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Pós-Graduação *Strictu Sensu* em Biologia Parasitária

ELVIA JANETH OSORIO ESPARZA

Os fatores de crescimento e a interleucina-4 convergem para ativar o fator de transcrição STAT6 e a expressão da arginase na leishmaniose visceral experimental

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária

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LISTA DE SIGLAS E ABREVIATURAS

AMPc	Adenosina monofosfato cíclico,
AP-1	Proteína ativadora-1
	do inglês: *activator protein-1*
ARNm	Ácido ribonucléico mensageiro
Arg-1	Arginase-1
Arg-2	Arginase-2
ATF-2	Fator ativador de transcrição 2
	do inglês: *activating transcription factor 2*
CBP	Proteína de ligação ao cAMP
CCR7	Receptor de quimiocina CCR7
C/EBP-β	CAAT/proteína de ligação potenciador-β
	do inglês: *CAAT/enhancer binding protein- β*
CCL5	Quimioquina (motivo C-C) do ligante5
CD	Conjunto de diferenciação
c-Fos	Proto-oncogene c-Fos
c-Jun	Proto-Oncogene c-Jun
CAT2B	Transportador catiônico de aminoácidos-2B
	do inglês: *Cationic aminoacid transporter-2B*
Cox-2	Citocromo c oxidase subunidade 2
CREB	Proteína de ligação ao elemento de resposta do AMPc
ERK1/2	Quinase regulada por sinal extracelular
EGFR	Receptor do fator de crescimento de epiderme
	do inglês: *Epidelmal growth factor receptor*
FGF	Fator de crescimento de fibroblasto
	do inglês: *Fibroblast growth factor*
FGFR	Receptor do fator de crescimento de fibroblasto
	do inglês: *Fibroblast growth factor receptor*
Fizz1	Proteína homóloga relacionada - Fizzy
FRS2	Receptor do substrato de fator de crescimento de fibroblasto 2
GAB1	Proteína de ligação associada ao receptor do fator de frescimento
G-CSF	Fator estimulante de colônias de granulócitos
	inglês: Granulocyte colony stimulating factor
GH	Hormônio do crescimento
	do inglês: *growth hormone*

GRB2	Proteínas de ligação do receptor de fator de crescimento 2 do inglês: *Growth factor receptor-bound protein 2*
Gr-1	Antígeno de diferenciação mielóide Gr-1
HB-EGF	Fator de crescimento de epiderme de ligação à heparina do inglês: *Heparin-binding epidermal growth factor-like growth factor*
HIF	Fator indutor de hipóxia do inglês: *Hypoxia-inducible factor*
HLA	Antígeno de histocompatibilidade de classe II
HIV	Vírus da imunodeficiência humana
ELK-1	Proteínas contendo o domínio ETS do inglês: *ETS domain-containing protein Elk-1*
Ephs	Receptor tipo efrina
IFN	Interferon do inglês: *Interferon*
IRF	Fator regulador de interferon do inglês: *Interferon regulatory factor*
IGF-I	Fator de crescimento semelhante à insulina do inglês: *Insulin-like growth factor-I*
I-R	Receptor de insulina do inglês: *Insulin receptor*
IGFBP	Proteína de Ligação do Fator de Crescimento Similar à Insulina do inglês: *Insulin-like Growth Factor Binding Protein*
IGF-IR	Receptor do Fator de Crescimento Similar à Insulina-I do inglês: *Insulin-like Growth Factor Receptor-I*
IL	Interleucina
JAK	Quinase Janus
iNOS	óxido nítrico sintetase induzida do inglês: *Inducible Nitric oxide*
IRS-1	Receptor do substrato de Insulina do inglês: *Insulin Receptor Sustrate*
KLF4	Fator Similar ao Krueppel-4 do inglês: *Krueppel-like factor 4*
LDLR	Receptor de Lipoproteína de Baixa Densidade do inglês: *Low-density Lipoprotein Receptor*
LPS	Lipopolissacarídeo
LV	Leishmaniose visceral

LXR	Receptor X do Fígado do inglês: *Liver X Receptor*
M1	Macrófagos M1
M2	Macrófagos M2
MAPK	Proteínas Quinase Ativada por Mitógenos do inglês: *Mitogen Activated Protein Kinase*
MDSC	Células mielóides supressoras
MEK1/2	Proteína Quinase de Dupla Especificidade Ativada por Mitógeno
MHC II	Complexo de Histocompatibilidade de Classe II
Mrc1	Receptor de Manose 1 de Macrófagos
MSP	Proteína Estimuladora do Macrófago do inglês: *Macrophage Stimulating Protein*
Myd88	Proteína de Resposta Primária de Diferenciação Mielóide
NF-κB	Fator Nuclear kappa-B
NK	Célula Natural Killer
NKT	Célula T Natural Killer
NOS2	óxido Nítrico Sintase 2
NRAMP1	Proteína 1 do Macrófago Associada à Resistência Natural do inglês*: Natural Resistance-Associated Macrophage Protein 1*
OAT	Ornitina Aminotransferase
ODC	Ornitina Decarboxilase
p38	Proteína Quinase Ativada por Mitogénio p38
PDGF	Fator de Crescimento de Plaquetas do inglês: *Platelet-Derived Growth Factor*
PDGF-R	Receptor do Fator de Crescimento de Plaquetas do inglês: *Platelet-Derived Growth Fator Receptor*
PI3K	Fosfatidil Inositol 3-Quinase do inglês: *Phosphatidyl Inositol 3-Kinase*
PPAR	Receptor Ativado por Proliferador do Peroxissomo do inglês: *Peroxisome Proliferator Activated Receptor*
PR	Receptor de Progesterona do inglês: *Progesterone Receptor*
PU.1	Fator de Caixa de Purina
qPCR	Reação em Cadeia de Polimerase em Tempo Real
RELM	Molécula Similar à Resistina do inglês: *Resistin-Like Molecule*
RonRTK	Receptor de Tirosina Quinase Ron do inglês: *Ron Receptor Tyrosine Kinase*

RTK	Receptor de Tirosina Quinase do inglês: *Tyrosine Kinase Receptor*
RXR	Receptor de Retinóide X do inglês: *Retinoid X Receptor*
SHP-1	Proteína-Tirosina-Fosfatase
SLC11A1	Portador de Soluto da Familia 11 Membro 1
STAT	Transdutor de Sinal e Ativador da Transcrição do inglês: *Signal Transducer and Activator of Transcription*
TCR	Receptor de células T
TGF	Fator de Transformação do Crescimento do inglês: *Transforming Growth Factor*
Th1	Linfócito T Auxiliar 1
Th2	Linfócito T Auxiliar 2
TLR	Receptores do Tipo Toll do inglês: *Toll-Like Receptor*
TNF	Fator de Necrose Tumoral do inglês: *Tumor Necrosis Fator*
Ub	Ubiquitina
v-AKT	Oncogene Viral Homólogo Murino 1
VDR	Receptor de Vitamina D do inglês: *Vitamin D Receptor*
VEGF	Fator de Crescimento Vascular e Endotelial do inglês: *Vascular Endothelial Growth Factor*
VEGFR	Receptor do Fator de Crescimento Vascular e Endotelial do inglês: *Vascular Endothelial Growth Fator Receptor*
YM1	Proteína Secretada Ym1

LISTA DE SÍMBOLOS

mL-	Mililitro
µL-	Microlitro

RESUMO

TESE DE DOUTORADO POS-GRADUAÇÃO EM BIOLOGIA PARASITÁRIA

Elvia Janeth Osorio Esparza

A expressão de arginase-1 (Arg1) está associada com a patogênese da leishmaniose visceral (LV), embora os mecanismos que norteiam a sua regulação na doença não sejam adequadamente conhecidos. Nesta tese foram explorados os mecanismos de regulação da Arg-1 no modelo experimental hamster, a fim de aplicar o conhecimento para o tratamento da doença. Encontrou-se que células infectadas in vitro com *Leishmania (Leishmania) donovani* expressaram Arg-1 e inhibiram a produção do óxido nítrico, o que contribuiu para o aumento da carga parasitária. A regulação da Arg1 dependeu do fator de transcrição STAT-6, que foi ativado de forma independente da IL-4. Para explorar outros mecanismos envolvidos na ativação de STAT-6 foi utilizada uma biblioteca quimica de 80 inibidores dos receptores tirosine quinases (RTK) e uma membrana microarranjo revestidas com anticorpos contra proteínas phosphoriladas dos RTK. Desta forma, foi possível identificar a ativação do receptor do fator de crescimento de fibroblasto-1 (FGFR-1) e o receptor do fator de crescimento semelhante à insulina tipo I (IGF-IR) nos macrofagos. Além disso, realizou-se uma caracterização mais detalhada da cascata de fosporilação canônica dessos receptores. Experimentos *in vitro* mostraram que macrófagos infectados com *L. donovani* aumentaram a transcrição de Arg1 quando foram tratados com os ligantes dos RTK, o FGF-2 e o fator de crescimento semelhante à insulina tipo 1 (IGF-I), sugerindo que estes RTK tinham um papel na regulação da Arg1 na LV. Quando inibidores químicos do FGFR-1 e IGF-IR foram testados em macrófagos infectados, a Arg1 e o STAT-6 foram inibidos sugerindo uma ligação entre essas vias. De fato, a estimulação com FGF-2 e IGF-I aumentou a fosforilação e a ativação transcripcional do STAT-6, e se observou um efeito aditivo quando a citocina IL-4 foi adicionada, demonstrando a interação positiva destas vias. A inibição do STAT-6 mediante interferência de RNA impediu ou diminuiu significativamente a Arg-1 induzida nos macrófagos infectados e estimulados com FGF-2 e por IGF-I. respectivamente, indicando um papel chave do STAT-6 na Arg-1 induzida pelos RTK. A relevância destes achados experimentais para os seres humanos foi avaliada em cultivos de sangue total de doadores sadios estimulados *in vitro* e no plasma de crianças durante a doença ativa, um mês, seis meses e até um ano após tratamento. Foi observado um aumento de Arg-1 após o estimulo *in vitro* com IL-4 e FGF-2 ou IGF-I, consistente com o achado no modelo hamster. Nos pacientes, os níveis plasmáticos de Arg-1 não mudaram com a evolução da doença após e o tratamento e o FGF-2 só apresentou um aumento associado com a resolução um ano após tratamento. Em conclusão, os sinais comuns ativados através do FGF-1R e IGF-IR convergem com a IL-4 para ativar STAT-6 e a transcrição de Arg-1 na LV experimental, mas determinar o papel destes fatores de crescimento na LV humana requer estudos adicionais. Este estudo propõe a base mecanicista da regulação da Arg1 mediada pela convergência da sinalização RTK e IL-4R ao STAT-6 na LV e sugere que os inibidores RTK são de utilidade potencial como tratamento adjuvante da doença.

INSTITUTO OSWALDO CRUZ

Growth Factors and IL-4 Cytokine Converge at STAT6 to Promote Arginase Expression in Experimental Visceral Leishmaniasis

ABSTRACT

PHD THESIS IN PARASITE BIOLOGY

Elvia Janeth Osorio Esparza

Arginase-1 (Arg1) expression is associated with the pathogenesis of visceral leishmaniasis (VL) but the mechanisms that lead to its up-regulation on disease are not completelly known. The objective of this thesis project was to explore the mecanistic basis of Arg-1 regulation in the experimental hamster model of VL with the aim to apply the knowledge to the treatment of the disease. We found that cells infected *in vitro* with *Leishmania donovani* expressed Arg1 and that nitric oxide production was inhibited, contibuting to the increased parasite burden. Arg-1 regulation was dependent of the transcription factor STAT-6, but the activation of this transcription factor was independent of IL-4 cytokine. To explore other mechanisms involved in activation of STAT-6 and the expression of Arg1 we used a chemical library of 80 tyrosine kinase receptor (RTK) inhibitors and a protein array with antibodies against phosphorylated RTKs. This allowed identify the activation of fibroblast growth factor receptor-1 (FGFR-1) and insulin-like growth factor receptor (IGF-IR) and the canonical down-stream signaling cascade. Macrophages infected in vitro with *L. donovani* increased transcription of Arg1 when treated with RTK ligands (FGF-2, IGF-I), suggesting a role of these RTKs in the regulation of the Arg1 in LV. Chemical inhibitors of the activated receptors were used to assess the effect on infected macrophages. We found that inhibitors of FGFR-1 and IGF-IR inhibited parasite burden, Arg1 and STAT-6, suggesting a connection between these routes. Furthermore, the simultaneous stimulation of RTKs with its ligands FGF-2 or IGF-I and IL-4 increased the phosphorylation and transcriptonal activation of STAT-6 and increased Arg1 in an additive manner, showing a positive interaction between these signaling cascades. STAT-6 inhibition by RNA interference prevented or significantly diminished the induction of Arg-1 in macrophages stimulated with FGF-2 and IGF-I respectively, indicating a key role of the STAT-6 on Arg-1 induced by RTKs. The relevance of these experimental findings to humans was evaluated in stimulated whole blood cultures of healthy donor's or plasma of children during active disease, 1 month, 6 months or after 1 year of treatment. We found that the Arg-1 induced by *in vitro* stimulation with IL-4 and FGF-2 or IGF-I, was consistent with the animal model. However, in the patients plasmatic Arg-1 did not change over the follow-up, while FGF-2 increased only after 1 year of treatment. We conclude that the signals activated through FGFR-1 and IGF-I converge to IL-4 to activate STAT-6 and induce Arg-1 in experimental VL. This study propose that the mecanistic basis of the regulation of Arg1 in VL is mediated by the convergence of RTK signaling pathways and IL-4R to STAT-6 and suggest the potential utility of RTK inhibitors as adjuvant treatment for the disease.

1. INTRODUÇÃO

1.1. Importância epidemiológica da leishmaniose visceral.

A leishmaniose visceral (LV) é uma infecção causada pelos parasitos intracelulares pertencentes ao domínio Eukaryota, Reino Protozoa (*Apud*, Goldfuss, 1818; R. Owen, 1858), Infra Reino Excavata (*Apud*, Cavalier-Smith, 2002), Filo Euglenozoa (*Apud*, Cavalier-Smith, 1981), Subfilo Saccostoma (*Apud*, Cavalier-Smith, 1998), Classe Kinetoplastea (*Apud*, Honigberg, 1963; L. Margulis, 1974), Ordem Trypanosomatida (*Apud*, Kent, 1880; Hollande, 1952), Família Trypanosomatidae (*Apud*, Doflein, 1901), Gênero Leishmania (*Apud*, Ross, 1903), Subgênero *Leishmania*, e pelas espécies *L. (L.) infantum* e *L. (L.) donovani* (Bañuls *et al*. 2007). A primeira ocorre no continente Americano, no Mediterrâneo, no Oriente Meio, na África Ocidental e na Ásia Central, emquanto que a *L. donovani* ocorre na Índia e na África (Van Griensven & Diro 2012). A LV é reconhecida como uma doença negligenciada pelo sistema de saúde, pois os focos endêmicos estão localizados em locais remotos habitados por populações pobres. Esta situação faz com que exista registro insuficiente dos casos e que os pacientes mais graves tenham dificuldades de acesso à assistência médica.

Cerca de 90% dos casos de LV no mundo ocorrem na Índia, Bangladesh, Sudão, Etiópia e o Brasil, com um total de 10,000-20,000 mortes no ano (Alvar *et al*. 2012), equivalente a 10% dos casos. O Brasil é o país com maior registro de casos na América do Sul, e apresenta uma taxa de 0.15 mortes no ano por 100.000

habitantes, equivalente a 8.1% de casos fatais nos anos 2010-2011 (Martins-Melo *et al*. 2014). A região do Brasil mais atingida pela LV é a região Nordeste onde ocorrem cerca de 50% dos casos. A incidência está relacionada com práticas da população e fatores, como condições precárias socioeconômicas, mudanças ambientais, assentamentos humanos não planejados, migrações de populações junto com cães e migração de outros reservatórios secundários do ambiente silvestre ao rural, além de desmatamento. Estes fatores em conjunto trazem como consequência a adaptação dos vetores insetos flebotomíneos e reservatórios do ambiente silvestre ao ambiente peridomiciliar e domiciliar, favorecendo, em conseqüência, a transmissão do parasito (Harhay *et al*. 2011, Desjeux 2004, Werneck 2010).

1.2. Características clínicas e fatores que determinam a susceptibilidade à leishmaniose visceral.

A LV pode ocasionar desde uma infecção subclínica até uma doença potencialmente fatal, dependendo de múltiplos fatores que medeiam a susceptibilidade do hospedeiro. Os indivíduos susceptíveis desenvolvem a doença caracterizada por aumento do fígado e baço, onde pode ser observada a replicação ativa de grande número de parasitos que são liberados no tecido pelos macrófagos infectados. A infecção que também é evidente na medula óssea e causa enfraquecimento sistêmico, pois causa redução de outras populações celulares sanguíneas como eritrócitos, causando anemia, linfopenia, e deficiências em plaquetas e neutrófilos, culminando finalmente com a imunossupressão (Kumar & Nylen 2012). Em estados

avançados da doença ocorrem manifestações clínicas como febre, perda de peso, hepatoesplenomegalia, hipergamaglobulinemia, hipoalbuminemia, caquexia, disfunção hepática, icterícia, ascite, escurecimento da pele ou kala-azar (originando o nome da doença de febre negra, em hindu), edema, desnutrição, diarreia, trombocitopenia, hemorragia da mucosa intestinal e septicemia (Costa *et al*. 2010, van Griensven & Diro 2012). Estima-se que o risco de morte aumenta 3 - 4 vezes quando as crianças apresentam manifestações tais como respiração rápida, sangue na mucosa e gengiva e infecções bacterianas concomitantes. Ao mesmo tempo quando a contagem de neutrófilos e plaquetas é baixa, o risco de morte pode aumentar 3 - 12 vezes (Sampaio *et al*., 2010).

A susceptibilidade das populações em risco está associada com fatores socioeconômicos e de meio ambiente com consequência de uma taxa de incidência maior nas crianças de sexo masculino (Martins-Melo *et al*. 2014).

Dentre os fatores de risco se encontraram associações significativas relativas ao contato mais frequente das crianças com reservatórios, principalmente cães, e vetores e à maturação incompleta do sistema imune (Oliveira *et al*. 2012, Rajabi *et al*. 2012;). A susceptibilidade ao desenvolvimento da LV também se associa com fatores do parasito que beneficiam a seleção de populações resistentes ao tratamento preconizado com o antimonial pentavalente (Ait-oudhia *et al*. 2011) ou por fatores genéticos. Por exemplo, a presença da variante HLA-DRB1-HLA-DQA1 na região do MHC II (Fakiola *et al*. 2013), e o alelo SLC11A1 (NRAMP1) em pacientes de Sudão (Mohamed *et al*., 2004). Embora os fatores citados acima sejam

importantes, a resposta imune do hospedeiro exerce um papel determinante nos diversos mecanismos que predispõem susceptibilidade a doença.

1.3. Resposta imune e susceptibilidade na leishmaniose visceral

No passado, a LV foi descrita como uma doença imunossupressora, pois os pacientes apresentavam falta de reatividade ao antígeno de Montenegro, os linfócitos não proliferaram como resposta à estimulação com antígenos de *Leishmania spp. in vitro*, e a resposta efetora aos antígenos de *Leishmania spp*. *in vitro* tinha um padrão ineficiente (Carvalho *et al*., 1985; Carvalho *et al*., 1994; Kumar & Nylen 2012). Não obstante estudos recentes mostraram, que contrário ao que se espera de uma doença immunossupressora, o sistema imune na LV está altamente ativado, pois produz abundantemente citocinas pro e antiinflamatórias no nível sistêmico (Nylen *et al*. 2007, Costa *et al*., 2010, Santos-Oliveira *et al*. 2011, Costa *et al*. 2013). Este conjunto de fenômenos tem sido avaliado recentemente de forma que a LV ativa é considerada atualmente uma doença inflamatória sistêmica com um padrão similar à sepse bacteriana, com o agravante que é acompanhada pela imunossupressão da resposta imune celular (Costa *et al*. 2010, Santos-Oliveira *et al*. 2011, Costa *et al*. 2013).

No homem, o IFN-ɣ tem sido associado com a resposta protetora, pois o tratamento combinado ao antimônio e IFN-ɣ acelerou a cura clínica em pacientes da Índia (Sundar *et al*. 1995). Entretanto, a resposta ao tratamento se associou com

incremento no soro de IFN-γ e com a diminuição da IL-10 e TGF-ß (Saha *et al*. 2007). Estas duas citocinas são caracteristicamente inibitórias da função do macrófago e bloqueiam a produção de metabólitos do oxigênio e do nitrogênio que atuam no controle parasitário. O desenvolvimento da resposta imune também é atingida mediante a diminuição na expressão de MHCII, de moléculas co-estimuladoras e na produção de IL-12 (Nylen *et al*. 2007). Quando células de sangue periférico em pacientes com LV são estimuladas *in vitro* com antígenos de *Leishmania ssp.* não produzem IL-2 nem IFN-ɣ (Carvalho *et al*., 1985), mas a resposta imune e a produção de IFN-ɣ pode ser recuperada mediante a neutralização de IL-10 e de IL-4 (Carvalho *et al*. 1994). A neutralização da IL-10 em células esplênicas cultivadas *ex vivo* eliminou o parasito em 73% dos pacientes, associada como a liberação no sobrenadante de TNF e IFN-γ (Gautam *et al*. 2011). Estes trabalhos põem em evidência o papel do IFN-ɣ na proteção mediada por células T e o efeito inibitório da resposta protetora mediada por IL10, TGF-β e IL-4 na LV humana.

Contrastando com a resposta imune deprimida citada acima, na LV também ocorrem níveis altos de citocinas Th1, IFN-ɣ e IL-12, e Th2 IL-10 no soro, assim como níveis mais elevados de citocinas foram associados com o risco de morte (Caldas *et al*. 2005; De Medeiros *et al*. 1998).

Embora, o padrão de resposta imune protetora na LV humana seja diferente da resposta sistêmica, as condições de doença resolutiva no humano podem ser ilustradas pelo estudo da resposta local resolutiva em camundongos infectados com

L. donovani. Eles apresentam uma formação eficiente de granuloma no fígado que envolve células residentes de Kupffer, monócitos, células T $CD4^+$ e T $CD8^+$ que em conjunto expressam IFN-ɣ, IL-12, IL-4 e níveis moderados de TNF (Engwerda *et al*. 2004). A produção de IFN-ɣ por células da resposta inata como células NK e NKT é importante nesta fase inicial de formação de granuloma e de controle do parasito, assim como na produção de IL-12 pelas células dendríticas e macrófagos. Em contraste, a doença no baço envolve a quebra na arquitetura normal do tecido induzida pela exagerada produção de TNF pelos macrófagos e de IL-10. Em consequência, a expressão do CCR7 é inibida nas células dendríticas, Inibindo a migração para a região da polpa branca que contém as células T (Melby *et al*. 2001, Engwerda *et al*. 2004).

Diversos fatores afetam o desenvolvimento da resposta imune na LV, por exemplo, as deficiências nutricionais (Malafaia G. 2009). Apesar de que se conhece pouco sobre os mecanismos envolvidos nesse campo, estudos experimentais sugerem que deficiências em proteína, zinco e ferro diminuem as células da resposta inata no nódulo linfático conduzindo à visceralização do parasito, com consequente deficit da resposta adquirida (Ibraim *et al*. 2013, Carrillo *et al*. 2014). Efeitos da má nutrição na resposta inata de pacientes foram notados pela diminuição de ligantes CD62L e CD11b, que levam a menor mobilização dos macrófagos e de neutrófilos nos pacientes mal nutridos, comparado com os bem nutridos. Os mal nutridos, ao mesmo tempo, tiveram menor produção de radicais oxidativos, IFN-ɣ e TNF quando foram estimulados com antígenos de *Leishmania* ssp. (Kumar *et al*. 2014).

Outros fatores extrínsecos para o hospedeiro que afetam seu *status* imune, como a HIV/AIDS e as infecções bacterianas concomitantes (Santos-Oliveira & Da-Cruz 2012) ou congênitas (Osorio *et al*. 2012) também afetam a gravidade da LV mediante mecanismos que envolvam uma resposta pró-inflamatória excessiva ou mediante a depressão da resposta imune adquirida.

1.4. O fenótipo do macrófago e susceptibilidade à leishmaniose visceral

Entre os fatores que determinam a susceptibilidade da resposta imune na LV, o macrófago exerce um papel crucial, pois, quando é permissivo à infecção, o parasito inicia os ciclos de replicação no fagolisosomo. A permissividade do macrófago está definida de acordo com os estímulos que recebe do meio ambiente e que determinam o seu estado de ativação, que é definido em dois fenótipos principais, resistente M1 ou susceptível M2 (Martinez *et al*. 2009). Ainda, é interessante que o fenótipo do macrófago não dependente de citocinas das células T, mas pode ser influenciado por elas. Estes estímulos, não dependentes de citocinas, podem ser originados por respostas a estresse, hormônios, dano tissular e fagocitoses, entre outros (Mills 2012).

Os macrófagos podem adquirir o fenótipo clássico M1 quando estimulados com ligantes de TLR ou IFN-ɣ, ou o fenótipo M2, derivado de ativação alternativa, quando estimulados com IL-4 e IL-13. Estes estados de ativação são análogos ao encontrado na polarização de células Th1 e Th2. Por definição, os macrófagos M1

quando estimulados com IFN-ɣ e LPS produzem grande quantidade de citocinas pró-inflamatórias (IL-12, TNF, IL-23), enquanto os macrófagos M2, quando estimulados com IL-4, IL-13 e TGF-β, produzem poucos níveis de citocinas pró-inflamatórias e elevados níveis da citocina anti-inflamatória IL-10 (Weisser *et al*. 2013). Tem sido hipotetizado que a resposta anti-inflamatória dos macrófagos M2, em certa medida, pode limitar o dano tissular ocasionado pela resposta inflamatória tipo Th1 excessiva, sem dúvida os macrófagos M2, ao estarem inativados pelas citosinas Th2, contribuem para a persistência do parasito (Stempin *et al*. 2010).

O parasito *Leishmania spp. per se* manipula o macrófago do camundongo e o leva à inativação, mesmo quando estão no ambiente Th1. Assim, o proteossoma dos macrófagos infectados com *L. donovani* degradam o STAT-1, através da qual as respostas ativadoras de produção de óxido nítrico, como expressão de MHCII e produção de IL-12 pelo macrófago são bloqueadas (Olivier *et al*. 2005, Forget *et al*. 2005). A eliminação do parasito requer a produção do óxido nítrico induzível (iNOS) nos macrófagos M1 a partir do substrato L-arginina e a ação da enzima óxido nítrico sintase 2 (NOS2). A enzima arginase compete pelo mesmo substrato L- arginina para produzir poliaminas, que promovem o crescimento do parasito nos macrófagos M2 (Stempin *et al*. 2010). Apesar de não existirem estudos que demonstrem a competição pela L-arginina nos humanos, no camundongo observou-se que o co-cultivo de macrófagos com células TCD4+ estimuladas com as citocinas Th1 (IFN-ɣ, TNF) e LPS ou Th2 (IL-4, IL-13, IL-10) pode determinar a expressão exclusiva e

competente dos ciclos metabólicos que produzem óxido nítrico ou arginase (Munder *et al*. 1999).

Os fenótipos M1 e M2 exercem outras funções opostas, os M1 inibem a proliferação celular e causam danos ao tecido, enquanto os M2 promovem a proliferação celular e a reparação do tecido (Mills 2012). A coexistência dos fenótipos M1 e M2 foi encontrada recentemente no baço de hamsters com LV crônica (Saldarriaga *et al*. manuscrito em preparação), mas ainda não se conhece a dinâmica da expressão deles, relacionada com a doença não resolutiva. Sabe-se que em lesões arterioescleróticas de camundongos LDLR -/- ambos tipos de macrófago coexistem, sendo que os M1 representam cerca de 40% e os M2 representam 25% do total da população (Kadl *et al*. 2010).

O fenótipo M2 tem sido objeto de outras classificações de acordo com as funções e as citocinas que os ativam, assim os denominados M2a são ativados com IL-4/IL-13, os M2b são ativados com complexos imunes e os M2c são ativados pelo TGF-β e IL-10 (Jang & Nair 2013). O fenótipo M1 possui uma ação microbicida e tumoricida intensa, enquanto os M2 estão envolvidos no controle de parasitos, promoção de remodelação de tecidos, progressão tumoral e funções imunoregulatórias (Sica & Mantovani 2012).

