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REMODELAMENTO PARENQUIMATOSO PULMONAR EM DOIS MODELOS EXPERIMENTAIS DE FIBROSE

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1. Fibrose Pulmonar - Matrix extracelular - IL-17 – Estudos experimentais.

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Dedicatória

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Epígrafe

"Somente hoje
Hoje a luz do presente
Dia como este dia
Em toda a vida
Terás este somente
Recorda isso
E atende todo o bem que desejes fazer
Prestação de serviço em socorro de alguém...
...Contando sempre aquilo que se fez
E dia igual a hoje, só terás uma vez"

Francisco de Paula Cândido Xavier

Sumário

1. Revisão da Literatura

A fibrose pulmonar é caracterizada pela deposição de matriz extracelular nos interstícios pulmonares como o axial, septal e/ou periférico em resposta crônica reparativa a um processo injuriante. A unidade alvéolo-capilar pode desta forma ser alterada e levar a perda da função pulmonar com eventual progressão para doença clinicamente detectável, definidas coletivamente de doenças pulmonares fibrosantes. Quando essa deposição de matriz extracelular é anormal, ou seja, não controlada, difusa e progressiva, forma-se um sub-grupo de doenças, as chamadas doenças pulmonares intersticiais difusas (*Difuse Interstitial Lung Disease*, DILD), termo comum a um extenso grupo de doenças pulmonares crônicas. Entretanto, a etiologia é determinada em aproximadamente um terço destas doenças intersticiais difusas, sendo que o restante são de causa desconhecida, levando ao diagnóstico de doenças idiopáticas (1-3). Isso impede uma abordagem terapêutica eficaz e resolutiva em algumas destas doenças com alta morbi-mortalidade, como por exemplo na Fibrose Pulmonar Idiopática. Os processos fibro-reparativos pulmonares de etiologia conhecida perfazem determinadas vias fisiopatológicas, percorrendo o elo entre etiologia e fibrose, como na tuberculose ou silicose. De modo análogo, é possível que as doenças pulmonares intersticiais difusas idiopáticas também sejam desencadeadas pela participação de pelo menos um estímulo etiológico injuriante em indivíduos susceptíveis. Após a lesão inicial, a cascata de eventos fisiopatológicos deve envolver controles epiteliais, imunológicos, genéticos, virais, vasculares e fibroblásticos (2, 4). Porém, estes estímulos injuriantes e suas vias fibrogênicas ainda estão a serem determinados. E apesar de apresentarem características semelhantes, as várias doenças pulmonares fibrosantes têm aspectos individuais, fazendo com que cada uma

delas seja estudada separadamente. A gravidade das doenças envolvidas e a evolução clínica comumente insatisfatória da maioria delas têm motivado pesquisas quanto à patogênese da fibrose pulmonar de muitos centros de pesquisas.

As alterações estruturais dessas patologias consistem no acometimento fibro-inflamatório das pequenas vias aéreas caracterizadas pelo espessamento do interstício septal, por deposição acentuada de matriz extracelular, infiltrado inflamatório, remodelamento bronquiolar com alteração das células epiteliais e endoteliais, dos septos alveolares e da rede vascular pulmonar (5, 6). Estudos histológicos, ultra-estruturais, funcionais e do lavado broncoalveolar são realizados em modelos experimentais na busca de semelhanças com as encontradas nos pacientes, com o intuito de compreender melhor os mecanismos evolutivos e etiopatogênicos destas doenças. Se por um lado, esses modelos tem permitido conhecer melhor o comportamento de tais doenças, por outro, tem levantado novas questões a serem investigadas.

Modelos Experimentais

Com o intuito de melhor compreender os mecanismos das doenças pulmonares fibrosantes, a utilização de modelos experimentais permite obter informações significativas quanto às alterações morfológicas, funcionais e bioquímicas. A reprodutividade e a possibilidade do estudo controlado tem resultado em uma maior compreensão da patogenia do processo (7, 8).

Fibrose Pulmonar induzida por Bleomicina (BLM).

É um modelo experimental clássico para estudo de mecanismos fisiopatológicos de fibrose pulmonar. Trata-se de um agente antimicrobiano com propriedades quimioterápicas, isolado de culturas de uma cepa de *Streptomyces verticullus (9).* É utilizado no tratamento inicial de linfomas, tumores testiculares e carcinoma de células escamosas. Dependendo da dose, ao longo do tempo, seu uso pode produzir inflamação e fibrose pulmonar com consequente redução da capacidade e volume pulmonar (10). Os primeiros trabalhos descrevendo a bleomicina, sua farmacologia e seus efeitos foram realizados em ratos (9, 11). Atualmente os roedores são os espécimes de eleição para os estudos de fibrose pulmonar, devido à facilidade de formação de colônias, manuseio e sensibilidade pulmonar (12). Os efeitos terapêuticos e citotóxicos da bleomicina são resultados de sua capacidade de fragmentar o DNA das células alvo, com conseqüente redução na síntese de RNA e proteínas. É possível que os danos pulmonares causados pela bleomicina estejam diretamente relacionados à formação de radicais livres de oxigênio por um mecanismo dependente de ferro (13). Além disso, a produção de radicais livres pode aumentar a lesão pulmonar por inativação de antiproteinases, rompimento de membranas e exposição da barreira alvéolo-capilar (14). Estes radicais agem como iniciadores, mantenedores e amplificadores da reação inflamatória (15). A quebra da fita de DNA seria, portanto, definida por três mecanismos associados e sinérgicos: a ligação simultânea da BLM com o DNA e o Fe^{+2}; a fácil oxidação do Fe^{+2} pelo oxigênio molecular; e a geração de radicais livres em íntima proximidade com o DNA (16, 17). O uso da BLM também é relacionado com o aumento seletivo de RNAs mensageiros

específicos que codificam a produção de proteínas como o procolágeno-alfa 1, a elastina e a fibronectina dos pulmões, que tem papel determinante na gênese da fibrose, e mediadores inflamatórios que acarretam em uma migração e ativação de neutrófilos e linfócitos semelhante ao estímulo infeccioso (18-22). A migração de polimorfonucleares ativados para o tecido pulmonar e a liberação de proteases lisossômicas destrói o colágeno e outras proteínas estruturais, estimulam a migração de linfócitos e ativação dos fibroblastos. A conseqüência histológica desta destruição é o espessamento septal por edema e por infiltração de células inflamatórias e deposição de colágeno. A lesão é heterogênea e a região axial e periférica é a mais intensamente comprometida com presença de mononucleares e neoformação intensa de colágeno. Nas regiões periféricas, as lesões são observadas ao longo da região subpleural posterior (23). A destruição alveolar pode ser observada pela presença de alterações das células alveolares e presença de material proteináceo nos espaços alveolares, até pelo total desarranjo estrutural com conseqüente substituição por tecido fibrótico.

Fibrose Pulmonar induzida por Paraquat (PQ).

Outro modelo experimental para estudo dos mecanismos de fibrose pulmonar é obtido com o paraquat. Os efeitos tóxicos do herbicida paraquat podem ser entendidos, em grande parte, por uma reação de oxido-redução, que compreende a transferência de um elétron para o NADP, com formação de NADPH celular e formas oxidadas potencialmente tóxicas, como o radical superóxido. O citocromo P450 redutase microsomal, atua como catalisador

dessa reação (24). O paraquat acumula-se ativamente nos pneumócitos tipo I e II, através de um mecanismo dependente de energia e mediado pelos canais seletivos de poliaminas. No interior dos pneumócitos tipo I ocorre a oxi-redução e auto-oxidação, mediadas pelo citocromo P450. O paraquat é reduzido por intermédio do NADPH e reage fortemente com o oxigênio para formar um radical livre de oxigênio (O^{2-}) (24). A interação entre o anion superóxido e o H_2O_2 induz a Reação de Haber-Weiss, tendo a formação de dois radicais hidroxil (OH^- e OH^+) e oxigênio, sendo a reação catalisada pela presença do íon ferro (Fe^{2+}) (25). Os radicais livres de oxigênio lesam diretamente as células, das paredes alveolares ativando o sistema mononuclear fagocitário, com eventual fibrose generalizada do tecido pulmonar. Em resumo, o mecanismo primário da toxicidade do paraquat baseia-se no ciclo de oxidações e reduções que o composto é susceptível de sofrer, e com a depleção da enzima superóxido dismutase, capaz de converter o íon superóxido em oxigênio. Esse mecanismo, sendo seletivo para as células epiteliais, causa a injúria inicial nos pneumócitos, precipitando edema intra-alveolar, vasodilatação e infiltrado inflamatório mononuclear e polimorfonuclear (26). Após três semanas observa-se a a proliferação fibroblástica com deposição de matriz extracelular no interstício pulmonar (26). Consistente com esses achados, tem sido demonstrado o aumento do nível de expressão do RNA mensageiro do colágeno tipo I e da hidroxiprolina (27, 28).

Resposta Fibrótica das Cepas Murinas dos modelos experimentais.

Diferentes cepas murinas apresentam respostas diversas na susceptibilidade ao desenvolvimento de fibrose pulmonar por drogas indutoras como a bleomicina (8). Desta forma, a cepa C57/B6J é mais susceptível a fibrose pulmonar do que a cepa Balb/c (29). Especificamente, nas fases intermediárias (14 dias) e tardias (30 dias), a cepa C57/B6J mostra significante aumento da deposição de colágeno pelo método da hidroxiprolina em relação a cepa Balb/c (29). Somando se a esses resultados, a taxa de síntese de colágeno em cultura celular dos pulmões foi de 210% maior no C57/B6J em relação ao Balb/c (29). Essas diferenças refletem provavelmente distintos padrões de expressão de citocinas, de proteases e anti-proteases, de ativação e inativação enzimática e em última análise pelo perfil genético peculiar de cada cepa, determinando por fim características específicas na composição de matriz extracelular e de elementos contrateis responsáveis pela fibrose pulmonar (30, 31).

Mecanismos de Lesão

Na doença pulmonar fibrosante experimental, a unidade epitélio-membrana basal, os capilares e o interstício septal encontram-se comprometidos (32, 33). O mecanismo de desenvolvimento das lesões compreende três etapas consecutivas. Primeiramente, o tecido pulmonar é danificado de alguma forma pelo agente conhecido (bleomicina, paraquat). Em segundo lugar, as paredes alveolares apresentam inflamação e finalmente, na terceira etapa, ocorre fibrose que começa no interstício pulmonar. Pelo

comprometimento do interstício pulmonar, estas entidades produzem diminuição da distensibilidade, redução dos volumes pulmonares e da troca gasosa conseqüente destas duas primeiras alterações e da redução da capacidade de difusão da barreira alvéolo-capilar espessada. Todas as doenças pulmonares fibrosantes remodelam a arquitetura pulmonar, comprometendo as diferentes estruturas pulmonares e determinando, desta maneira, alterações funcionais qualitativas e quantitativas. São as principais vias de lesão: epitelial, vascular, matriz extracelular e alteração imuno-celular.

