for number of cycles and temperatures) and 72°C for 30s, then 10 min at 72°C for final extension. PCR product was cleaned up using Edge Biosystems Ultra Kit (MFRS-DTR0850), spinning plates at 850g for 5 min, and then verified in a 1% agarose gel stained with ethidium bromide. Clean PCR product (1μL), BigDye® Terminator (Applied Biosystems) and forward primer (5μM/μL) were used to perform sequencing reaction. Conditions of sequencing

comprised 1 min at 95°C, then 35 cycles at 96°C for 10 s, 50°C for 5s and 60°C for 4 min. Sequencing product was cleaned up using Edge Biosystems Ultra Kit (MFRS-DTR0850), spinning plates at 850g for 5 min, and then placed in the 3730xl DNA Analyser. Results were then analysed by the Sequencher™ 5.0 – Build 7081 Software.

Table 1. Primer sequences and PCR conditions used for genotyping and sequencing of *TLR2* polymorphisms

Primers	Polymorphism	Primers	Annealing temperature	Cycles
TLR2	-16934A/T	F: 5`-AACAGAAATTTATCCATTCATGGTT-3` R: 5`-AGCAGTTTATTGTGAGAATGAGTTT-3`	55°C	45
TLR2	GT repeats	F: hex-5`-GCATTGCTGAATGTATCAGGGA-3` R: 5`-CTTGAGAAATGTTTTCTAGGC-3`	58.7°C	35

2.3. TLR-2, cytokines and transcription factor gene expression by real time PCR (qPCR).

To evaluate TLR-2, IFN-γ, IL-12, TNF-α, IL-17, IL-10, TGF-β and NF-κB mRNA expression, peripheral blood (20 mL - per standard procedure) was taken in heparized blood tubes, at a single time point from controls (*n*=20) and at three serial time points from patients (*n*=31) with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and T3), as previously defined. Peripheral blood mononuclear cells (PBMC) were obtained through a Histopaque® gradient separation method (Boyum 1968). The layer rich with lymphocytes and monocytes was aseptically removed and washed twice with PBS for 15 min at 1500 RPM. The cell suspension was re-suspended and the identification and viability of cells was determined by counting with Turk (50 μL aliquots of cell suspension with 50μL of the dye solution at 5%). Total RNA extraction from PBMC ($2x10^6$ Cells/mL) was made using Trizol Reagent (Invitrogen®), according to manufacturer's instructions. Concentration of total RNA

was determined by the absorbance values of samples at 260 nm. cDNA was synthesized from 1 μg of total RNA using the Reverse Transcriptase Super Script™ II (Invitrogen®). Thereafter enzyme SuperScript™ II RT (Invitrogen®) was added and conditions of PCR comprised 25°C for 5 minutes, 50°C for 50 minutes and 70ºC for 15 minutes. Soon after RNAse H (Invitrogen®) was added and incubated at 37ºC for 20 minutes. From cDNA obtained above, amplification was made according to the protocol of Applied Biosystems Power Sybr Green on the ABI Prims® 7300 Sequence Detector (Applied Biosystems). Product purity was confirmed by dissociation curve analysis. Gene expression was quantified relative to the values of the control group after adjusting for β-Actin. Primers are described in Table 2.

Table 2. Primers sequence for qPCR

Primer	Forward Sequence	Reverse Sequence
IFN-γ	5`-AAAAGAGTTCCATTATCCGCTACATC-3`	5`-GTTTTGGGTTCTCTCTTGGCTGTTA-3`
IL-12	5`-ACCTCCACCTGCCGAGAAT-3`	5`-CATGGTGGATGCCGTTCA-3`
TNF-α	5`-GGTTTGCTACAACATGGGCTACA-3`	5`-CCCCAGGGACCTCTCTCTAATC-3`
IL-10	5`-CTTGATGTCTGGGTCTTGGTTCT-3`	5`-GCTGGAGGACTTTAAGGGTTAACCT-3`
TGF-β	5`-AGGGCCAGGACCTTGCTG-3`	5`-CAAGGGCTACCATGCCAACT-3`
IL-17	5`-TTAGGC ACATGGTGGACAATCGG-3`	5`-ATGACTCCTGGGAAGACCTCA TTG-3`
TLR-2	5`-CTGAGCCTCGTCCATGGGCCACTCC-3`	5`-GGCCAGCAAATTACCTGTGTG-3`
NF-kB	5`-GGGAGGACGTAAAGGGATAG-3`	5`-GAAGAAGAGTCCTTTCAGCG-3`
β-actina	5`-GCTGGAAGGTGGACAGCGA-3`	5`-GGCATCGTGATGGACTCCG-3`

2.4. Cell surface expression of TLR-2 and co-expression TLR-2/4 by flow cytometry

PBMC obtained as above adjusted at a concentration of $1x10^6$ cells/mL were placed in Falcon tubes for flow cytometer (BD-Becton, Dickinson and Company) and centrifuged at 1700 rpm for 10 minutes at 4°C. Thereafter cells are resuspended in 1 mL of eletrolyte

solution (ISOTON II) and incubated with anti-TLR-4 monoclonal antibody conjugated with PE, with anti-TLR-2 conjugated with FITC , with anti-CD3 conjugated with PEDY647-clone UCHT1 (IgG1) and with anti-CD14 conjugated with PEDY647-clone MEM-15 (IgG1) for 15 minutes. For each test a control tube was used, in which the cells are incubated with isotype antibody controls labeled with fluorochromes of their tests. Cells were then centrifuged for 10 minutes at 1500 rpm for washing and resuspended in 1 ml ISOTON II, fixed with50µL of fixation solution containing 5% formaldehyde (BD-Becton, Dickinson and Company) and analyzed by flow cytometer model FACSCALIBUR™ (Becton Dickinson) using Cell Quest (Becton Dickinson) program according to manufacturer's instructions.

2.5. Plasma cytokine levels

Plasma samples were obtained from the same peripheral blood used for the genetic expression of cytokines from controls (*n*=20) and at three serial time points from patients with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and T3), as previously defined. Samples were maintained frozen (-80°C) until use. Quantikine®ELISA kits (R&D Systems®) were used, according to manufacturer's instructions, to measure IFN-γ, IL-12, TNF-α, IL-17, IL-10 and TGF-β plasma levels and method sensitivity were in accordance to each kit. Cytokine analysis was not possible in all 31 PTB patients, therefore the distribution of individuals among cytokines was: IFN-γ (*n=30)*, IL-12 (*n=30)*, TNF-α (*n=30)*, IL-17 (*n=30)*, IL-10 (*n=30)* and TGF-β (*n=29)*.

2.6. Statistical analysis

Comparisons between different genotypes in the control group and patient group were made using Mann-Whitney Test with two-tail P value. For the comparison between the three time points of the treatment in the group of patients (T1, T2 and T3), a Friedman Test (Nonparametric Repeated Measures ANOVA) was used. After this test, Dunn's Multiple Comparisons Test was applied. Correlations between variables were made using Spearman Test. Results were considered significant when $p<0.05$. Tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Demographics characteristics of PTB patients

Distribution among PTB patients regarding sex and age was 23 males with mean age of 48.8 years and 8 females with mean age of 38.9 years. PTB patients diagnosis was confirmed by sputum smear or culture positive for *M. tuberculosis* (n=2), sputum smear or culture positive for *M. tuberculosis* and image exams compatible with active TB (n=19) or by image exams compatible with active TB (n=10). All patients had respiratory symptoms consistent with dysphonea, cough and ventilation-dependent pain, and most of them presented constitutional symptoms, consistent with weight loss, fever and weakness at the beginning of the anti-TB treatment (T1). The symptoms were less frequent along treatment time line (T2) and at the end (T3) all patients were considered recovered. Characteristics of clinical-epidemiologic data and/or image exams after medical evaluation showed that all of our patients had a moderated form of active TB disease.

3.2. General immune response

In general, when compared to controls, PTB patients percentage of $CD3^{+}$TLR-2 was increased only at T2 time point and of $CD14^{+}$TLR-2, $CD14^{+}$TLR-2/4 and $CD3^{+}$TLR-2/4 were increased during anti-TB treatment (T1, T2 and T3) (data not shown). When compared to controls, plasma levels of IFN-γ, IL-12 and IL-10 were similar among PTB patients during anti-TB treatment, of IL-17 and TGF-β were increased among PTB patients only at the beginning of anti-TB treatment (T1) and of TNF-α were decreased among PTB patients during anti-TB treatment (T1, T2 and T3) (data not shown). When compared to controls, mRNA expression of IL-12, TNF-α and IL-17 were similar among PTB patients during anti-TB treatment (T1, T2 and T3), of IFN-γ, IL-10, NF-κB and TLR-2were increased among PTB patients during anti-TB treatment (T1, T2 and T3) and of TGF-β was increased only at T2 time point among PTB patients (data not shown).

3.3. Influence of *TLR-2* -16934A/T gene polymorphism on TRL-2 expression

TLR-2 -16934A/T gene SNP had no influence on TLR-2 mRNA expression (Figure 1A) and percentage of $CD14^{+}$TLR-2 cells (Figure 1B) of control individuals and PTB patients when AA genotype and T carriers were compared and between PTB patients time points within both genotypes (data not shown). Percentage of $CD14^{+}$TLR-2/4 cells was higher in PTB patients AA genotype then in T carriers only at T2 time point (p=0.04) and no difference in the controls between genotype AA and T carriers was found (Figure 1C) and between PTB patients time points within both genotypes (data not shown). When compared with T carriers percentage of $CD3^{+}$TLR-2 cells was increased in AA genotype controls (p=0.02) and PTB patients only at T2 time point (p=0.04) and at T3 time point PTB patients

T carriers had increased percentage of these cells when compared with AA genotype individuals (p=0.04) (Figure 1D). Analyses between time points showed higher expression of these cells at T2 time point when compared with T1, in AA genotype individuals (p<0.05) and in T carriers (p<0.05) (Figure 5A). Controls showed no difference in the percentage of $CD3^{+}$TLR-2/4 cells and PTB patients AA genotype had a higher percentage of these cells at T1 (p=0.03) and T3 ($p<0.05$) when compared with T carriers (Figure 1E). When PTB patients time points where compared AA genotype individuals had a higher expression of these cells at T3 when compared with T2 (p<0.05) and T carriers where increased at T3 (p<0.05) and T2 (p<0.05) when compared with T1 (Figure 5B).

3.4. Influence of *TLR-2* -16934A/T gene polymorphism on NF-κB expression

NF-κB mRNA expression on controls T carriers was higher than in those with the AA genotype (p=0.04). PTB patients AA genotype showed higher mRNA expression only at T1, when compared with T carriers (p=0.03) (Figure 2). PTB patients time point comparisons showed no differences within AA genotype and T carriers (data not shown).

3.5. Influence of *TLR-2* -16934A/T gene polymorphism on pro-inflammatory cytokine levels

TLR-2 -16934A/T gene SNP had no influence on IL-12 plasma levels of control individuals when AA genotype and T carriers were compared and PTB patients T carriers had a higher plasma level only at T3 (p=0.02) (Figure 3A). IL-12 mRNA expression was higher in controls with AA genotype than T carriers (p=0.02). PTB patients T carriers showed a higher expression at T2 when compared with AA genotype individuals (p=0.04)

(Figure 3B). There were no differences in IL-12 plasma and mRNA expression levels between time points of PTB patients within AA genotype and T carriers (data not shown).

T carriers showed higher IFN-γ levels when compared with AA genotype individuals in both study groups: controls (p=0.01), T1 (p=0.03), T2 ($p<0.05$) and T3 (p=0.02) (Figure 3C). IFN-γ mRNA expression had no difference when AA genotype and T carriers where compared in the controls and PTB patients T carriers had higher levels only at T1 ($p<0.05$) when compared with AA genotype individuals (Figure 3D). There were no differences in IFN-γ plasma and mRNA expression levels between time points of PTB patients within AA genotype and T carriers (data not shown).

Controls T carriers had higher TNF-α plasma levels when compared to AA genotype controls (p=0.03). PTB patients AA genotype showed higher plasma levels of this cytokine at T1 (p=0.02) and ($p<0.05$) T2 when compared to T carriers (Figure 3E). TNF-α mRNA expression had no differences in the control group when AA genotype and T carriers where compared and PTB patients AA genotype had a higher expression only at T1 ($p<0.05$) when compared with T carriers (Figure 3F). When PTB patients time points where compared AA genotype individuals had higher TNF-α plasma level ($p<0.05$) and mRNA expression ($p<0.05$) at T1 when compared with T3 (Figure 6A). PTB patients T carriers showed only higher plasma levels in T1 when compared with T2 ($p<0.05$) and T3 ($p<0.05$) (Figure 6B).

IL-17 plasma levels where not different in the control group when AA genotype and T carriers where compared. In the PTB patient group AA genotype had higher levels than T carriers at T2 (p=0.03) (Figure 3G). IL-17 mRNA expression was higher in controls AA genotype ($p<0.05$) and in PTB patients T carriers at T2 (p=0.03), when compared to T carriers and AA genotype individuals, respectively (Figure 3H). There were no differences in IFN-γ plasma and mRNA expression levels between time points of PTB patients within AA genotype and T carriers (data not shown).

3.6. Influence of *TLR-2* -16934A/T gene polymorphism on anti-inflammatory cytokine levels

Controls (p=0.02) and PTB patients at T1 (p=0.02), T2 ($p<0.05$) and T3 (p=0.03) with AA genotype had higher IL-10 plasma levels when compared with T carriers (Figure 4A). Controls ($p<0.05$) and PTB patients at T3 (p=0.03) also had a higher IL-10 mRNA expression when compared with T carriers. PTB patients T carriers had higher mRNA expression only at T1 ($p<0.05$) when compared with AA genotype (Figure 4B). When PTB patients time points where compared AA genotype individuals showed higher IL-10 plasma level at T1 when compared to T2 (p<0.05) and higher mRNA expression at T3 when compared with T1 (p<0.05) (Figure 6C). PTB patients T carriers showed a higher IL-10 mRNA expression at T1 when compared with T3 (p<0.05) (Figure 6D).

TGF-β plasma levels where not different when controls AA genotype and T carriers where compared. PTB patients AA genotype had higher levels at T1 when compared with T carriers (Figure 4C). TGF-β mRNA expression had no differences when AA genotype and T carriers where compared in both study groups (Figure 4D). There were no differences in TGF-β plasma and mRNA expression levels between time points of PTB patients within AA genotype and T carriers (data not shown).

3.7. TLR-2 expression correlates with NF-κB and cytokine levels according to *TLR-2* -169334A/T genotypes

Controls AA genotype showed a direct correlation of TLR-2 mRNA expression with IL-17 (r=0.82; p=0.02), IFN-γ (r=0.82; p=0.02), TNF-α (r=0.82; p=0.02) and TGF-β (r=0.81;

p=0.03) mRNA expressions and controls T carriers showed a direct correlation with TGF-β plasma level (r=0.61; p=0.03) and NF-κB (r=0.82; p=0.0006) mRNA expression. PTB patients AA genotype had a direct correlation with TNF-α plasma level at T2 (r=0.87; p<0.05) and IL-12 mRNA expression at T3 (r=0.90; p=0.04) and T carriers with IL-12 plasma level (r=0.57; p=0.02) at T2. An inverse correlation with plasma levels of TGF-β (r=-0.90; p=0.04) at T2 and of IL-10 (r=-0.55; p<0.05) at T3 was also seen in PTB patients T carriers.

PTB patients T carriers had a direct correlation of percentage of $CD14^{+}$TLR-2 cells with IFN-γ (r=0.52; p=0.01) at T1, with IL-17 (r=0.52; p=0.04) and IL-12 (r=0.54; p=0.03) mRNA expression at T3. These patients also showed an inverse correlation of these cells with IL-10 (r=-0.41; p<0.05) at T1 and TNF-α (r=-0.47; p=0.04) mRNA expression at T2. No correlations were seem in PTB patients AA genotype and controls AA genotype and T carriers (data not shown).

Percentage of $CD14^{+}$TLR-2/4 cells had a direct correlation with IL-12 plasma level (r=0.97; p=0.004) in controls AA genotype, NF-κB (r=0.82; p=0.0006) mRNA expression in controls T carriers and with IL-17 (r=0.52; p=0.04) and IFN-γ (r=0.54; p=0.03) mRNA expression in PTB patients T carriers at T1. In PTB patients AA genotype these cells also showed an inverse correlation with TNF-α (r=-0.90; p=0.04) mRNA expression at T3.

Percentage of $CD3^{+}$TLR-2 cells had a direct correlation with IL-17 (r=0.79; p=0.04), IFN-γ (r=0.79; p=0.03) and TNF-α (r=0.79; p=0.04) mRNA expression in controls AA genotype and with TNF-α (r=0.90; p=0.04) in PTB patients AA genotype at T2. These cells showed an inverse correlation with TNF-α plasma level (r=-0.86; p=0.01) in controls AA genotype, TNF-α (r=-0.42; p<0.05) and IL-10 (r=-0.64; p=0.009) mRNA expression in PTB patients T carriers at T1 and with IFN-γ (r=-0.40; p<0.05) mRNA expression in PTB patients AA genotype at T3.

Percentage of $CD3^{+}$TLR-2/4 cells had a direct correlation with IL-12 (r=0.93; p=0.002) mRNA expression in controls AA genotype and with NF-κB (r=0.87; p<0.05) mRNA expression at T1 and IL-12 plasma level (r=0.90; p=0.04) at T2 in PTB patients AA genotype. An inverse correlation was shown with IFN-γ (r=-0.57; p=0.01) and IL-10 (r=-0.54; p=0.01) plasma levels at T1 in PTB t carriers with NF-κB (r=-0.90; p=0.04) and with mRNA expression at T2 in PTB patients AA genotype.

3.8. NF-κB expression correlates with cytokine levels according to *TLR-2* -169334A/T genotypes

Our results showed an direct correlation of NF-κB mRNA expression with IL-10 (r=0.87; p<0.05) and TGF-β (r=0.87; p<0.05) mRNA expression at T3 of PTB patients AA genotype, with IL-10 plasma level at T2 (r=0.54; p=0.03), TGF-β plasma level (r=0.69; p=0.01) and IL-10 mRNA expression (r=0.60; p=0.01) at T3 of PTB patients T carriers. An inverse correlation was showed with IL-17 plasma level at T3 of PTB patients AA genotype (r=-0.90; p=0.04) and IL-12 plasma level at T1 of PTB patients T carriers (r=-0.42; p<0.05).