Os genes envolvidos na polarização do macrófago M2 mais representativos são o Mrc1, Fizz1, Chi3l3, Ym1, e genes ativados pela IL-10 mediados por STAT3 (Tgfb1, Mrc1). A função antagônica dos fenótipos M1 e M2 é evidenciada pela regulação negativa sobre os genes que determinam o fenótipo oposto. Assim, genes ativados

por KLF4/Stat6 (Arg-1, Mrc1, Fizz1, PPAR) inibem genes M1 (TNFα, Cox-2, CCL5, iNOS) mediante o sequestro de coativadores requeridos para a ativação do NF-κB. Os fatores induzíveis pela hipóxia HIF-1 e HIF-2 são expressos diferencialmente pelos macrófagos polarizados M1 ou M2, respectivamente, e regulam NOS2 (M1) e Arg-1 (M2), respectivamente (Sica & Mantovani 2012).

1.5. Arginase 1 está envolvida na patogênese da leishmaniose visceral

A enzima arginase (L-arginine ureahydrolase, AI, EC 3.5.3.1) hidrolisa o aminoácido L-arginina e produz L- ornitina e ureia. A Arginina foi descoberta cerca de 100 anos atrás, e a sua importância no sistema imune foi desvendada por estudos que demostraram que processos biológicos essenciais dos linfócitos T como proliferação, expressão do complexo TCR e o desenvolvimento de células T de memória dependiam deste aminoácido (Popovic *et al.*, 2007). Outro tipo de células, as células mielóides supressoras (MDSC), em condições basais, utilizam muito pouco este aminoácido, enquanto durante os processos patológicos incrementam o transporte deste aminoácido desde o meio extracelular ao interior da célula mediante a expressão do transportador catiônico CAT2B. Esse processo ocorre em consequência à depleção da arginina do meio e o impedimento da sua utilização pelos linfócitos (Popovic *et al.*, 2007).

A arginase catboliza a L-arginina e é produzida por células de origem mielóide como neutrófilos, células dendríticas e macrófagos e pelos linfócitos T. O macrófago possui duas isoformas de arginase, que catalisam a mesma reação bioquímica, mas

que diferem na expressão celular, regulação e localização subcelular. A Arg-1 é expressa pelo fígado no citoplasma da célula e é uma das enzimas componentes do ciclo da ureia que detoxifica a amônia nos mamíferos. A Arg-1 possui 322 aminoácidos que são idênticos em 58% com a isoforma Arg-2. A Arg-2 é expressa na mitocôndria e nos tecidos do rim, próstata, intestino e glândulas mamárias. Até há pouco tempo, a Arg-2 foi considerada uma enzima constitutiva (Munder 2009), enquanto a Arg-1 foi considerada a forma induzível pela ação de citocinas IL-4, IL-10, IL-13 e o receptor da IL-21 (Pesce *et al*. 2006). Não obstante, estudos recentes mostraram que a Arg-2 é regulada positivamente em macrófagos sujeitos a estímulos pró-inflamatórios como endotoxina (Ming *et al*. 2012); assim, sua contribuição às doenças crônicas inflamatórias esta sendo reavaliada.

A Arg-1 gera L-ornitina que está envolvida no ciclo metabólico da produção de poliaminas (putrescine, spermidine, spermine) via ornithina decarboxilase (ODC). As poliaminas são moléculas catiônicas pequenas que participam de uma variedade de funções celulares como proliferação e transporte da membrana. O metabolismo da L-ornitina via ornitina aminotransferase (OAT) gera a L-prolina, que é um componente essencial do colágeno. Em oposição ao ciclo da ureia, a L- Arginina serve como substrato da enzima óxido nítrico sintase (NOS2), gerando L-citrulina, óxido nítrico e outros reativos intermediários de nitrogênio, como peróxinitritos que contribuem para a eliminação de *Leishmania ssp*. Porém, a competição pelo substrato L-Arginina e a utilização preferencial do metabolismo da enzima arginase, tem como consequência o prejuízo do ciclo metabólico do óxido nítrico e o controle

parasitário (Munder 2009, Melby *et al*. 2001) (Figura 1). A dicotomia na regulação do óxido nitrico e da arginase é observada em outras espécies mais antigas em escala evolutiva, como, por exemplo, em leucócitos de peixes (Munder *et al*. 2009).

Os macrófagos humanos infectados *in vitro* com *L. donovani* ativam mecanismos de eliminação do parasito dependentes do oxigênio e independentes do oxigênio (radicais e ânions de oxigênio e nitrogênio) que funcionam coordenadamente na eliminação do parasito. Embora na infecção *in vitro* de PBMCs humanos aproximadamente 80% dos parasitos possam ser eliminados rapidamente mediante o H2O2 dos neutrófilos (Murray & Cartelli., 1983). O óxido nítrico não é detectado em sobrenadantes de macrófagos humanos infectados com *L. chagasi*, entretanto está aumentado em macrófagos humanos infectados *in vitro* com promastigotas de *L. chagasi* estimulados com IFN-ɣ. O inibidor do iNOS N(G)-monomethyl-L-arginine inibiu a eliminação do parasita mediada pelo IFN-ɣ, e o RNAm e a proteína do iNOs foram expressos em maior medida na medula óssea de pacientes com LV que nos controles (Gantt *et al*., 2001). Este conjunto de informação indica que os radicais de oxigênio e nitrogênio (O2-, NO) contribuem na eliminação de parasitas de *L. chagasi* em macrófagos humanos (Gantt *et al*., 2001).

O parasito *Leishmania spp.* também possui o gene da arginase, que é essencial para a produção de poliaminas que o mesmo utiliza para sua proliferação. O papel da arginase do parasito nos processos de patogênese foi evidenciado num estudo que demostrou que cepas isoladas de pacientes com doença mucosa apresentaram maior atividade de arginase, do que cepas isoladas de pacientes com leishmaniose

cutânea (Vendrame *et al*. 2010). A pesar do parasita possuir sua própria arginase, ele também tem a capacidade de utilizar as poliaminas da célula hospedeiro (Muleme *et al*. 2009), assim, a arginase da célula produz ornitina, que é então utilizada pelo parasito na via biossintética das poliaminas. Além disso, os parasitos não sintetizam L-Arginina e por isso obrigatoriamente utilizam a enzima da célula hóspede. A atividade comparativa da atividade de arginase da célula e do parasito mostrou que ela foi cerca de 80 vezes maior que a atividade de arginase dos amastigotas na lesão de camundongos (Modolell *et al*. 2009).

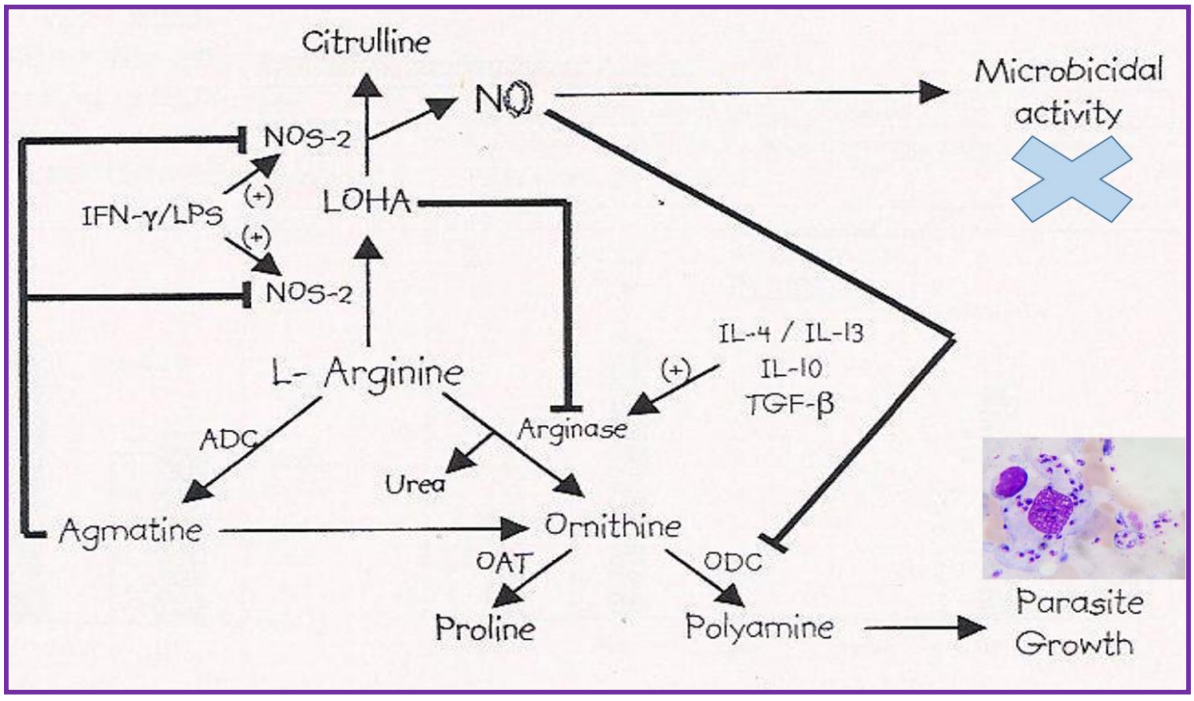

Figura 1. Metabolismo da L-arginina. A L-arginina é utlizada pela enzima arginase dirigindo a via metabólica à síntese de prolina (favorece o colágeno) ou poliaminas favorecem o crescimento de *Leishmania* spp. enquanto, esta via está ativada ocorre um desvio da via metabólica da óxido nítrico sintase, desfavorecendo a produção de óxido nítrico e eliminação do parasito (cortesia de Melby PC).

Por outro lado, monócitos de sangue periférico humano não expressaram Arg-1 quando foram estimulados com IL-4, mas eles expressaram outros marcadores de ativação alternativa como o ligante do carbohidrato galactose (lectina MGL) (Raes *et al*. 2005). Em contraste, macrófagos alveolares humanos expressaram Arg-1 quando foram estimulados simultaneamente com IL-4 e ativadores do AMP cíclico (Erdely *et al*. 2006), implicando que a regulação da Arg-1 humana requer sinais regulatórios mais complexos. De fato, a Arg-1 humana é encontrada em ambientes patológicos complexos como câncer (Rodriguez *et al*. 2008), filariose (Babu *et al*. 2009), tuberculose (Pessanha *et al*. 2012) e asma (North *et al*. 2009). Até então, os estudos ainda não avaliaram se a expressão de Arg-1 é dependente da IL-4 na LV humana, embora se saiba que a IL-4 encontra-se aumentada no plasma e no soro de pacientes com LV (Sundar *et al*. 1997).

Recentemente, verificou-se que a atividade de arginase encontrava-se elevada em lesões crônicas de leishmaniose cutânea e foi correlacionada com diminuição na expressão membranar de CD3ζ, CD4 e CD8 no sítio inflamatório (Abebe *et al*. 2012). Mesmo assim, a atividade da arginase foi maior nos leucócitos de sangue periférico de pacientes com LV ativa, estando correlacionada com a diminuição da expressão de CD3ζ (Abebe *et al*. 2013) e com a gravidade da doença em pacientes da Etiópia co-infectados com HIV (Takele *et al*. 2013).

A arginase é induzida em células mielóides murinas mediante citocinas Th2 e agentes inflamatórios, o que produz a inibição do óxido nítrico e induz fibrose e regeneração do tecido. Até agora a Arg-1 foi identificada nos neutrófilos de sangue

periférico humano, sendo liberada durante os processos inflamatórios. Em forma contrastante, se conhece que estas células produzem uma imunossupressão medida pela utilização do substrato L-Arginina que traz como consequência a depleção de linfócitos T (Munder 2009). O primeiro estudo a estabelecer a relação da Arg-1 com a resposta imune concluiu que os macrófagos poderiam ser ativados e expressar arginase gerando uma depleção de L-arginina no microambiente (Schneider *et al*. 1985). A expressão do transportador catiônico da L-Arginina na membrana celular CAT-2B é fundamental para o transporte da L-Arginina ao interior da célula para serem metabolizada pela enzima arginase. Este transportador pode ser regulado mediante a ativação clássica ou alternativa do macrófago, contribuindo para o direcionamento do metabolismo da L-arginina para a produção de óxido nítrico ou poliaminas, respectivamente (Munder 2009).

Numerosos estudos experimentais apontam a arginase como fator que contribui para a patogênese em camundongos infectados com *L. major*. A IL-4 regula positivamente a expressão de arginase e o crescimento do parasito nos macrófagos, fenômeno que depende da produção de poliaminas provenientes do metabolismo da arginase mediado receptor da IL-4 (Kropf *et al*. 2005, Holscher *et al*. 2006). Camundongos infectados com cepas de *L. braziliensis* isoladas de pacientes resistentes ao tratamento desenvolveram lesões maiores, associadas com maior expressão de Arg-1, e IL-4 no nódulo linfático (Costa *et al*. 2011), mas não se associaram com os níveis de outras citocinas como o IFN-γ, TNF, IL-10 e TGF-β.

Na infecção por *L. major*, o tratamento com nor-NOHA, inibidor competitivo da Arg-1, retarda o aparecimento da lesão e reduz a carga parasitária em camundongos susceptíveis Balb/c, enquanto a suplementação de ornitina aumenta os níveis de poliaminas e a expressão da Arg-1 em camundongos resistentes C57Bl/6 (Iniesta *et al*. 2005).

A arginase, além de diminuir a capacidade de produzir óxido nítrico pelo macrófago, pode contribuir para a patogênese devido a sua participação nos mecanismos de imunossupressão. Níveis baixos de arginina encontram-se associados com a presença de células mielóides supressoras (MDSC) em condições traumáticas e câncer. As MDSC diminuem arginina do meio ambiente e suprimem as funções de outras células que dependem desse aminoácido (Popovic *et al*., 2007; Rodríguez *et al*., 2006). Estudos experimentais demostraram que uma alta atividade de arginase na lesão de camundongos Balb/c infectados com *L. major* causa uma depleção local de L-arginina, produzindo uma diminuição na capacidade de proliferar e de produzir IFN-ɣ das células T da lesão (Modolell *et al*. 2009) enquanto a suplementação de L-arginina restaurou as funções efetoras do linfócito T e reduzindo a patologia e a carga parasitária nos animais tratados (Modolell *et al*. 2009). Considerando esses mecanismos, podemos hipotetizar que o mesmo fenômeno deve pode ocorrer na LV humana, já que uma das causas da imunossupressão na doença está relacionada com a depleção local de L-arginina pela enzima arginase, que conduz à inibição da expressão da cadeia CD3ζ que sinaliza o TCR (Wanasen & Soong, 2008).

Em contraste com os achados acima, envolvendo a Arg-1 em detrimento da resposta imune do hospedeiro, outros estudos mostram que a arginase do macrófago pode exercer um efeito protetor, mediado pelo controle da resposta Th1 exagerada induzida na inflamação. De fato, estudos demostraram um efeito protetor da Arg-1 do macrófago em algumas doenças infeciosas. Por exemplo, a infecção de camundongos com o nematódeo *Nippostrongylus brasiliensis* produz, uma infiltração de macrófagos ativados pela via alternativa na parede intestinal mediada por IL-4/IL-13/STAT6 (Zhao *et al*. 2008). A depleção daqueles macrófagos alternativamente ativados com lipossomas do fármaco clodronato, ou mediante inibição farmacológica da arginase do macrófago inibiu a expulsão de helmintos por reduzir a contratilidade do músculo liso ou mediante produtos do metabolismo da arginase (Zhao *et al*. 2008).

1.6. O hamster dourado como modelo experimental de leishmaniose visceral

O hamster dourado (*Mesocricetus auratus*) é o modelo por excelência para o estudo da leishmaniose visceral humana. O hamster quando é infectado com *L. donovani* ou *L. infantum*, desenvolve um conjunto de sinais e sintomas semelhantes ao que ocorrem no homem, tais como hepatoesplenomegalia, aumento progressivo da carga parasitária, hipoalbuminemia, hipergamaglobulinemia, pancitopenia, e inibição da resposta linfoproliferativa. A doença visceral crônica leva à morte de cerca 20% dos animais, porém, é considerado uma ferramenta útil para estudos de patogênese

e de teste de vacinas que buscam padrões de proteção (Melby *et al*. 2001, Fazani *et al*. 2011), e na descoberta de novos alvos terapêuticos (Osorio *et al*. 2011).

O camundongo apresenta uma infecção autocontrolada, que é resolvida em poucas semanas após a infecção, mediante um mecanismo mediado pela produção de IFN-ɣ pelas células Th1 e a produção de óxido nítrico pelas células infectadas (Iniesta *et al*. 2005). No contrario, os macrófagos de hamster e humanos produzem poucos níveis de óxido nítrico quando são infectados pelo parasita *L. donovani* e geram de forma concomitante citocinas de tipo Th1 (IFN-γ, IL-2 e TNF) e Th2 (TGF-ß e IL-10) (Melby *et al*. 2011). Embora isto leve a uma doença progressiva, a utilização do hamster como modelo experimental possui limitações pelo fato do escasso número de reagentes disponíveis no mercado para realizar estudos da resposta imune e de patogênese. Ainda mais, não existem cepas endogâmicas, ou modelos de deleção gênica comercial que possam ser usados com propósitos de pesquisa (Melby *et al*. 2001). No entanto, a disponibilização recente do genoma parcial do hamster nas bases de dados do NCBI, o desenvolvimento dos *softwares* para análise comparativa de sequências e o desenvolvimento de novas ferramentas tecnológicas certamente contribuirão para o avanço significativo na utilização deste modelo.

A optimização do modelo envolve o uso de condições experimentais similares à infecção natural. Na procura de evitar os inóculos altos utilizados nas infeções intracardíacas padrão ou intraperitoneais, onde são injetados 10^7–10^8 parasitos, foi avaliada a utilização de vetores para que a transmissão do parasito ocorresse de forma mais próxima da natural. Nessas condições, os parasitos inoculados pelo

vetor variaram entre 22.000 a 80.000 para *L. infantum* e 29.000 em *L. donovani*. Nesse modelo, o efeito foi o surgimento relativamente frequente de manifestações cutâneas nos infectados com *L. infantum* (42%). Em contraste, os infectados com *L. donovani* não desenvolveram lesões cutâneas, enquanto as cargas parasitárias desenvolvidas foram comparáveis às obtidas na inoculação cardíaca. As desvantagens estão relacionadas com a maior dificuldade para o manejo e manutenção da infecção vetorial, enquanto no hamster a evolução da doença foi mais lenta e apresentou maior perda de peso que nas condições tidas como padrão (Aslan *et al*. 2013).

1.7. Os receptores tirosina quinase e fatores de crescimento

A família de receptores tirosina quinase (RTK) compreende 58 tipos de receptores. Entre outros, podemos destacar o receptor do fator de crescimento da epiderme (EGFR), o receptor do fator de crescimento de plaquetas (PDGFR), o receptor do fator de crescimento do fibroblasto (FGFR), o receptor do fator de crescimento vascular e endotelial (VEGFR), receptores ephrin (Ephs), o receptor de insulina (I-R) e o receptor similar ao fator de crescimento similar à insulina (IGF). Estes receptores regulam funções na célula como proliferação, diferenciação, sobrevida, quimiotaxia, adesão celular, migração e inibição da apoptose (Hubbard *et al*. 2007).

Os humanos posuem 4 tipos de receptores FGFR que, quando ativados com os FGF, causam a proliferação de variados tipos celulares, incluindo células endoteliais,

células tronco e células epiteliais. Também estão envolvidos em processos de patogênese como angiogênese mesmo exercendo essa função com maior potência do que VEGF e PDGF. O FGF-2 é produzido por fibroblastos, macrófagos e células estromais e induz a expressão de genes relacionados com inflamação das células endoteliais consistentes com o ambiente pró-inflamatório. Os monócitos e os macrófagos estão relacionados ao fenômeno angiogênico dirigido pelo FGF-2 e estão associados com a produção de fatores de crescimento pró-angiogênicos (VEGF, FGF2, IL-1ß, IL-8, TNF), regulando a proliferação dos vasos sanguíneos (Presta *et al*. 2009).

A dimerização do receptor FGFR pela união do FGF-2 desencadeia a dimerização do receptor e subsequente recrutamento e ativação de proteínas intracelulares que ativam cascatas de fosforilação. As mais representativas são as cascatas das MAPK 1 and 2 (MEK1/2) e 1 e 3 (ERK1/2). Estas geram a fosforilação do fator de transcrição ELK1. A junção do complexo FRS2/GRB2/GAB1 induzido pela estimulação com FGF origina a ativação do PI3K e de proteínas efetoras como o v-AKT, p38, CREB1 e ativação do fator de transcrição ATF-2 (Hubbard *et al*. 2007).

Por outro lado, os IGF-IR exercem um papel importante na mitogênese, angiogênese, transformação e proteção de apoptose, estimulação somática de crescimento, além de promoção da proliferação e da diferenciação celular. Os IGF atuam de forma autócrina, parácrina e endócrina. O sistema IGF compreende os receptores I e II, com os respectivos fatores de crescimento similar a insulina 1 e 2

(IGF-1 e IGF-2) e 6 proteínas de alta afinidade que regulam a biodisponibilidade dos IGF (IGFBP) (Yu & Rohan 2000).

Após ligação dos IGF ao receptor, a atividade de tirosina quinase nos receptores conduz à fosforilação de vários substratos, incluindo os da família do receptor da insulina, como o substrato do receptor da insulina (IRS-1). Uma vez fosforiladas, estas proteínas ativam a sinalização intracelular de proteínas como a fosfatidil inositol 3-quinase (PI3K) e AKT iniciando a proliferação celular (Hubbard *et al*. 2007).

A ativação da cascada do MAPK, ERK tem sido relacionada com a patogênese da infecção por leishmânia. A infecção de macrófagos com *L. amazonesis* ativara as cascatas MAPK, ERK1/2 e essa ativação foi requerida, mas não foi suficiente para a indução da IL-10 pela célula. Ainda mais, a administração de U0126, inibidor do ERK, nos camundongos infectados resultou numa diminuição da lesão e redução no número de parasitos (Yang *et al*. 2007).

O efeito do fator de crescimento do fibroblasto na leishmaniose é desconhecido, pois não existem publicações no tema. Por outro lado, dispõe-se de mais informações sobre as implicações do IGF nos processos de patogênese que envolvem *Leishmania* e a célula hospedeira (Gomes *et al*. 2000, Reis *et al*. 2013). O IGF estimulou o crescimento de promastigotas de *L. chagasi* (Gomes *et al*., 1997) e a fosforilação de moléculas intracelulares do parasito, como também aumentou o número de parasitos vivos após a infecção (Gomes *et al*. 2000). Na célula hospedeira, o IGF favoreceu o crescimento de parasitos nos macrófagos infectados *in vitro*, associados com menor produção de óxido nítrico (Vendrame 2007). Outros

estudos sugerem que as citocinas Th1 inibem a expressão de IGF-I em macrófagos (Arkins *et al*. 1995, Reis *et al*. 2013) e revelaram o papel do IGF no crescimento dos parasitos no macrófago. Assim, o estimulo de macrófagos com IFN-ɣ atenua a expressão do IGF-I e a carga parasitária dos macrófagos, enquanto a adição de IGF-I recuperou a carga parasitária (Reis *et al*. 2013).

Dado o papel dos fatores de crescimento nas funções angiogênicas, a inibição dos RTK tem sido utilizada como estratégia para o tratamento de doenças crônicas inflamatórias. Só existe um estudo na literatura que demostrou que o tratamento de camundongos infectados com *L. donovani* com o inibidor de RTKs sunitinib maleato (Sm) evitou as manifestações clínicas de esplenomegalia e gerou uma restauração da microarquitetura esplênica, associada com incremento na frequência de células $TCD4^+$ produtoras do IFN-γ e TNF, aumentando a produção de óxido nítrico, embora o efeito não tenha atenuado a carga parasitária (Dalton *et al*. 2010).

Em contraste, o PDGF foi associado com a resposta inata protetora em camundongos infetados com *L. major*, pois o PDGF liberado pelas plaquetas resultou na liberação do CCL2 e, portanto, foram recrutados rapidamente monócitos ativados com a capacidade de eliminar os parasitas (Gonçalves *et al*. 2011).

1.8. Mecanismos de regulação da arginase

Estudos em camundongos infectados com *L. major* mostraram que a regulação da Arg1 na leishmaniose depende do compartimento de células T acompanhada pelo

aumento da citocina IL-4 e diminuição de IL-12 (Iniesta *et al*. 2005, Kropft *et al*. 2005). A expressão da Arg1 mediada por IL-4 exige a ação conjunta dos fatores de transcrição STAT-6 e do cofator C/EBP-β num fenômeno que também dependente da ubiquitinação do C/EBP-β (Gray *et al*. 2005, Ye *et al*. 2012). O AMP cíclico exerce o seu efeito aumentando a ligação do C/EBP-β ao promotor da Arg-1, porém atua cooperando na indução rápida da Arg-1 induzida pela ativação do IL-4-STAT-6 (Sheldon *et al*. 2013). Enquanto não é claro se o C/EBP-β liga-se diretamente ao promotor da Arg-1 (Ye *et al*. 2012) (Figura 2), o outro fator de transcrição o KLF4 une-se ao promotor da arginase, num processo que depende e requer a cooperação espacial de STAT-6. Em contraste, a ativação do STAT-6 pela IL-4 e a ação subsequente no promotor da Arg-1 não depende do fator de transcrição KLF4 (Liao *et al*. 2011).

A Arg-1 também pode ser induzida em macrófagos infectados mediante mecanismos que induzem a expressão dos receptores nucleares PPAR γ/δ. A ativação do PPAR pode ocorrer pela fagocitose de neutrófilos apoptóticos ou pela ação da IL-4 dependente do STAT-6 (Chawla *et al*. 2010). O PPAR γ/δ une-se próximo ao sitio de ligação do STAT-6 e C/EBPβ e exerce efeitos subsequentes na polarização do macrófago para o fenótipo M2, produção de arginase e inibição da produção de óxido nítrico (Chan *et al*. 2012) (Figura 1).

Além dos mecanismos dependentes do STAT-6, tem sido descritos outros mecanismos independentes do STAT-6 que ativam Arg-1. Por exemplo, macrófagos de camundongo infectados com *M. tuberculosis* aumentaram a expressão de Arg1

mediante mecanismos dependentes do C/EBPB-β e do adaptador do TLR Myd88 (El Kasmi *et al*. 2008). Estes autores demonstraram que a Arg-1 induzida mediante essa via causava efeitos similares na inibição da produção de óxido nítrico e no controle do parasito. Um estudo posterior determinou que o estímulo dependia do C/EBP-β, que leva à secreção de citocinas IL-6, G-CSF e IL-10 para ativar o STAT3 e transcrever Arg-1 (Qualls *et al*. 2010). Por outro lado, não se tem certeza se o STAT-3 liga-se diretamente ao promotor da Arg-1 ou atua mediante outras vias indiretas (Figura 2). O fator de transcrição PU.1, um fator de transcrição que atua na diferenciação em células mielóides, induz a expressão de Arg-1 mediante a ligação ao promotor perto dos sítios de ligação do STAT6-C/EBPβ (Pauleau *et al*. 2004). Tem sido proposto que a estimulação por citocinas Th2 PU.1, STAT-6 e C/EBPβ, e o coativador CBP atuam conjuntamente para ativar o promotor da Arg-1, o que daria início ao processo de transcrição (Pauleau *et al*. 2004). Outro mecanismo detectado em macrófagos associados às células tumorais e macrófagos residentes é mediado pelo receptor RonRTK, que ativa sequencialmente a via das MAPK e o fator de transcrição c-fos, os quais se unem ao sítio de ligação (AP-1) no promotor da Arg-1 (Figura 2) (Sharda *et al*. 2011). Os receptores LXRα são receptores nucleares que controlam a homeostase do colesterol, quando ativados por citocinas inflamatórias ou lipídios metabólicos. Eles ativam indiretamente Arg-1 mediante a indução da união do complexo PU.1-IRF8 ao promotor da Arg-1 (Pourcet *et al*. 2011) (Figura 2). De forma Interessante, foi proposto que o IRF-8 pode estar ativado por outro mecanismo que envolva a interação do IFN-ɣ com vitamina D. Assim, o complexo

VDR-STAT-1 aumenta o IRF-8 conduzindo à transcrição de Arg-1 (Ramagopalan *et al*. 2010, Pourcet *et al*., 2013). Estudos recentes no modelo hamster indicaram que, em certas condições de infecção *in vitro*, o IFN-ɣ modula a Arg-1 (Melby *et al*. manuscrito em preparação). A possível regulação da Arg-1 mediada por mecanismos que abrangem o IFN-ɣ é um mecanismo que potencialmente está envolvido na patogênese da leishmaniose e requer investigação.

