UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

PAPEL DAS CÉLULAS APOPTÓTICAS NA ATIVAÇÃO ALTERNATIVA DE MACRÓFAGOS E SEU EFEITO SOBRE A AGRESSIVIDADE DE CÂNCER DE PULMÃO DE NÃO PEQUENAS CÉLULAS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul como requisito para a obtenção do grau de Doutor em Ciências Biológicas – Bioquímica.

Porto Alegre

2015

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19/08/2015

Este trabalho foi realizado no Departamento de Bioquímica da UFRGS e no Laboratório de Cardiologia Molecular e Celular do Instituto de Cardiologia de Porto Alegre. Os recursos obtidos para a realização deste foram providos pelo projeto MCT/CNPq Universal 2008 476114/2008-0 e MCT/CNPq INCT – Translacional em Medicina (573671/2008-7), além de recursos advindos da CAPES e da FAPERGS.

Pai Nosso de Chico Xavier

Pai nosso que estás nos céus
Na luz dos sóis infinitos
Pai de todos os aflitos
Neste mundo de escarcéus

Santificado, Senhor
Seja teu nome sublime
Que em todo o universo exprime
Ternura, Concórdia e Amor

Venha ao nosso coração
O teu reino de bondade
De paz e de claridade
Na estrada da redenção

Cumpra-se o teu mandamento
Que não vacila nem erra
Nos céus, como em toda a Terra
De luta e de sofrimento

Evita-nos todo o mal
Dá-nos o pão no caminho,
Feito de luz, no carinho
De pão espiritual.

Perdoa-nos, Senhor
Os débitos tenebrosos
De passados escabrosos
De iniqüidade e de dor

Auxilia-nos também,
Nos sentimentos cristãos
A amar os nossos irmãos
Que vivem distantes do bem

Com a proteção de Jesus
Livra nossa alma do erro
Neste mundo de desterro
Distante da tua luz

Que o nosso ideal igreja
Seja o altar da Caridade
Onde se faça a vontade
De teu amor... Assim seja.

“Quando alguém lhe magoar ou ofender, não retruque. Não responda da mesma forma, apenas sinta compaixão daquele que precisa humilhar, ofender e magoar para sentir-se forte.”
Chico Xavier

“Não cruze os braços diante de uma dificuldade, pois o maior homem do mundo morreu de braços abertos!”

Bob Marley

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer a Deus e ao Mestre Jesus pela oportunidade dada nessa nova missão aqui na crosta terrestre. Pela caminhada cheia de luz e de realizações.

À minha querida e amada mãe, Janise Becker, que não se encontra mais entre nós no envoltório da carne. Tu, mãe, foste meu porto seguro, meu abrigo, meu amor para todo o infinito. Ensinaste-me os valores certos e sempre me guiaste no caminho do bem. A saudade que sinto parece que às vezes vai dilacerar meu coração, mas sei que tudo foi um até breve e não um adeus. “A saudade é um sentimento que, quando não cabe no peito, escorre pelos olhos”. Tenho certeza que teu espírito agora descansa na paz eterna de nosso Pai e que tu estarás sempre iluminando a minha caminhada.

Ao amor de minha vida, Renata Tôndolo Tavares, por todo o companheirismo, a paciência e o amor verdadeiro dedicado a mim. Ensinaste-me, durante esse tempo juntos, o valor da caridade e como ser uma pessoa melhor. Apoiaste-me em todos os momentos, dos de alegria aos de tormenta. Amo-te com todo o meu coração e espírito! Um amor puro, daqueles que, infelizmente, poucos conseguem na vida. Com certeza, se eu desencarnar primeiro, estarei te esperando no plano espiritual para continuarmos nossa caminhada juntos.

À minha querida e amada família, principalmente meu tios Joice e Pedro, Carlos Alberto e Jussara, Cuco e Beatriz e meus primos Fábio Becker, Paulo Becker e Manuela Becker. Vocês foram fundamentais num dos piores momentos da minha vida: a passagem da mãe. Em um curto intervalo de tempo, mostraram-me o verdadeiro valor da família, com muito carinho e apoio em todos os momentos. Amo vocês de todo o meu coração. Também aos meus avós maternos Ruben Becker e Cecy Krebs Becker. Mesmo não estando mais na Terra, tenho certeza de que vocês estão sempre conosco, guiando e iluminando o nosso caminho. Vô, a saudade de ti é imensa, ensinaste-me muita coisa nessa vida. Foste meu avô, meu pai e meu melhor amigo. Tenho certeza de que um dia nos reencontraremos.

Ao pessoal do Instituto de Cardiologia, pessoas simples, de grande coração e que fizeram meus dias muito mais felizes. À Melissa Markoski, por ter confiado em mim e pela grande amizade que temos. Ao Max Schaun, pela amizade, conversas, desabafos e muitas risadas. À Melissinha, por vários momentos de risadas e apoio. Ao pessoal do LEA – Thiago, Augusto e Rafael – pela amizade e companheirismo. Ao Luciano, por lavar os materiais! (hehehe) e pela eficiência do seu trabalho. À Ludmila, pessoa mais querida e carinhosa do mundo! Por fim, mas não menos importante, à Lucinara (cabeçuda), pela grande amizade que temos, pelas brincadeiras, pela ajuda, pelos conselhos e por ter me aturado todo esse tempo (no fundo, eu sei que ela me ama!...hahaha).

A todo o pessoal do laboratório 24 pelo carinho e amizade. Em especial ao Marco Antônio, meu grande amigo que me ajudou muito nessa caminhada, auxiliando no trabalho manual e intelectual. Marcão, tu é o cara! À Fernanda Lopes, uma grande amiga, pessoa muito divertida com um enorme coração. Ao Leonardo Motta que, mesmo tendo nos distanciado um pouco, sempre irá morar no meu coração, pois nossa amizade é muito valiosa. À Carol Müller, por toda a amizade e ajuda na imunohistoquímica.

À Florência e à Mariana Parisi, duas grandes pessoas dotadas de uma inteligência fora do padrão. Vocês foram fundamentais para que tudo isso fosse possível. Sem a ajuda de vocês, boa parte desse trabalho não teria sido realizado. Além da contribuição científica, vocês também estiveram presentes no pior momento da minha vida. Além disso, foram compreensivas num momento de devaneio da minha parte. Muito obrigado por tudo mesmo!

Aos meus queridos e amados amigos Eduardo Chiela, Gabriel Fries, Thiago Ribeiro, Maurício Rigo, Andrew Silva, Pablo Escuder, Paola Escuder, Manon Escuder, Eduardo Silva, Michele Campos, Rafael Serrão, Débora de Oliva e Fernanda Lemos. A amizade de vocês é uma dádiva divina de Deus, pois "amigos são irmãos que podemos escolher em vida". Vocês fizeram, fazem e farão sempre parte da minha vida. Levarei sempre todos vocês no meu coração!

Por fim, mas óbvio que não menos importante, ao meu grande amigo e orientador Fábio Klamt. A pessoa que me abriu as portas para realizar meu mestrado e doutorado. Aquele que me ajudou MUITO em todos os momentos ao longo desses seis anos de laboratório. Fábio, tu és mais que um orientador pra mim, tu és como um irmão mais velho a quem eu tenho um carinho e amor enorme! Tu me ajudaste nos melhores e nos piores momentos que tive, principalmente, em toda a situação da mãe até o desencarne dela. Foste meu amigo sempre, mesmo quando podias me dar a maior das "mijadas", tu me chamavas para conversar e tentar esclarecer minhas ideias. Se eu ficasse aqui escrevendo o quanto tu representa pra mim, daria outra tese...hehehe. Muito obrigado por tudo!!!!!

ÍNDICE

PARTE I

1. Resumo

O câncer de pulmão é o tipo de câncer mais letal em todo o mundo, contabilizando 1.3 milhões de mortes anualmente. O câncer de pulmão de não-pequenas células (CPNPC) representa aproximadamente 85% dos casos diagnosticados. Apesar de o conhecimento da biologia molecular destes tumores ter aumentado muito, o prognóstico do CPNPC continua desfavorável, com uma sobrevida média de 10 meses. Um dos motivos deste mau prognóstico é a dificuldade relacionada à detecção precoce, de modo que a maioria dos pacientes diagnosticados apresenta estágios avançados. A falta de avanço clínico se dá muitas vezes pela dificuldade de compreender o microambiente que circunda o tumor, o qual está relacionado, entre outros fatores, à capacidade metastática e a resistência à quimioterapia. Nesse contexto, o estabelecimento e o avanço do processo neoplásico resultam em uma estimulação da resposta imunológica do hospedeiro contra o avanço tumoral. Uma das células recrutadas são os macrófagos, os quais desempenham um papel fundamental tanto na resistência tumoral, quanto na progressão da neoplasia. Essas células apresentam uma plasticidade celular de amplo espectro – a qual dificulta a análise da influência dessas células em relação a determinadas patologias - podendo, de acordo com o estímulo ambiental, apresentarem propriedades anti ou pró-inflamatórias. Essa plasticidade confere ao macrófago uma característica que o faz ser central no estabelecimento do microambiente tumoral. Nesse contexto, sabe-se que os agentes quimioterápicos, e outras moléculas, induzem a morte celular, principalmente, pelo processo apoptótico, sendo que a remoção dessas células e dos corpos apoptóticos é realizada pelos fagócitos, ocorrendo, assim, uma modulação do sistema imune, principalmente em relação aos macrófagos. Portanto, o presente trabalho tem por objetivo analisar a influência dos macrófagos e da apoptose na sobrevida de pacientes com CPNPC, verificar se a fagocitose de células apoptóticas advindas de diferentes indutores apresentam diferentes capacidades imunomodulatórias sobre o macrófago e buscar ferramentas que auxiliem na caracterização dos fenótipos dos macrófagos. Para tanto, inicialmente, realizou-se uma meta-análise de dados clínicos correlacionando macrófagos e apoptose com o desfecho clínico de pacientes com CPNPC. Em seguida, induziu-se a apoptose na linhagem de CPNPC A549 com os indutores cisplatina (droga padrão-ouro na clínica), taurina cloramina (um oxidante biológico produzido por macrófagos e neutrófilos) e ácido bromopirúvico (um inibidor metabólico), sendo as células apoptóticas postas em contato com os macrófagos. Para verificar a modulação dos macrófagos, analisou-se as citocinas liberadas. Por fim, através de bioinformática e RT-PCR, criaram-se duas assinaturas gênicas de macrófagos. Nossos resultados mostraram que há discrepâncias quanto ao papel dos macrófagos e da apoptose no desfecho dos pacientes com CPNPC. Nas análises *in vitro*, observamos que existem diferenças entre a eficiência da fagocitose das células apoptóticas advindas do tratamento com taurina cloramina quando comparada aos demais tratamentos. Observou-se também que ocorre uma modulação diferencial nos macrófagos que fagocitaram as células apoptóticas advindas do tratamento com cisplatina e taurina cloramina. Por fim, as assinaturas gênicas criadas conseguiram caracterizar de forma robusta não somente os fenótipos dos macrófagos *in vitro*, mas também seus fenótipos em determinados contextos clínicos, além da capacidade de correlacionar genes com o desfecho de determinadas patologias.

2. ABSTRACT

Lung cancer is the most lethal type of cancer in the world, and is responsible for 1.3 million deaths annually. Non small cell lung cancer represents approximately 85% of all cases diagnosed. Even though the knowledge of the molecular biology of these tumors has increased, the prognosis of NSCLC continues to be unfavorable, with an overall survival of 10 months. One reason for this difficulty is the poor prognosis associated with early detection, since most of the patients diagnosed with this pathology have advanced stages. The lack of clinical progress occurs often due to the difficulty of understanding the microenvironment surrounding the tumor, which is related with metastatic capacity and resistance to chemotherapy. In this context, the establishment and advancement of the neoplastic process result in a stimulation of the host immune response against tumor. One of recruited immune cells are the, which plays a key role in tumor resistance or progression. These cells exhibit a broad spectrum of cellular plasticity (which results in the difficulty to correlate these cells in human pathologies) may, depending on the environmental stimulus, presenting anti or pro-inflammatory properties. This plasticity confers to macrophage a key role in the establishment of tumor microenvironment. It is known that chemotherapy agents, and other molecules induce cell death mainly by apoptosis, and the removal of these cells and apoptotic bodies by phagocytes modulates the immune system, especially macrophages. Therefore, this study aims to analyze the influence of macrophages and apoptosis in patients' outcome and survival in NSCLC, to verify whether the phagocytosis of apoptotic cells obtained from different inducers had to capacity to modulate differently the macrophages and lastly found tools that can help in the characterization of macrophges' phenotypes. For that, firstly was made a meta-analysis of clinical data correlating macrophages and apoptosis with clinical outcome of patients with NSCLC. Further, apoptosis was induced in NSCLC cell line A549 with the inducers Cisplatin (gold standard in clinic), Taurine Chloramine (physiological oxidant) and Bromopyruvic Acid (metabolic inhibitor), and the apoptotic cells were putted in contact with macrophages. To verify the macrophages' modulation, we analyzed the cytokines released. Lastly, using bioinformartics and RT-PCR, we constructed two gene signatures of macrophages. Our results showed discrepancies concerning the role of macrophages and apoptosis in NSCLC patients' outcome. In *in vitro* analysis, we observed that the phagocytosis efficiency of apoptotic cells induced by Taurine Chloramine was different when compared to the others treatments. We also observed a differential modulation of macrophages that phagocytosed the apoptotic cells obtained from the treatment of Cisplatin and Taurine Chloramine. Finally, the gene signatures created were capable to characterize robustly not only the macrophages' phenotypes *in vitro*, but also the phenotypes in clinical contexts, predicting the correlation between genes expression and diseases outcomes.

LISTA DE ABREVIATURAS

- BAI1: *"brain-specific angiogenesis inhibitor 1"*;
- CD14: "*cluster of differentiation 14"*;
- CD31: "*cluster of differentiation 31"*;
- CD36: "*cluster of differentiation 36"*;
- CD47: "*cluster of differentiation 47"*;
- CPNPC: câncer de pulmão de não pequenas células;
- ERN: espécies reativas do nitrogênio;
- ERO: espécies reativas do oxigênio;
- GAS-6: *"growth arrest-specific 6"*;
- GM-CSF: "g*ranulocyte macrophage colony-stimulating factor*";
- HMGB1: *"high mobility group box 1"*;
- IFNγ: interferon gama;
- IL-10: interleucina 10;
- IL-12: interleucina 12;
- IL-13: interleucina 13;
- IL-1β: interleucina 1β;
- IL-4: interleucina 4;
- LPC: "*lysophosphatidilcholine"*;
- LPS: lipopolissacarídeo;
- MΦ: macrófagos derivados de monócitos;
- MCP-1: *"monocyte chemoattractant protein-1"*
- M-CSF: "*macrophage colony-stimulation factor"*;
- MFG8: *"milk fat globule-EGF factor 8 protein"*;

- S1P: *"sphingosine-1-phosphate"*
- TAM: "*tumor associated macrophages"* (macrófagos associados ao tumor);
- TGF-β: "*transforming growth factor beta"*;
- TIM-4: *"T cell immunoglobulin mucin-4"*;
- TNF: "*tumor necrosis factor"*;
- VEGF: "*vascular endothelial growth factor"*

CAPÍTULO I

3. INTRODUÇÃO

3.1 Câncer de Pulmão.

Por volta do século XIX, o câncer de pulmão era uma doença considerada rara – possivelmente devido à dificuldade de diagnóstico - representando somente 1% de todos os cânceres vistos em autópsias no Instituto de Patologia da Universidade de Dresden na Alemanha. Em 1918, a porcentagem de incidência desse tumor aumentou para quase 10% e, em 1927, para mais de 14%. Ainda, notou-se também que, enquanto a maioria dos tumores de pulmão ocorria em homens, nas mulheres parecia haver um aumento constante. O que poderia ter causado esse aumento considerável na incidência dos tumores de pulmão? Naquela época, o livro "*The handbook*" discutiu alguns fatores etiológicos possíveis: aumento na poluição do ar causada pela emissão de gases e por poeira, o asfaltamento de ruas e rodovias, o aumento do tráfego de carros, o trabalho com benzeno e gasolina, a pandemia de influenza em 1918, dentre outros fatores. Entretanto, a incidência do câncer de pulmão aumentou também em países menos industrializados. Chegou-se a conclusão, então, que outro fator contribuiu para esse aumento na incidência: o consumo do cigarro (Witschi 2001). Por isso, uma nova edição do livro "*The handbook"*, em 1969, dedicou cerca de vinte e cinco páginas sobre o papel do cigarro na patologia do câncer de pulmão. Além disso, mencionou que a poluição do ar e os compostos químicos encontrados em certas ocupações – como o arsênico, asbestos, níquel e crômio nas minas – eram fatores preponderantes para o aumento da incidência do câncer de pulmão (Hecht 1999).

Atualmente, o câncer de pulmão é uma doença altamente letal. De acordo com o trabalho de Siegel *et al*., de 2014, o câncer de pulmão aparece em segundo lugar na estimativa de novos casos e em primeiro lugar na estimativa de mortes (Siegel, Ma et al. 2014). O percentual de pacientes que apresentam sobrevida superior a cinco anos varia entre 13 a 21% em países desenvolvidos e entre 7 a 10% em países em desenvolvimento. O principal fator de risco para o seu desenvolvimento é o tabagismo, pois os tabagistas têm cerca de 20 a 30 vezes mais risco de desenvolver carcinoma de pulmão quando comparados aos não fumantes (Jamnik, Santoro et al. 2009). Segundo dados do INCA, o câncer de pulmão/brônquios apresentou uma alta nos índices de mortalidade no período de 1979 - 2012. Ainda, numa comparação entre os Estados da federação, é o tipo de câncer que mais mata no Rio Grande do Sul, sendo que, em Porto Alegre, este tipo de tumor matou 3.059 pessoas no período de 2000-2004 (INCA 2014).

O câncer de pulmão, segundo a Organização Mundial de Saúde, é geralmente classificado em duas classes principais, baseado na sua biologia, terapia e prognóstico: câncer de pulmão de pequenas células (SCLC – do inglês *small cell lung cancer*), e câncer de pulmão de não pequenas células (NSCLC – do inglês *nonsmall cell lung cancer*). O de não pequenas células é responsável por aproximadamente 85% de todos os casos de câncer de pulmão e inclui dois tipos principais: o carcinoma de células não escamosas (incluindo o adenocarcinoma, o carcinoma de células grandes e outros tipos celulares) e o carcinoma de células escamosas (epidermóide). O subtipo histológico adenocarcinoma é o tipo mais comum de câncer de pulmão encontrando nos Estados Unidos e é o mais frequente tipo celular encontrado em tumores de pessoas não fumantes (Ettinger, Akerley et al. 2010).

A avaliação do prognóstico do paciente é um fator muito importante na seleção do tratamento adequado para cada caso, sendo que as variáveis associadas com o prognóstico podem ser divididas em algumas categorias: aquelas relacionadas ao tumor, como local primário e tipo celular; aquelas relacionadas ao paciente, como o "*perfomance status*" (essa escala é usada por médicos e pesquisadores para verificar como a doença está progredindo - ver anexo, tabela 1), comorbidade e o gênero; e os fatores ambientais, como a nutrição e a escolha e qualidade do tratamento. Estas variáveis podem ser úteis individualmente ou combinadas para formar um índice prognóstico múltiplo (Goldstraw, Ball et al. 2011). Ainda, a classificação anatômica, como descrita pela classificação TNM (ver anexos, tabela 2), constitui-se também em um dos mais importantes fatores prognósticos no câncer de pulmão de pequenas e não pequenas células (Goldstraw, Crowley et al. 2007).

No que diz respeito ao tratamento, o procedimento cirúrgico continua sendo a principal forma de tratamento para os estágios iniciais e da doença localizada. As terapias multimodais se tornaram um modelo para doenças regionalmente avançadas, em que pacientes com doença avançada e metastática são candidatos a tratamentos quimioterápicos paliativos, os quais apresentam evidências de melhoras na sobrevivência e qualidade de vida destes (Ramalingam and Belani 2008). A quimioterapia sistêmica também beneficia pacientes com estágios iniciais da doença e tem sido parte das estratégias terapêuticas multimodais para os estágios I e III de CPNPC (Arriagada, Bergman et al. 2004). Nesse contexto a quimioterapia baseada em agentes alquilantes (a base de platina) como um tratamento de primeira escolha para pacientes com CPNPC avançado foi reportado pela primeira vez em um ensaio

clínico randomizado publicado em 1988. Evidências mostrando a eficácia da quimioterapia usando agentes alquilantes foram demonstradas por uma meta-análise de todos os ensaios clínicos randomizados. Esta análise demonstrou que a quimioterapia baseada em cisplatina foi associada com uma taxa de sobrevivência de um ano (Ramalingam and Belani 2008). Devido a isso, os agentes alquilantes foram combinados com outros medicamentos no intuito de melhorar a sobrevida e a qualidade de vida dos pacientes. No entanto, a eficácia dessas drogas, principalmente os agentes alquilantes, é muito baixa. Em vista disso, novas abordagens estão sendo realizadas visando à melhora na resposta ao tratamento do tumor (Davies 2014). Por exemplo, o *erlotinib* (Tarceva®, OSI Pharmaceuticals, LLC, Farmingdale, NY, USA), o *afatinib* (Gilotrif®, Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT, USA), e o *crizotinib* (Xalkori®, Pfizer, Inc, New York, NY, USA) são terapias de primeira linha aprovadas para pacientes que expressam mutações pontuais no receptor de EGF (sigla em inglês para *"epidermal growth factor"*), ou que apresentem tumores positivos para rearranjo do ALK (sigla em inglês para *"anaplastic lymphoma kinase"*). Em relação aos pacientes com CPNPC de células não escamosas, estes podem ser tratados com *bevacizumab* (Avastin®, Genentech, South San Francisco, CA, USA) - um anticorpo monoclonal que inibe a angiogênese do tumor através da alteração da sinalização do EGFR, culminando em uma depleção de moléculas e nutrientes essenciais no microambiente tumoral (Davies 2014). No entanto, essas abordagens privilegiam uma pequena parcela dos pacientes com CPNPC sendo, assim, restritos a determinados fatores.

Percebe-se, portanto, que ainda há a necessidade de se buscar novos fármacos e novas terapias (como, por exemplo, a imunoterapia) no tratamento do câncer de pulmão de não pequenas células, com o objetivo de melhorar a qualidade de vida dos pacientes em tratamento e também de se alcançar a remissão completa do tumor.

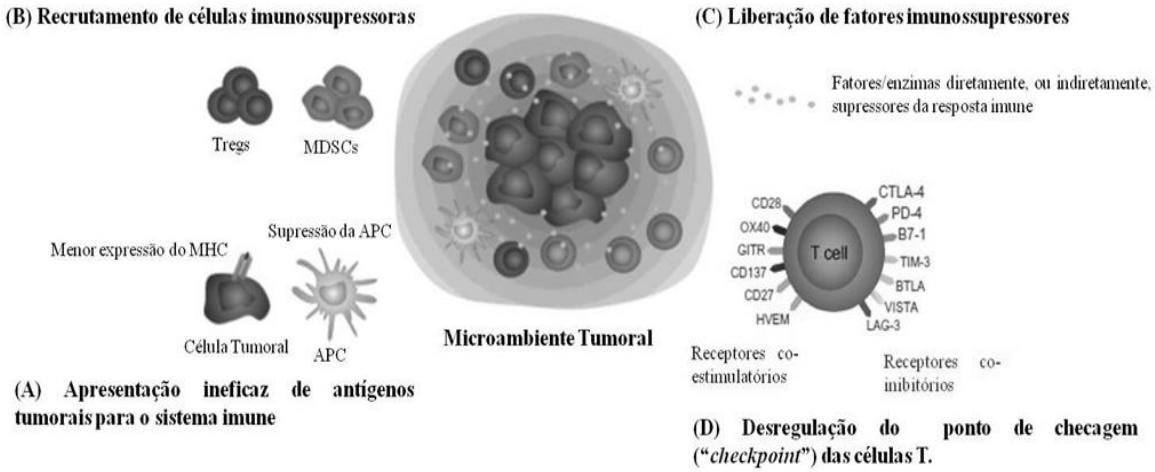
3.1.1 Imunoterapia: conceitos gerais e aplicação no Câncer de Pulmão

O sistema imune tem como função principal proteger o nosso corpo de danos causados pelos mais diversos patógenos. Além disso, esse sistema também serve para detectar e eliminar células aberrantes – com comportamento fora do padrão homeostático, incluindo as células tumorais, as quais podem potencialmente causar enormes danos ao organismo (Schreiber, Old et al. 2011). Esse sistema de proteção se torna bastante evidente pelos resultados obtidos em diversos estudos que mostram que pacientes com reduzidas funções imunológicas, como, por exemplo, em situações de imunodepressão ou imunossupressão, apresentam chances aumentadas de desenvolver tumores (Grulich, van Leeuwen et al. 2007). Essa ativação imune é mediada principalmente pelas células T, as quais reconhecem uma "exibição" anormal de epitopos das células tumorais no MHC de classe I, e coordenam, assim, a resposta imunológica contra os tumores. No entanto, se a resposta produzida for muito exacerbada, esta poderá acarretar em sérios danos teciduais. Para proteger o organismo contra possíveis efeitos danosos, o sistema imunológico apresenta múltiplos mecanismos para controlar a resposta imune – os chamados pontos de checagem ("*checkpoints"*)

(Arriagada, Bergman et al. 2004). Destarte, toda a ativação imunológica exige uma sintonia fina entre seus diferentes tipos celulares, com o objetivo de eliminar o agressor de forma extremamente eficiente e sem prejudicar o organismo.

Devido a grande importância do sistema imunológico no reconhecimento e eliminação tumoral, a investigação e a relação da resposta imune com o tumor têm se tornado um campo de vasta pesquisa. Em vista disso, surgiu, então, o termo imunoterapia, a qual consiste em um delineamento para restabelecer, estimular, ou aumentar a habilidade do sistema imune em reconhecer e eliminar as células tumorais (Garbe, Eigentler et al. 2011; Topalian, Hodi et al. 2012). Ainda, essa abordagem é baseada na premissa de que o sistema imune tem um papel chave na chamada imunovigilância e remoção das células tumorais (Yoon 2014). Ela, por isso, é diferente das terapias usuais, as quais apresentam como forma de tratamento alvos seletivos que bloqueiem o processo de crescimento tumoral, sua capacidade de proliferação, sua capacidade invasiva, etc. O objetivo da imunoterapia é o de auxiliar o sistema imune em reconhecer as células tumorais novamente como um organismo "estranho", de estimular a capacidade de resposta imunológica e de suprimir a inibição do sistema imune promovida pelo microambiente tumoral, o qual promove uma imunotolerância, contribuindo para a sobrevivência e crescimento do tumor (Yoon 2014). A figura 1 demonstra de forma resumida as estratégias usadas pelo tumor para evadir a resposta imune. Além disso, diversas estratégias estão sendo utilizadas para ativamente aumentar a resposta imune contra diversos tumores, incluindo a vacinação para estimular a produção de anticorpos, a depleção dos macrófagos associados ao tumor, as respostas baseadas nas células T, como o uso de inibidores dos pontos

de checagem ("*checkpoints"*) para reiniciar a resposta imune através delas, sendo que, em relação aos pontos de checagem, as vias do CTLA-4 (sigla em inglês para "*cytotoxic T-lymphocyte-associated antigen-4"*) , da PD-1 (sigla em inglês para "*programmed cell death protein-1"*) e da PD-L1 (sigla em inglês para *"programmed cell death- ligand 1"*) são as mais estudadas, por serem vias



que apresentam um papel fundamental em controlar a resposta imune mediada pelas células T.

Figura 1: Estratégias imunossupressoras e de evasão imune utilizada pelas células tumorais. *Adaptado de New modalities of cancer treatment for NSCLC: focus on immunotherapy. Cancer Management and Research 6:63.*

No que concerne ao NSCLC, embora historicamente este tipo de tumor não seja consideravelmente sensível às terapias baseadas no sistema imune, estudos clínicos já demonstraram que um alto número de células T $CD4^+$, $CD8^+$, *natural killers* e/ou células dendríticas na massa tumoral está associado a um aumento da sobrevida dos pacientes (Takanami, Takeuchi et al. 2001; Hiraoka, Miyamoto et al. 2006; Dieu-Nosjean, Antoine et al. 2008). Além disso, novos agentes antitumorais baseados na resposta imune, como o *Nivolumab* – um anticorpo contra PD-1 -, já estão em ensaios clínicos de fase II (Creelan 2014).

No entanto, os maus prognósticos associados ao NSCLC indicam que o sistema imunológico se torna incapaz de destruir completamente as células tumorais, possivelmente devido ao microambiente imunossupressor criado pelo tumor, permitindo, assim, que haja uma evasão e uma imunodulação pró-tumoral (Schneider, Kimpfler et al. 2011; Wang, Lu et al. 2011; Dasanu, Sethi et al. 2012). Em relação a esse tipo tumoral, verifica-se que grande parte dos trabalhos objetiva o reestabelecimento da resposta imune adaptativa, sem focar, de forma mais precisa e detalhada, nas células do sistema imune inato, como os macrófagos, por exemplo. Percebe-se, portanto, que estudos mais aprofundados sobre essa abordagem terapêutica no NSCLC se tornam necessários, no intuito de alavancar a associação da imunoterapia com as terapias atualmente utilizadas.

3.2 Macrófagos

Os macrófagos são células do nosso organismo que fazem parte da diferenciação final do sistema fagocítico mononuclear, o qual compreende também as células dendríticas e os monócitos circulantes (Cassetta, Cassol et al. 2011). A descoberta de sua capacidade fagocítica foi feita por Élie Metchnikoff, que ganhou o prêmio Nobel por essa descrição, fazendo com que ele propusesse que a chave para a imunidade seria o estímulo da fagocitose (Mosser and Edwards 2008; Nathan 2008). Assim, muita atenção foi dada apenas a relevância imunológica do macrófago. No entanto, sabe-se, no contexto atual, que os macrófagos desempenham funções além das imunológicas, como, por exemplo, a remodelação tecidual, angiogênese, progressão tumoral, processos fibróticos, dentre outras (Mosser and Edwards 2008; Sica, Larghi et al. 2008; Murray and Wynn 2011; Wynn, Chawla et al. 2013). Os macrófagos, por exemplo, removem aproximadamente 2×10^{11} eritrócitos a cada dia. Esta quantidade equivale a

aproximadamente 3 gramas de íon ferro e hemoglobina que são reciclados para que o indivíduo possa reutilizá-lo. Esse processo de remoção denota-se uma contribuição metabólica vital, a qual um indivíduo não sobreviveria sem ele. Além disso, os macrófagos também estão envolvidos na remoção dos "*debris*" celulares que são gerados durante o remodelamento de um tecido, fagocitando rapidamente e eficientemente as células que entraram no programa de apoptose. Os receptores que medeiam este processo homeostático de remoção incluem os receptores de captura, receptores de fosfatidilserina, receptores de trombospondina, integrinas e receptores do complemento. Além disso, a fagocitose que ocorre diariamente pelos macrófagos é independente de outras células do sistema imunológico (Kono and Rock 2008; Mosser and Edwards 2008). Devido a característica dos macrófagos de fagocitar células apoptóticas, muitos estudos estão sendo realizados, visto que esse processo também influencia na resposta imunológica do hospedeiro. A fagocitose das células apoptóticas tem a capacidade de modular o macrófago, suprimindo o processo inflamatório e a resposta imune, em parte através da liberação de citocinas anti-inflamatórias (Fadok, Bratton et al. 2000; Byrne and Reen 2002; Huynh, Fadok et al. 2002; Lauber, Blumenthal et al. 2004). Além disso, a produção de moléculas oxidantes durante o *"burst"* respiratório, principalmente em neutrófilos, como o ácido hipocloroso – HOCl – e o ânion hipoclorito (ClO^-), através da reação da enzima mieloperoxidase, também pode influenciar na modulação do macrófago. Por exemplo, o aminoácido taurina é extremamente abundante em células inflamatórias e possui ação protetora contra a auto destruição durante o processo de produção de agentes oxidantes. Sua reação com o HOCl forma um composto chamado Taurina-Cloramina, que tem a capacidade

de inibir a superprodução de ERO e, além disso, de modular o macrófago para um fenótipo anti-inflamatório (Marcinkiewicz, Grabowska et al. 1995; Chorąży, Kontny et al. 2002; Kim and Cha 2014). Apesar dessa visão clássica, estudos recentes demontraram que a taurina cloramina é um poderoso indutor de apoptose de células tumorais, sendo considerado um dos principais mecanismos fisiológicos na eliminação de tumores (Englert and Shacter 2002; Klamt and Shacter 2005). Por isso, esse composto apresenta um alvo potencial a ser estudado não somente na modulação da resposta imunológica, mas também como um agente de eliminação de células tumorais.

Os macrófagos são divididos em subpopulações, baseados na sua localização anatômica e fenótipo funcional, originando os macrófagos residentes teciduais e, como exemplos, têm-se os osteoclastos nos ossos, os macrófagos alveolares no pulmão, as células de Küpffer no fígado, dentre outras (Murray and Wynn 2011). A figura 2 representa, de forma geral, os diversos tipos de macrófagos e algumas de suas principais funções. O crescimento e a diferenciação dos macrófagos dependem das citocinas no microambiente, como o M-CSF e o GM-CSF, e das interações com o estroma dos órgãos hematopoiéticos. A interleucina-3, o KIT (receptor de tirosina cinase), as proteínas da família do fator de necrose tumoral e as moléculas relacionadas ao TNF contribuem para a diferenciação do macrófago, assim como os fatores de transcrição, como o PU.1 e outros membros da família ETS ("*E26 transformation-specific*") (Gordon 2003).

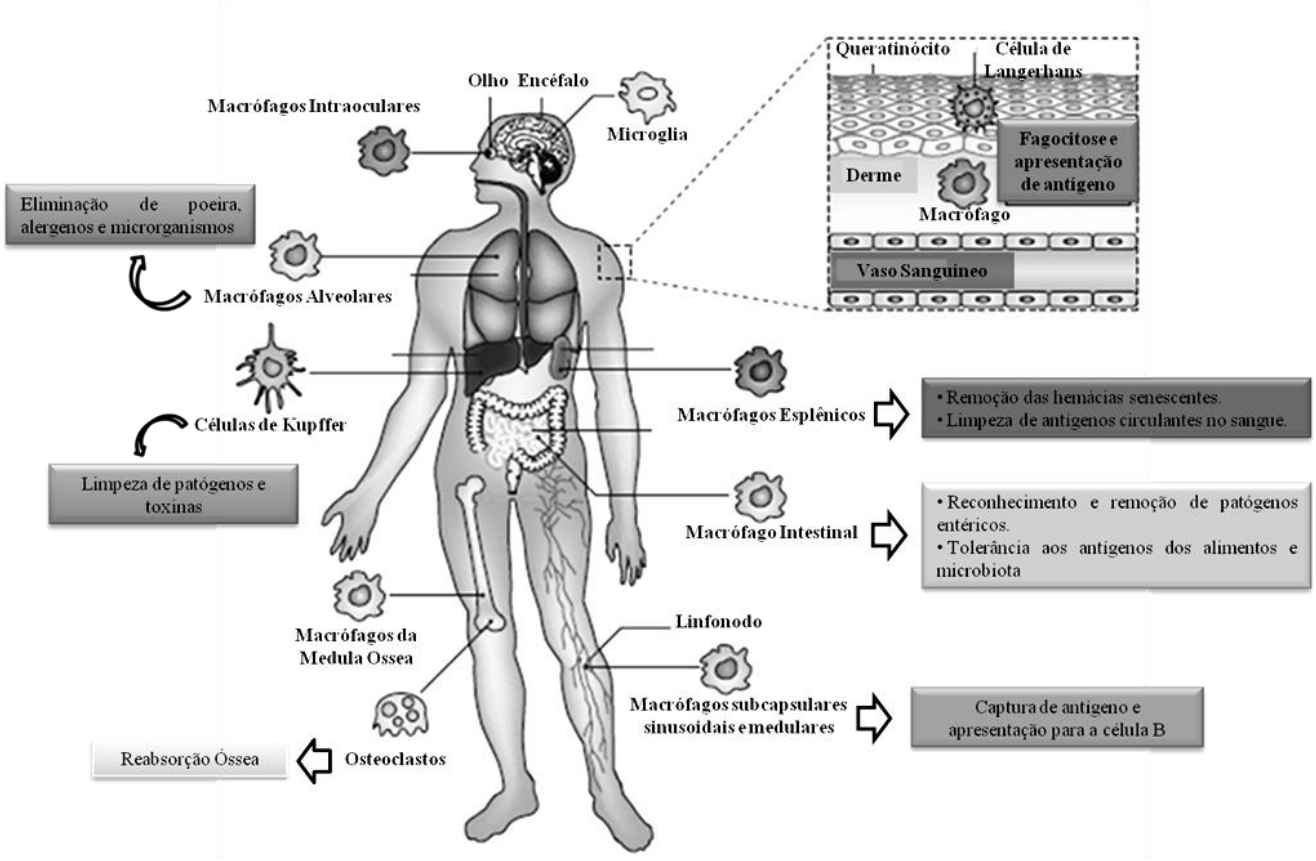


Figura 2: Funções dos macrófagos na homeostasia tecidual. *Adaptado de Protective and pathogenic functions of macrophage subsets*. *Nature Reviews Immunology, 2011.*

Os macrófagos também apresentam uma característica de fundamental importância no seu papel fisiológico: a plasticidade celular. A diversidade e a plasticidade são características bem conhecidas da linhagem monócitos-macrófagos (Mantovani, Sica et al. 2004; Mantovani, Biswas et al. 2013). Nos tecidos, os fagócitos mononucleares respondem aos estímulos ambientais (produtos microbianos, células danificadas, linfócitos ativados, etc.) com a aquisição de fenótipos funcionais distintos. Quando em resposta aos ligantes dos receptores tipo *Toll* e IFNγ ou IL-4/IL-13, os macrófagos ativam-se de maneira clássica (fenótipo M1) ou alternativa (fenótipo M2), respectivamente. Essa dicotomia M1/M2 foi espelhada na polarização Th1/Th2 dos linfócitos e representaria um *continuum* de ativação dessas células. Em geral, os macrófagos M1 (pró-inflamatórios) representam um fenótipo de alta produção de IL-12 e IL-

23 e baixa produção de IL-10, sendo eficientes produtores de ERO, ERN e citocinas pró-inflamatórias, como a IL-1β, o TNF e a IL-6, participando, assim, como uma célula indutora e polarizadora dos linfócitos T para o perfil Th1. Em contrapartida, os vários subfenótipos (M2a, M2b e M2c) dos macrófagos M2 (anti-inflamatórios) geralmente compartilham uma baixa produção de IL-12 e IL-23, com alta produção de IL-10, possuindo uma capacidade variada de produção de citocinas inflamatórias, a qual depende do sinal existente em abundância no microambiente. Além disso, em geral apresentam altos níveis de receptores de captura, receptores de manose e galactose e uma mudança no metabolismo da arginina (Mantovani, Sozzani et al. 2002; Mantovani, Sica et al. 2004; Mosser and Edwards 2008; Gordon and Martinez 2010; Mantovani, Biswas et al. 2013). Os macrófagos polarizados para o fenótipo M2 também apresentam regulação diferencial do sistema da IL-1: baixa produção de IL-1β e ativação de caspase I e alta expressão do antagonista do receptor de IL-1 (IL-1ra) e receptores *decoy* tipo II (Dinarello 2005). Por fim, os macrófagos M2 participam da resposta de polarização Th2, remoção de parasitas (Noël, Raes et al. 2004), diminuição da resposta inflamatória, remodelamento tecidual (Wynn 2004), angiogênese, progressão tumoral e imunorregulação (Biswas and Mantovani 2010). A figura a seguir apresenta de forma resumida os fenótipos M1 e M2, além dos subfenótipos M2.