Via Epitelial. A lesão das células epiteliais alveolares é um evento frequentemente observado nas doenças fibróticas pulmonares, sendo até agora um acontecimento pouco compreendido (34). Normalmente, a regeneração epitelial da parede alveolar é um evento crítico na restauração da integridade do parênquima alveolar. A falha na regeneração adequada pode levar a formação de uma cicatriz fibrótica irreversível e a perda da função pulmonar (35). A reepitelização imperfeita resultante da perda da capacidade proliferativa pode ser maior pelo aumento de apoptose ou pela inefetividade das células epiteliais para migrar ao sítio de lesão tecidual (35). A redução da capacidade de proliferação de pneumócitos tipo II e a impossibilidade destas células de diferenciar-se em pneumócitos tipo I são observadas nos processos de fibrose pulmonar humana e experimental (36). Os pneumócitos do tipo I são células vulneráveis a processos de injúria, ao contrário dos pneumócitos tipo II que são células de maior resistência (35, 37). Estas células proliferam e migram para recobrir a membrana basal exposta nos casos de lesão epitelial. Esta repopulação é feita caracteristicamente por células de aspecto

cuboidiforme que posteriormente se diferenciam em pneumócitos do tipo II e I para restabelecer desta forma a função epitelial. Estudos sugerem que os pneumócitos tipo II em regiões lesadas podem dividir-se em duas subpopulações distintas de acordo com a expressão de E-caderina, que é responsável pela diferenciação celular e morfogênese (38, 39). Uma subpopulação possuindo níveis baixos de E-caderina, maior resistência à injúria, maior poder proliferativo e maior atividade da telomerase, e outra com características contrárias a estas (39-41). As primeiras são supostamente a subpopulação de pneumócitos tipo II encarregados da regeneração do epitélio lesado e viriam a constituir células com características fisiopatológicas importantes nas doenças fibrosantes nas quais estariam caracteristicamente aumentadas e com alterações fenotípicas importantes (42, 43). Alterações nestas células, por conseguinte, podem comprometer a reepitelização alveolar dando lugar ao processo de fibrose. Por outro lado, o epitélio alveolar injuriado não apenas promove sinais ativadores para células mesenquimais, como também deixam de exercer sua função supressora sobre a multiplicação de fibroblastos, via produção de prostaglandinas E2 (PGE2) e derivados da ciclooxigenase COX–1 e principalmente COX–2, importante na produção dessa prostaglandina. Portanto, níveis reduzidos de PGE2 por injúria epitelial podem aumentar a proliferação, migração, contratibilidade e diferenciação dos fibroblastos para miofibroblastos e aumentar o processo fibrótico, mas a verdadeira participação destes processos ainda não é bem compreendida(44-48). A ativação das células alveolares epiteliais provoca uma resposta pró-fibrótica intensa, via liberação de fator de crescimento derivado de plaquetas, fator de crescimento transformador (TGF-β), fator de necrose tumoral (TNF-α),

endotelina-1, fator de crescimento conjuntivo tissular, interleucinas (IL-4, IL-13) e osteoponina, que podem contribuir no processo fibrótico nas diferentes doenças fibrosantes(49) e em modelos experimentais induzidos por bleomicina(4, 50, 51). Um tipo semelhante de ativação exagerada das células epiteliais é visto de forma caracteristicamente semelhante nos processos de proliferação celular neoplásicos, à custa do aumento dos processos de ativação enzimática da telomerase celular (52). Repetições teloméricas fornecem a cada célula somática do corpo um mecanismo “relógio” que evita a proliferação ilimitada de células aberrantes em tecidos adultos. Devido às limitações do processo de replicação do DNA, que não é capaz de replicar adequadamente as extremidades 3´ dos cromossomos, após sucessivas gerações celulares, ocorre a erosão ou encurtamento crítico dos telômeros. Por conseguinte, estas células são removidas de modo permanentemente do ciclo celular e param de se dividir, um processo chamado de senescência celular replicativa (52). Em teoria, tal mecanismo poderia oferecer alguma segurança contra proliferação celular excessiva de células anormais em tecidos somáticos. Estudos demonstraram que a atividade da telomerase está aumentada em fibroblastos de pulmões sob efeito de bleomicina, apresentando uma alta capacidade de proliferação (41, 53, 54). Esta proliferação exagerada de fibroblastos pode estar ligada ao descontrole ou lesão da célula epitelial responsável pela estimulação da proliferação de fibroblastos ou pela ativação descontrolada anormal de proteínas profibróticas talvez por defeitos celulares inerentes à ativação contínua da telomerase em áreas de injúria tecidual (55). Por outro lado, a própria falha na replicação celular pode levar a apoptose das células epiteliais desencadeando um processo de desativação dos

mecanismos reguladores da proliferação de fibroblastos aumentando o processo de depósito de fibras de colágeno (54). Uma melhor compreensão destes aspectos são importantes para o melhor entendimento dos processos fibróticos pulmonares.

Via Vascular. Outro aspecto importante na patogênese das doenças pulmonares fibrosantes é o processo de angiogênese observado nestas doenças e que nos últimos anos tem adquirido um papel essencial para alguns mecanismos dos processos de cicatrização e fibroproliferativos que contribuem com a deposição de matriz extracelular (55, 56). A lesão da célula endotelial pode ativar o sistema imune e induzir a proliferação de fibroblastos contribuindo com o processo de fibrose. É importante sinalizar que existem evidências de um remodelamento vascular importante nos pacientes tanto *in vivo (57)* como em modelos de fibrose pulmonar induzido por bleomicina (58). Estas evidências aparentemente tem um papel fundamental e crítico na patogênese deste tipo de fibrose progressiva, levando a uma excessiva produção de colágeno e conseqüente remodelamento tecidual que pode induzir a ativação do sistema imune talvez perpetuando um ciclo fatal de fibrose (59). Por outro lado, a ativação de proteínas derivadas de angiotensina pode ser responsável pela ausência de capilares, característica principalmente vista nas áreas de lesão inicial (focos de fibroblastos) dos processos fibróticos (59). Relatos recentes em modelos experimentais de fibrose pulmonar induzido por bleomicina demonstram que a própria angiotensina II está implicada em processos fibróticos com importante papel vasoconstritor e sua disfunção pode provocar aumento do tônus vascular mediante a ativação de seus receptores

AGTR tipo I e AGTR tipo II, interferindo na boa função endotelial ou aumentando a atividade dos miofibroblastos, provocando alterações vasculares, aumento da produção de colágeno e conseqüente perpetuação da fibrose (4, 60). Ao mesmo tempo, diversas evidências sugerem que existem deficiências na coagulação e na fibrinólise, e um desequilíbrio na produção de oxidantes e antioxidantes, que promovem a deposição extravascular de fibrina, presentes no compartimento alveolar dos casos de fibrose progressiva. Notadamente, células alveolares epiteliais parecem ter um papel fundamental na fisiopatologia por secretar fator tissular, o iniciador celular primário da cascata de coagulação, como o PAI-1 (inibidor do ativador de plasminogênio – 1) (61, 62), por outro lado existem trabalhos que indicam um aumento da produção de oxidantes, aumentando a expressão de NOS (*nitric oxide synthase*) nas células endoteliais (62). A matriz extracelular provisória formada pela fibrina e pela fibronectina depositada no espaço alveolar após a lesão inicial pode desencadear reparação fibrótica, o que resultaria no remodelamento do septo alveolar (63). Os vasos linfáticos também contribuem nos processos de fibrose, além do transporte de líquidos, proteínas do tecido, transporte de células imunes e absorção de lipídeos, as alterações destas estruturas, podem contribuir no desbalanço das proteínas e outras macromoléculas reguladoras vasculares contribuindo com o aumento do edema ou da fibrose de uma determinada área lesada (64). Com base em nestes conhecimentos se faz importante uma melhor compreensão em modelos experimentais das características vasculares e vásculo-linfáticas nos processos fibróticos pulmonares.

Via Matriz Extracelular. A excessiva e descontrolada deposição de proteínas da MEC causada pela proliferação e ativação dos fibroblastos chama a atenção, sendo um ponto crucial nos processos de fibrose pulmonar (65). Neste sentido, a regulação da biossíntese da matriz extracelular na fibrose pulmonar é alvo de muitos estudos. Mudanças arquiteturais intensas resultantes do aumento da espessura das paredes alveolares com perda das unidades alvéolos-capilares conduzem à insuficiência respiratória característica das doenças pulmonares fibrosantes (65). Uma característica chave do processo de fibrose pulmonar progressiva é a presença do aumento dos fibroblastos e miofibroblastos, evento crítico para o quadro da fibrose pulmonar (2, 4, 66). A partir do estabelecimento destas células nos alvéolos, modificações profundas ocorrem na morfologia do tecido pulmonar, sobretudo pela intensa deposição de componentes da matriz extracelular, principalmente colágeno. Há evidências que sugerem que os fibroblastos e miofibroblastos nestas áreas de proliferação são fonte principal para a expressão, depósito de matriz extracelular, ativação de interleucinas e células inflamatórias (2, 4, 67-69). Outros autores tentam explicar a patogênese da fibrose pulmonar sugerindo participação da predisposição genética sob influência ambiental, com estímulo do sistema imune. A ativação do sistema imunológico pode causar lesão do endotélio vascular e proliferação de fibroblastos que estimularia à produção de colágeno. Em geral, o processo de fibrogênese secundário à lesão do tecido pulmonar é caracterizado por deposição de uma grande quantidade de colágenos fibrilares na matriz extracelular e pela desorganização morfológica das mesmas (70-72). Numerosos estudos confirmaram uma produção aumentada de vários componentes do tecido conjuntivo, incluindo

colágenos I, III e VI, fibronectina, decorina e glicosaminoglicanos em processos fibrosantes (72, 73). Entre estes, os colágenos fibrilares e em particular o colágeno V tem sido implicado em vários processos de remodelamento e rejeição pulmonar nos últimos anos (74, 75). O colágeno V desempenha um papel fundamental nos processos de proliferação e reparação tecidual. A sua presença na membrana basal de vasos e em alguns tecidos mesenquimatosos é de extrema importância na ligação entre o colágeno IV da membrana basal e o conjuntivo frouxo dos órgãos (76), participa ativamente na interação dos componentes da MEC estabelecendo associação com outros tipos de colágeno, como os tipos I e III no interstício pulmonar (77, 78). Outro ponto importante no processo de fibrose é a participação da substância intersticial constituída pelos complexos de glicosaminoglicanas e pelas proteínas, denominadas proteoglicanas e glicoproteínas. A glicosaminoglicana mais comum e importante é o ácido hialurônico ou hialuronan, o qual apresenta várias funções principalmente ao influenciar a morfologia das fibrilas de colágeno, organizando e estabilizando-as (79). Sua interação com proteínas da matriz extracelular e com receptores da superfície celular podem regular muitos aspectos do comportamento das células, tais como a migração, a adesão célula-célula e a diferenciação celular (80, 81). Nas doenças fibrosantes pulmonares o ácido hialurônico pode aumentar a vida dos miofibroblastos e, por conseguinte perpetuar o contínuo depósito de fibras de colágeno na matriz extracelular. Estas alterações na síntese ou degradação do ácido hialurônico podem estar ligadas à alteração das hialuronidases, enzimas que degradam o ácido hialurônico (81-83). Os produtos originados da degradação do ácido hialurônico podem estimular a proliferação de células endoteliais, induzindo

neoangiogênese como visto nos processos neoplásicos (83). A digestão da matriz extracelular pela hialuronidases pode liberar fatores de crescimento contidos no microambiente, alguns desses como o fator de crescimento endotelial vascular (VEGF) e o fator básico de crescimento de fibroblasto (FGF-2) essenciais no crescimento celular e vascularização (83). Por outro lado, o ácido hialurônico de forma intacta pode atuar inibindo a neoangiogênese e consequentemente o crescimento desordenado das células (83). Alguns autores sugerem que citocinas como TGF-α e β, fator de crescimento derivado de plaquetas (PDGF), fator de crescimento epidérmico (EGF), fator de crescimento do tecido conjuntivo (CTGF), TNF-α e β, IL-1α e β, IL-4, IL-6, oncostatina e endotelina–1 regulem a proliferação de fibroblastos pulmonares e o depósito de matriz extracelular (84-86). Outros estudos evidenciam uma participação de outros fatores na regulação da produção de colágeno por citocinas o como o TNF-α e o interferon (IFN-γ) (87). Estudos também tem apresentado um dominante papel para IL-13 na patogênese da fibrose pulmonar. Uma maior expressão de IL-13 no pulmão provoca em camundongos significante fibrose pela via subepitelial, mesmo na ausência de estímulos inflamatórios adicionais, enquanto o tratamento com anticorpos anti-IL-13 reduzem a deposição de colágeno nos pulmões de animais que foram induzidos com bleomicina (2, 4, 88). É possível que estas substâncias da matriz extracelular juntamente com o colágeno V, tenham um papel importante no processo fibrótico pulmonar, modulando as propriedades biomecânicas das fibrilas de colágeno, gerando desestruturação e aumento da sua síntese, contribuindo no processo de fibrose.

O colágeno V é um componente da membrana basal, membrana sinovial, cartilagem articular hialina, placenta e ao redor das células musculares lisas vasculares (77, 78, 89). Ele difere dos outros colágenos por apresentar terminações NH2 e COOH na sua molécula e retenção de grande parte de N-propeptídeos na molécula madura, o que a torna mais imunogênica (77, 78, 90). Além disso, o colageno V não é normalmente exposto nos tecidos, já que ele é encontrado entre os colágenos I e III, formando fibras heterotípicas, exceto na córnea (78, 89), e assim, mantendo a integridade da matriz extracelular e regulando o diâmetro da fibra de colágeno (91-94). As fibras heterotípicas, de colágeno I e V, encobrem a região helicoidal da molécula de colágeno V, fazendo com que estes epítopos sejam escondidos e inacessíveis (78).

Estudos demonstram que a fibrose pulmonar, de pele e gastrointestinal provém de disfunção endotelial (95), ativação do sistema imune (96), proliferação de fibroblastos e síntese excessiva de colágeno V anormal (96-99). O colágeno V também está envolvido na patogênese de rejeição experimental de pulmão (74, 75, 100) e bronquiolite obliterante em pulmão de ratos por tolerância oral induzida por colágeno V (101), além de ter um importante papel nos processos de proliferação e remodelamento (102).