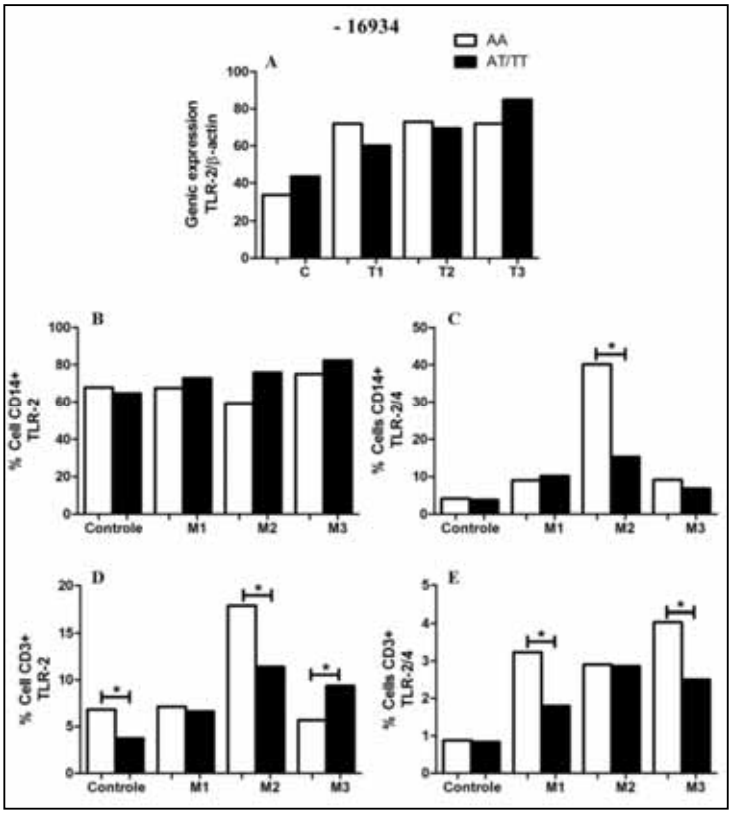

Figure 1. Influence of *TLR-2* -16934 gene SNP on TLR-2 mRNA expression (A) and cell surface expression (B, C, D, E) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: AA=5, AT/TT=15; T1: AA=5, AT/TT=23; T2: AA=5, AT/TT=19; T3: AA=5, AT/TT=16. * $p < 0.05$

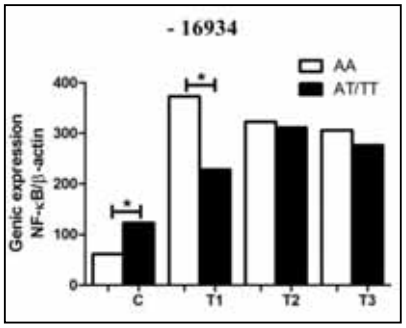

Figure 2. Influence of *TLR-2* -16934 gene SNP on NF-κB mRNA expression in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: AA=5, AT/TT=15; T1: AA=5, AT/TT=23; T2: AA=5, AT/TT=19; T3: AA=5, AT/TT=16. * $p < 0.05$

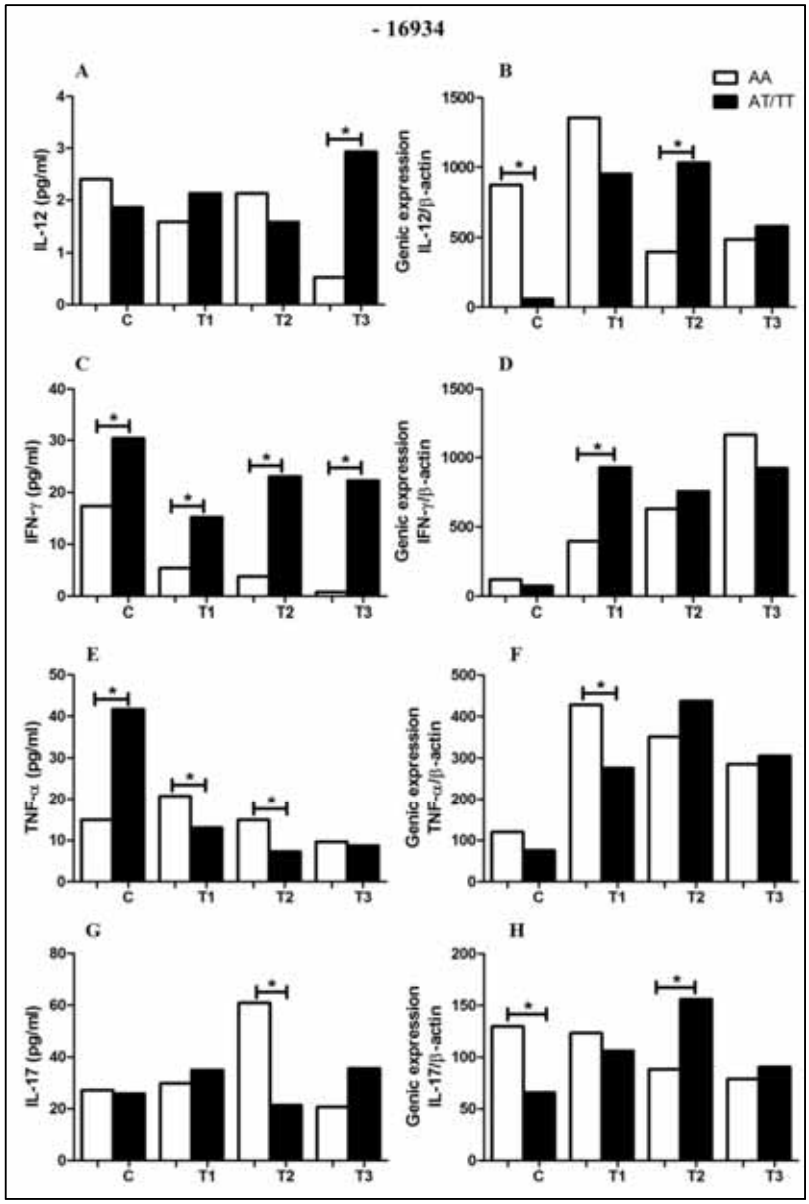

Figure 3. Influence of *TLR-2* -16934 gene SNP on pro-inflammatory cytokine plasma levels (A, C, E, G) and mRNA expression (B, D, E, H) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A, G): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=22; T2: AA=5, AT/TT=16; T3: AA=5, AT/TT=13; (B, D, F, H): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=23; T2: AA=5, AT/TT=19; T3: AA=5, AT/TT=16; (C): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=22; T2: AA=5, AT/TT=17; T3: AA=5, AT/TT=15; (E): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=22; T2: AA=5, AT/TT=18; T3: AA=5, AT/TT=15. * $p < 0.05$

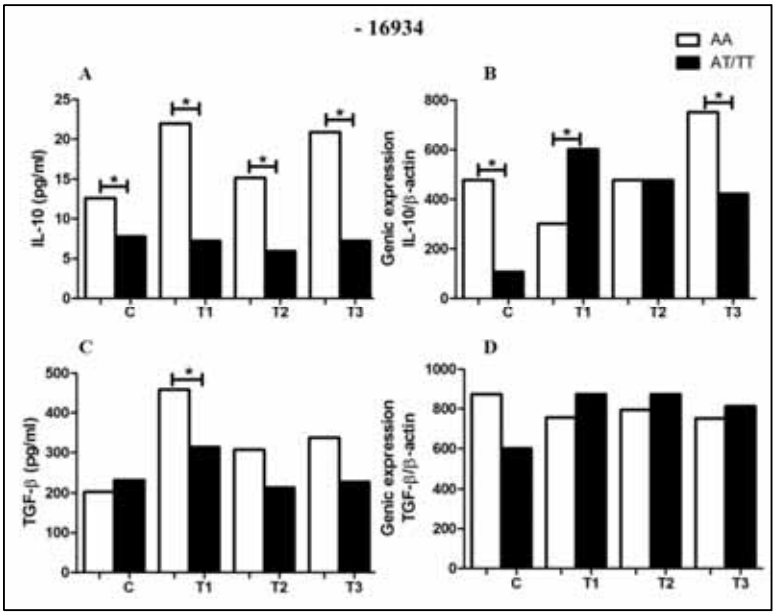

Figure 4. Influence of *TLR-2* -16934 gene SNP on anti-inflammatory cytokine plasma levels (A, C) and mRNA expression (B, D) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=22; T2: AA=5, AT/TT=16; T3: AA=5, AT/TT=13; (B,D): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=23; T2: AA=5, AT/TT=19; T3: AA=5, AT/TT=16; (C): C: AA=7, AT/TT=13; T1: AA=5, AT/TT=21; T2: AA=5, AT/TT=17; T3: AA=5, AT/TT=14. * $p < 0.05$

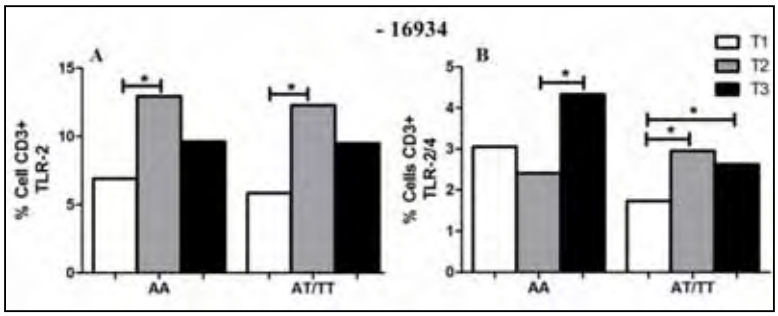

Figure 5. Influence of *TLR-2* -16934 gene SNP on cell surface expression of $CD3^{+}$TLR-2 cells (A) and $CD3^{+}$TLR-2/4 cells (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals: AA=3, AT/TT=15. * $p < 0.05$

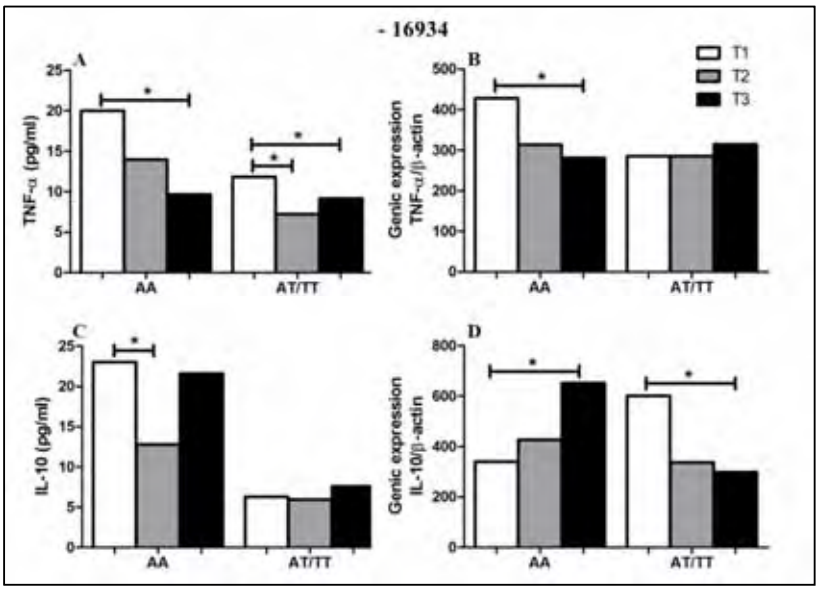

Figure 6. Influence of *TLR-2* -16934 gene SNP on cytokine plasma levels (A) and mRNA expression (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): AA=3, AT/TT=14; (B, D): AA=3, AT/TT=15; (C): AA=3, AG=12. * $p < 0.05$

3.9. Influence of *TLR-2* $(GT)_n$ polymorphism on TRL-2 expression

TLR-2 GT repeats gene SNP had no influence on TLR-2 mRNA expression (Figure 7A) and percentage of $CD14^+$TLR-2 cells (Figure 7B) and of $CD3^+$TLR-2 (Figure 7D) of control individuals and PTB patients when number of GT repeats were compared. There were no differences in $CD14^+$TLR-2 cells expression between time points of PTB patients within both genotypes (data not shown) and $CD3^+$TLR-2 expression of PTB patients with $(GT)_{\leq 19}$ was higher at T2 when compared with T1($p<0.05$) (Figure 11B) .Percentage of $CD14^+$TLR-2/4 cells was higher in PTB patients with $(GT)_{\leq 19}$ then $(GT)_{>19}$ at T1 time point ($p<0.05$), with $(GT)_{>19}$ then $(GT)_{\leq 19}$ at T2 ($p<0.05$) and no difference in the controls between number

of GT repeats was found (Figure 7C). When PTB patients time points where compared $(GT)_{\leq 19}$ individuals showed higher $CD14^{+}$TLR-2/4 cells expression at T1 ($p<0.05$) and T2 ($p<0.05$) when compared with T3 and $(GT)_{>19}$ individuals higher at T2 ($p<0.05$) and T3 ($p<0.05$) when compared to T1 (Figure 11A). Controls showed no difference in the percentage of $CD3^{+}$TLR-2/4 cells and PTB patients $(GT)_{\leq 19}$ had a higher percentage of these cells at T1 ($p=0.03$) and T3 ($p<0.05$) when compared with $(GT)_{>19}$ (Figure 1E). When PTB patients time points where compared $(GT)_{>19}$ individuals had a higher expression of these cells at T3 then at T1 ($p<0.05$) (Figure 11C) and no differences where seen in $(GT)_{\leq 19}$ individuals (data not shown).

3.10. Influence of *TLR-2* $(GT)_n$ polymorphism on NF-κB expression

NF-κB mRNA expression showed no difference when different numbers of GT repeats where compared in the controls group. PTB patients $(GT)_{\leq 19}$ had a higher mRNA expression only at T3 when compared to $(GT)_{>19}$ ($p<0.05$) (Figure 8). PTB patients time point comparisons showed no differences in PTB patients with $(GT)_{\leq 19}$ (data not shown). PTB patients with $(GT)_{>19}$ had higher mRNA expression at T2 when compared with T1 ($p<0.05$) (Figure 12).

3.11. Influence of *TLR-2* $(GT)_n$ polymorphism on pro-inflammatory cytokine levels

Individuals with $(GT)_{>19}$ showed higher IL-12 plasma levels in the control group ($p=0.03$) and at T1 of PTB patients ($p=0.02$) when compared with $(GT)_{\leq 19}$ individuals (Figure 9A). IL-12 mRNA expression was only higher in the controls with $(GT)_{\leq 19}$ when compared with $(GT)_{>19}$ individuals (Figure 9B). PTB patients time point comparisons showed higher IL-

12 plasma levels only in $(GT)_{\leq 19}$ individuals at T2 when compared to T1 ($p<0.05$) (Figure 13A). There were no differences in and mRNA expression between time points of PTB patients within both genotypes (data not shown).

IFN-γ plasma levels where higher in $(GT)_{\leq 19}$ controls ($p<0.05$) and $(GT)_{>19}$ PTB patients at T1 ($p=0.04$) and T2 ($p=0.04$) when compared to $(GT)_{>19}$ and $(GT)_{\leq 19}$ individuals, respectively (Figure 9B). No differences where seen in mRNA expression between number of GT repeats in both study groups (Figure 9C). There were no differences in IFN-γ plasma and mRNA expression levels between time points of PTB patients within both genotypes (data not shown).

Individuals with $(GT)_{>19}$ showed higher TNF-α plasma levels in the control group ($p=0.02$) and at T2 of PTB patients ($p<0.05$) when compared with $(GT)_{\leq 19}$ individuals (Figure 9E). TNF-α mRNA expression was higher in $(GT)_{\leq 19}$ controls ($p<0.05$) and $(GT)_{>19}$ PTB patients at T2 ($p=0.03$) when compared to $(GT)_{>19}$ and $(GT)_{\leq 19}$ individuals, respectively (Figure 9F). Comparison between time points of PTB patients showed that $(GT)_{\leq 19}$ individuals had higher TNF-α plasma levels at T1 ($p<0.05$) and T2 ($p<0.05$) when compared to T3 and that $(GT)_{>19}$ individuals had higher TNF-α plasma levels at T1 when compared to T3 ($p<0.05$) (Figure 13B). There were no differences in and mRNA expression between time points of PTB patients within both genotypes (data not shown).

Regarding IL-17 no differences in plasma levels were shown in both study groups (Figure 9G). IL-17 mRNA expression was higher only in $(GT)_{\leq 19}$ controls ($p<0.05$) (Figure 9H). There were no differences in IL-17 plasma and mRNA expression levels between time points of PTB patients within both genotypes (data not shown).

3.12. Influence of *TLR-2* $(GT)_n$ polymorphism on anti-inflammatory cytokine levels

IL-10 plasma levels where higher in $(GT)_{\leq 19}$ controls (p=0.03) and $(GT)_{>19}$ PTB patients at T2 (p=0.04) when compared to $(GT)_{>19}$ and $(GT)_{\leq 19}$ individuals, respectively (Figure 10A). IL-10 mRNA expression was also higher in $(GT)_{\leq 19}$ controls (p=0.04) and $(GT)_{>19}$ PTB patients at T1 ($p<0.05$) when compared to $(GT)_{>19}$ and $(GT)_{\leq 19}$ individuals, respectively (Figure 10B). Comparison between time points of PTB patients showed that $(GT)_{\leq 19}$ individuals had higher IL-10 plasma levels at T1 (p<0.05) and T2 (p<0.05) when compared to T3 (Figure 13C) and that $(GT)_{>19}$ individuals had higher mRNA expression at T1 when compared to T2 (p<0.05) (Figure 13D).

Controls had no difference in TGF-β plasma levels and PTB patients $(GT)_{>19}$ had higher plasma levels only at T1 (p=0.01) (Figure 10C). No differences in TGF-β mRNA expression were shown in both study groups (Figure 10D). Comparison between time points of PTB patients showed that $(GT)_{\leq 19}$ individuals had higher TGF-β plasma levels at T1 (p<0.05) when compared to T3 (Figure 13E). There were no differences in mRNA expression between time points of PTB patients within both genotypes (data not shown).

3.13. TLR-2 expression correlates with NF-κB and cytokine levels according to *TLR-2* $(GT)_n$

Direct correlations of TLR-2 mRNA expression with NF-κB mRNA expression (r=0.67; p=0.02) in controls $(GT)_{\leq 19,}$ with IL-10 plasma level (r=0.86; p=0.01), NF-κB (r=0.75; p=0.03) and IL-12 (r=0.72; p=0.04) mRNA expression in controls $(GT)_{>19}$ and with

IL-10 plasma level in PTB patients $(GT)_{>19}$ ($r=0.57$; $p<0.05$) at T2 was shown. PTB patients $(GT)_{\leq 19}$ at T2 had an inverse correlation with TGF-β plasma level ($r=-0.67$; $p=0.03$).