Há apenas indícios na literatura que mostraram uma ligação entre o RTK e a regulação da Arg1. A superexpressão do EGFR foi associada com Arg1 em processos patológicos como lesões granulomatosas em tuberculose disseminada (Bermudez *et al*. 1996). O EGFR ativado por o ambiente hipóxico regulou Arg-1 mediante a ativação do EGFR em células pulmonares endoteliais (Toby *et al*. 2010). O tratamento de células endoteliais pulmonares com LPS e TNF resultou na regulação de arginase 1 e 2 dependentes do EGFR sem afetar o NOS (Nelin *et al*. 2005). No entanto, estes trabalhos não indicam o mecanismo envolvido abaixo do receptor.

Indiretamente, o IGF tem sido associado à regulação de Arg-1 na leishmaniose. A pré-incubação de parasitos com IGF-I agravou as lesões cutâneas de camundongos Balb/c infectados com *L. amazonensis* (Gomes *et al*. 2000), e apresentaram maiores níveis de arginase e menor produção de NO na lesão (Vendrame *et al*. 2007, 2010). Os resultados advindos dessa tese deverão complementar esta informação e explorar pela primeira vez, um possível mecanismo de regulação Arg-1 mediada pela ativação dos fatores de transcrição STAT-6, através da via de sinalização dos

RTK na LV. A compreensão dos sinais que levam ao aumento da Arg-1 e aplicabilidade dos resultados para a doença humana, podem potencialmente fornecer novas ferramentas para o manejo da doença.

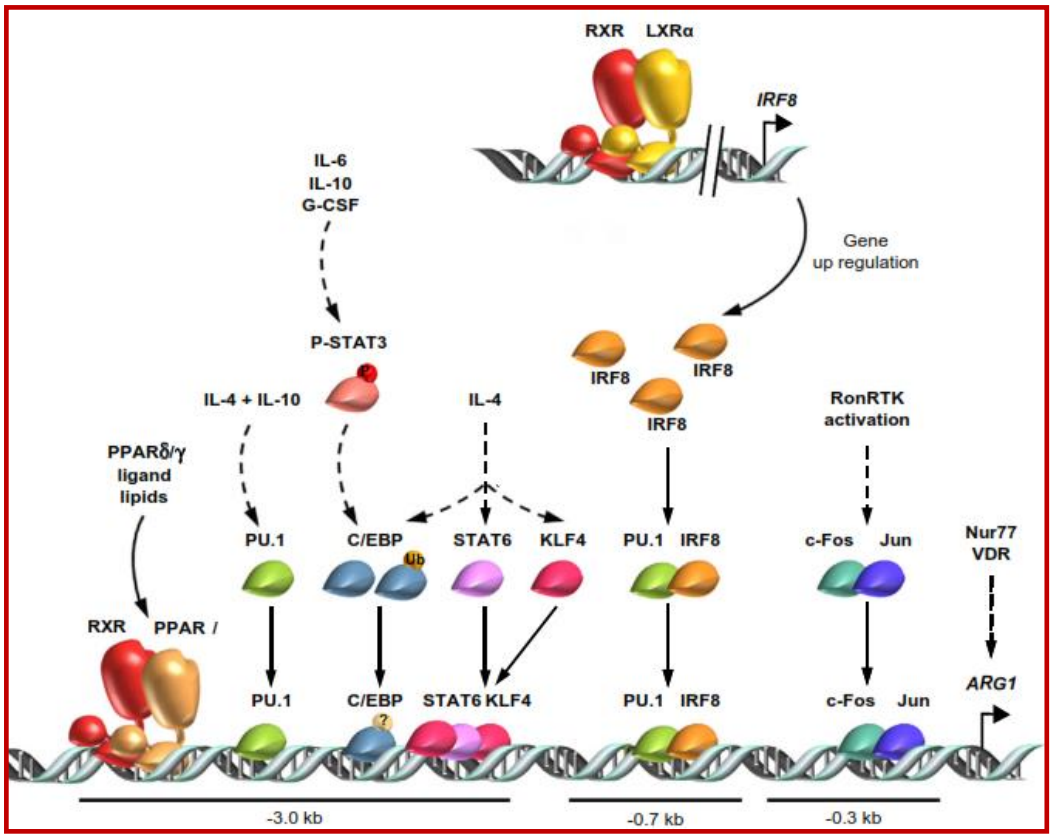

Figura 2. Modelo dos principais fatores de transcrição que regulam o promotor da arginase no camundongo (modelo baseado na revisão da literatura). O STAT-6 é essencial na ativação da Arg-1 mediada pela IL-4. Não obstante, a transcrição da arginase ocorre com a união dos cofatores CEBP/β ou mediante outros mecanismos pós-transcricionais. Ilustram-se os sítios de união ao promotor da Arg-1. C/EBP-β: CAAT/Proteína de Ligação Potenciador-β, IL: Interleucina, IRF: Fator Regulador de Interferon, KLF4: Fator Similar ao Krueppel-4, LXR: Receptor X do Fígado, PPAR: Receptor Ativado-Proliferador do Peroxissoma, PR: Receptor de Progesterona, P: fosfato, PU.1: Fator de Caixa de Purina1, RonRTK: Receptor Tirosina Quinase Ron, RXR: Receptor de Retinóide X, STAT: Transdutor de Sinal e Ativador da

Transcrição, Ub: Ubiquitina, VDR: Receptor da Vitamina D. Fonte: Artigo de revisão de literatura: Porcet & Pineda-Torra 2013.

1.9. JUSTIFICATIVA

O objetivo deste projeto é identificar mecanismos de regulação da Arginase que contribuem para o desenvolvimento de leishmaniose visceral. Existem lacunas de conhecimento acerca dos fenômenos associados com a evolução da LV progressiva como, por exemplo, carga parasitária elevada, esplenomegalia massiva, pancitopenia, imunossupressão e morte. O modelo hamster de doença crônica causada por *L. donovani* proporciona a oportunidade de estudar os mecanismos causais destas manifestações, pois, diferente do camundongo, o hamster apresenta característica da doença similar à infecção humana. Assim, o hamster desenvolve uma resposta exagerada de tipo Th1, mas o macrófago não responde a essa estimulação, e não produz níveis significativos de óxido nítrico, sendo parasitado abundantemente o que gera uma resposta imunossupressora. A enzima arginase tem sido associada com o acréscimo destes processos, já que determina o fenótipo do macrófago ativado pela via alterna mediante a utilização preferencial da L-arginina. Fenômeno que aumenta a carga parasitária, devido à redução da enzima óxido nítrico sintase e à inibição da ativação do linfócito. Apesar do papel regulatório que esta enzima exerce no controle parasitário e no sistema imune, os mecanismos que dirigem a sua expressão na LV são desconhecidos. Ainda mais, quando esta tese foi proposta se desconhecia se esta enzima estava associada com a LV humana.

Estudos experimentais mostraram que os fatores de transcrição STAT-6 e o STAT-3 são chaves na transcrição dos genes que geram a ativação pela via alternativa do macrófago e a resposta susceptível a *Leishmania spp.*, por tanto, o estudo das vias que contribuem para a regulação patológica da arginase mediante este fator de transcripção tornam-se importantes para o controle da doença. Este trabalho propõe avaliar se o STAT-6 está associado com a regulação patológica da Arg-1 na LV, e se exploraram as vias de que potencialmente derivaram nessa ativação. Porém esta tese explorou a hipótese de que a Arg-1 estava sendo regulada por STAT-6, e que fatores como as citocinas e os RTKs determinaram a ativação do STAT-6 na LV experimental. Também foi proposto avaliar se a enzima estava envolvida na doença humana, pois não há estudos que avaliaram esta possibilidade. Por tanto, foi proposto o analise de arginase em pacientes com LV em diferentes fases da doença, assim como também determinar se os fatores relacionados com a ativação dos RTKs no modelo experimental encontravam-se expressados nos pacientes com VL. Este trabalho é desenvolvido com o fim último de conhecer os fatores que abrangem a patogênese da LV com o fim de vislumbrar novas perspectivas terapêuticas para o controle da doença.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar se os mecanismos que determinam a regulação da arginase na leishmaniose visceral experimental dependem do fator de transcrição STAT-6 e se outros fatores afetam a regulação positiva da arginase no modelo experimental.

2.2. OBJETIVOS ESPECÍFICOS

Avaliar se a Arg-1 está envolvida na patogênese da leishmaniose visceral experimental e se é regulada pelo fator de transcrição STAT-6.

Demostrar que a cascata de sinalização RTK leva à ativação do STAT-6 e à expressão da Arg-1 na leishmaniose visceral experimental.

Validar os resultados obtidos no modelo experimental animal em pacientes com leishmaniose visceral.

3. MÉTODOS e RESULTADOS

3.1. DOCUMENTO 1:

Progressive visceral leishmaniasis is driven by dominant parasite-induced STAT6 activation and STAT6-dependent host arginase 1 expression. PLoS Pathog. 2012;8(1):e1002417

Neste estudo avaliou-se se o hamster com leishmaniose visceral (LV) experimental tinha uma inabilidade de controlar a replicação do parasito *L. donovani* causada por um estado de ativação pela via alternativa dos macrófagos. Os resultados mostraram que devido à baixa expressão de óxido nítrico sintetase (NOS) 2, os macrófagos dos hamsters foram direcionados para o fenótipo de ativação alternativa (M2). Nestes, a expressão de Arg1 e a produção de poliaminas foi dominante na LV progressiva. As manifestações clínicas no hamster com LV estiveram correlacionadas com a produção abundante da Arg-1, poliaminas e aumento da carga parasitária, e pouca produção de NOS induzível (iNOS) no baço. No entanto, distinto do paradigma predominante da ativação alternativa mediada por citocinas, encontramos que o STAT-6 foi ativado independentemente de uma resposta Th2 polarizada e determinou a expressão da Arg-1. A importância crítica da ativação da via alternativa direcionada pelo parasito em células infectadas foi demonstrada através do controle da infecção após a silenciamento transcricional do gene da Arg1 mediante RNA de interferência. Isto levou à maior geração de óxido nítrico e diminuiu em cerca de 50% a carga parasitária. Uma vez que expressão da Arg-1 induzida pelo parasita ocorreu na ausência de IL-4 exógena, consideramos a possibilidade de que a *L. donovani* ativou diretamente o STAT6. A interferência da expressão do STAT6 em células BHK resultou em redução da expressão da Arg-1 e no controle da infecção. Coletivamente, estes resultados indicaram que a infecção por *L. donovani* gera a expressão de Arg-1 de forma dependente do STAT6, num processo independentemente da IL-4. Embora a ação de IL-4, IL-13, IL-10 e IL-21 tenham contribuído para a apresentação de manifestações clínicas e aumento da Arg-1 e da carga parasitária na fase crônica.

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PLoS PATHOGENS

Progressive Visceral Leishmaniasis Is Driven by Dominant Parasite-induced STAT6 Activation and STAT6-dependent Host Arginase 1 Expression

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Abstract

The clinicopathological features of the hamster model of visceral leishmaniasis (VL) closely mimic active human disease. Studies in humans and hamsters indicate that the inability to control parasite replication in VL could be related to ineffective classical macrophage activation. Therefore, we hypothesized that the pathogenesis of VL might be driven by a program of alternative macrophage activation. Indeed, the infected hamster spleen showed low NOS2 but high arg1 enzyme activity and protein and mRNA expression ($p<0.001$) and increased polyamine synthesis ($p<0.05$). Increased arginase activity was also evident in macrophages isolated from the spleens of infected hamsters ($p<0.05$), and arg1 expression was induced by *L. donovani* in primary hamster peritoneal macrophages ($p<0.001$) and fibroblasts ($p<0.01$), and in a hamster fibroblast cell line ($p<0.05$), without synthesis of endogenous IL-4 or IL-13 or exposure to exogenous cytokines. miRNAi-mediated selective knockdown of hamster arginase 1 (arg1) in BHK cells led to increased generation of nitric oxide and reduced parasite burden ($p<0.005$). Since many of the genes involved in alternative macrophage activation are regulated by Signal Transducer and Activator of Transcription-6 (STAT6), and because the parasite-induced expression of arg1 occurred in the absence of exogenous IL-4, we considered the possibility that *L. donovani* was directly activating STAT6. Indeed, exposure of hamster fibroblasts or macrophages to *L. donovani* resulted in dose-dependent STAT6 activation, even without the addition of exogenous cytokines. Knockdown of hamster STAT6 in BHK cells with miRNAi resulted in reduced arg1 mRNA expression and enhanced control of parasite replication ($p<0.0001$). Collectively these data indicate that *L. donovani* infection induces macrophage STAT6 activation and STAT6-dependent arg1 expression, which do not require but are amplified by type 2 cytokines, and which contribute to impaired control of infection.

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Introduction

In humans, active visceral leishmaniasis (VL), caused by the intracellular protozoan *Leishmania donovani*, is a progressive, potentially fatal infection characterized by chronic, fever, hepatosplenomegaly, pancytopenia, and profound cachexia. VL remains a significant cause of morbidity and mortality in the developing world; hundreds of thousands of people have died in recent years in epidemics in Sudan and India.

Most experimental studies of infection with the visceralizing *Leishmania* (*L. donovani* and *L. infantum/chagasi*) have used the murine infection model. Mice are genetically resistant or susceptible to *L. donovani*, but even susceptible strains are able to contain the infection without overt disease [1]. *L. donovani*-infected mice mount a vigorous anti-leishmanial type 1 CD4+ and CD8+ T cell response that leads to control of the infection, primarily through the upregulation of inducible nitric oxide synthase 2 (iNOS or NOS2) and generation of nitric oxide (NO) in the spleen and liver [2–4]. Susceptibility in the murine *L. donovani* infection model is related to the expression of IL-10 [5,6] and TGF-β [7]. Notably, IL-4, which has a prominent role in the immunopathogenesis of murine *L. major* infection (reviewed in [8]), appears to have a limited role in the pathogenesis of murine *L. donovani* infection [9]. While study of the chronic, self-controlled infection in mice has been instrumental in dissecting mechanisms of immunity and susceptibility, this model may be limited in representing the mechanistic underpinnings of progressive visceral disease.

The underlying immunopathogenic mechanisms related to human VL have not been fully elucidated. In human VL there

Author Summary

Visceral leishmaniasis (VL), caused by the intracellular protozoan *Leishmania donovani*, is a progressive, potentially fatal infection found in many resource-poor regions of the world. We initiated these studies of an experimental model of VL to better understand the molecular and cellular determinants underlying this disease. We found that host macrophages or fibroblasts, when infected with *Leishmania donovani* or exposed to products secreted by the parasite, are permissive to infection because they fail to metabolize arginine to generate nitric oxide, the effector molecule needed to kill the intracellular parasites. Instead, the infected host cells are activated in a way that leads to the expression of arginase, an enzyme that metabolizes arginine to produce polyamines, which support parasite growth. This detrimental activation pathway was dependent on the parasite-induced activation of the transcription factor STAT6, but contrary to the previously accepted paradigm, did not require (but was amplified by) the presence of polarized Th2 cells or type 2 cytokines. Knockdown of host arginase or STAT6 enhanced control of the infection, indicating that this activation pathway has a critical role in the pathogenesis of the disease. Interventions designed to inhibit the STAT6-arginase-polyamine pathway could help in the treatment or prevention of VL.

is elevated expression of type 1 cytokines (IFN-γ and IL-12) in the plasma [10,11] and in the infected lymph node, bone marrow, and spleen [12–14]. Paradoxically, this robust type 1 cytokine response, which typically mediates control of intracellular pathogens, does not mitigate the relentlessly progressive disease in humans. Several cytokines known to impair macrophage-mediated killing of *Leishmania* [15] have been postulated to have a detrimental role in human VL [16,17]. The Th2 cytokines, interleukin (IL)-4 and IL-13, which play a prominent role in promotion of disease in some experimental models of *Leishmania* infection [8], were found to be increased in the serum of patients with active VL in some [18–21], but not all studies [22–24]. The importance of IL-10 in the pathogenesis of human VL is more clearly established [16]. Patients with VL have elevated levels of IL-10 in serum or plasma [22–25] and increased IL-10 mRNA expression in the spleen and bone marrow [13,14,19]. In vitro neutralization of IL-10 in peripheral blood mononuclear cell cultures from patients with VL resulted in enhancement of type 1 T cell responses to *Leishmania* antigens [12,26], and neutralization of IL-10 in splenic aspirates promoted parasite clearance [27]. Impairment of signaling pathways in human macrophages infected in vitro with *Leishmania* is also well described (reviewed in [28]) and may play a role in human VL by rendering the infected cells less responsive to activating stimuli.

In light of the ineffective killing of *L. donovani* in human VL, it is pertinent to consider the activation phenotypes of macrophages. Classically activated macrophages are primed by proinflammatory cytokines, most notably IFN-γ, and triggered by microbial products to produce antimicrobial mediators such as NO and reactive oxygen species. These macrophages play a critical role in the protection against intracellular pathogens such as *Leishmania* [2,29–35]. Macrophages exposed to type 2 cytokines (IL-4 and IL-13) were thought initially to be in a deactivated state because of blunting of the pro-inflammatory cytokine response, oxidative burst, or NO response [15]. However, it is now recognized that these macrophages are not paralyzed, but in fact display a different activation program. Alternatively activated macrophages (AAMs), as introduced by Gordon and colleagues in describing the phenotype of macrophages activated in the presence of IL-4 (and later IL-13) [36,37], fail to produce NO, have pronounced arginase activity (which competes with NOS2 for the common substrate arginine), and fail to control the intracellular replication of pathogens, including *Leishmania* [38,39]. Recently, a role for IL-21 in the amplification of arginase-producing alternatively activated macrophages has been identified [40]. Alternatively activated macrophages play an important role in dampening tissue inflammation, and mediating tissue repair and wound healing.

AAMs play a role in the pathogenesis of protozoal infections [41], and several lines of evidence from the murine *L. major* infection model identified an important role of AAMs in promoting *Leishmania* infection. First, the constitutive expression of arg1 was higher in macrophages from *L. major*-susceptible compared to resistant mice [42]. Second, arg1 induction correlated with lesion size in mice infected with *L. major* [43,44]. Third, AAMs failed to control the intracellular replication of pathogens, including *Leishmania* [38,39]. Fourth, the upregulation of arginase by IL-4, IL-10, and TGF-β was associated with impaired capacity to kill intracellular *L. major* [42–44]. Finally, inhibition of arginase decreased disease and parasite burden in *L. major* infected mice and macrophages [43,44]. The increased expression of host arg1 has two important downstream effects that can promote *Leishmania* infection: (1) arginase competes with NOS2 for the common substrate, arginine, thereby reducing the generation of the antimicrobial molecule NO; and (2) arginase activity leads to the generation of polyamines that can be scavenged through uptake receptors [45] to promote *Leishmania* growth [46].

Unfortunately, there remain significant deficits in our understanding of the molecular and cellular determinants underlying VL pathogenesis. The Syrian hamster (*Mesocricetus auratus*) affords a unique opportunity to address questions related to the pathogenesis of visceral leishmaniasis, because the clinicopathological features of the hamster model of VL mimic active human disease. We, and others, demonstrated that despite progressive disease hamsters with VL mount a vigorous type 1 cellular immune response [47–49], an immunological event that is typically associated with disease control and resolution. This paradoxical finding was reminiscent of the findings in humans [13,14], and suggested that the inability to control parasite replication could be related to ineffective IFN-γ-mediated induction of classical macrophage activation. Indeed, we found that the expression of NOS2 and production of NO, which is the primary mechanism by which mice control *Leishmania* infection [2,29–35], was low during the progressive course of disease in hamster VL [48,49]. Because of the low NOS2 expression in hamsters with VL we hypothesized that during progressive disease macrophages would default toward and/or be driven toward an alternatively activated phenotype. Indeed, the expression of arg1 and the production of polyamines was dominant in progressive VL. However, distinct from the prevailing paradigm of cytokine-mediated alternative activation of macrophages, we found that the *L. donovani*-induced activation of STAT6 and arg1 expression and polyamine production in macrophages and fibroblasts did not require the presence of a polarized Th2 response or synthesis of type 2 cytokines. The critical importance of the parasite-induced STAT6-arg1 pathway in infected cells was demonstrated by finding enhanced control of infection following either STAT6 or arg1 knockdown.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the Institutional Animal Care and Use Committee of the South Texas Veterans Health Care System.

Hamsters and mice

6-8 week old inbred Chester Beatty Syrian golden hamsters (*Mesocricetus auratus*) were obtained from our own established breeding colony at the South Texas Veterans Health Care System Veterinary Medical Unit. 6-week old BALB/c mice were obtained from Charles River Laboratories.

Isolation of primary macrophages and fibroblasts

Splenic macrophages were isolated from a spleen cell suspension by adherence to plastic culture dishes. Resident peritoneal macrophages were isolated from mice or hamsters by peritoneal lavage with DMEM containing 2% heat-inactivated fetal calf serum (HIFCS) and cultured in complete DMEM (DMEM plus 2mM glutamine, 1 mM Sodium pyruvate (Gibco), 1X MEM aminoacids solution (Sigma), 50 μM β-mercaptoethanol, 10 mM Hepes, 100 U/ml penicillin, and 100 mg/ml streptomycin) with 2–10% HIFCS. After overnight incubation, non-adherent cells were removed and adherent cells cultured in complete DMEM with 0.4% bovine serum albumin (BSA) or in Opti-MEM (Invitrogen) plus 1% HIFCS for in vitro infections. Hamster primary fibroblasts were obtained following the protocol described to isolate mouse splenic primary fibroblasts [50]. In brief, spleens were harvested from uninfected hamsters, treated with collagenase D to obtain a single cell suspension, and cultured in RPMI 1640 containing 10% of heat inactivated FBS, 10 mM Hepes, 1 mM sodium pyruvate (1 mm), 1X MEM aminoacids solution (Sigma), 100 IU/mL penicillin, 100 mg/mL streptomycin (Cellgro) and 50 mM 2-mercaptoethanol. The non-adherent cells were removed at 24, 48 and 72 h of culture and the adherent cells were cultured to reach confluence over 2 weeks. The fibroblast-like monolayer was detached with Trypsin/EDTA and cultured overnight in DMEM plus 2% FBS prior infection experiments. The purity of the fibroblast population was determined to be >80% using PE labeled-antibody against the ER-TR7 antigen (Santa Cruz) and flow cytometry.

Parasites and infection

Leishmania donovani (MHOM/SD/001S-2D) promastigotes were cultured as described previously [51]. Hamsters were infected by intracardial injection and mice by intravenous injection of 10^6 peanut agglutinin purified metacyclic promastigotes [51] of a *L. donovani* strain transfected with an episomal vector containing the luciferase (luc) reporter gene [52]. The parasite burden was measured in 100 mg of tissue homogenized in PBS and the luminescent counts were transformed to number of parasites by interpolation from a standard curve of luciferase activity and number of amastigotes. For in vitro infections, stationary phase promastigotes obtained from 5–6 day old cultures were either non-opsonized, or opsonized with fresh, complement-containing normal mouse or hamster serum (20% in DMEM), or freeze-thawed hamster serum obtained from 6-week infected hamsters (contained high titer of anti-*Leishmania* antibody) for 30 min at 37°C and 5% CO_2, washed with PBS and used immediately to infect the adherent primary macrophages or splenic fibroblasts, or serum-starved (2% HIFCS) BHK fibroblast cells. Cells were infected at a promastigote to host cell ratio of 10:1. Four hours after the infection extracellular parasites were removed by repeated (at least 3 times) washing with PBS and the infected cells cultured thereafter in the low-serum medium described above. For parasite burden determinations, the complete removal of extracellular parasites was verified by microscopic inspection. The level of infection after initial phagocytosis was assessed 4 hrs after infection in Giemsa-stained preparations enumerated by microscopy. Typically at 4 hrs post-infection there were 1–2 parasites per infected BHK cell (range 1 to 7) and >75% of cells were infected. In experiments where parasite killing was assessed an equivalent rate of initial (4 hr) infection was confirmed and the parasite burden determined 4–72 hrs after infection by luminometry.

Measurement of NOS2 and arginase enzymatic activity and arginase protein expression

NOS2 activity (NO production) was estimated by the measurement (Griess assay) of nitrites and nitrates in supernatants of unstimulated cells or cells stimulated with IFN-γ (10% v/v of hamster recombinant IFN-γ supernatants) plus 1 μg/mL lipopolysaccharide (LPS; *E. coli* serotype 0111:B4; Sigma) as described previously [48,49]. The enzymatic activity of arginase was determined in 100,000 adherent splenocytes [53], peritoneal macrophages, or BHK fibroblasts cultured for 24–48 hrs in complete DMEM supplemented with 0.4% BSA by measuring the rate of urea formation from L-arginine in the presence of 1-phenyl-1,2-propanedione-2-oxime (ISPF) [54,55]. Cells were stimulated with IL-4 (10% v/v) and/or LPS (1 μg/mL), or infected with *L. donovani* promastigotes or amastigotes. The measurement of arginase activity in tissue samples homogenized in PBS (5 mg/mL) was accomplished using the same method. Arg1 and GAPDH protein expression were determined by western blot after separation of 2 μg protein/lane from spleen homogenates and probing with 1 μg/mL goat anti-hamster arg1 polyclonal antibody or mouse anti-glyceraldehyde-3-phosphate dehydrogensase (Clone 6C5, Millipore) diluted 1:500 in 3% nonfat milk in TBS-T + 0.5M NaCl and incubated at 4°C for 2 hours. After washing the membrane the primary antibody was detected with HPRT-conjugated rabbit anti-goat antibody diluted 1:20,000 in 5% BSA in TBS-T for 1.5 hrs at room temperature. The anti-hamster arg1 antibody was produced by Genemed Synthesis, Inc. (San Antonio, TX) by immunizing goats with peptides (CFGTAREGNHKPGVDYLNNPPK and CGLVEKLKETVYDVKDY) derived from the hamster arg1 deduced amino acid sequence. The antibody did not react with *L. donovani* parasite lysates.

Determination of tissue and cellular polyamine content

Polyamines (putrescine, cadaverine, spermine, spermidine, N-acetylspermine, and N-acetylspermidine) were quantified in acid-extracted uninfected and infected hamster spleen, or uninfected and in vitro infected macrophages using High Performance Liquid Chromatography and fluorescence-based detection as described [56–58].

Sequences of hamster and *L. donovani* cDNAs

The cDNAs of the hamster arg1 (GenBank Accession number HM801029), arg2 (GenBank Accession number HM801027), STAT6 (GenBank Accession number HM801028), and *Leishmania donovani* arginase (GenBank Accession number DQ649412) were cloned and sequenced as we have described previously [49,59].

Determination of gene expression

The expression of hamster and mouse mRNAs and *L. donovani* arginase were determined in uninfected or infected tissue or cells by real time RT-PCR. In brief, the extracted RNA (RNeasy, Quiagen) was treated with DNAse (Turbo DNAse, Ambion), adjusted to 40–100 ng/μL and reverse transcribed in a final volume of 20 μL (High capacity reverse transcription kit, Applied Biosystems). 2–40 ng of reverse transcribed RNA was amplified with 400 nM of primers and 200 nM of Taqman probe in 15 μL of master mix (TaqMan, Universal PCR Master Mix, Applied Biosystem). The sequences (5′ to 3′) of the primers and probes (5′ 6-FAM and 3′ TAMRA Quencher) used to detect the specific hamster or mouse cDNAs are as follows: hamster and mouse arg1, forward, ACCTATGTGTCATTTGGGTGGA, reverse, GCAGATATGCAGGGAGTCACC, probe, TGCATGGGCAACCTGTGTCCTTTCT; hamster and mouse arg2, forward, AGCCTGGCAATAGGTACCATTA, reverse, TTCCAGATACAGTGGTGAGAGGT, probe, CCGGCACCGCCCAGATCTC; Hamster NOS2, forward, TGAGCCACTGAGTTCTCCTAAGG, reverse, TCCTATTTCAACTCCAAGATGTTCTG, probe, CGTGGACACTTCCTTTGTCTG TGCTCC; Mouse NOS2, forward, CCCAACAATACAAGATGACCCTAA, reverse, TCCAGGGATTCTGGAACATTCT, probe, ACCAAAATGGCTCCCCGCAGC; hamster STAT6, forward, GAAGCACCACTTTGCAACACA, reverse, GGCAGGTGACGGAACTCTTCT, probe, AGCTGGTGGCCACCATCAGACAAATAC; *L. donovani* arginase, forward, CGCGGACATCAACACTATGTCT, reverse, AAAGCACTCGGGAATGTTCTTG, probe, CTTGCACGGCTGCCCCTTATCGATC. The level of gene expression was determined by the comparative threshold method using uninfected BHK-21 cells (Syrian hamster fibroblast cell line) as a calibrator sample and the 18S ribosomal RNA (rRNA) gene (Applied Biosystems) as a reference (normalizer) gene. Hamster cytokine mRNA expression was determined as published [59].