Assim, pode haver pelo menos 2 mecanismos fisiopatológicos da participação do colágeno V no processo de fibrose pulmonar: 1) A ligação direta de epítopos do colágeno V em receptores celulares, aumentando a síntese de colágeno por fibroblastos e miofibroblastos; 2) Injúria prolongada poderia provocar lesão da membrana basal e exposição de epítopos internos de colágeno V, originando neoantígenos, podendo atuar no desencadeamento

de doenças auto-imunes. Isso pode ser corroborado pela presença de anticorpos anti-colágeno V no soro de coelhos imunizados com colágeno V (103) e pela ativação da resposta imune inata pelas células TH17 colágeno V específicas em disfunção primária no enxerto de transplate pulmonar (104). O estudo e a compreensão dos mecanismos vinculados com o aumento de fibras colágenas, das células que intervém na sua produção e da análise funcional nos mecanismos da fibrose pulmonar mostra-se como uma chave para compreender melhor a patogênese destas doenças pulmonares fibróticas.

Via Imuno-celular. A fibrose pulmonar é oriunda de um processo inflamatório crônico, que além da fibrose mostra um infiltrado mononuclear, cuja principal célula é o linfócito. Estes são atraídos ao local de injúria e são ativados por vários antígenos, sendo assim estimulados a produzirem citocinas e progredirem com o processo inflamatório. No entanto, a fibrose pulmonar nem sempre apresenta persistência da inflamação (18). E este fato é associado ao desenvolvimento de uma resposta celular TH2 (IL-4, IL-5, Il13 e IL21) (2, 4). No entanto, pela análise desses podemos encontrar evidências de outros mecanismos a este padrão de resposta, podendo ser um mecanismo paralelo ou subjacente.

Em contraste ao padrão TH1 e TH2 introduzido por Mosmann & Coffman (105) há 25 anos atrás, estabeleceu-se um novo padrão de células T auxiliares pela caracterização de fatores de diferenciação e transcrição únicos da chamada célula TH17 (106, 107). A descoberta da cadeia de citocina p19 no ano de 2000 permitiu identificar um nova citocina, chamada de IL-23, quando esta formava heterodímeros com a cadeia p40 da IL-12 (108). Assim, após estudos comprobatórios, ficou claro que a IL-23 e não a IL-12 esta envolvida

em doenças autoimunes e processos inflamatórios intensos. Em estudos de seguimento, mostrou-se que a IL-23 expande e mantém a diferenciação de células produtoras de IL-17 (109). Entretanto, o fator de diferenciação inicial foi demonstrado ser o TGF-β. Sob influência desta citocina, a celula T naive mostra expressão da forkhead box p3 (Foxp3), originando a célula T reguladora CD25+CD4+Foxp3+ (célula Treg), a qual é supressora da resposta imune. Mas sobre a ação da IL-6, pela via STAT3, há liberação da função da RORc, a qual é fisicamente associada a Foxp3 e também induzida pelo TGF-β (110-112). Então o TGF-β e a IL-6 induzem a RORc e este induz o programa transcricional da IL17 (113, 114). A IL-21 exerce o efeito da IL-6, quando o nível da IL-6 é baixo, mantendo e amplificando o reservatório de precursores de células IL-17, por exemplo na ausência de inflamação (115-117). O efeito da IL-6 ou IL-21 mais TGF-β sobre a RORc e STAT3 também promove a expressão do receptor de IL-23 (IL-23R) (110, 116, 118). A disponibilidade de IL-23 é essencial para completar e sustentar a diferenciação da célula TH17, sendo um fator limitante da resposta TH17 (119, 120). Como a IL-23 é produzida pelos macrófagos e células dendríticas, estas células apresentam o colágeno ao sistema imune e desencadeiam a resposta TH17, como demonstrado nos estudos iniciais sobre TH17 associados a doenças autoimunes (121-123). Após a indução pela RORc, a IL-17A (IL-17) é produzida na célula TH17. Várias outras células também produzem IL-17 (células T γδ, células NK, neutrófilos, células do sistema inato, eosinófilos) (124-126). E seus efeitos são proinflamatórios (127), induzindo a expressão de citocinas (TNF, IL-1β, IL-6, GMCSF, G-CSF), quimiocinas (CXCL1, CXCL8, CXCL10), metaloproteinases (128-130), fatores chaves no recrutamento, ativação e migração de neutrófilos (130, 131), além

de propriedades quimiotáxicas (132). O receptor de IL-17 (IL-17RA) pode formar um heterodímero com o receptor de IL-25 (IL-17RB) e assim pode contribuir tanto para uma resposta inflamatória TH17 dependente como uma resposta TH2 via sinalização pela IL-25 (129, 133-138). No entanto, a sinalização IL-25 induz a expressão de IL-13 e IL-5, os quais inibem a expressão de IL-23, fator chave da resposta IL-17 (137).

Desta forma, num estudo recente, a gravidade da Fibrose Pulmonar Idiopática é relacionada a diminuição das Tregs (139) e em estudos de fibrose hepática e pulmonar, mostra-se que a sinalização via IL-21 e IL-13 promove fibrose (140-144), porém a relação e/ou influência TH17 é desconhecida.

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3. Hipótese de Trabalho

Levantamos a hipótese que os modelos experimentais de doença pulmonar fibrosante induzidos por bleomicina e paraquat causam diferentes mecanismos de injúria do interstício pulmonar. Estes mecanismos perfazem vias fisiopatológicas, o mais próximo da fibrose pulmonar humana, que resultam em estabelecer o processo de remodelamento pulmonar. As vias devem envolver, após micro-injúria, a participação do colágeno V, como neoantígeno e/ou imunoestimulante, que ativam e/ou intensificam a resposta inflamatória conduzidas por células Th17, levando à fibrose e/ou sua perpetuação.

4. Objetivo

Analisar as vias fisiopatológicas de remodelamento parenquimatoso na fase tardia de modelos experimentais clássicos de fibrose pulmonar visando caracterizar o papel do Colágeno V e da IL-17 na sua patogênese.

5. Resumo

A fibrose pulmonar é a base patológica de uma variedade de doenças crônicas incuráveis. A IL-17A, uma glicoproteína secretada de células Th17, é uma citocina pró-fibrótica relacionada recentemente à síntese de colágeno V e fibrose pulmonar. O remodelamento parenquimatoso pulmonar da matriz extracelular pelo colágeno I e V, apoptose e resposta Th17 foi estudado em camundongos Balb/c, C57/B6J selvagens e com knockout para o receptor A da IL-17; para determinar as vias fisiopatológicas que prolongam a fase tardia do processo fibrótico induzido por bleomicina e paraquat. Microscopia eletrônica, imunofluorescência, imunohistoquímica, detecção in situ da apoptose, morfometria, reconstrução tridimensional e reação em cadeia da polimerase em tempo real foram usados para avaliar a quantidade, estrutura e cadeias moleculares dos tipos de colágenos, apoptose e células imunes. Verificamos um aumento da síntese e secreção do colágeno V que promove a perpetuação da fibrose pulmonar de maneira IL-17A dependente. Além disso, observou-se que marcadores críticos da resposta Th17 como IL17, STAT3, TGF-β, IL-6, IL-21, IL-23 e células T CD4+ foram significantemente aumentados em cepas susceptíveis a fibrose pulmonar e intensificadas na ausência do receptor A da IL-17. O aumento de marcadores Th17 resulta em um aumento de células T CD4+ através de uma resposta imune que efetivamente bloqueia a degradação do colágeno V, o qual contribui para o bloqueio da apoptose. Nosso estudo indica que o colágeno V participa da perpetuação da fibrose pulmonar por mecanismos IL-17 dependente e independentes, indicando o potencial alvo terapêutico do colágeno V e das vias de sinalização da resposta IL-17 no tratamento das doenças fibroproliferativas pulmonares.

Paravras-chaves: Fibrose Pulmonar Exprimental, Colágeno V, IL-17, Microscopia eletrônica, Reconstrução 3D, Imunohistoquímica, Biologia Molecular, Imunofluorescência

6. Abstract

Pulmonary fibrosis is the pathologic basis for a variety of incurable human chronic lung diseases. IL-17A, a glycoprotein secreted from IL-17–producing cells, has recently been shown to be a profibrotic cytokine involved in type V collagen synthesis and pulmonary fibrosis. Remodeling of the extracellular matrix by collagen I and V, cell death and Th-17 immune response were evaluated in Balb/c, wild and IL-17 receptor A knockout C57/B6J mice, to determine the pathways that prolong the late phase of the fibrotic process induced by bleomycin and paraquat. Electron microscopy, immunofluorescence, immunohistochemistry, in situ detection of apoptosis, morphometry, tridimensional reconstruction and a real-time PCR were used to evaluate the amount, structure and molecular chains of collagen types, apoptosis and immune cells. We verified increased synthesis and secretion of type V collagen that promoted the maintenance of pulmonary fibrosis in a IL-17A dependent manner. However, we observed that the critical Th17 markers, IL-17, STAT3, TGF-β, IL-6, IL-21, IL-23 and CD4+ T cells, were significantly increased in the fibrosis-susceptible strain and intensified in the absence of IL-17 receptor A. Increased Th17 markers resulted in an increase in CD4+ T cells in fibrotic lung tissue toward an immune response that effectively blocked degradation of collagen V, which contributes to block apoptosis. Our studies indicate that collagen V participates in the maintenance of pulmonary fibrosis in both Th-17 – dependent and -independent manners and that collagen V and the components of the Th-17 signaling pathway are potential therapeutic targets for the treatment of fibroproliferative lung diseases.

Keywords: Experimental pulmonary fibrosis, collagen V, IL-17, electron microscopy, 3D-reconstruction, immunohistochemistry, molecular biology, immunofluorescence.

7. Artigo Científico

Collagen V maintains Experimental Pulmonary Fibrosis in IL17–dependent and -independent pathways

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Abstract

Pulmonary fibrosis is the pathologic basis for a variety of incurable human chronic lung diseases. IL-17A, a glycoprotein secreted from IL-17–producing cells, has recently been shown to be a profibrotic cytokine involved in type V collagen synthesis and pulmonary fibrosis. Remodeling of the extracellular matrix by collagen I and V, cell death and Th-17 immune response were evaluated in BALB/c, Wild C57 and IL-17 receptor A knockout C57 mice, to determine the pathways that prolong the late phase of the fibrotic process induced by bleomycin and paraquat. Electron microscopy, immunofluorescence, immunohistochemistry, in situ detection of apoptosis, morphometry, tridimensional reconstruction and a real-time PCR were used to evaluate the amount, structure and molecular chains of collagen types, apoptosis and immune cells. We verified increased synthesis and secretion of type V collagen that promoted the maintenance of pulmonary fibrosis in a IL-17A dependent manner. However, we observed that the critical Th17 markers, IL-17, STAT3, TGF-β, IL-6, IL-21, IL-23 and CD4+ T cells, were significantly increased in the fibrosis-susceptible strain and intensified in the absence of IL-17 receptor A. Increased Th17 markers resulted in an increase in CD4+ T cells in fibrotic lung tissue toward an immune response that effectively blocked degradation of collagen V, which contributes to block apoptosis. Our studies indicate that collagen V participates in the maintenance of pulmonary fibrosis in both Th-17 – dependent and -independent manners and that collagen V and the components of the Th-17 signaling pathway are potential therapeutic targets for the treatment of fibroproliferative lung diseases.

Keywords: Experimental pulmonary fibrosis, collagen V, IL-17, electron microscopy, 3D-reconstruction, immunohistochemistry, molecular biology, immunofluorescence.

Introduction

Pulmonary fibrosis can be caused by a large number of lung diseases. The most common interstitial lung disease of unknown etiology is **Idiopathic Pulmonary Fibrosis (IPF)**, which has a dismal prognosis and, at present, no effective treatment option is available (American Thoracic and European Respiratory, 2002). IPF proliferation and activation of fibroblasts to myofibroblasts causes excessive and disordered deposition of matrix proteins, resulting in severe architectural changes of the alveolar walls (Baptista et al., 2006; Kuhn and McDonald, 1991). These lead the loss of functional alveolar capillary units and respiratory failure (Fukuda et al., 1995). Despite significant advances concerning the cellular and molecular mechanisms of pulmonary fibrosis, its etiopathogenesis remains unclear (Fernandez and Eickelberg, 2012).