Percentage of $CD14^{+}$TLR-2 cells had a direct correlation with IFN-γ plasma level ($r=0.84$; $p=0.002$) at T1 and with IL-12 ($r=0.70$; $p=0.03$) and TNF-α ($r=0.80$; $p=0.01$) mRNA expressions at T3 in PTB patients $(GT)_{\leq 19}$ and with IFN-γ mRNA expression ($r=0.57$; $p=0.01$) in PTB patients $(GT)_{>19}$ at T1. An inverse correlation was seen with TNF-α ($r=-0.54$; $p=0.02$) and TGF-β ($r=-0.77$; $p=0.0003$) plasma levels and IL-10 mRNA expression ($r=-0.46$; $p<0.05$) at T1 and with TNF-α ($r=-0.60$; $p=0.04$) plasma level at T2 in PTB patients $(GT)_{>19}$. No correlation was seen in the controls within both genotypes.

Percentage of $CD14^{+}$TLR-2/4 cells had a direct correlation with IL-10 plasma level ($r=0.59$; $p<0.05$) at T2 and IL-10 mRNA expression ($r=0.67$; $p=0.03$) at T3 in PTB patients $(GT)_{\leq 19}$ and with IFN-γ ($r=0.65$; $p=0.003$) and IL-17 ($r=0.48$; $p=0.04$) mRNA expression at T1 in PTB patients $(GT)_{>19}$. No correlation was seen in the controls within both genotypes.

Percentage of $CD3^{+}$TLR-2 cells had a direct correlation with IL-10 mRNA expression ($r=0.67$; $p=0.02$) of controls $(GT)_{\leq 19}$ and IL-10 plasma level ($r=0.59$; $p<0.05$) at T3 in PTB patients $(GT)_{\leq 19}$. PTB patients $(GT)_{>19}$ showed an inverse correlation with IL-10 mRNA expression at T3 ($r=-0.62$; $p=0.01$).

Percentage of $CD3^{+}$TLR-2/4 cells had a direct correlation with NF-κB mRNA expression in controls $(GT)_{\leq 19}$ ($r=0.74$; $p=0.01$) and with IFN-γ ($r=0.71$; $p=0.02$) and IL-10 ($r=0.95$; $p=0.00003$) mRNA expression at T3 in PTB patients $(GT)_{\leq 19}$. PTB patients $(GT)_{\leq 19}$ also showed an inverse correlation with IFN-γ plasma level at T1 ($r=-0.71$; $p=0.02$).

3.14. NF-κB expression correlates with cytokine levels according to *TLR-2* GT repeats

Our results showed an direct correlation of NF-κB mRNA expression with IL-10 (r=0.89; p=0.0005) and TNF-α (r=0.65; p=0.04) plasma levels at T1, IFN-γ plasma (r=0.75; p=0.01) and mRNA expression (r=0.74; p=0.02) levels and TGF-β mRNA expression (r=0.66; p=0.04) at T3 of PTB patients $(GT)_{\leq 19}$ and with IL-12 (r=0.77; p=0.03) and TGF-β mRNA expressions (r=0.79; p=0.02) of controls $(GT)_{>19}$.

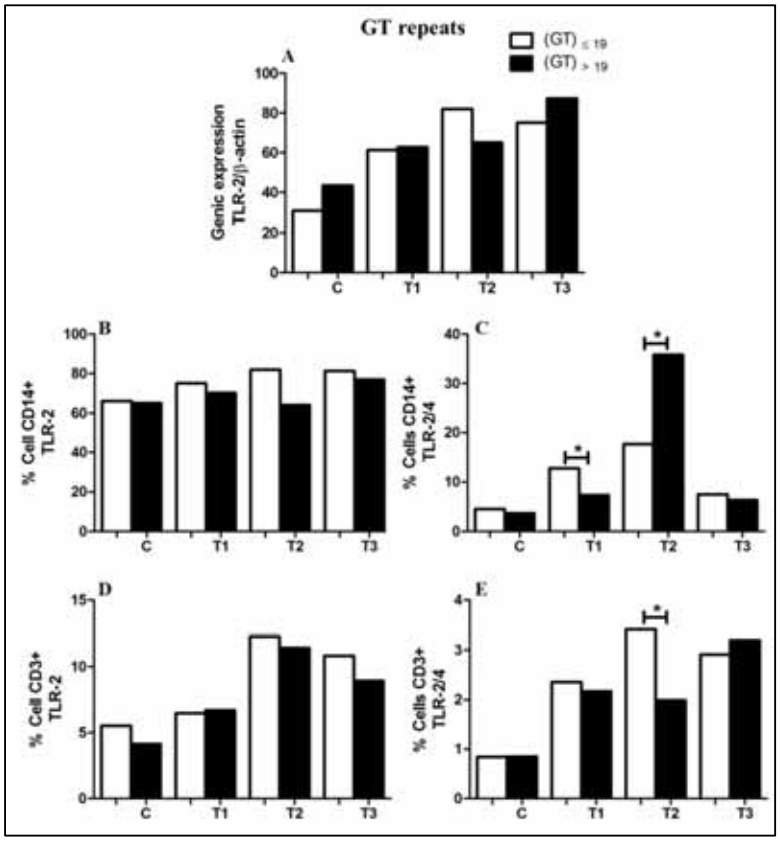

Figure 7. Influence of *TLR-2* GT repeats gene SNP on TLR-2 mRNA expression (A) and cell surface expression (B, C, D, E) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1:

$(GT)_{\leq 19}$=10, $(GT)_{>19}$=18; T2: $(GT)_{\leq 19}$=11, $(GT)_{>19}$=13; T3: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=11. * p < 0.05

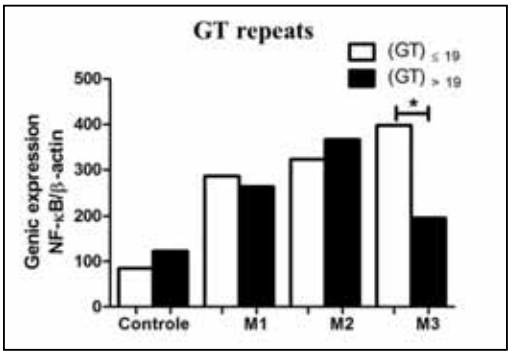

Figure 8. Influence of *TLR-2* GT repeats gene SNP on NF-κB mRNA expression in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=18; T2: $(GT)_{\leq 19}$=11, $(GT)_{>19}$=13; T3: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=11. * p < 0.05

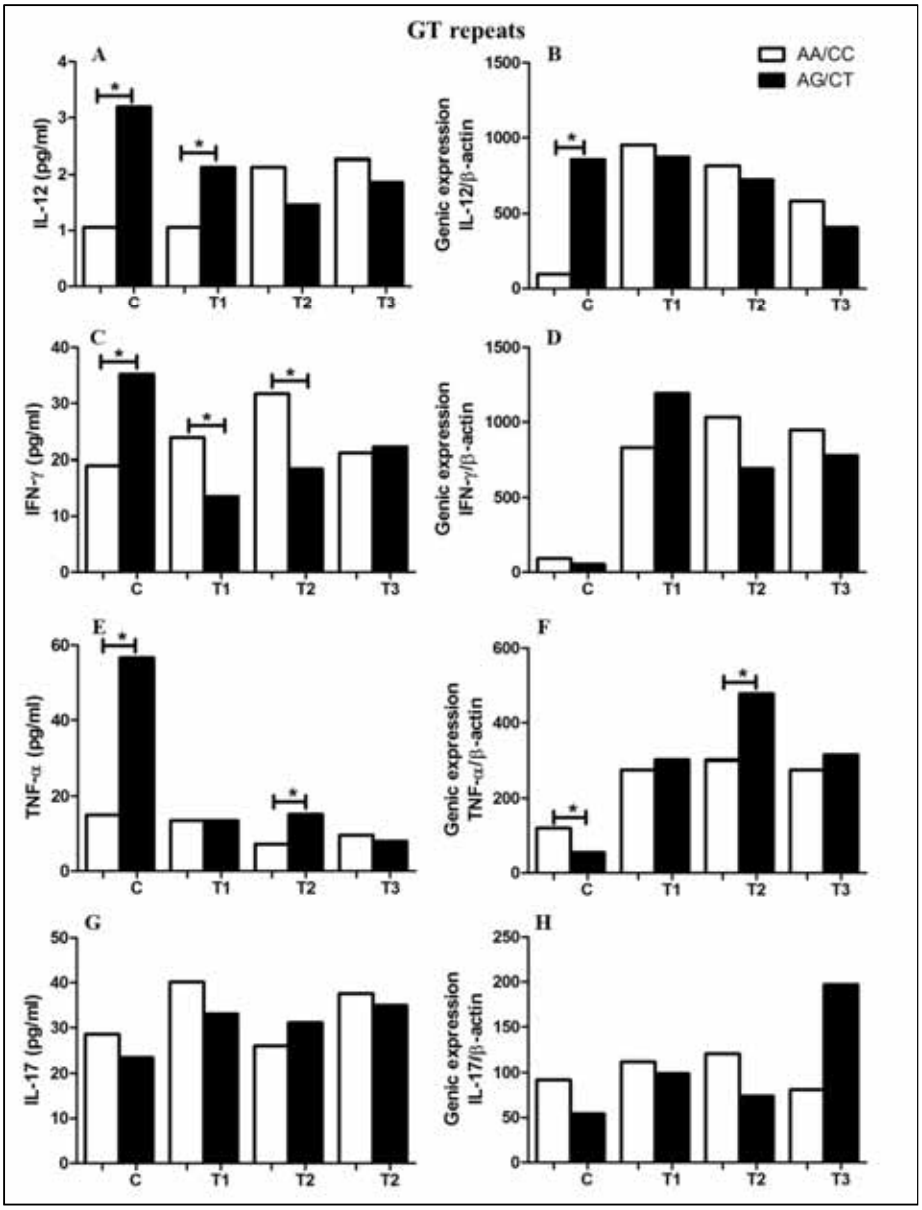

Figure 9. Influence of *TLR-2* GT repeats gene SNP on pro-inflammatory cytokine plasma levels (A, C, E, G) and mRNA expression (B, D, E, H) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A, G): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=17; T2: $(GT)_{\leq 19}$=9, $(GT)_{>19}$=12; T3: $(GT)_{\leq 19}$=8, $(GT)_{>19}$=10; (B, D, F, H): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=18; T2: $(GT)_{\leq 19}$=11, $(GT)_{>19}$=13; T3: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=11; (C): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=17; T2: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=12; T3: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=10. * $p < 0.05$

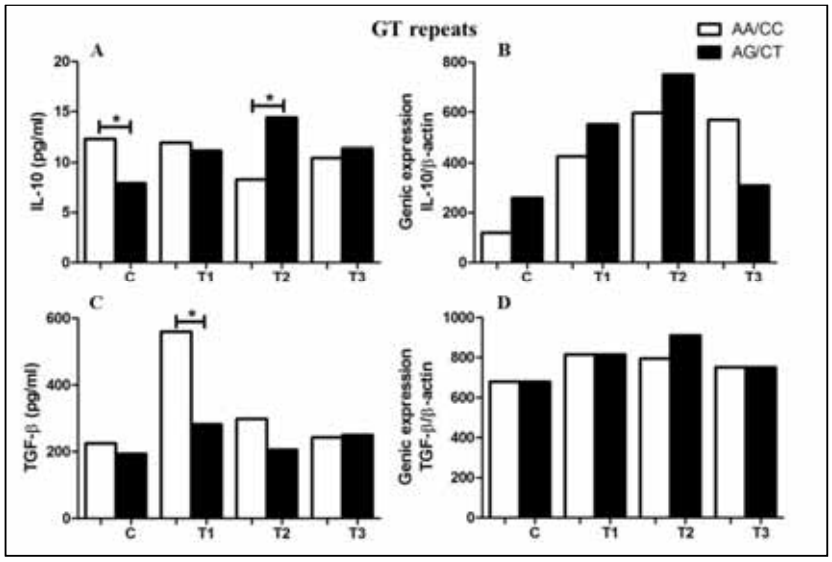

Figure 10. Influence of *TLR-2* GT repeats gene SNP on anti-inflammatory cytokine plasma levels (A, C) and mRNA expression (B, D) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=17; T2: $(GT)_{\leq 19}$=9, $(GT)_{>19}$=12; T3$(GT)_{\leq 19}$=8, $(GT)_{>19}$=10; (B,D): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=18; T2: $(GT)_{\leq 19}$=11, $(GT)_{>19}$=13; T3: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=11; (C): C: $(GT)_{\leq 19}$=12, $(GT)_{>19}$=8; T1: $(GT)_{\leq 19}$=9, $(GT)_{>19}$=17; T2: $(GT)_{\leq 19}$=10, $(GT)_{>19}$=12; T3: $(GT)_{\leq 19}$=9, $(GT)_{>19}$=10. * $p <$ 0.05

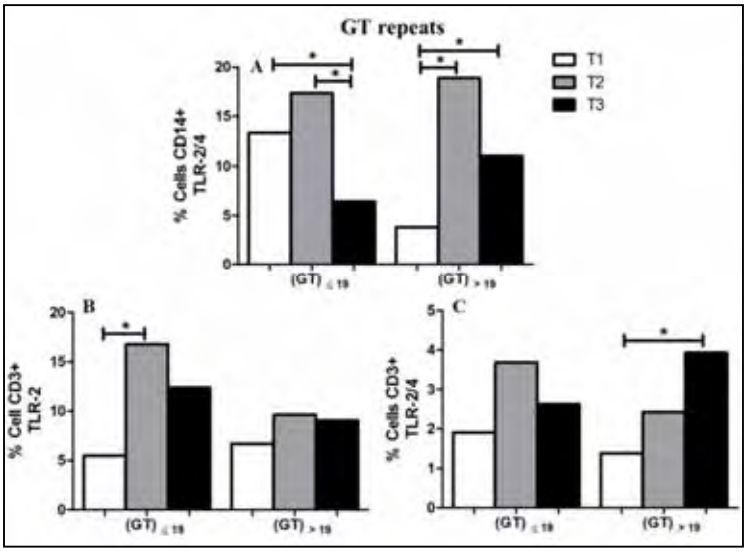

Figure 11. Influence of *TLR-2* GT repeats gene SNP on cell surface expression of $CD3^{+}$TLR-2 cells (A) and $CD3^{+}$TLR-2/4 cells (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals $(GT)_{\leq 19}$=9, $(GT)_{>19}$=10; (B. * $p <$ 0.05

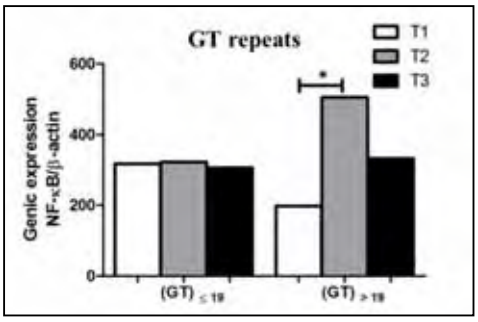

Figure 12. Influence of *TLR-2* GT repeats gene SNP on NF-κB mRNA expression in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals $(GT)_{\leq 19}$=9, $(GT)_{>19}$=10; (B. * $p < 0.05$

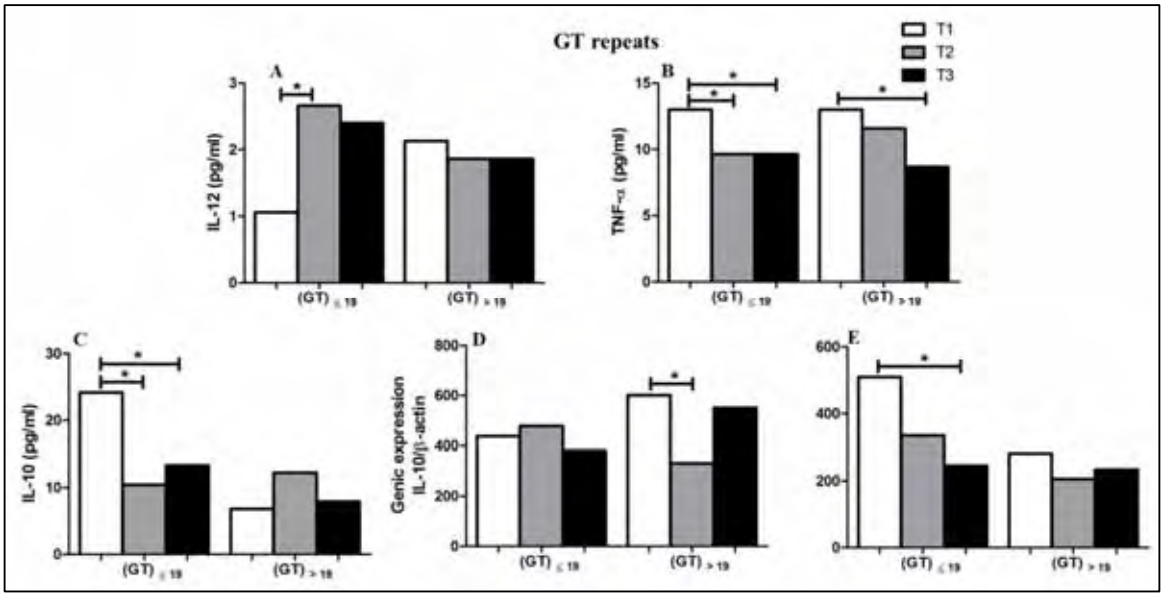

Figure 13. Influence of *TLR-2* GT repeats gene SNP on cytokine plasma levels (A) and mRNA expression (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A, C): $(GT)_{\leq 19}$=7, $(GT)_{>19}$=9; (B): $(GT)_{\leq 19}$=8, $(GT)_{>19}$=10; (D): $(GT)_{\leq 19}$=9, $(GT)_{>19}$=10; (E): $(GT)_{\leq 19}$=8, $(GT)_{>19}$=9. * $p < 0.05$

4. Discussion

From the estimated 2 billion individuals that have been initially infected with *Mycobacterium tuberculosis*, only 5% to 10% develop symptomatic TB. The reason why some infected individuals develop active disease, while others do not is not yet entirely understood (Kleinnijenhuis et al 2011). TLR2 is capable of recognizing pathogen-associated molecular patterns expressed by *M. tuberculosis*, such as 19-kDA lipoprotein, lipoarabinomannan, and soluble tuberculosis factor (Basu et al. 2007; Fricke et al. 2006; López et al. 2003; Means et al 1999). Stimulation of TLR2 by these products triggers activation of the transcription factor nuclear factor-κB (NF-κB), which induces transcription

of many genes encoding cytokines, chemokines, and adhesion molecules crucial to the inflammatory process (May and Ghosh 1998; Medzhitov et al. 1997). Given the central role of TLR-2 in the incitement of inflammation, polymorphisms in its gene might be involved in both infectious and inflammatory diseases (Yim et al. 2004). Here we evaluated the influence of *TLR2* -16934A/T and GT repeat polymorphisms on the immune response of PTB patients undergoing anti-TB treatment.