Generation of recombinant hamster IL-4 and IFN-γ

Hamster IL-4 was cloned [60] and inserted into the pMIB expression vector (Invitrogen) and a stably-transfected line was derived in insect SF9 cells with blastocidin selection. The bioactivity of the recombinant protein in blastocidin-free SF9-supernatants was confirmed using the STAT6-luciferase reporter assay (see below) and the supernatants were used at 10% v/v concentration. Recombinant hamster IFN-γ was generated and used to stimulate cells as described previously [48,49].

Measurement of STAT6 activation

Phospho-STAT6 was measured in resident peritoneal macrophages infected *in vitro* or in resident peritoneal macrophages obtained from hamsters with VL. In brief, after lysis of the red blood cells, macrophages were fixed with Phosflow fix buffer I (BD), permeabilized with Phosflow perm buffer III (BD), washed and blocked with 5% donkey serum, 2% BSA, and 0.05% sodium azide in PBS, and incubated overnight at 4°C with rabbit anti-human Phospho-STAT6 antibody (Tyr641, Cell Signaling) or isotype controls diluted 1:100 in blocking buffer. After washing, the cells were stained for 1 hr at room temperature with the secondary antibody (Texas red labeled donkey anti-Rabbit antibody) and the mean fluorescence intensity and percentage of positive cells was determined by flow cytometry (FacsAria, BD). The fluorescence of isotype controls from infected and uninfected cells was used to determine the threshold fluorescense.

STAT6 phosporylation was also determined by immunoprecipitation and western blotting. In brief, spleens were homogenized in RIPA buffer with protease and phosphatase inhibitors (Santa Cruz), the supernatants cleared with Protein A/G agarose (Santa Cruz), and incubated at 4°C overnight with 1 μg of anti-STAT6 polyclonal antibody (M-20, Santa Cruz). The STAT6/antibody complex was then immunoprecipitated with Protein A/G agarose, the protein released from the beads by heating at 95°C, and resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked with 5% BSA in TBS-T and incubated overnight at 4°C with 1 μg/mL of anti-phospho STAT6 antibody (Cell Signaling). After washing, the primary antibody was detected with HRP-conjugated anti-rabbit secondary antibody (Cell Signaling) and substrate (West Pico, Thermo Scientific) followed by chemiluminescent detection (ECL; Amersham). GAPDH expression was determined as described above.

STAT6 activation was measured by a reporter assay using the BHK-21 cell line transfected with the luciferase reporter plasmid $p(IE\text{-}IL4_{RE})_4$-LUC (a generous gift from Dr. Michael Berton, University of Texas Health Science Center, San Antonio, TX) that included 4 copies of a consensus STAT6 response element sequence. The p(IE-IL4RE)4-LUC reporter plasmid was initially used to determine the STAT6 domains required for IL-4-induced transcription [61] and has since been used extensively as a reporter for IL-4 induced STAT6 activation. The BHK-21 $[IL\text{-}4_{RE}]_4$ LUC cell line was regularly maintained and sub-cultured every 72 h in DMEM with 10% HIFCS and 2 μg/mL puromycin. When cells were to be infected they were cultured overnight in complete DMEM with 2% HIFCS without puromycin, seeded in 96-clear-bottom white plates (25,000/well), and infected at 10:1 parasite:cell ratio using opsonized *L. donovani* promastigotes. After 4 hr of infection, excess extracellular parasites were removed and the infected cells cultured in complete DMEM with 2% HIFCS. The STAT-6 reporter activity was determined by luminometry using 20 μL of 1X lysis buffer and 80 μL of luciferin substrate from Promega.

Knock down STAT-6 and arg1 by miRNAi

The expression of STAT6 and arg1 genes was knocked down using a vector-based approach. miRNAi inserts targeting the hamster STAT6 or arg1 sequences and containing the structural features of the pre-miRNA, were designed using the BLOCK-iT RNAi Designer program (Invitrogen) as follows: hamster arg1, top, TGCTGTGTATCAGCTGACTAT CATG TGTTTTGGCCACTGACTGACACATGATACAGCTGATACA, bottom, CCT GTGTATC AGCTGTATCATGTGTCAGTCAGTGGCCAAAA CACATGATAGTCA GCTGATACAC; hamster STAT6, top, TGCTGAACAGGATCTCCTTGTTGAA CGTTTTGGCCACTG ACTGACGTTCAACAGAGATCCTGTT, bottom, CCTG AACAGGATCTCTGTTGAA CGTCAGTCAGTGGCCAAAACGTTCAACAAGGAGATCCTGTTC. dsOligos were generated and cloned into pcDNA 6.2-GW/±EmGFP-miR according the manufacturer instruction (Block-it PolII miR RNAi Expression Vector kits, Version E, Invitrogen). The expression vector was purified with PureLink HiPure Plasmid DNA purification kit (Invitrogen) and transfected in low passage BHK-21 cells (obtained from ATCC; 150,000 cells per well/500 μL in 24 well plates) using 0.8 μg of plasmid and 1 μL of Lipofectamine 2000 in OptiMem (InVitrogen) as recommended in the instruction manuals. The pcDNA 6.2-GW/±EmGFP-miR-LacZ plasmid (Invitrogen), which expresses an irrelevant target sequence was used as a control in all the miRNAi experiments. The transfected cells were selected after 24 hr in DMEM with 10% HIFCS and 20 μg/ml of Blasticidin, and after 15 days of selection the stably transfected cells were cloned by limiting dilution and screened for

the level of gene knockdown using real time RT-PCR. The selected cells were expanded and the efficiency of gene knockdown was determined at the mRNA level by real time RT-PCR and at the protein level by western blot. STAT6- and arg1-knockdown cells were transferred to 12-well plates (250,000 cells/1 mL DMEM with 2% HIFCS and 20 μg/ml of blasticidin) and infected for 4h with 1:10 *L. donovani* opsonized promastigotes as above. The parasite burden was determined in equal numbers of cells at the specific time points by luminometry.

Statistical analyses

Comparison between experimental groups was performed using one-way ANOVA. A parametric or non-parametric test was selected according the distribution of the raw data, followed by a post-test analysis for multiple groups as appropriate. All analyses were conducted using GraphPad InStat version 3.00 software for Windows 95 (GraphPad Software, San Diego California USA).

Results

Dominant expression of arg1 during progressive VL

Systemic infection of hamsters with *L. donovani* resulted in progressive, lethal VL [47–49]. This contrasted sharply with *L. donovani* infection in the mouse, which did not cause lethal disease and had a significantly lower parasite burden compared to hamsters infected with the same number of parasites (Fig. 1A). We demonstrated previously that in the hamster model of VL, there was impaired macrophage activation and parasite killing [49]. This was accompanied by transcriptionally-mediated low NOS2 expression and NO production in the infected hamster compared to the infected mouse ([48,49] and Fig. 1B). We hypothesized that the low NO production would favor default toward an arginase-dominated metabolism of arginine at the site of infection. Indeed, the low expression of NOS2 in the hamster spleen was accompanied by high splenic arginase activity, measured by

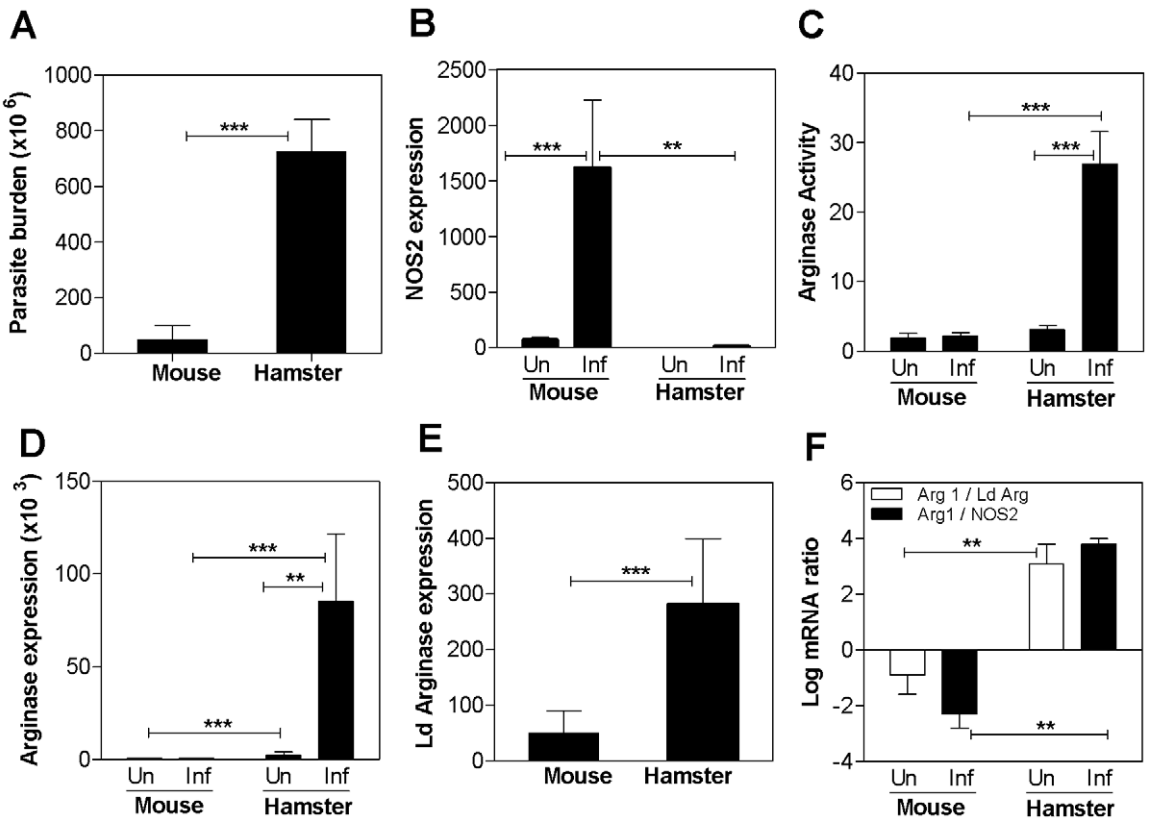

Figure 1. Dominant expression of arg1 in spleen tissue during progressive visceral leishmaniasis. A) Parasite burden in the spleens of hamsters and mice (n = 6 per group) infected with *L. donovani*-Luc for 6 weeks. The mean and standard deviation (SD) of the parasite burden, determined by luminometry and interpolation from a standard curve generated by enumeration and luciferase assay of amastigotes from the same tissue, is shown from a single experiment that is representative of 3 independent experiments. **B**) Expression of NOS2 mRNA in spleens of uninfected (Un) and 6-week *L. donovani* infected (Inf) mice and hamsters (n = 6 per group). The mean and standard error of the mean (SEM) of the fold-increase of NOS2 mRNA relative to BHK cells, determined by real time RT-PCR in 2 experiments is shown. **C**) Arginase activity in spleens of uninfected (Un) and 6-week *L. donovani* infected (Inf) mice and hamsters (n = 6 per group). The mean and standard deviation (error bars) of the tissue arginase activity, determined by assay of urea production, is shown from a single experiment that is representative of 3 independent experiments. **D**) Expression of arg1 mRNA in spleens of uninfected (Un) and 4-week *L. donovani* infected (Inf) mice and hamsters. The mean and standard deviation (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 7 animals, is shown from a single experiment that is representative of 2 independent experiments. **E**) Expression of *L. donovani* arginase mRNA in spleens of 4-week *L. donovani* infected mice and hamsters. The mean and standard deviation (error bars) of the fold-increase of *L. donovani* arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 7 animals, is shown from a single experiment that is representative of 2 independent experiments. **F**) Log_{10} ratio of hamster arg1 mRNA to *L. donovani* arginase mRNA (open bars) and log_{10} ratio of hamster arg1 mRNA to hamster NOS2 mRNA (filled bars) in the spleens of 4-week *L. donovani* infected mice and hamsters. The mRNA expression was determined by real time RT-PCR in groups of 7 animals and used to calculate the mean and SD of the log ratios. The ratios were calculated from the raw data shown in Figs. 1B, 1D, and 1E. The statistical significance of differences in each of the panels is identified by asterisks (** $p<0.01$; ***, $p<0.001$).
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enzymatic conversion of arginine to urea (Fig. 1C), and this pattern of high arginase activity/NOS2 expression was reversed in the infected mouse spleen (Figs. 1B and 1C).

To determine the source of the increased arginase activity we first analyzed the expression of parasite and host arginase mRNAs. We cloned the hamster arg1 and arg2 cDNAs and the *L. donovani* arginase cDNA and measured expression of these cDNAs by real time RT-PCR because the biochemical measurement of arginase did not discriminate between the mammalian isoforms and parasite arginase. Hamster arg2 expression was not increased in infected compared to normal tissue (see Fig. 2D), but there was a striking increase in hamster arg1 mRNA in the spleens of hamsters with progressive VL compared to uninfected hamsters and uninfected or infected mice (Fig. 1D). Additionally, infection did not upregulate splenic arg1 mRNA expression in mice (Fig. 1D). The expression of *L. donovani* arginase transcripts paralleled the

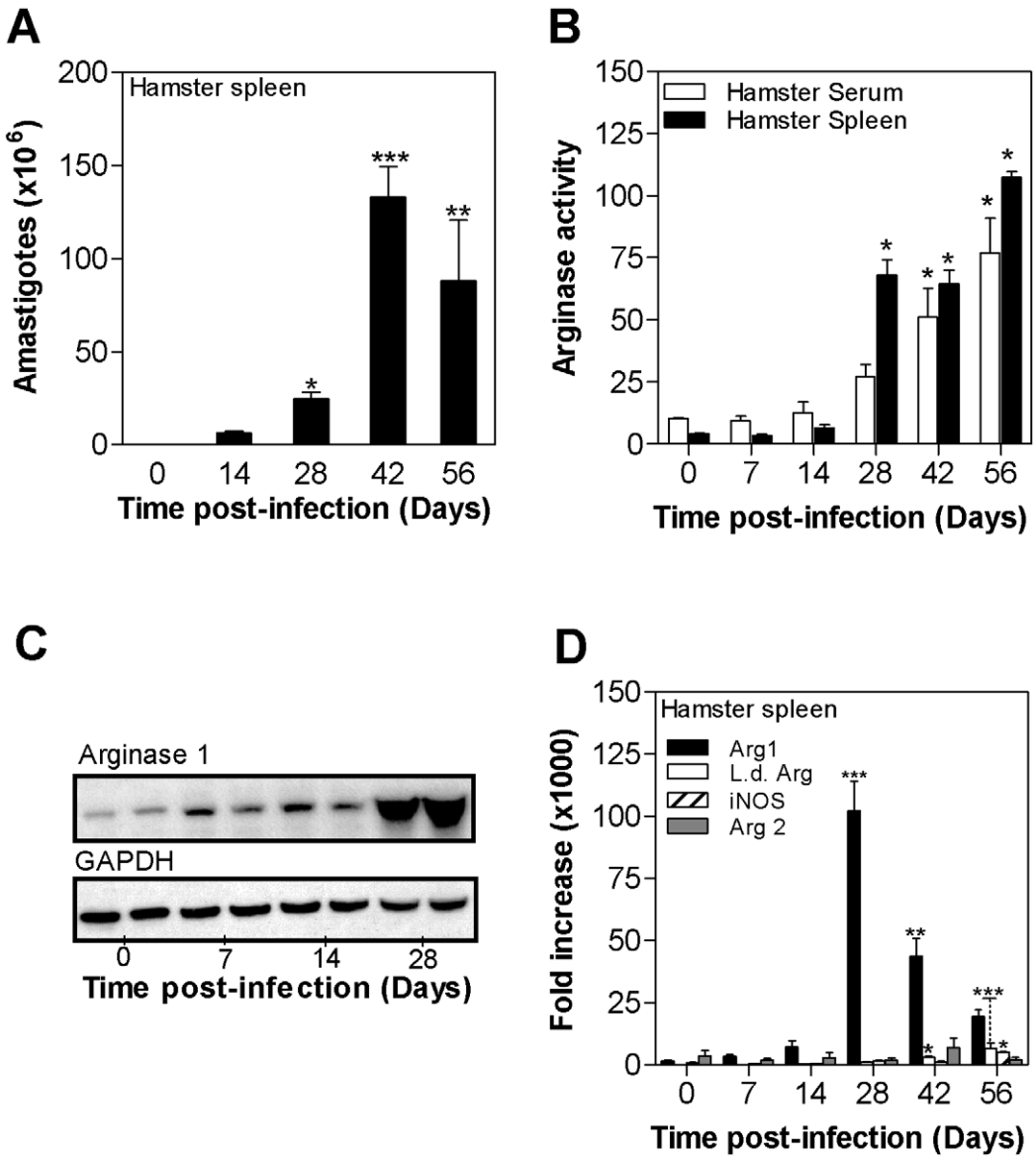

Figure 2. Kinetics of arginase expression in spleen tissue during progressive visceral leishmaniasis. A) Parasite burden in the spleens of hamsters (n = 6 per group) infected with *L. donovani* for 14, 28, 42, and 56 days. The mean and SEM (error bars) of the parasite burden, determined by luminometry and interpolation from an amastigote standard curve, is shown from a single experiment that is representative of 2 independent experiments. **B**) Arginase activity in the serum (open bars) and spleen tissue (filled bars) of uninfected hamsters (Day 0) and hamsters infected with *L. donovani* (n = 6 per group) for 7, 14, 28, 42, and 56 days. The mean and SD of the tissue arginase activity, determined by assay of urea production, is shown. **C**) Hamster arg1 protein expression determined by western blot in spleens of control hamsters (0 days post-infection) and hamsters infected with *L. donovani* for 7, 14, and 28 days. The expression of GAPDH is shown as a control for protein loading. Each lane contains splenic lysate from a single hamster, with two lanes per time point. The anti-arginase antibody did not react with parasite arginase by immunoblot. **D**) Time course of expression of hamster arg1 mRNA (filled bars), *L. donovani* arginase mRNA (empty bars), hamster NOS2 (iNOS) mRNA (hatched bars), and hamster arg2 mRNA (hatched bars) in spleens of control hamsters (0 days post-infection) and hamsters infected with *L. donovani* for 7, 14, 28, 42, and 56 days. The mean and standard deviation (error bars) of the fold-increase of arginase mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from a single experiment that is representative of 3 independent experiments. The statistical significance of differences in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
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splenic parasite burden in infected mice and hamsters (Fig. 1E), but was a relatively minor contributor to the overall expression of arginase in hamsters (Fig. 1F; Fig. 2B). When the expression of parasite arginase and host NOS2 was compared to host arg1 a striking difference between mice and hamsters was evident. The level of hamster arg1 was more than 1000-fold greater than the level of parasite arginase in infected spleen tissue, whereas the ratio was inverted in the infected mouse spleen with parasite arginase being greater than host arg1 expression (Fig. 1F, open bars). Similarly, the host arg1 to NOS2 ratio was high in the infected hamster spleen, but the ratio was reversed in the infected mouse spleen (Fig. 1F, filled bars).

Kinetics studies revealed a sharp increase in the visceral parasite burden of L. donovani infected hamsters relatively late in the course of infection (Fig. 2A) that was accompanied by increased arginase activity in the serum and spleen (Fig. 2B). Dramatically increased expression of arg1 protein (Fig. 2C) and mRNA (Fig. 2D) was also evident in the spleen of infected hamsters. Unexpectedly, we found discordance between the level of hamster arg1 mRNA expression and arg1 protein expression and arginase enzyme activity late in the course of infection; after the peak at 28 days post-infection, the mRNA expression decreased but the level of protein and enzyme activity remained high. Hamster arg2 mRNA was not expressed in the spleen throughout the course of infection, and hamster NOS2 mRNA was increased only slightly at day-56 of infection (Fig. 2D).

Several polyamines are end-products of arginine metabolism through the action of arginase and ornithine decarboxylase [56–58]. We confirmed the downstream effect of increased arg1 expression in hamsters with VL by demonstrating increased putrescine and spermidine in infected compared to uninfected spleen tissue (Fig. 3A), and increased putrescine, spermine, and spermidine in the infected liver (Fig. 3B). In infected hamsters there was no increase in the tissue content of acetylspermine and acetylspermidine, or cadaverine, which is a product of lysine metabolism. The tissue polyamine content in the spleen and liver of mice was relatively low compared to the hamster tissue, and it was not increased in infected compared to uninfected mice (Fig. S1).

Cytokines that promote alternative macrophage activation are upregulated late in the course of VL

The dominant expression of hamster arg1 in progressive VL suggested that IL-4, IL-10, IL-13, or IL-21, or a combination of these cytokines, which are known to promote alternative macrophage activation (reviewed in [36]), might be driving the expression of arg1. Since the type 2 cytokines induce arginase expression through a STAT6-dependent pathway, we first investigated whether there was evidence of STAT6-inducing activity in the serum and spleens of hamsters with VL. Using a STAT6 reporter assay, we found that there was a significant increase in STAT6-inducing activity in the serum (56% increase; $p<0.05$) obtained from hamsters with active VL (56 days post-infection) compared to uninfected controls. These findings led us to consider that the type 2 cytokines might contribute to the increased expression of arg1 in VL. After systemic infection with *L. donovani*, a relatively silent phase during the first ~3 weeks of infection was followed by increased expression of IL-4, IL-10, IL-13, and IL-21 (Fig. 4). The increase in these cytokines was coincident with, but did not appear to precede, the dramatic increase in parasite burden and arginase activity (see Figs. 2 and 5A).

L. donovani induces arg1 expression in infected macrophages

The increase in splenic arginase activity over the course of infection was paralleled by a similar increase in arginase activity in macrophages isolated from the spleens of hamsters infected with *L. donovani* (Fig. 5A). The reciprocal expression of arg1 and NOS2 mRNAs was also observed in splenic macrophages isolated from infected mice and hamsters. Splenic macrophages isolated from 4-week infected hamsters had a high arg1 to NOS2 ratio, but this ratio was reversed in splenic macrophages isolated from 4-week infected mice (Fig. 5B). Furthermore, resident peritoneal macrophages from hamsters with VL showed increased arginase activity compared to peritoneal macrophages from uninfected animals, but arginase activity in peritoneal macrophages from mice infected with the same number of parasites was not increased (Fig. 5C). An increase in *L. donovani*-induced arginase activity (Fig. 5D) and arg1 mRNA (Fig. 5E) was also evident in hamster peritoneal macrophages infected in vitro with *L. donovani* promastigotes. Opsonization of *L. donovani* promastigotes with either fresh hamster serum containing complement or hamster serum containing anti-*Leishmania* antibodies did not influence the parasite-mediated induction of arginase activity (Fig. 5D). Congruent with the findings of increase polyamine synthesis in infected hamster tissues (see Fig. 3), the downstream effect of the increased arginase activity

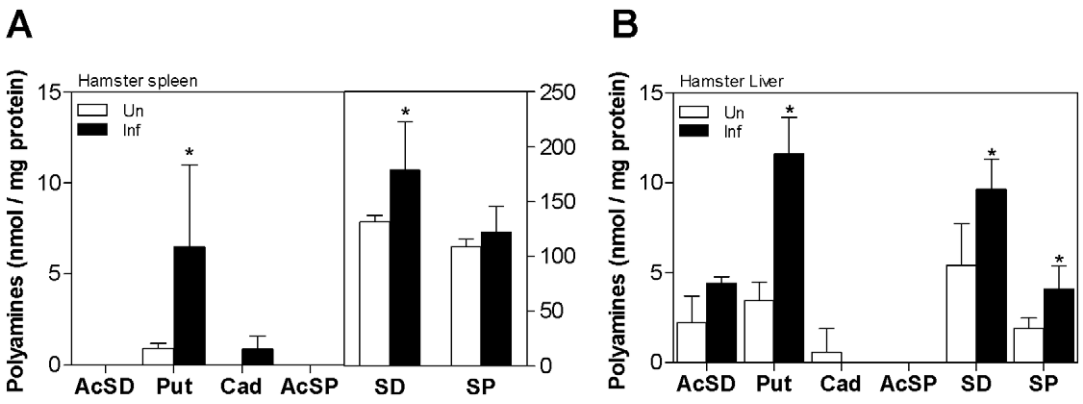

Figure 3. Polyamine content in spleen and liver tissue in *L. donovani* infected hamsters. The concentration of polyamines in spleen (**A**) and liver (**B**) tissue samples from groups of 6 uninfected hamsters (open bars) and 56 day infected hamsters (filled bars) is expressed as the mean and SD (error bars) of nmol polyamine per mg protein. The data shown are from a single experiment that is representative of 2 independent experiments. The statistical significance of differences in each of the panels is identified by an asterisk (*, $p<0.05$).
doi:10.1371/journal.ppat.1002417.g003

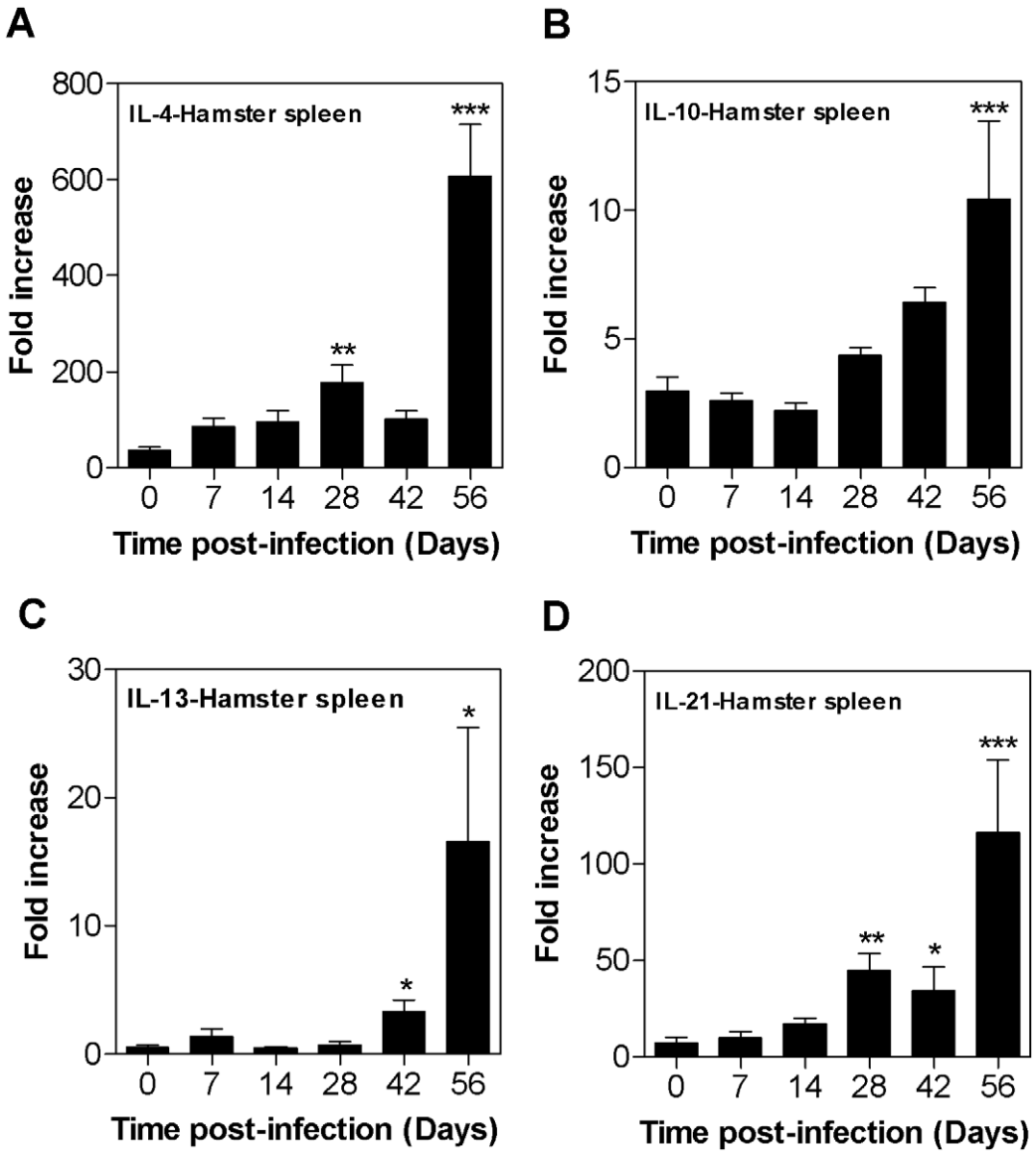

Figure 4. Kinetics of cytokine expression in spleen tissue during progressive visceral leishmaniasis. Expression of hamster IL-4 (**A**), IL-10 (**B**), IL-13 (**C**), and IL-21 (**D**) mRNAs in the spleens of control hamsters (0 days post-infection) and hamsters infected with *L. donovani* for 7, 14, 28, 42, and 56 days. The mean and standard deviation (error bars) of the fold-increase of cytokine mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from a single experiment that is representative of 2 independent experiments. The statistical significance of differences in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
doi:10.1371/journal.ppat.1002417.g004

in infected hamster macrophages was evident by increased polyamine (putrescine, spermidine, and spermine) synthesis compared to uninfected cells (Fig. 5F).