Recently, it has been suggested that type V collagen plays an important role in remodeling of several fibrosing lung diseases, including pulmonary sclerosis (Parra et al., 2009; Parra et al., 2010), bronchiolitis obliterans syndrome (Braun et al., 2009; Garippo et al., 2007), usual interstitial pneumonia (Parra et al., 2006) and the microenvironment of lung cancer (Souza et al., 2010). Collagen V is abundant in the skin, placenta and lungs and is essential for tissue elasticity and compliance (Linsenmayer et al., 1993; O'Sullivan et al.,

2003). It is a minor collagen and a cryptogenic antigen that, together with collagens I and III, constitutes the heterotypic fibrils and is not normally recognized by the immune system in normal collagen fibril structure (Birk et al., 1990; Linsenmayer et al., 1993). The injury and repair process can cause exposure of collagen V, which has been linked to the Th17 response, contributing to the pathogeneses of fibrosing lung diseases (Braun et al., 2009; Burlingham et al., 2007; Mi et al., 2011; Nakashima et al., 2012). The Th17 pattern is characterized by CD4+T cells producing IL-17, a potent proinflammatory cytokine (Bettelli et al., 2007; Park et al., 2005). Several cytokines, such as TGF-β, IL-6, IL-21 and IL-23 participate in the differentiation, amplification and stabilization of Th17 cells, respectively (Bettelli et al., 2008).

Many different approaches to modeling pulmonary fibrosis have been employed by investigators providing research tools for the study of new pathways and to test hypotheses. Among these, the most commonly used include exposure to bleomycin (Gauldie and Kolb, 2008; Moore and Hogaboam, 2008) and paraquat (Honore et al., 1994; Ishida et al., 2006; Satomi et al., 2007). However, a major pitfall with these methods is that the murine lung, particularly Balb/c and C57, are able to recover quickly from this single injury and the disease findings are reversible to near normal lung in many studies by six weeks (Chung et al., 2003). However, despite these limitations, the bleomycin and paraquat models have undoubtedly been extremely important in deciphering which mechanisms prolong or recover pulmonary fibrosis in Balb/c and C57 mouse strains. Thus the mechanisms that prolong the fibrotic process in the late phase in certain strains may participate in the progression of pulmonary fibrosis. Using bleomycin and paraquat models of pulmonary

fibrosis, our group investigated whether IL-17A directly regulates type V collagen synthesis via immune mechanisms in BALB/c, Wild C57 and IL-17 receptor A knockout C57 mice. We observed increased synthesis and secretion of type V collagen that promoted the maintenance of pulmonary fibrosis in a IL-17A dependent manner. We also verified that increased IL-17 resulted in an increase in CD4+ T cells in fibrotic lung tissue toward an immune response that effectively blocked apoptosis and degradation of collagen. Our studies indicate that collagen V participates in the maintenance of pulmonary fibrosis in both Th17-dependent and -independent manners and that collagen V and the components of the IL-17A signaling pathway are potential therapeutic targets for the treatment of fibroproliferative lung diseases.

Material and Methods

Experimental Design

Male mice, aged 4 to 6 weeks-old, were divided into four groups and their respective controls: i) Paraquat-induced BALB/c mice (PQ Balb; n=15, control=10); ii) Bleomycin-induced BALB/c mice (BLM Balb; n=15, control=10); iii) Bleomycin-induced wild C57 Black/6J mice (C57; n=10, control=5); iv) Bleomycin-induced IL17 receptor A knockout C57 Black/6J mice (IL17-KO C57; n=8, control=5) (kindly donated by Dr. João Santana da Silva, USP, Ribeirão Preto, Brazil). The mice were housed under a constant 12 h light/ dark cycle in a temperature controlled room, with *ad libitum* access to food and water, in accordance with the Ethical Guidelines for Animal Experiments of the National Institutes of Health. All procedures were reviewed and approved by the

institutional review board, the Research Ethics Committee of Botucatu Medical School, São Paulo State University, under protocol no. 721/09. The bleomycin-induced groups were administered a single intratracheal instillation of 2 U/kg of bleomycin. A single dose of Paraquat (15mg/Kg) were injected intraperitoneally in PQ Balb group. After 21 days, the mice were sedated, anaesthetized (intraperitoneal 10mg/kg ketamine, Park-Davis and 0.3mg/Kg xylazine, Rompum, Bayer, Brazil), exsanguinated via the abdominal aorta to minimize blood spillage into the pulmonary cavity and then rinsed free of blood with saline solution. The left lung was quickly removed under sterile conditions to perform primary cell cultures from tissue explants. The right lung was inflated in situ through the right bronchus at a pressure of 15 mmH_2O, to calculate the mouse tidal volume, and fixed with 10 ml/kg (0.2 ml) of buffered formalin for 6 h. The lungs were then stored in 70% ethanol for 24 h at ambient temperature and embedded in paraffin. Histological sections (5 μm thick) were obtained and then stained with hematoxylin-eosin and submitted for immunohistochemistry and immunofluorescent.

Histochemistry, immunohistochemistry, immunofluorescence and in situ detection of apoptosis

Histochemistry was performed to characterize total collagen fibers in peribronchiolar and peripheral pulmonary areas using Picro Sirius Red Stain. Quantification was performed by image analysis following the standard protocol of our laboratory, as described in detail by Silva et al (Silva et al., 2012). The images were processed using the program Image Pro-Plus, version 7.0 (Media Cybernetics Inc., Bethesda, MD, USA). For each tissue specimen, a range of 8-

10 slides were analyzed at 400x magnification. The final results were expressed as a percentage of the stained area per total peribronchiolar or total septal area.

Immunohistochemical analysis was used to evaluate IL-17-related markers and immune cells in peripheral septal parenchymal, as previously described by Garippo et al (Garippo et al., 2006). Tissue microarray was constructed from all paraffin blocks with 3 core tissue from each sample. We used the following antibodies: CD20, B-Cell (Clone L26, Dako Corporation Carpinteria, USA, 1:600); CD3 (Leu-4, T3, 1:600), CD4 (CD45RO, clone OPD4, 1:400); CD8 (Clone C8/144B, 1:100); IL-17 SC7927, 1:500); STAT3 (SC482; 1:400); IL-6; TGFbeta (SC146, 1:200); IL-21 (SC17647, 1:100) ; IL-23 (SC21079, 1:100) ;; FGF-1 (SC1884, 1:500; IL-13 (SC1776, 1:400); TNF-alfa (SC1348, 1:200), and CD83 (SC14592, 1:600). The markers were quantified by a conventional stereological method, as recommended by ATS/ERS standards for quantitative assessment of lung structure (Hsia et al., 2010).

Immunofluorescence was used to discriminate collagen types I, III and V in septal interstitium and image analysis was used to quantify these using 3 μm paraffin-embedded sections, as previously described by Parra et al (Parra et al., 2010).

In situ hybridization was used to identify apoptosis in lung parenchyma via TUNEL (+) nuclear expression by deoxynucleotidyl transferase (TdT) end labeling (TUNEL) (Boehringer Mannheim, Mannheim, Germany).

Primary cell cultures from tissue explants and RT-PCR of collagen

Following the quickly removal of the left lung from the mouse using a sterile technique, it was placed in a Petri dish and cut into small pieces (less

than 0.5 mm). Working in a sterile hood, DMEM/F12 medium containing 10% fetal bovine serum with penicillin/streptomycin/amphotericin was added and tissue pieces were dispersed along the bottom of the 25cm^2 flask. The flasks were maintained at 37°C in a cell culture incubator with 5% CO^2-95% air. The medium was changed every 2 to 3 days until the cells achieved subconfluence (i.e., 90% confluent). At this point, the fibroblast cells were trypsinized and transferred to two other flasks. For this experiment, the fibroblast cells from passage 3 to 4 were used until fusiform-shaped cells were observed. The adherent fibroblast cells were trypsinized again and total RNA was isolated using Trizol reagent to evaluate the biomolecular profile of collagen types I, III and V by real-time polymerase chain reaction (RT-PCR), as previously described by Parra et al (Parra et al., 2010). Primer sets were designed based on the coding region closer to the 3' end of the gene using Applied Software: ACTB Forward: GGCTCTTTTCCAGCCTTCCT, Reverse: GTCTTTACGGATGTCAACGTCACA; Col1A1 Forward: GGCCTTGGAGGAAACTTTGC, Reverse: GCACGGAAACTCCAGCTGAT; Col3A1 Forward: CCTGGCTCCCCTGGAATCT, Reverse: CCATTCCTCCCACTCCAGACT; COL5A1 Forward: AGCACCACTGTTACCTCCAA, Reverse: AGGAAGTCCTTTCTCTCCAG.

Confocal and Electron Microscopy

Ultrastructural immunohistochemical localization of collagen types was performed by Immunogold electron microscopy, with ultrathin mouse lung sections fixed in 4% paraformaldehyde and embedded in Lowicryl HM-20 resin, following the protocol established by our laboratory (Prota et al., 2010).

To visualize the distribution of the collagens, providing additional and useful morphological data for a clearer understanding of the interaction between them, we performed confocal laser scanning microscopy, a technique for obtaining high-resolution optical images known as optical scanning sectioning, which permits three-dimensional reconstructions of topologically complex collagen, as our group previously described (Parra et al., 2010; Santana et al., 2010).

Heart measurements

The heart was dissected and the right left ventricular wall thickness ratio was calculated as an index of right ventricular hypertrophy. The largest slice was taken using a Canon camera at a height of 15cm. The images were analyzed by Image J software using four distributed measurements in the ventricular walls. An average ventricle thickness ratio was calculated for each case using the measurements from the four distinct positions on the ventricle cross section.

Statistical analysis

The data were expressed as mean ± standard deviation. All statistical procedures were performed using SPSS software v.13.0 (SPSS, Inc., Chicago, IL 2004). The data were assessed using the *t* test and ANOVA with Tukey or Dunnett post-hoc tests for multiple comparisons, when normality of the data and homogeneity of variances were satisfied. A *P* value of less than 0.05 was considered statistically significant.

Results

Septal fibroblast cells determine resistance or susceptibility to pulmonary fibrosis.

In the late stage of experimental pulmonary fibrosis, resolution of the fibrotic process can occur, as observed in the resistant Balb strain. However, persistence of the fibrotic process can also occur, as verified in the susceptible strain C57.

To determine the microanatomical compartment responsible for the perpetuation of pulmonary fibrosis, we evaluated total collagen, one of the final products of the fibroblast cell during the fibrotic process, using the histochemical technique Picrosirius and morphometric analysis in Balb and C57 mouse tissues. We demonstrated that the total peribronchiolar collagen did not differ between the two treated strains (Figure 1). In contrast, a significant increase in total collagen was observed in peripheral septal parenchyma of the treated C57 groups (Figure 1).

To validate these findings, primary culture of fibroblast cells from control and treated mice were established to assess the gene expression of type I collagen *in vitro*. Collagen I, the main structural protein of pulmonary interstitium during fibrotic process, was also significantly increased in the C57 groups compared with the Balb groups (Figure 2).

The expression of collagen I in peripheral lung parenchyma is IL-17-independent in the fibrosis-susceptible strain.

Type I collagen appears to be the principal collagen at later stages in the murine models of pulmonary fibrosis and the alveoli-capillary junction is widened by the addition of collagen fibers to alveolar-capillary membrane. Additionally, pulmonary fibrosis has been associated with the Th17 pattern.

To test the relation between collagen I and the Th17 pattern in peripheral septal parenchyma, we evaluated the expression of collagen I *in situ* by immunofluorescence in C57 and IL17 KO C57 mice. The protein expression in the fibrosis-susceptible strain was significantly higher than controls and similar to the bleomycin-treated groups (Figure 2). Surprisingly, in the fibrosis-resistant strain, the expression of collagen I was lower than in controls. These data corroborate our findings with Picrosirius-stained peripheral collagen and cultured fibroblasts.

Collagen V expression is linked to susceptibility to pulmonary fibrosis in an IL17-dependent pathway.

Pulmonary interstitial remodeling resulted in extracellular matrix degradation and neoformation with exposure to normally cryptic proteins, including collagen V, which has been associated with autoimmunity and the Th17 pattern.

To determine the role of collagen V and IL-17 response in susceptibility or resistance in late stage pulmonary fibrosis *in situ*, we evaluated collagen V using immunofluorescence in wild Balb and C57 group and IL-17 knockout mice. Surprisingly, we determined a significantly lower expression of collagen V

in BLM Balb mice and a stepwise increase in the expression of collagen V between the C57, control IL-17 KO C57 and IL-17 KO C57, respectively (Figure 3).