The -16934A/T locus at promotor region of *TLR2* is located next to an evolutionary conserved sequence and of several putative transcriptional factor binding sites, including NF-κB and IFN regulating factors. This fact indicates that this region is transcriptionally relevant (Nardone et al. 2004; Oh et al. 2009). In the 5'-untranslated region of *TLR2*, a highly polymorphic (GT)n dinucleotide repeat has been reported with numbers of GT repeats varying from 12 to 28 (Yim et al. 2004).

We found no differences in TLR-2 mRNA expression between -16943AA genotype and -16934T carriers and between different $(GT)_n$ repeats. We showed that AA genotype individuals had higher surface expression of $CD14^+$TLR-2/4, CD3+TLR-2 and CD3+TLR-2/4 cells. Our results do not agree with a study that measured TRL-2 surface expression on both granulocytes and $CD14^+$ cells in healthy donors and found no correlation between AA, AT or TT genotypes (Veltkamp et al. 2007, Oh et al. 2009).

Our results also showed that $(GT)_{\leq 19}$ PTB patients have higher $CD14^+$TLR-2/4 cells at the beginning of treatment and CD3+TLR-2/4 cells in the middle. PTB patients $GT)_{>19}$ had higher levels of $CD14^+$TLR-2/4 cells only at the middle of treatment. We found no differences on $CD14^+$TLR-2 cells for both *TLR2* polymorphisms studied and this result does not agree with study showing that TRL-2 expression on $CD14^+$ cells was lower in healthy individuals with shorter alleles (Aleman et al. 2004; Krutzik et al. 2003). Another study

showed no influence of $(GT)_n$ on TRL-2 surface expression on the same cells (Veltkamp et al. 2007)

Since TLR-2 protein expression on human monocytes is transcriptionally regulated our result suggests that TLR2 -16934 and $(GT)_n$ polymorphisms may have a transcriptional impairment for TRL-2 which reflects on the reduced surface expression. Our results do not agree with others that shown previously that TLR-2 protein expression on human monocytes correlating with mRNA levels (Armstrong et al. 2004; Flo et al. 2001).

The essential role of TLR-2 against mycobacterial infection *in vivo* has been shown by rapid fatality and higher burden of mycobacteria in TLR2-deficient mice. Therefore, it is reasonable to hypothesize that a subtle reduction in the expression of TLR-2 could likewise make humans more prone to the development of TB (Drennan et al. 2004; Heldwein et al. 2003; Reiling et al 2002).

We found that NF-κB expression was higher in controls -16934T carriers and the opposite in PTB patients at the beginning of the treatment. TLR2 $(GT)_n$ also influenced NF-κB expression, but only in PTB patients at the end of the treatment, with higher levels seen in PTB patients $(GT)_{\leq 19}$. A lower expression of NF-κB could lead to reduced cytokine production (Fewerda et al. 2008).

Our study showed that -16934T carriers produce more IL-12 at the end and IFN-γ during all anti-TB treatment and lower levels of TNF-α, IL-10 and TGF-β during all treatment and IL-17 at the middle, when compared with AA genotype individuals. IL-12 promotes differentiation of CD4+ T cells into Th1 cells that are potent IFN-γ producers. IFN-γ has a pivotal role in the activation of anti-mycobacterial activities of macrophages, and hence considered crucial for protection against tuberculosis (Flynn and Chan 2001). It seems that individuals -16934T carriers tend to have a more pro-inflammatory profile than individuals carrying AA genotype. Persistence of low IFN-γ production from middle to the

end of therapy suggests that AA genotype individuals may underlie their increased risk for reactivation of a latent PTB focus. Such an inadequate IFN-γ production may result in failure of macrophage activation, which could lead to active disease progression (Vidyarani et al. 2006).

TNF-α is important for containing infection and preventing dissemination but has also been implicated in immunopathological response and is often a major factor in host-mediated destruction of lung tissue (Moreira et al. 1997). Although our results showed higher TNF-α level in AA genotype than in -16934T carriers, the production was similar to normal levels. In agreement with our results lower TNF-α level was reported in -16934T carriers after evaluating PBMC from normal individuals stimulated with TLR-2 agonists (Veltkamp et al. 2007). Another study using whole blood culture did not found influence of *TLR2* -16934A/T genotypes on TNF-α level (Oh et al. 2009).

IL-17 appears to be critical to the induction of *M. tuberculosis*–specific memory response and the mediation of protection against challenge infections and during vaccinations, although Th17 cells are not as important as Th1 cells in mediating protection against primary *M. tuberculosis* infection (Khader et al. 2005; Khader et al. 2007; Umemura et al. 2007; Wozniak et al. 2006). To our knowledge this is the first report to evaluate *TLR2* polymorphisms influence on IL-17 levels.

We found that -16934AA genotype produce higher levels of IL-10 and TGF-β. IL-10 is considered to be an anti-inflammatory cytokine and directly inhibits $CD4^+$ T cell responses, as well as by inhibiting antigen presenting cells (APC) function after infection with mycobacteria (Fenhalls et al. 2000). TGF-β is able to have pro and anti-inflammatory functions depending on its concentration. At high levels it will deactivate macrophages and can impaired TNF-α production and in lower levels acts as a chemotaxis factor for monocytes and will induce TNF-α secretion (Hirsch et al. 1994; Kehrl et al. 1986; Lezini et al. 1977). To

our knowledge this is the first report to evaluate *TLR2* polymorphisms influence on TGF-β levels. So, we can speculate that IL-10 and TGF-β could contribute to low IFN-γ level found in -16934AA genotype. There are no reports of *TLR-2* 16934A/T influence on IL-10 and TGF-β

We also reported that controls $(GT)_{>19}$ have higher IL-12, IFN-γ, TNF-α and lower IL-10 levels, which will result in an pro-inflammatory profile. PTB patients $(GT)_{>19}$ had a different profile with higher IL-12 at the beginning, TNF-α and IL-10 at the middle and lower IFN-γ at the beginning and middle and TGF-β levels at the beginning of anti-tuberculosis treatment. Our results are in disagreement with other study that observed no correlation between the production of IFN-γ and $(GT)_n$ and higher TNF-α and IL-12 in individuals with lower number of repeats $(GT)_{\leq 20}$, after stimulation with TLR-2 agonists (Veltkamp et al. 2007). Although different $(GT)_n$ may induce difference on cytokine profile, it seem that this difference do not impair the immune response against active tuberculosis, since even though $(GT)_{>19}$ induces lower production of IFN-γ, the levels were similar to control individuals and tended to increase during the treatment. To our knowledge this is the first study to report influence of $(GT)_n$ on IL-17, IL-10 and TGF-β.

The present study suggests that -16934A/T and GT repeats polymorphisms in *TLR2* gene can influence differential TLR-2, NF-κB and cytokine levels during anti-TB treatment. We also suggest that PTB patients with *TLR2* -16934AA genotype may have a worst outcome of the disease, since they have a lower IFN-γ, an essential cytokine to initiate the protective immunity against active TB. This association could not be made in our study due to the low number of patients evaluated. Since TLR-2 plays a major role in initiating immune response against *M. tuberculosis* polymorphisms in *TLR2* gene are important to better understand protective immune responses and may serve as genetic risk markers for TB susceptibility.

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V- Capítulo 2

***TLR4* Asp299Gly/Thr399Ile SNP influence cytokine plasma and mRNA expression levels of pulmonary tuberculosis patients undergoing anti-tuberculosis treatment.**

Abstract

Tuberculosis (TB) is a leading public health problem in the world and development of active disease depends on the relationship between the bacillus and the host. The fact that only 10% of people infected with *M. tuberculosis* develop clinical tuberculosis (TB) suggests that genetic factors play a role in the pathogenesis of this disease. Since TLR-4 is essential to initiate innate response, regulating the expression of pro-inflammatory cytokines and genes that control cell survival and apoptosis, the aim of this study was to verify the influence of *TLR4* Asp299Gly/Thr399Ile polymorphism on the immune profile of Brazilian patients with pulmonary TB (PTB) under anti-TB treatment at different time points of anti-TB treatment: T1 (beginning), T2 (3 months) and T3 (end). For this we genotyped Asp299Gly/Thr399Ile polymorphisms and evaluated the immune response of PTB during the time of anti-TB treatment. The present study suggests that *TLR4* Asp299Gly/Thr399Ile polymorphism is associated with differential TLR-4 and cytokine levels during anti-TB treatment and that the TLR-4 action could be through the activation of signaling pathway other than that of the NF-κB. Since PTB patients with A/G-C/T haplotype have a lower IFN-γ, we suggest that they may have higher chances to reactivate a TB focus. TLR-4 plays a major role in initiating the immune response against *M. tuberculosis* thus polymorphisms in *TLR4* could be important to better understand protective immune responses and may serve as genetic risk markers for TB susceptibility.

Keywords: pulmonary tuberculosis, polymorphisms, cytokines, anti-tuberculosis treatment, TLR-4

1. Introduction

TLR-4 is a transmembrane pattern recognition receptor that plays a key role to innate immunity by inducing inflammatory responses to its main ligand, LPS (Kaufmann 2002). This receptor can also interact with both heat labile soluble mycobacterial factor and whole viable *Mycobacterium tuberculosis* (*M. tuberculosis*) to initiate responses (Means et al. 1999; Tsuji et al. 2000). TLR-4 signals through adaptor proteins such as myeloid differentiation factor 88 (MyD88) which will activate downstream effectors, like nuclear factor κB (NF-κB), which regulates the expression of pro-inflammatory cytokines and genes that control cell survival and apoptosis (Kawai et al. 1999; Lee et al. 2003; Paik et al. 2003). Qualitative or quantitative changes of this nuclear transcription factor may cause changes in the regulation of gene transcription of inflammatory cytokines, leading to changes in production of these mediators (Motsinger-Reif1 et al. 2010, Sutmuller et al. 2006).

TLR4 gene shows a greater variation in the sequence that encodes the extracellular domain signaling than in the coding region of the cytoplasmic domain (Ma et al 2007).It has been demonstrated that two non-synonymous polymorphisms (SNP) at *TLR4* gene 299 and 399 can induce a decrease in the activity of NF-κB, compared to the wild type gene of *TLR4*, suggesting that these SNPs may influence the phenotype, leading to a low production of cytokines during the immune response. It is also known that *TLR4* Asp299Gly polymorphism seems to impair the immune response to LPS, even when presented in heterozygous form (Ferwerda et al 2008).

Tuberculosis (TB) is a leading public health problem in the world and development of active disease depends on bacillus host interplay. Only 10% of people infected with *M. tuberculosis* develop clinical tuberculosis (TB) which suggests that genetic factors play a role in the pathogenesis of this disease (Kleinnijenhuis et al. 2008).

Since TLR-4 is essential to initiate protective response to TB and many studies reported association of SNPs in its gene with TB in activity, but few have evaluated its influence in modulating the immune response. Thus the aim of this study was to verify the influence of *TLR-4* Asp299Gly/Thr399Ile polymorphism on the immune profile of Brazilian patients with pulmonary TB (PTB) under anti-TB treatment.

2. Material and Methods

2.1. Study population

The study group enrolled 31 Brazilian patients attending the Infectious and Parasitic Diseases Services at Botucatu Mecdical School University Hospital – UNESP, Botucatu Teaching Health Centre and Primary Healthcare units of Botucatu and surrounding region with pulmonary TB diagnose confirmed by sputum smear or culture positive for *M. tuberculosis*, or else by clinical-epidemiologic data, laboratory and image exams compatible with active tuberculosis. Patients with pulmonary tuberculosis concurrent with other active granulomatous disease or HIV positive were excluded. All patients diagnosed with pulmonary TB received treatment for six months, using different combinations of the four first-line drugs: isoniazid, ethambutol, pyrazinamide and rifampicin. For the evaluation of immunological function, patients samples were collected based on the anti-tuberculosis treatment time line, defined as T1: after diagnosis and with no more than one month of treatment; T2: with three months of treatment; and T3: with six months of treatment. As normal controls (C), we studied 20 health care workers from Botucatu Medical School (Botucatu, São Paulo, Brazil), 9 males (mean age 40.4 years) and 11 females (mean age 34.1 years), without clinical complaints and with no history of TB disease, autoimmune disease

and other infectious disease. All controls were tuberculin test (PPD) positive (hardering ≥ 5mm). All patients and controls agreed to participate in the study, after due clarification and signing of the written informed consent.

2.2. SNP Genotyping

Herin, we studied the *TLR-4* Asp299Gly/Thr399Ile polymorphisms. For this proposal peripheral blood (5 mL - per standard procedure) was drawn in EDTA, from patients (*n*=31) with pulmonary tuberculosis and controls (*n*=20) groups at base line T0, and genomic DNA was extracted from leukocytes employing DNAzol commercial reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantification and purity determination of the extracted DNA was determined on a spectrophotometer (reading at 260, 280 and 320nm). Amplification of the genomic regions of interest was performed by PCR using 50µg of DNA, Master Mix (Invitrogen) and primers (20µM) (F: 5`-GCCTGTGCAATTTGACCATTG-3`; R: 5`-GAGTATGAGAAATGTCAAGGT-3`). Conditions of PCR comprised 10 min of initial denaturation at 95°C, then 35 cycles of 94°C for 30s, annealing at 55°C for 30s and 72°C for 30s, then 10 min at 72°C for final extension. PCR product was cleaned up using Edge Biosystems Ultra Kit (MFRS-DTR0850), spinning plates at 850g for 5 min, and then verified in a 1% agarose gel stained with ethidium bromide. Clean PCR product (1µL), BigDye® Terminator (Applied Biosystems) and forward primer (5µM) were used to perform sequencing reaction. Conditions of sequencing comprised 1 min at 95°C, then 35 cycles at 96°C for 10 s, 50°C for 5s and 60°C for 4 min. Sequencing product was cleaned up using Edge Biosystems Ultra Kit (MFRS-DTR0850), spinning plates at 850g for 5 min, and then placed in the 3730xl DNA Analyser. Results were then analysed by the Sequencher™ 5.0 – Build 7081 Software.

2.3. TLR-4, cytokines and transcription factor gene expression by real time PCR (qPCR).

To evaluate TLR-4, IFN-γ, IL-12, TNF-α, IL-17, IL-10, TGF-β and NF-κB mRNA expression, peripheral blood (20 mL - per standard procedure) was taken in heparized blood tubes, at a single time point from controls (*n*=20) and at three serial time points from patients (*n*=31) with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and T3), as defined previously. Peripheral blood mononuclear cells (PBMC) were obtained through a Histopaque® gradient separation method (Boyum 1968). The layer rich with lymphocytes and monocytes was aseptically removed and washed twice with PBS for 15 min at 1500 RPM. The cell suspension was re-suspended and the identification and viability of cells was determined by counting with Turk solution (50 μL aliquots of cell suspension with 50μL of the dye solution at 5%). Total RNA extraction from PBMC ($2x10^6$ Cells/mL) was made using Trizol Reagent (Invitrogen®), according to manufacturer's instructions. Concentration of total RNA was determined by the absorbance values of samples at 260 nm. cDNA was synthesized from 1 μg of total RNA using the Reverse Transcriptase Super Script™ II (Invitrogen®). Reaction was incubated in a thermocycler for 5 minutes at 65°C and then transferred immediately to ice. Thereafter enzyme SuperScript™ II RT (Invitrogen®) was added and conditions of PCR comprised 25°C for 5 minutes, 50°C for 50 minutes and 70ºC for 15 minutes. Soon after RNAse H (Invitrogen®) was added and incubated at 37ºC for 20 minutes. From cDNA obtained above, gene amplification was made according to the protocol of Applied Biosystems Power Sybr Green on the ABI Prims® 7300 Sequence Detector (Applied Biosystems). Product purity was confirmed by dissociation curve analysis. Gene expression

was quantified relative to the values of the control group after adjusting for β-Actin. Primers are described in Table 1.

Table 1. Primers sequence for qPCR

Primers	Forward Sequence	Reverse Sequence
IFN-γ	5`-AAAAGAGTTCCATTATCCGCTACATC-3`	5`-GTTTTGGGTTCTCTCTTGGCTGTTA-3`
IL-12	5`-ACCTCCACCTGCCGAGAAT-3`	5`-CATGGTGGATGCCGTTCA-3`
TNF-α	5`-GGTTTGCTACAACATGGGCTACA-3`	5`-CCCCAGGGACCTCTCTCTAATC-3`
IL-10	5`-CTTGATGTCTGGGTCTTGGTTCT-3`	5`-GCTGGAGGACTTTAAGGGTTAACCT-3`
TGF-β	5`-AGGGCCAGGACCTTGCTG-3`	5`-CAAGGGCTACCATGCCAACT-3`
IL-17	5`-TTAGGC ACATGGTGGACAATCGG-3`	5`-ATGACTCCTGGGAAGACCTCA TTG-3`
TLR-4	5`-GTGCTGGGACACCACAACAATCACC-3`	5`-TGCAATGGATCAAGGACCAGAGGC-3`
NF-kB	5`-GGGAGGACGTAAAGGGATAG-3`	5`-GAAGAAGAGTCCTTTCAGCG-3`
β-actina	5`-GCTGGAAGGTGGACAGCGA-3`	5`-GGCATCGTGATGGACTCCG-3`

2.4. Cell surface expression of TLR-4 and co-expression of TLR-2/4 by flow cytometry

PBMC obtained as above adjusted at a concentration of $1x10^6$ cells/mL were placed in Falcon tubes for flow cytometer (BD-Becton, Dickinson and Company) and centrifuged at 1700 rpm for 10 minutes at 4°C. Thereafter cells are resuspended in 1 mL of eletrolyte solution (ISOTON II) and incubated with anti-TLR-4 monoclonal antibody conjugated with PE, with anti-TLR-2 conjugated with FITC , with anti-CD3 conjugated with PEDY647-clone UCHT1 (IgG1) and with anti-CD14 conjugated with PEDY647-clone MEM-15 (IgG1) for 15 minutes. For each test a control tube was used, in which the cells are incubated with isotype antibody controls labeled with fluorochromes of their tests. Cells were then centrifuged for 10 minutes at 1500 rpm for washing and resuspended in 1 ml ISOTON II, fixed with50µL of fixation solution containing 5% formaldehyde (BD-Becton, Dickinson and Company) and

analyzed by flow cytometer model FACSCALIBUR™ (Becton Dickinson) using Cell Quest (Becton Dickinson) program according to manufacturer's instructions.

2.5. Plasma cytokine levels

Plasma samples were obtained from the same peripheral blood used for the genetic expression of cytokines from controls (*n*=20) and at three serial time points from patients with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and T3), as defined previously. Samples were maintained frozen (-80°C) until use. Quantikine®ELISA kits (R&D Systems®) were used, according to manufacturer's instructions, to measure IFN-γ, IL-12, TNF-α, IL-17, IL-10 and TGF-β plasma levels and method sensitivity were in accordance to each kit. Cytokine analysis was not possible in all 31 PTB patients, therefore the distribution of individuals among cytokines was: IFN-γ (*n=30)*, IL-12 (*n=30)*, TNF-α (*n=30)*, IL-17 (*n=30)*, IL-10 (*n=30)* and TGF-β (*n=29)*.