Parasite-induced arg1 requires de novo protein synthesis but not parasite internalization

We next determined if *de novo* protein synthesis was required for the parasite-induced increase in arg1 mRNA. To do this we established an in vitro infection model in the BHK hamster fibroblast cell line, since primary macrophages were killed by the protein synthesis inhibitor cycloheximide, even at very low doses, and no hamster macrophage cell lines were available. We first demonstrated that, like hamster macrophages (Fig. 5D), hamster primary splenic fibroblasts expressed arg1 in response to in vitro infection with *L. donovani* (Fig. 6A). Furthermore, we showed that BHK fibroblasts could be infected with *L. donovani* (confirmed by microscopical examination and co-localization with phagolysosomal staining; see Fig. S2), and were capable of metabolizing arginine through either the NOS2 or arginase pathways. BHK fibroblasts generated NO in response to classical activation stimuli (IFN-γ/LPS) and expressed arginase activity in response to LPS as was described for macrophages from murine rodents [62] (Fig. 6B). Surprisingly, IL-4 alone did not induce arginase activity in BHK fibroblasts. We next showed that like hamster macrophages, in vitro infection of BHK fibroblasts resulted in parasite-induced arg1 mRNA expression (Fig. 6C). The parasite-induced arg1 expression was amplified by concomitant exposure to exogenous recombinant hamster IL-4 (Fig. 6C). The induction of arg1 expression was also evident in BHK cells infected with spleen-derived purified amastigotes (Fig. 6D). Dose titration studies

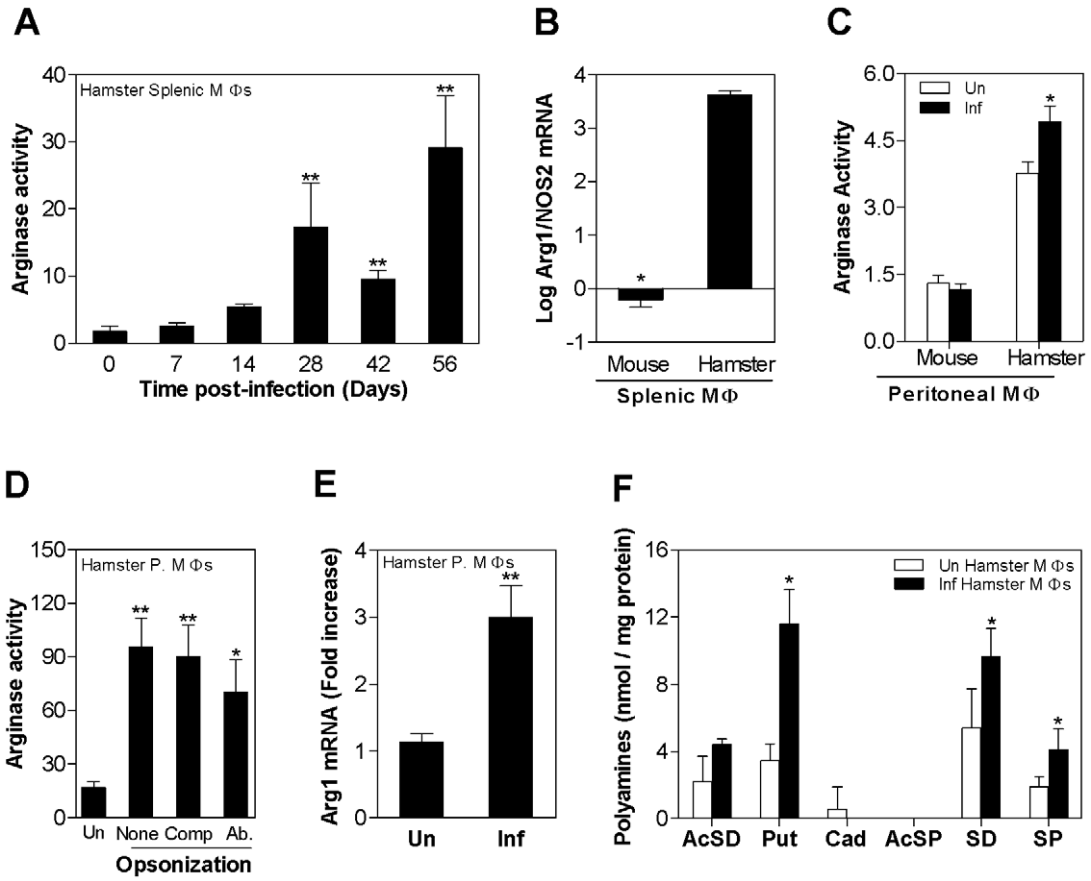

Figure 5. Arg1 expression and polyamine content in *L. donovani* infected macrophages. **A**) Arginase activity in macrophages isolated by adherence from single-cell suspensions from the spleens of control hamsters (0 days post-infection) and hamsters infected with *L. donovani* for 7, 14, 21, 42, and 56 days. The mean and SD (error bars) of the arginase activity in 100,000 cells, determined by assay of urea production, is shown from a single experiment that is representative of 2 independent experiments. **B**) Log_{10} ratio of host arg1 to NOS2 mRNA in splenic macrophages of 4-week *L. donovani* infected mice and hamsters. The mRNA expression was determined by real time RT-PCR in groups of 5 animals and used to calculate the log ratios, shown as the mean and SD (error bars) from a single experiment that is representative of 2 independent experiments. The ratios were determined from the following raw data: Arg1 mRNA fold-increase with reference to the BHK cell calibrator (mean ± SD): uninfected mice, 5.7±5.9; uninfected hamster, 3,367±2,858, infected mice, 8.5±10; infected hamster, 131,984±56,407; iNOS mRNA fold-increase with reference to BHK cell calibrator (mean ± SD): uninfected mice, 133.34±182.4; uninfected hamster, 1.03±0.3, infected mice, 2,984±4,535; infected hamster, 19.56±10.87). **C**) Arginase activity in peritoneal macrophages isolated from mice and hamsters that were uninfected (open bars) or infected in vitro with *L. donovani* for 48 hrs (filled bars). The mean and SD (error bars) of the arginase activity, determined by assay of urea production, is shown from a single experiment that is representative of 2 independent experiments. **D**) Arginase activity in hamster peritoneal macrophages that were uninfected (Un) or infected in vitro with *L. donovani* (1:5 macrophage:parasite ratio). Parasites were either unopsonized (None), opsonized with normal fresh complement-containing hamster serum (Comp), or opsonized with freeze-thawed hamster serum containing anti-Leishmania antibody (Ab). The mean and standard deviation (error bars) of the arginase activity in 200,000 cells of 6 different samples determined by assay of urea production, is shown from a single experiment that is representative of 2 experiments. Statistical comparisons are made to the control group. **E**) Expression of hamster arginase mRNA in hamster peritoneal macrophages that were uninfected (Un) or infected in vitro with *L. donovani* stationary-phase promastigotes for 24 hrs (Inf). The mean and SEM (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 samples per experiment, is shown as data pooled from 4 independent experiments. **F**) The concentration of polyamines in uninfected (open bars) and 48-hour in vitro infected hamster peritoneal macrophages (filled bars) (n = 6 per group) is expressed as the mean and SD (error bars) of nmol polyamine per mg protein. The data shown are from a single experiment that is representative of 2 independent experiments. The statistical significance of differences in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
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identified cycloheximide concentrations that effectively blocked protein synthesis but did not significantly reduce cell viability in BHK cells. Parasite-induced arg1 transcription was blocked by non-toxic concentrations of cycloheximide (CHX) when the CHX was present during the first 12 hrs of exposure to the *Leishmania* (Fig. 6E), indicating that early *de novo* synthesis of either an autocrine or paracrine protein, was required for arg1 mRNA expression. However, infected BHK cells did not express IL-4 mRNA (by real-time RT-PCR the C_T value for GAPDH was 22.9±0.09 and the C_T value for IL-4 was below the threshold limit of detection), and expressed very low basal levels of IL-13 that did not increase with infection (after correction for GAPDH expression the fold-increase relative to the BHK calibrator was 1.14 ± 0.16 for uninfected cells and 0.98±0.2 for infected cells; $p = 0.16$), suggesting that the parasite-induced arg1 expression was independent of type 2 cytokine synthesis. The parasite-induced transcription of arg1 did not require the internalization of parasites, since separation of promastigotes from hamster cells with a 0.4 μ membrane did not abrogate the parasite-induced increase in arg1 mRNA expression (Fig. 6F).

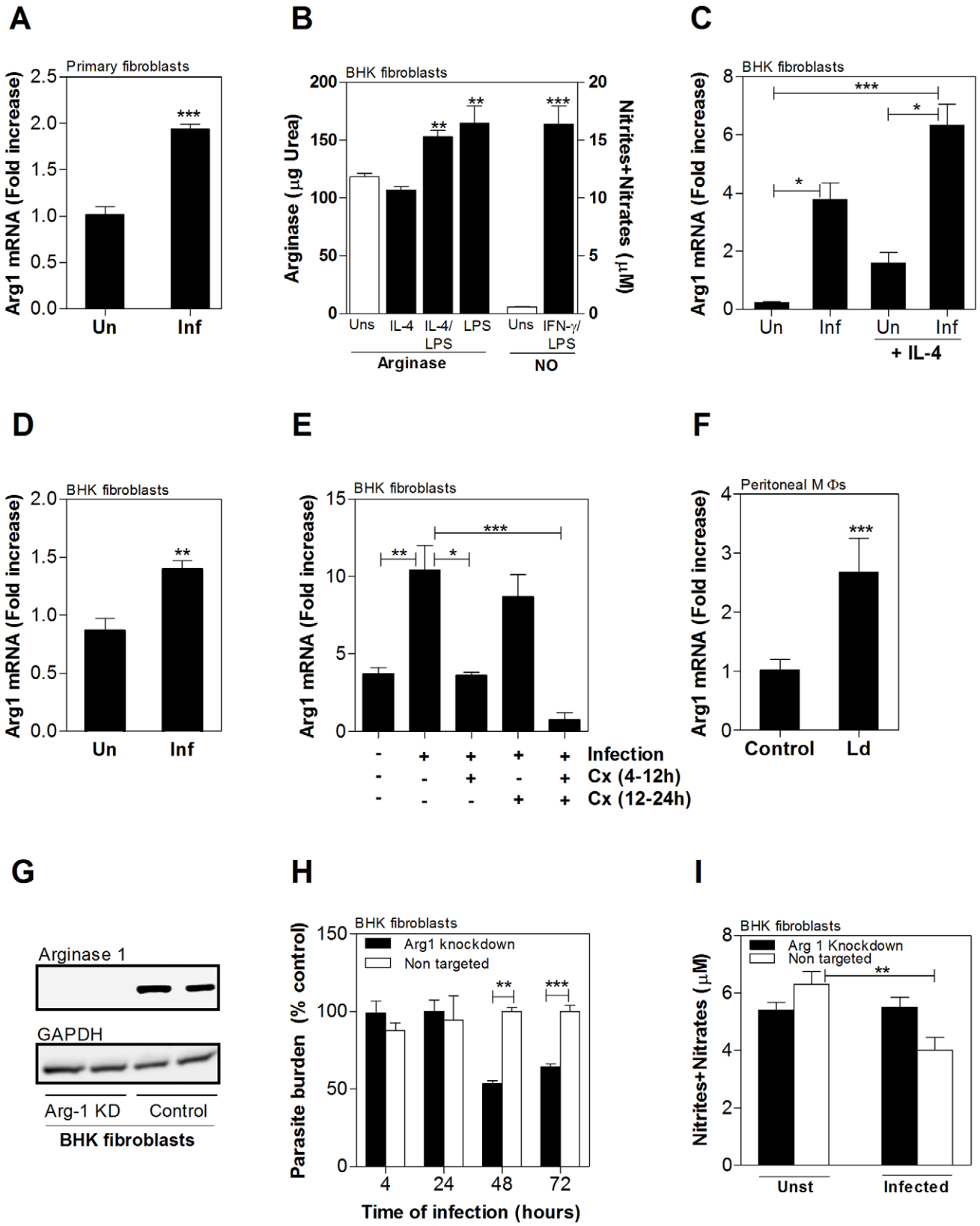

Figure 6. Parasite-induced host arg1 expression impairs macrophage anti-leishmanial activity. A) Expression of hamster arginase mRNA in primary hamster splenic fibroblasts that were uninfected (Un) or infected in vitro with *L. donovani* stationary-phase promastigotes for 24 hrs (Inf). The mean and standard deviation (error bars) of the fold-increase of arg1 mRNA relative to normal BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from data pooled from 3 independent experiments. **B**) Arginase activity and NO production in BHK fibroblasts. Arginase activity was determined stimulated with IL-4 or IFN- γ respectively. Arginase activity was determined in BHK cells that were unstimulated (Uns) or

stimulated for 48h with IL-4 (10% v/v), LPS (1 μg/mL), or both. The mean and standard deviation (error bars) of the arginase activity of 6 different stimulated samples compared to that of non stimulated cells determined by assay of urea production, is shown from a single experiment that is representative of 2 experiments. NO production was estimated by the measurement of nitrites + nitrates in supernatants of unstimulated cells (Uns) and cells stimulated with IFN-γ (10% v/v of hamster recombinant IFN-γ supernatants) plus 1 μg/mL LPS for 48h. The mean and standard deviation (error bars) of the nitrites/nitrates of 6 different samples determined by Griess assay is shown from a single experiment that is representative of 2 experiments. **C**) Expression of hamster arginase mRNA in hamster BHK cells that were uninfected (Un) or infected in vitro with *L. donovani* stationary-phase promastigotes for 24 hrs (Inf) and cultured with or without recombinant hamster IL-4 (10% v/v supernatant) or sham supernatant. The mean and standard deviation (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from data pooled from 3 independent experiments. **D**) Expression of hamster arginase mRNA in hamster BHK fibroblasts that were uninfected (Un) or infected in vitro with *L. donovani* tissue-derived amastigotes for 24 hrs (Inf). The mean and standard deviation (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from data pooled from 2 independent experiments. **E**) Effect of cycloheximide (CHX) on parasite-induced hamster arg1 mRNA expression in BHK cells was determined by exposing cells to CHX (20 μg/mL) early (4–12 hrs) and/or late (12–24 hrs) during a 24-hr *L. donovani* infection. The mean and SD (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 samples, is shown from data from a single experiment that is representative of 2 independent experiments. **F**) Induction of expression of hamster arg1 in peritoneal macrophages by soluble parasite factors was determined by culturing hamster peritoneal macrophages with stationary phase promastigotes (1:10 ratio) separated by a 0.4 μ pore size membrane (Falcon). The mean and SEM (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR in groups of 6 animals, is shown from data pooled from 3 independent experiments. **G**) miRNAi-mediated arg1 knockdown in BHK cells. Arg1 protein in BHK cells stably transfected with a miRNAi vector targeting arg1 (Arg-1 KD) or stably transfected with a miRNAi vector coding a control sequence (Control) determined by Western blot using a specific polyclonal antibody raised against hamster arg1. Representative of 2 different blots. **H**) Effect of miRNAi-mediated knockdown on parasite burden was determined in BHK cells that were transfected with a non-targeting miRNAi vector (control) or a vector specific to hamster arg1. The transfected cells were infected with *L. donovani* metacyclic promastigotes and the mean and standard deviation (error bars) of the parasite burden at 4, 24, 48 and 72 hrs post-infection, determined by luminometry, is shown from a single experiment that is representative of 2 independent experiments. There was no difference in parasite burden between the two groups at 4 and 24 hrs post-infection. **I**) Arg1 knockdown did not induce NO production in BHK cells infected with *L. donovani*. NO production in BHK cells stably transfected with a miRNAi vector targeting Arg1 (Arg1 knockdown) or transfected with an irrelevant miRNAi vector (Non targeted). The mean and standard deviation (error bars) of the nitrites/nitrates released in the supernatant of cells after 48h infection with *L. donovani* was determined by Griess assay. Data are from a single experiment that is representative of 3 independent experiments. (**p<0.001). The statistical significance of differences in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
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Knockdown of host arg1 promotes parasite killing without enhanced NO production

Since arginase and NOS2 compete for the same substrate, arginine, we reasoned that inhibition of arginase might reverse the low NO production observed in activated hamster macrophages and lead to enhanced parasite killing. Treatment with the arginase inhibitor norNOHA resulted in a significant dose-dependent reduction in arginase activity (Fig. S3, panel A) and parasite burden (Fig. S3, panel B) in peritoneal macrophages infected in vitro, and a reduction in parasite burden in ex vivo cultured spleen cells isolated from infected hamsters (Fig. S3, panel C). However, we found that norNOHA demonstrated a dose-dependent killing of amastigotes purified from infected hamster spleens (Fig. S3, panel D) and axenically cultured *L. donovani* promastigotes (Fig. S3, panel E), suggesting that it was inhibiting parasite arginase. Therefore, to investigate the role of arginase in parasite replication without the potential confounding influence of parasite arginase activity, we used the BHK infection model to knockdown host arg1. Transfection of BHK cells with an arg1-specific miRNAi vector resulted in >90% reduction in arg1 mRNA expression ($p = 0.002$) and reduction of arg1 protein to a level undetectable by western blot (Fig. 6G). *L. donovani* infected BHK cells that expressed the arg1-specific miRNAi vector were found to have an equivalent parasite burden at 4 and 24 hrs post-infection but significantly reduced parasite burden at 48 hrs ($p<0.01$) and 72 hrs ($p<0.001$) post-infection compared to non-transfected cells or cells transfected with a non-targeting miRNAi construct (Fig. 6H). The approximately 50% decrease in parasite burden following arg1 knockdown suggests that either there is residual arginase expression (undetectable by western blot) that is sufficient to promote some parasite survival, or more likely that arginase is not the only determinant of parasite survival and replication in this model. This enhancement of parasite killing by knockdown of arg1 in BHK cells was not accompanied by greater parasite-induced NO production (Fig. 6I), even though BHK cells were fully capable of generating NO (see Fig. 5B). Thus it would appear that parasite-induced macrophage arg1 contributes to *L. donovani* replication through mechanisms other than reduction of NO production.

STAT6 activation drives parasite-induced arg1 expression in VL

The expression of phosphorylated STAT6 was increased in the spleen tissue of hamsters over the course of in vivo infection with *L. donovani* (Fig. 7A). To dissect the role of STAT6 in *L. donovani*-induced arg1 expression we used the in vitro infection model of the BHK hamster fibroblast cell line as described above. Using an in vitro reporter assay we found that exposure of hamster fibroblasts to metacyclic *L. donovani* promastigotes activated STAT6 in a dose-dependent manner (Fig. 7B). The STAT6 activation was not influenced by parasite opsonization with complement-containing serum (Fig. S4). Parasite-induced STAT6 phosphorylation was further confirmed by flow cytometry in infected BHK cells (Figs. 7C and 7D), in splenic macrophages isolated from hamsters with VL (Fig. 7E), and in hamster peritoneal macrophages infected in vitro with *L. donovani* promastigotes (Fig. 7F). The percent of macrophages that showed parasite-induced STAT-6 phosphorylation was relatively lower than the percent positive activated by IL-4 (Fig. 7D). Knockdown of hamster STAT6 in BHK cells using miRNAi resulted >90% decrease in mRNA expression (Fig. 7G) and reduction of STAT6 protein to a level undetectable by western blot (Fig. 7H). The significance of parasite-induced STAT6 activation was confirmed by showing a 92% reduction in arg1 mRNA expression when STAT6 was knocked-down (similar to the reduction obtained with arg1-specific knockdown) compared to a control non-targeting miRNAi ($p<0.001$; Fig. 7G) and enhanced control of intracellular parasite replication in BHK cells (equivalent infection after 4 hrs but significantly reduced parasite burden at 24, 48, and 72 hrs post-infection compared to non-transfected cells or cells transfected with a non-targeting miRNAi construct ($p = 0.001$; Fig. 7H). Collectively, these data indicate that parasite-induced STAT6 activation drives expression

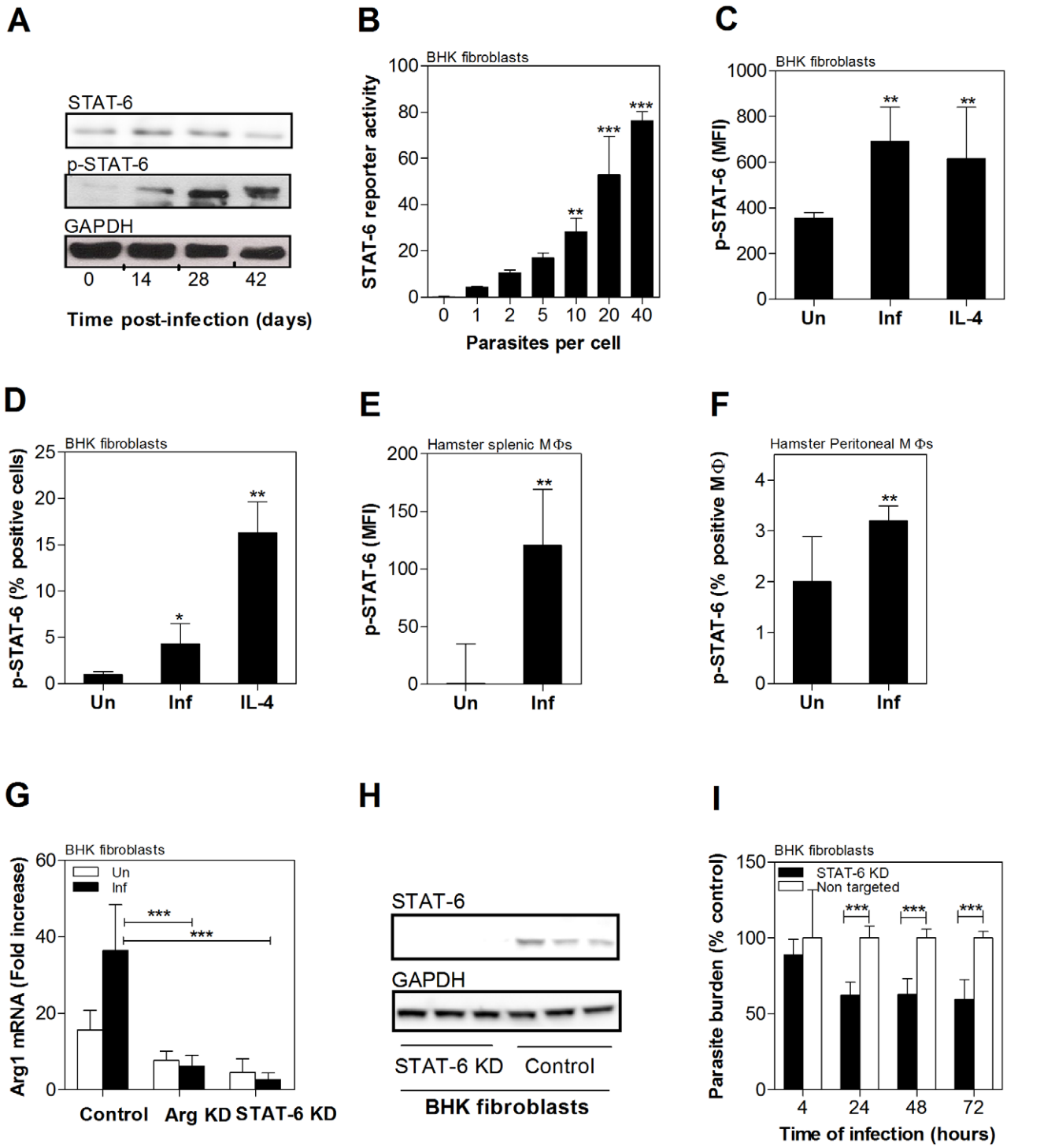

Figure 7. Role of parasite-induced STAT6 activation in host arg1 expression and *L. donovani* infection. A) Phosphorylated STAT6 in spleens of hamsters infected with *L. donovani* determined by immunoprecipitation followed by western blot of whole splenic lysates. The samples (before immunoprecipation) were probed with anti-STAT6 anti-GAPDH antibodies to confirm equivalent protein loading. The blot shown is from tissue from a single animal, representative of 4 independent experiments. **B**) Dose-dependent induction of STAT6 activation by *L. donovani* in BHK cells transfected with a STAT6-luciferase reported vector. Data are presented as the mean and standard deviation (error bars) of the relative light units in uninfected (0 parasites) cells and cells exposed to 1–40 parasites per cell over 48 hrs of culture. Shown is data from a single experiment that is representative of 2 independent experiments. **C-D**) Phosphorylation of STAT6 in hamster peritoneal macrophages exposed to *L. donovani* (10 parasites per cell) or IL-4 (10% v/v supernatants) measured as fold-increase of mean fluorescence intensity (MFI) (**C**) or percent positive cells (**D**) by flow cytometry. Data are presented as the mean and standard deviation (error bars) and are from a single experiment that is representative of 2 independent experiments. **E–F**) Phosphorylation of STAT6 in hamster BHK cells exposed to *L. donovani* (10 parasites per cell) measured as fold-increase of mean fluorescence intensity (MFI) (**E**) or percent positive cells (**F**) by flow cytometry. Data are presented as the mean and standard deviation (error bars) and are from a single experiment that is representative of 2 independent experiments. **G**) Effect of miRNAi-mediated STAT6

knockdown on arg1 and STAT6 mRNA expression. BHK cells were transfected with a non-targeting miRNAi vector (control) or a vector specific to hamster STAT6 and the expression of arg1 and STAT6 mRNA determined by real time RT-PCR in uninfected (open bars) and 24-hr infected cells (filled bars). The data are presented as the mean and standard deviation (error bars) of the fold-increase of arg1 mRNA relative to BHK cells, determined by real time RT-PCR from a single experiment, representative of 2 independent experiments. **H**) miRNAi-mediated STAT-6 knockdown in BHK cells. The expression of STAT-6 protein in BHK cells stably transfected with a miRNAi vector targeting STAT-6 (STAT-6 KD) or transfected with a miRNAi vector coding a control sequence (Control) was determined by western blot using an anti-STAT6 antibody. An anti-GAPDH antibody was used to confirm equivalent protein loading. Data are from a single blot representative of 2 independent experiments. **I**) Effect of miRNAi-mediated STAT6 knockdown on parasite burden was determined in BHK cells that were transfected with a non-targeting miRNAi vector (control) or a vector specific to hamster STAT6. The transfected cells were infected with *L. donovani* metacyclic promastigotes and the mean and standard deviation (error bars) of the parasite burden at 4, 24, 48, and 72 hrs post-infection, determined by luminometry, is shown as data pooled from 2 independent experiments. There was no difference in parasite burden between the two groups at 4 hrs post-infection. The statistical significance of differences between groups in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
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of host arg1, which in turn contributes to intracellular parasite replication and/or survival.

Discussion

We initiated these studies because there is a deficit in our understanding of the molecular and cellular determinants underlying the pathogenesis of VL. We used a hamster model of VL because the clinicopathological features of this model closely mimic active human VL, and insights gained from this model may enhance our understanding of the immunopathogenesis of human disease. In the studies we present here we identified a program of alternative macrophage activation that is evident in the spleens of hamsters with VL, and in *L. donovani* infected macrophages. Characteristic of this phenotype is the dominant expression of host arg1 over NOS2 in infected hamster spleen tissue and macrophages. Here we show that *L. donovani* induces host arg1 expression through a mechanism that involves parasite-induced STAT6 activation, but different from the prevailing paradigm of alternative activation in cutaneous leishmaniasis [42–44], occurs even in the absence of a polarized type 2 cytokine response.

The tissue-level dominance of arginase over NOS2-mediated arginine metabolism is driven by increased host arg1 but not arg2 transcription. Furthermore, hamster arg1, which is expressed by macrophages in the infected spleen, is dominant over the level of parasite arginase even though parasite arginase expression increases throughout the course of infection. This contrasts sharply with what we found in the murine model of non-progressive *L. donovani* infection where host arg1 expression is not dominant, and parasite arginase appears to have a relatively greater contribution to the total arginase activity. Similarly, in the murine model of *L. major* infection, parasite arginase contributes significantly to the overall cellular arginase activity [63]. While it is logical that *L. donovani* arginase contributes to the pathogenesis of VL, our data suggest that it has a significantly lesser role than host arg1. Quantification of its contribution to the pathogenesis of VL in this model will require studies using an enzyme inhibitor selective for parasite arginase or arginase-deficient parasites.