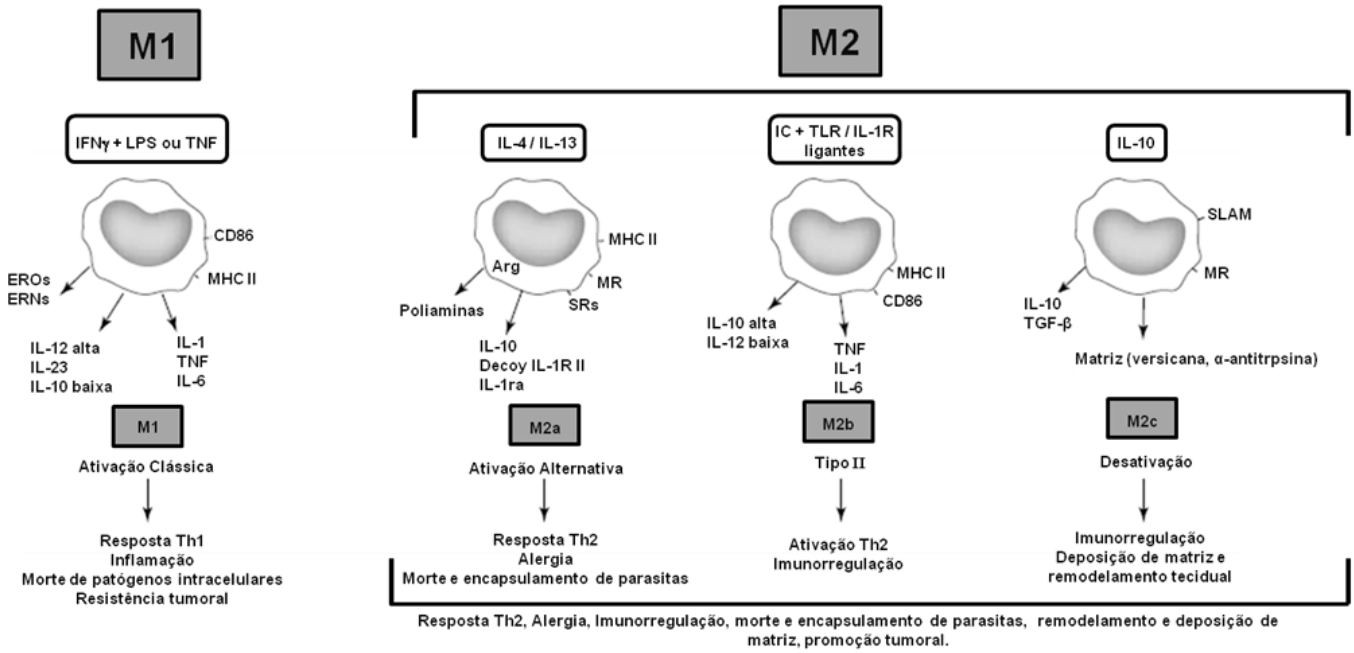


Figura 3: Indutores e principais características dos diferentes fenótipos e subfenótipos dos macrófagos. IL-1ra, antagonista do receptor de IL-1; MR, receptor de manose; SR, receptor de captura; SLAM, molécula sinalizadora e ativadora linfocítica. *Adaptado de The chemokine system in diverse forms of macrophage activation and polarization. Trends in Immunology, 2004.*

Os macrófagos são muito influenciados pelo microambiente em que se encontram, apresentando um espectro ("*spectrum")* de ativação devido à sua plasticidade. Isso denota que os macrófagos não podem ser simplesmente fenotipados como M1 ou M2. Por isso, atribuir a ele fenótipos somente de acordo com o estímulo dos linfócitos Th1 ou Th2 se torna muito simplório. Por exemplo, sabe-se, atualmente, que as células T regulatórias também modulam o fenótipo do macrófago, uma vez que essas células produzem IL-10, TGF-β e glicocorticoides, os quais não preenchem claramente o contexto de resposta Th1/Th2; no entanto, algumas características do perfil Th2 podem ser vistas nos macrófagos modulados por IL-10, TGF-β e glicocorticoides como, por exemplo, uma alta expressão do receptor de manose, indução de IL-10 pelo próprio macrófago, um aparente antagonismo do estímulo clássico (M1), com baixa produção de citocinas pró-inflamatórias e uma diminuição da produção de ERO e ERN (Martinez and Gordon 2014). Por isso, torna-se extremamente difícil caracterizar o perfil dos macrófagos em determinados microambientes, tornando

a descrição da ativação dos macrófagos controversa e confusa. Além disso, a falta de consenso sobre a definição de ativação dos macrófagos em experimentos *in vitro* e *in vivo* dificulta sua caracterização de várias maneiras, incluindo o fato de que muitos pesquisadores consideram ainda que haja apenas dois tipos de macrófagos ativados - M1 e M2. Por essas razões, renomados pesquisadores na área dos macrófagos sugeriram que os ativadores usados fossem bem definidos, e que os macrófagos fossem caracterizados de acordo com o indutor utilizado (por exemplo, M(IFNγ) para macrófagos ativados com essa molécula) (Murray, Allen et al. 2014).

Percebe-se, portanto, que há uma lacuna a ser preenchida em relação à caracterização do macrófago. Por isso, a criação de ferramentas que auxiliem na caracterização do seu perfil, tanto *in vitro*, quanto *in vivo,* faz-se necessária para a melhor compreensão do papel dessas células na fisiologia e nas patologias humanas. Ainda, seu papel chave na progressão tumoral faz com que o macrófago seja um importante alvo a ser considerado não somente pelo seu papel na modulação imunológica, mas também pela sua ação de reestabelecimento da homeostasia tissular que, no microambiente tumoral, auxilia no desenvolvimento do tumor.

3.2.1 Macrófagos Associados ao Tumor

O microambiente tumoral apresenta uma complexidade muito alta devido à presença de um grande número de células e, consequentemente, de uma grande quantidade de diferentes moléculas produzidas. Em vista disso, o microambiente que circunda o tumor não é constituído somente pelas células neoplásicas, mas também pelas células residentes no local, células hematopoiéticas, neutrófilos, mastócitos e, predominantemente, macrófagos. Estes, então, denominam-se macrófagos associados ao tumor (TAM) e, em conjunto com as demais células, produzem um microambiente único, o qual modifica as propriedades das células tumorais (Ramanathan and Jagannathan 2014). Ainda, os tumores sólidos representam um exemplo extremo de desregulação tecidual, com múltiplas características, incluindo hipóxia e altas taxas de morte celular, necessitando, assim, da ajuda direta dos macrófagos, com o intuito de reestabelecer a homeostasia tecidual. No contexto tumoral, entretanto, essa resposta, ao invés de reestabelecer a homeostasia, auxilia o tumor no seu crescimento através de programas angiogênicos, remodelamento tecidual, sobrevivência ectópica de células malignas e desenvolvimento de microambientes imunossupressores que atenuam a atividade citotóxica dos linfócitos T $CD8^+$ (Ruffell, Affara et al. 2012). No que diz respeito ao pulmão, o macrófago alveolar - que é responsável, principalmente, por remover as impurezas aspiradas pelo trato respiratório superior - também mostrou uma função ineficiente de resposta homeostática contribuindo para a promoção tumoral (Siziopikou, Harris et al. 1991; Pouniotis, Plebanski et al. 2006). A inibição de citocinas pró-inflamatórias secretadas pelos macrófagos alveolares foi demonstrada em soros com níveis elevados de IL-10.

Essa inibição favorece a proliferação e diferenciação do tumor, e isso é consistente com a supressão das funções anticâncer em estágios avançados de câncer de pulmão. Além disso, a secreção de IL-10 pelos macrófagos alveolares demonstrou inibir a produção de interleucina-2 – responsável pela proliferação das células T (Almatroodi, McDonald et al. 2014). Portanto, complexo ambiente tumoral modula fortemente os macrófagos, uma vez que estas células possuem diversidade e plasticidade funcional, sendo este ambiente, então, capaz de induzir os macrófagos a adquirirem propriedades pró ou anti-inflamatórias (Jinushi and Komohara 2015).

Sabe-se que, além dos macrófagos residentes teciduais, os monócitos circulantes são os precursores dos macrófagos recrutados para um determinado local. Em murinos, os monócitos inflamatórios $CCR2^{+}Ly6C^{+}$ são os principais precursores dos TAM, sendo recrutados dentro do tecido tumoral pela quimiocina CCL2 (Qian, Li et al. 2011). Dentro do microambiente tumoral, os monócitos são induzidos a diferenciarem em macrófagos pró-tumorais através de uma intrincada rede de moléculas solúveis, como, por exemplo, o M-CSF, o GM-CSF e citocinas imunossupressoras: IL-4, IL-10, TGF-β, etc (Sica, Saccani et al. 2000; Sinha, Clements et al. 2005; Flavell, Sanjabi et al. 2010). Além disso, após a exposição a fatores produzidos pelas células tumorais, como, por exemplo, IL-10, prostaglandina E2, CCL2 e o fator de estimulação de colônia 1 (CSF1, sigla em inglês), muitas evidências apontam que os TAM são capazes de se polarizar em macrófagos imunossupressores (Ruffell, Affara et al. 2012). Ainda, o metabolismo energético do tumor também tem a capacidade de influenciar o microambiente tumoral, visto que as células neoplásicas utilizam a via glicolítica de forma muito mais acentuada que as células não neoplásicas

(Moreno-Sánchez, Rodríguez-Enríquez et al. 2007). Nesse contexto, o ácido lático liberado pelas células tumorais promove a diferenciação de macrófagos que passam a expressar altos níveis de VEFG-A e arginase-I, os quais contribuem para o crescimento tumoral. A figura 4 apresenta as principais interelações entre as células tumorais e os TAM (Jinushi and Komohara 2015), elucidando a ativação pró-tumoral (M2) desses macrófagos.

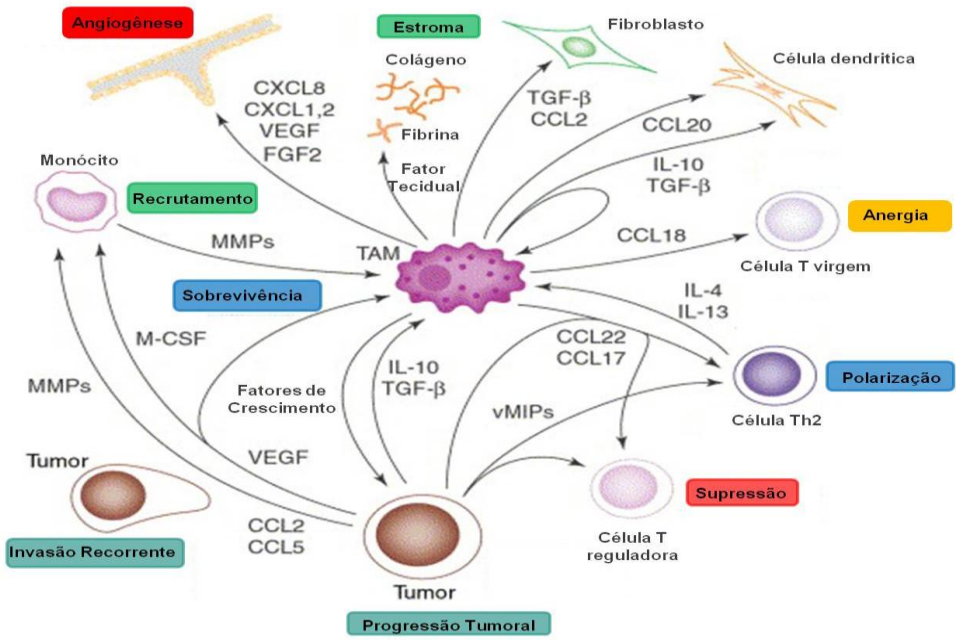


Figura 4: Principais interações entre as células tumorais e os macrófagos associados ao tumor, criando um microambiente propício à progressão tumoral. FGF2, "*fibroblast growth factor*";vMIPs: *"virus macrophage inflammatory protein". Adaptado de Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in Immunology, 2002.*

A hipótese de que os TAM adquirem um fenótipo M2-*like* ainda é bastante controversa, pois se sabe que estes apresentam subpopulações heterogêneas que possuem alta plasticidade e flexibilidade, permitindo a eles se adaptarem aos diferentes microambientes tumorais (Biswas and Mantovani 2010). De fato, Franklin e colaboradores obtiveram, de modelos de câncer de mama, um fenótipo particular para uma população de macrófagos –

CD11b(baixo)/MHCII^{+}/VCAM^{+}, com um perfil genético não compartilhado com ambos os macrófagos M1 e M2 (Franklin, Liao et al. 2014). Destarte, isso corrobora ainda mais a grande complexidade envolvida na modulação fenotípica dos macrófagos.

Os TAM podem, direta ou indiretamente, contribuir para a sobrevivência do tumor, crescimento e metástase e, por isso, estas células podem ser um alvo potencial no que diz respeito às terapias antitumorais. Possíveis estratégias que envolvem os TAM incluem a redução do número de macrófagos e/ou aumento da atividade tumoricida dessas células (Wahl and Kleinman 1998). Vários tipos de terapias antitumorais, como o uso de cisplatina, paclitaxel e radiação ionizante, promovem o recrutamento de monócitos/macrófagos para o local em que se encontra o tumor através da indução da expressão de CSF1 e interleucina 34 (DeNardo, Brennan et al. 2011), contribuindo, assim, para o aumento do número de macrófagos na região intratumoral e, consequentemente, com sua modulação pelo microambiente. Allavena e colaboradores demonstraram que a trabectidina, um agente antitumoral aprovado para determinados tipos de sarcoma, exerce resposta contra o tumor através da redução de monócitos e macrófagos. Ainda, certos tipos de agentes citotóxicos, como a 5-fluorouracila, taxanos e docetaxel, apresentam propriedades únicas por diminuir os macrófagos com fenótipo M2 e, ao mesmo tempo, aumentar a quantidade de macrófagos com fenótipo M1 na região intratumoral (Kodumudi, Woan et al. 2010; Vincent, Mignot et al. 2010; Germano, Frapolli et al. 2013). Além disso, alguns agentes quimioterápicos, como a oxaliplatina e a doxorubicina, levam a um processo conhecido como morte celular imunogênica das células tumorais em determinados cânceres, culminando, assim, com liberação de mediadores

inflamatórios (Krysko, Garg et al. 2012; Kroemer, Galluzzi et al. 2013). Então, presume-se que uma elevada morte celular imunogênica, mediada por determinadas drogas, pode apresentar um alto impacto ao restringir a capacidade pró-tumoral dos TAM. Em contrapartida, estudos mostram que algumas drogas com propriedades antitumorais polarizam os macrófagos para um fenótipo pró-tumor (Jinushi and Komohara 2015). Por exemplo, a droga *Imatinib* tem a capacidade de polarizar os macrófagos para um fenótipo M2, através da ativação do fator de transcrição C/EBP nas células tumorais que entraram em apoptose (Cavnar, Zeng et al. 2013). Além disso, um recente ensaio clínico randomizado de fase III, em pacientes com câncer colorretal, mostrou que a adição do *cetuximab* – um anticorpo monoclonal contra o receptor de EGF, que, *in vitro*, evidenciou a propriedade de modular os macrófagos para o fenótipo M2 – junto à quimioterapia resultou na diminuição da sobrevida livre de progressão quando comparado à quimioterapia sozinha (Pander, Heusinkveld et al. 2011). Ainda nesse contexto, a produção de anticorpos contra antígenos específicos podem ter efeitos modulatórios nas propriedades funcionais dos TAM. Por exemplo, o número de TAM provém um melhor valor prognóstico em pacientes com linfoma não-Hodkin que receberam terapia com anticorpo monoclonal anti-CD20 (*rituximab*) junto à quimioterapia (Taskinen, Karjalainen-Lindsberg et al. 2007). Além disso, o tratamento com anticorpos agonistas anti-CD40 aumentou a atividade antitumoral do *gemcitabine* através da infiltração de macrófagos M1 dentro do tecido tumoral, aumentando a resposta imune contra o tumor em pacientes com câncer pancreático (Beatty, Chiorean et al. 2011; Vonderheide and Glennie 2013). Por fim, tendo um enfoque mais específico em relação aos TAM, o recente sucesso clínico dos inibidores do receptor de CSF1 para

malignidades humanas pode estimular o desenvolvimento de estratégias futuras cujo alvo seja os TAM. No entanto, modular a resposta imune deve ser feita com muita precaução, visto que a inibição dos receptores do CSF1 podem causar efeitos adversos, como o surgimento de infecções oportunistas e prejuízo do reparo tecidual, visto que o CFS1 é indispensável para os macrófagos conseguirem manter a homeostasia e protegerem contra os patógenos. Por isso, torna-se necessário focar no desenvolvimento de drogas que sejam específicas para os TAM como, por exemplo, inibidores da angiotensina-II, de CCL2, de IL-13 e de prostaglandina E2, além de alvos terapêuticos objetivando a IL-6, TGF-β e metabólitos associados ao câncer - como o lactato - podem ser úteis para impedir o recrutamento dos monócitos para o ambiente tumoral, além de dificultar a polarização dos macrófagos para o fenótipo M2 (Cassetta, Cassol et al. 2011; Germano, Frapolli et al. 2013; Jinushi and Komohara 2015).

Apesar de parecer bem consolidada *in vitro* a participação dos macrófagos no desenvolvimento do câncer, ainda não há resultados consolidados em relação a essas células e o desfecho e prognóstico dos pacientes com CPNPC, possivelmente devido a grande complexidade envolvida na ativação e, consequentemente, na plasticidade dos macrófagos *in vivo*. Além disso, poucos estudos mostraram a influência de uma terapia tendo os TAM como alvo, além do impacto do tipo de morte celular causada pelos principais quimioterápicos utilizados na clínica no desfecho e prognóstico dos pacientes com CPNPC.

3.3 Morte Celular Programada

A morte celular programada constitui um evento chave no desenvolvimento do indivíduo, assim como na manutenção da homeostasia tecidual. Em 1964, foi proposto o termo "morte celular programada" para designar um tipo de morte celular que ocorre de forma não acidental (Lockshin and Williams 1964). Em 1972, Kerr, Wyllie e Currie sugeriram o termo apoptose para indicar esse tipo de morte celular (Kerr, Wyllie et al. 1972). Após o reconhecimento do processo apoptótico como um mecanismo celular fundamental, a biologia da apoptose continuou a ser investigada através da avaliação das alterações morfológicas e bioquímicas características (Wyllie, Kerr et al. 1980), a natureza das vias intracelulares (Hale, Smith et al. 1996), a complexa biologia molecular de genes e elementos efetores (Baker and Reddy 1996), a sua relação no desenvolvimento embrionário (Brill, Torchinsky et al. 1999), o seu papel na homeostase celular e o seu envolvimento na patogênese de várias doenças (Thompson 1995; Mollazadeh, Bazzaz et al. 2015) tais como doenças autoimunes, infecções parasitárias, doenças neurodegenerativas, lesões isquêmicas e o câncer (a tabela 3, em anexo, mostra, de forma resumida, as condições envolvendo a apoptose) (Raffray 1997; Anazetti and Melo 2007; Sayers 2011; Scatena 2012).

A apoptose pode ser dividida classicamente em duas vias: a via intrínseca e a via extrínseca. Na via extrínseca, os receptores de morte são membros da superfamília de genes do TNF que consistem em mais de 20 proteínas com um largo espectro de funções biológicas, incluindo a regulação da morte e sobrevivência celular, diferenciação e regulação do sistema imune (Walczak and Krammer 2000; Ashkenazi 2002). Os receptores de morte são definidos por um

domínio citoplasmático, com cerca de 80 aminoácidos, chamados de "domínio de morte", o qual apresenta um papel crucial na transmissão dos sinais de morte extracelulares para as vias de sinalização intracelulares. Os receptores de morte mais bem caracterizados incluem o CD95 (APO-1/Fas), receptor de TNF tipo 1 (TNFRI) e TRAIL-R1 e R2. Os ligantes correspondentes da superfamília do TNF compreendem, por exemplo, os ligantes de CD95 (CD95L), TNFα, linfotoxina-α – sendo estes dois últimos ligantes de TNFRI - e ligantes do TRAIL (Fulda and Debatin 2006). Em contrapartida, a via intrínseca – ou via mitocondrial – é ativada pela liberação do citocromo *c* da mitocôndria para o citosol. Uma vez liberado, o citocromo *c* interage com Apaf-1, ATP, e pró-caspase-9 para formar o apoptossomo. Este, então, cliva e ativa a caspase-9, a qual estimula a atividade das caspases-3, 6 e 7, culminando com o processo apoptótico (Adrain and Martin 2001). A permeabilidade da membrana mitocondrial ao citocromo *c* é determinada pela razão relativa de mediadores pró e anti-apoptóticos. Quando moléculas pró-apoptóticas, como BAX e/ou Bak, são translocadas a partir da membrana mitocondrial, promove-se o aumento da disponibilidade do citocromo *c* para formar o apoptossomo. As proteínas BAX e Bak alteram a permeabilidade da membrana mitocondrial permitindo, assim, a saída do citocromo *c* para o citosol e, consequentemente, a ativação das vias de sinalização apoptóticas. Pelo fato dessas proteínas terem a capacidade de se inserir em membranas, hipotetizou-se que estas poderiam inserir-se na membrana mitocondrial criando, assim, poros ou canais os quais promoveriam o extravasamento do conteúdo da matriz da mitocôndria. A ligação dos fatores pró-apoptóticos com os anti-apoptóticos – como, por exemplo, Bcl-2, Bcl-xL e Mcl-1 – neutralizam os efeitos anti-apoptóticos. Por isso, a razão relativa de mediadores pró e anti-apoptóticos determina a quantidade relativa de

citocromo *c* disponível para formar o apoptossomo (Hengartner 2000; Huerta, Goulet et al. 2007). Ainda, a ativação da via mitocondrial de morte celular também resulta da liberação de SMAC/DIABLO e Omi/HTRA-2. O papel desses fatores é o de neutralizar as ações dos inibidores de apoptose, como cIAP1, cIAP2, e XIAP (Du, Fang et al. 2000; Baldwin 2001). Por fim, a figura abaixo mostra de maneira geral as duas vias de ativação clássica da apoptose, e a comunicação que pode existir entre elas.

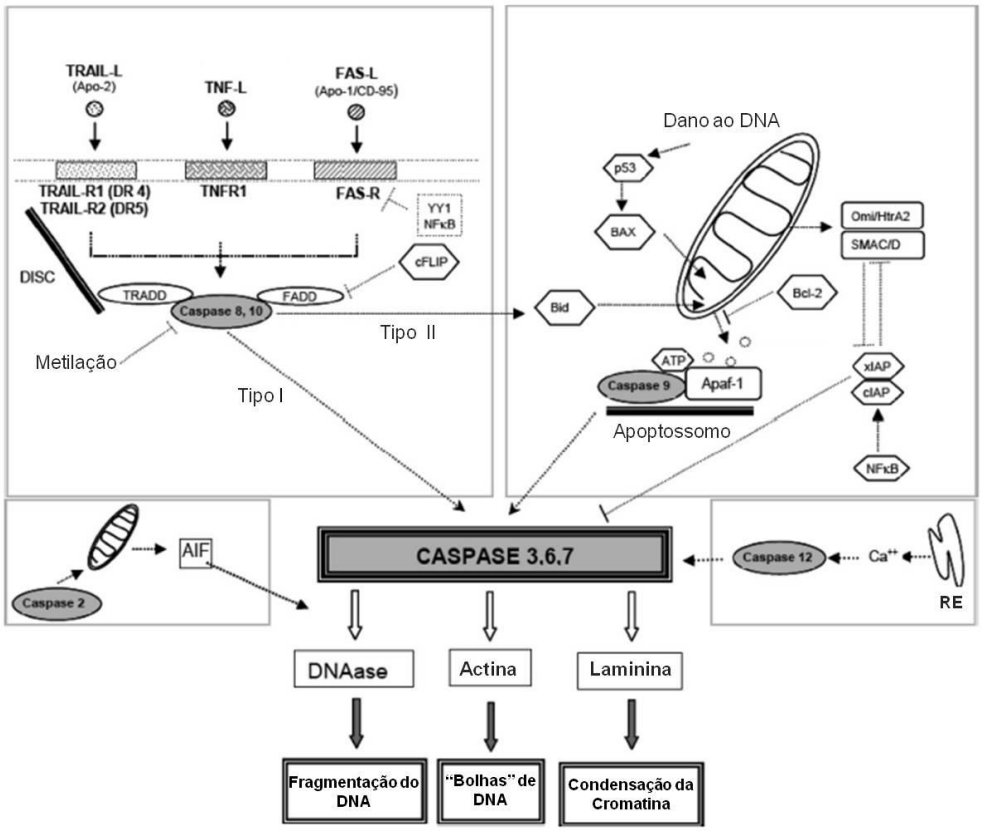
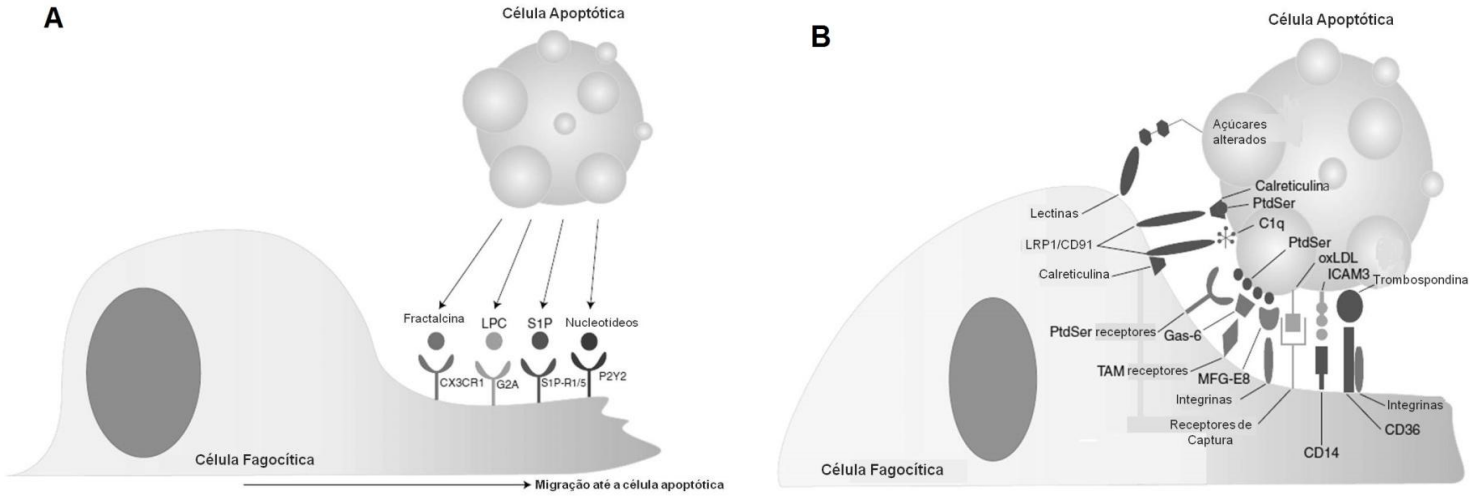


Figura 5: Visão geral das duas principais vias de indução da apoptose e suas etapas em comum. RE: retículo endoplasmático. *Adaptado de Screening and Detection of Apoptosis. Journal of Surgical Research, 2007.*

O mecanismo de morte celular por apoptose pode ser reconhecido por características morfológicas muito marcantes e coordenadas. De um modo geral, a apoptose é um fenômeno bastante rápido: ocorre uma retração da célula e perda da aderência com a matriz extracelular. As organelas celulares mantêm a sua morfologia, com exceção, em alguns casos, das mitocôndrias, que podem

apresentar ruptura da membrana externa. A cromatina sofre condensação e se concentra junto à membrana nuclear, que se mantém intacta. A seguir, a membrana celular forma prolongamentos ("*blebs*") e o núcleo forma fragmentos - a fragmentação internucleossômica do DNA, em que uma endonuclease é ativada e produz fragmentos de DNA de tamanhos variáveis, mas sempre múltiplos de 200 pares de base (Grivicich, Regner et al. 2007) - envoltos pela membrana nuclear. Os prolongamentos da membrana celular aumentam de número e tamanho e rompem, originando estruturas com conteúdo celular. Estas porções celulares envoltas pela membrana celular são denominadas corpos apoptóticos, os quais são rapidamente fagocitados pelos fagócitos. Os estágios dinâmicos que ligam as células apoptóticas aos fagócitos, nos quais se incluem os macrófagos, são divididos, por conveniência, em fases definidas de acordo com as interações e vias de sinalizações específicas ativadas. Essas fases são usualmente chamadas de três erres (3Rs): reconhecimento, resposta e remoção (Gregory and Pound 2010). A fase de reconhecimento compreende os processos migratórios dos fagócitos mononucleares em resposta às moléculas liberadas pelas células apoptóticas, como, por exemplo, o fator lipídico lisofosfatidilcolina (LPC) (Lauber, Bohn et al. 2003), a quimiocina CX_3CL1 (também conhecida como fractalcina) (Truman, Ford et al. 2008) e os nucleotídeos ATP e UTP (Elliott, Chekeni et al. 2009), assim como outras moléculas, como a S1P e o MCP-1 (Gude, Alvarez et al. 2008; Kobara, Sunagawa et al. 2008). A fase de reconhecimento também envolve uma forte ligação molecular entre a superfície da célula apoptótica - a qual contém moléculas sinalizadoras – e a superfície dos fagócitos, processo este atualmente chamado de sinapse apoptótica. Mudanças na composição e na topologia molecular da membrana plasmática das células apoptóticas envolvem a perda de sinais que evitam a fagocitose (conhecidos como

"*dont-eat me signals"*) - como CD31 e CD47 -, e a exposição de sinais pró-fagocíticos (conhecidos como "*eat-me signals"*), sendo a exposição do fosfolipídio aniônico fosfatildilserina (PS) para a face externa da membrana plasmática o sinal-chave para a ativação do processo de fagocitose. A sinapse fagocítica é fortemente formada quando há interações que envolvem os receptores de reconhecimento de padrão de fagócitos tais como CD14 (Devitt, Moffatt et al. 1998), os receptores de captura - como CD36 e integrinas $\alpha_v\beta_3$ (Savill, Hogg et al. 1992) -, os receptores de PS (BAI1, TIM-4, e estabilina -2), as moléculas ligantes de PS (MFG-E8 (lactaderina) e Gas-6 – as quais se ligam a PS na célula apoptótica e nas integrinas α_v ou Mer nos fagócitos), e, com isso, as vias de resposta à fagocitose são ativadas. Estas envolvem a ativação de duas vias de sinalização mediadas por Rac que culminam na remoção da célula apoptótica (Gregory and Pound 2011). Torna-se importante ressaltar que, sem a exposição da fosfatidilserina, a fagocitose não ocorre, mesmo que as outras moléculas da sinapse fagocítica estejam presentes (Ravichandran 2010). A figura abaixo mostra os principais sinais liberados pela



célula apoptótica e a sinapse apoptótica que ocorre entre o fagócito e ela.

Figura 6: Visão geral das moléculas sinalizadoras liberadas pelas células em apoptose (A) e as principais moléculas de reconhecimento expressas nelas e nos fagócitos (B). *Adaptado de Clearing the*

Dead: Apoptotic Cell Sensing, Recognition, Engulfment, and Digestion. Cold Spring Harbor Perspectives in Biology, 2013.

A descoberta do processo apoptótico tornou-se de grande importância para o estudo do câncer, pois este pode ser visto como o resultado de uma sucessão de mudanças genéticas durante as quais as células normais são transformadas em células malignas (Wong 2011). Por isso, os agentes antitumorais utilizados na prática clínica têm, por objetivo, induzir a regressão e remissão tumoral através de mecanismos que induzam a morte das células tumorais. Em relação a isso, sabe-se que a ocorrência da apoptose é elevada em tumores pelos tratamentos bem estabelecidos, como a radiação, quimioterapia citotóxica e remoção hormonal. De fato, com o avanço no conhecimento do controle da apoptose em nível molecular, estendeu-se o potencial da apoptose, em termos oncológicos, além de uma mera explicação para a deleção tumoral. A descoberta do gene de supressão tumoral *p53*, e que a apoptose pode ser regulada pelos produtos de certos oncogenes, revelou-se de grande importância para a melhor compreensão e manipulação da indução do processo apoptótico (Kerr, Winterford et al. 1994). Em contrapartida, sabe-se que os tumores apresentam mecanismos de evasão da morte celular, os quais apresentam um papel vital na carcinogênese. Existem muitas maneiras pelas quais as células malignas adquirem resistência ao processo apoptótico. De forma geral, os mecanismos de evasão podem ser divididos em: i) desbalanço das proteínas anti e pró-apoptóticas, ii) redução na atividade das caspases e iii) inativação das vias de sinalização de morte celular (Wong 2011). A figura 7 mostra as principais vias de evasão da apoptose pelas células tumorais.

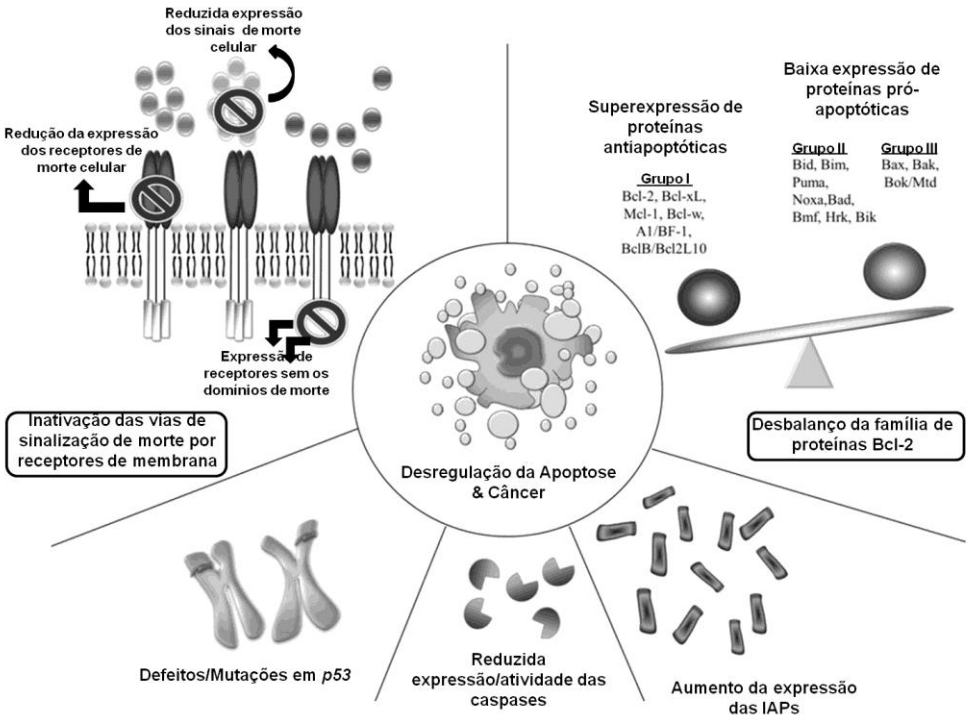


Figura 7: Principais vias de evasão apoptótica tumoral. *Adaptado de Apoptosis in cancer: from pathogenesis to treatment. Journal of Experimental & Clinical Cancer Research, 2011.*

Além da capacidade de evasão da resposta tumoral, outro aspecto deve ser considerado em relação à apoptose: sua capacidade ou não de estimular a resposta imune. A morte celular imunogênica – a qual se caracteriza pela liberação de ATP, HMGB1, estresse de retículo e exposição de calreticulina na membrana plasmática - não somente promove a retração tumoral, mas também tem a capacidade de ativar a resposta imunológica antitumor. Sabe-se que uma morte celular não imunogênica modula as células do sistema imune, principalmente os macrófagos, promovendo a liberação de fatores de reconstrução tecidual e modulando a resposta imunológica para um fenótipo anti-inflamatório (Kepp, Tesniere et al. 2009; Krysko, Garg et al. 2012; Kroemer, Galluzzi et al. 2013). Por isso, promover a morte tumoral ativando as vias de apoptose pode não se constituir um efetivo tratamento antitumoral quando não se é analisada de forma mais profunda o tipo de morte celular promovida por um agente quimioterápico. Ainda, o tumor pode regredir com o

tratamento; porém, a seleção de células resistentes e a alteração do microambiente para um contexto anti-inflamatório pode ser decisivo para promover a progressão tumoral, visto que a fagocitose promovida pelos fagócitos das células apoptóticas tem um papel chave no desenvolvimento do tumor. Dessa forma, a compreensão mais aprofundada dos mecanismos ativados pelos agentes quimioterápicos e a escolha de um tratamento baseado no tipo de morte a qual a célula é induzida podem auxiliar na melhor escolha do agente antitumoral a ser utilizado na prática clínica. Portanto, torna-se necessário o estudo mais aprofundado da modulação promovida pela morte celular apoptótica induzida não somente por drogas quimioterápicas, mas também por moléculas produzidas no nosso organismo e outras moléculas promissoras sobre as células que compõem o microambiente tumoral, principalmente os macrófagos.