2.6. Statistical analysis

Comparisons between different genotypes in the control group and patient group were made using Mann-Whitney Test with two-tail P value. For the comparison between the three time points of the treatment in the group of patients (T1, T2 and T3), a Friedman Test (Nonparametric Repeated Measures ANOVA) was used. After this test, Dunn's Multiple Comparisons Test was applied. Correlations between variables were made using Spearman Test. Results were considered significant when $p<0.05$. Tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Demographic characteristics of PTB patients

Distribution among PTB patients regarding sex and age was 23 males with mean age of 48.8 years and 8 females with mean age of 38.9 years. PTB patients diagnosis was confirmed by sputum smear or culture positive for *M. tuberculosis* (*n*=2), sputum smear or culture positive for *M. tuberculosis* and image exams compatible with active TB (*n*=19) or by image exams compatible with active TB (*n*=10). All patients had respiratory symptoms consistent with dysphonea, cough and ventilation-dependent pain, and most of them presented constitutional symptoms, consistent with weight loss, fever and weakness at the beginning of the anti-TB treatment (T1). The symptoms were less frequent along treatment time line (T2) and at the end (T3) all patients were considered recovered. Characteristics of clinical-epidemiologic data and/or image exams after medical evaluation showed that all of our patients had a moderated form of active TB disease.

3.2. General immune response

In general, when compared to controls, PTB patients percentage of $CD14^{+}$TLR-4 cells were similar during anti-TB treatment (T1, T2 and T3), of $CD14^{+}$TLR-2/4, $CD3^{+}$TLR-2/4 and $CD3^{+}$TLR-4 were increased during anti-TB treatment (T1, T2 and T3) (data not shown). When compared to controls, plasma levels of IFN-γ, IL-12 and IL-10 were similar among PTB patients during anti-TB treatment, of IL-17 and TGF-β were increased among PTB patients only at the beginning of anti-TB treatment (T1) and of TNF-α were decreased among PTB patients during anti-TB treatment (T1, T2 and T3) (data not shown). When compared to

controls, mRNA expression of IL-12, TNF-α and IL-17 were similar among PTB patients during anti-TB treatment (T1, T2 and T3), of IFN-γ, IL-10, NF-κB and TLR-4 were increased among PTB patients during anti-TB treatment (T1, T2 and T3) and of TGF-β was increased only at T2 time point among PTB patients (data not shown).

3.3. Influence of *TLR-4* Asp299Gly/Thr399Ile gene polymorphism on TRL-4 expression

Regarding *TLR4* Asp299Gly/Thr399Ile loci we obtained only A/G-C/T and A/A-C/C diplotypes. Only one PTB patient carrying AA/CT diplotype was found which was not included in our analysis. Thus, herein we conducted analysis comprising A/G-C/T and A/A-C/C comparisons.

Controls A/G-C/T had higher TLR-4 mRNA expression then A/A-C/C individuals ($p<0.05$) and no differences were seen in the PTB patients group when diplotypes (Figure 1A) and different time points where compared (data not shown). No difference were shown for the percentage of $CD14^{+}$TLR-2 cells in both study groups when diplotypes (Figure 1B) and different PTB patients time points where compared (data not shown). Percentage of $CD14^{+}$TLR-2/4 cells was higher only in PTB patients A/G-C/T then in A/A-C/C individuals only at T1 ($p=0.04$) No difference in the controls between diplotypes (Figure 1C) and between PTB patients time points within both genotypes was found (data not shown). PTB patients A/G-C/T had increased $CD3^{+}$TLR-2 cells only at T2 time point ($p=0.04$) and No difference in the controls between diplotypes was found (Figure 1D). Analyses between time points showed higher expression of these cells only in A/A-C/C individuals at T3 when compared with T1 and T2 in ($p<0.05$) (Figure 5A). Controls showed no difference in the percentage of $CD3^{+}$TLR-2/4 cells and PTB patients A/G-C/T had a higher percentage of these cells at T2 ($p<0.05$) when compared with A/A-C/C diplotype (Figure 1E). When PTB

patients A/A-C/C time points where compared, T3 had a higher expression of these cells then T1 (p<0.05) (Figure 5B).

3.4. Influence of *TLR-4* Asp299Gly/Thr399Ile gene polymorphism on NF-κB expression

Only controls AG /CT showed a higher NF-κB mRNA expression when compared to A/A-C/C haplotype (p=0.008) (Figure 2). When PTB patients A/A-C/C time points where compared, showed higher mRNA expression at T2 than at T1 (p<0.05) (Figure 6).

3.5. Influence of *TLR-4* Asp299Gly/Thr399Ile gene polymorphism on pro-inflammatory cytokine levels

There were no differences on IL-12 plasma levels on the control group. PTB patients A/G-C/T showed higher levels at T2 (p=0.03) and T3 (p=0.04) when compared with A/A-C/C individuals (Figure 3A). IL-12 mRNA expression was higher only in controls AG/AC when compared with A/A-C/C individuals (Figure 3B). There were no differences in IL-12 plasma and mRNA expression levels between time points of PTB patients within both haplotypes (data not shown).

PTB patients A/A-C/C showed higher plasma levels of IFN-γ at T2 (p=0.04) and T3 (p=0.04) when compared with A/G-C/T individuals (Figure 3C). No differences on IL-12 plasma levels in the control group (Figure 3C), on mRNA expression in both study groups (Figure 3D) and on PTB patients time points within both haplotypes were found (data not shown).

Only controls A/G-C/T showed higher TFN-α plasma levels when compared to A/A-C/C individuals (p<0.05) (Figure 3E). TFN-α mRNA expression was elevated in controls

A/A-C/C (p=0.04) and in PTB patients A/G-C/T at T2 (p<0.05), when compared with A/G-C/T and A/A-C/C haplotypes, respectively (Figure 16F). When PTB patients A/A-C/C time points where compared, showed higher mRNA expression at T3 then at T1 (p<0.05) and T2 (p<0.05) (Figure 7).

IL-17 plasma levels were higher at T2 (p=0.03) and T3 (p=0.03) of PTB patients A/G-C/T when compared with A/A-C/C individuals (Figure 3G). No differences on IL-12 plasma levels in the control group (Figure 16C), on mRNA expression in both study groups (Figure 3H) and on PTB patients time points within both haplotypes were found (data not shown).

3.6. Influence of *TLR-4* Asp299Gly/Thr399Ile gene polymorphism on anti-inflammatory cytokine levels

IL-10 plasma levels were higher at T2 (p=0.03) of PTB patients A/G-C/T when compared with A/A-C/C individuals (Figure 4A). No differences on IL-12 plasma levels in the control group (Figure 17A), on mRNA expression in both study groups (Figure 4B) and on PTB patients time points within both haplotypes were found (data not shown).

TGF-β plasma levels were higher in controls A/A-C/C (p<0.05) and PTB patients A/G-C/T at T1 (p<0.05) when compared with A/G-C/T and A/A-C/C haplotypes individuals, respectively (Figure 4C). Controls (p=0.03) and PTB patients A/G-C/T at T1 (p<0.05) had higher mRNA expression then A/A-C/C haplotypes individuals (Figure 4D). There were no differences in TGF-β plasma and mRNA expression levels between time points of PTB patients within both haplotypes (data not shown).

3.7. TLR-2 expression correlates with NF-κB and cytokine levels according to *TLR-4* Asp299Gly/Thr399Ile genotypes

Controls A/A-C/C diplotype showed a direct correlation of TLR-2 mRNA expression with NF-κB mRNA expression (r=0.70; p=0.002). Direct correlations were also seen with TNF-α plasma levels at T1 (r=0.54; p=0.01) and TNF-α (r=0.68; p=0.001) and TGF-β (r=0.46; p=0.04) mRNA expressions at T2 of PTB patients A/A-C/C diplotype and with TNF-α plasma (r=0.95; p<0.05) and IL-17 (r=0.95; p<0.05) and IL-10 (r=0.95; p<0.05) mRNA expressions at T2 of PTB patients A/G-C/T diplotype. Inverse correlation were seen with TGF-β plasma level at (r=-0.50; p=0.02) T1 and IL-17 at T3 (r=-0.55; p=0.04) mRNA expressions in PTB patients A/A-C/C diplotype and with IL-17 (r=-0.95; p<0.05) and TGF-β (r=-0.95; p<0.05) plasma levels at T2 in PTB patients A/G-C/T diplotype (r=0.82; p=0.02).

Percentage of $CD14^{+}$TLR-2 cells showed a direct correlation with TGF-β plasma level in controls A/G-C/T (r=0.49; p=0.04) and with TNF-α plasma level at T1 in PTB patients A/G-C/T (r=0.87; p<0.05). These cells had an inverse correlation with IL-10 mRNA expression in controls A/A-C/C (r=-0.64; p=0.01), with IL-17 plasma level in controls A/G-C/T (r=-1; p=0.000001) and with IFN-γ (r=-0.43; p<0.05) and TGF-β (r=-0.50; p=0.01) at T1 and with IFN-γ (r=-0.45; p<0.05) at T2 of PTB patients A/A-C/C.

Percentage of $CD14^{+}$TLR-2/4 cells had a direct correlation with TGF-β plasma level in controls A/G-C/T (r=0.52; p=0.04) and with IL-17(r=0.46; p=0.03) and IFN-γ (r=0.52; p=0.01) mRNA expression at T1 of PTB patients A/A-C/C. An inverse correlation of these cells was seen with IL-12 plasma level in controls A/G-C/T (r=-1; p=0.00001), with IFN-γ plasma level at T1 of PTB patients A/A-C/C (r=-1; p=0.00001), with IL-10 mRNA expression at T1 (r=-0.45; p=0.04) and with IL-17 plasma level at T3 (r=-1; p=0.00001) of PTB patients A/G-C/T.

Percentage of $CD3^{+}$TLR-2 cells had a direct correlation with TGF-β mRNA expression at T1 (r=0.95; p=0.01) and IL-12 plasma level at T2 (r=0.95; p<0.05) of PTB

patients A/G-C/T. An inverse correlation of these cells was shown with IL-17 plasma level in controls A/A-C/C (r=-0.52; p=0.03), with IFN-γ plasma level in controls A/G-C/T (r=-1; p=0.000001), with IFN-γ plasma level at T1 of PTB patients A/A-C/C (r=-0.54; p=0.01), with IL-17 plasma level at T1 (r=-0.87; p<0.05) and TNF-α at T2 (r=-0.95; p<0.05) of PTB patients A/G-C/T.

Percentage of $CD3^{+}$TLR-2/4 cells had a direct correlation with NF-κB (r=0.58; p=0.01) mRNA expression in controls A/A-C/C, with TGF-β mRNA expression at T1 (r=0.95; p=0.01) and IL-12 plasma level at T2 (r=0.95; p<0.05) of PTB patients A/G-C/T. Inverse correlation of these cells were seen with IL-17 plasma level in controls A/A-C/C (r=-0.62; p=0.01), with IFN-γ plasma level in controls A/G-C/T (r=0.95; p<0.05), with IFN-γ plasma level (r=-1; p=0.000001) and IL-10 mRNA expression (r=-0.52; p=0.01) at T1 and IL-17 plasma level at T2 (r=-0.61; p=0.01) of PTB patients A/A-C/C, with IL-17 plasma level at T1 of PTB patients A/G-C/T (r=-0.87; p<0.05).

3.8. NF-κB expression correlates with cytokine levels according to *TLR-4* Asp299Gly/Thr399Ile genotypes

NF-κB mRNA expression showed direct correlations with IL-10 plasma levels (r=0.60; p=0.01) and TNF-α mRNA expression (r=0.60; p=0.01) at T2, with IL-12 (r=0.50; p=0.04) and IL-10 (r=0.48; p=0.04) mRNA expressions at T3 of PTB patients A/A-C/C diplotype and with IL-12 mRNA expression at T1 (r=0.90; p=0.04) of PTB patients A/G-C/T diplotype. Inverse correlation were seen with TNF-α plasma level in controls A/G-C/T diplotype (r=-1; p=0.000001) and IL-17 mRNA expression at T1 (r=-0.90; p=0.04) in PTB patients A/G-C/T diplotype.

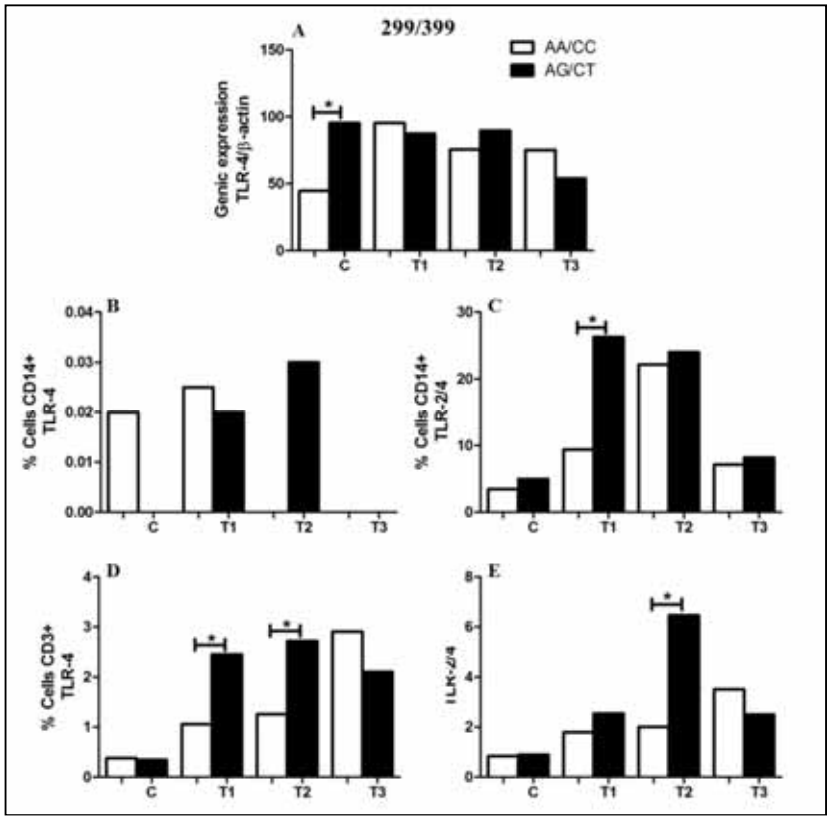

Figure 1. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on TLR-2 mRNA expression (A) and cell surface expression (B, C, D, E) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=22, A/G-C/T =5; T2: A/A-C/C=19, A/G-C/T =4; T3: A/A-C/C=18, A/G-C/T =2. * $p < 0.05$

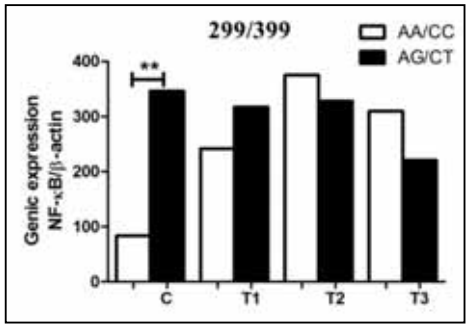

Figure 2. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on NF-κB mRNA expression in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=22, A/G-C/T =5; T2: A/A-C/C=19, A/G-C/T =4; T3: A/A-C/C=18, A/G-C/T =2. ** p=0.008

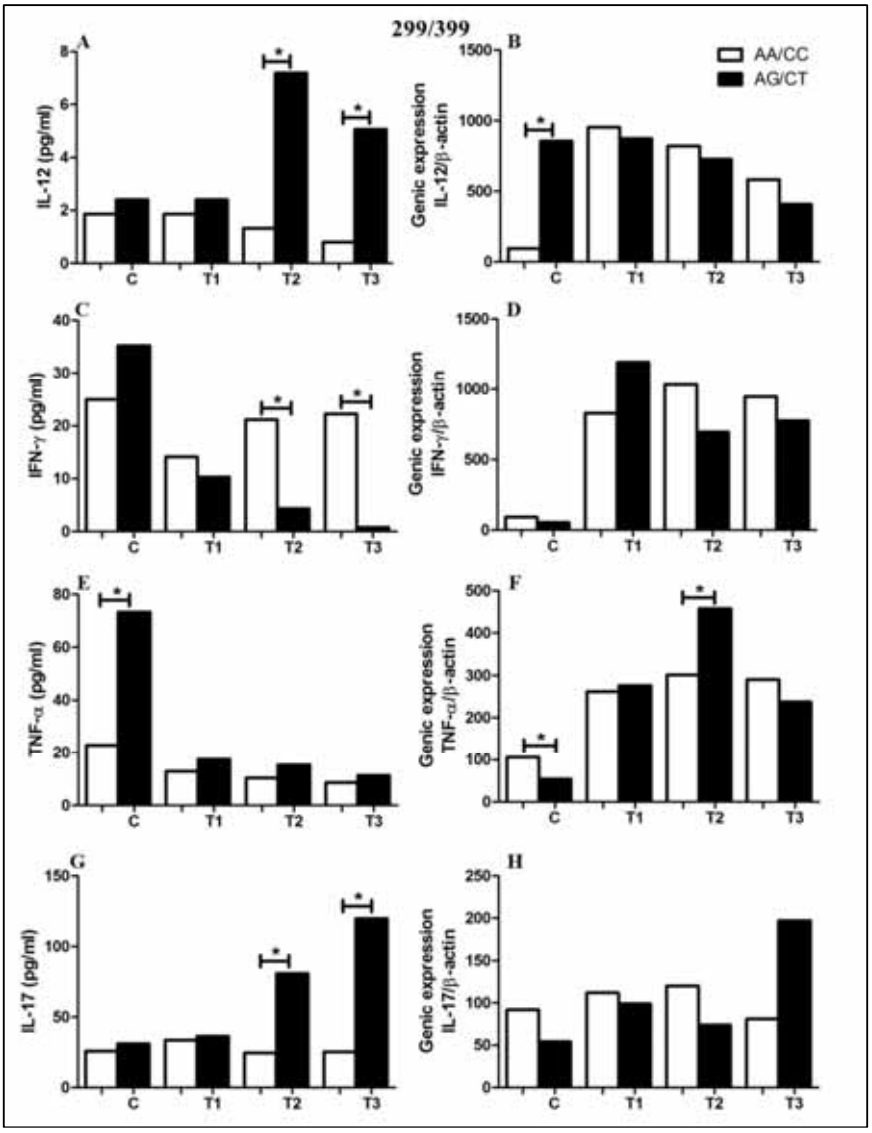

Figure 3. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on pro-inflammatory cytokine plasma levels (A, C, E, G) and mRNA expression (B, D, E, H) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A, G): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=21, A/G-C/T =5; T2: A/A-C/C=18, A/G-C/T =4; T3: A/A-C/C=17, A/G-C/T =2; (B, D, F, H): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=22, A/G-C/T =5; T2: A/A-C/C=19, A/G-C/T =4; T3: A/A-C/C=18, A/G-C/T =2; (C): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=21, A/G-C/T =5; T2: A/A-C/C=17, A/G-C/T =4; T3: A/A-C/C=17, A/G-C/T =2. * $p < 0.05$