To validate our results, we evaluated collagen V by gene expression, immunogold labeling electron microscopy and confocal analysis. Collagen V gene expression showed downregulation in the Balb group and upregulation in IL-17 KO C57 group (Figure 3). Collagen V was located as sparse gold particles along the fibrils, predominantly associated with loosely formed aggregates distinct from the collagen I fibrils in the IL-17-KO-C57 group (Figure 4). Confocal microscopy revealed irregular deposits of collagen V in the basement membrane of the alveolar-capillary unit with intermittent distribution in the fibrosis-susceptible strain (Figure 5).

The upregulation of Th17-related markers in fibrosis-susceptible strains is amplified in the absence of the IL-17 receptor A.

The formation of the IL-17 response is complex, involving multiple critical cytokines and other IL-17-related markers, CD4+ T cells and five different IL17-receptors, one of which is the main receptor mediating the TH17 pattern, receptor A.

To establish the profile of Th17-related markers, we evaluated these *in situ* using an amplified immunohistochemical panel. The critical Th17 markers, IL-17, STAT3, TGF-β, IL-6, IL-21, IL-23 and CD4+ T cells, were significantly increased in the fibrosis-susceptible strain and intensified in the absence of IL-17 receptor A (Figures 6 and 7). Similar expression patterns were observed of FGF-2 and IL13, both key mediators of tissue fibrosis (Figure 7). However,

TNF-α, an initial inflammatory mediator of pulmonary fibrosis, showed no significant differences between the groups, corroborating our findings concerning the late stage of experimental pulmonary fibrosis (Figure 8).

Resistance to late stage pulmonary fibrosis is drug-independent.

Animal models of pulmonary fibrosis can be the result of drug-induced toxicity. Depending on the drug, its toxicity can activate distinct pathways to establish the characteristic fibrosis of each model. Paraquat exposure leads to the production of reactive oxygen species (ROS) through a process of redox cycling and bleomycin forms a complex with ferrous ions and molecular oxygen to produce single- and double-strand breaks in DNA (scission).

To determine the effect of bleomycin and paraquat toxicity in the late stage of fibrotic process, we evaluated the protein expression of total collagen, Th17-related markers and collagen I and V in Balb mice. There was no difference in peribronchiolar and peripheral septal fibrosis were determined between the groups treated with bleomycin and paraquat using picrosirius staining and morphometric analysis (Figure 1). No difference in collagen I and V protein expression was determined between the groups treated with bleomycin and paraquat by RT-PCR and immunofluorescence (morphometric analysis) (Figures 2 and 3). However, in both treated groups, a reduction collagen I and V in relation to their own controls was observed, together with an increase in CD8+ T cells (Figure 8).

Reduction in autophagy-associated cell death in the late stage in the fibrosis-susceptible strain contributes to decreased collagen degradation in the alveolar epithelial cells and vascular remodeling

A complicated interaction between epithelial cells, fibroblast activation with extracellular matrix deposition in the form of collagen V, IL-17 response and vascular cells seem to be regulated by a wide variety of angiogenesis promoters, growth factors and apoptosis, resulting in the induction of vascular remodeling, pulmonary hypertension and right ventricular hypertrophy.

To determine the degree of chronic pulmonary hypertension and the pulmonary apoptotic index, we evaluated the right left ventricular wall thickness ratio and performed the TUNEL assay with in situ hibridization. A significant reduction in apoptosis was verified in the fibrosis-susceptible strain and the paraquat-treated group. In contrast, an increase in the right left ventricular wall thickness ratio only occurred in the IL-17 KO C57 group compared with the BLM Balb group, while the IL-17 KO C57 group showed a tendency toward increase compared with its control (Figure 9).

Discussion

Experimental pulmonary fibrosis models have been developed to study the evolution of fibrotic responses that more closely resemble idiopathic pulmonary fibrosis (Gauldie and Kolb, 2008); however, they have failed to specifically mimic its irreversible and progressive condition (Moeller et al., 2008). Different strains show distinct spatiotemporal patterns of fibrotic phenotype in late stage disease (Moeller et al., 2008), which have been

implicated in the genetic background (Barth et al., 2002; Kaminski et al., 2000), in cytokines patterns (Phan and Kunkel, 1992) and in proteases/anti-proteases and specific extracellular matrix compositions (Kolb et al., 2002). Although these pathophysiological events that permit the perpetuation of pulmonary fibrosis in susceptible strains have not been completely elucidated, they activate the myofibroblast, the major source of the extracellular matrix and collagen I, which causes remodeling (Phan, 2002).

In this study, bleomycin and paraquat models were used to investigate whether IL-17A directly regulates type V collagen synthesis via immune mechanisms in fibrosis-resistant Balb/c mice, fibrosis-susceptible C57 and IL-17 receptor A knockout C57 mice. In our study, we observed no difference in peribronchiolar collagen between fibrosis-resistant and fibrosis-susceptible strains in the late stage; rather the difference between these strains, previously reported in the literature, was due to deposition of peripheral septal collagen, indicating that the microanatomical location of the myofibroblast plays a critical role in parenchymal remodeling. The activation of myofibroblast can be modulated by IL-17 response (Hata et al., 2002) and a critical role for IL-17 in pulmonary fibrosis has been suggested by increased IL-17 in the bronchoalveolar lavage fluid of patients with IPF and in bleomycin-induced pulmonary fibrosis (Gasse et al., 2011; Mi et al., 2011; Wilson et al., 2010). We tested the response of myofibroblasts to IL-17 signaling using IL-17 receptor A knockout mice. Surprisingly, no differences in deposition of total and type I collagen *in situ* in the peripheral septal parenchyma were verified between IL17RA knockout and wild fibrosis-susceptible strains, despite the increased protein expression of IL-17 and IL-17-related markers in peripheral septal

parenchyma. These data demonstrate that the absence of IL-17 receptor A promotes a response in which IL-17 is required to maintain and perpetuate the same pulmonary fibrosis in the fibrosis-susceptible strain.

We were able to demonstrate that the expression of collagen V in septal peripheral parenchyma was increased in the fibrosis-susceptible strain, with irregular collagen V deposits in the basement membrane, which were decreased in the fibrosis-resistant strain. These findings suggest that increased synthesis and secretion of type V collagen promotes the maintenance of pulmonary fibrosis in a IL-17A-dependent manner. However, we determined that the critical Th17 markers, IL-17, STAT3, TGF-β, IL-6, IL-21, IL-23 and CD4+ T cells, were significantly increased in the fibrosis-susceptible strain and intensified in the absence of IL-17 receptor A. Increased Th17 markers resulted in a enhancement immune response in fibrotic lung tissue toward an immune response that effectively blocked apoptosis and degradation of collagen V. Similar findings were previously demonstrated by our group (Garippo et al., 2007; Parra et al., 2010; Parra et al., 2006) and others(Braun et al., 2010; Braun et al., 2009). In our experiments, the absence of IL-17 signaling by IL-17 Receptor A Knockout C57 mice determines an exacerbation of collagen V expression followed by the overexpression of IL-17 and other IL-17-related-markers, such as TGFbeta, IL-6, IL-23 and CD4+ T cell. Thus, the absence of IL-17 receptor A could be related to collagen V overexpression, which promotes potentiation of the IL-17 response. IL-17 may stimulate the maintenance of collagen I production by myofibroblast at the same levels of unchanged IL-17 signaling, probably through other receptors, thus perpetuating pulmonary fibrosis in strains susceptible to fibrosis in the late stage.

Collagen V, myofibroblastic activation and endothelial apoptosis were investigated by Parra et al. (Parra et al., 2012; Parra et al., 2007; Parra et al., 2006) in the vascular remodeling of interstitial lung diseases. More recently, differential expression genes of endothelial and vascular smooth muscle cell proliferation were demonstrated in IPF (Patel et al., 2012) and an imbalance between angiogenic and angiostatic factors promote a distinct apoptotic pattern with an aberrant vascular architecture (Farkas et al., 2011). These studies support our findings in the late stage, in which we verified a decrease in apoptosis in the pulmonary interstitium in the fibrosis-susceptible strain and increased right ventricular wall thickness in the IL-17 KO C57 group compared with BLM Balb, together with increased collagen V deposition, indicating an IL-17-independent mechanism of perpetuation of pulmonary fibrosis.

We conclude that collagen V fiber density and IL-17 offers the potential to maintain the remodeled extracellular matrix, suggesting that strategies aimed at preventing high collagen V synthesis, or local responses to high IL-17 stimulation, could have a useful impact in pulmonary fibrosis treatment.

Acknowledgments

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Legend of Figures

Figure 1. HE- and picrosirius red-stained sections and morphometric analysis. The columns represent the BLM/PQ-treated Balb/C57 groups. The lines represent the treated groups, controls and morphometric analysis (%area), respectively; 40x. All the results are presented as mean and associated standard errors and different small letters (a–c) denote a significant difference between groups ($p<0.05$).

Figure 2. Immunofluorescence and RT-PCR of collagen I. The columns represent the study groups. The lines represent the controls and treated groups and morphometric analysis (%area), respectively; 40x. RT-PCR of collagen I after primary cell cultures from lung explants**.** All the results are presented as mean and associated standard errors and different small letters (a–b) denote a significant difference between groups ($p<0.05$).

Figure 3. Immunofluorescence and RT-PCR of collagen V. The columns represent the study groups. The lines represent the controls and treated groups and morphometric analysis (%area), respectively; 40x. RT-PCR of collagen V after primary cell cultures from lung explants**.** All the results are presented as mean and associated standard errors and different small letters (a–b) denote a significant difference between groups ($p<0.05$).

Figure 4. Confocal Microscopy of Collagen Fibrillogenesis. The columns represent collagens I, III and V. The lines represent the treated groups; 40x.

Figure 5 - Immunogold Labeling Electron Microscopy. Note sparse gold particles along the fibrils (arrows) in the IL-17 KO C57 group.

Figure 6. Immunohistochemical staining of IL-17-related markers and their morphometric analyses. The columns represent the BLM/PQ-treated Balb/C57 groups. The lines represent the treated groups, controls and morphometric analysis (%area), respectively; 40x. All the results are presented as mean and associated standard errors and different small letters (a–b) denote a significant difference between groups ($p<0.05$).

Figure 7. Immunohistochemical staining of FGF-2 and other IL-17-related markers and their morphometric analyses. The columns represent the BLM/PQ-treated Balb/C57 groups. The lines represent the treated groups, controls and morphometric analysis (%area), respectively; 40x. All the results are presented as mean and associated standard errors and different small letters (a–e) denote a significant difference between groups ($p<0.05$).

Figure 8. Immunohistochemical staining of other markers and their morphometric analyses. The columns represent the BLM/PQ-treated Balb/C57 groups. The lines represent the treated groups, controls and morphometric analysis (%area), respectively; 40x. All the results are presented as mean and associated standard errors and different small letters (a–d) denote a significant difference between groups ($p<0.05$).

Figure 9. Immunohistochemical staining of TUNEL and its morphometric analysis. The columns represent the BLM/PQ-treated Balb/C57 groups. The lines represent the treated groups, controls and morphometric analysis (%area),

respectively; 40x. The right left ventricular wall thickness ratio was calculated as an index of right ventricular hypertrophy. All the results are presented as mean and associated standard errors and different small letters (a–b) and asterisk (*) denote a significant difference between groups ($p<0.05$).

Figure 10. Proposed pathophysiological scheme of the interaction between collagen V and IL-17 signaling. The absence of the IL-17 receptor enhances the expression of collagen V and IL-17 response to maintain the same production of type I collagen in the fibrosis-susceptible strain.