4. OBJETIVOS

Verificar se diferentes indutores apoptóticos modulam, através da fagocitose das células apoptóticas, os fenótipos dos macrófagos, além de buscar novos métodos e marcadores de caracterização dessas células.

4.1 Objetivos Específicos

1. Analisar, através de meta-análise, a influência do índice apoptótico e dos macrófagos na sobrevida e desfecho dos pacientes com CPNPC.
2. Determinar a densidade de células apoptóticas (caspase-3-clivada^{+}), de macrófagos (MΦ) (CD68^{+}), de fenótipo M1 (CD86^{+}) e M2 (CD206^{+}) de MΦ por imunohistoquimica em uma coorte retrospectiva de biópsias de CPNPC e avaliar, por meio de curvas de sobrevida tipo Kaplan-Meier, se estes parâmetros influenciam na sobrevida (desfecho) destes pacientes.
3. Verificar o tipo de morte celular promovida pelo tratamento com Taurina Cloramina, Cisplatina e Ácido-3-Bromopirúvico na linhagem humana de CPNPC A549;
4. Caracterizar *in vitro* a diferenciação das linhagens humanas U-937 e THP-1 em macrófagos, além de realizar o cultivo primário (MDM), procedendo à comparação entre linhagens diferenciadas e MDM;
5. Avaliar a eficiência da fagocitose dos corpos obtidos de diferentes indutores, e o perfil de secreção de citocinas dos diferentes fenótipos dos macrófagos não expostos e expostos aos corpos derivados de células apoptóticas advindas dos diferentes tratamentos;

6. Obtenção de estratégias que sejam capazes de auxiliar na caracterização dos fenótipos dos macrófagos em diferentes contextos biológicos;

PARTE II

CAPÍTULO II

HISTOLOGY AND HISTOPATHOLOGY

Cellular and Molecular Biology

HISTOLOGY AND HISTOPATHOLOGY is an international journal, the purpose of which is to publish original works in English in histology, histopathology and cell biology; high quality is the overall consideration. Its format is the standard international size of 21 x 27.7 cm. One volume is published every year (more than 1,600 pages, approximately 100 original works and 50 reviews). Each volume consists of 12 numbers published monthly online. The printed version of the journal includes 4 books every year; each of them compiles 3 numbers previously published online. It is indexed in PubMed, ISI WoK, Biosis, CABLE, Center for Clinical Computing, Chemical Abstracts Service, Current Awareness in Biological Sciences, Current Contents, Euroscience, Excerpta Medica, Medline, Research Information Systems, Science Citation Index, etc. The price per volume, including postage by surface mail, is 650 euros (or an equivalent amount in USD). **Impact factor: 2.236**. Journal Citation Report® 2013, published by Thomson Scientific. **SJR: 1.107**, according to the information contained in Scopus® database 2013.
Printed by Jiménez-Godoy, S.A. Murcia. Spain

Nesse trabalho, buscamos compreender a importância clínica dos macrófagos e do índice apoptótico no prognóstico e na sobrevida dos pacientes com CPNPC. Para tanto, fizemos uma revisão sistemática dos dados clínicos publicados que correlacionaram o índice apoptótico e/ou a densidade dos macrófagos e suas polarizações com a sobrevida e o desfecho dos pacientes com câncer de pulmão de não pequenas células. Nossos resultados mostraram que há uma discrepância entre o papel dos macrófagos e do índice apoptótico na sobrevida e desfecho dos pacientes, pois, enquanto alguns trabalhos demonstraram um pior prognóstico associado a essas duas variáveis, outros mostraram um melhor prognóstico. Ainda, houve trabalhos que mostraram não haver correlação entre estas com o desfecho e sobrevida dos pacientes com CPNPC. Postulamos que a obtenção de resultados discrepantes na literatura pode estar associada aos marcadores usados para caracterizar o fenótipo dos macrófagos e, principalmente, aos utilizados para detectar o processo apoptótico. Por fim, sugerimos a análise combinada do índice apoptótico com todos os fatores que envolvem os macrófagos (densidade, microlocalização anatômica e fenótipos).

Histol Histopathol (2014) 29: 21-31

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

The prognostic impact of tumor-associated macrophages and intra-tumoral apoptosis in non-small cell lung cancer

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Summary. Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung malignancies. Tumor-associated macrophages (TAM) are abundant components of NSCLC. Although under certain conditions TAM can kill tumor cells, they can also act as tumor promoters secreting a variety of factors that directly stimulate tumor invasion and metastasis. TAM presents two distinct phenotypes: the classically activated (or M1) phenotype, which is highly pro-inflammatory (phagocytic and cytotoxic), and the alternatively activated (or M2) phenotype, which has anti-inflammatory and pro-tumoral properties. The polarization status of TAM depends on stimulating factors from the tumor microenvironment, and some *in vitro* evidence implies that the phagocytosis of apoptotic bodies derived from tumoral cells is a key factor in M1/M2 modulation, raising the question of whether the evaluation of the apoptotic index (AI) and macrophage polarization have a prognostic role in NSCLC patient survival. The present article systematically reviewed the published series of clinical data that correlated the AI and/or macrophage densities and polarization status (M1/M2) with the outcome of non-small cell lung cancer patients. Even though an overwhelming body of clinical data support that TAM's density, micro-anatomical localization, phenotype and intra-tumoral AI are independent predictors of survival time, no study to date has been conducted to evaluate the impact of these parameters altogether in NSCLC patient outcome. Joint analysis of these biologic factors in future studies might reveal their prognostic value in the management of NSCLC cases.

Key works: Tumor-associated macrophages, Non-small cell lung cancer, Apoptotic Index, Clinical outcome, Prognosis

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Introduction

Lung cancer is the leading cause of cancer deaths throughout the world and is divided into 2 distinct clinical categories, small cell and non-small cell lung carcinoma (NSCLC). NSCLC account for approximately 80% of all diagnosed lung cancer cases (Kawai et al., 2008; Sulpher et al., 2013). Despite numerous clinical trials of promising drugs, no major breakthrough has been made in NSCLC management in the last decades (Passaro et al., 2012; Tong and Taira, 2012). Reflecting that, the prognosis of NSCLC is still poor, with a 5-year survival probability of 49% for early stages, and less than 1% for advanced stages (Siegel et al., 2012). Unfortunately, since most cases are diagnosed with advanced pathologic (p)-stages of disease, curative pulmonary resection is no longer a therapeutic option and multimodality treatment became the indicative management of disease (Al-Shibli et al., 2009). To improve patients' prognosis, it is important to establish biological markers and processes that determine tumors' aggressiveness and predict response toward a particular therapeutic treatment (Tanaka et al., 1999; Muller et al., 2011). Ongoing studies are searching for NSCLC biomarkers that could provide the potential benefits of

prognosis, and could lead to important applications, such as drug targeting (Castro et al., 2010; Muller et al., 2011). For example, the treatment and diagnosis of NSCLC has been revolutionized by the development of targeted agents (e.g.: the FDA-approved targeted drugs *Erlotinib* and *Gefitinib* for patients harboring specific *EGFR* mutations) (Heist and Engelman, 2012). Following these new advances in therapies, a better understanding of the role of local microenvironment in tumor promotion and progression might be helpful to establish new strategies against NSCLC.

The tumor microenvironment is composed of proliferating neoplastic cells, a vascular network, the extracellular matrix produced by fibroblasts and infiltrated immune cells (Schmieder et al., 2012). Solid tumors are composed by a large mass of immune cells that could reach approximately 60% of total cells, contributing to a unique chronic inflammatory microenvironment that influences both negatively and positively the biological properties of tumor tissue (Fig. 1) (Pollard, 2004; Grivennikov et al., 2010). One of these inflammatory cells presented in high amounts in tumors are the macrophages (MΦ). Tumor-associated macrophages (TAM) have complex functions in their interaction with neoplastic cells because of their capacity to polarize into two different phenotypes (M1 or M2) (Mantovani et al., 2002). The M1 (or classically activated) phenotype of macrophages is thought to be induced *in vitro* by interferon-γ, in combination with lipopolysaccharide (LPS) and/or tumor necrosis factor (TNF)-α. M1 macrophages are associated with the expression of TNF-α, interleukin (IL)-12, IL-1,

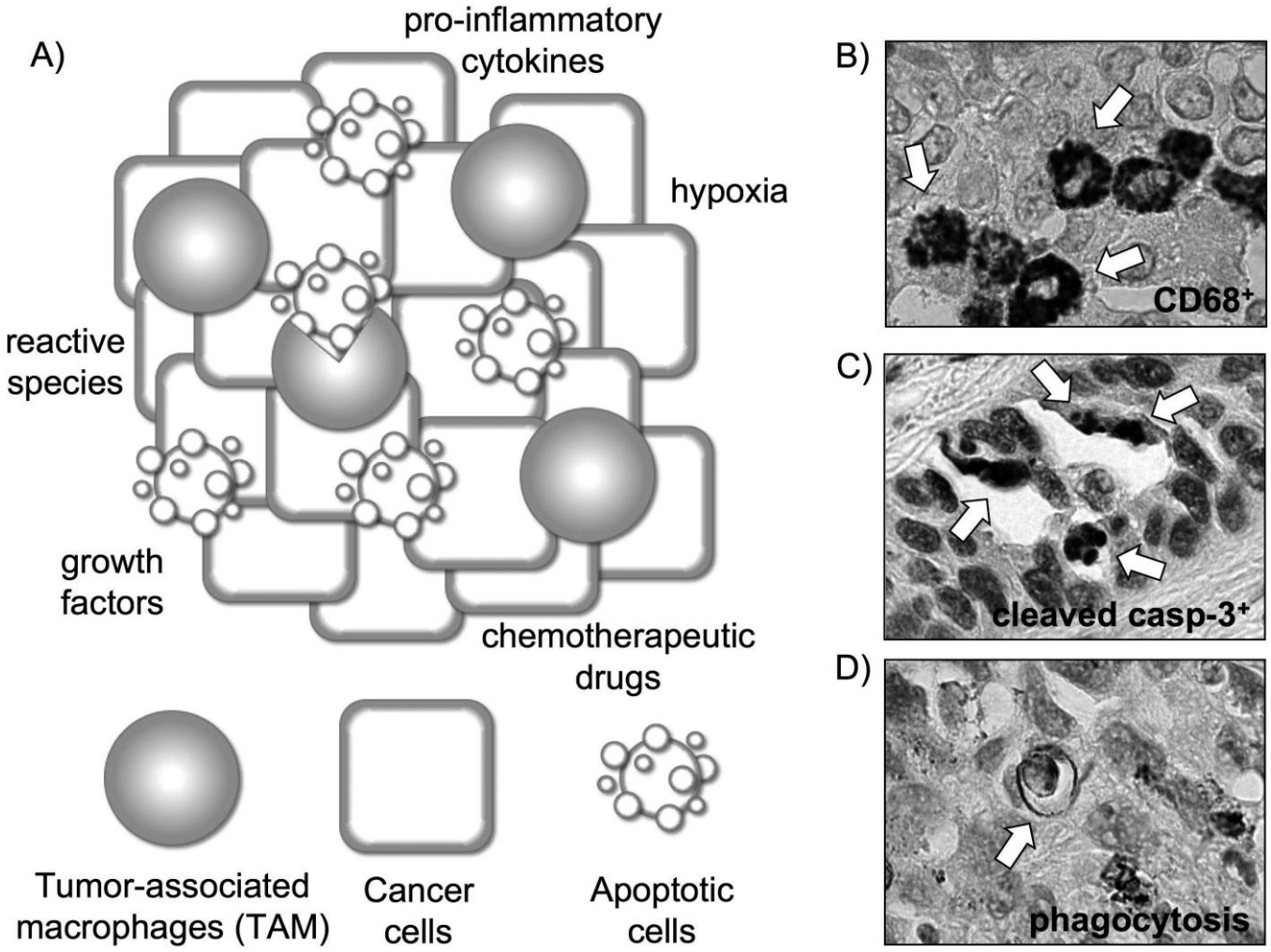

Fig. 1. Interactions between TAMs and viable/apoptotic NSCLC cells in tumor islets **(A).** The abundance of CD68-positive TAM cells (**B**, arrows), intratumoral apoptosis (identified with cleaved/active caspase-3 antibody) (**C**, arrows) and the phagocytosis of apoptotic bodies by CD68-positive TAM cells in tumor islets (**D**, arrow) are shown. Representative images were obtained by immunohistochemistry of formalin-fixed paraffin-embedded NSCLC tissues.

inducible nitric oxide synthase (iNOS), and are responsible for a pro-inflammatory and cytotoxic response against tumoral cells (Mantovani et al., 2004). On the other hand, M2 (also known as alternatively activated) macrophages are known to be modulated by IL-4, IL-13 and IL-10, and are associated with tumor progression by secreting molecules like vascular endothelial growth factor (VEGF) and transforming growth factor (TFG-ß) (Biswas et al., 2008; Ohri et al., 2009; Qian and Pollard, 2010; Ruffell et al., 2012). In addition, M2 macrophages are described to be the predominant phenotype of TAM in solid tumors (Anderson and Mosser, 2002).

Even though the role of different macrophage phenotypes in tumor progression has been extensively reviewed (Anderson and Mosser, 2002; Gordon, 2003; Sica et al., 2006; Biswas et al., 2008; Mosser and Edwards, 2008; Gordon and Martinez, 2010) and most studies suggest that TAM are associated with poor clinical prognosis, some contradictory data can be found in the literature even for the same histological type of tumors. Specifically, in lung cancer, studies demonstrated a positive, negative and inconclusive prognostic significance of TAM densities (Zhang et al., 2012). Therefore, the prognostic value of TAM for patients with lung tumors remains controversial. Indeed, several works support a dual role for macrophages in the regulation of tumor proliferation and immune control, which indicates that more studies are necessary to address the role that the local tissue microenvironment plays in determining the macrophage phenotype (Kataki et al., 2002).

Multiple characteristics of solid tumors, including hypoxia and abundant cell death, such as apoptosis or necrosis, influence macrophage functions (Fig. 1) (Ruffell et al., 2012). Immune response and chemotherapeutic agents used regularly in NSCLC management, such as cisplatin and carboplatin, induce cell death in different ways. In immune response, production of TNF-α and other pro-inflammatory cytokines induce cell death by stimulation of death receptors in the tumoral cell membrane (involved in the so-called extrinsic apoptotic pathway), and nitric oxide ($NO^{\cdot}$) and other reactive species (RS) induce programmed cell death by increasing the oxidative stress inside the cell (Weigert and Brüne, 2008). On the other hand, cisplatin and carboplatin induce DNA damage, leading the target cells to commit apoptosis by activating the mitochondrial (intrinsic) pathway (Siddik, 2003). In addition, hypoxic cores inside the tumor also elicit tumor destructive reactions, leading mainly to a necrotic type of cell death.

Typically, cells dying by apoptosis are thought to be promptly phagocytized by mechanisms that fail to incite pro-inflammatory or immune reaction (Fig. 1) (Gregory and Devitt, 2004). Indeed, signaling molecules and membrane receptor in apoptotic cells (e.g.: externalized phospholipid phosphatidylserine) are recognized by macrophages, generating a cascade of cell signals leading to the phagocytosis of apoptotic bodies. This process is believed to be responsible for a M1-to-M2 shift in macrophage phenotype, which will then secrete anti-inflammatory mediators, most notably TGF-ß but also IL-10, prostaglandin E2 (PGE2), lactoferin and VEGF (Gregory and Pound, 2011). Thus, many lines of *in vitro* data have suggested that intratumoral phagocytosis of apoptotic cells by macrophages could be to a large extent responsible for the modulation of immune response, especially the M2 polarization of TAM (Reiter et al., 1999; Weigert et al., 2007; Weigert and Brüne, 2008) . This critical event has been associated with dampening of the immune responses, leading to tumor promotion, progression, and metastasis (Sica et al., 2008). So, the macrophage polarization to the M2 phenotype appears to be a key event in tumoral progression, and it seems that phagocytosis of apoptotic cells is a very important element of this polarization.

For these reasons, the apoptotic index (AI) and TAM polarization in solid tumors could have an intrinsic relationship with tumor progression, influencing patient outcome and overall survival (Törmänen et al., 1995; Tanaka et al., 1999; Kim et al., 2008; Ohri et al., 2009; Dworakowska et al., 2009). Therefore, the major goal of this work is to review and compile the available clinical data in the literature that correlates the apoptotic index and/or macrophage densities and the polarization status (M1 or M2) with the outcome of non-small cell lung cancer patients. We aimed to evaluate the clinical relevance of these important biological processes, highlighting the clinical use of these parameters for future improvement in the management of NSCLC patients.

Macrophage polarization in non-small cell lung cancer patient prognosis: a clinical update

Many studies have been conducted trying to elucidate the role of macrophages in tumor growth and their prognostic value (Leek et al., 1996; Shimura et al., 2000). One recent study concluded that high TAM density seems to be associated with a worse overall survival in patients with gastric, urogenital and head and neck cancers, and with better overall survival in patients with colorectal cancer (Zhang et al., 2012). In lung cancer, their analysis showed conflicting results about the prognostic significance of counting TAM in tumor tissues (Zhang et al., 2012). Thus, the prognostic value of TAM quantification for patients with lung tumors remains controversial.

For this reasons, we reviewed the medical literature searching for prospective/retrospective clinical studies that evaluated not just the impact of macrophage densities (cell number), but also their stromal and/or parenchymal (tumor islets) micro-anatomical localization and polarization (M1/M2) status, with the NSCLC patient survival rate (overall survival). The characteristics of these studies, performed in formalin-fixed paraffin-embedded tissue, are summarized in Table

1 and discussed below.

Macrophage densities

Trying to elucidate the importance of macrophage density on patient survival/outcome, Arenberg and collaborators (Arenberg et al., 2000), using immunohistochemistry (IHC) detection of HAM-56+ cells as macrophage marker, performed a prospective study with 15 consecutive patients who had undergone thoracotomy for suspected primary bronchogenic carcinoma. The authors followed the patients for an average of 76 months and found that those patients who died (n=7) had significantly higher numbers of macrophages than did those who remained free of recurrence (n=8) (92.3±19.8 vs. 49.2±6.6 macrophages/x400 magnification field, respectively) (P<0.05). Corroborating these findings, Chen and collaborators (Chen et al., 2003) demonstrated that the median survival for patients with high density of TAM (≥162 macrophages/x200 magnification field, 16 months, n=18) was significantly shorter when compared to patients with low density (<162 macrophages/X200 magnification field, 45 months, n=17) (log-rank test, P=0.025). Their study was performed in a retrospective cohort of 35 patients who had undergone curative resection of early p-stage (I, II or IIIa) of NSCLC cases, using IHC of CD68+ cells as macrophage marker. The same group, in a follow-up study (Chen et al., 2005)

Table 1. Characteristics of the eligible studies that evaluated the clinical significance of macrophage (MΦ) densities, micro-anatomical localization and M1/M2 phenotypes as prognostic biomarkers in NSCLC.

Description	Population	Size	Diagnostic technique	Analytic result	P	Reference
Prospective association of MΦ density with patient outcome	Patients undergoing thoracotomy	15	IHC of $HAM56^+$	High MΦ density is negatively correlated with survival	<0.05	Arenberg et al., 2000
Retrospective association of MΦ density with patient outcome with 3 year follow-up	Patients with primary NSCLC who underwent surgery	117	IHC of $CD68^+$	No correlation on 3-years survival	NS	Toomey et al., 2003
Retrospective association of MΦ density with patient outcome	Surgically resected (Stage I-IIIa)	35	IHC of $CD68^+$	High MΦ density is negatively correlated with probability of survival	=0.024	Chen et al., 2003
Retrospective association of MΦ densities with 5-years patient survival (stromal vs. islets)	Stage I-IV	High MΦ in tumor islet and 175	IHC of $CD68^+$ stromal are a predictor of survival		<0.001	Welsh et al., 2005
Retrospective association of MΦ density with patient outcome	Stage I-IIIb	41	IHC of $CD68^+$	MΦ negatively correlated with survival	<0.05	Chen et al., 2005
Retrospective association of MΦ with the clinical outcome (stromal vs. islets)	Stage IV	199	IHC of $CD68^+$	High MΦ in islets is a predictor of survival	<0.001	Kawai et al., 2008
Retrospective association of MΦ density with patient survival (stromal vs. islets)	(Stage I-IV)	144	IHC of $CD68^+$	High MΦ density in islets is a predictor of survival	<0.001	Kim et al., 2008
Retrospective association of MΦ (M1 or M2) densities in islet or stroma with patient survival	Poor (7-months) vs. extended (92-months) survival (Stage I-IV)	40	IHC of $CD68^+$/HLA-DR^+, $iNOS^+$, $MRP8/14^+$, TNF-α^+ for M1 and $CD68^+$/$CD163^+$ for M2	High MΦ (M1) density in islets is a predictor of survival	<0.001	Ohri et al., 2009
Retrospective association of MΦ density in islet or stroma with patient survival	Surgically resected (Stage I-IIIa)	335	IHC of $CD68^+$	No correlation on 16-years patients' survival	NS	Al-Shibli et al., 2009
Retrospective association of MΦ density in islet or stroma with patient survival	(Stage I-IV)	99	IHC of $CD68^+$	High MΦ density in islets and low in stroma is a predictor of patients' survival	<0.001	Dai et al., 2010
Retrospective association of MΦ (M1 or M2) densities in islet or stroma with patient survival	Short (1-year) vs. long (5-year) survival (Stage I-IV)	100	IHC of $CD68^+$/HLA-DR^+ (M1) and $CD68^+$/$CD163^+$ (M2)	High MΦ M1 density in islets is a predictor of survival	<0.001	Ma et al., 2010
Retrospective association of M2 phenotype and patient outcome	Stage Iab-IIIa	170	IHC of $CD68^+$/$CD204^+$ for M2	High MΦ M2 density phenotype is a predictor of survival	=0.007	Ohtaki et al., 2010
Retrospective association of M1/M2 phenotypes and patient outcome	Patients undergoing lobectomy or pneumonectomy	65	IMF of $CD68^+$/$iNOS^+$ for M1 and $CD68^+$/$CDMRR^+$ for M2	High TAMs and M2 density phenotype is a predictor of survival	<0.001	Zhang et al., 2011
Prospective association of abundance and distribution of immune cells with patient outcome	Surgically resected ($T_{1-3}N_{0-2}M_0$)	65	IHC of $CD68^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, $S100^+$, $CD1a^+$	High MΦ density is a predictor of survival	=0.03	Da Costa Souza et al., 2012

MΦ, macrophages; M1, classically-activated macrophages; M2, alternatively-activated macrophages; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; IMF, immunofluorescence; NS, no significant.

confirmed that the median relapse-free survival for patients with high density of TAMs (≥163 macrophages/x200 magnification field, 7 months, n=21) was also significantly shorter than for patients with a low density of TAMs (<163 macrophages/x200 magnification field, 26 months, n=20) (log-rank test, P=0.018). Despite the small size of the cohorts (the median sample size was 31 patients), all these studies found a significantly shorter relapse-free survival of patients with high TAM density.

In contrast to these findings, the recent study of da Costa Souza (2012) showed that the 5-year survival rate in patients with high macrophage density is correlated with high probability of survival (median of 4.5% of cells as cut-off point, estimated median survival of 76 vs. 30 months for the high-risk group) (log-rank test, P=0.02). They performed a prospective study with 65 patients using IHC of CD68+ cells. Moreover, Toomey and collaborators (Toomey et al., 2002) used a retrospective cohort of 117 patients who had undergone curative surgery and showed that the density of CD68+ cells was not associated with a 3-year survival time (Cox-regression analysis, P=0.24). They found an average of 10-50 macrophages/x400 magnification field (of around 500 tumor cells in total). These studies reinforced previous inconsistent findings (Zhang et al., 2012), and suggested that other biological/clinical variables should be included to strengthen the prognostic role of TAM in NSCLC.

Stromal vs. tumor islets macrophages' densities

One important biological feature that should be considered is the micro-anatomical localization of macrophages. Solid tumors are known to have distinct but interdependent compartments: the parenchyma of neoplastic cells (tumor islets, or nests) and the stroma that, in many tumors, is separated by a basal lamina (Tlsty and Coussens, 2006). The stroma comprises nonmalignant supporting tissue, including connective tissue, blood vessels, and inflammatory cells. Solid tumors require the stroma to grow beyond a minimal size of 1 to 2 mm, since it provides the vascular supply that tumors require for obtaining nutrients, gas exchange and waste disposal. This compartment may also limit the influx of inflammatory cells or may limit the egress of tumor cells (invasion) (Elgert et al., 1998; Welsh et al., 2005). Hence, the evaluation of TAM distribution between compartments could have important prognostic implications.

Embracing this idea, Welsh and collaborators (Welsh et al., 2005) evaluated in a retrospective cohort of 175 patients with NSCLC who had undergone resection with curative intent (p-stages I, II and IIIa), the parenchymal (tumor islets) and stromal densities of macrophages (Table 1). Stromal $CD68^+$ macrophages presented a median of 174 cells/mm^2 (ranging from 5 to 3,310) and tumor islets a median of 131 cells/mm^2 (ranging from 1 to 891). They also determined the islet/stromal ratio of MΦ number in tumor samples. Kaplan-Meier survival curve and Cox-regression analysis showed that high macrophage numbers in islets (>174 cells/mm^2, 2,244 days vs. <174 cells/mm^2, 334 days, P<0.001) and high islet/stroma ratio (2,147 vs. 312 days, P<0.001) emerged as independent favorable prognostic indicators, whereas, in IIIa p-stage, high macrophage density in stroma reflected a poor prognosis (224 vs. 936 days, P=0.022). In accordance with these results, Kim and collaborators, using a retrospective study of NSCLC 144 patients who had undergone curative intended surgery, found that patients with high tumor islet macrophage density survived longer compared to patients with low tumor islet macrophage density (P=0.0002), concluding that a high number of tumor islet macrophages was an independent favorable prognostic factor for patients with resected NSCLC (Kim et al., 2008). The same results were obtained in a retrospective study performed by Dai and collaborators, with IHC detection of CD68+ cells in 99 NSCLC patients with different p-stages (I-IV). They confirmed the favorable prognosis represented by high islet/stromal macrophage ratio and the unfavorable prognosis of patient with a high density of macrophages in the stroma (Dai et al., 2010). Furthermore, the study of Kawai and collaborators performed with a retrospective cohort of 199 NSCLC patients in advanced stage IV of disease, also showed that a high islets/stromal macrophage ratio was associated with better prognosis (Kawai et al., 2008). They also found that high stromal macrophage density was associated with poor prognosis. In contrast to these previous studies, Al-Shibli, using IHC of CD68+ in a retrospective cohort of 335 patients, showed that macrophage density/micro-anatomical localization did not have any statistical correlations with patient disease specific survival (Al-Shibli et al., 2009). This was the first work that showed no relationship between macrophage density/micro-anatomical localization and patient outcome/survival in NSCLC.

The studies addressed until now have some contradictory results about macrophage micro-anatomical localization and NSCLC patient outcome/survival. Nevertheless, they overall suggest that high macrophage densities in islets and stroma could be a prognostic marker in NSCLC. Therefore, the micro-anatomical localization analysis of TAMs could be a new approach in clinical predicting studies.

Macrophage phenotypes

The previous works mentioned showed that TAMs have an intrinsic relationship with NSCLC patient overall survival time in according with their density *per se* or their micro-anatomical localization. Beyond these features, macrophage polarization is suggested as a key factor in tumor progression and metastasis (Mantovani et al., 2002; Lewis and Pollard, 2006; Sica et al., 2006; Allavena et al., 2008; Qian and Pollard, 2010). It is well known that macrophages can polarize to a M1 (classic)

or M2 (alternative) phenotype. Then, the puzzle of how macrophage density in different micro-anatomical localization could be associated with better or worse prognosis in NSCLC may be solved by the investigation of the M1/M2 polarization status of TAMs.

In 2009, Ohri and collaborators, based on the marked survival advantage of NSCLC patients presenting a high number of macrophages in their tumor islets, identified CD68+ macrophages expressing M1 (iNOS, MRP8/14, TNF-α, and HLA-DR) or M2 (CD163 and VEGF) double-staining markers in islets and stroma of surgically resected tumors from 20 patients with extended survival (ES) (median 92.7 months) and 20 with poor survival (PS) (median 7.7 months) (Ohri et al., 2009). Their study also confirmed that a high macrophage count in tumor islets was correlated with a better overall survival (median of 33.95 cells/mm^2 in ES group vs. 4.02 cells/mm^2 in PS group, P<0.001) but, more than that, the data demonstrated the relevance of macrophage phenotype in NSCLC patients' overall survival. Interestingly, more than 70% of islet TAMs were positive for M1 markers in the ES group. They concluded that the survival advantage conferred by macrophages infiltrated in tumor islets was related to their cytotoxic (M1) antitumor activity. Moreover, Ma and collaborators conducted a study to determine whether the micro-localization of M1/M2 macrophages densities are associated with NSCLC patient overall survival time (Ma et al., 2010). Their retrospective cohort was stratified into patients with an average of 1-year survival (short survival group) (n=50) and patients with an average of 5-year survival (long survival group) (n=50), and tumor biopsies were double-stained IHC for M1 (CD68+/HLA-DR+ cells) and M2 (CD68+/CD163+ cells). Even though the overall analysis of Ma's study indicates that approximately 70% of TAMs are M2 macrophages, the long survival group also presented a significantly higher M1 macrophage count in the NSCLC tumor islets and stroma as compared to short survival group (approximately 70 cells/mm^2 vs. 17 cells/mm^2, P<0.001), thus establishing it as an independent predictor of patient survival time. More interestingly, the M1/M2 macrophage ratio in the tumor islets and in stroma from long survivors was 9- and 2-fold higher when compared to short survival patients, respectively. In this context, evaluating specifically the prognostic role of stromal M2 macrophage density, Ohtaki and collaborators found a significant association between the number of CD204+ macrophages and poor outcome (Ohtaki et al., 2010). Their study was based on recent evidence that demonstrated high expression of CD204 in M2 macrophages (Komohara et al., 2008; Kawamura et al., 2009; Ohtaki et al., 2010). They used a retrospective cohort of 170 patients with lung adenocarcinoma who had undergone surgery with curative intent (I-IIIa stages). Moreover, they also evaluated the expression level of several cytokines and found an association between IL-10 and monocyte chemoattractant protein-1 (MCP-1/CCL2), both molecules involved with the tumor-promoting phenotype of TAMs, with the number of CD204+ cells. The authors suggested that MCP-1 and IL-10 derived from tumor cells or stromal cells induce differentiation, accumulation, and migration of M2 macrophages into lung cancer tissue, outlining the macrophages that promote tumor progression (Ohtaki et al., 2010).

With similar intent, Zhang and collaborators (2011) used immunofluorescence staining determination of CD68+ cells, macrophage mannose receptor-positive (MRR+, as a specific M2 marker) and inducible nitric oxide synthase-positive (iNOS+, as a specific M1 marker) cells in tumor biopsies from a retrospective cohort of 65 patients with lung adenocarcinoma who had undergone either lobectomy or pneumoctomy. Kaplan-Meier curves showed that high TAM counts (>102 vs. ≤102 cells per high magnification field, P<0.001) and high M2 phenotype (>82 vs. ≤82 cells per high magnification field, P<0.001) were associated with poor survival. In the lung adeno-carcinomas sample, they showed that an overall percentage of 79% (±16.27%) were of M2 TAMs (representing a hazard ratio of 4.28, P=0.038). Moreover, total macrophage count and M2 polarized TAM numbers were significantly associated with p-TNM staging: high number and M2 TAMs were associated with advanced stages (III and IV) when compared with initial stages (I and II). Furthermore, they analyzed the cytokine profile of the lung adenocarcinoma compared to a benign lung lesion and found that lung adenocarcinoma had a high expression of IL-4 and IL-10 (anti-inflammatory cytokines), and low expression of interferon-gamma and IL-12 (pro-inflammatory cytokines), suggesting that during tumor progression the M1 macrophages could be shifting to an alternative phenotype in response to micro-environmental changes.

Taking into account all the clinical-pathological information available in the abovementioned studies (Table 1), it became clear that tumor-associated macrophages have pro-tumorigenic as well as anti-tumorigenic contributions in lung tumors. Furthermore, our meta-data analysis suggests that the joint analysis, including the discrimination between macrophage count in tumor islets and/or stroma, in combination with the M1/M2 polarization status, is a more useful clinical information for the oncologist and an important step towards predicting NSCLC patient outcome. Differences in these biological parameters could be responsible for the conflicting results described in previous reports. Altogether, these data suggest that TAM influence on patients' prognosis depends on the balance between M1/M2 phenotypes in tumor islets (e.g.: high M1 density in tumor islets in early clinical stages I-IIIa represents favorable prognosis, in spite of high M2 density in tumor islets in advanced stages IIIb-IV, which reflects an unfavorable outcome) (Table 1), implying that several components and factors of the micro-environment of the tumor tissue (cytokines, growth factors, pO_2, oxidants levels, chemotherapeutic agents,

intracellular components and/or apoptotic bodies) could be key players in this modulation. Therefore, due to their intrinsic relationship with NSCLC progression and patient outcome/survival, the evaluation of TAMs has the potential to be incorporated in clinics as a prognostic biomarker in non-small cell lung cancer cases. At this point, we are going to explore the clinical available data addressing the role of intra-tumoral apoptosis (as percentages of apoptotic cells and bodies among tumor cells) in TAM polarization and/or in the prediction of NSCLC patient survival time.

Apoptosis in the tumor milieu: A major factor for TAM polarization and unfavorable outcome in NSCLC?

In the human lung, due to the intense cell death caused by acute injury (such as cigarette smoking), or within the pro-inflammatory micro-environment where solid lung tumors have developed (with chemotherapeutic agents and hypoxic cores), the initial responses to unwanted/damaged cells involves the recruitment of professional phagocytes such as macrophage cells (Savill, 1997; Serhan and Savill, 2005). Macrophages are pivotal players in the removal of dead cells (Savill, 1997; Serhan and Savill, 2005), preventing the secondary inflammatory responses mediated by the release of intracellular components (Hodge et al., 2002). The histologic determination of the amount of apoptosis in tumor islets has been established as a key event in the balance between cell proliferation/cell death processes during tumor progression (Gonzalez et al., 2001; Mantovani et al., 2002; Siddik, 2003), being useful information for predicting response to chemotherapy (Lankelma et al., 1999; Moertel et al., 1992). More importantly, as explored in the introductory section, several in vitro experiments suggest that secreted compounds or integral receptors and molecules in the surface of apoptotic cells can trigger the modulation from anti- to a pro-tumoral phenotype of innate immune cells (Gregory and Devitt, 2004; Weigert et al., 2007; Biswas et al., 2008; Gregory and Pound, 2011). Moreover, the molecular pathways of apoptosis are controlled by genes that either promote (e.g.: Bax, Fas, Bid) or inhibit (e.g.: Survivin, Bcl-xL, Bcl-2) the activation of the caspase cascade, whose levels have already been investigated and reported to be correlated with better or worse patient outcome (Estrov et al., 1998; Volm and Koomägi, 2000; Singhal et al., 2005). Despite all these implications of apoptosis and cancer described above, the significance of apoptosis in NSCLC as a biologic marker, especially as a prognostic factor, remains controversial. In this section, we search the literature focusing specifically on the available medical data that has evaluated the role of apoptotic index (percentage of apoptotic cells or apoptotic bodies per 100 viable tumor cells) in patient outcome (Table 2). We did not aim to evaluate the prognostic role of pro- or anti-apoptotic genes/protein levels in NSCLC tumor tissues, since this biological information could mainly reflect the resistance/sensitivity of tumor cells to undergo apoptosis.

Trying to associate whether apoptosis has a relationship with patient outcome in non-small cell lung

Table 2. Characteristics of the eligible studies that evaluated the clinical significance of the Apoptotic Index (AI) as a prognostic biomarker in NSCLC.