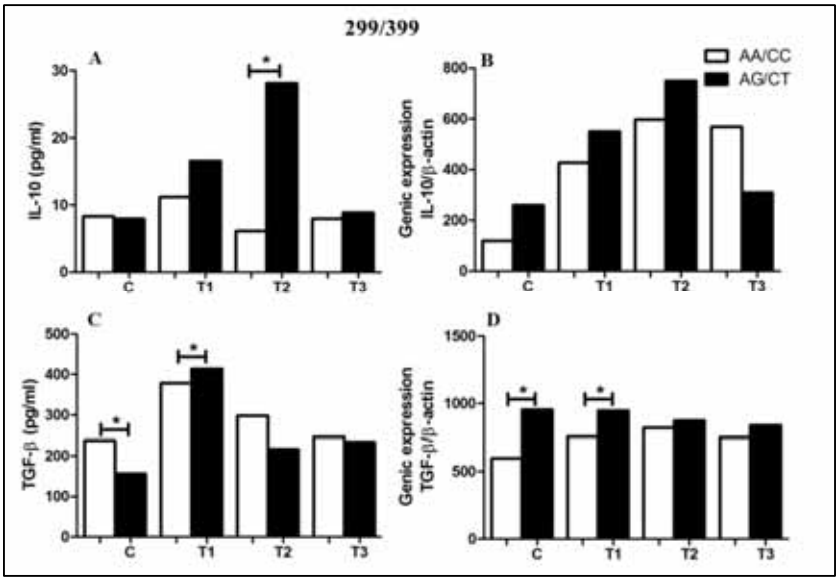

Figure 4. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on anti-inflammatory cytokine plasma levels (A, C) and mRNA expression (B, D) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=21, A/G-C/T=5; T2: A/A-C/C=16, A/G-C/T=4; T3: A/A-C/C=15, A/G-C/T =2; (B,D): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=22, A/G-C/T =5; T2: A/A-C/C=19, A/G-C/T =4; T3: A/A-C/C=18, A/G-C/T =2; (C): C: A/A-C/C=17, A/G-C/T =3; T1: A/A-C/C=20, A/G-C/T =5; T2: A/A-C/C=17, A/G-C/T =4; T3: A/A-C/C=16, A/G-C/T=2. * $p < 0.05$

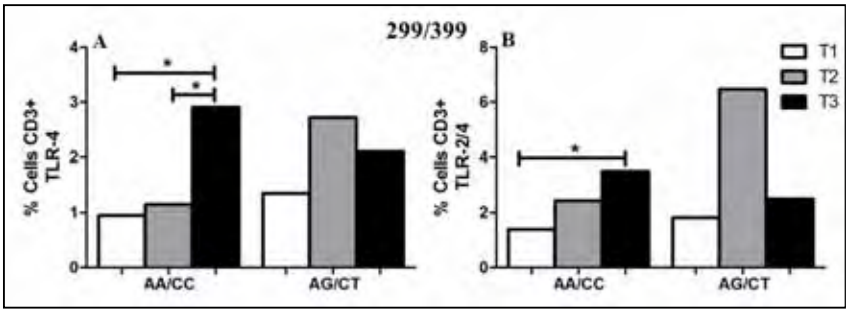

Figure 5. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on cell surface expression of $CD3^{+}$TLR-2 cells (A) and $CD3^{+}$TLR-2/4 cells (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six

months). Data are shown as median. Number of individuals in (A, B): A/A-C/C=16, A/G-C/T =2. * $p < 0.05$

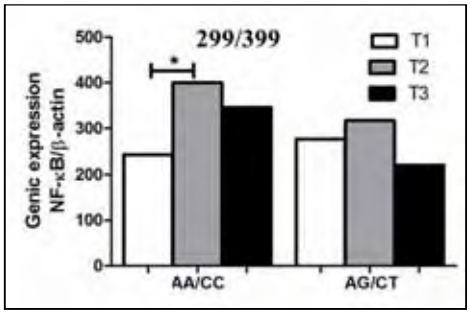

Figure 6. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on NF-κB mRNA expression in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals: A/A-C/C=16, A/G-C/T =2. * $p < 0.05$

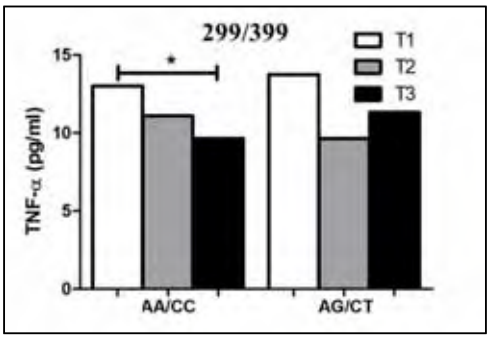

Figure 7. Influence of *TLR-4* Asp299Gly/Thr399Ile gene SNP on cytokine plasma levels (A) and mRNA expression (B) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals A/A-C/C=15, A/G-C/T=2. * $p < 0.05$

4. Discussion

TLR-4 is essential to initiate innate responses since it can interact with both heat labile soluble mycobacterial factor and whole viable *M. tuberculosis* (Means et al. 1999;

Tsuji et al. 2000). Two nonsynonymous polymorphisms of *TLR4* gene have been described with minor allele frequencies higher than 0.05. These are a A/G transition causing an Asp→Gly substitution at 299 residue, and a C/T transition causing the Thr399Ile protein change. Besides, and these polymorphisms polymorphisms are in linkage disequilibrium and A/A-C/C, A/G-C/T diplotypes are observed in high frequency in the population. (Fewerda et al. 2008).

Every second of every day, *M. tuberculosis* infects another human being somewhere in the world (WHO Publications 2011). Despite this, only around 1 in 10 of those infected will ever develop active disease, which raises a question: why do some individuals develop disease, while others do not? (Doherty and Arditi 2004) Since genetic factors might play a role in the pathogenesis of this disease we evaluated the influence of *TLR4* Asp299Gly/Thr399Ile on the immune response of PTB patients during anti-TB treatment.

Our results showed that control individuals with A/G-C/T diplotype had higher TLR-4 genic expression when compared with A/A-C/C individuals and no significant differences on TLR-4 cell expression. Although not significant our study showed that normal individual A/A-C/C had a tendency to present higher $CD14^{+}$TLR-2, which is in agreement with another study evaluating expression in normal children (Tulic et al 2007). We also observed that PTB patients, in general, had no difference in TLR-4 gene expression, but that A/G-C/T combined genotype had higher expression of $CD14^{+}$TLR-2/4, $CD3^{+}$TLR-4 and $CD3^{+}$TLR-2/4 cells than A/A-C/C patients.

Since an induction signal is required to up-regulate TLR-4 translocation to cell surface our results suggest that in PTB patients A/A-C/C carriers the receptor fails to translocate to the cell surface and is trapped within the cell. Low surface TRL-4 expression could confer poor capacity to bind ligand following exposure leading to an impaired cytokine response

(Kurt-Jones et al. 2000; Tulic et al 2007). To our knowledge this is the first report of this *TLR4* functional action on PTB patients.

We also showed that normal individuals carrying A/G-C/T diplotype had higher mRNA levels of NF-κB when compared to A/A-C/C but none diplotype were lower than normal *TLR4*. Our results do not agree with other studies using transfected cells that showing that any of the *TLR4* diplotypes have a decreased NF-κB activity compared with normal *TLR4* (Arbour 2000; Fewerda et al. 2008; Schwartz 2001; Schwartz 2002). A lower expression of NF-κB could lead to reduced cytokine production (Fewerda et al. 2008). Even with significant correlation, since our results showed a reduced TLR-4 cell surface expression and cytokine production in A/A-C/C genotype PTB patients and no differences in NF-κB activity, we suggest that the TLR-4 action could be through the activation of another signaling pathway.

Regarding cytokine profile our results showed that different *TLR4* Asp299Gly/Thr399Ile combined genotypes can induce different plasma levels of cytokines. Interesting data was that PTB patients A/G-C/T diplotype had high IL-12, IL-17, IL-10 and TGF-β and low IFN-γ plasma levels when compared with A/A-C/C in patients within three months of treatment. Our results also showed that A/G-C/T normal individuals have higher IL-12 gene expression, with is not in agreement with study that evaluated A/G-C/T normal children that reported lower IL-12 mRNA expression following LSP induction (Tulic et al. 2007).

IL-12 is induced following phagocytosis of *M. tuberculosis* by macrophages and dendritic cells, which leads to development of a protective Th1 response with production of IFN-γ (Raja 2004). Regarding this, production of higher levels of IL-12 should induce higher IFN-γ levels, which was not seen in our study. Since IFN-γ is a key cytokine in activation of macrophages for mycobacterial stasis and killing, persistence of low IFN-γ production from

middle to the end of therapy suggests that A/G-C/T genotype individuals may underlie their increased risk for reactivation of a latent PTB focus. Such an inadequate IFN-γ production may result in failure of macrophage activation, which could lead to active disease progression (Vidyarani et al. 2006).

IL-17 appears to be critical to the induction of *M. tuberculosis*–specific memory response and the mediation of protection against challenge infections and during vaccinations, although Th17 cells are not as important as Th1 cells in mediating protection against primary *M. tuberculosis* infection (Khader et al. 2005; Khader et al. 2007; Umemura et al. 2007; Wozniak et al. 2006). It seems that IL-17 had a pro-inflammatory effect in A/G-C/T haplotype PTB patients, since higher levels of IL-17 and lower levels of IFN-γ, and all of these individuals were considered cured at the end of the therapy. To our knowledge this is the first report to evaluate *TLR4* influence on IL-17 levels.

IL-10 is considered to be an anti-inflammatory cytokine and directly inhibits $CD4^+$ T cell responses, as well as by inhibiting antigen presenting cells (APC) function of cells infected with mycobacteria (Rojas et al. 1999). Inhibition of the pro-inflammatory response could result in impaired IFN-γ production, as seen in our A/G-C/T genotype PTB patients. Our results don't agree with studies using e*x vivo* stimulation of whole blood or isolated monocytes which revealed reduced IL-10 release in individuals A/G-C/T (Dheus et al. 2008; Koch et al. 2011). Still in disagreement with our results, studies evaluating normal individuals after LPS stimulation and respiratory syncytial virus (RSV) patients showed no influence of *TLR4* on IL-10 level (Douville et al. 2010; Taudorf et al. 2008).

TGF-β is able to have pro and anti-inflammatory functions depending on its concentration. At high levels it will deactivate macrophages and can impaired TNF-α production and in lower levels acts as a chemotaxis factor for monocytes and will induce TNF-α secretion (Hirsch et al. 1994; Kehrl et al. 1986; Lezini et al. 1977). In our study TGF-

β had probably an anti-inflammatory response at the beginning and had a role on the induction of fibrosis from middle to end of treatment, since our results showed higher TGF-β levels and lower TNF-α.To our knowledge this is the first report to evaluate *TLR4* influence on TGF-β levels.

Our study found no influence of the *TLR4* Asp299Gly/Thr399Ile polymorphism on TNF-α level in PTB patients. This result agrees with other studies with *ex vivo* and *in vivo* LPS stimulation, RSV patients and patients undergoing elective cardiac surgery which showed no influence of *TLR4* on TNF-α level (Douville et al. 2010; Koch et al. 2011; Taudorf et al. 2008). Still not in agreement with our results, sepsis and pneumonia patients A/A-C/C showed a stronger induction of TNF-α following LPS-stimulation compared with patients A/G-C/T (Kumpf et al. 2010). Our results also showed higher plasma levels of TNF-α in the controls A/G-C/T, which agrees with other study which showed that normal individuals with the AG genotype had higher TNF-α in response to LPS (Fewerda et al. 2007; Kumpf et al. 2010).

Since TNF-α is important for walling off infection and preventing dissemination by granuloma formation, low levels of this cytokine, as seen in our PTB patients in our general results, could impaired the containment of the bacilli and increase a risk of reactivation, as shown in rheumatoid arthritis patients who were undergoing anti-TNF-α therapy (Feldmann and Maini 2001; Maini et al. 1999).

Study that evaluated association of *TLR4* Asp299Gly/Thr399Ile with hyporesponsiveness to inhaled LPS showed that immune response was due to different cells and that it is possible that any functional effect of this gene is determined by the cell lineage or maturity in which TLR-4 is expressed (Newport at al. 2004). This fact could explain functional studies with different results regarding the *TLR4* Asp299Gly/Thr399Ile polymorphism.

The present study suggests that *TLR4* Asp299Gly/Thr399Ile polymorphism is associated with differential TLR-4 and cytokine levels during anti-TB treatment. Besides TLR-4 action could be through the activation of signaling pathway other than that of the NF-κB. Since all of our PTB patients were considered cured after treatment it seems that the low levels of IFN-γ and TNF-α were enough to resolve the active disease and that other cytokines and immune mechanisms could also be involved in this process. We were no able to make any association with disease outcome due to the low number of patients evaluated and to the fact that they had all a moderate type of PTB. Since PTB patients with A/G-C/T haplotype have a lower IFN-γ, we suggest that they may have higher chances to reactivate a TB focus. Since TLR-4 plays a major role in initiating immune response against *M. tuberculosis* polymorphisms in *TLR4* gene could be important to better understand protective immune responses and may serve as genetic risk markers for TB susceptibility.

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VI- Capítulo 3

Cytokine polymorphisms and their influence in cytokine levels in Brazilian patients with pulmonary tuberculosis during anti-tuberculosis treatment.

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Abstract

Tuberculosis is a major public health problem and it is well known that cytokines play an essential role during the immune response during active tuberculosis. Reasons why only 5-10% of the infected individuals develop active tuberculosis are still unknown. Cytokine genes have been described in association with altered levels of cytokines. Therefore the aim of this study was to verify if *IFNG, IL12B, TNF, IL17A, IL10 and TGFB1* gene polymorphisms influence the immune response of Brazilian patients with pulmonary tuberculosis at different time points of anti-tuberculosis treatment: T1 (beginning), T2 (3 months) and T3 (end). For this we genotyped cytokine polymorphisms and evaluated the immune response of pulmonary tuberculosis patients during the time of anti-tuberculosis treatment. *IFNG* +874 T allele and *IFNG* +2109 A allele both at T2 and T3were associated with higher IFN-γ levels. *IL12B* +1188 C allele was associated with higher IL-12 levels at T2. *TNF* -308 A allele was associated with higher TNF-α levels in the controls. *IL17A* A allele at rs7747909 was associated with higher IL-17 levels at T3. *IL10* -819 T allele was associated with higher IL-10 levels during all time of treatment. *TGFB1* +29 CC genotype was associated with higher TGF-β levels at T2. The present study suggests that *IFNG* +874T/A, *IFNG* +2109A/G, *IL12B* +1188A/C, *IL10* – 819C/T and *TGFB1* +21C/T are associated with differential cytokine levels in pulmonary tuberculosis patients and may play a role in the initiation and maintenance of acquired cellular immunity to tuberculosis and in the outcome of the active disease and the anti-tuberculosis treatment.

Keywords

Pulmonary tuberculosis, single nucleotide polymorphisms, cytokine, anti-tuberculosis

treatment

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is an obligatory aerobic, intracellular pathogen, which has a predilection for the lung (Raja 2004). Macrophages initiate defense performing phagocytosis of bacilli and regulating immune response mediated by pro-inflammatory cytokines such as TNF-α. T cells and natural killer (NK) secretes IFN-γ which activates alveolar macrophages to produce reactive intermediates from nitrogen and oxygen, which inhibit the growth and promote the death of mycobacteria (Kaufmann 2002). IL-12, produced mainly by macrophages and dendritic cells, has a key role in the immune response to *M. tuberculosis*, making a link between innate and adaptive immunity. Moreover, it induces T and NK cells to produce pro-inflammatory cytokines such as IFN-γ and TNF-α and also regulates the production of IL-17 (Hoeve et al. 2006; Kaufmann 2002). Synergism of IFN-γ, IL-12, TNF-α and IL-17 will activate macrophages, stimulating these cells to eliminate the intracellular pathogen, as a major effector mechanism of cellular immune response (Sahiratmadja et al. 2007).

Despite the protective effect of Th1 response against tuberculosis certain cytokines such as TNF-α are correlated with the immunopathogenesis of the disease (van Crevel et al. 2002). To prevent tissue damage active tuberculosis is associated with decreased Th1 and increased production and action of suppressing cytokines produced by Th2 and T regulatory (Treg) cells, IL-10 and TGF-β, respectively, which act by deactivating macrophages, modulating pro-inflammatory cytokines and reducing the antigen presenting function of T cells (Flynn and Chan 2001). TGF-β, also participates in the induction of fibrosis (Toossi and Ellner 1998).

Tuberculosis (TB), as well as other infectious diseases, has a complex character since various aspects of parasite-host interaction contribute to the occurrence of the outcome. In this scenario there is an important contribution of human genetic susceptibility to disease after exposure to *M. tuberculosis* (Bellamy 2003; Casanova and Abel 2002; Remus et al. 2003). Cytokines have a key role in the defense against mycobacteria and their genes might be considered candidates for host susceptibility to the onset of active TB.

The association between cytokines polymorphisms with susceptibility to TB has been reported, however, few studies have investigated their role in modulating the immune response during pulmonary tuberculosis (PTB) treatment. Therefore the aim of this study was to verify the influence of *IFNG, IL12B, TNF, IL17A, IL10 and TGFB1* gene polymorphisms in the immune response of Brazilian patients with PTB under anti-TB treatment.

2. Material and Methods

2.1. Study population

The study group enrolled 31 Brazilian patients attending the Infectious and Parasitic Diseases Services at Botucatu Mecdical School University Hospital – UNESP, Botucatu Teaching Health Centre and Primary Healthcare units of Botucatu and surrounding region with PTB diagnose confirmed by sputum smear or culture positive for *M. tuberculosis*, or else by clinical-epidemiologic data and laboratory and image exams compatible with active TB. Patients with PTB concurrent with other active granulomatous disease or HIV positive were excluded. All patients diagnosed with PTB

received treatment for six months, using different combinations of the four first-line drugs: isoniazid, ethambutol, pyrazinamide and rifampicin. For the evaluation of immunological function, patients samples were collected based on the anti-TB treatment time line, defined as T1: after diagnosis and with no more than one month of treatment; T2: with three months of treatment; and T3: with six months of treatment. Patients distribution in each time line: T1 (n=28); T2 (n=24); T3 (n=21), and with the three time points T1, T2 and T3 (n=19). As normal controls (C), we studied 20 health care workers from Botucatu Medical School (Botucatu, São Paulo, Brazil), 9 males (mean age 40.4 years) and 11 females (mean age 34.1 years), without clinical complaints and with no history of TB disease, autoimmune disease and other infectious disease. All controls were tuberculin test (PPD) positive (hardering ≥ 5mm). All patients and controls agreed to participate in the study, after due clarification and signing of the written informed consent.