The consequences and significance of the increased host arg1 expression in the progression of VL is underscored by several findings. First, during progressive infection there was an increase in tissue polyamines, which are end products of arginase- and ornithine decarboxylase-mediated metabolism of arginine. Polyamines, synthesized by the parasite or scavenged from host cells through uptake receptors [45], promote *Leishmania* growth [46]. The importance of polyamines is underscored by their critical role in the growth of a number of other protozoa, including *Trypanosoma brucei*, *T. cruzi*, *Toxoplasma* and *Plasmodium* [64–66]. Second, the increase in host arg1 transcription and enzyme activity paralleled the increase in the visceral parasite burden. This corroborates the findings in the murine model of *L. major* infection [42–44]. However, late in the course of hamster VL there is a relative decrease in the expression of arg1 mRNA but sustained arginase protein expression and enzyme activity, which is consistent with the previous notion that arg1 is also post-transcriptionally regulated [67]. Third, as noted above, we found that at the site of visceral infection (spleen) in hamsters there is dominant expression of arg1, such that the arg1 to NOS2 ratio in hamsters with progressive disease was thousands-fold greater than the ratio observed in mice, which are able to control the infection. The non-induced arg1 mRNA expression in the *L. donovani* infected mouse spleen in our study was different from the 4.8-fold increase reported in a recent study [68]. This difference may be due in part to infection with a different *L. donovani* strain (Indian vs. East African) and use of a much larger inoculum in the study by Biswas, et al. [68]. Nevertheless, the expression of arg1 in the *L. donovani* infected mouse is dramatically less than the increase observed in the hamster model of progressive VL. This dominant expression of arg1 in hamster VL lead to disease by production of parasite-promoting polyamines, or by driving arginine metabolism away from NOS2 and production of the anti-leishmanial effector molecule NO (which is already expressed at a low level). Fourth, and most significantly, targeted knockdown of host arg1 mRNA led to enhanced capacity to control intracellular parasite replication. Although other investigators demonstrated that chemical inhibition of arginase enhanced control of *Leishmania* infection [42,44], we found that the arginase inhibitor nor-NOHA mediated a host-independent anti-parasitic effect, presumably by direct inhibition of parasite arginase (which was also recently demonstrated for *L. mexicana* arginase [69]), and therefore could not be used to distinguish an effect of host arginase independent of parasite arginase in our model. This increased control of infection mediated by targeted arg1 knockdown did not appear to be driven by enhanced production of NO by the isolated infected macrophage, suggesting that the parasite-induced arginase has a pathological effect through the increase in polyamines that promote parasite growth. This is consistent with the findings in *L. major* infection [44].

As stated previously, alternatively activated macrophages, as introduced by Gordon and colleagues, display a unique phenotype when activated in the presence of IL-4 or IL-13 [36,37]. The gene expression profile of these macrophages includes reduced expression of NOS2, and increased expression of a number of unique genes, including arg1, which are transcriptionally activated by IL-4 through a STAT6-dependent mechanism [70,71]. STAT6 is considered to be the central regulator of alternative macrophage activation [36]. While STAT6 may be activated by other stimuli such as IL-15, platelet-derived growth factor, kit ligand, and leptin, the canonical pathway for STAT6 activation is through IL-4 or IL-13 (reviewed in [72]). With this understanding, it is not surprising that the prevailing paradigm is that alternative activation of macrophages during murine *L. major* infection is a downstream effect of the dominant Th2 polarization and type 2

cytokine production seen in this model [42–44]. Indeed, the IL-4/IL-13/IL-4Rα/STAT6 signaling pathway has a well-established role in the pathogenesis of cutaneous *L. major* and *L. mexicana* infection in mice [73,74]. However, these studies in the murine model focused on the effects of STAT6 deficiency in the T cell compartment without addressing the role of STAT6 in macrophages [74,75]. We found that exposure of macrophages or fibroblasts to *L. donovani* led to activation of STAT6 (measured using an in vitro reporter assay, flow cytometry of infected macrophages and fibroblasts, and western blotting of phospho-STAT6) even in the absence of T cell signals or exogenous type 2 cytokines. The parasite-induced host arg1 expression was completely abolished by miRNAi-mediated knockdown of STAT6, and interruption of this pathway either by STAT6 or arg1 knockdown enhanced the control of intracellular parasite replication.

Although it has not been thoroughly studied, fibroblasts can be polarized by exposure to IL-4, resulting in STAT6 activation [76] and expression of arginase and other markers that are expressed by alternatively activated macrophages [77,78]. *Leishmania* are known to infect fibroblasts [79,80], and stromal cells are increasingly being recognized as modulators of host defense (reviewed in [81]). The finding of arg1 expression in splenic fibroblasts from hamsters with VL, and in fibroblasts infected in vitro with *L. donovani*, suggests that the "alternatively activated" phenotype extends to splenic stromal cells in VL, which are likely to contribute to the pathogenesis of the disease. The fact that IL-4 alone did not induce arginase activity in BHK fibroblasts supports the notion that the *Leishmania*-induced arg1 is driven through an IL-4-independent pathway in this cell.

Although early *de novo* synthesis of either an autocrine or paracrine protein was required for arg1 mRNA expression, this yet to be identified factor did not appear to be IL-4 or IL-13 since there was no increase in endogenous expression of these cytokines in the in vitro infected cell culture where parasite-induced STAT6 activation and arg1 expression were evident. This is not to say that signaling through IL-4/IL-13/IL-4Rα/STAT6 has no role in the pathogenesis of VL. The addition of exogenous IL-4 to the in vitro infection model clearly amplified the parasite-induced arg1 expression, and our in vivo data suggest that the prominent type 2 cytokine expression that is evident late in the course of VL serves to amplify arg1 expression and the alternative activation phenotype, and thus contributes to the relentlessly progressive infection. In human visceral and cutaneous leishmaniasis, there is increased expression of the type 2 cytokines at the site of chronic and severe infection [13,14,23,82–84], but the role of these cytokines in the pathogenesis of human infection has not been fully defined. Other signaling molecules could also interface with this pathway to influence arg1 expression. A number of co-activator proteins, including, p100, CBP/p300, SRC-1, RNA pol II, PU.1, and C/EBP, are recruited with STAT6 to form a complex enhancer element in the promoter to initiate in arg1 transcription [85–87]. Of particular note, C/EBPβ may be activated by the IL-10/STAT3 pathway [88,89]. The potential for synergistic interaction of the IL-4/IL-13/STAT6 and IL-10/STAT3 pathways was not fully appreciated until the recent work of Biswas, et al, who found that IL-10 expressed in the spleens of mice infected with *L. donovani* induced the upregulation of IL-4Rα, which was required for arg1 expression [68]. Identification of co-activators and additional pathways that contribute to STAT6-dependent *L. donovani*-induced arg1 expression in this model of progressive VL is currently under investigation.

A number of host factors other than type 2 cytokines can induce a macrophage activation profile that overlaps the classic IL-4- and IL-13-induced alternative macrophage activation [36], and could be contributing as an autocrine or paracrine factor in the *L. donovani*-induced arginase activity. These include the STAT3-activating cytokines IL-10, IL-6, and G-CSF [90,91], TGF-β [46], cAMP [92], and PGE2 [93]. Of these, IL-10 is particularly noteworthy for several reasons: (1) IL-10 is increased in patients [13,14,19,22-25] and hamsters with VL ([60] and current work), (2) in vitro neutralization of IL-10 in cultures of peripheral blood mononuclear cells from patients with VL led to recovery of suppressed Th1 responses [12,26], (3) parasite replication in human macrophages was enhanced by exposure to recombinant IL-10 [94], (4) neutralization of IL-10 in serum from patients with VL cultured with *in vitro* infected macrophages, or in cultured splenic aspirates of patients with VL resulted in reduced parasite burden [19,27], and (5) IL-10 deficient mice show increased resistance to experimental *L. donovani* infection [5,6]. As noted above, the recently described synergy between IL-4/STAT6 and IL-10/STAT3 in the murine model of *L. donovani* infection [68] suggests that IL-10 could augment the STAT6-dependent arg1 expression in the hamster model.

The role of arginase in the pathogenesis of human VL is uncertain. Polarization of isolated human macrophages by exposure to IL-4 in vitro does not lead to upregulation of arginase activity or arg1 expression [95]. However, the presence of alternatively activated monocytes/macrophages and arginase expression has been found in some human disease states. Human filarial infection is associated with enhanced expression of a number of genes related to alternative activation in peripheral blood mononuclear cells, including arg1 [96]. Arginase activity and arg1 expression were also increased in peripheral blood mononuclear cells from patients following traumatic tissue injury [97]. The expression of arginase by myeloid cells in the human tumor microenvironment is well established [98,99]. Studies of the role of AAMs and arginase in the pathogenesis of human VL are certainly warranted.

While alternatively activated macrophages have been described in a number of protozoan and metazoan infections (reviewed in [100,101]), rarely has a parasite antigen or product been found to directly induce arginase in isolated macrophages. We found that STAT6 activation and arg1 transcription could be initiated through parasite-derived soluble factors and did not require parasite contact or internalization by the host cell. Stempin et al [39] found that the cruzipain antigen from *Trypanosoma cruzi* directly induced the expression of host arignase. Recently, a proteophosphoglycan produced by *L. mexicana* within the sand fly vector was shown to induce arginase activity in inflammatory macrophages and enhance intracellular parasite replication [102]. In addition, *Toxoplasma gondii* was found to activate STAT6 directly [103], and induce arginase through TLR-dependent, but STAT6-independent pathway [104]. Lastly, it was demonstrated that the Ym1, another marker of alternative activation, was induced in macrophages exposed to a helminth antigen [105]. Work is underway to identify the soluble *L. donovani* factor(s) that induce macrophage arg1.

Collectively, these data lead us to propose a new model in which ineffective classical macrophage activation in experimental VL, which is reminiscent of human VL, is associated with, and perhaps enables, the emergence of a dominant program of STAT6-dependent alternative macrophage (and fibroblast) activation with impaired control of parasite replication. The metabolism of arginine through the arginase-polyamine pathway not only redirects arginine away from the generation of NO by NOS2, but also favors the production of polyamines, which promote parasite replication. That the type 2 cytokines can drive alternative

macrophage activation is without question [36,106], however, the greatest significance of our findings is that *L. donovani* can activate macrophage and fibroblast STAT6 and induce arg1 expression without synthesis of endogenous IL-4 or IL-13 or stimulation by exogenous cytokines. We postulate that in progressive VL these cytokines serve as an amplification factor for macrophages that have already started down the alternative activation pathway through interaction with *L. donovani* or a soluble parasite factor or factors. This notion is supported by the dramatic increase in parasite burden that accompanies the type 2 cytokine response first evident several weeks into the course of infection. Further dissection of the pathway by which *L. donovani* drives host arg1 expression may identify unique pathogenic mechanisms and targets for therapeutic intervention.

Supporting Information

Figure S1 Polyamine content in spleen and liver tissue in *L. donovani* infected mice. The concentration of polyamines in spleen and liver from groups of 5 uninfected mice (open bars) and 5 infected mice (filled bars) is expressed as the mean and standard deviation (error bars) of nmol polyamine per mg protein. The data shown are from a single experiment that is representative of 2 independent experiments. There were no statistically significant differences between the uninfected and infected tissue samples.
(TIF)

Figure S2 *L. donovani* infection of BHK cells. BHK cells were infected at 10:1 ratio with *L. donovani promastigotes* for 4 hours and then the extracellular parasites were removed by washing 3 times with PBS and once with 0.1% trypsin/EDTA (Gibco) in PBS for 3 min at 37°C. After the last wash the BHK monolayer was detached with 0.25% trypsin and the cells collected by centrifugation at 400 x g for 5 min. The pelleted cells were adjusted to 200,000 cells/200 µL of culture medium, transferred to 4-well chamber slides, and incubated for 24 h at 37°C, 5% CO_2. (A-B) Intracellular amastigotes were imaged at 40× magnification after nuclear labeling with 2 µg/mL Hoechst 33342 (Molecular Probes, Invitrogen) for 5 min. A BHK nucleus is shown by an arrowhead and amastigotes are identified by arrows. In some instances the amastigotes are oriented so that the kinetoplast DNA is clearly visible. (C) *L. donovani* amastigotes in BHK cells imaged at 100X magnification after staining with Hoechst 33342 and (D) 100 nM Lysotracker Red DND-99 (Molecular Probes, Invitrogen) to stain the phagolysosome. (E) Overlay of the Hoechst and Lysotracker Red stained images using the NIS-Elements Software (Nikon) to confirm the intraphagolysosmal location of the amastigotes.
(TIF)

Figure S3 Anti-*Leishmania* activity of the inhibitor nor-NOHA (Nω-hydroxy-nor-Arginine). (A) Arginase activity was measured in supernatants of *L. donovani*-infected hamster peritoneal macrophages after incubation with or without nor-NOHA for 48 h. The data is shown as the mean and standard deviation (error bars) of the arginase activity determined by assay of urea production in 100,000 cells. Statistical differences are shown between untreated and treated samples. (B) Number of amastigotes in *L. donovani*-infected hamster peritoneal macrophages after incubation with or without nor-NOHA for 48 h. The data is shown as the mean and standard deviation (error bars) of the number of parasites determined by luminometry. (C) Number of amastigotes in splenic macrophages isolated from hamsters at 15 days post-infection and untreated or treated *ex vivo* for 48 h with nor-NOHA. The data is shown as the mean and standard deviation (error bars) of the number of parasites determined by luminometry. (D) Number of amastigotes (purified from infected hamster spleen) after 24 h of *in vitro* culture with or without nor-NOHA (seeded at 100,000 amastigotes/100 µL). The data is shown as the mean and standard deviation (error bars) of the number of parasites determined by luminometry. (E) Number of promastigotes after 24h of *in vitro* culture with or without norNOHA (seeded at 100,000 amastigotes/100 µL). The data is shown as the mean and standard deviation (error bars) of the number of parasites determined by luminometry. The data shown for each of the panels is from a single experiment representative of at least 2 independent experiments. The statistical significance of differences between groups in each of the panels is identified by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
(TIF)

Figure S4 Effect of opsonization on *L. donovani*-induced STAT-6 activation. BHK cells transfected with a STAT6-luciferase reported vector were exposed or not to unopsonized, or complement opsonized (fresh hamster serum), or heat killed L. donovani promastigotes**.** Data are presented as the mean and standard deviation (error bars) of the relative light units in uninfected (Un) cells and cells exposed to 10 parasites per cell over 48 hrs of culture. Shown is data from a single experiment that is representative of 2 independent experiments. The statistical significance of differences between uninfected and parasite-exposed groups is identified by asterisks (*, $p<0.05$).
(TIF)

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Author Contributions

Conceived and designed the experiments: EYO OS LH CVB BLT PCM. Performed the experiments: EYO WZ CE LH BLT. Analyzed the data: EYO WZ CE LH CVB BLT PCM. Contributed reagents/materials/analysis tools: LH CVB. Wrote the paper: EYO BLT PCM.

References

1. Barbosa Junior AA, Andrade ZA, Reed SG (1987) The pathology of experimental visceral leishmaniasis in resistant and susceptible lines of inbred mice. Braz J Med Biol Res 20: 63–72.
2. Murray HW, Nathan CF (1999) Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J Exp Med 189: 741–746.
3. Squires KE, Schreiber RD, McElrath MJ, Rubin BY, Anderson SL, et al. (1989) Experimental visceral leishmaniasis: role of endogenous IFN-gamma in host defense and tissue granulomatous response. J Immunol 143: 4244–4249.
4. Stern JJ, Oca MJ, Rubin BY, Anderson SL, Murray HW (1988) Role of L3T4+ and LyT-2+ cells in experimental visceral leishmaniasis. J Immunol 140: 3971–3977.
5. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001) IL-10 mediates susceptibility to Leishmania donovani infection. Eur J Immunol 31: 2848–2856.
6. Murray HW, Moreira AL, Lu CM, DeVecchio JL, Matsuhashi M, et al. (2003) Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis. J Infect Dis 188: 458–464.

7. Wilson ME, Recker TJ, Rodriguez NE, Young BM, Burnell KK, et al. (2002) The TGF-beta response to Leishmania chagasi in the absence of IL-12. Eur J Immunol 32: 3556–3565.
8. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol 2: 845–858.
9. Kaye PM, Curry AJ, Blackwell JM (1991) Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. J Immunol 146: 2763–2770.
10. Cenini P, Berhe N, Hailu A, McGinnes K, Frommel D (1993) Mononuclear cell subpopulations and cytokine levels in human visceral leishmaniasis before and after chemotherapy. J Infect Dis 168: 986–993.
11. Hailu A, van der Poll T, Berhe N, Kager PA (2004) Elevated plasma levels of interferon (IFN)-gamma, IFN-gamma inducing cytokines, and IFN-gamma inducible CXC chemokines in visceral leishmaniasis. Am J Trop Med Hyg 71: 561–567.
12. Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, et al. (1993) Interleukin 10 production correlates with pathology in human Leishmania donovani infections. J Clin Invest 92: 324–329.
13. Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S (1998) Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis 177: 815–818.
14. Karp CL, el-Safi SH, Wynn TA, Satti MM, Kordofani AM, et al. (1993) In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma [see comments]. J Clin Invest 91: 1644–1648.
15. Bogdan C, Nathan C (1993) Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. Ann N Y Acad Sci 685: 713–739.
16. Nylen S, Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28: 378–384.
17. Wilson ME, Jeronimo SM, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing Leishmania species. Microb Pathog 38: 147–160.
18. Babaloo Z, Kaye PM, Eslami MB (2001) Interleukin-13 in Iranian patients with visceral leishmaniasis: relationship to other Th2 and Th1 cytokines. Trans R Soc Trop Med Hyg 95: 85–88.
19. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, et al. (2007) Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med 204: 805–817.
20. Sundar S, Reed SG, Sharma S, Mehrotra A, Murray HW (1997) Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. Am J Trop Med Hyg 56: 522–525.
21. Zwingenberger K, Harms G, Pedrosa C, Omena S, Sandkamp B, et al. (1990) Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon-gamma production. Clin Immunol Immunopathol 57: 242–249.
22. Ansari NA, Saluja S, Salotra P (2006) Elevated levels of interferon-gamma, interleukin-10, and interleukin-6 during active disease in Indian kala azar. Clin Immunol 119: 339–345.
23. Hailu A, van Baarle D, Knol GJ, Berhe N, Miedema F, et al. (2005) T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with Leishmania donovani. Clin Immunol 117: 182–191.
24. Kurkjian KM, Mahmutovic AJ, Kellar KL, Haque R, Bern C, et al. (2006) Multiplex analysis of circulating cytokines in the sera of patients with different clinical forms of visceral leishmaniasis. Cytometry A 69: 353–358.
25. Caldas A, Favali C, Aquino D, Vinhas V, van Weyenbergh J, et al. (2005) Balance of IL-10 and interferon-gamma plasma levels in human visceral leishmaniasis: implications in the pathogenesis. BMC Infect Dis 5: 113.
26. Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, et al. (1994) Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol 152: 5949–5956.
27. Gautam S, Kumar R, Maurya R, Nylen S, Ansari N, et al. (2011) IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis 204: 1134–1137.
28. Olivier M, Gregory DJ, Forget G (2005) Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev 18: 293–305.
29. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, et al. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 375: 408–411.
30. MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. Annu Rev Immunol 15: 323–350.
31. Green SJ, Meltzer MS, Hibbs JB, Jr., Nacy CA (1990) Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine-dependent killing mechanism. J Immunol 144: 278–283.
32. Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S (1990) Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. J Immunol 144: 4794–4797.
33. Liew FY, Li Y, Moss D, Parkinson C, Rogers MV, et al. (1991) Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. Eur J Immunol 21: 3009–3014.
34. Stenger S, Thuring H, Rollinghoff M, Bogdan C (1994) Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major. J Exp Med 180: 783–793.
35. Stenger S, Donhauser N, Thuring H, Rollinghoff M, Bogdan C (1996) Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. J Exp Med 183: 1501–1514.
36. Gordon S, Martinez FO (2010) Alternative activation of macrophages: mechanism and functions. Immunity 32: 593–604.
37. Stein M, Keshav S, Harris N, Gordon S (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 176: 287–292.
38. Iniesta V, Gomez-Nieto LC, Corraliza I (2001) The inhibition of arginase by N(omega)-hydroxy-l-arginine controls the growth of Leishmania inside macrophages. J Exp Med 193: 777–784.
39. Stempin C, Giordanengo L, Gea S, Cerban F (2002) Alternative activation and increase of Trypanosoma cruzi survival in murine macrophages stimulated by cruzipain, a parasite antigen. J Leukoc Biol 72: 727–734.
40. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF, Jr., et al. (2006) The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest 116: 2044–2055.
41. Raes G, Beschin A, Ghassabeh GH, De Baetselier P (2007) Alternatively activated macrophages in protozoan infections. Curr Opin Immunol 19: 454–459.
42. Iniesta V, Gomez-Nieto LC, Molano I, Mohedano A, Carcelen J, et al. (2002) Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular Leishmania parasites. Parasite Immunol 24: 113–118.
43. Iniesta V, Carcelen J, Molano I, Peixoto PM, Redondo E, et al. (2005) Arginase I induction during Leishmania major infection mediates the development of disease. Infect Immun 73: 6085–6090.
44. Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, et al. (2005) Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. Faseb J 19: 1000–1002.
45. Colotti G, Ilari A (2011) Polyamine metabolism in Leishmania: from arginine to trypanothione. Amino Acids 40: 269–285.
46. Iniesta V, Carlos Gomez-Nieto L, Molano I, Mohedano A, Carcelen J, et al. (2002) Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular Leishmania parasites. Parasite Immunol 24: 113–118.
47. Gifawesen C, Farrell JP (1989) Comparison of T-cell responses in self-limiting versus progressive visceral Leishmania donovani infections in golden hamsters. Infect Immun 57: 3091–3096.
48. Melby PC, Chandrasekar B, Zhao W, Coe JE (2001) The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like response. J Immunol 166: 1912–1920.
49. Perez LE, Chandrasekar B, Saldarriaga OA, Zhao W, Arteaga LT, et al. (2006) Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol 176: 5519–5528.
50. Rahimi F, Hsu K, Endoh Y, Geczy CL (2005) FGF-2, IL-1beta and TGF-beta regulate fibroblast expression of S100A8. FEBS J 272: 2811–2827.
51. Sacks DL, Melby PC (2001) Animal models for the analysis of immune responses to leishmaniasis. Curr Protoc Immunol Chapter 19(Unit 19): 12.
52. Roy G, Dumas C, Sereno D, Wu Y, Singh AK, et al. (2000) Episomal and stable expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol 110: 195–206.
53. Osorio Y, Travi BL, Renslo AR, Peniche AG, Melby PC (2011) Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. PLoS Negl Trop Dis 5: e962.
54. Herzfeld A, Raper SM (1976) The heterogeneity of arginases in rat tissues. Biochem J 153: 469–478.
55. Corraliza IM, Campo ML, Soler G, Modolell M (1994) Determination of arginase activity in macrophages: a micromethod. J Immunol Methods 174: 231–235.
56. Gilbert RS, Gonzalez GG, Hawel L, 3rd, Byus CV (1991) An ion-exchange chromatography procedure for the isolation and concentration of basic amino acids and polyamines from complex biological samples prior to high-performance liquid chromatography. Anal Biochem 199: 86–92.
57. Hawel L, 3rd, Tjandrawinata RR, Fukumoto GH, Byus CV (1994) Biosynthesis and selective export of 1,5-diaminopentane (cadaverine) in mycoplasma-free cultured mammalian cells. J Biol Chem 269: 7412–7418.
58. Hawel L, 3rd, Byus CV (2002) A streamlined method for the isolation and quantitation of nanomole levels of exported polyamines in cell culture media. Anal Biochem 311: 127–132.
59. Espitia CM, Zhao W, Saldarriaga OA, Osorio Y, Harrison LM, et al. (2010) Duplex real-time reverse transcriptase PCR to determine cytokine mRNA expression in a hamster model of New World cutaneous leishmaniasis. BMC Immunol 11: 31.
60. Melby PC, Tryon VV, Chandrasekar B, Freeman GL (1998) Cloning of Syrian hamster (Mesocricetus auratus) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infect Immun 66: 2135–2142.

61. Rothman P, Li SC, Gorham B, Glimcher L, Alt F, et al. (1991) Identification of a conserved lipopolysaccharide-plus-interleukin-4-responsive element located at the promoter of germ line epsilon transcripts. Mol Cell Biol 11: 5551–5561.
62. Salimuddin, Nagasaki A, Gotoh T, Isobe H, Mori M (1999) Regulation of the genes for arginase isoforms and related enzymes in mouse macrophages by lipopolysaccharide. Am J Physiol 277: E110–117.
63. Muleme HM, Reguera RM, Berard A, Azinwi R, Jia P, et al. (2009) Infection with arginase-deficient Leishmania major reveals a parasite number-dependent and cytokine-independent regulation of host cellular arginase activity and disease pathogenesis. J Immunol 183: 8068–8076.
64. Landfear SM (2011) Nutrient transport and pathogenesis in selected parasitic protozoa. Eukaryot Cell 10: 483–493.
65. Ramya TN, Surolia N, Surolia A (2006) Polyamine synthesis and salvage pathways in the malaria parasite Plasmodium falciparum. Biochem Biophys Res Commun 348: 579–584.
66. Seabra SH, DaMatta RA, de Mello FG, de Souza W (2004) Endogenous polyamine levels in macrophages is sufficient to support growth of Toxoplasma gondii. J Parasitol 90: 455–460.
67. Morris SM, Jr. (2009) Recent advances in arginine metabolism: roles and regulation of the arginases. Br J Pharmacol 157: 922–930.
68. Biswas A, Bhattacharya A, Kar S, Das PK (2011) Expression of IL-10-triggered STAT3-dependent IL-4Ralpha is required for induction of arginase 1 in visceral leishmaniasis. Eur J Immunol 41: 992–1003.
69. Riley E, Roberts SC, Ullman B (2011) Inhibition profile of Leishmania mexicana arginase reveals differences with human arginase I. Int J Parasitol 41: 545–552.
70. Pauleau AL, Rutschman R, Lang R, Pernis A, Watowich SS, et al. (2004) Enhancer-mediated control of macrophage-specific arginase I expression. J Immunol 172: 7565–7573.
71. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, et al. (2001) Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. J Immunol 166: 2173–2177.
72. Hebenstreit D, Wirnsberger G, Horejs-Hoeck J, Duschl A (2006) Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev 17: 173–188.
73. Noben-Trauth N, Lira R, Nagase H, Paul WE, Sacks DL (2003) The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to Leishmania major. J Immunol 170: 5152–5158.
74. Stamm LM, Raisanen-Sokolowski A, Okano M, Russell ME, David JR, et al. (1998) Mice with STAT6-targeted gene disruption develop a Th1 response and control cutaneous leishmaniasis. J Immunol 161: 6180–6188.
75. Dent AL, Doherty TM, Paul WE, Sher A, Staudt LM (1999) BCL-6-deficient mice reveal an IL-4-independent, STAT6-dependent pathway that controls susceptibility to infection by Leishmania major. J Immunol 163: 2098–2103.
76. Nabeshima Y, Hiragun T, Morita E, Mihara S, Kameyoshi Y, et al. (2005) IL-4 modulates the histamine content of mast cells in a mast cell/fibroblast co-culture through a Stat6 signaling pathway in fibroblasts. FEBS Lett 579: 6653–6658.
77. Mora AL, Torres-Gonzalez E, Rojas M, Corredor C, Ritzenthaler J, et al. (2006) Activation of alveolar macrophages via the alternative pathway in herpesvirus-induced lung fibrosis. Am J Respir Cell Mol Biol 35: 466–473.
78. Yu B, Koga T, Urabe K, Moroi Y, Maeda S, et al. (2002) Differential regulation of thymus- and activation-regulated chemokine induced by IL-4, IL-13, TNF-alpha and IFN-gamma in human keratinocyte and fibroblast. J Dermatol Sci 30: 29–36.
79. Bogdan C, Donhauser N, Doring R, Rollinghoff M, Diefenbach A, et al. (2000) Fibroblasts as host cells in latent leishmaniosis. J Exp Med 191: 2121–2130.
80. Hespanhol RC, de Nazare CSM, Meuser MB, de Nazareth SLMM, Corte-Real S (2005) The expression of mannose receptors in skin fibroblast and their involvement in Leishmania (L.) amazonensis invasion. J Histochem Cytochem 53: 35–44.
81. Kaye P, Scott P (2011) Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol 9: 604–615.
82. Caceres-Dittmar G, Tapia FJ, Sanchez MA, Yamamura M, Uyemura K, et al. (1993) Determination of the cytokine profile in American cutaneous leishmaniasis using the polymerase chain reaction. Clin Exp Immunol 91: 500–505.
83. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, et al. (1994) Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. Infect Immun 62: 837–842.
84. Pirmez C, Yamamura M, Uyemura K, Paes-Oliveira M, Conceicao-Silva F, et al. (1993) Cytokine patterns in the pathogenesis of human leishmaniasis [see comments]. J Clin Invest 91: 1390–1395.
85. Albina JE, Mahoney EJ, Daley JM, Wesche DE, Morris SM, Jr., et al. (2005) Macrophage arginase regulation by CCAAT/enhancer-binding protein beta. Shock 23: 168–172.
86. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM, Jr. (2005) Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene 353: 98–106.
87. Valineva T, Yang J, Palovuori R, Silvennoinen O (2005) The transcriptional co-activator protein p100 recruits histone acetyltransferase activity to STAT6 and mediates interaction between the CREB-binding protein and STAT6. J Biol Chem 280: 14989–14996.
88. Robb BW, Hershko DD, Paxton JH, Luo GJ, Hasselgren PO (2002) Interleukin-10 activates the transcription factor C/EBP and the interleukin-6 gene promoter in human intestinal epithelial cells. Surgery 132: 226–231.
89. Tanaka N, Hoshino Y, Gold J, Hoshino S, Martiniuk F, et al. (2005) Interleukin-10 induces inhibitory C/EBPbeta through STAT-3 and represses HIV-1 transcription in macrophages. Am J Respir Cell Mol Biol 33: 406–411.
90. Schreiber T, Ehlers S, Heitmann L, Rausch A, Mages J, et al. (2009) Autocrine IL-10 induces hallmarks of alternative activation in macrophages and suppresses antituberculosis effector mechanisms without compromising T cell immunity. J Immunol 183: 1301–1312.
91. Qualls JE, Neale G, Smith AM, Koo MS, DeFreitas AA, et al. (2010) Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. Sci Signal 3: ra62.
92. Morris SM, Jr., Kepka-Lenhart D, Chen LC (1998) Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol 275: E740–747.
93. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, et al. (2005) Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med 202: 931–939.
94. Vouldoukis I, Becherel PA, Riveros-Moreno V, Arock M, da Silva O, et al. (1997) Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation. Eur J Immunol 27: 860–865.
95. Raes G, Van den Bergh R, De Baetselier P, Ghassabeh GH, Scotton C, et al. (2005) Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. J Immunol 174(6561; author reply): 6561–6562.
96. Babu S, Kumaraswami V, Nutman TB (2009) Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis 199: 1827–1837.
97. Ochoa JB, Bernard AC, O'Brien WE, Griffen MM, Maley ME, et al. (2001) Arginase I expression and activity in human mononuclear cells after injury. Ann Surg 233: 393–399.
98. Rodriguez PC, Ochoa AC (2008) Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 222: 180–191.
99. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, et al. (2004) Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 64: 5839–5849.
100. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A (2004) Alternatively activated macrophages during parasite infections. Trends Parasitol 20: 126–133.
101. Stempin CC, Dulgerian LR, Garrido VV, Cerban FM (2010) Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals. J Biomed Biotechnol. pp 683485.
102. Rogers M, Kropf P, Choi BS, Dillon R, Podinovskaia M, et al. (2009) Proteophosophoglycans regurgitated by Leishmania-infected sand flies target the L-arginine metabolism of host macrophages to promote parasite survival. PLoS Pathog 5: e1000555.
103. Ahn HJ, Kim JY, Ryu KJ, Nam HW (2009) STAT6 activation by Toxoplasma gondii infection induces the expression of Th2 C-C chemokine ligands and B clade serine protease inhibitors in macrophage. Parasitol Res 105: 1445–1453.
104. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, et al. (2008) Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol 9: 1399–1406.
105. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL, et al. (2008) Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. Faseb J 22: 4022–4032.
106. Goerdt S, Politz O, Schledzewski K, Birk R, Gratchev A, et al. (1999) Alternative versus classical activation of macrophages. Pathobiology 67: 222–226.