Description	Population	Size	Diagnostic technique	Analytic result	P	Reference
Retrospective association of basal AI (≤1.5% vs. >1.5%) with patient outcome	Surgically resected (Stage I-IIIa)	75	TUNEL$^+$ cells	High apoptotic index correlated with shortened survival	<0.01	Törmänen et al., 1995
Retrospective association of AI and patient survival	Surgically resected (Stage I-III)	178	TUNEL$^+$ cells	No correlation with patient survival	NS	Stammler and Volm, 1996
Retrospective association of basal AI with patient outcome (<1.0 vs. >1.0% of median)	Surgically resected (Stage I-IIIa)	173	Morphological analysis of H&E slides	No correlation on 5-years overall survival	NS	Komaki et al., 1996
Retrospective association of AI ranges (<0.5%; 0.5-1.1%; 1.1-2.5%; ≥2.5%) with patient outcome	Surgically resected (Stage I-IIIa)	236	TUNEL$^+$ cells with H&E confirmation	High apoptotic index is a predictor of survival	=0.003	Tanaka et al., 1999
Retrospective association of AI in squamous cell carcinoma with patient survival	Patients with squamous cell carcinoma	134	Morphological analysis of H&E or anti-ASP	High apoptotic index correlated with shortened survival	=0.036	Gosh et al., 2001
Retrospective association of basal AI with patient outcome (<1.4 vs. ≥1.4%)	Surgically resected (Stage I-IIIa)	50	TUNEL$^+$ cells with H&E confirmation	High apoptotic index correlated with shortened survival	=0.03	Dworakowska et al., 2005
Retrospective association of basal AI with patient outcome (median of 0.8% vs. 25th and 75th percentile)	Surgically resected (Stage I-IV)	170	TUNEL$^+$ cells with H&E confirmation	No correlation on 5-years survival	NS	Dworakowska et al., 2009

AI, apoptosis index; NSCLC, non-small cell lung cancer; H&E, Hematoxylin and eosin-stain; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling assay; anti-ASP, antibody against apoptosis-specific protein; SQC, squamous cell carcinoma; AdC, adenocarcinoma; LCC, large cell carcinoma; NS, no significance.

carcinomas, Törmänen and collaborators evaluated, in a retrospective cohort of 75 patients, the apoptotic index (AI) using terminal deoxynucleotidyl transferase dUTP nick end labeling assay-positive (TUNEL+) cells (Törmänen et al., 1995). Their data showed that patients with high AI had a shortened survival as compared to patients with low AI (>1.5% cells vs. ≤1.5% cells; log rank test of P<0.01). Furthermore, in a multivariate analysis, enhanced apoptosis showed a 1.9-fold risk for shortened survival (95% CI, 1.04-3.60; P<0.05). Interestingly, the percentage of apoptotic cells in lung carcinoma was significantly higher in poorly differentiated (grade III) carcinomas than in low-grade carcinomas (grades I-II) (P<0.02), as described for others types of tumors (Hirvikoski et al., 1999; Yamasaki et al., 1997; Lipponen et al., 1994), although no association with the p-TNM status was observed. In contrast, the study from Stammler and Volm found no relationship between apoptotic index (according with a median value) and patient survival in NSCLC (P=0.22) (Stammler and Volm, 1996). Their study was performed with a retrospective cohort of 176 patients, using TUNEL+ cells to evaluate apoptotic index (AI=apoptotic cells/viable tumor cells x 100). The apoptotic index was in the range of 0.0 to 1.7% for all cases (mean value ± standard deviation: 0.37±0.29; median: 0.25) (Stammler and Volm, 1996).

Komaki et al. (1996), in a retrospective cohort of 173 patients, evaluated the apoptotic index (AI ranged from 0.2% to 2.8%, with a median of 1.0%), using haematoxylin and eosin (H&E) staining to score apoptotic cells as having characteristic heavily condensed aberrant nuclei with homogeneous dark basophilia. Even though the overall 5-year survival was not statistically different between patients in regard to high or low apoptotic index (Table 2), a high level of apoptosis was associated with the worst survival of patients with adenocarcinomas (AdC) and large cell carcinomas (LCC) (P<0.001). Additionally, patients with high apoptosis showed significantly better 5-year overall (P=0.008) survival in the squamous cell carcinomas (SQC) group. Multivariate analysis showed that apoptosis was a significant predictor of 5-year distant metastasis (P=0.01). Based on these result, the authors suggested that this observation may have an effect on the treatment selection for the subset of Nl NSCLC patients.

In the article of Tanaka and collaborators, detailed examination of AI in lung cancer tissue identified two borderline values that determined postoperative prognosis, establishing the biologic and clinical significance of AI in NSCLC (Tanaka et al., 1999). In a retrospective cohort of 236 consecutive patients with pathologic (p)-stage I to IIIa NSCLC, who underwent complete tumor resection and mediastinal lymph node dissection without any preoperative therapy, AI was evaluated by TUNEL+ cells (with haematoxylin counterstaining) and expressed as number of apoptotic cells/1000 cells. The mean AI found for all 236 patients studied (adjusted for % of viable tumor cells) was 1.88±0.14%, with a median of 1.1%. No significant correlation was observed between AI and sex, performance status (PS), histologic type, or p-stage. All patients were divided into four AI groups using the 25^{th}, 50^{th}, and 75^{th} percentile values (0.5, 1.1, and 2.5%, respectively) (AI<0.5%, 0.5≤AI<1.1%, 1.1≤AI<2.5%, AI≥2.5%), and the 5-year survival rate was 74.7%, 51.6%, 57.8% and 83.2%, respectively. Their findings demonstrated that the prognosis of these moderate AI groups (0.5≤AI<1.1%, 1.1≤AI<2.5%) was significantly worse compared with low AI group (<0.5%). Interestingly, patients with the highest AI (≥2.5%) had the most favorable prognosis. AI proved to be an independent prognostic factor in NSCLC.

Ghosh et al. (2001), in a retrospective cohort of 134 patients with squamous cell carcinoma, used haematoxylin/eosin staining and apoptosis specific protein (ASP) immune quantification to evaluate apoptotic index (AI=number of apoptotic cells/10.000 malignant cells). The value of the AIs obtained by H&E staining for all 134 cases (adjusted for % of viable tumor cells) ranged from 0.024 to 1.455%, with a mean of 0.302 (SD, 0.24; median, 0.22). In all cases the count obtained by anti-ASP staining was higher than that obtained by H&E staining, but the two sets of AIs correlated very strongly (R^2=0.9839; P<0.001). Patients were grouped into high and low apoptosis groups based on the AI of the tumor (>0.5% was regarded as high). Overall analysis of their series of SCCs of the lung showed patients whose tumors had a low AI surviving longer. The mean survival of patients was 109 and 172 weeks, respectively (P=0.036). They concluded that AI, as measured by histological techniques, could be used as a prognostic guide in squamous lung cancer cases. In addition, Dworakowska et al. (2005), in a pilot study performed with a retrospective cohort of 50 patients, also assessed the prognostic relevance of apoptotic index (using TUNEL-stained cells with serial H&E counterstained sections) in non-small cell lung cancer patients. The mean and median AI (adjusted for % of viable tumor cells) calculated for all 50 patients was 1.4% and 0.9%, respectively. Median survival for patients with lower (<1.4%) and higher (≥1.4%) AI was 43 months and 22 months, respectively, with a 5-year survival probability of 60 and 25%, respectively (P=0.03). Multivariate analysis showed that the only variable associated with shortened overall and disease-free survival was AI (P=0.03, HR=2.9, 95% CI 1.95-3.86), concluding that AI is a major influence in NSCLC survival. The most important finding of Dworakowskas' study is the negative prognostic impact of high AI in NSCLC patients.

Finally, attempting to verify the robustness of their previous findings, Dworakoska and collaborators (Dworakowska et al., 2009) re-evaluated the prognostic role of AI in a larger group of 170 NSCLC cases. The apoptotic index (AI) grading was expressed as the number of TUNEL+ cells (with haematoxylin/eosin counterstain), comparing the 25th, 50th and 75th

percentile groups (mean AI in 168 positive cases was 1.2±1.0 SD, with median, 25th and 75th percentile of 0.8, 0.5 and 1.4%, respectively) (AI<0.5%, 0.5≤AI<0.8%, 0.8≤AI<1.4%, AI≥1.4%) (values adjusted for % of viable tumor cells). Different from what was obtained in their pilot study, no association between the apoptotic index and patient 5-year/overall survival in NSCLC was found.

So far, based on the presence of positive (Tanaka et al., 1999), negative (Törmänen et al., 1995; Tanaka et al., 1999; Ghosh et al., 2001; Dworakowska et al., 2005) and non-significant (Komaki et al., 1996; Stammler and Volm, 1996; Dworakowska et al., 2009) studies addressing the prognostic significance of apoptotic index in non-small cell lung cancer, the clinical association of the apoptotic index with patient outcome/survival in NSCLC is still controversial. It is important to emphasize that apoptosis appears to be a rare event in routine histological tissue sections because the prompt phagocytic clearance mechanism effectively narrows the window of observational opportunity (Kerr et al., 1972). However, there is a strong association between high apoptotic index (AI values greater that 1.16±0.6%) and a lower overall survival. Quantitative aggregation of the survival results demonstrated that the mean overall survival of patients with low AI compared to high AI was 35.09±10.0 vs. 23.01±6.3 months (P<0.05) and a 5-year overall survival probability of 49.75% vs. 31.02% (P=0.027), respectively. Median sample size for the abovementioned studies (n=7) was 144±64 patients (range=50-236). These studies reinforce that joint analysis of several factors, such as proliferative index, metastasis, or TAM densities in tumor islets, and AI, might provide additional prognostic information in NSCLC patients. Therefore, the apoptotic index has potential to be a biomarker in NSCLC.

Conclusion

Much effort has been made to try to find and establish useful biomarkers in lung cancer that could guide physicians to decide the best treatment for patients. Taking into account all potential bias that exists between different studies evaluated here, our meta-data analysis showed that macrophage density, their micro-anatomical localization and phenotype, and the apoptotic index are all effective biological parameters to predict patient survival, independently. However, no study has addressed the prognostic role of these biological variables in combination. Several authors (Mantovani et al., 2002, 2004; Biswas et al., 2008; Qian and Pollard, 2010; Ruffell et al., 2012) already showed the intrinsic relationship between macrophages and tumor development and progression, probably because of their capability to shift from a pro-inflammatory and anti-tumoral (M1) to anti-inflammatory and pro-tumoral (M2) phenotype. An interesting observation is that TAMs in tumor islets seem to change their polarization status from M1 to M2 during tumor promotion (early to advanced p-stages). The histologic determination of the amount of apoptosis in tumor islets has already been established as a useful clinical parameter correlated with tumor cell proliferation (Siddik, 2003; Gonzalez et al., 2001; Mantovani et al., 2002), and to predict the response to chemotherapeutic regimen (Moertel et al., 1992; Lankelma et al., 1999). Since the process of phagocytosis of apoptotic bodies derived from dying tumor cells is a key factor in shifting macrophages' phenotype in the tumor milieu, future studies should be designed to address the prognostic impact of TAMs (densities, micro-anatomical localization and phenotype) in combination with the intra-tumoral apoptosis in non-small cell lung cancer.

Acknowledgements. The Brazilians funds MCT/CNPq Universal (470306/2011-4), PRONEX/FAPERGS (1000274), PqG/FAPERGS (2414-2551/12-8), PRONEN/FAPERGS (1120325) and MCT/CNPq INCT-TM (573671/2008-7).

Disclosure. The authors have stated that they have no conflicts of interest.

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Accepted August 9, 2013

CAPÍTULO III

ARTIGO A SER SUBMETIDO NO PERIÓDICO CLINICAL CANCER RESEARCH

Clinical Cancer Research

About *Clinical Cancer Research*

***Clinical Cancer Research (CCR)* (Print ISSN: 1078-0432; Online ISSN: 1557-3265) is published twice monthly, one volume/year, by the American Association for Cancer Research, Inc. (AACR), 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404.Phone: (215) 440-9300 or Toll Free: (866) 423-3965; Fax: (267) 646-0590; E-mail:pubs@aacr.org.**

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Nesse trabalho buscamos compreender o efeito da fagocitose de corpos de células apoptóticas de CPNPC advindos de diferentes indutores na modulação dos fenótipos dos macrófagos. Para tanto, caracterizamos os fenótipos macrófago-*like* de duas linhagens celulares humanas – U937 e THP-1 -, tanto no que diz respeito à diferenciação (parâmetros como morfologia, proliferação e produção de espécies reativas), quanto à polarização dessas células com os indutores IFNγ, LPS e IL-4 (parâmetros como viabilidade, produção de espécies reativas, liberação de citocinas e expressão gênica) Ainda, caracterizamos o perfil de liberação de citocinas e de expressão gênica de macrófagos derivados de monócitos humanos (MDM). Para obtermos os corpos apoptóticos advindos de diferentes indutores, procedemos ao tratamento da linhagem humana de CPNPC A549 com Cisplatina, Taurina Cloramina e Ácido-3-Bromopirúvico. Em seguida, analisamos a eficiência da fagocitose desses corpos e avaliamos a capacidade de modulação da fagocitose destes no fenótipo dos macrófagos sem ativação, ativados com IFNγ + LPS ou IL-4. Nossos resultados mostraram que o comportamento das linhagens celulares diferenciadas é muito semelhante aos MDM, excetuando-se a eficiência da fagocitose. Por fim, mostramos que existem diferenças imunomodulatórias da fagocitose de corpos apoptóticos provindos de diferentes indutores nos fenótipos dos macrófagos.

Intratumoral apoptosis and macrophages have prognostic impact in NSCLC and Apoptotic Bodies from diverse inducers modulates differently the polarization status of macrophages

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ABSTRACT

Non Small Cell Lung Cancer (NSCLC) is the most cause of death in worldwide. The understanding of their microenvironment is extremely important to find new agents that could kill effectively NSCLC cells. Macrophage is largely associated with tumor prognosis. We used MDM, THP-1 and U-937 cell lines to evaluate the influence of different apoptotic inducers in macrophage modulation phenotype. Moreover, we analyzed the prognostic impact of apoptosis and macropahges in a 40 patients cohort in NSCLC innitial stages I and II, we analyzed the correlation of macrophage density, microanatomical localization, phenotype, and apoptosis in patients' survival time. Moreover, we analyzed the influence of apoptotic bodies in macrophage modulation. Our results showed that $CD68^+$ total and in tumoral stroma were associated with better prognosis. Moreover, high apoptosis was correlated with worse prognosis. On the other hand, $CD206^+$ cells (total, in stroma and parenchyma) and $CD68^+$ in parenchyma had no association with patients' outcome. Also, apoptosis has impact in patients' prognosis. Moreover, we observed that apoptotic bodies obtained from different inducers modulates differently the macrophage polarization status.

Keywords: Apoptosis, Macrophages Polarization, Non Small Cell Lung Cancer, Patients' Outcome

INTRODUCTION

Lung cancer is the most frequently diagnosed cancer and the most common cause of cancer mortality worldwide (Jemal, Bray et al. 2011; Siegel, Ma et al. 2014). Nearly 85% of lung cancer cases are non-small cell lung cancer (NSCLC) for which the predicted 5-year survival rate is 15% (Chen, Fillmore et al. 2014). NSCLC is currently defined by pathological characteristics and the two predominant histological phenotypes are adenocarcinoma (ADC; ~50% of cases) and squamous cell carcinoma (SCC; ~40% of cases) (Langer, Besse et al. 2010). Long-term survival is most likely when disease is early diagnosed, but this is extremely rare, due to inaccurate diagnostic methods, and its capacity to generate early metastasis within the lungs and then to distant organs and because NSCLC is a silent disease(Detterbeck, Mazzone et al. 2013). The advanced stages of the disease presents symptoms and have poorly prognosis (5-year survival rate of almost 2%) (Heist and Engelman 2012) and the treatment choice is ineffective in most cases (Raez, Kobina et al. 2010). In an attempt to better understand this cancer behavior, approaches and concepts from fields such as developmental biology, stem cell biology and immunology have deepened our knowledge of tumor development, cellular heterogeneity and interactions between the lung tumor and its surrounding microenvironment (Chen, Fillmore et al. 2014).

Macrophages (Mφ) are pivotal cells of the immune system that participate in pleiotropic actions (Wynn, Chawla et al. 2013). These cells participate of immune response against pathogens and tumor, with the production of oxidants, like nitric oxide ($NO^{\bullet}$) and the anion hypochlorite (OCl^{-}) from nitric oxide synthase and myeloperoxidase activities, contributing to an inflammatory context. On the other hand, macrophages are essential to tissue repair, cleaning of dead cells and modulation of immune response with the release of anti-inflammatory cytokines. Then, sub-populations of Mφ exist within a continuum of diverse interconvertible phenotypic spectrums designated in the literature for simplicity as M1 (classically activated) or M2 (alternatively activated) (Mantovani, Sica et al. 2004; Biswas, Chittezhath et al. 2012). In solid tumors, tumor associated macrophages (TAM) are an important component of immune cells that contributes strongly to compose the tumor microenvironment (Qian and Pollard 2010; Ramanathan and Jagannathan 2014). TAM are known to present a M2-like phenotype, which releases pro-tumoral molecules, like IL-10, TGF-β, angiogenic factors and molecules responsible for tumor progression and metastasis (Mantovani, Sozzani et al. 2002; Mantovani, Sica et al. 2004; Gordon and Martinez 2010). Besides their well established *in vitro* association with tumor progression, the correlation of macrophages with NSCLC patients' outcome is still controversial (Becker, Muller et al. 2013). This could be associated with the complexity of *in vivo* scenarios, like micro-anatomical localization and polarization spectrum of macrophages (Gordon and Martinez 2010; Biswas, Chittezhath et al. 2012; Becker, Muller et al. 2013). Then, a better

understand of TAM behaviors are extremely important, since macrophages can be modulated by a diverse molecules and cellular processes. For example, ingestion of apoptotic cells can result in the production of anti-inflammatory cytokines (Lauber, Blumenthal et al. 2004; Gregory and Pound 2011). Indeed, the majority of publications demonstrate that the complex process of apoptotic cell clearance actively suppresses the initiation of inflammation by releasing of anti-inflammatory cytokines and tolerogenic properties that suppress innate and adaptive anti-tumor and immune responses. In this context, apoptosis contributes to oncogenesis through recruitment and appropriate polarization of TAM that support tumor growth and evolution (Lauber, Blumenthal et al. 2004; Ravichandran and Lorenz 2007; Gregory and Pound 2011). On the other hand, some works showed that apoptosis could stimulate the immune response and inflammation depending of the stimuli (Kepp, Tesniere et al. 2009; Krysko, Garg et al. 2012; Kroemer, Galluzzi et al. 2013). In colorectal cancer the chemotherapeutic agent oxaliplatin induces cell death through apoptosis which stimulate antigen presentation and release of inflammatory cytokines majorly by dendritic cells (Kroemer, Galluzzi et al. 2013). This type of apoptosis is named immunogenic cell death, which is characterized by reticulum stress, exposition of calreticulin in the membrane of apoptotic cells/apoptotic bodies and release of HMGB1 (high mobility group box 1) and ATP (Tesniere, Apetoh et al. 2008; Tesniere, Panaretakis et al. 2008; Krysko, Garg et al. 2012; Kroemer, Galluzzi et al. 2013). Then, find molecules that stimulate this specific type of cell death is extremely important in an attempt to modulate the immune response to fight neoplasic cells, specially the macrophages.

Therefore, our main objective in this work was to verify whether different apoptotic inducers in NSCLC cancer cell line can modulate differently the macrophages' phenotypes. Moreover, we analyzed the clinical relevance of macrophages' density, micro-anatomical localization, phenotypes and apoptotic index in NSCLC patients' outcome.

MATERIAL AND METHODS

Ethics

The research programme, including studies on archival and stored materials, was approved by the Research Ethics Committee of the HCPA.

Cell Cultures and Macrophage Differentiation

PBMC from healthy individuals (n = 5) were isolated by Histopaque® gradient (d = 1.077) (Sigma Aldrich, MO, USA) according to manufacturer's instructions. Monocytes were cultured in RPMI-1640 media (Invitrogen, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen) (RPMI 10% FBS) at 37^{o}C in 5% CO_2 humidified air. For *in vitro* differentiation of monocytes derived macrophages (MDM), monocytes were incubated with RPMI (10% FBS) supplemented with Macrophage Colony Stimulating Factor (M-CSF) (50 ng/mL) (Peprotech, USA) for 7 days. For differential polarization, Mφ were

supplemented with IFNγ (20 ng/mL) (Peprotech, USA) and LPS (100 ng/mL) (Sigma-Aldrich) or IL-4 (20 ng/mL) (Peprotech, USA) for additional 24 h, respectively.

The THP-1 (human acute monocytic leukemia) and U-937 (human histiocytic lymphoma) cells lines were obtained from Rio de Janeiro Cell Bank (www.bcrj.org.br). A549 NSCLC cell line was obtained from ATCC. All cells were maintained in RPMI-1640 media as mentioned above. THP-1 and U-937 cell lines were differentiated to macrophages-like phenotype using phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), 20 nM for 72 h. PMA treated cells were polarized for additional 24 h by incubation with IFNγ (20 ng/mL) + LPS (100 ng/mL) or IL-4 (20 ng/mL) (Baek, Haas et al. 2009).

Proliferation Analysis and Reactive Species (RS) Measurement

Undifferentiated and PMA-differentiated THP-1 and U-937 cells were seeded in a 24-well plate in a density of 10^5 cells for 24, 48 and 72 hours. MTT analysis was performed to evaluate cellular proliferation rate. Undifferentiated, PMA-differentiated and activated (with IFNγ+LPS, IL-4 or IL-13) U-937 and THP-1 cells were incubated with 100 µM of DCFH-DA (Sigma-Aldrich® Co.) for 1 hour at 37°C. A kinetic measurement was made for 1 hour in a SpectraMax m2^e microplate reader (Molecular Devices®, USA) at 37°C. RS graphs were constructed based in the variation of initial and final measurement (Δ), relativized by MTT evaluation and calculated as fold increase.

Cytometric Bead Array (CBA)

The CBA methodology was made according with manufacturer protocol (BD-Biosciences®, Brazil). Briefly, MDM and differentiated U-937 cell was stimulated with the classical inducers mentioned above for 24 hours, and the culture medium was replaced. After another 24 hours, the culture medium was collected to cytometer analyses.

Cell Death Induction, Analysis and Apoptotic Cells Achievement in A549 NSCLC Cell Line

Apoptosis of A549 cells was induced with Cisplatin (Sigma®), Taurine Chloramine (TnCl) and 3-Bromopyruvic Acid (Sigma®). Cells were treated with 40 µM of Cisplatin for 24 hours, 3 mM of Taurine Chloramine for 4 hours, and 2 mM of 3-Bromopyruvate Acid for 2 hours. The supernatant was centrifuged at 600 rpm for 10 minutes to remove viable cells. After, the supernatant was centrifuged at 1600 rpm for 7 minutes to obtain the apoptotic bodies. Apoptosis and necrosis were characterized by double staining with annexin V/fluorescein isothiocyanate (FITC; BD Bioscience) and propidium iodide (PI) according with the manufacture. Flow cytometry was performed using a single-cell gate. The data were analyzed in a FITC/PE dot plot to quantify the percentage of annexin V^+/PI^- cells (early apoptosis), annexin V^+/PI^+ cells (late apoptosis) and annexin V^-/PI^+ cells (necrotic cells).

Measurement of Phagocytosis Efficacy

Apoptotic A549 cells were stained with CellTrace™ CFSE (Carboxyfluorescein succinimidyl ester) Cell Proliferation Kit (Molecular Probes®) according with

manufacture protocol. Cells were centrifuged at 1600 rpm for 7 minutes, washed three times with Hanks Balanced Salt Solution (HBSS) and then resuspended in RPMI supplemented with 10% of SFB. The apoptotic cells were placed on well with differentiated MDM, U-937 and THP-1 cells at a ratio of 5:1 and incubated at 37°C for 3 h for cell lines and 1 hour and 30 minutes for MDM. The wells were washed with Phosphate Buffer Saline (PBS) to remove all nonphagocytosed cells. Cells were detached from the wells by adding a solution of 10 mM of EDTA and incubated for 10 minutes at 37°C. The wells were mixed vigorously to remove all cells (verified by phase contrast microscopy), which were then centrifuged and resuspended in PBS. Cells were analyzed by flow cytometry to determine the percentages of green fluorescent macrophages that had engulfed the apoptotic labeled cells.

Treatment of Macrophages with Apoptotic Cells Obtained from Different Inducers.

Differentiated MDM, THP-1 and U-937 were not activated, activated with IFNγ+LPS or IL-4 as mentioned above. The apoptotic cells obtained from different stimuli were labeled priorly with CFSE-DE and placed on the top of the differentiated MDM, U-937 and THP-1 phagocytes at a protein ratio of 5:1, and incubated at 37°C for 3 h for cell lines, and 1 hour and 30 minutes for MDM. The wells were washed three times with HBSS to remove all nonphagocytosed cells. Fresh medium was added, and cells were incubated for 24 hours at 37°C. The supernatant was used for CBA measurement and conditioned medium assay.

Patient cohort and clinicopathological review

Formalin-fixed paraffin embedded NSCLC tumors from patients diagnosed between 2003 and 2005 were obtained from the Pathology Service at the Hospital de Clinicas de Porto Alegre (HCPA), Brazil. Table 1 summarizes the characteristic of cohort. The pathological diagnoses were extracted from these clinical records, reviewed for the blocks studied and classified by two independent pathologists at collaborating institute, according to World Health Organization criteria. Information such as gender, age, histological type, NSCLC staging and patient outcome were collected. Inclusion criteria were non-small cell lung primary tumor and clinical follow-up of at least 5 years available. Survival was determined from the date of diagnosis to the date of death or last contact. The research programme, including studies on archival and stored materials, was approved by the Research Ethics Committee of the HCPA.

Immunohistochemistry

The corresponding archived paraffin-embedded specimens were sectioned into 4 μm slices, deparaffinized and antigen retrieval was performed in a water bath for 30 minutes with sodium citrate buffer (pH 6.0). Endogenous peroxidases were blocked with 5% hydrogen peroxide in methanol. To avoid nonspecific background staining, slides were incubated for one hour with 1% bovine serum albumin (BSA) (Sigma®) in PBS. Rabbit polyclonal anti-CD68, anti-CD86, anti-CD206 and cleaved caspase-3 primary antibodies (Abcam®) (diluted 1:200 in 1% BSA for CD68 and CD86, and 1:100 for CD206) was incubated

overnight at 4ºC. After incubation, HRP-labeled polymer conjugated (Invitrogen®) was added and incubated for 45 minutes, rinsed, exposed to a solution of diaminobenzidine (0.06%) for 5 minutes and then rinsed in running water. Next, they were dehydrated with alcohol, cleared in xylene and mounted. Negative controls were obtained performing the same protocol above described, with the omission of primary antibody, representing in optical density (O.D.) measurements the background staining value. The brownish-color was considered to be a positive expression of cells. Apoptosis was measured by SignalStain® Apoptosis (Cleaved Caspase-3) IHC Detection Kit from Cell Signaling®.

Statistical Analyses

DCF production of undifferentiated and differentiated macrophage-like phenotype was analyzed by *Student t test*. For CBA and DCF production of polarized macrophages and Cell Viability was used *1 way ANOVA followed by Tukey's test*. Phagocytosis efficiency and CBA analysis of macrophages' phenotype exposed to apoptotic cells were analyzed by *2 way ANOVA*. Comparisons of cytokine release between the different macrophages' phenotypes ((MIFNγ+LPS) and M(IL-4)) were analyzed by *Paired t test*.

RESULTS

Correlation between the Macrophage Density, Phenotypes, and Apoptosis in patients' survival time

A cohort of 40 patients in stages I and II (Table 1) with NSCLC was submitted to immunohistochemical assay for CD68 (*pan* macrophage marker), CD206 (M2 marker) and Cleaved Caspase-3 (apoptosis marker) to verify the influence of macrophage density, polarization status and apoptosis in the outcome of patients with NSCLC. Moreover, we analyzed whether macrophage microanatomical localization (stroma or parenchyma) was also associated with the overall survival of patients. We observed that high total $CD68^+$ cells and $CD68^+$ cells in tumoral stroma were associated with better prognosis in early stages of lung adenocarcinoma (Figure 1A, $P < 0.05$). On the other hand, $CD68^+$ in parenchyma plus $CD206^+$ total, in stroma and parenchyma had no correlation with patients' survival time (Figure 1B). We also analyzed the correlation of apoptotic index in patients' outcome (cleaved casp-3^+ cells, Figure 1C), and observed that high levels of apoptosis are correlated with worse prognosis.

Characterization of U-937 and THP-1 macrophage-like phenotype

In an attempt to better characterize and analyze the differentiation of macrophage-*like* phenotype, we stimulated U-937 and THP-1 cells with PMA. This differentiation showed a strong difference in morphological features of U-937 and THP-1 cells (Figure 2A and 3A). In addition, we analyzed the proliferation parameter and RS production in undifferentiated and differentiated cells. As expected for proliferation, cells stimulated with PMA stopped their growth when compared with nonstimulated cells. Moreover, the RS production in differentiated cells was 10-fold higher in U-937 cells and 5-fold higher in THP-1 than in undifferentiated cells (Figure 2A and 3A). After, we analyzed the effect of polarization of

differentiated cells treating them with IFNγ+LPS or IL-4, and compared to not polarized cells. Our results showed that treatment with IFNγ+LPS increased the production of RS in both cells lines. This is in accordance with an expected behavior of pro-inflammatory stimulation. For viability analysis, only in U-937 cells the polarization with IFNγ+LPS showed reduction in this parameter (Figure 2B and 3B). Furthermore, we analyzed the immunological cytokine production of U-937 and THP-1 macrophage-like cells polarized with the inducers mentioned above. As expected for IL-1β, IL-6, IL-12 and TNF-α, the production of these cytokines was higher in macrophage-like U-937 cells stimulated with IFNγ+LPS (Figure 2D). However, in macrophage-like THP-1 cells, we only observed an increase in production of IL-8 in IFNγ+LPS and, surprisingly, in IL-4 stimulated cells (Figure 3C). Previous data from our group showed that CXCL9, CXCL10, IL-15, IL-1β and TNF-α are good gene markers for IFNγ+LPS polarization, and CCL13, CCL17, ALOX15, TGF-β and F13A1 for IL-4 polarization. LastlyThen, to better characterize PMA treated U-937 cells,we made a RT-PCR analysis for IFNγ+LPS and IL-4polarizationin U-937 cells forusing five differentthese gene markers for IFNγ+LPS or IL-4 polarization. The result obtained was in accordance with the expected, when polarization with IFNγ+LPS showed a high expression for CXCL9, CXCL10, IL-15, IL-1β and TNF-α. On the other hand, IL-4 polarization resulted in high expression of CCL13, CCL17, ALOX15, TGF-β and F13A1. Previous data of our group showed similar results to THP-1 and MDM cells for RT-PCR analysis. Altogether, our results demonstrated that treatment with PMA and polarization with well established inducers produces cells with macrophage-like features and behaviors.

Cell Death induction in A549 NSCLC cell line

To verify the type of cell death promoted by different inducers, we treated A549 cells with Cisplatin, taurine chloramine and bromopyrivuc acid (3-BP) as described in Materials and Methods section (Figure 4A). In addition, we were concerned in obtain the highest fraction of apoptotic cells in early apoptosis, aiming to avoid the late apoptosis *in vitro* artifact. We observed by flow cytometry analysis that treatment with Cisplatin for 24 hours resulted in almost 70% of apoptotic cells in early apoptosis. Moreover, we obtained only 10% of apoptotic cells in late apoptosis, and 5% of cells in necrotic cell death (Figure 4B). The treatment with the oxidant Taurine Chloramine for 4 hours resulted in almost 50% of apoptotic cells in early stage and 20% of late apoptosis, with no necrotic cells (Figure 3B). Lastly, treatment with the glycolysis inhibitor 3-BP for 2 hours resulted in almost 50% of cells in early and late apoptosis (Figure 3C). The cells analyzed were only the detached. Our results showed that, except to 3-BP, our treatments resulted in high proportion of cells in early apoptosis, which are in accordance with the objective of this work. In contrast, as showed in all flow cytometry analyses, we obtained cells that were not marked with propidium iodide and/or annexin V, suggesting another mechanism of cell death.

Phagocytosis Efficiency Measurement of Cell Lines and MDM

Phagocytosis of apoptotic cells are well established as a modulator of immune response. The Figure 5A summarizes the approach to verify phagocytosis efficiency in MDM and differentiated cell lines. THP-1 and U-937 macrophage-like cells had a very low rate of phagocytosis (less than 2%) of apoptotic cells stimulated with the different inducers (Figure 4C and D) after 3 hours of incubation. We did not extend the time of incubation to avoid the change of early apoptosis / late apoptosis ratio (data not shown). In contrast, the phagocytosis capacity in MDM was different from cell lines. We chose a different time of contact of MDM with apoptotic cells exactly to avoid the change in apoptosis proportion. Figure 4B shows that Taurine Chloramine had very low phagocytosis inducing efficiency for all phenotypes when compared to Cisplatin and 3-BP. These results showed that apoptosis induced with Taurine Chloramine influences the phagocytic capacity of macrophages over apoptotic cells. Moreover, the phagocytosis efficiency of M(IL-4) macrophages was higher in apoptotic cells obtained from the treatment with Cisplatin when compared with 3-BP. Altogether, these results showed that cell lines have different behavior for phagocytosis capacity when compared with MDM, and the MDMs' phagocytic capacity of apoptotic bodies can be modulate by the inducer.

Cytometric Bead Array Measurement of MDM Not Exposed and Exposed to Apoptotic Cells Obtained from Different Inducers

In an attempt to understand more precisely the effect of the phagocytosis of apoptotic cells in macrophages' polarization status, we analyzed by CBA measurement the levels of cytokines released from MDM not exposed and exposed to apoptotic cells. First we compared the basal levels of cytokines in M0 phenotype with M(IFNγ+LPS) and M(IL-4) of MDM obtained from 5 different donors. Figure 6A shown the cytokine release pattern of M0 compared with M(IFNγ+LPS). We observed some unexpected results for pro-inflammatory cytokines. Only IL-6 showed an increased production when macrophages were stimulated with IFNγ+LPS. On the other hand, when we compared M0 with M(IL-4), we did not observed any differences in cytokine release of macrophages unstimulated and stimulated with IL-4 (Figure 6B). Moreover, we also did not find difference in cytokine release of M(IFNγ+LPS) when compared with M(IL-4) (Figure 7A). Although we did not find consistent differences between cytokine releases of macrophages' phenotypes, we analyzed the cytokine profile of macrophages exposed to apoptotic cells. Figure 7B showed that macrophages exposed to apoptotic cells obtained from Cisplatin treatment had an increase in the release of IL-12 and IL-10 in M0 phenotype. On the other hand, macrophages exposed to apoptotic cells obtained from Taurine Chloramine treatment increased the production of IL-6 in M(IL-4) phenotype. This result demonstrates that macrophages exposed to apoptotic cells obtained from diverse inducers have differences in cytokine release profile.

DISCUSSION

Macrophages are pivotal cells that contribute to tumor progression and metastasis (Fridlender, Jassar et al. 2013; Giraldo, Becht et al. 2014; Ramanathan

and Jagannathan 2014). Moreover, they are fundamental to tumor microenvironment because of the release not only of cytokines, but also others factors like TGF-β and VEGF. An important feature of macrophage is their capacity to phagocyte apoptotic cells which strongly contribute to modulate macrophages' phenotype. For example, the cytolysis of tumor cells by activated macrophages is inhibited by the ingestion of apoptotic but not necrotic cells (Reiter, Krammer et al. 1999). This might explain the surprising cohabitation of macrophages and malignant cells in many tumors. Blockade of the ingestion of cancer cells undergoing apoptosis by macrophages in the tumor might therefore be a new therapeutic approach in malignancy, disengaging an undesirable inhibition on macrophage cytotoxic capacity (Savill and Fadok 2000). Then, our main goal of this work was to analyze whether different apoptotic bodies generated by distinct inducers could modulate differently the macrophages' polarization. First, we analyzed the clinical relevance of both parameters (macrophages and apoptosis) in patients' outcome and survival. Previous data from our group showed a discrepancy concerning the role of macrophages and apoptosis in NSCLC patients' outcome (Becker, Muller et al. 2013). We observed in a cohort of early stages of NSCLC (I and II) that total $CD68^+$ macrophages and in stroma were associated with better survival. Previous data from literature showed that macrophages change their phenotype in the evolution of NSCLC (Zhang, Yao et al. 2011). Then, macrophages in initial stages could be more pro-inflammatory, contributing to tumor resistance, and the modulation of macrophages by tumor microenvironment could be not totally implemented. Furthermore, we also analyzed the effect of apoptosis in patients' survival and observed that high apoptosis is associated with worse prognosis. Previous work using TUNEL and H&E or anti-ASP technique in stages I-IIIa and squamous cells carcinoma, also found that high apoptotic index is correlated with worse prognosis in NSCLC (Törmänen, Eerola et al. 1995; Ghosh, Crocker et al. 2001; Dworakowska, Jassem et al. 2005).

High proliferation in tumor milleu is also associated with high apoptosis (Van Slooten, Van de Vijver et al. 1998), which could explain this inverse relationship between high apoptotic index and worse prognosis. Moreover, once macrophage has the ability to engulf apoptotic bodies, maybe these cells could have been modulated to a pro-tumoral phenotype, contributing to tumor progression.

Because macrophage can infiltrate different microanatomical compartments of tumor (Ohri, Shikotra et al. 2009; Ma, Liu et al. 2010) and have a strong plasticity (Biswas and Mantovani 2010; Khallou-Laschet, Varthaman et al. 2010; Sica and Mantovani 2012; Lohmeyer, Herold et al. 2015) plus the fact that apoptosis is occurring in all tumor stages, we analyzed the correlation of these parameters in patients' outcome and survival. Analyzed together, we did not find a correlation between them (data not shown). Maybe increasing the number of the patients in the analysis we can observe the relationship between these parameters.

To better understand the effect of apoptosis in macrophage polarization, we differentiated and characterized to a macrophage-like phenotype two human cells lines (U-937 and THP-1). We

observed that both cell lines had features very similar to primary culture (MDM). However, the phagocytic capacity of differentiated cell lines is distinct from MDM, when cell lines had a very low phagocytic activity. Because of that, we exclude the cell lines of our phagocytosis modulation analysis.