Figures

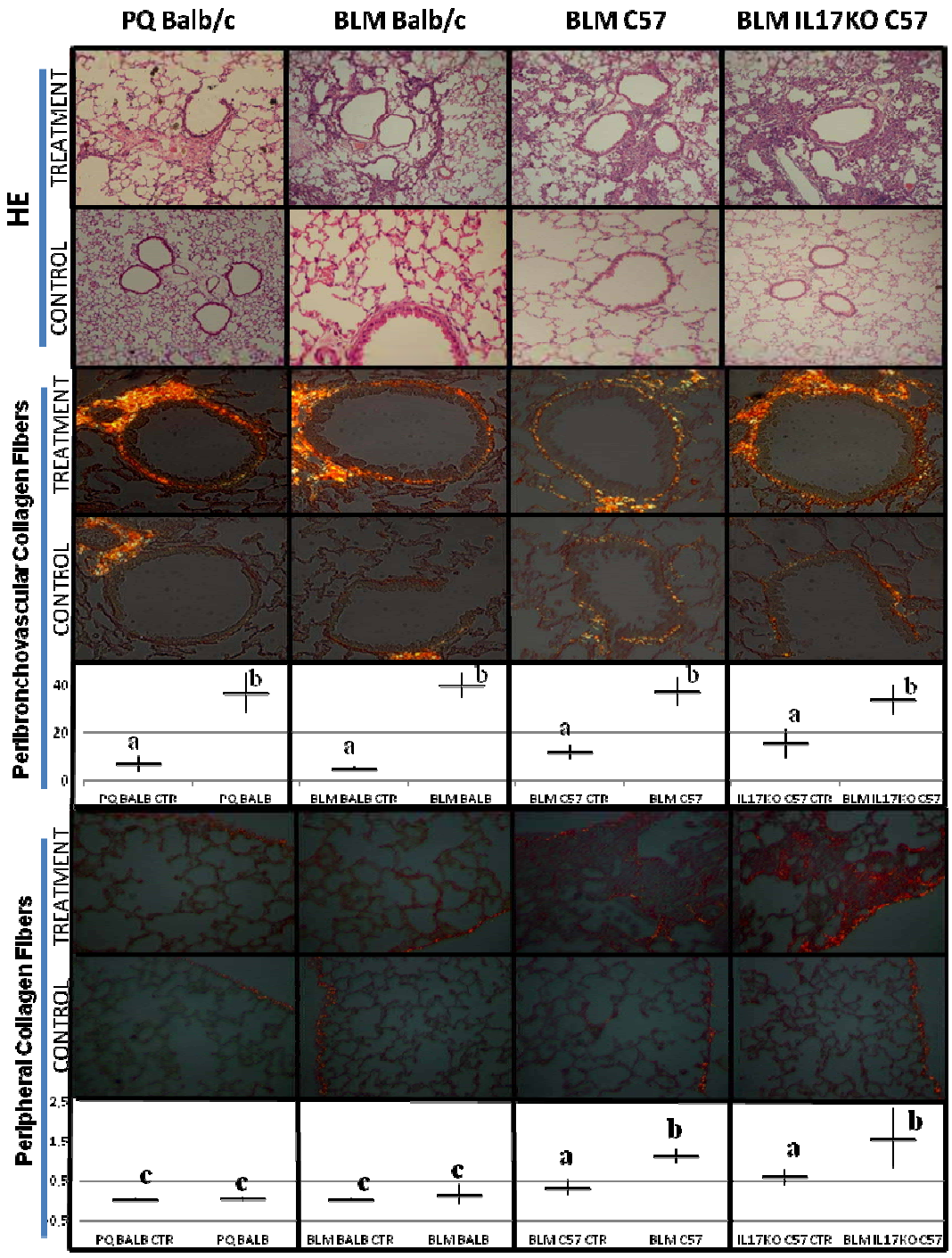
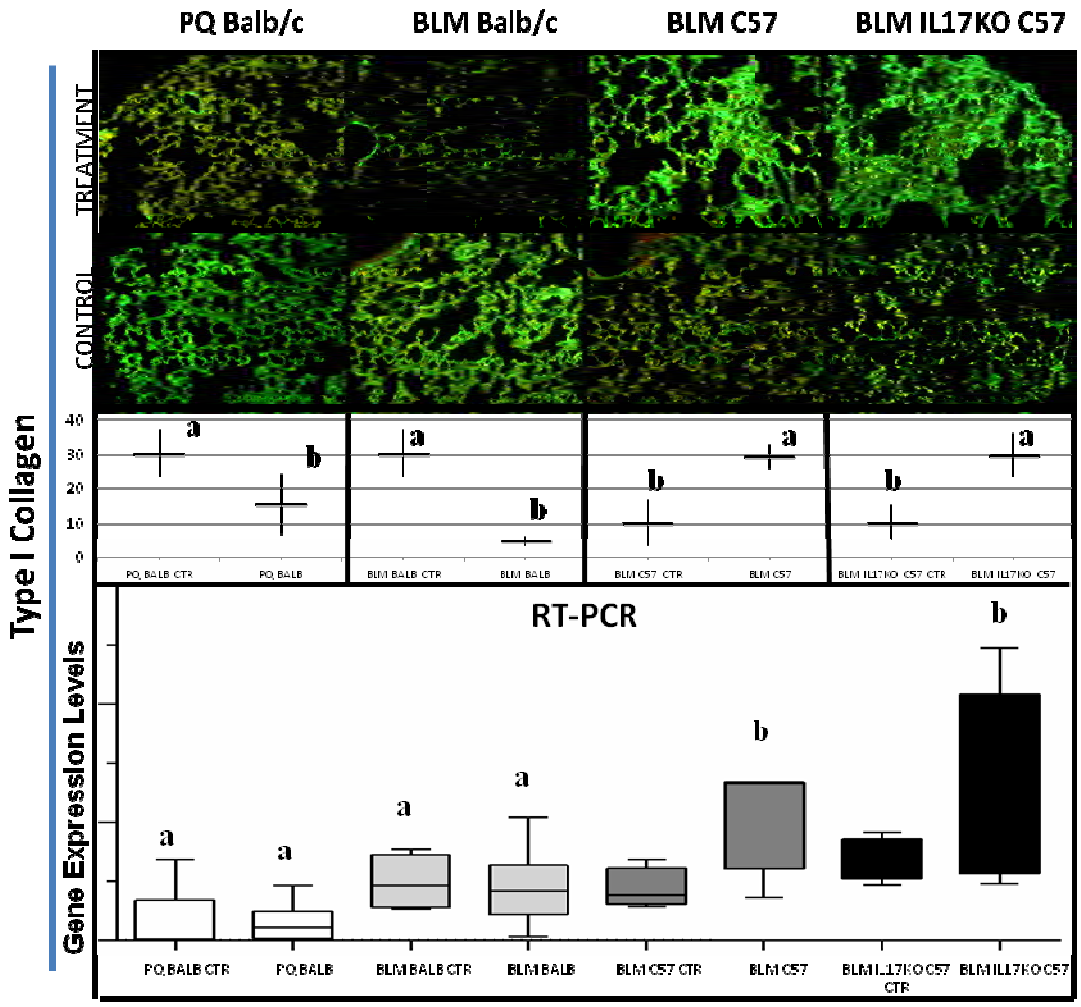


Figure 1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BLM C57
BLM IL17KO C57
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CONTROL
Type I Collagen
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30
20
10
0
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b
a
b
b
a
b
a
PQ BALB CTR
PQ BALB
BLM BALB CTR
BLM BALB
BLM C57 CTR
BLM C57
BLM IL17KO C57 CTR
BLM IL17KO C57
RT-PCR
Gene Expression Levels
a
a
a
a
b
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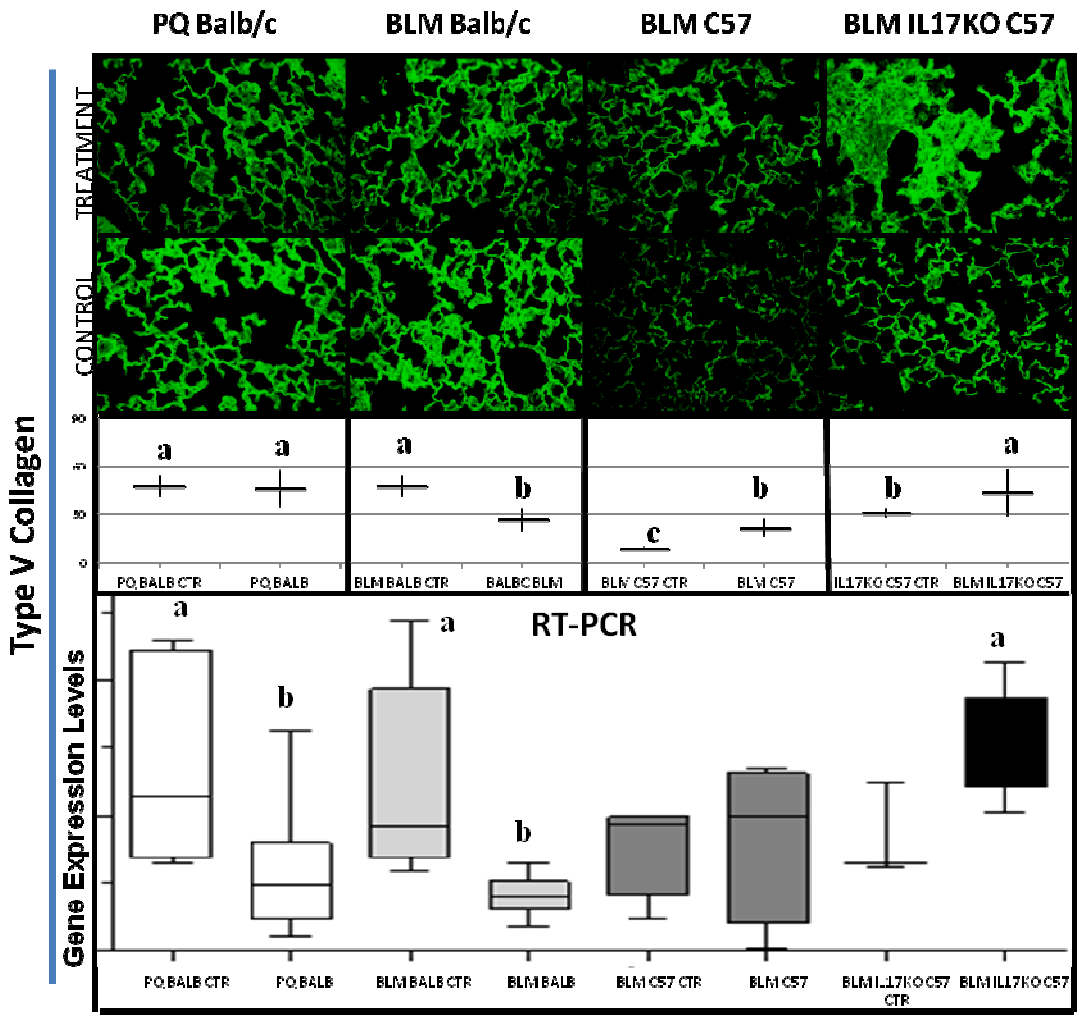
Figure 2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BLM Balb/c
BLM C57
BLM IL17KO C57
TREATMENT
CONTROL
Type V Collagen
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a
b
b
c
b
a
PQ.BALB CTR
PQ.BALB
BLM BALB CTR
BALBC BLM
BLM C57 CTR
BLM C57
IL17KO C57 CTR
BLM IL17KO C57
Gene Expression Levels
RT-PCR
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b
a
b
a
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Figure 3