3.2. DOCUMENTO 2:

Growth factor and Th2 cytokine signaling pathways converge at STAT6 to promote arginase expression in progressive experimental visceral leishmaniasis. PLoS Pathog. Aceito para publicação. 2014.

O trabalho prévio experimental mostrou que os hamsters infectados com *L. donovani* tinham altos níveis da enzima Arg-1 que conduziram à maior susceptibilidade associada à ativação alternativa do macrófago. Os resultados desse estudo indicaram que a infecção por *L. donovani* ativou o fator de transcrição STAT-6, e que o STAT-6 foi requerido para induzir Arg-1 nas células infectadas. No entanto, a contribuição de outros fatores co-estimuladores na indução máxima da Arg-1 foi indicada pela resposta tardia do gene (acima de 16 horas, mesmo sob estímulo de IL-4) e pela necessidade da síntese protéica *de novo*. Portanto, o estudo dos fatores que potencialmente contribuem para a regulação patológica da arginase mediada por STAT-6 tornam-se importantes para tentar entender o controle da doença. Dados preliminares indicaram que as citocinas Th2 e os receptores tirosina quinase (RTK) estavam envolvidos na regulação patológica da Arg-1, portanto este trabalho explorou a hipótese de que a Arg-1 na LV estava sendo regulada por aqueles fatores num processo dependente do fator de transcrição STAT-6.

PPATHOGENS-D-13-02456R1

Growth Factor and Th2 Cytokine Signaling Pathways Converge at STAT6 to Promote Arginase Expression in Progressive Experimental Visceral Leishmaniasis

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Abstract

Host arginase 1 (arg1) expression is a significant contributor to the pathogenesis of progressive visceral leishmaniasis (VL), a neglected tropical disease caused by the intracellular protozoan *Leishmania donovani*. Previously we found that parasite-induced arg1 expression in macrophages was dependent on STAT6 activation. Arg1 expression was amplified by, but did not require, IL-4, and required *de novo* synthesis of unknown protein(s). To further explore the mechanisms involved in arg1 regulation in VL, we screened a panel of kinase inhibitors and found that inhibitors of growth factor signaling reduced arg1 expression in splenic macrophages from hamsters with VL. Analysis of growth factors and their signaling pathways revealed that the Fibroblast Growth Factor Receptor 1 (FGFR-1) and Insulin-like Growth Factor 1 Receptor (IGF-1R) and a number of downstream signaling proteins were activated in splenic macrophages isolated from hamsters infected with *L. donovani*. Recombinant FGF-2 and IGF-1 increased the expression of arg1 in *L. donovani* infected hamster macrophages, and this induction was augmented by IL-4. Inhibition of FGFR-1 and IGF-1R decreased arg1 expression and restricted *L. donovani* replication in both *in vitro* and *ex vivo* models of infection. Inhibition of the downstream signaling molecules JAK and AKT also reduced the expression of arg1 in infected macrophages. STAT6 was activated in infected macrophages exposed to either FGF-2 or IGF-1, and STAT6 was critical to the FGFR-1- and IGF-1R-mediated expression of arg1. The converse was also true as inhibition of FGFR-1 and IGF-1R reduced the activation of STAT6 in infected macrophages. Collectively, these data indicate that the FGFR/IGF-1R and IL-4 signaling pathways converge at STAT6 to promote pathologic arg1 expression and intracellular parasite survival in VL. Targeted interruption of these pathological processes offers an approach to restrain this relentlessly progressive disease.

Author Summary

Visceral leishmaniasis (VL), caused by the intracellular protozoan *Leishmania donovani*, is a progressive infection that is particularly common in impoverished populations of the world. People die from this disease unless it is treated. We used an experimental infection model that mimics the clinical and pathological features of human VL to study how the parasite causes this severe disease. We found that host macrophages infected with *Leishmania donovani* are activated in a way that leads to the expression of arginase, an enzyme that counteracts the cell's mechanisms that control the infection. This disease-promoting activation pathway was driven by the convergence of growth factor and cytokine signaling pathways and activation of the transcription factor STAT6. Chemical inhibition of signaling through the fibroblast growth factor receptor-1 (FGFR-1) or insulin-like growth factor-1 receptor (IGF-IR), or genetic knockdown of STAT6 led to reduced expression of arginase and enhanced control of the infection by macrophages. This indicates that the growth factor signaling pathways together with the cytokine pathways promote this disease. Interventions designed to disrupt this signaling could help in the treatment of VL.

Introduction

Visceral leishmaniasis (VL), caused by the intracellular protozoan *Leishmania donovani or L. infantum,* is one of the "Neglected Tropical

Diseases" that impacts the poor of the world. Active VL is characterized by a relentlessly progressive infection with cachexia, massive splenomegaly, pancytopenia and ultimately death. VL ranks second to malaria in deaths caused by a protozoal pathogen; mortality is reported in up to 10-20% of patients, even with treatment [1]. The determinants of susceptibility and progressive disease are incompletely defined. However, it is clear that ineffective cellular immune function, dictated by the nature of cytokine response and polarization of macrophages [2], plays a critical role. Macrophages, the primary target of intracellular *Leishmania* infection, may take on distinct phenotypes in response to parasite signals and inflammatory stimuli within the infected microenvironment. Classically activated (M1) macrophages respond to IFN-γ and microbial products by generating antimicrobial molecules that effectively kill *Leishmania* and other intracellular pathogens [3,4]. Central to the killing of intracellular parasites is the production of nitric oxide by the action of inducible nitric oxide synthase 2 (NOS2) on the substrate L-arginine. In contrast, alternatively activated or M2 macrophages, which are typically generated by exposure to type 2 cytokines (IL-4, IL-13), fail to produce antimicrobial effector molecules to kill intracellular pathogens and serve to dampen inflammation and promote wound healing [5,6].

The activation status of macrophages in human VL has not been directly investigated. However, the progressive nature of the infection in the face of strong expression of IFN-γ [7-10], suggests that there is ineffective classical activation. The concomitant production of IL-4/IL-13 and IL-10 [7,8,11-14], which are known to impair macrophage leishmanicidal activity, may polarize macrophages toward a disease-promoting M2 phenotype. Neutralization of IL-10 in *ex vivo* splenocyte cultures from patients with VL promoted parasite clearance [15], but the importance of IL-4 and/or IL-13 in the pathogenesis of human VL is not clear. Additionally, *Leishmania*-driven subterfuge of a number of signaling pathways can render the macrophage less responsive to activating stimuli and more permissive to infection [16].

We have used the hamster model of VL, which closely mimics the clinicopathological features of human VL, to dissect the mechanisms by which *L. donovani* causes progressive disease. We demonstrated, similar to human VL, that progressive, lethal disease occurred in the face of what would be considered a protective type 1 cytokine response [17,18]. Despite high expression of IFN-γ, it was ineffective in mediating classical activation of M1 macrophages and control of *Leishmania* infection. In fact we found that splenic macrophages from hamsters with VL were polarized to a M2-like phenotype with dominant expression of host arginase 1 (arg1) [2]. *L. donovani* triggered arg1 expression through a STAT6-dependent mechanism, but surprisingly it did not require type 2 cytokines [2]. Arginase contributes to intracellular *Leishmania* replication by competing with NOS2 for the substrate arginine (thereby reducing NO production), and by driving the generation of polyamines, which promote parasite growth [2,19,20]. M2-like macrophages and arginase have also been implicated in the pathogenesis of experimental cutaneous leishmaniasis [19-23] and infections with other intracellular pathogens [24-27]. Furthermore, there is accumulating evidence that arginase has a role in the pathogenesis of human disease. Although, polarization of isolated human macrophages by exposure to IL-4 *in vitro* did not lead to upregulation of arginase activity or arg1 expression [28], the presence of M2-like monocytes/macrophages and arginase expression has been found in cancer [29,30], filariasis [25], tuberculosis [31,32], and traumatic tissue injury [33]. Elevated arginase activity was also recently reported in the lesions of patients with chronic cutaneous leishmaniasis [34] and arginase expression in peripheral blood leukocytes was found to be a marker of active VL [35].

In this work we have investigated the mechanisms of the pathological upregulation of arg1 in the hamster model of progressive VL. We discovered that the expression of arg1 in *L. donovani* infected macrophages is driven by activation of fibroblast growth factor receptor (FGFR) and insulin-like growth factor-1 receptor (IGF-IR). Inhibition of these growth factor signaling pathways led to reduced arg1 expression and enhanced control of parasite replication. Furthermore, signaling molecules downstream of the growth factor receptors converged with IL-4 signaling to promote STAT6 activation and arg1 expression in VL. The intersection of these pathways leads to subversion of macrophage effector function and impaired host defense against VL.

RESULTS

Receptor tyrosine kinases (RTK) are involved in parasite-induced arginase expression. We

previously determined that *L. donovani* induced STAT6-dependent, host arg1 expression. Host arginase expression promoted parasite replication, so we sought to understand the mechanisms by which it was expressed in VL. Arg1 transcription required the *de novo* synthesis of protein [2] suggesting that transcription of arg1 involved signaling pathway(s) other than just direct phosphorylation of STAT6. We postulated that the newly synthesized protein could mediate its effect through RTK signaling pathways, which regulate inflammation and wound repair [36,37]. Both of these processes are important functions of M2 macrophages. Therefore, we screened a library of 80 RTK inhibitors for inhibition of *L. donovani*-induced arginase transcription in an *ex vivo* model of infected splenocytes isolated from hamsters with VL [38]. Inhibitors of the Epidermal Growth Factor Receptor and Platelet-derived Growth Factor Receptor signaling pathways reduced arg1 transcription by >50% (Table 1). Because the RTK signaling pathways are overlapping and broad, and inhibitors of some growth factor receptors were not included in the inhibitor library, we used a RTK antibody array to further define the participation of specific RTKs in VL. We found that Fibroblast Growth Factor Receptor (FGFR) 1 and 2 and other molecules known to participate in growth factor signaling (Insulin receptor substrate 1 (IRS-1), v-akt murine thymoma viral oncogene homolog 1 and 2 (AKT 1/2), Mitogen-activated protein kinase (MAPK)-3, and Signal transducer and activator of transcription (STAT)-1, and STAT-3 were activated in splenic macrophages from hamsters infected with *L. donovani* (Table 2). Collectively, these data indicated that signaling through growth factor receptor pathways could contribute to the parasite-induced expression of host arg1.

Growth factors induce arg1 in *L. donovani* infected hamster macrophages. A significant increase in arg1 mRNA expression was observed in *L. donovani* infected hamster bone marrow-derived macrophages (BMDM) exposed to the recombinant growth factors FGF-2, IGF-1, and PDGF (Fig. 1A). Growth factor-induced arg1 was particularly evident in infected compared to uninfected macrophages, and it was equivalent to, or greater than, IL-4-induced arg1. Arginase protein activity was also significantly increased in *L. donovani* infected BMDM exposed to FGF-2, IGF-1, and PDGF (Fig. 1B). EGF did not consistently induce a significant increase arg1 mRNA or protein. Together, these data suggested that *L. donovani* infection of macrophages led to enhanced arg1 transcriptional responsiveness to multiple growth factors.

The FGF signaling pathway is activated in the spleens of hamsters infected with *Leishmania donovani*. Analysis of the FGF and IGF-1 signaling pathways in splenic macrophages from hamsters with VL by immunoblotting confirmed the finding of the antibody screening array (Figs. 2 and 3). There was no evidence for activation of other growth factor signaling pathways in VL (see Fig. S1 and S2). Our finding that inhibition of EGFR reduced arg1 mRNA expression (Table 1), when neither increased ligand expression nor receptor activation could be demonstrated, suggested that basal activity of EGF/EGFR modulated arg1 expression through an effect on downstream signaling. As we demonstrated previously [2], arg1 protein expression was increased in macrophages isolated from the spleens of hamsters with VL starting at 14 days post-infection (Fig. 2A). Of the growth factor receptor ligands, only FGF-2 expression was increased in splenic macrophages (Fig. 2B, Fig. S1, and Fig. S2) and it was accompanied by increased phosphorylation of $Tyr^{653/654}$ of the FGFR-1 (Fig. 2C) relative to overall receptor protein expression (Fig. 2D). The increase in both FGF-2 and its phosphorylated receptor paralleled the expression of arg1 in the splenic macrophages. Multiple molecules involved in the signaling cascade downstream of FGFR (shown in the diagram in Fig. 2M) were activated, including members of the PI3K/AKT pathway [GAB (Fig. 2E), PI3K (Fig. 2F)] and the MAPK/ERK pathway [c-RAF (Fig. 2G), ERK1/2 (Fig. 2H)]. Activation of p38 MAPK (Fig. 2I), that leads to activation of the transcription factor ATF-2 (Fig. 2J) and the cyclic AMP response element-binding protein (CREB) (Fig 2K) was observed at 14 days post-infection but was then down-modulated at 28 days post-infection. This suggested that sustained activation of these signaling molecules was not required for the expression of arg1 throughout the course of VL (Fig. 2A). The mechanism(s) through which these molecules are down regulated is unknown. Activation of STAT3, which was evident throughout the course of VL (Fig. 2L), may be a consequence of increased IL-10 production (Fig. S4A and reference [2]) or growth factor signaling (Fig. S4D) [39].

The IGF-1 signaling pathway is activated in the spleens of hamsters with VL. We were

unable to detect increased expression of IGF-1 or IGF-2 in the spleen or plasma of hamsters with VL (Fig. S1; data not shown). However, by immunoblot we found increased expression of the IGF-1R after 14 days post-infection (Fig. 3A), and somewhat unexpectedly the beta (cytoplasmic) domain of the IGF-1 receptor, which mediates intracellular signaling, was phosphorylated at these time points (Fig. 3B). We confirmed these findings in BMDM exposed *in vitro* to *L. donovani* where parasite-induced IGF-1R phosphorylation was evident between 20 minutes and 24 hrs of exposure, and enhanced expression of IGF-1R protein was present at 24 hrs after infection (Fig. 3C). A number of the activated signaling molecules downstream of FGFR overlap with the canonical IGF-1R signaling pathway (compare data in Fig. 2 with schematic in Fig. 3L). Additionally, other pathway members, including IRS-1 (Fig. 3D), SHC (Fig. 3E), AKT (Fig. 3F), p70S6K (Fig. 3G), and GSK3β (Fig. 3H) were activated, as were the downstream transcription factors c-FOS (Fig. 3I) and c-Jun (Fig. 3J). When all of the activated signaling molecules were subjected to network analysis (Ingenuity Pathway Analysis) both the FGFR and IGF-1R pathways were found to be significantly upregulated in splenic macrophages during the course of VL ($p<10^{-7}$; Fig. 3K).

Inhibition of the FGFR and IGFR-1 and downstream signaling molecules decreases arg1 expression and parasite burden in infected macrophages. Treatment of *L. donovani*-infected hamster BMDMs over 24 hrs of infection with an inhibitor of FGFR-1 resulted in a significant dose-dependent reduction of arg1 mRNA expression (Fig. 4A) and parasite burden (Fig. 4B) without affecting cell viability (Fig. 4C). Notably the concentration of FGFR inhibitor required to inhibit parasite replication was higher than the concentration that reduced arg1 expression. This suggests that growth factor signaling supported parasite growth/survival through additional arg1-independent mechanisms, or that residual arginase activity at the lower inhibitor concentration is enough to support parasite growth. The latter possibility is consistent with our previous finding that >90% arg1 knockdown led to approximately 50% reduction of parasite load [2]. The FGFR inhibitor also blocked the expression of arg1 mRNA (Fig. 4D) and protein (Fig. 4E), and reduced the parasite burden (Fig. 4F) without affecting cell viability (Fig. 4G) in ex *vivo* cultured spleen cells from infected hamsters. Similar effects were found by inhibition of IGF-1R. In the *in vitro* infection model, IGF-1R inhibition reduced parasite-induced expression of host arg1 mRNA (Fig. 5A) and the intracellular parasite load (Fig. 5B), without decreasing cell viability (Fig. 5C). Similarly, the inhibitor reduced arg1 mRNA (Fig. 5D) and protein (Fig. 5E), and reduced the parasite burden (Fig. 5F) without affecting cell viability (Fig. 5G) in *ex vivo* cultured spleen cells from infected hamsters. The FGFR and IGF-1R inhibitors did not have a direct effect on the viability of *L. donovani* cultured promastigotes (Fig. S3), suggesting that the effect of receptor inhibition was through modulation of the host cell. Inhibition of JAK, which plays a key role in the phosphorylation of STAT proteins following cytokine and growth factor signaling, dramatically reduced arg1 transcription in *ex vivo* cultured splenocytes from infected hamsters (Fig. 5H). To a lesser degree, inhibition of the protein AKT, which is involved in signal transduction downstream of the IGF-1 and FGF receptors, also decreased Arg-1 expression (Fig. 5H). Both the AKT and JAK inhibitors significantly reduced parasite load (Figs. 5I and 5J).

Cytokines amplify the *L. donovani*- and growth factor-induced expression of arginase 1. Since cytokines (IL-4 and IL-10) are known to stimulate the expression of arginase [5,6], and we demonstrated that growth factors also induced arginase (Fig. 1), we investigated the potential for amplification of arg1 expression in macrophages by simultaneous exposure to these stimuli (all of which are expressed in the spleen during VL (reference [2] and Figs. 2 and 3). The *L. donovani*-induced expression of arg1 in BMDM was modestly amplified by IL-4 but not IL-10 at the mRNA level (Fig. 6A), but neither significantly amplified the arg1 protein (Fig. 6B). However, IL-4 and IL-10 dramatically enhanced the FGF-2-induced arg1 mRNA (Fig. 6C), and IL-4 (but not IL-10) enhanced FGF-2-induced arg1 protein (Fig. 6D) expression in infected macrophages. IL-4 did not amplify IGF-1-induced arg1 mRNA expression in infected BMDMs (Fig. 6E) but augmented arg1 protein expression (Fig. 6F). Similar to IL-10 and FGF-2, IL-10 enhanced IGF-1-induced arg1 mRNA but not protein expression. A trend of an additive effect of IL-4 and the growth factors was also found in splenic macrophages from infected animals exposed to the cytokine and growth

factors *ex vivo* (Fig. 6G). The additive effect of IL-4 and growth factors in the induction of arg1 expression prompted us to consider that there may be cross-regulation of receptor expression. We found that the expression of IL-13Rα1, but not IL-4Rα, was upregulated in splenic macrophage from hamsters with VL (Fig. 6H) and in BMDMs infected with *L. donovani* (Fig. 6I). Addition of FGF-2 or IGF-1 to infected macrophages did not further increase the expression of either of these receptor components (data not shown and Fig. 6I). IL-10Rα expression (along with IL-10) was also increased in splenic macrophages from infected hamsters (Fig. S4A) and in *in vitro* infected BMDMs (Fig. S4B), but FGF-2 or IGF-1 did not augment IL-10 or IL-10Rα expression (Fig. S4B). These data, coupled with the data shown in Figs. 2 and 3, suggest that the cytokine-mediated amplification of growth factor driven arg1 could occur by either increased IL-4-mediated signaling through upregulated type II receptor (IL-13Rα1) expression [40] or through activation of signaling proteins (e.g. Jak-1, STAT6, IRS-1, PI3K, AKT) common to the two pathways.

STAT6 is required for *L. donovani* induced expression of arg1 in macrophages. We previously demonstrated that STAT6 was required for *L. donovani*-induced arg1 expression in fibroblasts [2]. Here we confirmed that siRNA-mediated knockdown of STAT6 mRNA (Fig. 7A) and protein (see Fig. 8F and 8I) in *in vitro* infected macrophages led to reduced arg1 mRNA (Fig. 7B) expression, and improved control of parasite replication (Fig. 7C). Similarly, knockdown of STAT6 (75% reduction) in *ex vivo* cultured splenic macrophages from infected hamsters led to significantly reduced arg1 mRNA expression (Fig. 7D). These data confirm the critical importance of STAT6 in the parasite-driven expression of arg1 in macrophages in VL.

Growth factors activate STAT6 and increase STAT6-dependent arg1 expression. Since STAT6 had a critical role in parasite-induced arg1 transcription, activation of growth factor signaling was evident in *L. donovani* infection, and there was an additive effect of IL-4 and growth factors in the induction of arg1 expression, we wanted to know if the FGF-2- and/or IGF-1-induced arg1 expression was dependent on the activation of STAT6. In a STAT6 reporter assay (hamster fibroblast cell line; reference [2]), we found that recombinant FGF-2 and IGF-1 induced STAT6 activation, which was blocked when cells were pre-treated with an inhibitor of the corresponding growth factor receptor (Figure 8A). In the fibroblast cell line, exposure to parasites had a relatively weak effect on STAT6 activation, probably because at this parasite dose the cells are infected at a very low level. The growth factor-induced activation of STAT6 in macrophages was confirmed by detection of phosphorylated STAT6 in immunoprecipitated lysates of splenic macrophages from *L. donovani* infected hamsters exposed *ex vivo* to recombinant FGF-2 or IGF-1 (Fig. 8B). Parasite-induced STAT6 activation was abrogated completely by an IGF-1R inhibitor and partially by an FGFR inhibitor (Fig. 8C). Conversely, siRNA-mediated knockdown of STAT6 mRNA in infected, FGF-2-treated BMDM (Fig. 8D) identified the requirement for STAT6 in the FGF-2-induced expression of arg1 mRNA (Fig. 8E) and protein (Fig. 8F). Similarly, siRNA-mediated knockdown of STAT6 in infected IGF-1-treated BMDM (Fig. 8G) identified the contribution of, but not absolute requirement for, STAT6 in the IGF-1-induced expression of arg1 mRNA (Fig. 8H) and protein (Fig. 8I). Collectively these data identify the critical importance of growth factor signaling in the parasite-induced activation of STAT6, and of STAT6 in the IGF-1 and FGF-2 driven expression of arg1 in *L. donovani* infected macrophages.

IL-4 and growth factors have an additive effect in the activation of STAT6. Since simultaneous exposure of infected macrophages to IL-4 and FGF-2 or IGF-1 led to enhanced arginase expression, and the growth factor- and cytokine-induced expression of arg1 was dependent on STAT6, we reasoned that there might be enhanced activation of STAT6 in cells exposed to both IL-4 and growth factors. Stimulation of the reporter cells with either growth factors (see also Fig. 8A) or IL-4 activated STAT6. There was evidence of an additive effect when the growth factor and cytokine were combined (Figs. 9A and 9B). By immunoblotting, STAT6 phosphorylation was amplified when IL-4 was combined with the growth factors (Figs. 9C and 9D). Inhibition of FGFR and IGF-1R activation led to decreased IL-4-induced STAT6 activation (Fig. 9E). Taken together, these data indicate that bi-directional crosstalk between the growth factor and IL-4 signaling pathways converges at STAT6 to drive arg1 expression in VL.

Discussion

In an experimental model of progressive VL, we demonstrated previously that parasitized macrophages were polarized to an M2-like phenotype [2], characteristic of macrophages at a site of chronic injury and wound healing [5,6], and were massively expanded in the spleen [2,38]. These macrophages had dominant expression of arg1, which promoted parasite growth. The *L. donovani*-induced macrophage arg1 expression did not require, but was amplified by, type 2 cytokines [2]. In this work we focused our attention on the mechanisms through which pathological arg1 expression occurs in VL. We discovered that FGF-2 and IGF-1 signaling pathways were activated in splenic macrophages from animals with progressive VL. These growth factors, which may be produced by macrophages, fibroblasts, or endothelial cells [41-43], induced macrophage arg1 expression. Inhibition of FGFR1 and IGF-1R signaling led to both reduced arg1 expression and improved control of intracellular *L. donovani* infection. Parasite-induced FGFR and IGF-1R signaling converged with the canonical type 2 cytokine signaling pathway through STAT6 activation to induce arg1 expression. Simultaneous exposure of macrophages to growth factors and IL-4, as would occur in the spleen during VL, enhanced the activation of STAT6 and expression of arg1. The interplay of STAT6 and growth factor signaling was confirmed by demonstrating that FGF-2- and IGF-1-induced arg1 expression was abrogated by knockdown of STAT6, and conversely, that inhibition of growth factor signaling reduced parasite- and IL-4-mediated STAT6 activation and arg1 expression.

Arginase expression contributes to the pathogenesis of cutaneous *L. major* infection in mice [19-23] and progressive experimental VL caused by *L. donovani* [2]. Its expression in blood leukocytes was also found to be a marker of active VL in patients from Ethiopia [35]. In that study the blood leukocytes that produced arginase were found in the mononuclear cell fraction but expressed CD15 so were identified as low-density granulocytes. Those cells were not further characterized, and we have not evaluated expression of arg1 in granulocytes in our model of experimental VL. Therefore, it remains to be determined if there is a fundamental difference in the source of arg1 in experimental and human VL, or if further characterization of the cell populations will resolve the apparent difference. The disease-promoting effect of arg1 may be mediated through several mechanisms. First, arg1 metabolizes arginine such that this substrate is not available for the generation of the antimicrobial effector molecule, nitric oxide, by the action of inducible nitric oxide synthase. Second, arg1 expression leads to the production of polyamines, which promote intracellular *Leishmania* growth [2,19,20]. Lastly, local depletion of arginine leads to impaired anti-leishmanial T cell responses [44]. The relative contributions of each of these effects on the pathogenesis of VL remain to be determined.