Taurine is one of the most abundant nonessential amino acid in mammals and its intracellular concentration ranges from 10 to 70 mM in human phagocytic cells. Upon inflammation, taurine undergoes halogenation in phagocytes and is converted to Taurine Chloramine (TnCl) . In the activated neutrophils, TnCl is produced by reaction with hypochlorite (HOCl) generated by the halide-dependent myeloperoxidase system (MPO). This compound is released from activated neutrophils following their apoptosis and inhibits the production of inflammatory mediators (Weiss, Klein et al. 1982; Kim and Cha 2014). This oxidant showed very interesting results for phagocytosis efficiency in MDM. Previous data from literature showed that the oxidant hydrogen peroxide (H_2O_2) inhibit the phagocytosis of MDMof MDM not itself, but from a change in the apoptotic bodies in a mechanism that which is not associated with structural changes in phosphaditilserine (Anderson, Englert et al. 2002). Then, this physiological oxidant released from activated neutrophils in infections also causes a disturbance in phagocytosis of apoptotic cells. Anderson et al., (Anderson, Maylock et al. 2003) showed that protein S, a protein present in the serum, binds to phosphatydilserine and is essential for phagocytosis. Therefore, Taurine Chloramine could interact with this protein, causing a disturbance in this binding, impairing phagocytosis. However, whether have a protein which is modified in plasma membrane in this process is still unknown. In the context of macrophage modulation by phagocytosis, a molecule that promotes tumor death, but impair the phagocytosis, could be a new candidate to be useful in cancer management.

To analyze the effect of different apoptotic inducers in macrophage modulation, we treated A549 cells with three different agents and analyzed the apoptosis profile. Previous data from literature showed that Taurine Chloramine induces cell death in Burkitt lymphoma cell line JLP-119 almost by apoptosis (Klamt and Shacter 2005). We observed that Taurine Chloramine induces cell death exclusively by apoptosis in A549 cells. Bromopyruvic Acid is a highly reactive electrophilic alkylating agent, and can be expected to nonspecifically affect a large number of macromolecules. In line with this property, the literature reports many targets of Bromopyruvic Acid (Shoshan 2012). These targets include, but are not restricted to, hexokinase-II (HK-II) (Mathupala, Ko et al. 2009) and GAPDH (Dell'Antone 2009; Ganapathy-Kanniappan, Geschwind et al. 2009; Ganapathy-Kanniappan, Vali et al. 2010) in the glycolytic pathway, and mitochondrial succinate dehydrogenase (Pereira Da Silva, El-Bacha et al. 2009), the endoplasmic reticulum (Ganapathy-Kanniappan, Vali et al. 2010) and the lysosomes (Dell'Antone 2006). This compound also induces cell death exclusively by apoptosis. For NSCLC cell lines, no previous report in literature had demonstrated the cell death profile of this agent. Cisplatin is one of the most potent antitumor agents known, displaying clinical activity against a wide variety of

solid tumors. Its cytotoxic mode of action is mediated by its interaction with DNA to form DNA adducts, primarily intra-strand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis (Siddik 2003). For this chemotherapeutic agent, we observed a high proportion of early apoptosis and a few cells in necrosis. This result was critical to our analysis, because we were concerned to avoid the *in vitro* artifact associated to the release of potential intracellular pro-inflammatory content found in the late apoptotic process.

In an attempt to understand the role of phagocytosis in macrophage modulation, we exposed the macrophages to apoptotic bodies derived from A549 apoptotic cells generated from different inducers. Before that, we analyzed the profile of cytokine released by macrophages not exposed to apoptotic cells. The majority of reports in literature show that macrophages polarized with IFNγ+LPS produces large amounts of IL-12, IL-6 and IL-1β and the polarization with IL-4 cause a decrease in the amount released of these cytokines (Gordon 2003; Mantovani, Sica et al. 2004; Mosser and Edwards 2008). Nevertheless, we only observed difference in IL-6 levels for M0 x M(IFNγ+LPS) comparison. Previous data from literature showed that MDM stimulated with the well established inducer could not change their cytokine release profile (Daigneault, Preston et al. 2010). However, other works showed that MDM stimulated with LPS raise their production of IL-1β, IL-8 and TNF-α (Fadok, Bratton et al. 1998; Kurosaka, Watanabe et al. 1998; Seow, Lim et al. 2013). These data reinforce the complex behavior of human macrophages. Furthermore, these controversial results highlight the differences between human and mouse macrophages. Engulfment of apoptotic cells by macrophages can modulate their phenotype from pro-inflammatory to an anti-inflammatory profile, which can contribute to cancer progression and metastasis (Fadok, Bratton et al. 1998; Kurosaka, Watanabe et al. 1998; Gregory and Pound 2011), so we investigated the role of apoptotic bodies obtained from different agents in macrophages' modulation. Our results showed that Taurine Chloramine raise in 14-fold the release of IL-6 in M(IL-4) macrophages, and Cisplatin raise in 4 and 1.5 fold the production of IL-10 and IL-12 in M0 macrophages, respectively. Then, we showed that have differences in macrophage modulation that had phagocytosed apoptotic cells obtained from different inducers. In the context of drug screening to treat cancer, these findings are extremely important, because they suggest that researchers should not only investigate the drug mechanism of cell death, but also the capacity of a new drug to modulate directly or indirectly the macrophages inside the tumor microenvironment. Therefore, more studies are necessary to verify the role of phagocytosis of apoptotic cells in cancer development and metastasis.

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suppressed in macrophages by complement protein C5a. The Journal of Immunology. 2013;191:4308-16.

Table 1: Cohort Baseline Characteristics.

Characteristics	***Total***

Cohort, n	42 (100%)
Age, years	64.8 ± 9.3
Sex	
Men	17 (40,5%)
Women	25 (59.5%)
Tumor Staging	
IA	10 (23.81%)
IB	14 (33.33%)
IIA	10 (23.81%)
IIB	7 (16.67%)
undetermined	1 (2.38%)
Smoking	
Smoker	31 (73.8%)
Non-smoker	11 (26.2%)

FIGURE 1

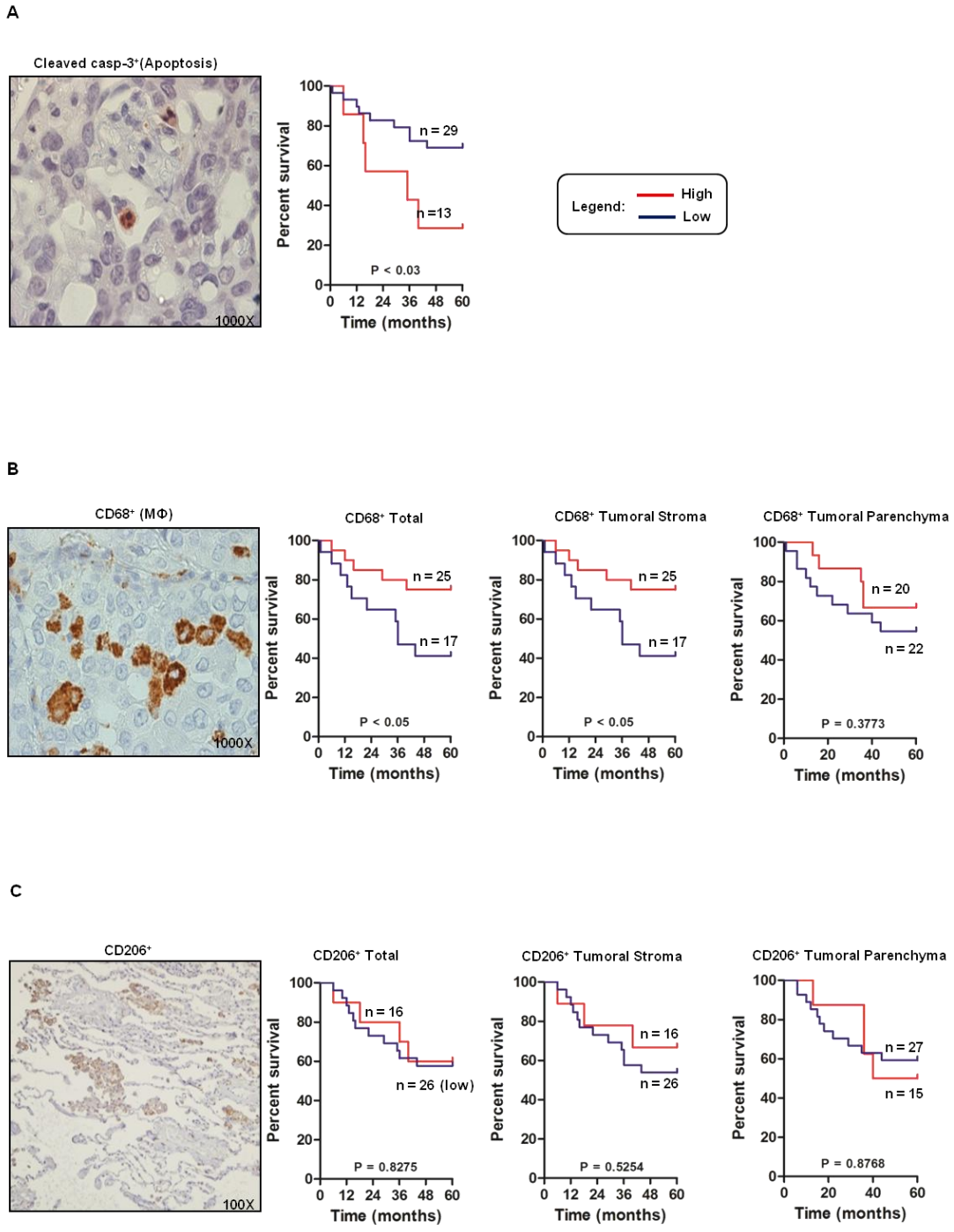

Figure 1: Analysis of apoptosis and macrophage involvment in NSCLC patients' survival. A) Analysis of apoptosis percentage involviment in NSCLC patients' survival. **B)** Analysis of total $CD68^{+}$ cells and their microanatomical localization in NSCLC patients' survival. **C)** Analysis of total CD206+ cells ("M2" phenotype) and their microanatomical localization in NSCLC patients' survival.

FIGURE 2:

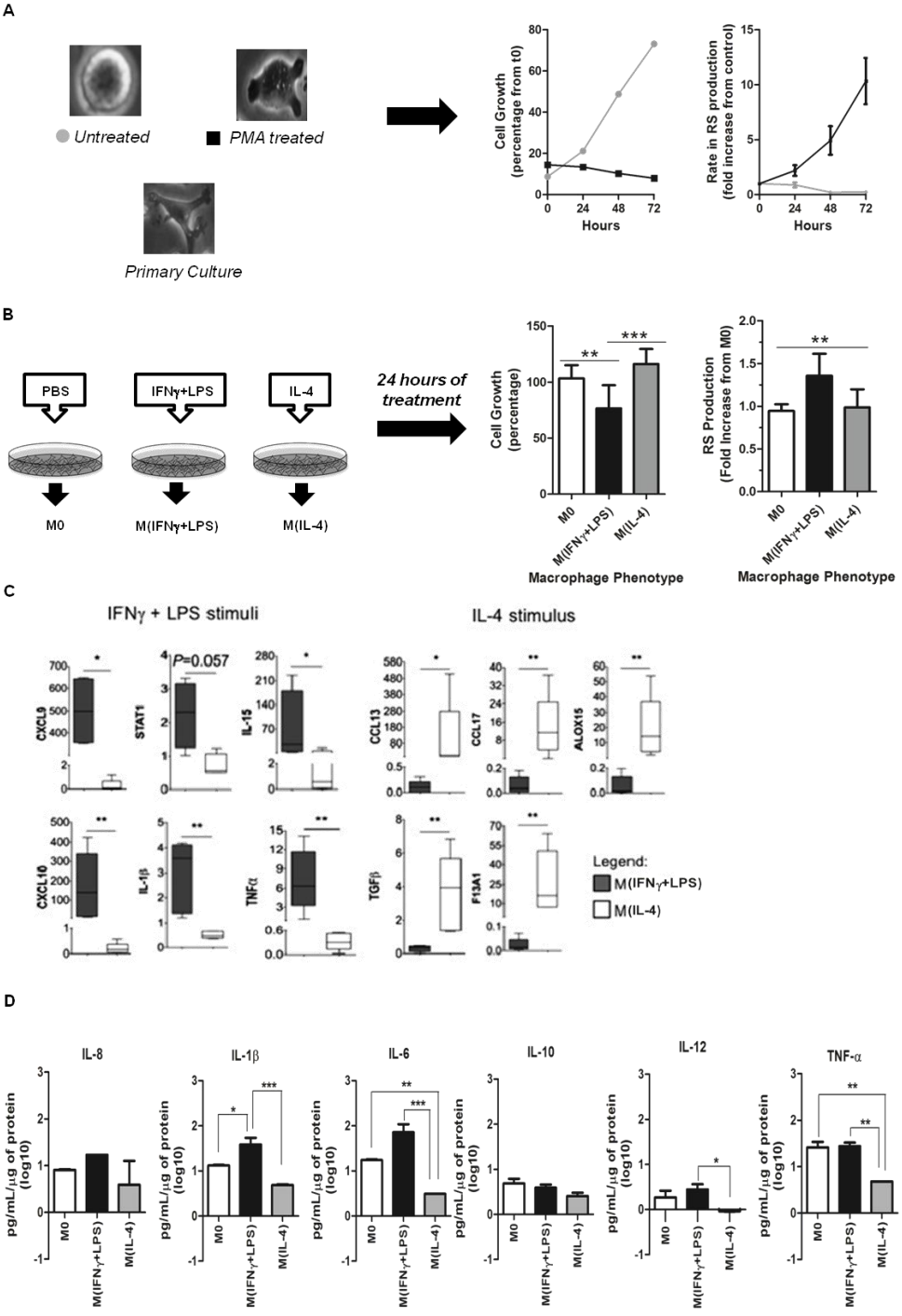

Figure 2: Morphological, Biochemical and Immunological characterization of U-937 cells. A) Morphological features of U-937 (left), proliferation analysis (center) and RS production (right). **B)** Schematical representation of macrophage-like stimula (left), cell viability (center) and RS production (right) after stimulation. **C)** RT-PCR analysis of U-937 macrophage-like cells induced with IFNγ+LPS or IL-4. **D)** Cytokine bead assay of U-937 macrophage-like cells unstimulated and stimulated with IFNγ+LPS or IL-4. Data was obtained from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

FIGURE 3:

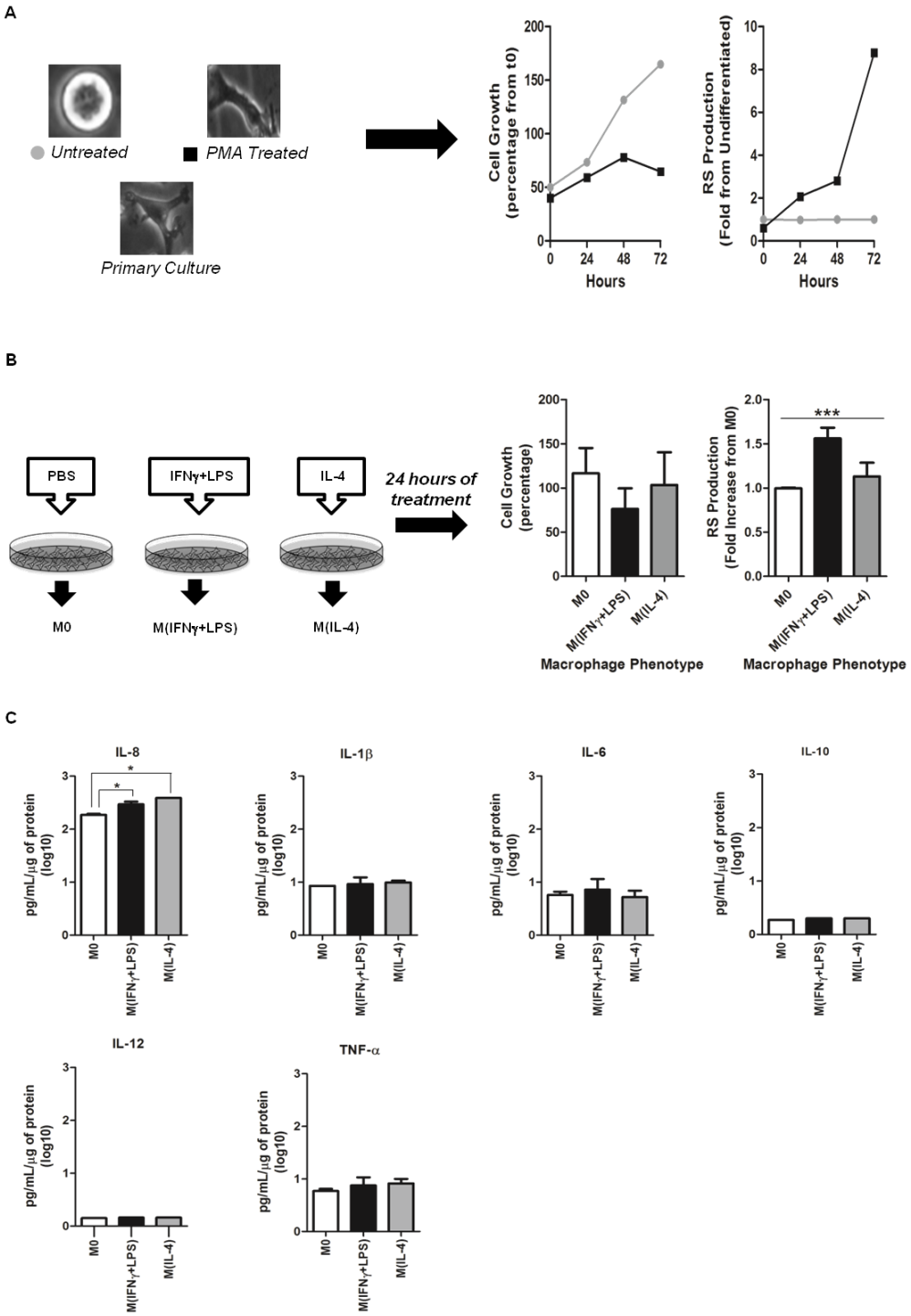

Figure 3: Morphological, Biochemical and Immunological characterization of THP-1 cells. A) Morphological features of THP-1 cells (left), proliferation analysis (center) and RS production (right). **B)** Schematical representation of macrophage-like stimula (left), cell viability (center) and RS production (right) after stimulation. **C)** Cytokine bead assay of THP-1 macrophage-like cells unstimulated and stimulated with IFNγ+LPS or IL-4. Data was obtained from three independent experiments. * P<0.05, *** P<0.001

FIGURE 4:

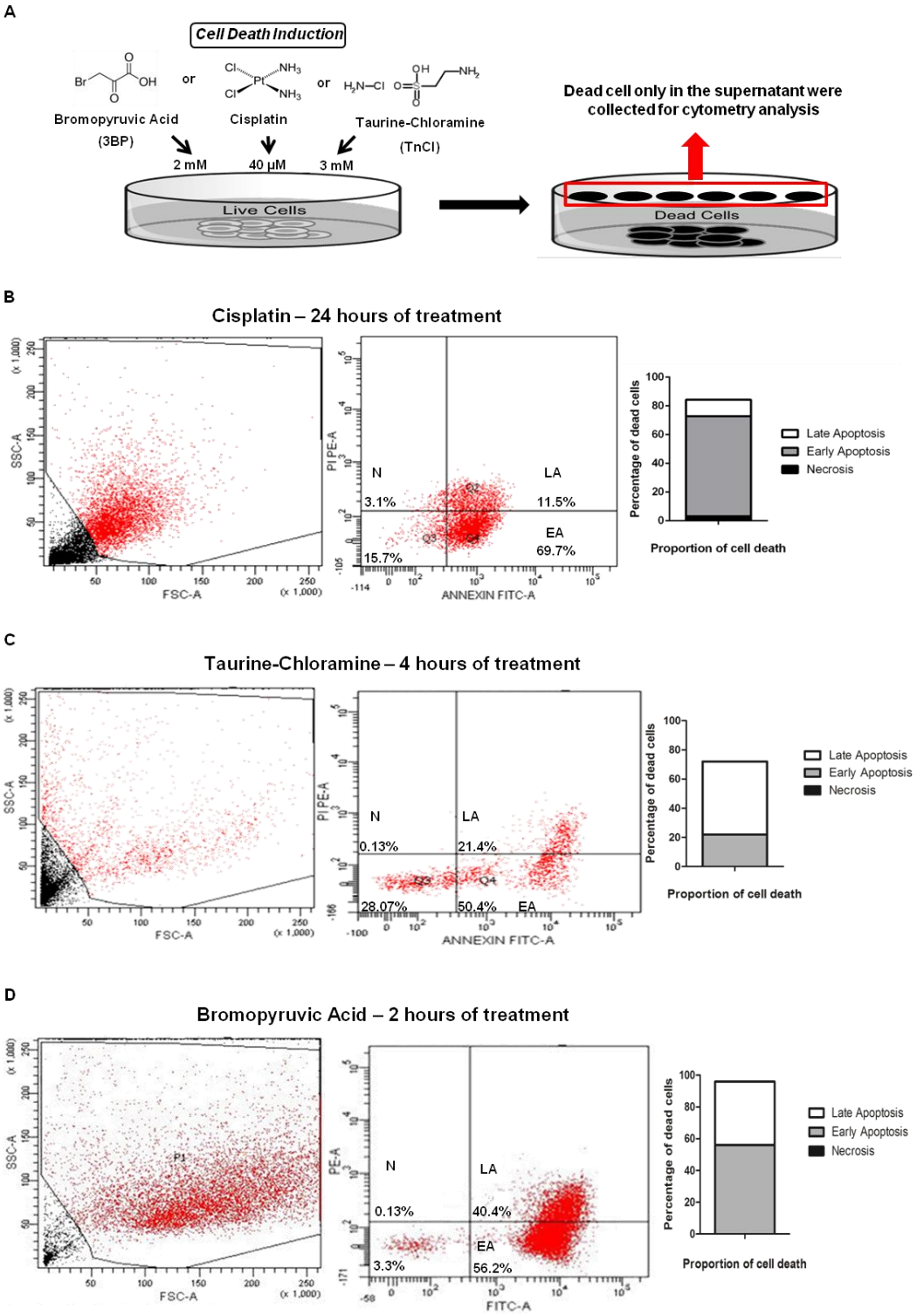

Figure 4: Cell Death profile of NSCLC A549 treated with different inducers. A) Schematical representation of cell death induction. Cytometrical cell death analyses with Annexin V and Propidium Iodide (PI) of A549 cells treated with Cisplatin **(B),** Taurine Chloramine **(C)** and Bromopiruvic Acid **(D)**. Data are presented as mean from three independent experiments.

FIGURE 5:

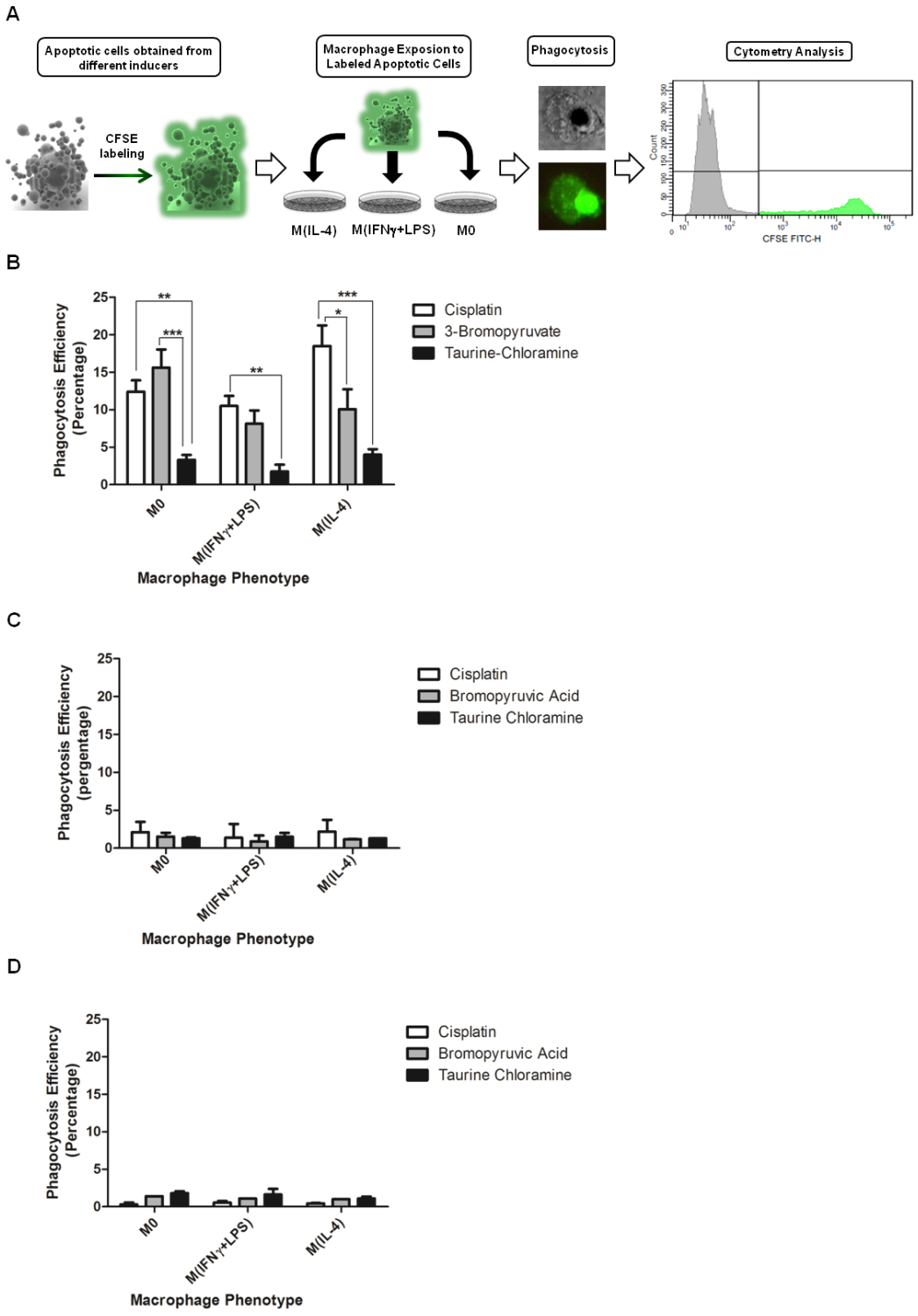

Figure 5: Phagocytosis Efficiency Measurement. A) Schematical representation of phagocytosis efficiency assay **B)** MDM efficiency **C)** U-937 macrophage-like efficiency **D)** THP-1 macrophage-like efficiency. Data are expressed as percentage of three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

FIGURE 6:

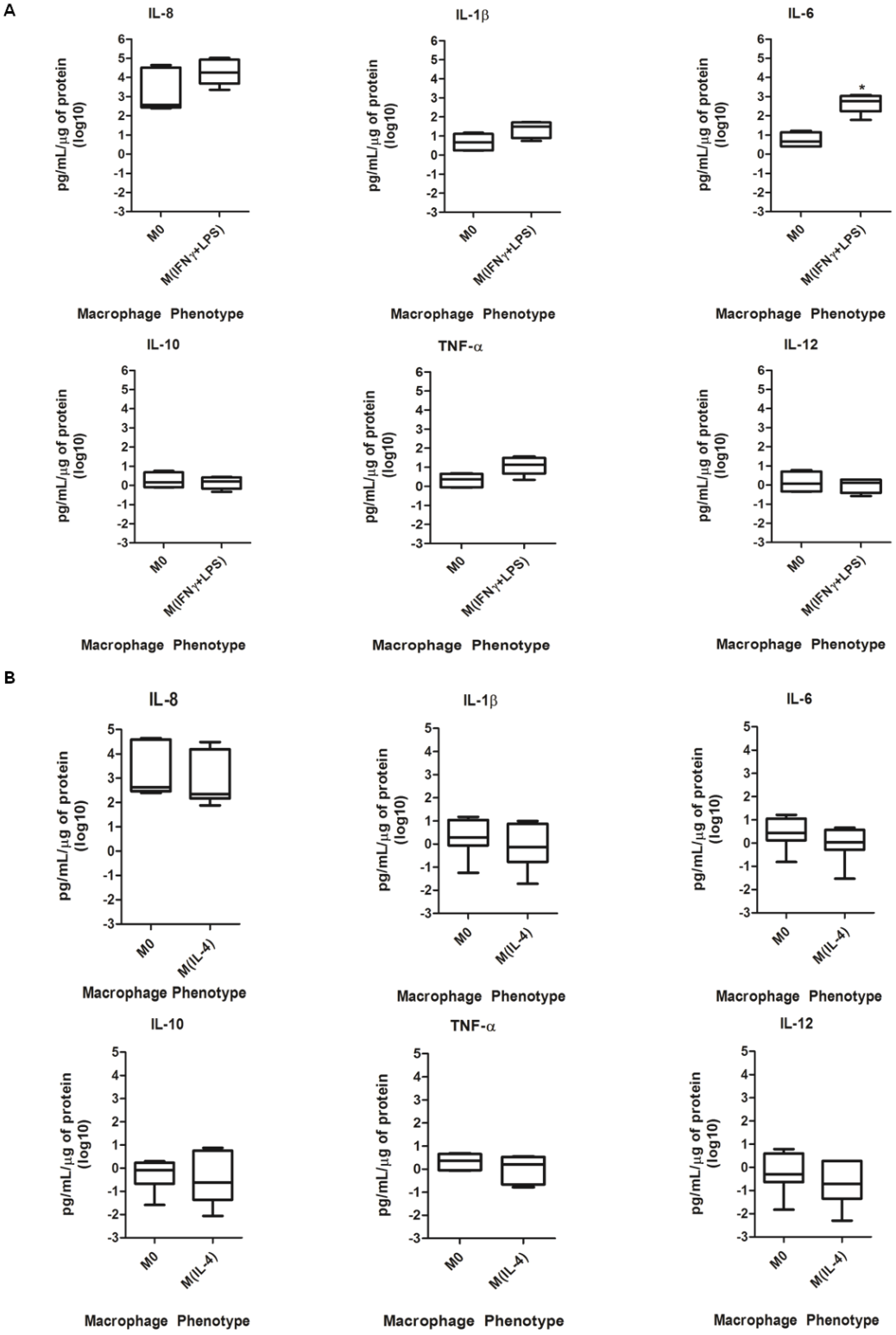

Figure 6: CBA measurement of MDM. A) CBA measurement and comparison Before-After between M0 x M(IFNγ + LPS). **B)** CBA measurement and comparison Before-After between M0 x M(IL-4). Data are presented as median plus S.D. of five independent experiments. * P<0.05.

FIGURE 7:

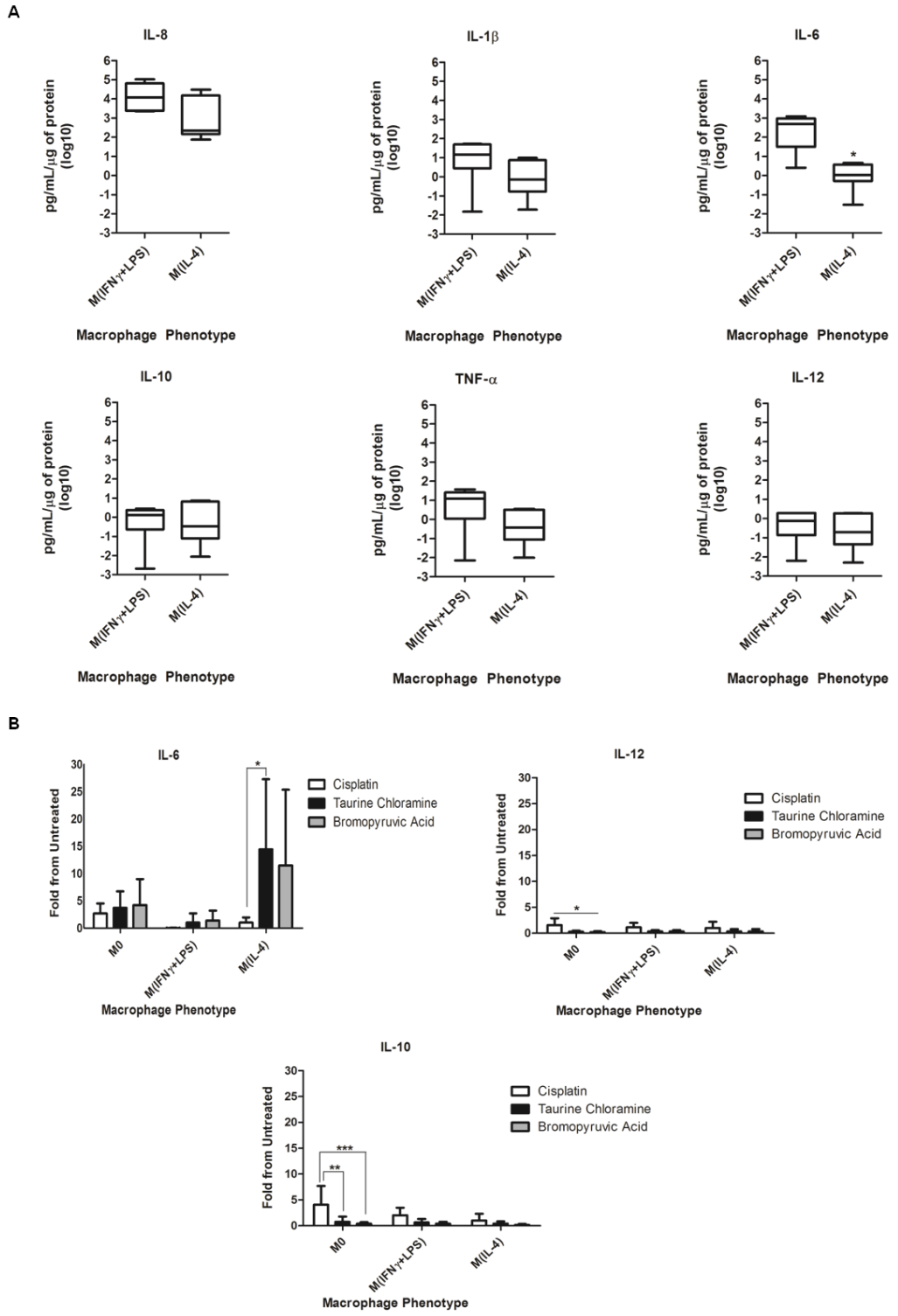

Figure 7: CBA measurement of MDM and MDM after exposure to Apoptotic Cells. A) CBA measurement and comparison between M(IFNγ + LPS) x M(IL-4). **B)** CBA measurement and comparison of MDM exposed to Apoptotic Cells relative to their respective unexposed phenotype. Data are presented as fold change plus S.D. of five independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

CAPÍTULO IV

ARTIGO ACEITO PARA PUBLICAÇÃO NO PERIÓDICO SCIENTIFIC REPORTS – NATURE PUBLISHING GROUP

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The 2014 Impact Factor for *Scientific Reports* is 5.578, according to the 2014 Journal Citation Reports®Science Edition (Thomson Reuters, 2015). This places *Scientific Reports* 5th among all multidisciplinary science primary research journals.

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Abbreviation

The correct abbreviation for abstracting and indexing purposes is *Sci. Rep*.

Nesse trabalho objetivamos construir duas assinaturas gênicas dos diferentes fenótipos dos macrófagos não somente para auxiliar na caracterização desses fenótipos, mas também para melhor compreender o papel dos macrófagos no contexto clínico de algumas patologias humanas. Para isso, pesquisamos na literatura trabalhos de microarray que usaram macrófagos derivados de monócitos humanos polarizados com os principais indutores, como IFNγ, LPS, TNF-α, IL-4 e IL-13. Após critérios rigorosos de seleção, obtivemos duas assinaturas gênicas com 106 genes para o fenótipo M(IFNγ + LPS, TNF-α) e 58 genes para o fenótipo M(IL-4, IL-13). Para verificar a robustez dessas assinaturas, desafiamo-las, através de análises de enriquecimento gênico, em diferentes condições como, por exemplo, em linhagens celulares polarizadas, em diferentes cenários que mimetizaram condições patológicas e em determinadas patologias humanas. Ainda, selecionamos dessas assinaturas 5 genes para cada fenótipo e mostramos a expressão gênica em condições *in vitro*. Nossos resultados mostraram que as duas assinaturas enriqueceram no fenótipo esperado em praticamente todas as condições às quais estas foram desafiadas e, ainda, os genes selecionados mostraram maior expressão nos fenótipos esperados. Demonstramos também uma possível funcionalidade da nossa lista, em que obtivemos genes consenso nos enriquecimentos das assinaturas em situações de infecção viral, bacteriana e na asma. Assim, demonstramos, através de análises de regressão logística, genes que possivelmente estejam correlacionados com o prognóstico de doenças altamente prevalentes como, por exemplo, infecção por HIV, dengue, sepse em crianças e asma.

SCIENTIFIC REPORTS

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Integrated Transcriptomics Establish Macrophage Polarization Signatures and have Potential Applications for Clinical Health and Disease

Received: 05 March 2015
Accepted: 23 July 2015
Published: xx xx xxxx

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Growing evidence defines macrophages (Mφ) as plastic cells with wide-ranging states of activation and expression of different markers that are time and location dependent. Distinct from the simple M1/M2 dichotomy initially proposed, extensive diversity of macrophage phenotypes have been extensively demonstrated as characteristic features of monocyte-macrophage differentiation, highlighting the difficulty of defining complex profiles by a limited number of genes. Since the description of macrophage activation is currently contentious and confusing, the generation of a simple and reliable framework to categorize major Mφ phenotypes in the context of complex clinical conditions would be extremely relevant to unravel different roles played by these cells in pathophysiological scenarios. In the current study, we integrated transcriptome data using bioinformatics tools to generate two macrophage molecular signatures. We validated our signatures in *in vitro* experiments and in clinical samples. More importantly, we were able to attribute prognostic and predictive values to components of our signatures. Our study provides a framework to guide the interrogation of macrophage phenotypes in the context of health and disease. The approach described here could be used to propose new biomarkers for diagnosis in diverse clinical settings including dengue infections, asthma and sepsis resolution.