2.2. SNP Genotyping

Seven SNPs were studied: *IFNG* + 874T/A; *IFNG* +2109A/G; *IL12B* +1188A/C; *TNF* -308G/C; *IL17A* rs7747909; *IL10* -819C/T; and *TGFB1* +29C/T.

For this proposal peripheral blood (5 mL - per standard procedure) was drawn in EDTA, from patients (*n*=31) with PTB and controls (*n*=20) groups at base line, and genomic DNA was extracted from leukocytes employing DNAzol commercial reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantification and purity determination of the extracted DNA was determined on a spectrophotometer (reading at 260, 280 and 320nm). Amplification of the genomic regions of interest was performed by PCR using 20 to 50 ηg of DNA, recombinant Taq

DNA polymerase, 0.2 mM of each dNTP (deoxy-nucleotide - adenine, guanine, thymine or cytosine - triphosphate), 0.3 to 1 mM concentration of each of specific primers, appropriate buffer, ultrapure water. The *IFNG* +874T/A was genotyped by PCR-ARMS as described by Pravica (2000). The SNP +2109A/G creates a restriction site for the enzyme *AciI* and was genotyped by a previously reported PCR-RFLP method (Henri et al. 2002). The *IL12B* +1188 genotyping was reached using a PCR-RFLP method in accordance with García-González (2005). Fluorescence-based TaqMan technology (Applied Biosystems) was applied to produce genotypes of *IL17A* and *TGFB1 polymorphisms* according to the manufacturer's instructions. *TNF* -308A/G genotyping was reached using a PCR-RFLP method in accordance with Wilson (1992). TGFB1 +29C/T genotyping was reached using a PCR-RFLP method in accordance with Santos (2002).

2.3. Cytokines gene expression by real time PCR (qPCR)

To evaluate IFN-γ, IL-12, TNF-α, IL-17, IL-10 and TGF-β mRNA expression, peripheral blood (20 mL - per standard procedure) was taken in heparinized blood tubes, at a single time point from controls (n=20) and at three serial time points from patients (n=31) with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and T3), as defined previously. Peripheral blood mononuclear cells (PBMC) were obtained through a Histopaque® gradient separation method (Boyum 1968). The layer rich with lymphocytes and monocytes was aseptically removed and washed twice with PBS for 15 min at 1500 RPM. The cell suspension was re-suspended and the identification and viability of cells was determined by counting with Turk solution (50μL aliquots of cell suspension with 50μL of the dye solution at 5%). Total RNA extraction from PBMC

($2x10^6$ Cells/mL) was made using Trizol Reagent (Invitrogen®), according to manufacturer's instructions. Concentration of total RNA was determined by the absorbance values of samples at 260 nm. cDNA was synthesized from 1μg of total RNA using the Reverse Transcriptase Super Script™ II (Invitrogen®) and RNAse H (Invitrogen®) as manufactures instructions. From cDNA obtained above, gene amplification was made according to the protocol of Applied Biosystems Power Sybr Green on the ABI Prims® 7300 Sequence Detector (Applied Biosystems). Product purity was confirmed by dissociation curve analysis. Gene expression was quantified relative to the values of the control group after adjusting for β-Actin. Primers are described in Table 1.

Table 1. Primers sequence for qPCR

Primers	Forward Sequence	Reverse Sequence
IFN-γ	5`-AAAAGAGTTCCATTATCCGCTACATC-3`	5`-GTTTTGGGTTCTCTCTTGGCTGTTA-3`
IL-12	5`-ACCTCCACCTGCCGAGAAT-3`	5`-CATGGTGGATGCCGTTCA-3`
TNF-α	5`-GGTTTGCTACAACATGGGCTACA-3`	5`-CCCCAGGGACCTCTCTCTAATC-3`
IL-17	5`-TTAGGC ACATGGTGGACAATCGG-3`	5`-ATGACTCCTGGGAAGACCTCA TTG-3`
IL-10	5`-CTTGATGTCTGGGTCTTGGTTCT-3`	5`-GCTGGAGGACTTTAAGGGTTAACCT-3`
TGF-β	5`-AGGGCCAGGACCTTGCTG-3`	5`-CAAGGGCTACCATGCCAACT-3`
β-actina	5`-GCTGGAAGGTGGACAGCGA-3`	5`-GGCATCGTGATGGACTCCG-3`

2.4. Plasma cytokine levels

Plasma samples were obtained from the same peripheral blood used for the genetic expression of cytokines from controls (*n*=20) and at three serial time points from patients with PTB, based on the anti-tuberculosis treatment time line (T1, T2 and

T3), as defined previously. Samples were maintained frozen (-80°C) until use. Quantikine®ELISA kits (R&D Systems®) were used, according to manufacturer's instructions, to measure IFN-γ, IL-12, TNF-α, IL-17, IL-10 and TGF-β plasma levels and method sensitivity were in accordance to each kit. Cytokine analysis was not possible in all 31 PTB patients, therefore the distribution of individuals among cytokines was: IFN-γ (*n=30)*, IL-12 (*n=30)*, TNF-α (*n=30)*, IL-17 (*n=30)*, IL-10 (*n=30)* and TGF-β (*n=29)*.

2.5. Statistical analysis

Comparisons between different genotypes in the control group and patient group were made using Mann-Whitney Test with two-tail P value. For the comparison among the three time points of the treatment in the group of patients (T1, T2 and T3) Friedman Test was used and Dunn's Multiple Comparisons Test was applied as a post test. Results were considered significant when $p<0.05$. Tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Demographic characteristics of PTB patients

Distribution among PTB patients regarding sex and age was 23 males with mean age of 48.8 years and 8 females with mean age of 38.9 years. PTB patients diagnosis was confirmed by sputum smear or culture positive for *M. tuberculosis* (*n*=2), sputum

smear or culture positive for *M. tuberculosis* and image exams compatible with active TB (*n*=19) or by image exams compatible with active TB (*n*=10). All patients had respiratory symptoms consistent with dysphonea, cough and ventilation-dependent pain, and most of them presented constitutional symptoms, consistent with weight loss, fever and weakness at the beginning of the anti-TB treatment (T1). The symptoms were less frequent along treatment time line (T2) and at the end (T3) all patients were considered recovered. Characteristics of clinical-epidemiologic data and/or image exams after medical evaluation showed that all of our patients had a moderated form of active TB disease.

3.2. General immune response during anti-TB treatment

In general, when compared to controls, plasma levels of IFN-γ, IL-12 and IL-10 were similar among PTB patients during anti-TB treatment, of IL-17 and TGF-β were increased among PTB patients only at the beginning of anti-TB treatment (T1) and of TNF-α were lower among PTB patients during anti-TB treatment (T1, T2 and T3) (data not shown). When compared to controls, mRNA expression of IL-12, TNF-α and IL-17 were similar among PTB patients during anti-TB treatment (T1, T2 and T3), of IFN-γ and IL-10 were increased among PTB patients during anti-TB treatment (T1, T2 and T3) and of TGF-β was increased only at T2 time point among PTB patients (data not shown).

3.3. Influence of *IFNG* +874T/A and +2109A/G gene polymorphisms on IFN-γ plasma level and mRNA expression

IFNG +874T/A gene SNP PTB patients T carriers patients had significant higher plasma and mRNA expression levels of IFN-γ when compared to individuals with AA genotype at T2 (p=0.04; p=0.03) and T3 (p=0.04; p=0.03) time points of the treatment (Figures 1A and 1B). When we compared all three time points AA genotype PTB patients at T1 presented significant higher plasma levels than T2 (p<0.05) and T3 (p<0.05) (Figure 8A) and no differences for mRNA expression (Figure 8B). T carriers at T2 presented significant higher mRNA expression than T1 (p<0.05) (Figure 8B) and no differences on the IFN-γ plasma level (Figure 8A). There was no influence of this polymorphism in the control group (Figures 1A and 1B).

Results for the *IFNG* +2109A/G gene SNP showed no difference between the genotypes in the controls. Genotype AA at T2 (p=0.04) and T3 (p=0.02) time points of PTB patients had significant higher levels of IFN-γ than AG genotype PTB patients (Figure 2A). Comparisons between AG genotype PTB patients time points, using the same individuals, showed that T1 had significant higher levels of IFN-γ than T2 (p<0.05) and T3 (p<0.05). There were no differences between time points of PTB patients with AA genotype (Figure 8C). No significant difference for mRNA expression was seen between genotypes in the controls and in PTB patients, although patients with AA genotype tended to present a higher expression when compared to AG individuals (Figure 2B). There were also no differences between time points of PTB patients for mRNA expression within both genotypes (data not shown). In our study group we did not find individuals with the GG genotype.

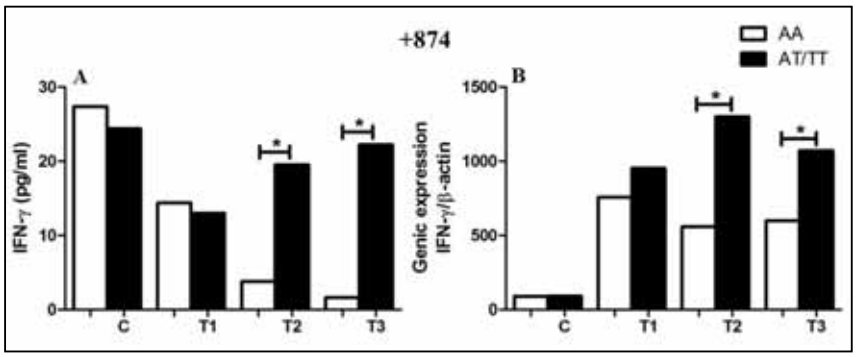

Figure 1. Influence of *IFNG* +874T/A gene SNP on IFN-γ plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: AA=7, AT/TT=13; T1: AA=12, AT/TT=15; T2: AA=12, AT/TT=10; T3: AA=10, AT/TT=10; (B): C: AA=7, AT/TT=13; T1: AA=12, AT/TT=16; T2: AA=12, AT/TT=11; T3: AA=10, AT/TT=11. * $p < 0.05$

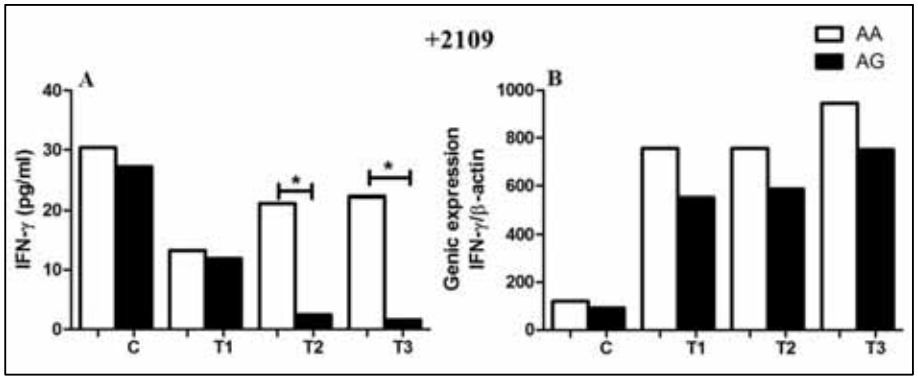

Figure 2. Influence of *IFNG* +2109A/G gene SNP on IFN-γ plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: AA=9, AG=11; T1: AA=19, AG=8; T2: AA=14, AG=8; T3: AA=14, AG=6; (B): C: AA=9, AG=11; T1: AA=19, AG=9; T2: AA=15, AG=9; T3: AA=14, AG=7. * $p < 0.05$

3.4. Influence of *IL12B* +1188A/C gene polymorphism on IL-12 plasma level and mRNA expression

IL12B +1188 gene SNP allele C carriers had significant higher plasma levels of IL-12 than individuals with AA genotype in the control group (p=0.04) and in T2 (p=0.03) time point of PTB patients (Figure 3A). IL-12 mRNA expression analysis showed no difference when AA genotype and C carriers were compared in controls. However at T2 time point of PTB patients C carriers expressed more IL-12 mRNA expression than individuals with AA genotype (p<0.05) (Figure 3B). There were also no differences between time points of PTB patients for IL-12 plasma and mRNA expression levels within both genotypes (data not shown).

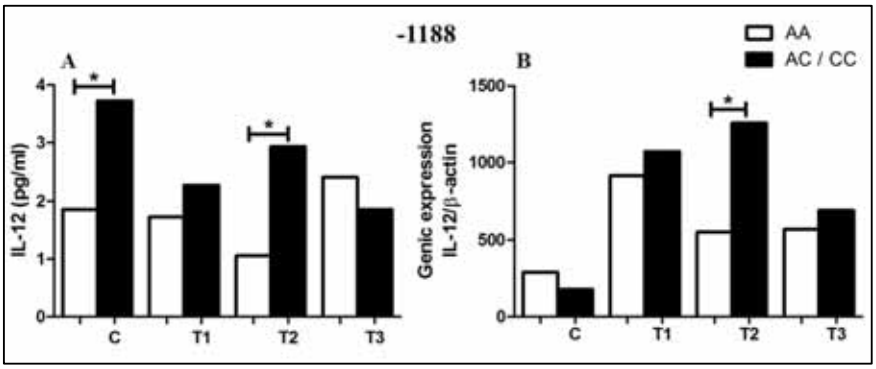

Figure 3. Influence of *IL12B* +1188A/C gene SNP on IL-12 plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: AA=10, AC/CC=10; T1: AA=11, AC/CC=16; T2: AA=12, AC/CC=9; T3: AA=9, AC/CC=9; (B): C: AA=10, AC/CC=10; T1: AA=12, AC/CC =16; T2: AA=13, AC/CC =11; T3: AA=10, AC/CC =11. * $p < 0.05$

3.5. Influence of *TNF* -308G/C gene polymorphism on TNF-α plasma level and mRNA expression

Control individuals with AG genotype had significant higher plasma levels of TFN-α than those with GG genotype (p=0.04). There was no difference between genotypes in the PTB patients group (Figure 4A). The analyses between time points of GG genotype PTB patients showed higher levels of TFN-α at T1 when compared with T3 (p<0.05). No differences were seen between time points of AG genotype PTB patients (Figure 8D). *TNF* -308G/C gene SNP did not influence TNF-α mRNA expression between controls. At T1, PTB patients with AG genotype had significant higher mRNA expression then those with GG genotype (p=0.02) (Figure 4B). There were also no differences between time points of PTB patients for mRNA expression within both genotypes (data not shown). In our study group we did not find individuals with the AA genotype.

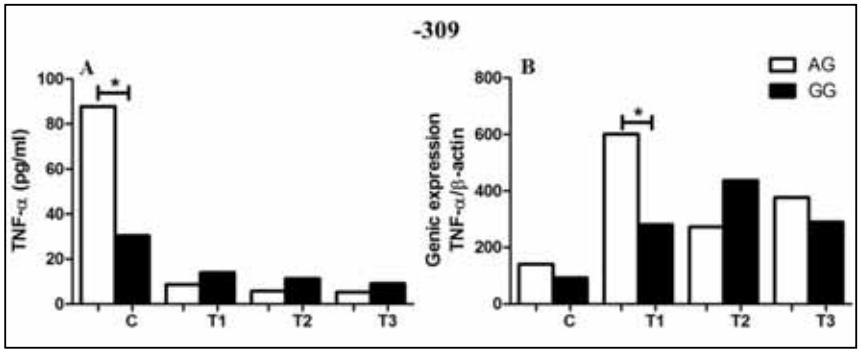

Figure 4. Influence of *TNF* -308G/C gene SNP on TNF-α plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: AG=5, GG=15; T1: AG =7, GG=20; T2: AG =4, GG =29; T3: AG =3, GG =17; (B): C: AG =5, GG =15; T1: AG =7, GG=21; T2: AG =4, GG =20; T3: AG =3, GG =18. * p < 0.05

3.6. Influence of *IL17A* rs7747909 gene polymorphism on IL-17A plasma level and mRNA expression

IL17 SNP rs7747909 did not influence IL-17 plasma levels between controls. PTB patients A carriers had significant higher IL-17 plasma levels only at T3 time point (p=0.04) (Figure 5A). Control individuals A carriers had significant higher IL-17 mRNA expression than those with GG genotype (p=0.04). PTB patients A carriers had significant higher IL-17 mRNA expression at T2 (p<0.05) and T3 (p=0.04) time points than those with GG genotype (Figure 5B). There were no differences between time points of PTB patients for IL-17 plasma and mRNA expression levels within both genotypes (data not shown).

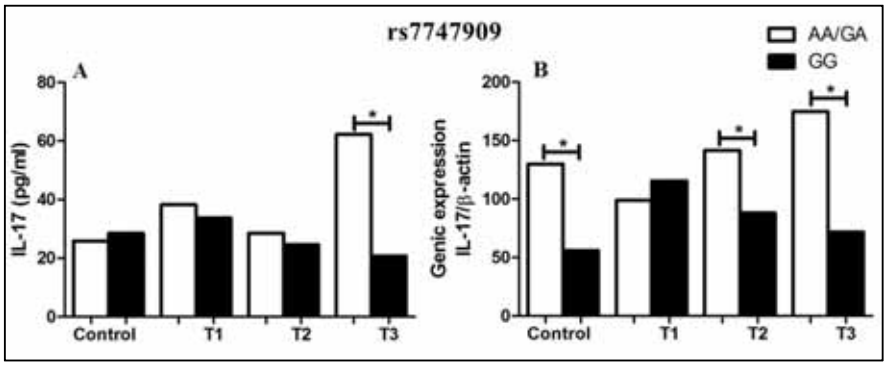

Figure 5. Influence of *IL17* SNP rs7747909 on IL-17 plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in each group: (A): CAA/AG=14; CGG=6; T1AA/AG=12; T1GG=15; T2AA/AG=11; T2GG=10; T3AA/AG=8; T3GG=10; (B): CAA/AG=14; CGG=6; T1AA/AG=12; T1GG=16; T2AA/AG=12; T2GG=12; T3AA/AG=9; T3GG=12. * p < 0.05

3.7. Influence of *IL10* -819C/T gene polymorphism on IL-10 plasma level and mRNA expression

There was no difference in IL-10 plasma levels in the controls between genotype CC and T carriers. PTB patients T carriers had significant higher levels of IL-10 when compared with those with CC genotype in all time points of treatment T1 (p<0.05), T2 (p=0.03) and T3 (p<0.05) (Figure 6A). IL-10 -819 SNP had no influence on IL-10 mRNA expression in the control group and PTB patients when CC genotype and T carriers were compared (Figure 6B). There were also no differences between time points of PTB patients for IL-10 plasma and mRNA expression levels within both genotypes (data not shown).