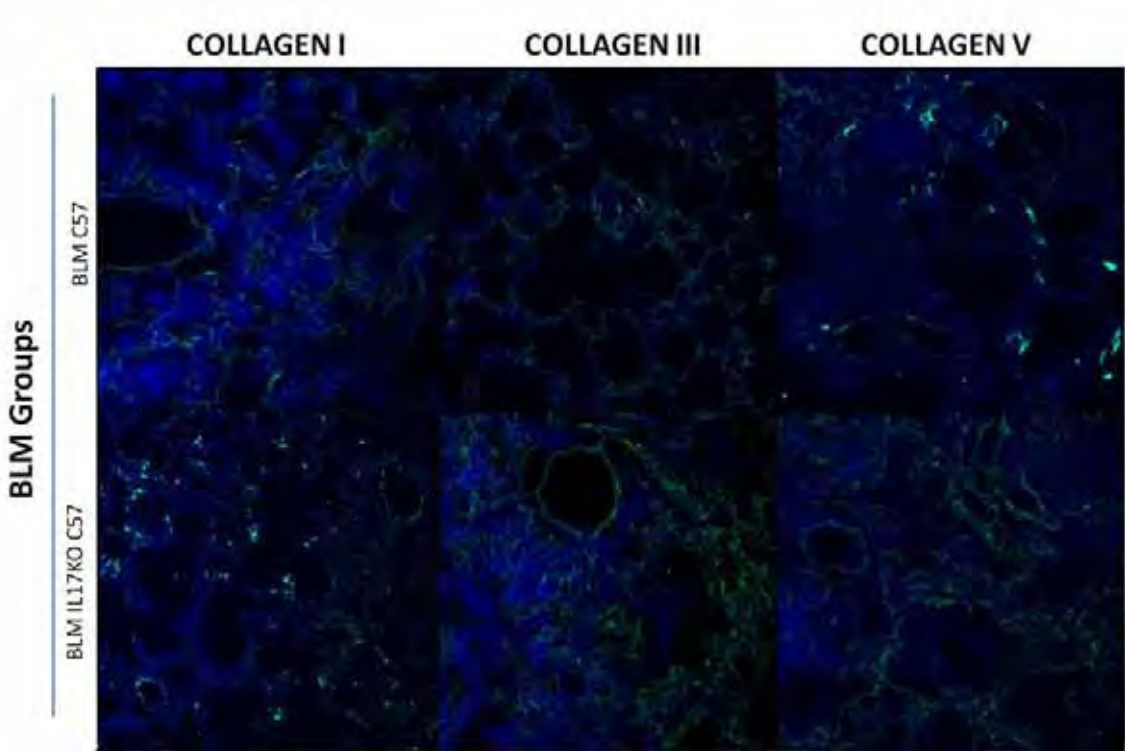

COLLAGEN I
COLLAGEN III
COLLAGEN V
BLM Groups
BLM C57
BLM IL17KO C57

Figure 4

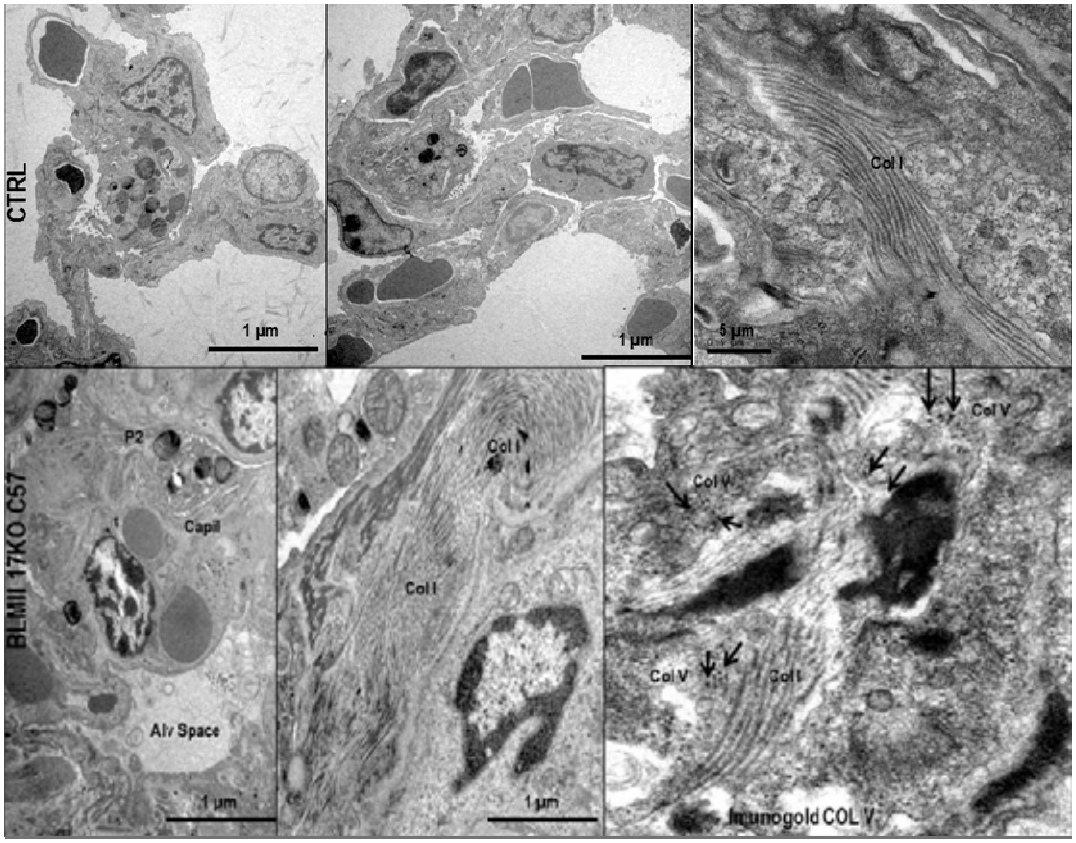
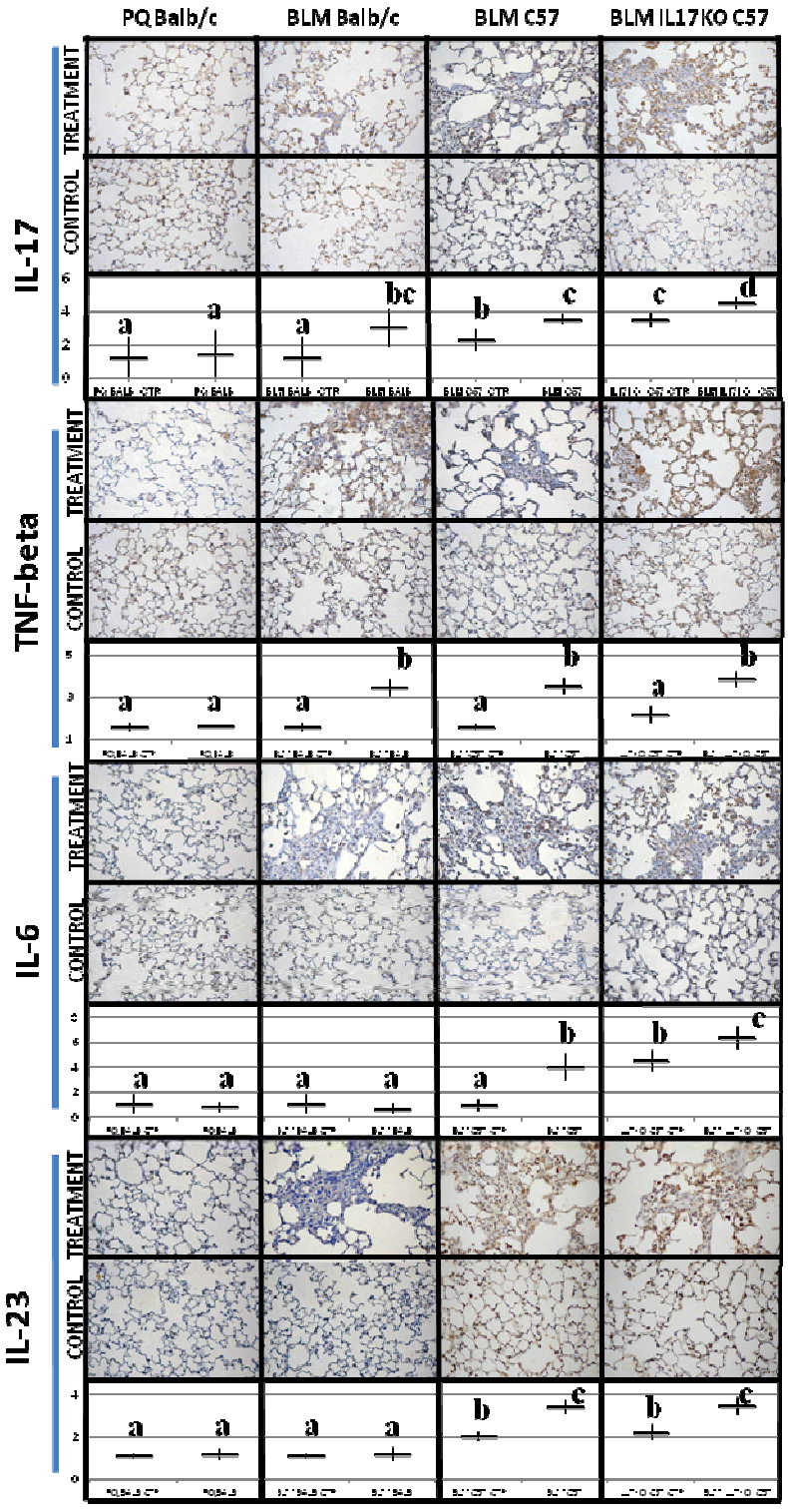

CTRL
1 µm
1 µm
Col I
5 µm
BLMII 17KO C57
P2
Capill
Alv Space
1 µm
Col I
1 µm
Col V
Col V
Col V
Col I
Immunogold COL V

Figure 5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BLM C57
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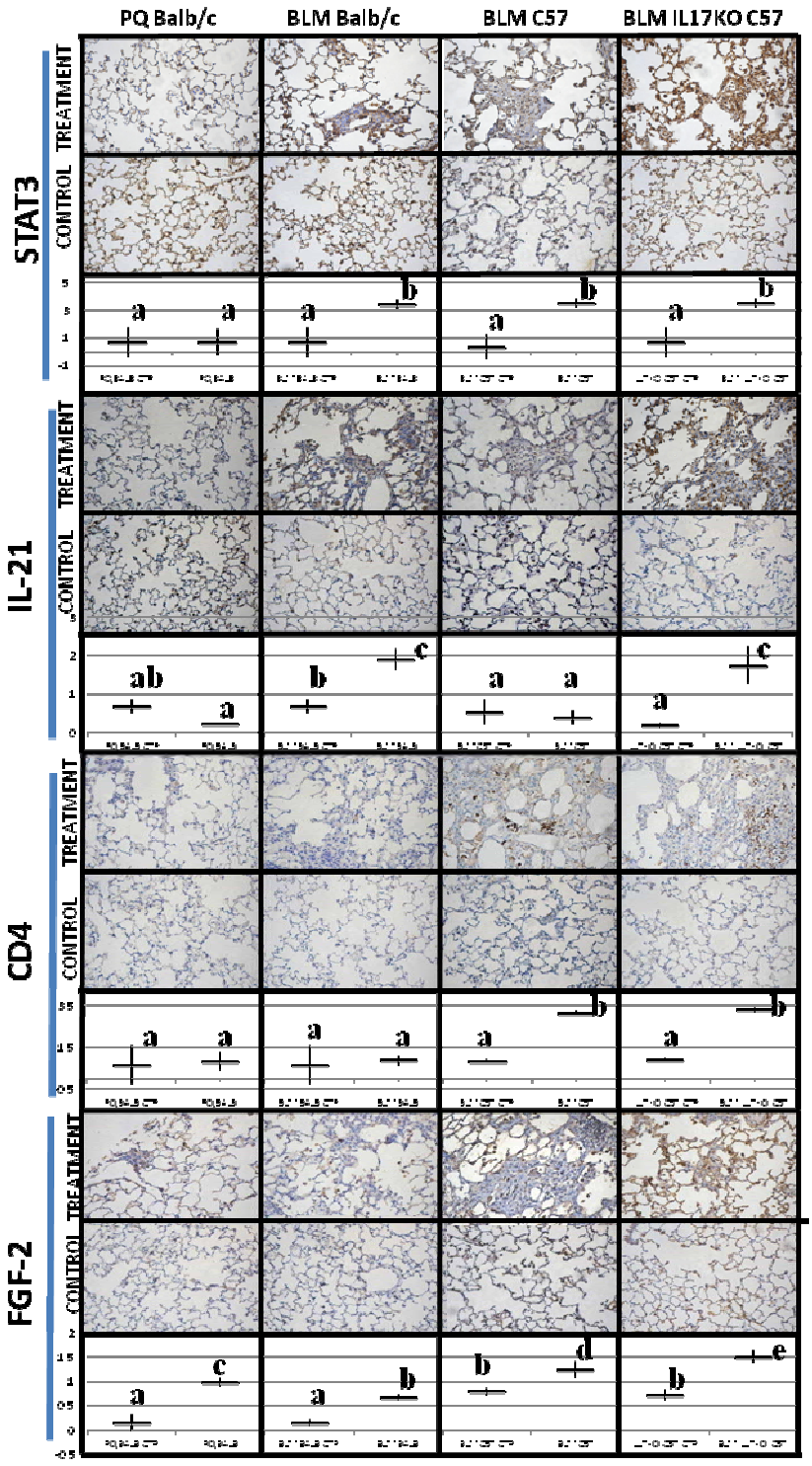
IL-17
TNF-beta
IL-6
IL-23
TREATMENT
CONTROL

Figure 6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STAT3
IL-21
CD4
FGF-2
TREATMENT
CONTROL