Macrophages (Mφ) are pivotal cells of the immune system that participate in pleiotropic actions[1]. Microenvironmental signals promote the development of Mφ subsets that secrete specific cytokines and perform distinct functions in regulating and resolving immunity, perpetuation of inflammation[2–5], or

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Research Article

Integrated Transcriptomics Establish Macrophage Polarization Signatures and have Potential Applications for Clinical Health and Disease

Running title

Transcriptome framework of Mφ signatures in health and disease

Authors

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Brazil; [7]Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indianapolis (IN), 46202, USA; Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis (IN), 46202, USA; [8]Biomedical Research Institute, PUCRS, 90619-900, Porto Alegre (RS), Brazil.

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Abstract (200 words)

Growing evidence defines macrophages (Mφ) as plastic cells with wide-ranging states of activation and expression of different markers, which are time and location dependent. Different from the initially proposed M1/M2 dichotomy, diversity have been extensively demonstrated as characteristic features of monocyte-macrophage differentiation, pointing to the difficulty on defining complex profiles by a limited number of genes. Since the description of macrophage activation is currently contentious and confusing, the generation of a simple and reliable framework to categorize major Mφ phenotypes in the context of complex clinical conditions would be extremely relevant to unravel different roles played by these cells in pathophysiological scenarios. In the current study, we integrated transcriptome data using bioinformatics

tools to generate gene expression profiling of macrophages. Doing so, we created two macrophage molecular signatures. We validated our signatures in *in vitro* experiments and in clinical samples. More importantly, we were able to attribute prognostic and predictive values to components of our signatures. Our study provides a framework to guide the interrogation of macrophage phenotypes in the context of health and disease. The approach described here could be used to propose new gene markers for diagnosis in diverse clinical settings including dengue infections, asthma and sepsis resolution.

Keywords: monocyte-derived macrophages; classically activated macrophages; alternatively activated macrophages; macrophages' phenotype signature; polarization profile; integrated transcriptome analysis.

Introduction

Macrophages (Mφ) are pivotal cells of the immune system that participate in pleiotropic actions(Wynn, Chawla et al. 2013). Microenvironmental signals promote the development of Mφ subsets that secrete specific cytokines and perform distinct functions in regulating and resolving immunity, perpetuation of inflammation(Pollard 2009; Jaiswal, Chao et al. 2010; Nathan and Ding 2010; Biswas, Chittezhath et al. 2012), or as lately suggested regulating blood supply and metabolism(Mantovani, Biswas et al. 2013; Jantsch, Binger et al. 2014). Sub-populations of Mφ exist within a continuum of diverse interconvertible phenotypic spectrums designated in the literature for simplicity as M1 (classically activated), or M2a, M2b and M2c (collectively termed alternatively activated). They have overlapping functions that can be modulated by inducers including hematopoietic growth factors and cytokines (*e.g.*: Macrophage Colony Stimulating Factor (M-CSF) or Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)) or small metabolites such as glucose, lipids or sodium chloride(Mantovani, Sica et al. 2004; Kostyk, Dahl et al. 2006; Allavena, Sica et al. 2008; Mantovani and Locati 2009; Biswas, Chittezhath et al. 2012; Haschemi, Kosma et al. 2012; Hucke, Eschborn et al. 2014). To add more complexity to this issue, Mφ exhibit plasticity across various diseases and can switch their phenotype depending on the *in vivo* environment and stage of the disease. As an example, more than 9 different gene signatures based on distinct spectrum of transcriptional programs have been described recently for human macrophage(Xue, Schmidt et al. 2014). Moreover, the inconsistency found in the diversity of terminology, activation inducers and markers used to describe Mφ subsets, enhance the complexity when comparisons of different studies are required for consensus(Murray, Allen et al. 2014).

Since different Mφ subsets are profoundly involved in the development and outcome of many of the so called "Western diseases" (*e.g.*: autoimmune diseases, atherosclerosis, cancer, microorganisms infections and asthma) and are key cells in controlling normal physiological processes(Pollard 2009; Balhara and Gounni 2012; Biswas, Chittezhath et al. 2012; Ruffell, Affara et al. 2012; Moore, Sheedy et al. 2013; da Rocha, De Bastiani et al. 2014; Ramanathan and Jagannathan 2014), here we question whether a restricted set of marker molecules could be helpful to define a particular functional phenotype encountered in the context of diseases. With this in mind, the presence of a minimum set of specific markers that describe a particular phenotype dependent on the inducers used to generate the Mφ, could be seen as a valuable tool to classify and study defined Mφ subsets found in a specific context, in order to develop precise targeted Mφ immunotherapies.

In the present study we explored the use of bioinformatics' tools to analyze and resume published transcriptome data in predefined *in vitro* conditions for Mφ activation. A robust phenotype signature, herein named M(IFNγ + LPS, TNFα) and M(IL-4, IL-13), was

obtained from the analysis of responsive genes in three pre-selected datasets where human monocyte-derived macrophages (MDM) were challenged under classical activators (IFNγ + LPS, TNFα) or alternative inducers (IL-4 or IL-13). The expression of some selected markers was confirmed by real time quantitative polymerase chain reaction (RT-qPCR) in *in vitro* MDM derived from healthy human peripheral blood mononuclear cells (PBMC) and in commonly used differentiated human cell lines (THP-1 and U-937). Finally, we validated our list using independent original microarray datasets of clinical cohorts in the context of different diseases. In this regard, we were able to attribute a minimum set of molecular biomarkers that corresponded to defined Mφ phenotypes among milieus of specific diseases. Our signatures effectively identified classically M(IFNγ + LPS,TNFα) and alternatively M(IL-4, IL-13) activated Mφ in clinical controlled sets. More importantly, we demonstrated prognostic and predictive values of selected biomarkers associated with diseases in diverse clinical settings such as dengue infections, asthma and sepsis resolution.

Results

Generation of M(IFNγ + LPS,TNFα) and M(IL-4, IL-13) Gene Signatures

Heterogeneous sources of cells, experimental inducers and markers are used to describe phenotypes and responses of polarized Mφ creating an enormous amount of conflicting data(Murray, Allen et al. 2014). To systematically evaluate data from defined experimental conditions, as classically and alternatively activated Mφ, we specifically chose *in vitro* datasets reporting explicit description of experimental standard conditions. In this regard, we integrated gene expression profiling from three independent human datasets (GSE5099(Solinas, Schiarea et al. 2010); GSE35449(Beyer, Mallmann et al. 2012); GSE36537(Mehraj, Textoris et al. 2013)) that used peripheral blood mononuclear cells as the source for differentiation into MDM with M-CSF and polarized *in vitro* with IFNγ + LPS or TNFα, IL-4 or IL-13 (Supplementary Table S1). The protocol design is illustrated in Fig.1a.

Using *GEO2R* tool analysis in each selected dataset we obtained two differentially expressed gene signatures that we termed the M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) phenotypes (Fig. 1b), following the guidelines recently suggested by Murray et al.(Murray, Allen et al. 2014). Afterward, with an inclusion criteria of *P* value ≤ 0.0001 (Fig. 1b, vertical line is the cut-off) and presence in all three datasets (red dots in Fig. 1b), our selection resulted in 106 M(IFNγ + LPS, TNFα) and 58 M(IL-4 or IL-13) differentially expressed genes (Supplementary Table S2 and S3). Finally, signatures of both phenotypes were depicted as graphic models with nodes representing gene products, clustered according to seven previously reported functional subdivisions(Murray, Allen et al. 2014). (Fig.1c). These gene signatures are comprised of established enzymes

(*ALOX15*), cytokines (*TNFα* and *TGFβ*), chemokines (*CCL13*, *CCL17*, *CXCL9* and *CXCL10*), receptors and transcription factors (*STAT1*), and less explored genes such as LAG-3 (lymphocyte-activation gene 3), LAMP3 (lysosomal-associated membrane protein 3), *FZD2* (frizzled class receptor 2) and *CLIC2* (chloride intracellular channel 2) among others. In order to illustrate differential gene expression responses to inducers, the obtained M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene networks were subsequently plotted as representative topological responses using landscape analysis with ViaComplex® software(Castro, Filho et al. 2009). *X* and *y*-axis represent gene signature networks and the *z*-axis indicates an illustrative expression response to IFNγ + LPS or IL-4 inducers (Fig. 1c).

In Vitro Validation of M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) Gene Signatures

To experimentally validate our *in silico* Mφ signatures, we used standard M-CSF conditions to differentiate human Mφ from peripheral $CD14^+$ blood monocytes obtained from five healthy donors. These cells were activated/polarized into the M(IFNγ + LPS) or M(IL-4) phenotypes. Figure 2a (left) shows RT-qPCR results from MDM stimulated with IFNγ + LPS. In agreement with the literature, we observed increased expression of selected targets of M(IFNγ + LPS, TNFα), such as *CXCL9*, *CXCL10*, *IL-1β*, *IL-15*, *STAT1* and *TNFα*. In the same way, RT-qPCR analysis of IL-4-induced MDM revealed increased expression of the selected targets of M(IL-4, IL-13), such as *ALOX15*, *CXCL13*, *CXCL17* and *F13A1*, with no significant upregulation of *TGFβ* (Figure 2a). Similar results were obtained from the cultured THP-1 cell line (Fig. 2b) and U-937 cells (manuscript in preparation).

Application of M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) Signatures to Discriminate Clinical Settings and Controlled Conditions

To evaluate the robustness of our gene signatures in discriminating between macrophage phenotypes, we analyzed the performance of our M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene signatures in datasets from previously published studies that used controlled clinical conditions with the GSEA tool. Table 1 summarizes the description of the selected clinical studies where a macrophage polarization phenotype could be expected: *i*) the classically activated phenotype, using retrieved macrophages from bronchoalveolar lavage (BAL) in patient treated with lipopolysaccharide (LPS) (GSE40885(Reynier, De Vos et al. 2012)) and *ii*) the alternatively activated phenotype, using lung biopsies from asthmatic patients (GSE41649 (Chamberland, Madore et al. 2009)). As expected, genes from the M(IFNγ + LPS, TNFα) set were significantly enriched in the LPS treated group (Fig. 3a). In addition, enrichment analysis of the asthmatic subjects was also significant in the M(L-4, IL-13) signature (Fig. 3b). Figure 3a and 3b (right) illustrate differential gene expression responses of the M(IFNγ +

LPS, TNFα) and M(IL-4, IL-13) gene networks in both conditions. These results are consistent with the role played by LPS-induced IFNγ in pro-inflammatory macrophages or IL-4 driving an alternative activation state in the clinical context of asthma(Humbert, Durham et al. 1996; Gordon 2003; Mantovani, Sica et al. 2004).

We also tested 8 additional GEO datasets that used *in vitro* MDM or human cell lines under controlled culture environment, retrieving the expected results from polarization. As expected and in light of our findings, the M(IFNγ + LPS, TNFα) signature was enriched in genes altered under intracellular parasites and bacterial, or viral infection, as well as under pro-inflammatory conditions (LPS or IFNγ). By contrast, the M(IL-4, IL-13) gene signature was enriched in the context of IL-4, IL-10 or IL-13 stimulation (Supplementary Table S4).

Together, our results indicate that our macrophage signatures could characterize microarrays from *in vivo* specific pathological scenarios that take into account the complexity of the tissue components, as well as in *in vitro* factors that impact cultured primary human cells and human cell lines.

Disease Outcomes Predictions and Functional Correlation in Complex Clinical Pathologies

As macrophages play a key role in determining the activation or resolution of immune responses and can determine the fate of a disease(Becker, Muller et al. 2014), we evaluated the capacity of our macrophage phenotype signatures to anticipate the patient outcome or response to specific drug treatment (prognostic and predictive behavior of markers) based on the enrichment analysis of selected genes. We asked whether our M(IFNγ + LPS, TNFα) signature could be used to predict the complication of dengue infection into a hemorrhagic outcome, the drug response in HIV positive patients, and sepsis resolution in children.

To do so, we considered new microarray datasets in order to first identify a responsive set of genes presented in our macrophage signatures clustered by different pro-inflammatory scenarios (*e.g.*: viral and bacterial infections). We retrieved 6 datasets derived from viral and 6 from bacterial infections (Supplementary Table S5). For each analysis set, based on GSEA, we obtained a significant responsive gene list, considering an inclusion criterion for genes present in the core enrichment in more than 80% of cases. This procedure retrieved 12 genes in our M(IFNγ + LPS, TNFα) signature responsive to viral and 35 genes to bacterial infections (protocol design presented in Fig. 4). With the virus and bacteria gene lists, we performed logistic regression analyses in order to associate gene expression with different disease outcomes. In this regard, we expressed our results as odds ratio (OR), which typically represents a measure of association between a predictor and an outcome. In our settings, a particular gene and a specific disease outcome prediction (*e.g.*: live versus death, disease severity or treatment response).

As depicted in Fig. 4a, when comparing controlled versus hemorrhagic dengue (GSE18090, unpublished data), we found that the individual expression of *ISG15*, *OASL*, *OAS2* and *TNFSF10* can be used to anticipate the complication status of dengue infection into a hemorrhagic outcome. Further combined analyses when all 4 genes were tested altogether was not able to improve OR. Similarly, the expression of IFITM1 can discriminate the response of patients to HIV treatment (GSE52900(Wu, Sasse et al. 2013)). From our bacterial infection list, we found that the expression of *CD40* was a predictor of death in septic children (GSE26440(Wynn, Cvijanovich et al. 2011)).

We then searched for clinical conditions that could benefit from the same experimental approach using genes associated with the M(IL-4, IL-13) phenotype, such as asthma and pulmonary fibrosis (Supplementary Table S6), and constructed another list of genes with the GSEA tool (Figure 4b). Data analysis enabled 2 comparisons and setup a threshold of 2/2 retrieved genes to be in the final list. In this new list, we obtained 7 genes. We performed logistic regression analyses and compared healthy versus severe asthmatic patients (GSE27876, unpublished data). We found that the expression of *RAMP1* was associated with the severity of asthma. In the same way (GSE43696(Voraphani, Gladwin et al. 2014)), the expression of three other genes *FSCN1*, *PSTG1* and *SPINT2* revealed an association with the development of severity of asthma (Data is summarized in Fig. 4b). Importantly, in all the 6 clinical datasets tested, we found a consistency in that the specified expressed genes were predicted by our created list M(IFNγ + LPS, TNFα) or M(L-4, IL-13).

Discussion

Growing evidence defines macrophages as plastic cells with wide-ranging states of activation and concomitant expression of different markers, which are time and location dependent(Biswas, Chittezhath et al. 2012; Martinez and Gordon 2014). Because of pleiotropic actions of signaling through recognition receptors, secretion of cytokines, their essential role in activation of immunity or in resolution and the relation to disease outcome, published data has identified macrophages as key players in tissue homeostasis. Indeed, different from the initially proposed M1/M2 dichotomy(Mills, Kincaid et al. 2000), plasticity and diversity have been extensively demonstrated as characteristic features of monocyte-macrophage differentiation(Biswas and Mantovani 2010; Martinez and Gordon 2014; Murray, Allen et al. 2014; Yang, Zhang et al. 2014), pointing to the difficulty on defining complex profiles by a small and limited number of genes(Martinez and Gordon 2014). In this regard, conflicting and oversimplified data have been used to define different subsets. Thus, the generation of a simple and reliable framework to categorize major Mφ phenotypes in the context of complex clinical conditions would be extremely

relevant to unravel different roles played by these cells in pathophysiological scenarios.

In the current study, we integrated transcriptome data using bioinformatics tools to generate gene expression profiling of macrophages, activated under precise and specific conditions. Doing so, we created two macrophage molecular signatures from specific defined *in vitro* induced phenotypes, namely M(IFNγ + LPS, TNFα) and M(IL-4, IL-13). In line with these findings and the complexity associated with a wide range of evidence not easy to summarize, we validated our macrophage molecular signatures *in vitro* and in clinical samples from published microarray data. More importantly, we were also able to attribute prognostic and predictive values to some components of our signatures.

As expected, our study confirms that pro-inflammatory inductors such as IFNγ, LPS and TNFα are able to induce a macrophage phenotype distinct from that induced by cytokines as IL-4 and IL-13(Martinez, Gordon et al. 2006; Murray, Allen et al. 2014; Xue, Schmidt et al. 2014). We confirmed the expression of well-established markers of classically (*e.g.*, CD80, CXCL9, CXCL10, IL-15, ITGAL, TNFS10 and STAT1) and alternatively (*e.g.*, ALOX15, CCL13, CCL17, F13A1, PTGS1) activated macrophages markers that support the validity of our gene lists. In addition, our strategy retrieved several genes not previously used as M(IFNγ + LPS, TNFα) or M(IL-4, IL-13) markers that could be explored in the macrophage biology field.

In this regard, unexplored gene markers such as LAG-3, LAMP3, OPTN (optineurin) and PIN-1 (peptidylprolyl cis/trans isomerase, NIMA-interacting 1) were highlighted in the M(IFNγ + LPS, TNFα) molecular signature. For example, LAG-3 is a high affinity ligand for MHC class II present in lymphocytes. Activated *LAG-3*$^{+}$ lymphocytes present at sites of inflammation may reduce the differentiation of monocytes into macrophages or fully competent antigen-presenting dendritic cells, thus limiting the magnitude of the ongoing T-cell immune responses(Buisson and Triebel 2005). Another newly retrieved gene was *LAMP3*, a well-established marker of mature dendritic cells(Johansson, Corripio-Miyar et al. 2012). Also, *OPTN* has a known role in vesicle trafficking and bacterial handling. However, their expression and role in human M(IFNγ + LPS, TNFα) macrophages remains unexplored. Interestingly, a recent work described the association of pro-inflammatory cytokine release deficiency in macrophages with reduced *OPTN* expression in a subset of patients with Crohn's disease(Smith, Sewell et al. 2015). Finally, we were not able to find any study addressing the association between *PIN-1* and macrophages. As these examples, several other genes retrieved in the M(IFNγ + LPS, TNFα) list have not yet been explored as potential macrophage markers. Thereby, our list suggest a great variety of genes to be studied and used as new M(IFNγ + LPS, TNFα) macrophage markers.

In addition, the M(IL-4, IL-13) molecular signature retrieved genes such as *FZD2*, *CLIC2*, *EMILIN2* (elastin microfibril interfacer 2), *CDR2L* (cerebellar degeneration-related protein 2-like), *CMTM8* (CKLF-like MARVEL transmembrane domain containing 8) that has not been fully explored as potential human macrophage markers. In this sense, *FZD2* has a potential role in macrophage-regulated angiogenesis, as proposed by Newman et al.(Newman and Hughes 2012). *CLIC2* is a chloride intercellular channel that is not reported as M(IL-4, IL-13) marker, but few evidences suggest that nuclear translocation of *CLIC4* regulates macrophage deactivation(Malik, Jividen et al. 2012). Another poorly explored gene in human macrophages, *EMILIN2* gene was not explored, but one study with mouse macrophages established a correlation between EMILIN2 protein and thrombosis(Sa and Hoover-Plow 2011). Finally to our knowledge, there are no association between *CDR2L* and *CMTM8* genes and macrophages.

Because we were interested in exploring the potential application of our framework in profiling macrophages' phenotypes, we generated logistic regression models to associate the consensus markers with diseases outcomes. In this regard, we show that a set of selected markers is able to predict patients' outcomes when dissimilar pathologies were grouped according to infectious (*e.g.:* dengue, HIV, and sepsis) or non-infectious conditions (*e.g.*: asthma) (Figure 4).

Our initial analysis of viral infection responsive genes derived from our M(IFNγ + LPS, TNFα) signature retrieved *TNFSF10*, *ISG15*, *OAS2* and *OASL*. For example, we found that the expression levels of *TNFSF10* (tumor necrosis factor (ligand) superfamily, member 10, commonly known as TRAIL), obtained from the M(IFNγ + LPS, TNFα) signature, could discriminate the severe cases of hemorrhagic dengue. The protective role of *TNFSF10*, as apoptosis inducer, in dengue severity or symptoms' complications has already been identified(Warke, Martin et al. 2008; Arias, Valero et al. 2014). Serum from patients with dengue had significantly increased TNFSF10 protein levels(Limonta, Torrentes-Carvalho et al. 2014). Its antiviral action has been demonstrated in various cells, including in *ex vivo* infected monocytes and macrophages(Wong, Chen et al. 2012). Another gene that was shown to protect from dengue severity was Interferon-Stimulated Gene 15 (*ISG15*) (Okumura, Lu et al. 2006; Lenschow, Lai et al. 2007). Regardless, our logistic regressions analyses demonstrate that the expression of *ISG15* can predict the course of dengue patients into a more severe outcome. Indeed, a recent published study(Hishiki, Han et al. 2014) with infected cultured cell lines implicated *ISG15* in dengue virus 2 replication inhibition. Moreover, two members of the OAS (2,5-oligoadenylate synthetase) gene family, *OAS2* and *OASL*, also contribute to the antiviral response. Two recent studies have demonstrated an association between *OAS2* haplotypes and differential susceptibility to clinical

outcomes of dengue virus infection (Alagarasu, Honap et al. 2013; Thamizhmani and Vijayachari 2014). In addition, other members of the OAS gene family, as *OAS1* p42, *OAS1* p46, and *OAS3* p100 have been shown to have antiviral effects in dengue complication (Lin, Yu et al. 2009). Therefore, these IFN-induced proteins may play important roles in the antiviral response and can be addressed as promising targets poorly explored in the literature for the management of dengue infection.

Other applications of our molecular signature could be to anticipate clinical response to treatment. In this aspect, we found that *IFITM1* (interferon-induced transmembrane 1) is associated with better response to HIV treatment, presenting an OR of 9.4. *IFITM1* has antiviral action already demonstrated in highly pathogenic human virus(Brass, Huang et al. 2009) and has been associated with cell-to-cell HIV-1 transmission(Ding, Pan et al. 2014), but how *IFITM1* contributes to treatment resistance is still unclear. We went further and analyzed the bacterial infection responsive genes derived from our M(IFNγ + LPS, TNFα) signature and applied in predicting sepsis outcome. We found that the expression of *CD40*, a molecular target that had already been linked with inflammation(Barbe-Tuana, Klein et al. 2006), have prognostic role for septic patients. Altogether these results emphasize the robustness of our genes signatures and demonstrate that our analysis framework was able to predict and validate the presence of already known genes or find new candidates to further explore association between expression, biological function and clinical outcome.

In the same context, we applied our M(IL-4, IL-13) signature to predict outcomes in asthma cohorts. Asthma is a heterogeneous disease that is classified phenotypically as mild, moderate, or severe(Meyers 2010). We found that RAMP1, *FSCN1*, *PSTG1* and *SPINT2* genes could be associated with the development of severity of asthma. However, no studies showed an association of *FSCN1* (fascin actin-bundling protein 1) and *SPINT2* (serine peptidase inhibitor, Kunitz type, 2) with the evolution of asthma severity.

In conclusion, we proposed a consensus collection of markers describing major macrophages' activation phenotypes, namely M(IFNγ + LPS, TNFα) and M(IL-4, IL-13), able to characterize robustly controlled *in vitro* and *in vivo* scenarios for macrophage induction. Since the description of macrophage activation is currently contentious and confusing(Martinez and Gordon 2014; Murray, Allen et al. 2014), our study provides a framework to guide the interrogation of macrophage phenotypes in the context of health and disease. Despite further studies being necessary to understand the role played by retrieved genes, the approach described unraveled new gene candidate markers for diverse clinical settings such as dengue infections, asthma and sepsis resolution.

Methods

Ethics

This study was approved by the Ethics Committee of PUCRS (No. 06/03537). All participants provided written informed consent for blood collection and research.

Microarray Datasets and Macrophage Phenotypes' Signatures

Microarray expression profiles were extracted from public available Gene Expression Omnibus (GEO) NCBI database (http://www.ncbi.nlm.nih.gov/geo/). The selected GSE datasets are presented in Supplementary Table S1. Search criteria included Medical Subject Heading (MeSH) terms for "monocytes-derived macrophages" and "*Homo sapiens*" and "macrophages polarization". GEO2R tool www.ncbi.nlm.nih.gov/geo/geo2r was used to identify differential gene expression to obtain classically activated M(IFNγ + LPS,TNFα) and alternatively activated M(IL-4, IL-13) phenotypes signatures (Barrett, Wilhite et al. 2013). We extracted two lists of genes with a significant different expression in the two polarized Mφ discrete phenotypes with adjusted *P*-value ≤ 0.0001 Student's *t*-test and Benjamini & Hochberg false-discovery rate (FDR) correction. Finally, an integrated gene list for each phenotype was created where all the pre-selected differentially expressed genes that were consistently present in the three datasets were included (protocol design in Figure 1a).

Gene Set Enrichment Analyses (GSEA) and Network Construction

We explored the robustness of our gene signatures using GSEA method (http://www.broadinstitute.org/gsea/index.jsp)(Subramanian, Tamayo et al. 2005). In this regard, genes are ranked based on the correlation between their expression and their class distinction, M1-*like* or M2-*like*, by using any suitable metric. In that case, the method evaluates if an *a priori* defined set of genes (*e.g.:* M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene signatures) is randomly distributed or is primarily associated with a tested class (PMID: 16199517). Expression networks of differential signatures graphs and illustrative landscape maps were constructed and edited using free academic Medusa® (https://sites.google.com/site/medusa3visualization) (Hooper and Bork 2005), and ViaComplex® (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex)(Castro, Filho et al. 2009) softwares.

Logistic Regression

Logistic regression models associate binary responses with continuous variables. Specifically, we applied a bias-reduced logistic regression to test gene expressions against defined pathological outcomes in an attempt to identify promising markers from our macrophage signatures. Firth's penalized-likelihood logistic regression was originally developed to reduce the bias of maximum likelihood estimates and provide a good solution to monotone likelihoods (http://www.meduniwien.ac.at/user/georg.heinze/techreps/tr2_2004.pdf) (http://www.jstor.org/stable/2336755?o

rigin=JSTOR-df&seq=1#page_scan_tab_contents).
The regressions were modeled using R statistical environment (http://www.R-project.org).

Cell Cultures and Macrophage Differentiation

PBMC from healthy individuals (n = 5) were isolated by Histopaque® gradient (d = 1.077) (Sigma Aldrich, MO, USA) according to manufacturer's instructions. Monocytes were purified from freshly isolated PBMC using monoclonal CD14 antibody-conjugated microbeads (Miltenyc Biotec, Germany). Purity was > 98%. Monocytes were cultured in RPMI-1640 media (Invitrogen, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen) (RPMI 10% FBS) at 37°C in 5% CO_2 humidified air. For *in vitro* differentiation of MDM, monocytes were incubated with RPMI (10% FBS) supplemented with Macrophage Colony Stimulating Factor (M-CSF) (50 ng/mL) (Peprotech, USA) for 7 days. For differential polarization, Mφ were supplemented with IFNγ (20 ng/mL) (Peprotech, USA) and LPS (100 ng/mL) (Sigma-Aldrich) or IL-4 (20 ng/mL) (Peprotech, USA) for additional 18 h, respectively.
The THP-1 (human acute monocytic leukemia) and U-937 (human histiocytic lymphoma) cells lines were obtained from Rio de Janeiro Cell Bank (www.bcrj.org.br) and maintained in RPMI-1640 media as mentioned above. Cell lines were differentiated using phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), 20 nM and 10 nM for THP-1 or U-937 cells, for 72 h. PMA treated cells were polarized for additional 24 h by incubation with IFNγ (20 ng/mL) + LPS (100 ng/mL) or IL-4 (20 ng/mL) or IL-13 (20 ng/mL) for M(IFNγ + LPS), M(IL-4) or M(IL-13) phenotypes.

RNA Isolation and RT-qPCR

Gene expression analysis was performed using gene-specific primers designed with IDT Design Software (Integrated DNA Technologies Inc., CA, USA) (Supplementary Table S7). Total RNA (1.2 µg) was isolated from M(IFNγ + LPS) and M(IL-4) cells using Trizol Reagent (Invitrogen). Reverse transcription was performed with M-MLV Reverse Transcriptase (Sigma-Aldrich) and random nonamers (Sigma-Aldrich) primers. Real-time PCR reactions were carried out in Step One Plus real-time cycler (Applied-Biosystem, NY, USA) using Taq Polimerase (Sigma-Aldrich) and SYBR green. Gene expression was quantified by the comparative cycle threshold method (ΔΔCT) and normalized using the housekeeping gene TATA binding protein (TBP). Melting curves were used to monitor unspecific amplification products.

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Acknowledgements

Brazilians funds MCT/CNPq Universal (445457/2014-7), MCT/CNPq Universal (479305/2009-9), PRONEX/FAPERGS (1000274), PRONEM/FAPERGS (11/2032-5), PqG/FAPERGS (2014-2551/12-8), MCT/CNPq INCT-TM (573671/2008-7) and FAPERGS/MS/CNPq/SESRS/PPSUS (1121-2551/13-8) provided the financial support without interference in the ongoing work. MB, MADB, MMP and FMBT received a fellowship from CAPES. FK received a fellowship from MCT/CNPq (303613/2008-4).

Authors' contribution

Conceived and designed the experiments: MB*, MDB*, MMP*, MAAC, MK, FMBT, FK. Performed the experiments: MB, MDB, and MMP. Analyzed the data: MB, MDB, MMP, MAAC, MK, FMBT, and FK.

Contributed reagents/materials/analysis tools: FTCRG, MMM, FMBT, FK. Wrote the paper: MB, MDB, MMP, MAAC, MK, FMBT, and FK.

Competing financial interest

The authors declare that they have no competing financial interest.

FIGURE LEGENDS

Figure 1

Macrophage phenotypes signatures construction and gene network representation. a) Protocol design for M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene signatures. b) Volcano plots representation of differential expression analyses. Red dots are genes present in all three datasets with adjusted P value ≤ 0.0001. c) M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene networks (left) and their illustrative topological representation (landscape analysis) showing changes in relative gene expression after IFNγ + LPS or IL-4 stimuli (right) (see Supplementary Table S2 & S3 for the complete list of retrieved genes).

Figure 2

In vitro validation of selected genes from M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) signatures. a) RT-qPCR from human MDM activated with 50 ng/mL of M-CSF for 7 days and stimulated with IFNγ (20 ng/mL) + LPS (100 ng/mL) or IL-4 (20 ng/mL) for additional 18 h. b) RT-qPCR from THP-1 (human acute monocyte leukemia cell line) differentiated with 20 ng/mL PMA for 3 days and stimulated with IFNγ (20 ng/mL) + LPS (100 ng/mL) or IL-4 (20 ng/mL) for additional 24 h. Data represent median and IQR (interquartile range) of five independent experiments normalized to TATA binding box protein (TBP). Data was considered statistically significant for *($P \leq 0.05$) and ** ($P \leq 0.01$) (Mann-Whitney U test).

Figure 3

Validation of M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) signatures under controlled clinical settings. a) M(IFNγ + LPS, TNFα) signature response of alveolar macrophages after LPS instillation in the lung based on Gene Set Enrichment Analysis (GSEA) (left) and topological representation (landscape analysis) (right). b) M(IL-4, IL-13) signature response of bronchial biopsy from asthmatic patients based on GSEA (left) and landscape analysis (right).

Figure 4

Prognostic and predictive values of selected components derived from M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) signatures in complex clinical settings. Protocol design to select consensus responsive genes in infectious (a) and non-infections (b) conditions to interrogate different clinical datasets. The input lists for the consensus analysis comprised of 106 M(IFNγ + LPS, TNFα) and 58 M(IL-4, IL-13) genes. M(IFNγ + LPS, TNFα) list was interrogated for association with viral and bacterial (6 independent gene expression signatures each) infections, retrieving 12 and 35 consensus gene markers, respectively. M(IL-4, IL-13) list was interrogated for association with non-infectious conditions (2 independent gene expression signatures), retrieving 7 consensus gene markers (see Supplementary Table S5 & S6 for the complete description of datasets). Prognostic or predictive values of these markers were assessed by logistic regression analysis using selected clinical cohorts. Data were expressed as Odds Ratio (OR).

Table 1: Gene Set Enrichment Analysis (GSEA) of M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) gene networks in clinical samples.

GEO ID	Cohort Description	Experimental Groups	Reference
GSE40885	Sterile saline was instilled into a lung segment by bronchoscope, followed of instillation of LPS into the contralateral lung for 6 hours. After, a bilateral bronchoalveolar lavagewas performed and transcriptional profiling was done on alveolar Mφ.	Alveolar Mφ from saline (n = 7) vs. LPS-treated (n = 7).	Reynier, F. et al. Gene expression profiles in alveolar macrophages induced by lipopolysaccharide in humans. Molecular Medicine 2012 (18):1303-1311.
GSE41649	Transcriptional profiling of bronchial biopsy of healthy and asthmatic subjects.	Healthy (n = 4) vs. asthmatic patients (n = 4).	Chamberland, A. et al. A comparison of two sets of microarray experiments to define allergic asthma expression pattern. Experimental lung research 2009 (35): 399-410.

Abbreviations: IFNγ, interferon-gamma; IL-4, interleukin-4; IL-13, interleukin-13; LPS, lipopolysaccharide; M ϕ , macrophages; TNFα, tumor necrosis factor-alpha.