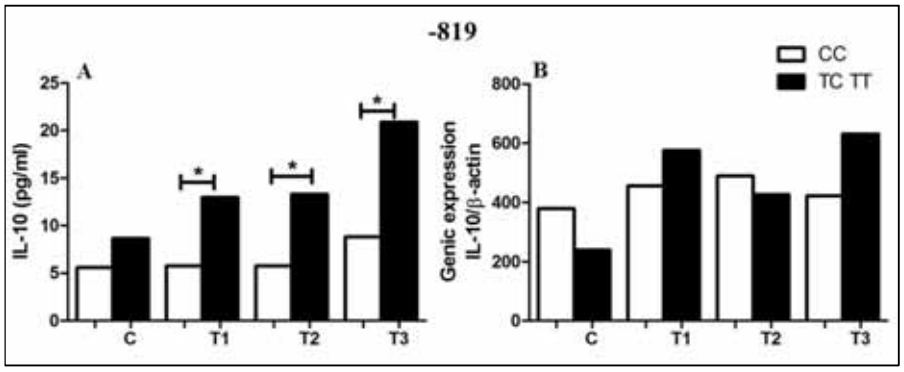

Figure 6. Influence of *IL10* -819C/T gene SNP on IL-10 plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients group at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): C: CC=8, TC/TT=12; T1: CC =11, TC/TT =16; T2: CC =9, TC/TT =12; T3: CC =9, TC/TT =9; (B): C: CC =8, TC/TT =12; T1: CC=12, TC/TT =17; T2: CC =10, TC/TT =14; T3: CC =10, TC/TT =11. * $p < 0.05$

3.8. Influence of *TGFB1* +29C/T gene polymorphism on TGF-β plasma level and mRNA expression

There was no difference in TGF-β plasma levels in the control group between both genotypes. PTB patients CC genotype had higher TGF-β plasma levels when compared with T carriers only at T2 time point (p=0.02) (Figure 7A). The analyses between time points of PTB patients CC genotype showed higher TGF-β plasma levels at T1 when compared to T3 (p<0.05) (Figure 8E). No difference in mRNA expression was seen in both controls and PTB patients within both genotypes (Figure 7B). There were also no differences between time points of PTB patients for mRNA expression within both genotypes (data not shown).

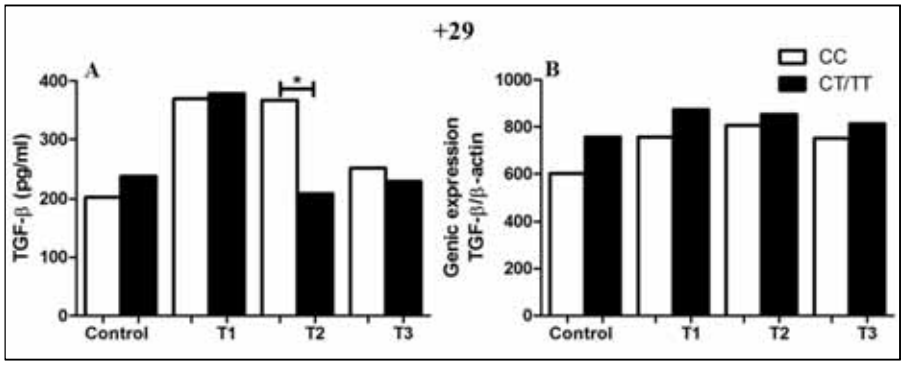

Figure 7. Influence of *TGFβ1* +29C/T gene SNP on TGF-β plasma levels (A) and mRNA expression (B) in the control group (C) and PTB patients at three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in each group: (A): CCC=5; CCT/TT=15; T1CC=7; T1CT/TT=19; T2CC=6; T2CT/TT=16; T3CC=5; T3CT/TT=14; (B): CCC=5; CCT/TT=15; T1CC=7; T1CT/TT=21; T2CC=6; T2CT/TT=28; T3CC=5; T3CT/TT=16. * $p < 0.05$

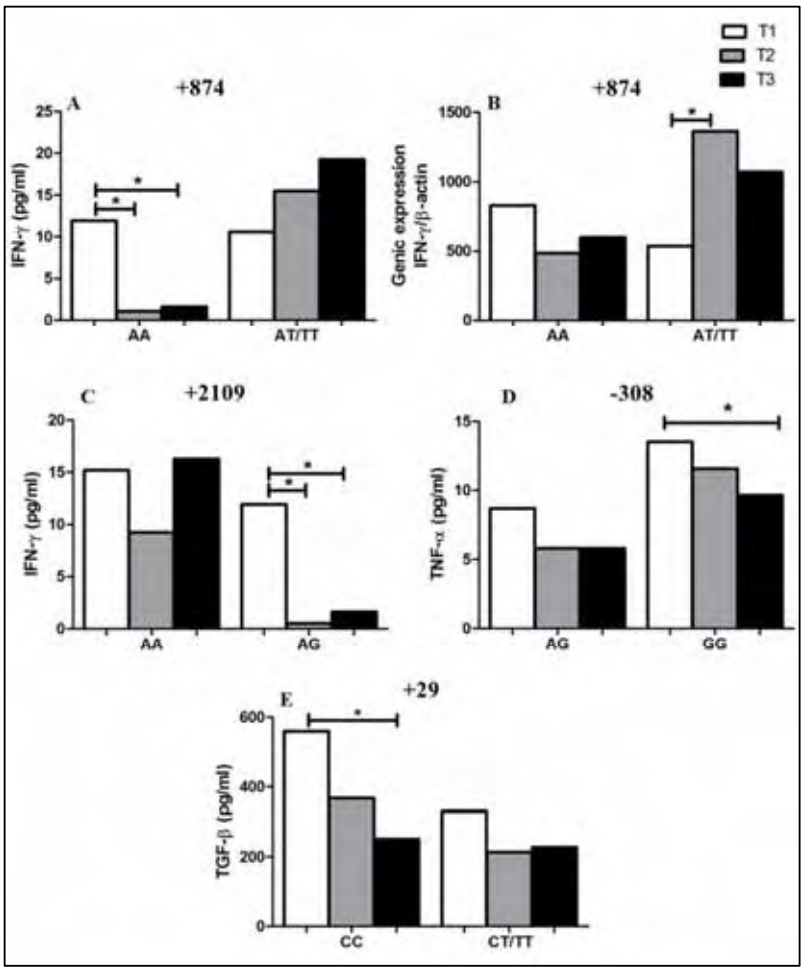

Figure 8. Influence of *IFNG* +874T/A gene SNP on IFN-γ plasma levels (A) and mRNA expression (B), *IFNG* +2109A/G gene SNP on IFN-γ plasma levels (C), *IL10* gene -819C/T SNP on IL-10 plasma levels (D) and TGFB1 +29C/T gene SNP on TGF-β plasma levels (E) in PTB patients that have all three time points of the treatment: T1 (after diagnosis and first month), T2 (three months) and T3 (six months). Data are shown as median. Number of individuals in (A): AA=9, AT/TT=8; (B): AA=10, AT/TT=9; (C): AA=11, AG=6; (D): AG=3, GG=15; (E): CC=5, CT/TT=12. * $p < 0.05$

4. Discussion

It is well known that cytokines play an essential role during the immune response to active TB disease and changes in their levels may lead to abnormal or

ineffective immune response, as seen in human infection by *M. tuberculosis* (Flynn and Chan 2001). The genetic component that contributes to susceptibility and progression of PTB probably involves an interaction between multiple alleles located on different genes and chromosomes (Hill 1998). Since a number of cytokine genes have been described in association with altered levels of cytokine (Pavrica et al. 2000; Yilmaz et al.2005), we evaluated the influence of cytokines SNP on immune response of PTB patients undergoing anti-TB treatment.

Our results showed that PTB patients T carriers had higher plasma and mRNA expression levels of IFN-γ in the middle and end of anti-TB treatment. IFN-γ is a key cytokine in activation of macrophages for mycobacterial stasis and killing (Kaufmann 2002). Studies have shown that *IFNG* +874A/T influence IFN-γ production by providing binding site for NF-κB and is associated with susceptibility to TB (Lopez-Maderuelo et al 2003; Pavrica et al. 2000; Sallakci et al. 2007) and it could have functional consequences for the transcription of IFN-γ production (Heinemeyer et al. 1998; Pavrica et al. 1999).

Study in Spain showed that genotype AA had the lowest IFN-γ production in PBMC culture after stimulation with PPD in PTB patients at the time of diagnosis and after completion of therapy (Lopes-Maderuelo et al. 2003), which agrees with our results at the end of anti-TB treatment. Still in agreement with our results, studies showed that TT genotype of normal and PTB individuals produce higher IFN-γ in response to mycobacterial antigens (Ansari et al 2009; Sallakci et al. 2007). In another functional study patients with tuberculosis carrying the genotype +874AA showed significantly lower IFN-γ plasma levels than those with +874AT and +874TT genotypes (Vallinoto et al. 2010).

Other studies that evaluated IFN-γ serum levels in occult HBV infection and production in PBMC cultures in acute sickness patients, cutaneous and normal individuals stimulated with PLS, PHA or *M. leprae* antigens also support our findings of higher IFN-γ levels in PTB patients T carriers (Arababadi et al. 2011; Cardoso et al. 2010; Matos et al. 2007; Vollmer-Conna et al. 2008)). Other study showed the relation between *IFNG* polymorphisms and IFN-γ transcription, reporting an association of allele 2 of a polymorphic microsatellite marker (which shows an absolute correlation with the *IFNG* +874 T allele), which are in agreement with our results (Pavrica et al. 2000).

Some studies evaluating tuberculosis patients in initial stages of disease and normal controls found no differences between genotypes of *IFNG* +874 SNP in IFN-γ production in PBMC culture under *M. tuberculosis* H37Rv, culture filtrate antigen (CFA) of *M. tuberculosis* and phytohemagglytinin (PHA) stimulus (Selvaraj et al. 2008, Vidyarani et al. 2006). Other study found no influence of *IFNG* +874 locus on mRNA expression during *Helicobacter pillory* infection (Rad et al. 2012). These different findings may be due to ethnic differences in genotypic frequencies among various populations. This result is in complete agreement with genetic epidemiologic data that T allele is associated with protection against TB (Pacheco et al. 2008). This same allele is also associated with resistance to leprosy (Cardoso et al. 2010).

Another polymorphism for the *INFG* gene located at 2,109 bp downstream from the translation start site in the third intron, was reported to be involved in transcriptional regulation of IFN-γ gene but its contribution is still unclear (Henri et al. 2002; Liu at al. 2006). To our knowledge this is the first report regarding the functional effect of *INFG* +2109A/G on IFN-γ plasma and mRNA expression levels. Ours results showed that PTB patients AG genotype had lower plasma levels at the end of the treatment.

The persistence of low IFN-γ production from middle to end of therapy suggests the presence of a genetic defect in IFN-γ production in patients with *IFNG* +874 AA genotype and *INFG* +2109 AG genotype that may also underlie their increased risk for reactivation of a latent PTB focus. Decreased production of IFN-γ in PTB patients when compared with healthy controls have been reported (Lin at al. 1996; Vidyarani et al. 2006; Zhang et al. 1995) and it may be due to the initial T cell anergy seen in the disease. Such an inadequate IFN-γ production may result in failure of macrophage activation, which could lead to active disease progression (Vidyarani et al. 2006).

IL-12 is important in mediating protective immunity against TB. A SNP in the 3'UTR of *IL12B* gene (+1188A/C) coding for IL-12p40 is known to modulate IL-12p40 levels. The present study showed that *IL12B* +1188 AA genotype is associated with lower IL-12 plasma levels in normal controls and in TB patients at 3 months of anti-TB treatment. Our results agree with other study that evaluated PTB patients and normal controls (Selvaraj et al 2008).

Studies found that *IL12B* +1188 C allele is associated to lower IL-12p40 production by PBMC from healthy individuals stimulated with C3 binding glycoprotein, LPS or PPD (Balcewicz-Sablinska et al. 1998; Davoodi-Semiromi 2002; Stanilova and Miteva 2005). Arababadi (2011) did not found significant difference in IL-12 serum level in AA and AC genotypes in occult HBV infection.

IL-12 production is induced following phagocytosis of *M. tuberculosis* by macrophages and dendritic cells, which leads to development of a Th1 response with production of IFN-γ (Raja 2004). Since IL-12p40 is a component of both IL-12p70 and IL-23 and regulates initiation and maintenance of acquired cellular responses to TB, low IL-12p40 levels in AA genotype individuals might have a role in limiting chronic

inflammation (Cooper et al. 2006). Discordance between results may be due to ethnic differences in ethnicity and designs of the studies.

TNF-α plays an important role in granuloma formation in tuberculosis (Sharma et al. 2010). The promoter region of the *TNF* gene is highly polymorphic and our evaluation of the *TNF* -308G/A locus showed that normal individuals with AG genotype had higher TNF-α plasma levels and PTB patients with the same genotype these levels tended to be higher, though not significant. There are conflicting results regarding *TNF* -308A/G. Some authors demonstrated increased TNF-α production of *TNF* -308 allele A carriers in LPS stimulated PBMC, whole blood cultures of leprosy patients after LPS and *M. leprae* stimulation and paracocidioidomycosis patients, while others failed to show any effect of this SNP on TNF-α production after LPS stimulation *in vivo* or *in vitro*, in HVC patients and in tuberculosis patients (Bouma et al. 1996; Bozzi et al. 2006; Cardoso et al. 2011; Chen et al. 2007; Louis et al. 1998; Sharma et al. 2010; Taudorf et al. 2008).

An *in vitro* expression study has indicated that *TNF-308G/A* SNP has direct effects on TNF-α gene regulation, and A allele at this locus may lead to a higher expression level (Wilson et al. 1997). These findings agree with our results that demonstrated that PTB patients AG genotype at the beginning of anti-TB treatment have higher TNF-α mRNA expression then GG genotype individuals.

Since TNF-α is important for walling off infection and preventing dissemination by granuloma formation, low levels of this cytokine, as seen in our PTB patients in our general results, could impair the containment of the bacilli and increase the reactivation risk, as shown in rheumatoid arthritis patients who were undergoing anti-TNF-α therapy (Feldmann and Maini 2001; Maini et al. 1999).

IL-17 is a potent inflammatory cytokine induced by *M. tuberculosis* infection. To our knowledge this is the first study to verify the functional effect of the *IL17A* rs7747909 polymorphism on IL-17A plasma and mRNA expression levels. Our results showed that A carriers at rs7747909 produce higher plasma levels of IL-17A at the end of therapy and, in general, PTB patients produced higher levels than normal controls. Although Th17 cells are not as important as Th1 cells in mediating protection against primary *M. tuberculosis* infection, IL-17 appears to be critical to the induction of *M. tuberculosis*–specific memory response and the mediation of protection against challenge infections and during vaccinations (Khader et al. 2005; Khader et al. 2007; Umemura et al. 2007; Wozniak et al. 2006). Our results suggest that PTB patients have no impairment to produce IL-17A.

IL-10 is known to have deactivating properties and undermines Th1 response. About 50% of the observed variability of IL-10 secretion is explained by genetic factors (Opdal 1994). Our results showed that *IL10* -819 T carriers had higher plasma levels of IL-10 during the 6 months of anti-Tb treatment. Other study evaluating PTB patients showed no influence of this SNP in IL-10 levels (Selvaraj et al. 2008). Also no impact of *IL10* -819 locus was found on IL-10 serum level of controls and patients with HCV infection or in normal individuals PBMC culture stimulated with LPS and PPD (Chen et al 2007; Yilmaz et al. 2005). Study with leprosy showed that *IL10* -819 T carriers produce lower levels of IL-10 when compared with non-carriers (Pereira et al. 2009).

Study evaluating *H. pillory* patients showed that SNPs at promoter region of *IL10* influence IL-10 mRNA expression with GCC haplotype carriers at *IL10 -1082G/-819C/-592C* is associated with high and ATA carriers with low IL-10 mRNA expression (Rad et al 2012). In our study *IL10 -819* genotypes had no influence on IL-10 mRNA expression.

Published data suggest that IL-10 inhibits synthesis of IFN-γ by T cells and that production of IL-10 has been associated with anergy in tuberculosis (Boussiotis et al. 2000; Sánchez et al. 1994). Our results suggest that PTB patients T carriers could have more difficult in building a protective response towards active TB.

TGF-β is present in the granulomatous lesions of TB patients and has important anti-inflammatory effects, including deactivation of macrophage and downregulation of IFN-γ and TNF-α release (Ruscetti et al. 1993). Our results showed that *TGFB1* +21 CC genotype in PTB patients have higher plasma levels of TGF-β. These results are consistent with other studies approaching cancer and myocardial infarction (Grainger et al. 1999; Dunning et al. 2003 ; Yokota et al. 2000). Part of the ability of macrophages to inhibit mycobacterial growth may depend on the relative influence of IFN-γ and TGF-β in any given focus of infection, since at low concentrations, act as chemotactic factor for monocytes and induces secretion of IL-1α and TNF-α, and in high concentration, inactivate macrophages, inhibits the expression and function of receptors for IFN-γ, IL-1α and IL-2 and decreases the production of TNF-α, parallel events related to increase of intracellular mycobacterial growth (Lasco et al. 2005; Numerof et al. 1988).

The present study suggests that *IFNG* +874T/A, *IFNG* +2109A/G, +1188A/C, *IL10* – 819C/T – 819 and *TGFB1* +21C/T are associated with differential cytokine levels in PTB patients and may play a role in the initiation and maintenance of acquired cellular immunity to TB and in the outcome of the active disease and the anti-tuberculosis treatment. Our word lacks association of the influence of cytokine SNPs in TB outcome due to the low number of patients and to the fact that all patients had a moderate type of PTB. Since cytokines play a major role in immunity to TB, profile of cytokines determined by the respective functional SNP and/or other closely linked

genes might be crucial for protective immune responses and may serve as genetic risk markers of TB susceptibility.

5. Conclusion

In this study we demonstrated that cytokine SNPs can induce different cytokine levels in PTB patients during anti-TB treatment and these levels could be important to the outcome of the treatment. Future studies with a larger population and different forms and severity stages of TB will help to better understand why many are infected by the mycobacterial bacilli but only 10% develop active disease.

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7. Trabalho submetido

Este trabalho foi submetido para publicação na revista "**Infection, Genetics and Evolution**", como comprova e-mail abaixo:

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