Figure 7

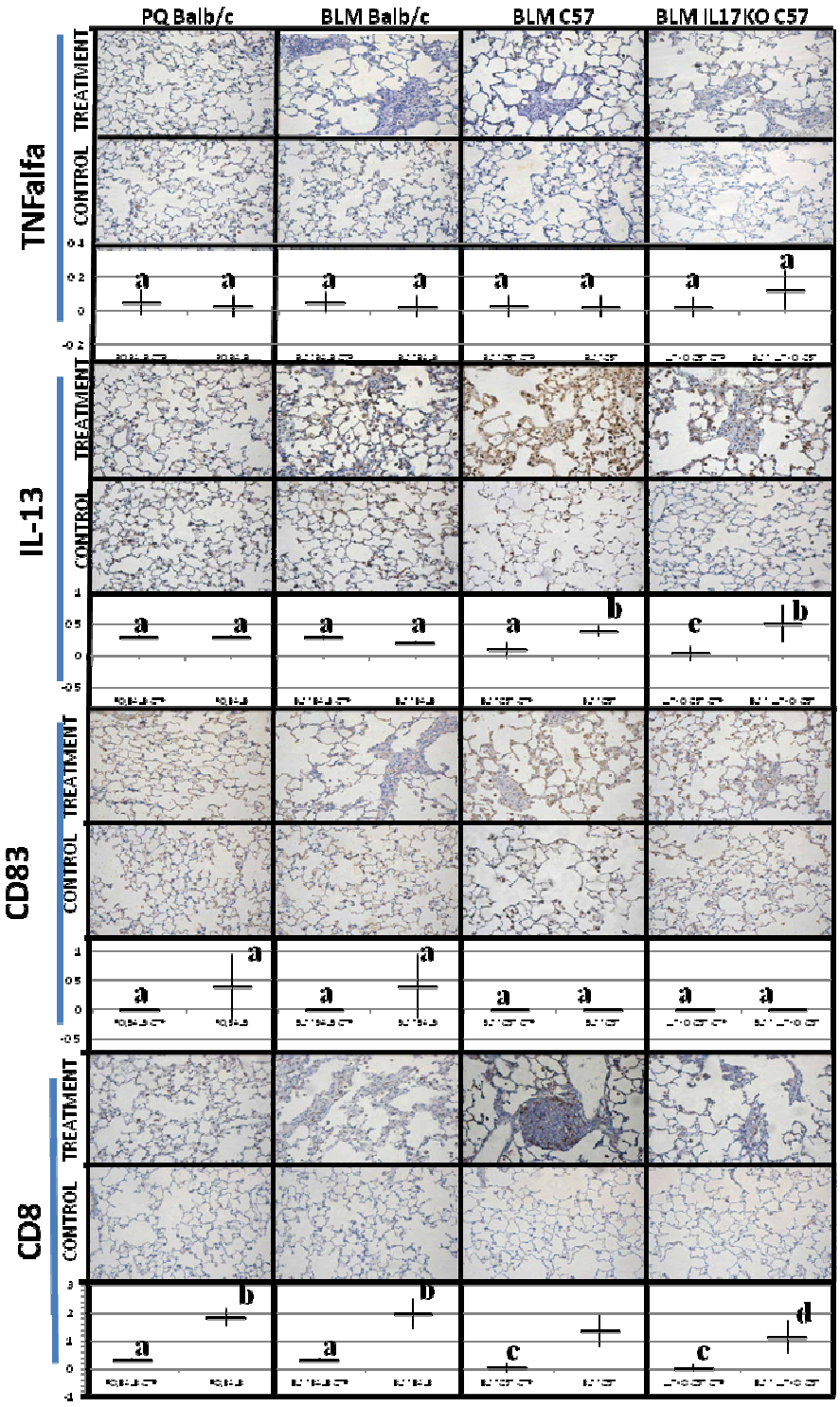
PQ Balb/c
BLM Balb/c
BLM C57
BLM IL17KO C57
TNFalfa
IL-13
CD83
CD8
TREATMENT
CONTROL

Figure 8

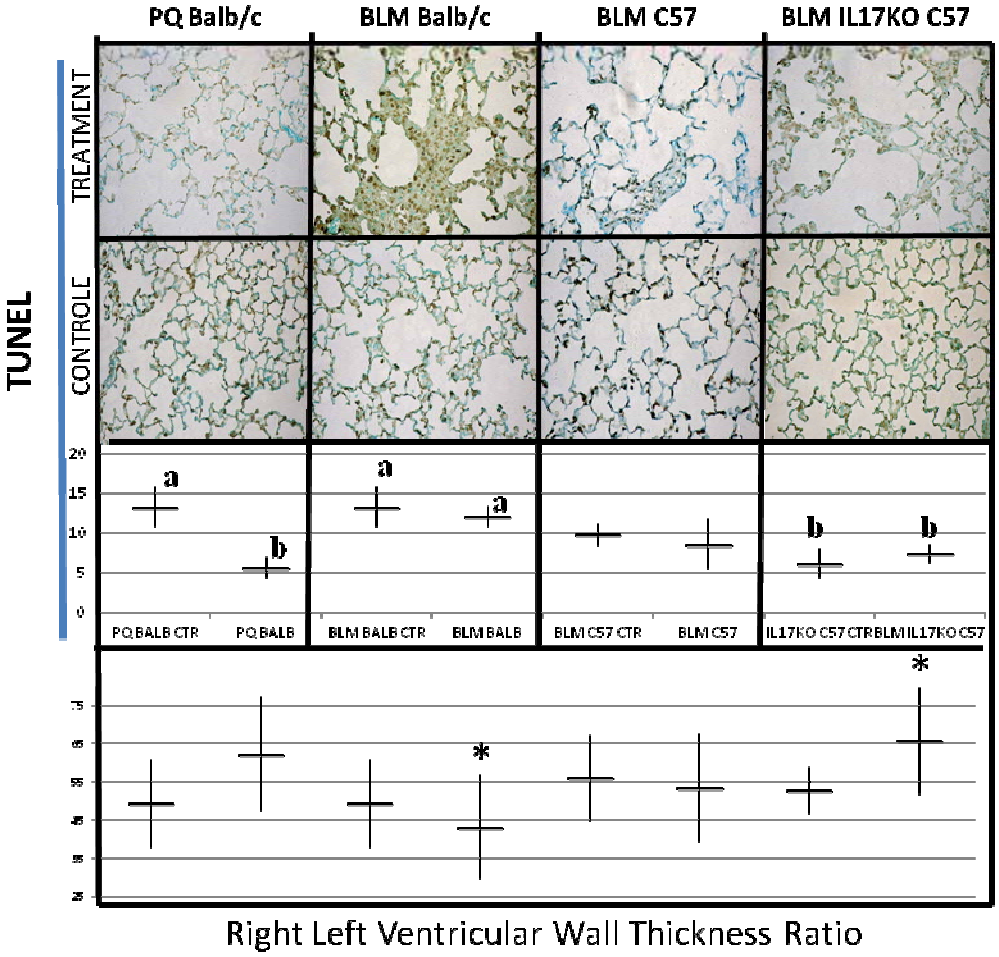
PQ Balb/c
BLM Balb/c
BLM C57
BLM IL17KO C57
TUNEL
TREATMENT
CONTROLE
20
15
10
5
0
a
a
a
b
b
b
PQ BALB CTR
PQ BALB
BLM BALB CTR
BLM BALB
BLM C57 CTR
BLM C57
IL17KO C57 CTR
BLM IL17KO C57
*
*
Right Left Ventricular Wall Thickness Ratio

Figure 9

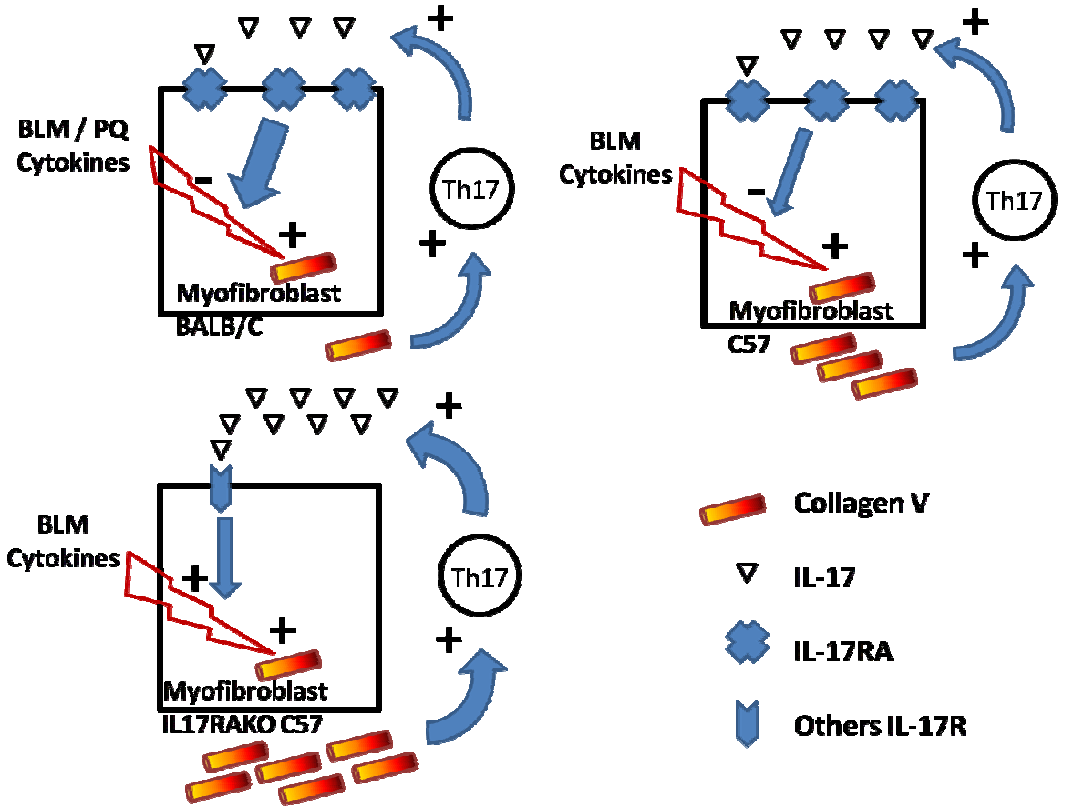

Figure 10

Reference

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8. Conclusão

A via fisiopatológica, na qual o colágeno V induz uma resposta IL-17, participa da modulação do remodelamento pulmonar, por interação dos receptores de IL-17 no padrão de deposição de matriz extracelular dos modelos experimentais susceptíveis a fibrose pulmonar. O colágeno V e a IL-17 são alvos potenciais para a manutenção do remodelamento extracelular, podendo sugerir que estratégias terapêuticas direcionadas a inibição ou alterações destes mecanismos podem ter um importante impacto na tratamento da fibrose pulmonar.

9. Anexos

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CERTIFICADO

CERTIFICAMOS que o Protocolo n.º 721 obre o Projeto de Pesquisa "Remodelamento parenquimatoso pulmonar em dois modelos experimentais de fibrose", a ser conduzido por Alexandre Todorovic Fabro, orientado pela Profª Drª Vera Luiza Capelozzi, com a colaboração dos Prof.s Drs. Cláudia Aparecida Rainho- Walcy P. Rosolia Teodoro - Edwin Roger Parra - Natalino Hajime Yoshinari, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os camundongos provenientes de Biotério convencional sem condições de emitir Atestado de Sanidade.

Tão logo seja apresentado o Certificado do CEMIB-UNICAMP, substituiremos o presente certificado.

Projeto de Pesquisa Aprovado em 26 de março de 2.009

Prof. Drª Regina Helena G. Martins
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

São Paulo, Brazil, 11 October, 2012

To Prof. Dr

J. M. Cruse

Editor-in-Chief

Experimental Molecular Pathology

Please find enclosed the manuscript **“Collagen V maintains Experimental Pulmonary Fibrosis in IL17–dependent and -independent pathways”**, which is being submitted for possible publication in Experimental Molecular Pathology. The work represents original investigation in the field of Pulmonary Fibrosis, allowing better understanding it pathogenesis, and then opening new perspectives of pathways in this severe disease. The manuscript will not be submitted to another Journal before your decision.

yours faithfully,

Alexandre Todorovic Fabro, MD

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To whom it may concern **1/24/12**

Report on Alexandre Todorovic Fabro activities as guest scientist at Institute of Pathology, Medical University of Graz

Mr. Alexandre Todorovic Fabro has joined as guest scientist the activities of the Research Unit on Molecular Lung and Pleura Pathology at the Institute of Pathology, Medical University of Graz, Austria, from January 14 2011 to December 23 2011. During this period, Mr. Fabro assessed qualitatively and quantitatively pathophysiological markers of pulmonary fibrosis in mice and human tissues using standard H&E and Movat staining, as well as immunohistochemical techniques to determine epithelial mesenchymal transition and stem cells. This work continued studies carried out during Mr. Fabro's project at São Paulo - Botucatu University.

During this period Mr. Fabro has acquired a good proficiency in German and participate actively in one international course and one meeting:

- Postgraduate course – Lung, Pleural and mediastinal diseases, Graz, Austria (July 11-16 2011);
- European Respiratory Society Annual Congress in Amsterdam (September, 24-28 2011).

I hope that this period of research and the discussions in the Unit will be of help for the further carrier of Mr. Fabro. We very much appreciated his enthusiasm and open-mindedness, as well as his lively and friendly character that led him immediately to integrate with the Unit's activities. Thus, I confidently hope that collaboration with Mr. Fabro and with the Pathology Dept.-Pathology of São Paulo- Botucatu University will go on.

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