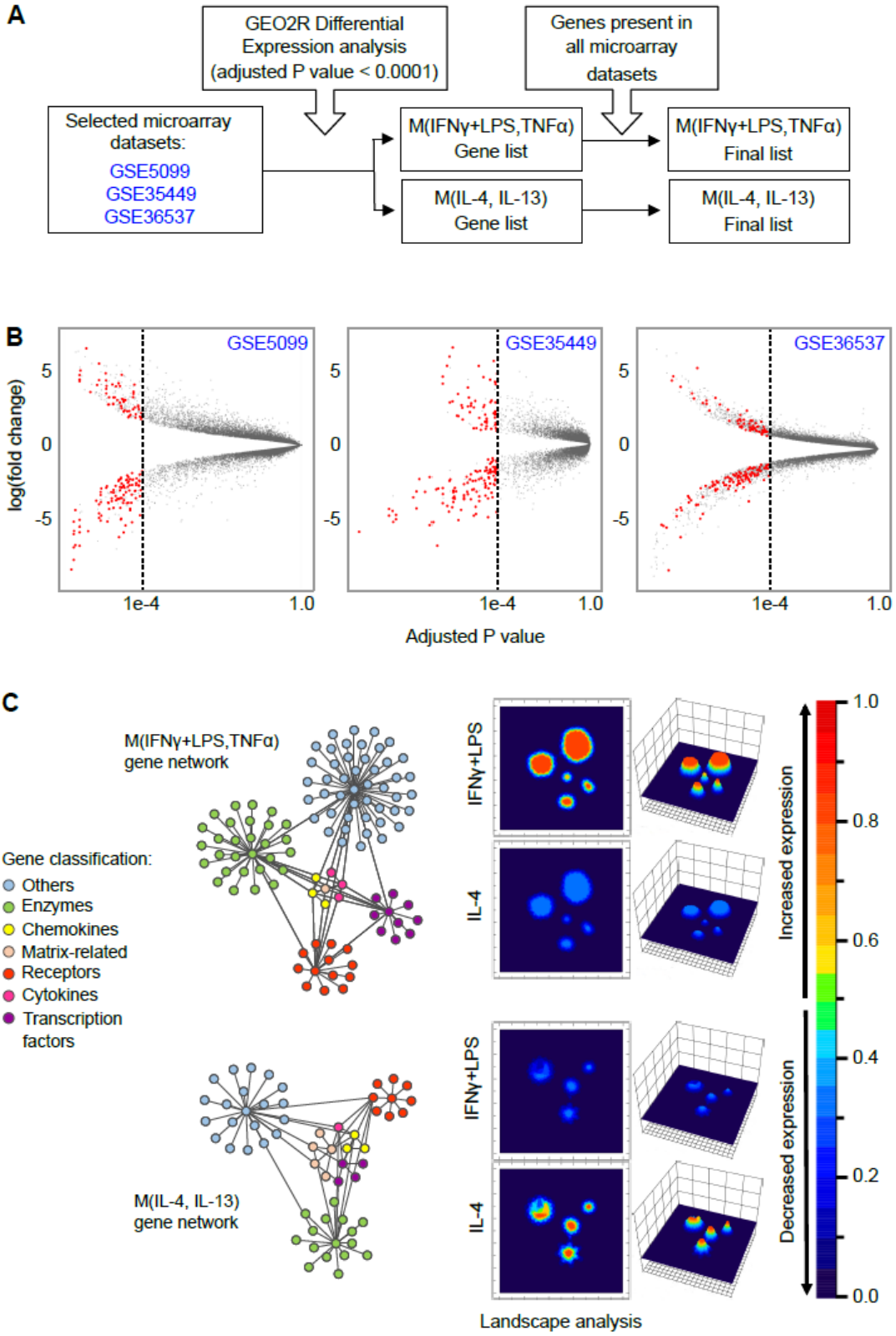

A
GEO2R Differential Expression analysis (adjusted P value < 0.0001)
Genes present in all microarray datasets
Selected microarray datasets:
GSE5099
GSE35449
GSE36537
M(IFNγ+LPS,TNFα) Gene list
M(IFNγ+LPS,TNFα) Final list
M(IL-4, IL-13) Gene list
M(IL-4, IL-13) Final list
B
GSE5099
GSE35449
GSE36537
log(fold change)
5
0
-5
1e-4
1.0
Adjusted P value
C
M(IFNγ+LPS,TNFα) gene network
Gene classification:
Others
Enzymes
Chemokines
Matrix-related
Receptors
Cytokines
Transcription factors
M(IL-4, IL-13) gene network
IFNγ+LPS
IL-4
Increased expression
Decreased expression
1.0
0.8
0.6
0.4
0.2
0.0
Landscape analysis

Figure 1

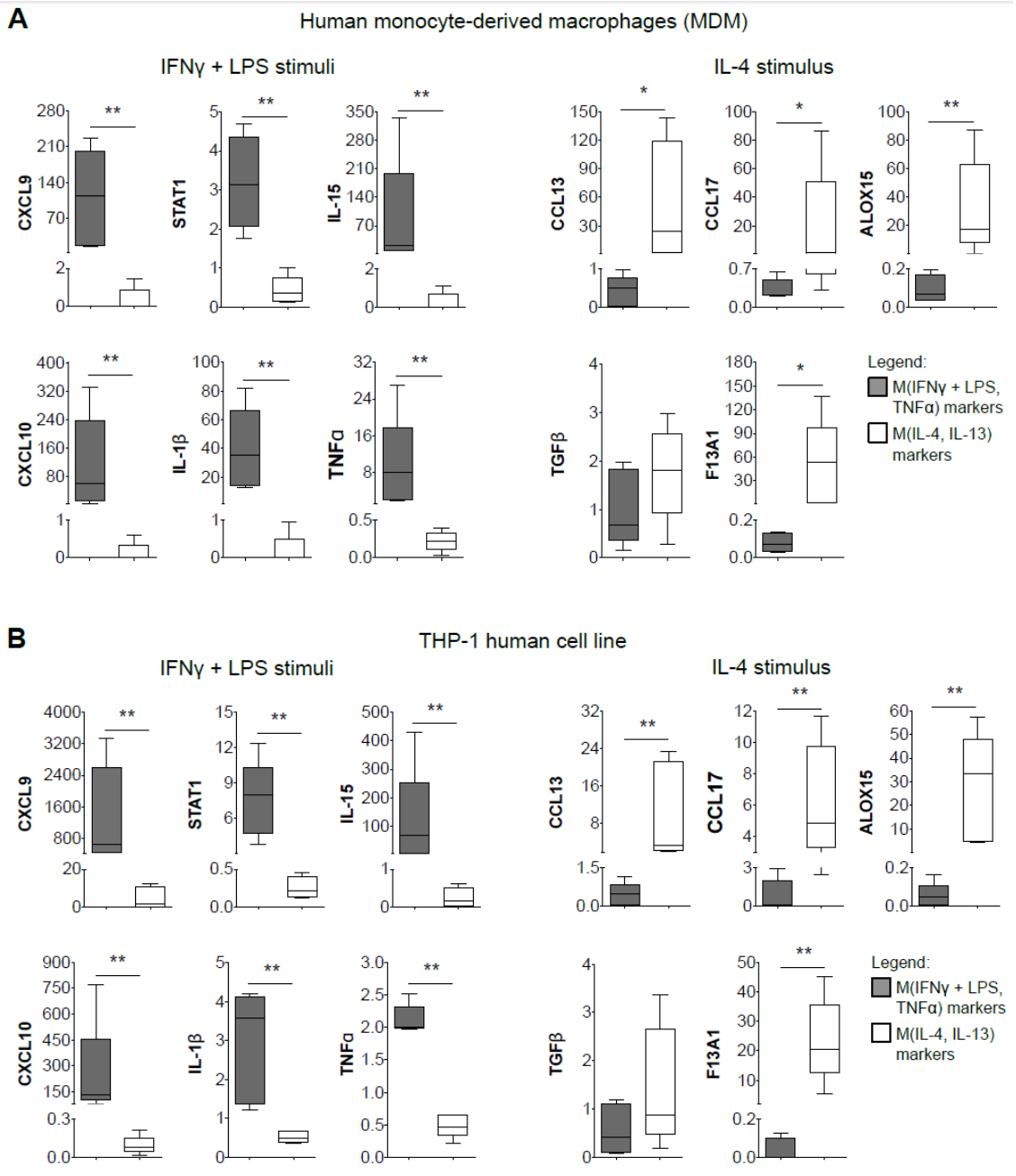
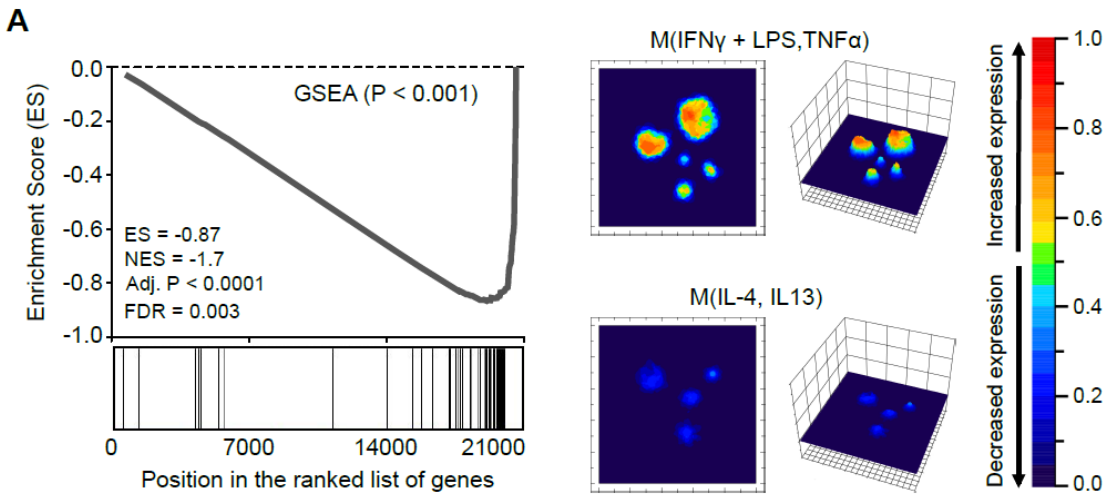
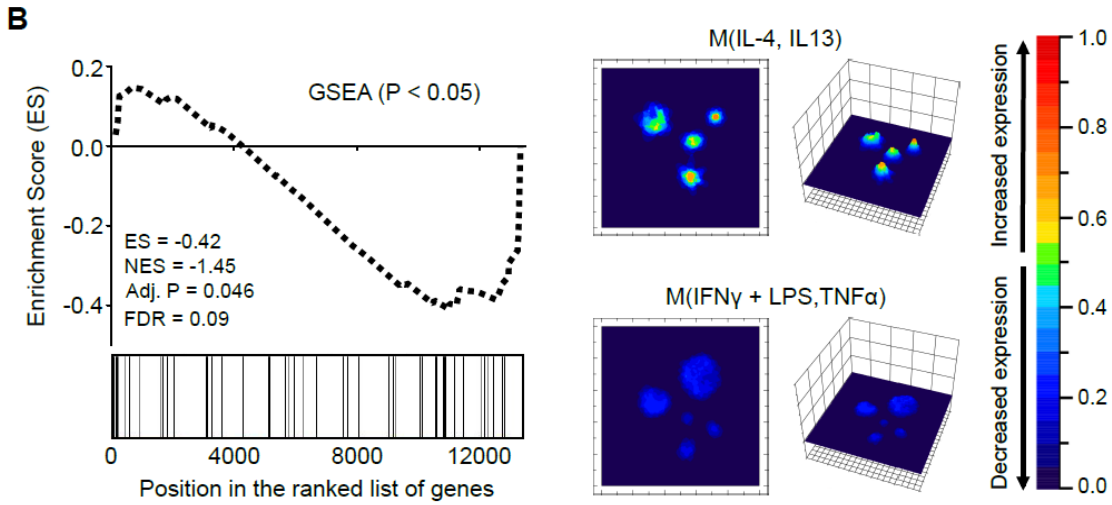

A
Human monocyte-derived macrophages (MDM)
IFNγ + LPS stimuli
IL-4 stimulus
CXCL9
STAT1
IL-15
CCL13
CCL17
ALOX15
CXCL10
IL-1β
TNFα
TGFβ
F13A1
Legend:
M(IFNγ + LPS, TNFα) markers
M(IL-4, IL-13) markers
B
THP-1 human cell line
IFNγ + LPS stimuli
IL-4 stimulus
CXCL9
STAT1
IL-15
CCL13
CCL17
ALOX15
CXCL10
IL-1β
TNFα
TGFβ
F13A1
Legend:
M(IFNγ + LPS, TNFα) markers
M(IL-4, IL-13) markers

Figure 2

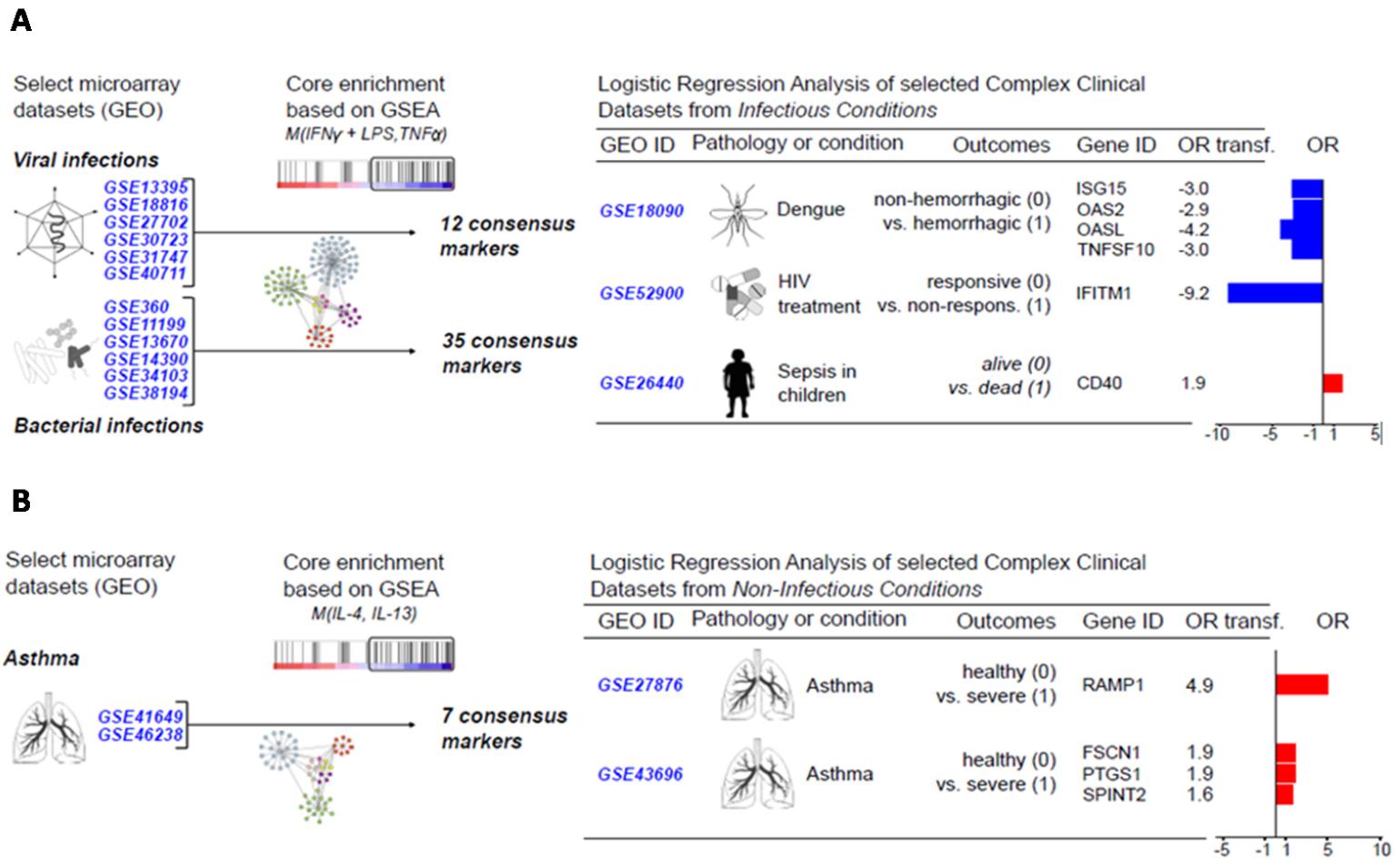


M(IFNγ + LPS,TNFα) gene network in LPS-treated samples (GSE40885)



M(IL-4, IL-13) gene network in Asthmatic samples (GSE41649)

Figure 3


A
Select microarray datasets (GEO)
Core enrichment based on GSEA
M(IFNγ + LPS,TNFα)
Viral infections
GSE13395
GSE18816
GSE27702
GSE30723
GSE31747
GSE40711
12 consensus markers
GSE360
GSE11199
GSE13670
GSE14390
GSE34103
GSE38194
35 consensus markers
Bacterial infections
Logistic Regression Analysis of selected Complex Clinical Datasets from Infectious Conditions
GEO ID
Pathology or condition
Outcomes
Gene ID
OR transf.
OR
GSE18090
Dengue
non-hemorrhagic (0) vs. hemorrhagic (1)
ISG15
OAS2
OASL
TNFSF10
-3.0
-2.9
-4.2
-3.0
GSE52900
HIV treatment
responsive (0) vs. non-respons. (1)
IFITM1
-9.2
GSE26440
Sepsis in children
alive (0) vs. dead (1)
CD40
1.9
-10
-5
-1
1
5

B
Select microarray datasets (GEO)
Core enrichment based on GSEA
M(IL-4, IL-13)
Asthma
GSE41649
GSE46238
7 consensus markers
Logistic Regression Analysis of selected Complex Clinical Datasets from Non-Infectious Conditions
GEO ID
Pathology or condition
Outcomes
Gene ID
OR transf.
OR
GSE27876
Asthma
healthy (0) vs. severe (1)
RAMP1
4.9
GSE43696
Asthma
healthy (0) vs. severe (1)
FSCN1
PTGS1
SPINT2
1.9
1.9
1.6
-5
-1
1
5
10

Figure 4

Supplementary Table 1: Summary description of datasets used to create the M(IFNγ + LPS, TNFα) and M(IL-4, IL-13) signatures.

GEO ID	Summary description
GSE5099	Peripheral monocytes were induced to mature macrophages with M-CSF. Cells were then activated with IFNγ and LPS or IL-4.
GSE35449	Analysis of human M0, M1 (IFNγ, LPS and TNFα stimulus), and M2 (IL-4 and IL-13 stimulus)-like macrophages derived from peripheral blood.
GSE36537	MDM were stimulated with IFNγ to M1-like phenotype and IL-4 for M2-like phenotype.

Abbreviations: IFNγ, interferon-gamma; IL-4, interleukin-4; IL-10, interleukin-10; IL-13, interleukin-13; LPS, lipopolysaccharide; M0, unstimulated macrophage; M-CSF, macrophage colony stimulation factor; MDM, monocyte derived macrophages.

Supplementary Table 2: Primers used for M(IFNγ + LPS) and M(IL-4) analyses in MDM and cell lines.

GENE	Access	Primers Sequences		Amplicon
		Forward	Reverse	
CXCL9	NM_002416.1	GGACTATCCACCTACAATCCTTG	TTTTAATCAGTTCCTTCACATCTGC	147 pb
CXCL10	NM_001565.3	ACTCTAAGTGGCATTCAAGGAG	ACGTGGACAAAATTGGCTTG	135 pb
IL1-beta	NM_000576.2	TGGCCCTAAACAGATGAAGTG	GAAGGTGCTCAGGTCATTCTC	199 pb
IL-15	NM_172175.2	CTGATCATCCTAGCAAACAACAG	GGACAATATGTACAAAACTCTGCAA	127 pb
STAT1	NM_007315.3	TGACTCAAAATTCCTGGAGCAG	AGGTCATGAAAACGGATGGTG	150 pb
TNF-alpha	NM_000594.3	TCTCTCTAATCAGCCCTCTGG	GCTTGAGGGTTTGCTACAAC	94 pb
ALOX15	NM_001140.3	GAGGTCAGGTTCCCTTGTTAC	CTTCTCTCTTCCAGCTCTTCTTC	137 pb
CCL13	NM_005408.2	CAGAGGCTGAAGAGCTATGTG	CGGCCCAGGTGTTTCATATAA	137 pb
CCL17	NM_002987.2	CCCCTTAGAAAGCTGAAGACG	TCAAGGCTTTGCAGGTATTTAAC	149 pb
F13A	NM_000129.3	TCCTGGAGTAACAAGACCAATG	CATACACATGTCTCAGGGAGTC	149 pb
TGF-beta	NM_000660.5	TGCTGTCTCCATGTTTGAT	TCTCTGCTCCCCACCTCTA	86 pb

Suplemmentary Table 3: M(IFNγ + LPS, TNFα) gene list.

GENE	Description	Gene Classification
ADAM28	ADAM metallopeptidase domain 28	Matrix
AIM2	absent in melanoma 2	Others
ANKRD22	ankyrin repeat domain 22	Others
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	Enzyme
APOL1	apolipoprotein L, 1	Others
APOL3	apolipoprotein L, 3	Others
BATF2	basic leucine zipper transcription factor, ATF-like 2	Transcription factor
C1R	complement component 1, r subcomponent	Enzyme
C1S	complement component 1, s subcomponent	Enzyme
CCL19	chemokine (C-C motif) ligand 19	Chemokine
CD38	CD38 molecule	Receptor
CD40	CD40 molecule, TNF receptor superfamily member 5	Receptor
CD80	CD80 molecule	Receptor
CFB	complement factor B	Others
CLEC4D	C-type lectin domain family 4, member D	Receptor
CXCL10	chemokine (C-X-C motif) ligand 10	Chemokine
CXCL9	chemokine (C-X-C motif) ligand 9	Chemokine
CYBB	cytochrome b-245, beta polypeptide	Others
DUSP10	dual specificity phosphatase 10	Enzyme
DUSP6	dual specificity phosphatase 6	Enzyme
ETV7	ets variant 7	Transcription factor
FAM49A	family with sequence similarity 49, member A	Others
FAM65B	family with sequence similarity 65, member B	Others
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	Recptor
FPR2	formyl peptide receptor 2	Receptor
GADD45G	growth arrest and DNA-damage-inducible, gamma	Others
GBP1	guanylate binding protein 1, interferon-inducible,	Others

	67kDa	
GBP2	guanylate binding protein 2, interferon-inducible	Others
GBP4	guanylate binding protein 4	Others
GBP5	guanylate binding protein 5	Others
GCH1	GTP cyclohydrolase 1	Enzyme
GK	glycerol kinase 3 pseudogene; glycerol kinase	Enzyme
GPR84	G protein-coupled receptor 84	Receptor
GUCY1A3	guanylate cyclase 1, soluble, alpha 3	Enzyme
HERC5	hect domain and RLD 5	Enzyme
HESX1	HESX homeobox 1	Transcription factor
HLA-F	major histocompatibility complex, class I, F	Receptor
IFI27	interferon, alpha-inducible protein 27	Others
IFI35	interferon-induced protein 35	Others
IFI44L	interferon-induced protein 44-like	Others
IFIH1	interferon induced with helicase C domain 1	Receptor
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	Others
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	Others
IFITM1	interferon induced transmembrane protein 1 (9-27)	Others
IFITM2	interferon induced transmembrane protein 2 (1-8D)	Others
IL15	interleukin 15	Cytokine
IL15RA	interleukin 15 receptor, alpha	Receptor
IL32	interleukin 32	Cytokine
INHBA	inhibin, beta A	Others
IRF1	interferon regulatory factor 1	Transcription factor
IRF7	interferon regulatory factor 7	Transcription factor
ISG15	ISG15 ubiquitin-like modifier	Others
ISG20	interferon stimulated exonuclease gene 20kDa	Enzyme
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	Receptor

ITGB7	integrin, beta 7	Receptor
LAG3	lymphocyte-activation gene 3	Others
LAMP3	lysosomal-associated membrane protein 3	Others
LIMK2	LIM domain kinase 2	Enzyme
LRRK2	leucine-rich repeat kinase 2	Enzyme
MUC1	mucin 1, cell surface associated	Others
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	Others
NAMPT	nicotinamide phosphoribosyltransferase	Enzyme
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Transcription factor ??
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	Enzyme
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	Enzyme
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	Enzyme
OASL	2'-5'-oligoadenylate synthetase-like	Enzyme
OPTN	optineurin	Transcription factor
PAG1	phosphoprotein associated with glycosphingolipid microdomains 1	Others
PARP14	poly (ADP-ribose) polymerase family, member 14	Enzyme
PCNX	pecanex homolog (Drosophila)	Others
PDE4B	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	Enzyme
PIM1	pim-1 oncogene	Enzyme
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	Others
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	Enzyme
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Enzyme
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	Others
RCN1	reticulocalbin 1, EF-hand calcium binding domain	Others
RHBDF2	rhomboid 5 homolog 2 (Drosophila)	Others
RSAD2	radical S-adenosyl methionine domain containing 2	Others

SAT1	spermidine/spermine N1-acetyltransferase 1	Enzyme
SCO2	SCO cytochrome oxidase deficient homolog 2 (yeast)	Others
SEPT4	septin 4	Others
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Others
SLAMF7	SLAM family member 7	Receptor
SLC22A15	solute carrier family 22, member 15	Others
SLC25A28	solute carrier family 25, member 28	Others
SLC31A2	solute carrier family 31 (copper transporters), member 2	Others
SLC6A12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	Others
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	Others
SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	Others
SNX10	sorting nexin 10	Others
SOCS3	suppressor of cytokine signaling 3	Trancription Factor
SOD2	superoxide dismutase 2, mitochondrial	Enzyme
STAT1	signal transducer and activator of transcription 1, 91kDa	Trancription Factor
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	Trancription Factor
STX11	syntaxin 11	Others
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Others
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	Others
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	Cytokine
TRIM69	tripartite motif-containing 69	Enzyme
UBE2L6	ubiquitin-conjugating enzyme E2L 6	Enzyme
USP18	ubiquitin specific peptidase 18	Enzyme
VAMP5	vesicle-associated membrane protein 5 (myobrevin)	Others
WARS	tryptophanyl-tRNA synthetase	Enzyme

XRN1	5'-3' exoribonuclease 1	Enzyme

Suplemmentary Table 4: M(IL-4, IL-13) gene list.

GENE	Description	Gene classification
ADAM19	ADAM metallopeptidase domain 19 (meltrin beta);	Matrix
ALOX15	arachidonate 15-lipoxygenase;	Enzyme
ARRB1	arrestin, beta 1;	Transcription factor
BZW2	basic leucine zipper and W2 domains 2;	Others
CARD9	caspase recruitment domain family, member 9;	Others
CCL13	chemokine (C-C motif) ligand 13;	Chemokine
CCL17	chemokine (C-C motif) ligand 17;	Chemokine
CCL23	chemokine (C-C motif) ligand 23;	Chemokine
CD1A	CD1a molecule;	Receptor
CD1C	CD1c molecule;	Receptor
CD1E	CD1e molecule; T-cell surface glycoprotein CD1e;	Receptor
CDR2L	cerebellar degeneration-related protein 2-like	Others
CHN2	chimerin (chimaerin) 2; GTPase-activating protein for p21-rac.	Others
CLEC4A	C-type lectin domain family 4, member A;	Receptor
CLIC2	Chloride intracellular channel 2;	Others
CMTM8	CKLF-like MARVEL transmembrane domain containing 8	Cytokine
CRIP1	cysteine-rich protein 1 (intestinal);	Others
CTSC	cathepsin C; Thiol protease. endopeptidase.	Enzyme
DUSP22	Dual specificity phosphatase 22;	Enzyme
EMILIN2	elastin microfibril interfacer 2;	Matrix
ESPNL	espin-like	Others
F13A1	coagulation factor XIII, A1 polypeptide;	Matrix
FOXQ1	forkhead box Q1	Transcription Factor

FSCN1	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus);	Others
FZD2	frizzled homolog 2 (Drosophila); Receptor for Wnt proteins.	Receptor
GALNTL4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4;	Enzyme
GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	Enzyme
GPD1L	glycerol-3-phosphate dehydrogenase 1-like;	Enzyme
GSTP1	glutathione S-transferase pi 1;	Enzyme
ITM2C	integral membrane protein 2C	Others
KCNK6	potassium channel, subfamily K, member 6;	Others
MAOA	monoamine oxidase A;	Enzyme
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1;	Enzyme
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3;	Enzyme
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase;	Enzyme
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A;	Receptor
NMNAT3	nicotinamide nucleotide adenylyltransferase 3; n	Enzyme
OSBPL7	oxysterol binding protein-like 7	Others
P2RY11	purinergic receptor P2Y, G-protein coupled, 11;	Receptor
PALLD	palladin, cytoskeletal associated protein; a variety of cell types.	Others
PAQR4	progestin and adipoQ receptor family member IV	Receptor
PELP1	proline, glutamate and leucine rich protein 1;	Transcription factor
PLAU	plasminogen activator, urokinase;	Enzime
PON2	paraoxonase 2;	Enzime
PPP1R14A	protein phosphatase 1, regulatory (inhibitor) subunit 14A;	Others
PTGS1	prostaglandin-endoperoxide synthase 1 (	Enzyme
RAMP1	receptor (G protein-coupled) activity modifying protein 1	Receptor
REPS2	RALBP1 associated Eps domain containing 2	Others
RGS18	regulator of G-protein signaling 18	Others

RRS1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae);	Others
S100A4	S100 calcium binding protein A4	Others
SEC14L5	SEC14-like 5 (S. cerevisiae)	Others
SHPK	sedoheptulokinase	Enzyme
SPINT2	serine peptidase inhibitor, Kunitz type, 2	Others
TGFBI	transforming growth factor, beta-induced, 68kDa;	Matrix
TMEM97	transmembrane protein 97	Others
VCL	vinculin; Involved in cell adhesion.	Matrix
ZNF789	zinc finger protein 789;	Transcription factor

Supplementary Table 5: Features and results obtained of GSE datasets that used classical activators and different conditions.

GEO ID	Comparison	Sample Size	Result	P-value
GSE360	Monocyte-derived macrophages (MΦ) were exposed to five different pathogens - *Mycobacterium tuberculosis* and to phylogenetically distinct protozoan *Leishmania major, L. donovani, Toxoplasma gondii* and helminth *Brugia malayi.*	MΦ unstimulated (2 samples) x MΦ stimulated with five different pathogens (2 samples)	M(IFNγ + LPS, TNFα) network genes were enriched in macrophages exposed to pathogens	P < 0.001
GSE1432	Unstimulated microglia cells x microglia cells stimulated with IFN-gamma for 1h and 24h	4 samples for control, 1h and 24h	M(IFNγ+LPS, TNFα) genes were enriched in microglia cells stimulated with IFN-gamma in both times	P < 0.001 for both times
GSE15038	PMA stimulated only x PMA and LPS stimulated U937 cells	1 for both samples	M(IFNγ + LPS, TNFα) genes were enriched in LPS stimulated cells	P = 0.001
GSE25289	Unstimulated microglia cells x stimulated with Poly I:C	5 samples for both	M(IFNγ + LPS, TNFα) genes were enriched in Poly I:C	P < 0.001

GSE29628	Control x THP-1 cells infected with different *W-Beijing Mycobacterium tuberculosis* strains of different sublineages	1 for control and 13 for *M. tuberculosis*	M(IFNγ + LPS, TNFα) genes were enriched in *M. tuberculosis* stimulated cells	P < 0.001
GSE32164	Unstimulated MDMs (M0) x Stimulated MDMs with IL-4	3 for both samples	M(IL4, IL-13) genes were enriched in IL-4 stimulated macrophages	P < 0.001
GSE49240	MDMs stimulated cells; IL-4 x IFN-gamma; MDMs stimulated cells; IL-10 x IFN-gamma	2 for IL-4, Il-10 and IFN-gamma	M(IFNγ + LPS, TNFα) genes were enriched in IFN-gamma stimulated cells in both comparisons. M(IL-4, IL-13) genes were enriched in IL-4 and IL-10 stimulated cells	P < 0.001 for all comparisons
GSE51446	MDMs stimulated cells; IL-4 x LPS; MDMs stimulated cells; IL-10 x LPS; MDMs stimulated cells; IL-13 x LPS	1 for IL-4, IL-10, IL-13 and LPS	M(IFN-γ + LPS, TNF-α) genes were enriched in LPS stimulated cells. M(IL-4,IL13) genes were enriched in IL-4, IL-10 and IL-13 stimulated cells	P < 0.001 for LPS x IL-4 P < 0.05 for LPS x IL-10 and IL-13.

Abbreviations: IL-4, interleukin-4; IL-10, interleukin-10; IL-13, interleukin-13; LPS, lipopolysaccharide; M. tuberculosis, *Mycobacterium tuberculosis*; MDM, monocyte-derived macrophage; PMA, phorbol myristate acetate; T. gonddi, *Toxoplasma gondii*.

Supplementary Table 6: Description of datasets used to create the virus and bacterial lists.

GEO ID	Description
	Bacterial Datasets
GSE360	MDMs (MΦ) generated *in vitro* from the same individual blood donors were exposed to five different pathogens, and gene expression profiles were assessed by microarray analysis. Responses to Mycobacterium tuberculosis and to phylogenetically distinct protozoan (Leishmania major, L. donovani, Toxoplasma gondii) and helminth (Brugia malayi) parasites were examined, each of which produces chronic infections in humans yet vary considerably in the nature of the immune responses they trigger.
GSE11199	Peripheral-blood leucocytes were separated from buffy coats of three healthy blood donors and cells were differentiated for 14 days before use. Differentiated macrophages were infected with H1N1 and H5N1 at a multiplicity of infection (MOI) of two. Total RNA was extracted from cells after 1, 3, and 6h post-infection, and gene expression profiling was performed using an Affymetrix Human Gene 1.0 ST microarray platform.
GSE13670	Human monocyte-derived macrophages (hMDMs) were separated from fractions of peripheral blood mononuclear cells (PBMCs) obtained from the blood of healthy donors. Control and S. aureus-exposed macrophages were incubated at 37C for 8, 24, or 48 hours
GSE14390	HAM was exposed to B. anthracis Sterne spores at an MOI of 1 for 6 hours. RNA was extracted from HAM and analyzed by the Affymetrix Human Genome U133 Plus 2.0 Array. The transcriptional profile of Bacillus anthracis spore-treated HAM was compared with mock infected cells, and differentially expressed genes were identified.
GSE38194	MDMs (4 × 10.5 cells per assay) were incubated with *O. tsutsugamushi* at a bacterium-to-cell ratio of 20:1 for 8 hours. RNA samples (four samples per experimental condition) were processed for microarray analysis.
	Viruses Datasets
GSE13395	An HIV-1 spreading infection was established in primary human macrophages. RNA was extracted from both viral- and mock-infected macrophages cultures over 7 days and hybridized to Affymetrix HG-U95Av2 GeneChips for analysis.
GSE18816	Peripheral-blood leucocytes were separated from buffy coats of three healthy blood donors and cells were differentiated for 14 days before use. Differentiated macrophages were infected with H1N1 and H5N1 at a multiplicity of infection (MOI) of two. Total RNA was extracted from cells after 1, 3, and 6h post-infection, and gene expression profiling was performed using an Affymetrix Human Gene 1.0 ST microarray platform.
GSE27702	Human monocytes were isolated from buffy coats of unrelated, healthy German blood donors and human macrophages were obtained by cultivating isolated monocytes in Teflon bags in RPMI-1640 medium, supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% human AB serum provided by the Department of Transfusion Medicine, University of Muenster (Germany). Medium was substituted every 3 days, and cells were used in experiments on Day 7. After, Macrophages were infected with low (PR/8) and high pathogenic influenza viruses (FPV and H5N1).

GSE30723	Primary cultured cells were infected with or without influenza virus PR/8 at moi of 0.5. At 4h and 24h, total RNA were isolated for microarray experiments.
GSE31747	Primary human macrophages from three donors (D1, D2 and D3) were harvested at 1 h and 6 h after in vitro exposure to purified Ebola virions and compared to RNA from mock-exposed cells derived from the same donors.
GSE40711	Human MDM were mock-infected, or infected with H5N1 strains IDN3006 (low cytokine inducer), VN3028IIcl2 (high cytokine inducer), or IDN3006/cl2PA at a multiplicity of infection (MOI) of 2. At 6 hours post-infection (hpi), the cells were harvested and subjected to microarray analysis (three technical replicates per each group).

Abbreviations: HAM, human alveolar macrophages; MDM, monocyte-derived macrophages.

Supplementary Table 7: Summary description of datasets used to create asthma list.

GEO ID	Summary Description
GSE41649	This dataset aimed to demonstrate the relevance and validity of microarrays in the definition of allergic asthma expression pattern. The authors compared the transcript expressions of bronchial biopsy of 2 different microarray experiments done 2 years apart, both including non-allergic healthy and allergic asthmatic subjects (n = 4 in each experiment). The results demonstrated the relevance of microarray experiments using bronchial tissues in allergic asthma.
GSE46238	Sputum cells collected before (visit 2) and after (visit 4) allergen challenge in asthma patients were isolated and RNA purified for analysis on gene expression arrays.

PARTE III

DISCUSSÃO

Nesse trabalho buscamos não somente compreender se diferentes indutores apoptóticos induziam diferentes modulações nos macrófagos, mas também tentar obter ferramentas que pudessem auxiliar a caracterização dos diferentes fenótipos dessas células. Para isso, inicialmente verificamos o papel dos macrófagos no contexto clínico através de meta-análise. No âmbito da compreensão dos mecanismos moduladores dos macrófagos, objetivamos entender como a fagocitose de corpos advindos de células apoptóticas promovem a modulação dessas células. Para tanto, induzimos a morte celular com diferentes indutores (Taurina Cloramina, Cisplatina e Ácido-3-Bromopirúvico) na linhagem de CPNPC A549, procedemos à fagocitose dos corpos apoptóticos e analisamos o perfil de liberação de citocinas. Por fim, no intuito de possibilitar uma melhorar caracterização fenotípica dos macrófagos tanto *in vitro*, como *in vivo*, usamos ferramentas de bioinformática para padronizar duas assinaturas gênicas dos dois principais fenótipos dos macrófagos (M1-*like* e M2-*like*) e, além de verificamos sua robustez em análises de enriquecimento e através de RT-PCR, também utilizamos essas duas assinaturas para predizer genes que possam estar envolvidos no desfecho de determinadas patologias.

Os macrófagos são células do sistema imunológico que desempenham uma função crucial para o estabelecimento e progressão tumoral (Mantovani, Sozzani et al. 2002; Mosser and Edwards 2008; Gordon and Martinez 2010). Devido a essa importância, realizamos uma busca na literatura clínica para avaliar a real influência dos macrófagos e da apoptose no desfecho e sobrevida de pacientes com câncer de pulmão

de não pequenas células. Observou-se que, apesar de parecer bem consolidado o papel dos macrófagos para a progressão tumoral, havia discrepâncias em relação a real influência dessas células na sobrevida dos pacientes. Viu-se também que os diferentes fenótipos do macrófago e sua localização microanatômica são variáveis fundamentais na análise da sobrevida dos pacientes. Ainda, a correlação desses parâmetros com o estadiamento da neoplasia pulmonar é de suma importância no âmbito da compreensão do papel dos macrófagos, visto que a evolução tumoral promove mudanças importantes no microambiente que circunda o processo neoplásico. Por isso, observar se os macrófagos mudam seu fenótipo e, além disso, em qual compartimento eles se encontram (parênquima tumoral ou estroma), ao longo do desenvolvimento tumoral, é de suma importância para se analisar de forma concisa o papel dessas células no estabelecimento e na progressão da neoplasia pulmonar. No entanto, a falta de marcadores confiáveis para analisar o fenótipo dos macrófagos é um viés importante de confusão na hora de se fazer as análises. Conforme observamos, diferentes estudos disponíveis utilizaram marcadores distintos para proceder à análise. Por exemplo, alguns autores utilizaram, como marcação para o fenótipo pró-inflamatório (M1), a enzima óxido nítrico sintase induzível (iNOS) (Ohri, Shikotra et al. 2009; Zhang, Yao et al. 2011). Sabe-se que essa enzima não é exclusiva dos macrófagos, podendo marcar também células tumorais contribuindo, assim, para um possível viés na hora da análise dos dados. Essa dificuldade em se obter marcadores mais fidedignos se deve ao fato de que os macrófagos são células extremamente plásticas, variando em um espectro de ativação muito amplo de acordo com o microambiente. Por exemplo, apesar de apresentarem propriedades anti-inflamatórias, os macrófagos polarizados com IL-4 apresentam características diferentes daqueles polarizados por complexos imunes, apresentando marcadores de superfície diferentes entre eles (Mantovani, Sica et al.

2004; Murray, Allen et al. 2014) como, por exemplo, a expressão do receptor de manose em macrófagos estimulados com IL-4, e a expressão de CD86 nos estimulados com complexos imunes. Além disso, a capacidade de fagocitar células apoptóticas e corpos apoptóticos pelos macrófagos também promove a modulação fenotípica destes (Lauber, Blumenthal et al. 2004; Gregory and Pound 2011). Portanto, apesar da grande dificuldade em caracterizar, na metodologia da imunohistoquímica, os diferentes fenótipos dos macrófagos, nós utilizamos marcadores que avaliamos serem os melhores dentre os utilizados, baseando-se nos diversos trabalhos da nossa meta-análise.

Devido ao fato de que a fagocitose de corpos derivados de células apoptóticas promove a imunomodulação dos macrófagos (Lauber, Blumenthal et al. 2004; Ravichandran 2010; Gregory and Pound 2011), analisou-se a relevância do índice apoptótico na sobrevida dos pacientes com CPNPC. Apesar de termos encontrado discrepâncias também em relação a esse parâmetro, a maioria dos trabalhos analisados mostrou que, quanto maior o índice apoptótico, pior era o prognóstico e o desfecho dos pacientes. Isso pode estar relacionado ao fato de que, associado ao processo da apoptose, esteja ocorrendo uma alta taxa proliferativa das células neoplásicas (Van Slooten, Van de Vijver et al. 1998). Além disso, a fagocitose das células e dos corpos apoptóticos, possivelmente, possa estar modulando o fenótipo dos macrófagos para um perfil anti-inflamatório, o qual contribuiria para a progressão da neoplasia. Nessas análises, também verificamos que há discrepâncias em relação aos marcadores de apoptose utilizados, sendo que a técnica de TUNEL foi a mais utilizada. Apesar de bem estabelecida, essa técnica não confirma de forma satisfatória que a célula entrou no programa apoptótico, visto que esta mede a fragmentação do DNA e isso não ocorre exclusivamente em células apoptóticas, pois células necróticas, por exemplo, também o fragmentam (Kraupp, Ruttkay-Nedecky et al. 1995; Ribble, Goldstein et al. 2005). Em

vista disso, utilizamos como marcador de apoptose a caspase-3-clivada, no intuito de observamos com maior fidedignidade o processo apoptótico. Por fim, verificamos que nenhum trabalho havia associado todas as variáveis em questão (densidade de macrófagos, microlocalização anatômica, fenótipos e apoptose) com a sobrevida e desfecho dos pacientes com CPNPC.

Com o objetivo de elucidar de forma mais concisa o papel dos macrófagos e da apoptose no contexto clínico dos pacientes com CPNPC, analisou-se, através de imunohistoquímica, todas as variáveis citadas anteriormente em uma coorte de estágios iniciais da patologia (I e II). Nossos resultados mostraram que uma alta densidade de macrófagos ($CD68^+$) e sua localização no estroma tumoral estão relacionadas a um melhor prognóstico. Isso poderia estar relacionado ao fato de que os macrófagos, nos estágios iniciais da neoplasia, poderiam ter uma característica pró-inflamatória e, portanto, de resistência à progressão tumoral. Em contrapartida, a análise dos macrófagos $CD206^+$ (marcação "M2") não apresentou correlação com o desfecho e a sobrevida dos pacientes com CPNPC, possivelmente devido ao fato de que, no início do processo neoplásico, a modulação fenotípica dos macrófagos e do sistema imune pelo microambiente tumoral, e pelo próprio tumor, ainda não esteja completamente estabelecida. No que diz respeito à apoptose, nossa análise mostrou que, quanto maior a porcentagem de células apoptóticas, pior era o prognóstico do paciente. Conforme explicitado anteriormente, isso poderia estar relacionado ao fato de que uma maior taxa de apoptose tumoral estaria relacionada a uma maior taxa proliferativa neoplásica. Ainda, uma elevada quantidade de células apoptóticas implicaria em uma maior fagocitose pelos macrófagos o que, ao longo do curso da patologia, poderia modular seu fenótipo para o contexto anti-inflamatório e pró-tumoral. Isso somente reforça o fato de se proceder às análises dos macrófagos e da apoptose ao longo da evolução da

neoplasia, pois já foi demonstrado na literatura que a mudança fenotípica do macrófago está correlacionada com o estágio da neoplasia pulmonar (Zhang, Yao et al. 2011). Além disso, no intuito de implantar os macrófagos e a apoptose como possíveis biomarcadores prognósticos não somente para o câncer de pulmão, mas também para outras neoplasias, as análises deveriam ser feitas correlacionando todas as variáveis supracitadas. Em nossa análise, nós verificamos se havia essa correlação através da análise por *Log-rank (Mantel-Cox) Test*; no entanto, não encontramos significância, possivelmente pelo pequeno número amostral e por analisarmos somente casos iniciais da patologia, uma vez que há mudanças importantes no contexto neoplásico ao longo da evolução da patologia. Portanto, analisar os estágios iniciais e avançados, sob a ótica da progressão da doença é de fundamental importância para uma melhor compreensão dessas variáveis (densidade de macrófagos e apoptose) envolvendo todo o contexto de formação, estabelecimento e progressão do processo neoplásico.

Conforme descrito anteriormente, a fagocitose de células e corpos apoptóticos constitui-se em um fenômeno que, ao contrário do que se pensava anteriormente, exerce uma influência ímpar na modulação da resposta imunológica do hospedeiro (Lauber, Blumenthal et al. 2004; Kepp, Tesniere et al. 2009; Gregory and Pound 2011; Kroemer, Galluzzi et al. 2013). Por exemplo, a fagocitose de corpos derivados de células apoptóticas tumorais pelos macrófagos associados ao tumor (TAM) pode modular o contexto da resposta imune para um perfil anti-inflamatório e, ainda, de angiogênese e remodelação tecidual (Gregory and Pound 2011). Além disso, um tema bastante presente na literatura científica atual é sobre a capacidade imunomodulatória do processo apoptótico, em que, dependendo do estímulo indutor da apoptose, a fagocitose das células e corpos apoptóticos modula de forma diferente a resposta dos fagócitos. Por exemplo, células de carcinoma colorretal tratadas com o agente quimioterápico

oxaliplatina induzem à morte celular chamada de "imunogênica", pois a fagocitose dessas células promove a liberação de citocinas pró-inflamatórias principalmente pelas células dendríticas (Kroemer, Galluzzi et al. 2013). Então, a capacidade de resistência tumoral pode estar associada com o tipo de indutor de morte celular utilizado. Nesse contexto, buscamos compreender se diferentes indutores de apoptose poderiam modular diferentemente os fenótipos dos macrófagos no contexto da fagocitose. Utilizamos como drogas de escolha a cisplatina (agente alquilante), a taurina cloramina (oxidante fisiológico derivado de neutrófilos ativados) e ácido bromopirúvico (inibidor da via glicolítica), visto que são drogas indutoras do processo apoptótico, mas que apresentam mecanismos diferentes de ação. Ainda, buscamos estabelecer modelos de macrófagos humanos "*in vitro"* a partir de células tumorais (U-937 e THP-1), além do cultivo primário (MDM), para a melhor compreensão do fenômeno imunomodulador da fagocitose. Apesar de as linhagens diferenciadas apresentarem características muito semelhantes ao cultivo primário, como a morfologia, a parada da proliferação, a produção de interleucinas pró-inflamatórias e a expressão gênica de marcadores considerados pró e anti-inflamatórios, sua capacidade fagocítica é muito inferior à cultura primária, inviabilizando a análise da influência da fagocitose nessas células. No entanto, a observação de que as linhagens diferenciadas apresentam comportamentos semelhantes ao cultivo primário constitui-se em um dado de grande importância, pois poderá ser um facilitador da análise de comportamentos fenotípicos e genotípicos dos macrófagos a diversas variáveis que possam ser estudadas, visto que o cultivo celular de células tumorais humanas é muito menos trabalhoso do que o procedimento de cultivo primário. Além disso, torna-se muito mais relevante para a pesquisa biomédica analisar macrófagos-"*like*" humanos em detrimento de macrófagos de camundongos, pois se sabe que os macrófagos murinos apresentam características que divergem muito dos

macrófagos humanos. Como exemplo, pode-se citar a produção de óxido nítrico, em que, nos macrófagos murinos, é muito mais elevada do que nos macrófagos humanos. Além disso, há discrepâncias em relação à atividade de arginase, em que há atividade dessa enzima em macrófagos murinos e, nos macrófagos humanos, não há atividade (Bogdan 2002; Mestas and Hughes 2004; Schneemann and Schoeden 2007; Ingersoll, Spanbroek et al. 2010). Portanto, as duas caracterizações das linhagens THP-1 e U-937 serão de grande contribuição para futuras análises celulares.

Sabe-se que o crescimento descontrolado de tumores sólidos provoca a morte celular e, além disso, o regime quimioterápico utilizado para esses tipos de neoplasias também busca induzir a morte celular (da Silva, de Oliveira et al. 1996; Fuertes, Castilla et al. 2003). No entanto, para o câncer de pulmão de não pequenas células, a quimioterapia nos estágios mais avançados da doença apresenta uma resposta muito insatisfatória. Essa conjectura pode estar associada não somente à resistência tumoral, mas também ao fato de que talvez a morte celular causada por esses agentes promova a geração de células apoptóticas – as quais seriam fagocitadas - que poderiam modular o macrófago para um fenótipo pró-tumoral. Por essa razão, buscamos entender como a fagocitose de células apoptóticas obtidas de diferentes indutores poderiam modular os macrófagos. Para tanto, utilizamos cisplatina, taurina cloramina e ácido bromopirúvico. Ainda, denota-se de fundamental importância a obtenção de células em apoptose inicial, Isso se deve ao fato de que a célula em apoptose tardia tem perda na sua integridade de membrana (Herrmann, Voll et al. 1998; Darzynkiewicz, Bedner et al. 2009; Fernandez-Boyanapalli, McPhillips et al. 2010) e, com isso, poderia liberar moléculas intracelulares que ocasionariam um efeito pró-inflamatório, contribuindo como um viés para as nossas análises. Por isso, buscamos a melhor dose e o melhor tempo para se obter o maior número de células em apoptose inicial. Nossos resultados mostraram que

o ácido bromopirúvico e a taurina cloramina induziram morte celular exclusivamente apoptótica. Trabalhos anteriores já demonstraram que a taurina cloramina, apesar de muito se conhecer sobre seu papel protetor intracelular, também tem a capacidade de promover a oxidação de outras moléculas – em especial os resíduos de cisteína e metionina em proteínas, induzindo a morte celular através do processo apoptótico (Klamt, Zdanov et al. 2009). Embora já se tenha demonstrado que outros N-cloroaminoácidos causam morte celular em A549 (Robaszkiewicz, Bartosz et al. 2010), nenhum outro trabalho mostrara especificamente a morte celular causada pela taurina cloramina nessa linhagem celular. Além disso, com relação ao ácido-3-bromopirúvico, o tipo e o perfil de morte causado por ele, nessa linhagem, também não tinha sido caracterizado. Em seguida, buscamos verificar se a eficiência da fagocitose para os corpos derivados de células apoptóticas providas desses diferentes indutores era semelhante. De forma surpreendente, observamos que as células apoptóticas advindas do tratamento com taurina cloramina (média de 3,03%) eram menos fagocitadas quando comparadas às dos outros tratamentos (média de 11,28% para o Ácido Bromopirúvico e 13,8% para a Cisplatina). Um estudo de Anderson et al. (Anderson, Englert et al. 2002) mostrou que células apoptóticas obtidas pelo tratamento com peróxido de hidrogênio eram menos fagocitadas pelos macrófagos (MDM). Além disso, eles mostraram que não houve modificação na fosfatidilserina e, portanto, a falha no processo da fagocitose devia-se a outro fator. Outro estudo desse mesmo grupo demonstrou que a proteína S – uma proteína do soro dependente de vitamina K – é essencial para a fagocitose das células apoptóticas, uma vez que esta se liga a fosfatidilserina e, assim, promove a fagocitose (Anderson, Maylock et al. 2003; Uehara and Shacter 2008). Dessa forma, possivelmente a taurina cloramina esteja promovendo a oxidação dessa proteína dificultando, assim, a fagocitose das células apoptóticas. Pelo fato da taurina ter uma

alta concentração intracelular em leucócitos – aproximadamente 50 mM –, de a taurina cloramina ser secretada principalmente por neutrófilos ativados no combate ao tumor (Weiss, Klein et al. 1982; Kontny, Maśliński et al. 2003; Schuller-Levis and Park 2004) e do seu potencial de modulação imunológica como, por exemplo, propriedades anti-inflamatórias (Kim and Cha 2014), esta molécula apresenta um forte alvo potencial para estudos futuros. Ainda, o fato de induzir seletivamente a morte celular apoptótica, aliado à diminuição da capacidade fagocítica dos macrófagos, faz com que a taurina cloramina possa ter um papel ainda não elucidado, pois, se está ocorrendo uma menor taxa fagocítica, consequentemente poderá também haver uma menor modulação dos macrófagos por esse processo. Além disso, células que não são fagocitadas imediatamente após o início do processo apoptótico perdem sua integridade de membrana, liberando o conteúdo intracelular e, dessa forma, promovendo um contexto pró-inflamatório (Herrmann, Voll et al. 1998; Darzynkiewicz, Bedner et al. 2009; Fernandez-Boyanapalli, McPhillips et al. 2010).

A polarização dos macrófagos *in vitro* com indutores bem estabelecidos como, por exemplo, interferon-γ (IFN-γ) e LPS para um fenótipo mais pró-inflamatório e IL-4 para um anti-inflamatório, apresentaria um perfil de secreção de citocinas o qual se esperaria um determinado padrão. Por exemplo, da polarização com interferon-γ e LPS, esperar-se-ia um aumento da produção de IL-12, IL-8, IL-6, IL-1β, dentre outros (Gordon 2003; Mantovani, Sica et al. 2004). No entanto, quando analisamos a produção de citocinas pró-inflamatórias nas linhagens diferenciadas, verificamos que o comportamento dessas células frente ao estímulo com IFN-γ+LPS apresentou um padrão aquém daquele esperado, no qual a linhagem U-937 mostrou responder melhor a essa polarização, com aumento da produção de IL-6, IL-1β e TNF-α. Com relação aos MDM, o resultado foi ainda mais surpreendente, pois observamos somente diferença na

comparação M0 x M(IFNγ+LPS) e M(IFNγ+LPS) x M(IL-4) em relação à interleucina-6. Diagneautl e colaboradores demonstraram que os MDM podem secretar pequenas quantidades de determinadas citocinas mesmo quando ativados com determinados indutores (Daigneault, Preston et al. 2010). Além disso, a alta variabilidade que observamos entre os doadores pode ter contribuído para que não obtivéssemos diferenças em determinadas comparações. No entanto, mesmo não encontrando muitas diferenças no contexto da polarização dos macrófagos, procuramos verificar o efeito da fagocitose dos corpos derivados de células apoptóticas advindas dos diferentes indutores no perfil de liberação de citocinas. Nossos resultados demonstraram que, quando não polarizados, os macrófagos que fagocitaram as células apoptóticas produzidas pelo tratamento com cisplatina tiveram um aumento na produção de IL-10, uma interleucina imunossupressora. Em contrapartida, quando analisamos, nesse mesmo contexto, a produção de IL-12, uma interleucina pró-inflamatória, observou-se um discreto aumento na sua produção. Mesmo sabendo que é muito difícil termos um macrófago M0 no contexto fisiológico tumoral, esse resultado se denota de grande importância, visto que observamos que existem diferenças entre os indutores apoptóticos no contexto da modulação dos macrófagos pela fagocitose. Quando analisamos a produção de IL-6, encontramos um resultado ainda mais interessante: vimos que há uma diferença entre a cisplatina e a taurina cloramina no fenótipo M(IL-4), em que há uma produção aumentada nos macrófagos que fagocitaram as células apoptóticas induzidas pela taurina cloramina. Embora se saiba que, no contexto do microambiente neoplásico, existem diversos de fatores que contribuem para a modulação dos macrófagos, esse resultado pode suscitar a hipótese de que os macrófagos anti-inflamatórios, os quais são abundantes nos estágios mais avançados da neoplasia, ao entrar em contato com células mortas pela taurina cloramina, produzam

mais IL-6 contribuindo ainda mais para a progressão tumoral. Essa hipótese baseia-se no efeito pleotrópico dessa interleucina (Yao, Fenoglio et al. 2010; Yao, Huang et al. 2014). No que concerne à resposta imune contra patógenos e estresse agudo, a IL-6 atua como uma potente citocina pró-inflamatória (Yao, Huang et al. 2014). Contudo, o aumento ou a expressão desregulada dessa citocina contribuiu significativamente para a progressão tumoral (Yao, Huang et al. 2014). A IL-6 tem sido associada a um pior prognóstico no câncer de pulmão e também aos sintomas relacionados à neoplasia, como caquexia, anemia, dentre outros (Yao, Huang et al. 2014). Além disso, em linhagens de CPNPC, sua alta secreção pode causar resistência aos inibidores do EGFR, incluindo o *erlotinib* e o *gefitinib* (Yao, Fenoglio et al. 2010). Portanto, apesar dos seus efeitos pró-inflamatórios, a IL-6 também atua como um fator de progressão e resistência neoplásica e, nesse contexto, o aumento da liberação desta, pelos macrófagos, poderia se tornar um fator de pior prognóstico. No que concerne ao ácido-3-bromopirúvico, não encontramos diferenças, quando comparado aos outros indutores, na secreção de citocinas dos macrófagos que fagocitaram as células apoptóticas obtidas do tratamento com esse composto. Apesar disso, compostos que promovam alterações no metabolismo energético celular são muito promissores no combate aos tumores, visto que as células neoplásicas são muito dependentes desse metabolismo, principalmente no que diz respeito à via glicolítica. Nesse contexto, o ácido-3-bromopirúvico surge como um promissor candidato para tratamento dos tumores (Shoshan 2012). Por exemplo, um trabalho de Gong e colaboradores demonstrou a eficiência do ácido-3-bromopirúvico como um efetivo agente antitumoral em células de hepatoma, tanto *in vitro*, como *in vivo* (Gong, Wei et al. 2014). Ainda, observamos que o tratamento da linhagem A549 com esse composto induziu, em apenas duas horas, uma massiva morte celular. Em resumo, nossos resultados mostraram que há diferenças entre as células apoptóticas

advindas de diferentes indutores não somente na capacidade de fagocitose dos macrófagos, mas também no perfil de liberação de citocinas por estas células. Isso demonstra que, na hora de avaliar a capacidade antitumoral de um composto, faz-se necessária a avaliação do tipo de morte causada por ele e também da capacidade imunomodulatória deste, principalmente no que diz respeito aos macrófagos, pois estes têm uma forte influência na construção do microambiente tumoral.

Embora tenhamos conseguido com sucesso observar diferenças no padrão de liberação de citocinas pelos macrófagos expostos a diferentes corpos derivados de células apoptóticas, a caracterização fenotípica dessas células tanto *in vivo*, como *in vitro*, ainda é bastante complexa (Sica and Mantovani 2012; Becker, Muller et al. 2013). No intuito de auxiliar nessa intricada caracterização dos fenótipos dos macrófagos, buscamos, através de ferramentas de bioinformática, construir assinaturas gênicas robustas para os fenótipos dos macrófagos. Após diversas análises e escolhas de critérios de seleção, obtivemos duas assinaturas gênicas para os fenótipos M(IFNγ+LPS, TNF-α) e M(IL-4, IL-13), com 106 e 58 genes, respectivamente. Cabe ressaltar que, apesar de parecer um número alto de genes, eles são resultado de uma seleção de mais de quarenta mil genes em cada trabalho. Ao testarmos e validarmos essas duas assinaturas, verificamos que elas eram robustas e que caracterizavam consistentemente esses dois fenótipos dos macrófagos. Outro dado importante foi que, quando analisamos, através de enriquecimento gênico, o trabalho GSE51446, verificamos que, apesar de ter o valor de *n* de apenas um para cada indução, nossa assinatura M(IL-4, IL-13) enriqueceu para MDM tratados com IL-10, o que demonstra que essa assinatura tem o potencial de caracterizar macrófagos polarizados com outros indutores além da IL-4 e IL-13. Além disso, em todas as análises em que os macrófagos foram estimulados com IFNγ, TNF-α e LPS, sejam sozinhos, sejam combinados, a assinatura

M(IFNγ+LPS, TNF-α) enriqueceu sempre no fenótipo esperado. Então, de posse dessas assinaturas gênicas, escolhemos cinco genes M2 e seis M1, no intuito de validar, no contexto *in vitro*, os dados obtidos com a análise de bioinformática. Nossos resultados demonstraram que, tanto as linhagens celulares diferenciadas e polarizadas, quanto os MDM polarizados, apresentaram um perfil de expressão gênica de acordo com o esperado. Portanto, as assinaturas gênicas dos fenótipos dos macrófagos que encontramos e escolhemos podem auxiliar muitos pesquisadores a caracterizar e compreender o papel dos macrófagos em diversos cenários biológicos. Nesse contexto, nossas assinaturas não somente podem ser utilizadas para caracterizar os macrófagos *in vitro*, mas também para caracterizar determinados contextos clínicos como, por exemplo, a asma. Ainda, na busca de outras funcionalidades para a nossa assinatura, verificamos que esta também pode ser utilizada com a finalidade de prever se determinados genes podem ou não contribuir para o desfecho de determinadas patologias. Portanto, com essas duas assinaturas bem estabelecidas, buscamos tentar dissipar a dificuldade de caracterização dos macrófagos seja para avaliar o papel dos macrófagos em diversas patologias, seja na busca por novos marcadores que distingam de forma mais fidedigna os fenótipos dos macrófagos.

Nossos resultados demonstraram que o tipo de indutor apoptótico utilizado é de fundamental importância para a modulação dos macrófagos. Então, ao se criarem novas moléculas antitumorais, denota-se de suma importância que sejam analisados alguns parâmetros: o tipo de morte celular induzida, a eficiência da fagocitose dessas células mortas e o quanto essas moléculas poderiam influenciar na modulação dos macrófagos, uma vez que estas células são de fundamental importância para a progressão, resistência e metástases tumorais, além de contribuírem fortemente para o estabelecimento do microambiente neoplásico. Verifica-se, portanto, que o estudo do comportamento dos

macrófagos é vital não somente para a compreensão da implantação e evolução do processo neoplásico, mas também na busca por novos fármacos antitumorais.

CONCLUSÃO

O câncer de pulmão é um dos tipos de tumores que mais mata no mundo todo (Chen, Fillmore et al. 2014; Siegel, Ma et al. 2014). Dentre seus subtipos, o de não pequenas células é responsável por 85% dos casos (Chen, Fillmore et al. 2014). Além disso, o subtipo histológico adenocarcinoma é o mais prevalente. Apesar de todo o avanço tecnológico, seu tratamento ainda é muito ineficaz, pois esse tipo de neoplasia tem uma detecção muito tardia e, muitas vezes, os tumores desenvolvem resistência aos quimioterápicos (Detterbeck, Mazzone et al. 2013). Por isso, a busca por novos fármacos e novas estratégias de tratamento se faz extremamente necessária. Nesse contexto, a compreensão do microambiente tumoral é de suma importância. Uma das células responsáveis por auxiliar na implantação desse microambiente são os macrófagos, os quais, muitas vezes, produzem moléculas que auxiliam na progressão e metástase tumoral (Gordon and Martinez 2010; Wynn, Chawla et al. 2013). Devido à sua plasticidade, os macrófagos podem ser modulados por uma grande gama de fatores como, por exemplo, a fagocitose de corpos apoptóticos (Fernandez-Boyanapalli, McPhillips et al. 2010; Gregory and Pound 2011). Então, devido a grande letalidade do câncer de pulmão, a baixa ineficácia dos tratamentos atuais para os estágios mais avançados, a participação fundamental dos macrófagos na constituição do microambiente neoplásico e sua capacidade de modulação por corpos apoptóticos, objetivamos nesse trabalho verificar a influência do índice apoptótico e dos macrófagos na sobrevida e desfecho dos pacientes com CPNPC, além de verificar se diferentes indutores da apoptose produzem diferentes corpos apoptóticos modulando os macrófagos diferencialmente pela fagocitose destes. Nossos resultados demonstraram, inicialmente, que ainda não há um consenso da real influência do índice apoptótico e dos macrófagos na sobrevida e no desfecho dos pacientes com CPNPC. Ainda, nenhum

trabalho demonstrou a correlação dessas variáveis no desfecho/sobrevida dos pacientes com CPNPC. Por isso, buscamos verificar o papel da apoptose e dos macrófagos na sobrevida dos pacientes com CPNPC em uma coorte de estágios iniciais da patologia. Mostramos que a apoptose apresenta uma correlação na sobrevida, uma vez que uma alta densidade apresenta pior prognóstico. Quanto aos macrófagos, estes também apresentam uma correlação na sobrevida, em que uma alta densidade dos macrófagos está associada a um melhor prognóstico. Além disso, a microlocalização anatômica dessas células também apresenta uma correlação, visto que pacientes com uma alta densidade de macrófagos localizados no estroma tumoral apresentam pior prognóstico. No entanto, quando analisadas em conjunto, essas variáveis não demonstrram ter fator prognóticos no CPNPC.

Verificamos também que o tratamento com as três diferentes moléculas, nos tempos e doses escolhidos, culminou com a morte celular predominantemente apoptótica. No que concerne à caracterização das linhagens e dos MDM, verificamos que as linhagens U937 e THP-1 diferenciadas apresentam características morfológicas, proliferativas e bioquímicas diferentes das células não diferenciadas, além da polarização dessas células apresentar diferentes perfis bioquímicos e de secreção de citocinas. Além disso, mostramos que as linhagens diferenciadas não somente apresentam um perfil de expressão gênica bastante análogo aos MDM, mas também possuem características morfológicas muito semelhantes a estes. Para analisar a influência dos corpos derivados de células apoptóticas advindos de diferentes tratamentos, nós avaliamos a eficiência da fagocitose e o perfil de citocinas dos macrófagos expostos e não expostos aos corpos. Nossos resultados mostraram que existe uma diferença não somente na eficiência da fagocitose dos corpos providos de diferentes tratamentos, mas também no perfil de liberação de citocinas dos macrófagos

expostos a estes corpos. No entanto, mais estudos são necessários para que se possa compreender não só o papel dos macrófagos na progressão tumoral, mas também a influência de determinados compostos na imunomodulação e consequente progressão neoplásica. Por fim, devido a grande complexidade de caracterização dos fenóitpos dos macrófagos humanos *in vitro*, mas, principalmente, *in vivo*, buscamos, por meio de bioinformática e análises de RT-PCR, estratégias que pudessem auxiliar nessa caracterização. Através da obtenção de duas assinaturas gênicas, mostramos que estas são capazes de caracterizar os macrófagos tanto *in vitro*, como em diversas patologias clínicas, mostrando o grande potencial que estas assinaturas podem ter para elucidar o papel dos macrófagos em diversos contextos biológicos.

Verifica-se, portanto, que os macrófagos são células que desempenham uma enorme gama de funções não somente na manutenção da homeostasia, mas também na severidade e no avanço de determinadas patologias, como o processo neoplásico. Por isso, os macrófagos apresentam um alto potencial de estudo para que possamos compreender de forma mais precisa o envolvimento dessas células nas patologias humanas.

PERSPECTIVAS

Para a finalização deste trabalho, temos as seguintes perspectivas:

- Realizar RT-PCR dos macrófagos derivados de monócitos humanos (cultivo primário) expostos às células apoptóticas advindas dos diferentes indutores.

- Aumentar o número amostral da coorte, além de analisar a influência de todos os parâmetros relacionados aos macrófagos, e da apoptose, em coorte de pacientes com câncer de pulmão de não pequenas células em estágios mais avançados da doença.

- Avaliar o perfil, em termos de imunogenicidade, da morte celular induzida pela cisplatina, taurina cloramina e ácido bromopirúvico, sozinhos e em combinação, na linhagem de adenocarcinoma A549.

- Analisar a influência do meio condicionado, com 0,5% de Soro Fetal Bovino, dos macrófagos, na viabilidade da linhagem A549.

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ANEXOS

Tabela 1: *ECOG PERFORMANCE STATUS.*

Grau	ECOG (*Eastern Cooperative Oncology Group)*
0	Totalmente ativo. Capaz de realizar todas as performances pré-doença sem restrições.
1	Restrições em relação a atividades extenuantes. Capaz de realizar trabalhos de natureza leve ou sedentária.
2	Ambulatorial e capaz de se cuidar, mas incapaz de realizar qualquer atividade laboriosa.
3	Capaz de alguns autocuidados limitados. Confinado à cama ou à cadeira mais de 50% das horas em que o indivíduo está acordado.
4	Completamente incapaz. Totalmente confinado à cama ou à cadeira.
5	Morto

Adaptado de: Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am. J. Clin. Oncol. 5:649-655, 1982.

Tabela 2: Estadiamento do Câncer de Pulmão

T—Tumor Primário
TX: O tumor primário não pode ser avaliado, ou o tumor é comprovado pela presença de células malignas no escarro ou de lavagem brônquica, mas não visualizado por imagem ou broncoscopia.
T0: Sem evidência de tumor primário;
Tis: Carcinoma *in situ*
T1: Tumor de três centímetros ou menos em sua maior dimensão, rodeado pelo pulmão ou pleura visceral, sem evidência broncoscópica de invasão mais proximal do que o brônquio do lobo (ou seja, não no brônquio principal).
T1a: Tumor de 2 cm ou menor em sua maior dimensão;
T1b: Tumor maior do que 2 cm, mas não maior do que 3 cm em sua dimensão maior.
T2: Tumor maior do que 3 cm, mas não maior do que 7 cm em sua dimensão maior, ou sem qualquer uma das seguintes características: envolvendo o brônquio principal, 2cm ou mais distal da Carina; invasão visceral da pleura; associação com atelectasia ou pneumonite obstrutiva que se estende para a região hilar, mas não envolve a pulmão inteiro.
T2a: Tumor maior do que 3 cm, mas não maior do que 5 cm em sua dimensão maior;
T2b: Tumor maior do que 5 cm, mas não maior do que 7 cm em sua dimensão maior;
T3: Tumor maior do que 7 cm, ou que invade qualquer uma das seguintes estruturas: caixa torácica (incluindo o sulco superior do tumor), diafragma, nervo frênico, pleura

mediastinal, pericárdio parietal; ou tumor no brônquio principal com menos de 2 cm da carina, mas envolvimento desta, associado a atelectasia ou pneumonite crônica do pulmão inteiro, ou nódulo(s) tumorais separados no mesmo lobo pulmonar.

T4: Tumor de qualquer tamanho que invada qualquer uma das seguintes estruturas: mediastino, coração, grandes vasos, traqueia, nervo laringeal recorrente, esôfago, corpo vertebral, carina, nódulos tumorais separados em localização ipsilateral do lobo.

N—Linfonodos Regionais

NX: Os linfonodos regionais não podem ser avaliados.

N0: Sem metástase nos linfonodos;

N1: Metástase nos gânglios ipsilaterais peribrônquicos e/ou ipsilaterais hilares linfáticos e gânglios intrapulmonares, incluindo a participação por extensão direta.

N2: Metástase em nódulos linfáticos (s) mediastinal e/ou subcarinal ipsilateral;

N3: Metástase em mediastino contralateral, hilar contralateral, escaleno ipsilateral ou contralateral, ou linfonodo (s) supraclavicular (es);

M—Metástases distantes

M0: Sem metástase distante;

M1: Com metástase distante;

M1a: Nódulo tumoral separado (s) em um lobo contralateral; tumor com nódulos pleurais, maligno da pleura, ou com derrame pericárdico;

M1b: Metástase distante;

Os agrupamentos de fase resultantes são:

Carcinoma oculto: TX, N0, M0
Stage 0: Tis/N0/M0
Stage IA: T1a,b/N0/M0
Stage IB: T2a/N0/M0
Stage IIA: T2b/N0/M0; T1a,b/N1/M0; T2a/N1/M0
Stage IIB: T2b/N1/M0; T3/N0/M0
Stage IIIA: T1a,b, T2a,b, N2/M0; T3/N1, N2/M0; T4/N0, N1/M0
Stage IIIB: T4/N2/M0; qualquer T/N3/M0
Stage IV: Qualquer T, qualquer N/M1

Fonte: American Joint Committee on Cancer. Site: cancerstaging.org., acesso em 18/02/2015.

Tabela 3: Condições fisiológicas e patológicas envolvendo o mecanismo da apoptose.

Condições Fisiológicas
Morte celular programada no desenvolvimento embrionário com o objetivo de modelamento tecidual.
Involução fisiológica como, por exemplo, descamação do endométrio, regressão das glândulas mamárias após a amamentação.
Destruição normal das células acompanhada pela substituição por proliferação, como no epitélio do intestino.
Involução tímica durante o avançado da idade.

Condições Patológicas
Indução de morte celular por drogas quimioterápicas.
Morte celular induzida pela citotoxidade promovida pelos linfócitos T $CD8^+$.
Progressiva morte celular e depleção das células $CD4^+$ em doenças autoimunes.
Morte celular em doenças neurodegenerativas, como o Alzheimer e Parkinson.
Morte celular decorrente de doenças cardíacas, como o infarto do miocárdio.
Algumas formas de morte celular induzida por vírus, como hepatite B e C.
Morte celular induzida por agentes danosos, como radiação e hipóxia.

Adaptado de Apoptosis in cancer: from pathogenesis to treatment, Journal of Experimental & Clinical Cancer Research 2011.

PROJETO VENCEDOR DO PRÊMIO BD Biosciences Pesquisador SBI 2013

Caracterização das células apoptóticas na ativação alternativa de macrófagos e o efeito sobre a agressividade de câncer de pulmão de não-pequenas células

Florencia María Barbé-Tuana

MATHEUS BECKER FREITAS
Curriculum Vitae

Julho/2015

Matheus Becker Freitas

Curriculum Vitae

Dados pessoais

Nome Matheus Becker Freitas
Filiação Maurício Aristóteles Freitas e Janise Becker
Nascimento 31/08/1982 - Porto Alegre/RS - Brasil
Carteira de Identidade: 5056433641 SJS - RS - 25/08/2008
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Formação acadêmica/titulação

2011 Doutorado em Ciências Biológicas (Bioquímica).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: PAPEL DAS CÉLULAS APOPTÓTICAS NA ATIVAÇÃO ALTERNATIVA DE MACRÓFAGOS E SEU EFEITO SOBRE A AGRESSIVIDADE DE CÂNCER DE PULMÃO DE NÃO-PEQUENAS CÉLULAS
Orientador: Fábio Klamt
Bolsista da: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2009 - 2011 Mestrado em Ciências Biológicas (Bioquímica).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Papel da Proteína Cofilina-1 na Resistência à Cisplatina em Câncer de Pulmão de Não-Pequenas Células, Ano de obtenção: 2011
Orientador: Fábio Klamt
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

2013 Especialização em CURSO DE ESPECIALIZAÇÃO EM ANÁLISES CLÍNICAS.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2005 - 2009 Graduação em Biomedicina.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Avaliação do polimorfismo dinucleotídico (GT)n da região promotora da enzima heme oxygenase I em pacientes com artrite idiopática juvenil
Orientador: José Artur Bogo Chies

Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

Graduação interrompida em Administração em Sistemas e Serviços de Saúde.
Universidade Estadual do Rio Grande do Sul, UERGS, Porto Alegre, Brasil
Ano de interrupção: 2004

Formação complementar

2013 - 2013 Curso de curta duração em Curso de Português Extensivo.
Centro de Treinamento em Concursos, CETEC, Brasil

2013 - 2013 Curso de curta duração em Interpretação de Exames Laboratoriais.
Portal Educação, PE, Brasil

2008 - 2008 Extensão universitária em Curso de Cultivo de Células Tronco.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2007 - 2007 Extensão universitária em Ferramentas de Bioinformática Aplicada a Imuno.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2007 - 2007 Extensão universitária em Atualização em Imunologia.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2005 - 2005 Extensão universitária em I Curso de Manipulação Genética.
Hospital de Clínicas de Porto Alegre, HCPA, Porto Alegre, Brasil

2005 - 2005 Extensão universitária em Atividade de Extensão do Departamento de Química.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Atuação profissional

1. Hospital de Clínicas de Porto Alegre - HCPA

Vínculo institucional

2004 - 2005 Vínculo: Celetista , Enquadramento funcional: Auxiliar Administrativo II , Carga horária: 40, Regime: Integral

2. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2011 - Atual Vínculo: Livre , Enquadramento funcional: Bolsista de Doutorado , Carga horária: 40, Regime: Dedicação exclusiva

2009 - 2011 Vínculo: Livre , Enquadramento funcional: Bolsista de Mestrado, Regime: Dedicação exclusiva

3. Universitário Escola Técnica - UNITEC

Vínculo institucional

2010 - 2014 Vínculo: Livre , Enquadramento funcional: Professor , Carga horária: 12, Regime: Parcial

Áreas de atuação

1. Imunologia Aplicada
2. Genética Molecular e de Microorganismos
3. Fisiologia Celular
4. Genética
5. Bioquímica
6. Biofísica

Prêmios e títulos

2013 Concurso BD Biosciences Pesquisador SBI, Sociedade Brasileira de Imunologia

Producão

Produção bibliográfica
Artigos completos publicados em periódicos

1.

2. MULLER, C. B., BASTIANI, M. A., **BECKER, MATHEUS**, CASTRO, M. A., FRANCA, F. S., BRANCO, M. A.
Potential crosstalk between cofilin-1 and EGFR pathways in cisplatin resistance of non-small-cell lung cancer. OncoTarget. , v.28, p.3531 - 3539, 2015.

3. **BECKER, M**., MULLER, C. B., BASTIANI, M. A., KLAMT, F.
The prognostic impact of tumor-associated macrophages and intra-tumoral apoptosis in non-small cell lung cancer.. Histology and Histopathology. , v.29, p.21 - 31, 2014.

4. **BECKER, MATHEUS**, BASTIANI, MARCO ANTÔNIO, MÜLLER, CAROLINA BEATRIZ, MARKOSKI, MELISSA M., CASTRO, MAURO ANTÔNIO A., Klamt, Fábio High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas. Tumor Biology. , v.35, p.1164-6 - 2013.

5. Zanotto-Filho, Alfeu, Delgado-Cañedo, Andrés, Schröder, Rafael, **Becker, Matheus,** Klamt, Fábio, Moreira, José Cláudio Fonseca
The pharmacological NFkB inhibitors BAY117082 and MG132 induce cell arrest and apoptosis in leukemia cells through ROS-mitochondria pathway activation. Cancer Letters (Print). v.288, p.192 - 203, 2010.

Orientações e Supervisões

Orientações e supervisões em andamento

Dissertações de mestrado: co-orientador

1. José Inácio Gonzalez Solari. **Avaliação da combinação quimioterápica na indução de morte imunogênica no tratamento do Câncer de Pulmão de Não Pequenas Células**. 2015. Dissertação (Ciências Pneumológicas) - Universidade Federal do Rio Grande do Sul