The role of growth factors in modulation of arg1 expression and macrophage function in response to *Leishmania* or other pathogens has received little attention. The induction of arginase expression is classically a type 2 cytokine (IL-4/IL-13)- and STAT6-driven process [5], although some parasites or parasite products have been shown to directly induce an M2-like macrophage phenotype [2,20,45]. Since growth factors modulate inflammation and tissue repair [46-50], processes in which M2 macrophages have an integral part, it is not surprising that there would be interconnections between growth factors, type 2 cytokines, and M2 polarization. The tissue remodeling [51,52], accumulation of macrophages [38,52-55] and collagen deposition/fibrosis [38,54] observed in the spleens in experimental and human VL are processes that suggest growth factors may contribute to VL pathology. Cytosolic IGF-1 was found increased in *L. major* infected murine macrophages [56], and IGF-1 induced parasite arginase in *L. amazonensis* infected macrophages [57]. Although we cannot exclude the potential contribution of parasite arginase in the IGF-1 and FGF-2-mediated effects on macrophages, we found previously that *L. donovani* arginase contributed little to the overall arginase expression at the site of infection in this model of progressive VL [2]. The increased expression of FGF-2 and evidence of signaling through the IGF-1 and FGF receptors to our knowledge had not been described previously in VL. Surprisingly, robust IGF-1R phosphorylation was evident in the infected spleen in the absence of increased IGF-1, suggesting cross-activation by FGF-2 [58] or by an unknown host or parasite-derived factor. We think cross-activation by FGF-2 is unlikely in the case of VL since we did not find IGF-1R phosphorylation in BMDMs infected with *L. donovani* and treated with FGF-2 for 20 minutes to 48 hours post-infection (data not shown). Of note, it was reported previously that *Leishmania* expressed

an ortholog of FGF-2 [59] so conceivably other parasite-produced growth factor orthologs could be driving the activation of IGF-1R in the absence of host IGF-1. Insulin-like growth factor binding proteins (IGFBPs) or IGFBP proteases [60] could also be modulating the local availability and activity of IGF-1 during the infection.

From this work we have begun to understand the mechanistic basis for the interplay of *L. donovani*, IL-4 and growth factors in the induction of arg1. IL-4, which is increased in the spleen during VL in humans and hamsters [2,12], amplifies the parasite- and growth factor-induced expression of arg1. IL-10 appears to have a more limited role in that it upregulates arg1 mRNA, but not protein expression, in infected macrophages, and does not amplify the growth factor effect. Similarly, in *L. donovani*-infected mice, IL-10 does not directly induce macrophage arginase, but contributes indirectly to its expression by upregulating the type I IL-4 receptor [61]. FGF-2- and IGF-1 enhance expression of arg1 in *L. donovani* infected macrophages, but have a more modest effect on uninfected macrophages. Thus, the concomitant expression of IL-4 and growth factors in the infected spleen provide an environment highly suited for arg1 expression. IL-4 and IL-13 were shown previously to induce the expression of macrophage IGF-1 [62] and coincident expression of type 2 cytokines and IGF-1 was demonstrated in experimental helminth infection [63]. The amplification of growth factor-induced arg1 by IL-4 in experimental VL is not associated with growth factor-mediated upregulation of the type 1 IL-4 receptor (IL-4rα). Although we found that *L. donovani* infection increased expression of IL-13Rα1, which partners with IL-4rα to transduce a signal via IL-4 or IL-13 [40], this receptor is thought to be a less-potent driver of M2 macrophage activation than is IL-4 signaling through the type I receptor. Furthermore, IRS-1/2, which we found strongly activated in VL, is activated primarily via IL-4 signaling through the type I rather than the type II receptor [64,65]. Collectively, these data suggest that the growth factor/IL-4-mediated amplification of arg1 expression results from an effect downstream of the IL-4 receptor. Since IL-13 [2] and its receptor are also increased in the spleen during VL, they may also contribute to the induction of arg1.

The body of work presented here supports the conclusion that the signaling pathways downstream of the growth factor and IL-4 receptors converge at STAT6 to drive pathological arg1 expression. Figure 10 illustrates our current working model for the expression of arg1 in VL. IGF-1 is known to activate STAT6 through an IRS-1/2-dependent pathway. IL-4, which is also an activator of IRS-1/2 [64], can amplify this effect [66,67]. To our knowledge FGF-2 had not been shown previously to activate STAT6. The pathway through which the growth factors activate STAT6-dependent arg1 transcription in VL remains to be fully elucidated, but for the reasons noted above, IRS-1/2 and JAKs, which are activated in splenic macrophages during VL, are likely key intermediates (see Fig. 10). The downstream activation of other transcription factors (CREB, STAT-3) and signaling molecules, including PI3K/AKT, ERK, p38 MAPK, and GSK3β, are also likely to directly or indirectly contribute to the growth factor induced macrophage polarization and arg1 expression. Notably, p38 MAPK and downstream transcription factors (CREB and ATF-2) are only transiently upregulated so do not account for the sustained increase in arg1 expression over time (reference [2] and this work). Down-modulation of the p38 pathway, however, may contribute to the survival and local expansion of splenic arginase-expressing macrophages [68]. *Leishmania* infection of macrophages was shown previously to activate the PI3K/AKT pathway, which is a critical regulator of the IL-10 and IL-12 response [69-71]. *L. donovani*-induced production of IL-10 by macrophages involved activation of the PI3K/AKT pathway and downstream phosphorylation-mediated inactivation of GSK-3beta and phosphorylation of CREB [72]. Our data suggest that parasite-induced arg1 is driven at least in part through activation of the same pathway that mediates production of IL-10 by macrophages. However, arg1 and IL-10 expression appear to result from parallel rather than interdependent processes because IL-10 was not a strong inducer of arg1 and did not amplify growth factor-induced arg1 as did IL-4. Also the inhibition of the AKT pathway, which drives IL-10 production, had a less dramatic effect in the down regulation of arg1 than the inhibition of JAKs with the consequent block in STAT activation. Taken together, these data indicate that the expression of arg1 downstream of the growth factor/PI3K/AKT pathway, which is enhanced by IL-4/STAT6 signaling, is an additional mechanism of parasite-mediated subversion of macrophage effector function. Further work is

needed to definitively determine the role of IL-10 and STAT3 in this process.

The pathological signaling through the IGF-1R and FGFR that leads to arginase expression in progressive VL is a potential target for adjunctive chemotherapy. Therapies targeting these pathways have recently emerged for a number of proliferative diseases, in particular hematopoietic malignancies and solid tumors (reviewed in [73,74]). Our *ex vivo* data suggest that inhibition of FGFR or IGF-1R signaling could have therapeutic potential. Furthermore, it was previously demonstrated in a murine model of *L. donovani* infection that *in vivo* administration of a receptor tyrosine kinase inhibitor, when combined with conventional anti-leishmanial chemotherapy had a therapeutic effect [75]. Future pre-clinical studies of FGFR and IGF-1R inhibitors, alone or in combination with current anti-leishmanial therapies, are warranted.

In summary, we determined that the convergence of FGFR/IGFR and IL-4 signaling pathways is responsible for the expression of arg1 in disease-promoting macrophages during chronic progressive VL. FGF-2 and Th2 cytokines [2] are produced in the spleen and lead to activation of the FGFR and STAT6 in infected splenic macrophages. Although the infection does not appear to increase IGF-1 production, the IGF-1R is activated on splenic macrophages through a yet to be identified host or parasite factor. Activation of the FGF and IGF-1 receptors leads to phosphorylation of downstream signaling molecules such as IRS1/2, PI3K, and AKT, which lead to expression of IL-10 [72] and converge with downstream components of the IL-4R pathway to drive arg1 expression. Activation of these pathways, along with the parallel effects of IL-10 in subverting macrophage function [16,76,77], plays an important role in the pathogenesis of VL. Targeted interruption of these pathological processes offers an approach to restrain this relentlessly progressive disease.

Materials and Methods

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas (protocol number 1101004).

Hamsters. 6-8 week old Syrian golden hamsters (Mesocricetus auratus) were obtained from Harlan Laboratories.

Parasites and infection. *L. donovani* (MHOM/SD/001S-2D) promastigotes were cultured as described previously [78]. Hamsters were infected by intracardial injection of 106 peanut agglutinin purified metacyclic promastigotes [78]. For in vitro infections, stationary phase promastigotes were washed with PBS and used immediately to infect hamster BMDMs. Cells were infected at a promastigote to macrophage ratio of 2:1 and cultured thereafter in complete medium (CM) composed of DMEM supplemented with 1 mM sodium pyruvate (Gibco), 1X MEM amino acids solution (Sigma), 10 mM HEPES buffer (Cellgro), and 100 IU/mL penicillin/100mg/mL streptomycin solution (Cellgro), which was supplemented with 2% heat inactivated fetal bovine serum (HIFBS). When infecting BMDMs at this ratio all parasites were internalized so that no extracellular parasites could be observed by light microscopy at 24 hrs post-infection.

Isolation of bone marrow derived macrophages. Bone marrow cells were flushed from normal hamster femurs and adjusted to 8x106/mL in RPMI with 10% HIFBS, 50 μ M β-mercaptoethanol (Sigma), and supplemented with 20 ng/mL recombinant human macrophage-colony stimulating factor (M-CSF) (R&D Systems). After 3 days of culture the medium was changed and at 6-7 days of culture the cell monolayer (>95% macrophages as determined by microscopy) was washed 3 times with PBS and detached with Trypsin/EDTA (Gibco) and cell scraping. The cells were starved of M-CSF or serum in CM with 2% HIFBS overnight before the assays.

Measurement of arginase. Arg1 expression and arginase enzymatic activity in BMDM was determined at 24 hrs or 48 hrs by real-time RT-PCR or by production of urea, respectively, as described previously [2]. For Western blot a goat anti-hamster arg1 polyclonal antibody was used [2]. The antibody used for detection of hamster arg1 did not react with L. donovani parasite lysates. The cells were left unstimulated or exposed to recombinant human Epidermal Growth Factor (EGF), mouse Insulin-like Growth factor-1 (IGF-1), human Platelet-Derived Growth Factor (PDGF) (Cell Signaling), human Fibroblast Growth Factor basic (heparin stabilized) (Sigma), 0.3-2.5% recombinant hamster IL-4 conditioned medium (equivalent to 3-25 IU/mL determined by STAT6 reporter

bioassay) [2], or human IL-10 (R&D Systems) and/or infected with *L. donovani* promastigotes at 1:2 macrophage:parasite ratio. The activity of human IL-10 on hamster cells was verified using hamster BMDMs transiently transfected with a STAT3 lentiviral reporter construct (Cignal Lenti-reporter, SA Biosciences).

Quantitative RT-PCR. Real time RT-PCR for arg1 and STAT6 mRNA was performed as described [2].

Screening of Receptor Tyrosine Kinase (RTK) inhibitors. Spleen cells from 28-day *L. donovani* infected hamsters were cultured *ex vivo* as described previously [38] and treated for 24 hrs with each inhibitor from a library of 80 RTK inhibitors (Biomol International, Inc.) at twice the dose reported to cause 50% inhibition. Total RNA was isolated and the level of arg1 transcription determined by real time PCR as described [2].

Screening for activated RTKs. An RTK antibody array (PathScan Array, Cell Signaling), which contains antibodies against 28 phospho-RTKs and 11 key signaling nodes of the RTK pathways, was used to identify RTKs activated by *L. donovani* infection. The mean dot-spot chemiluminescent intensity of splenic macrophages (n=4) from infected hamsters (28 days post-infection) was compared to that of 4 uninfected hamsters by densitometry analysis (GeneTools Analysis Software, Syngene).

Chemical inhibition of growth factor receptors. BMDMs were seeded in white clear bottom 96-well plates at 20,000 cells per well in CM and pre-treated with Fibroblast Growth Factor Receptor-1 inhibitor (PD166866; CAS 192705-79-6; Calbiochem) or Insulin-like Growth Factor Receptor inhibitor (PPP; CAS 477-47-4; Calbiochem). After 1-2 hrs the medium containing the inhibitor was discarded and the cells infected with *L. donovani* promastigotes for 20 min. Medium containing fresh inhibitors was then added back to the infected cells and the cells collected at 24h post-infection for measurement of arg1 expression and parasite burden. Parasite load was determined by measurement of luciferase activity from luciferase-transfected parasites as described previously [38] or by real time RT-PCR using primers and a Taqman probe against the conserved sequence of the 18S gene of *Leishmania* [79] (forward primer: TTACCACCTTACGTA TCTTTTCTATTCG; reverse primer: AAAACAGAAAACGTGCTGAGG AT; Taqman probe: FAM-CT TTACCGGCCACCCACGGGA-TAMRA). Similar experiments were performed with adherent spleen cells cultured *ex vivo* from hamsters infected with *L. donovani* (21 days post-infection) as described [38]. The viability of treated cells was assessed in parallel experiments (20,000 cells / well / 100 µL in 96-well white plates) by luminometric measurement of ATP (Cell Titer Glo Assay, Promega).

Measurement of growth factor receptor activation in hamsters infected with *L. donovani*. To confirm the results of the PathScan Array, we immunoprecipitated (IP) selected growth factor receptors from fresh lysates of splenic macrophages isolated from infected hamsters using cross-reacting anti-mouse/human/rat growth factor receptor antibodies (Table S1). Following cell lysis in RIPA buffer supplemented with protease/phosphatase inhibitors (Santa Cruz) the protein concentration of total cell lysates was adjusted to 3 µg/300 µL buffer and the IP procedure was followed according the manufacturer's instructions using protein A/G agarose (Santa Cruz). In brief, pre-cleared samples were incubated with the anti-receptor antibody overnight at 4°C on an Orbital shaker, then 20 µL protein A/G agarose was added to the Ag-antibody complex and incubated for 4 hr at 4°C. The protein A/G/antibody complex was precipitated by centrifugation, washed 3 times with PBS, suspended in 50 µL of 1X LDS running buffer (Invitrogen) and the antibodies released from the agarose beads by heat (100°C, 5min). After resolving 20 µL of sample by SDS PAGE the separated proteins were transferred to nitrocellulose membranes, blocked with TBS-T 5% milk with 1 mM sodium orthovanadate (Na_3VO_4) and incubated overnight at 4°C with the anti-phospho RTK in TBS-T with 0.4% BSA or TBS-T 3% milk with 1 mM Na_3VO_4.

Measurement of growth factors in hamsters infected with *L. donovani*. Growth factor receptor ligands were measured in plasma or spleen homogenates from uninfected or infected hamsters. IGF-I and PDGF-β were measured by ELISA using anti-rat/mouse IGF-I and anti-rat/mouse PDGF-β using ELISA kits (R&D Systems). Epidermal Growth Factor, heparin-binding EGF-like growth factor (HB-EGF), Epiregulin and Amphiregulin were measured by immunoprecipitation/western blot using antibodies reactive against the mouse/rat/human proteins (Santa Cruz).

Identification of activated signaling proteins. Splenic macrophages isolated by adherence

from infected or uninfected hamsters were lysed and suspended in RIPA buffer containing 1X protease/phosphatase inhibitors. Lysates were stored at -80°C and used within 2 months. Ten µg of total protein was suspended in 1X LDS sample buffer and separated by SDS-PAGE in pre-cast gels (NuPage, Bis-Tris 4-12%). The separated proteins were transferred to nitrocellulose membranes using the iBlot system (20V, 9min) (Invitrogen). Then membranes were incubated with primary antibody (Table S1) either in TBS-T with 0.4% BSA or TBS-T with 3% milk and 1mM Na_3VO_4 followed by the secondary antibody conjugated to HRP. The reaction was detected with enhanced chemiluminescent substrate (West Pico; Thermo Scientific) and captured with a Chemi X T4 camera (G BOX, SynGene) and analyzed with Gene Tools analysis Software (SynGene). The fold change of protein expression was calculated by densitometry analysis of western blot bands of infected samples (at 7, 14 and 28 days post-infection) with reference to uninfected samples.

Transcriptional activation of STAT6. STAT6 activity was determined in the hamster BHK-21 cell line stably transfected with the luciferase reporter plasmid p(IE-IL4$_{RE}$)$_4$-LUC as described previously [2]. p-STAT6 was detected by immunoprecipitation of cell lysates (5x10^6 cells / 300 µL RIPA with phosphatase inhibitors) with 1 µg of STAT6 capture antibody (M-20, Santa Cruz Biotechnology) at 4ºC. overnight. Protein A/G immunoprecipitated complexes were washed 4 times with PBS, eluted by heat 5 min 100 ºC in 50 µl of 1X LDS loading buffer and detected by SDS-page using anti-p-STAT6 antibody (# 9361, Cell Signaling, 1:1000 TBS-T, 0.4% BSA, 4ºC, overnight), anti-rabbit HRP conjugate, and West Pico substrate (Thermo Scientific) as above.

Knockdown of STAT6 in hamster BMDMs. Stealth RNAi sequences were designed *in silico* using the BLOCK-iT RNAi Designer (Life Technologies) and chosen based on the sequences spanning 2 regions that were successfully targeted in knockdown of STAT6 previously [2] as follows: region 1: top, UGGCCACCAUCAGACAAAUACUUCA; bottom, UGAAGUAUUUGUCUGAUGGUGGCCA; region 2: top, CACAGUUCAACAAGGAGAUCCUGUU; bottom,
AACAGGAUCUCCUUGUUGAACUGUG (each duplex synthetized and annealed by Life Technologies). Hamster BMDMs were differentiated for 6 days with 20 ng/mL of recombinant human M-CSF (R&D Systems) and plated overnight (250,000 cells per well in 24-well plates and 500 µL CM with 10% HIFBS). For transfection, 25 nM of each stealth duplex (239 and 1451) targeting hamster STAT6 was mixed in a volume of 100 µL Optimem with 0.9 µL of Lipofectamine RNAiMAX (Invitrogen) according the manufacturer's instruction. A non-targeting oligonucleotide (low GC, Invitrogen) was used as a control. Then the culture medium was discarded and 500 µL of Optimem (Invitrogen) with 10% HIFBS without antibiotics was added to the cell monolayer together with 100 µl of the transfection mix to achieve a final concentration of 8.3 nM of siRNAi oligos in 600 µL per well. The next day the transfection medium was changed for fresh Optimem with 10% HIFBS without antibiotics. At 48 hr post-transfection cells were serum starved in CM overnight, and stimulated with either *L. donovani* promastigotes or growth factors at 72 hr of transfection. Both STAT6 knockdown efficiency and arg1 transcription was measured 24h later by real time RT-PCR and Western blot.

Statistical Analysis. Comparison between groups was typically performed using ANOVA. A parametric or non-parametric test was selected according the distribution of the raw data, followed by a post-test analysis for multiple groups (e.g. Dunnett's Multiple Comparison Test) as appropriate. Paired t test and Wilcoxon signed rank test were used to identify differences between inhibitors and vehicle controls. All analyses were conducted using GraphPad InStat version 3.00 software for Windows 95 (GraphPad Software, San Diego California USA). *P* values of <0.05 were considered significant.

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References

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7: e35671.
2. Osorio EY, Zhao W, Espitia C, Saldarriaga O, Hawel L, et al. (2012) Progressive visceral leishmaniasis is driven by dominant parasite-induced STAT6

activation and STAT6-dependent host arginase 1 expression. PLoS Pathog 8: e1002417.
3. Green SJ, Nacy CA, Meltzer MS (1991) Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. J Leukoc Biol 50: 93-103.
4. Liew FY, Li Y, Moss D, Parkinson C, Rogers MV, et al. (1991) Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. Eur J Immunol 21: 3009-3014.
5. Gordon S, Martinez FO (2010) Alternative activation of macrophages: mechanism and functions. Immunity 32: 593-604.
6. Martinez FO, Helming L, Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27: 451-483.
7. Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S (1998) Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis 177: 815-818.
8. Karp CL, el-Safi SH, Wynn TA, Satti MM, Kordofani AM, et al. (1993) In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma [see comments]. J Clin Invest 91: 1644-1648.
9. Gidwani K, Jones S, Kumar R, Boelaert M, Sundar S (2011) Interferon-gamma release assay (modified QuantiFERON) as a potential marker of infection for Leishmania donovani, a proof of concept study. PLoS Negl Trop Dis 5: e1042.
10. Singh OP, Gidwani K, Kumar R, Nylen S, Jones SL, et al. (2012) Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. Clin Vaccine Immunol 19: 961-966.
11. Hailu A, van Baarle D, Knol GJ, Berhe N, Miedema F, et al. (2005) T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with Leishmania donovani. Clin Immunol 117: 182-191.
12. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, et al. (2007) Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med 204: 805-817.
13. Sundar S, Reed SG, Sharma S, Mehrotra A, Murray HW (1997) Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. Am J Trop Med Hyg 56: 522-525.
14. Zwingenberger K, Harms G, Pedrosa C, Omena S, Sandkamp B, et al. (1990) Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon-gamma production. Clin Immunol Immunopathol 57: 242-249.
15. Gautam S, Kumar R, Maurya R, Nylen S, Ansari N, et al. (2011) IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis 204: 1134-1137.
16. Olivier M, Gregory DJ, Forget G (2005) Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev 18: 293-305.
17. Melby PC, Chandrasekar B, Zhao W, Coe JE (2001) The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like response. J Immunol 166: 1912-1920.
18. Perez LE, Chandrasekar B, Saldarriaga OA, Zhao W, Arteaga LT, et al. (2006) Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol 176: 5519-5528.
19. Iniesta V, Gomez-Nieto LC, Corraliza I (2001) The inhibition of arginase by N(omega)-hydroxy-l-arginine controls the growth of Leishmania inside macrophages. J Exp Med 193: 777-784.
20. Stempin CC, Dulgerian LR, Garrido VV, Cerban FM (2010) Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals. J Biomed Biotechnol 2010: 683485.

21. Iniesta V, Carlos Gomez-Nieto L, Molano I, Mohedano A, Carcelen J, et al. (2002) Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular Leishmania parasites. Parasite Immunol 24: 113-118.
22. Iniesta V, Carcelen J, Molano I, Peixoto PM, Redondo E, et al. (2005) Arginase I induction during Leishmania major infection mediates the development of disease. Infect Immun 73: 6085-6090.
23. Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, et al. (2005) Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. Faseb J 19: 1000-1002.
24. Stempin C, Giordanengo L, Gea S, Cerban F (2002) Alternative activation and increase of Trypanosoma cruzi survival in murine macrophages stimulated by cruzipain, a parasite antigen. J Leukoc Biol 72: 727-734.
25. Babu S, Kumaraswami V, Nutman TB (2009) Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis 199: 1827-1837.
26. Benoit M, Barbarat B, Bernard A, Olive D, Mege JL (2008) Coxiella burnetii, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. Eur J Immunol 38: 1065-1070.
27. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, et al. (2008) Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol 9: 1399-1406.
28. Raes G, Van den Bergh R, De Baetselier P, Ghassabeh GH, Scotton C, et al. (2005) Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. J Immunol 174: 6561; author reply 6561-6562.
29. Rodriguez PC, Ochoa AC (2008) Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 222: 180-191.
30. Rodriguez NE, Chang HK, Wilson ME (2004) Novel program of macrophage gene expression induced by phagocytosis of Leishmania chagasi. Infect Immun 72: 2111-2122.
31. Mattila JT, Ojo OO, Kepka-Lenhart D, Marino S, Kim JH, et al. (2013) Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. J Immunol 191: 773-784.
32. Pessanha AP, Martins RA, Mattos-Guaraldi AL, Vianna A, Moreira LO (2012) Arginase-1 expression in granulomas of tuberculosis patients. FEMS Immunol Med Microbiol 66: 265-268.
33. Ochoa JB, Bernard AC, O'Brien WE, Griffen MM, Maley ME, et al. (2001) Arginase I expression and activity in human mononuclear cells after injury. Ann Surg 233: 393-399.
34. Abebe T, Hailu A, Woldeyes M, Mekonen W, Bilcha K, et al. (2012) Local increase of arginase activity in lesions of patients with cutaneous leishmaniasis in ethiopia. PLoS Negl Trop Dis 6: e1684.
35. Abebe T, Takele Y, Weldegebreal T, Cloke T, Closs E, et al. (2013) Arginase activity - a marker of disease status in patients with visceral leishmaniasis in ethiopia. PLoS Negl Trop Dis 7: e2134.
36. Muller AK, Meyer M, Werner S (2012) The roles of receptor tyrosine kinases and their ligands in the wound repair process. Semin Cell Dev Biol 23: 963-970.
37. Porta C, Larghi P, Rimoldi M, Totaro MG, Allavena P, et al. (2009) Cellular and molecular pathways linking inflammation and cancer. Immunobiology 214: 761-777.
38. Osorio Y, Travi BL, Renslo AR, Peniche AG, Melby PC (2011) Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. PLoS Negl Trop Dis 5: e962.
39. Zong CS, Chan J, Levy DE, Horvath C, Sadowski HB, et al. (2000) Mechanism of STAT3 activation by insulin-like growth factor I receptor. J Biol Chem 275: 15099-15105.
40. Jiang H, Harris MB, Rothman P (2000) IL-4/IL-13 signaling beyond JAK/STAT. J Allergy Clin Immunol 105: 1063-1070.
41. Lu H, Huang D, Saederup N, Charo IF, Ransohoff RM, et al. (2011) Macrophages recruited via CCR2

produce insulin-like growth factor-1 to repair acute skeletal muscle injury. FASEB J 25: 358-369.
42. Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. Physiol Rev 83: 835-870.
43. Yun YR, Won JE, Jeon E, Lee S, Kang W, et al. (2010) Fibroblast growth factors: biology, function, and application for tissue regeneration. J Tissue Eng 2010: 218142.
44. Modolell M, Choi BS, Ryan RO, Hancock M, Titus RG, et al. (2009) Local suppression of T cell responses by arginase-induced L-arginine depletion in nonhealing leishmaniasis. PLoS Negl Trop Dis 3: e480.
45. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A (2004) Alternatively activated macrophages during parasite infections. Trends Parasitol 20: 126-133.
46. Puzik A, Rupp J, Troger B, Gopel W, Herting E, et al. (2012) Insulin-like growth factor-I regulates the neonatal immune response in infection and maturation by suppression of IFN-gamma. Cytokine 60: 369-376.
47. Jimenez-Sousa MA, Almansa R, de la Fuente C, Caro-Paton A, Ruiz L, et al. (2010) Increased Th1, Th17 and pro-fibrotic responses in hepatitis C-infected patients are down-regulated after 12 weeks of treatment with pegylated interferon plus ribavirin. Eur Cytokine Netw 21: 84-91.
48. Mlambo NC, Hylander B, Brauner A (1999) Increased levels of transforming growth factor beta 1 and basic fibroblast growth factor in patients on CAPD: a study during non-infected steady state and peritonitis. Inflammation 23: 131-139.
49. Skevaki CL, Psarras S, Volonaki E, Pratsinis H, Spyridaki IS, et al. (2012) Rhinovirus-induced basic fibroblast growth factor release mediates airway remodeling features. Clin Transl Allergy 2: 14.
50. Tourdot S, Mathie S, Hussell T, Edwards L, Wang H, et al. (2008) Respiratory syncytial virus infection provokes airway remodelling in allergen-exposed mice in absence of prior allergen sensitization. Clin Exp Allergy 38: 1016-1024.
51. Engwerda CR, Ato M, Cotterell SE, Mynott TL, Tschannerl A, et al. (2002) A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection. Am J Pathol 161: 429-437.
52. Yurdakul P, Dalton J, Beattie L, Brown N, Erguven S, et al. (2011) Compartment-specific remodeling of splenic micro-architecture during experimental visceral leishmaniasis. Am J Pathol 179: 23-29.
53. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, et al. (2004) The immunopathology of experimental visceral leishmaniasis. Immunol Rev 201: 239-253.
54. Veress B, Omer A, Satir AA, El Hassan AM (1977) Morphology of the spleen and lymph nodes in fatal visceral leishmaniasis. Immunology 33: 605-610.
55. Woodruff AW, Topley E, Knight R, Downie CG (1972) The anaemia of kala-azar. Br J Haematol 22: 319-329.
56. Reis LC, Ramos-Sanchez EM, Goto H (2013) The interactions and essential effects of intrinsic insulin-like growth factor-I on Leishmania (Leishmania) major growth within macrophages. Parasite Immunol 35: 239-244.
57. Vendrame CM, Carvalho MD, Rios FJ, Manuli ER, Petitto-Assis F, et al. (2007) Effect of insulin-like growth factor-I on Leishmania amazonensis promastigote arginase activation and reciprocal inhibition of NOS2 pathway in macrophage in vitro. Scand J Immunol 66: 287-296.
58. Yoshinouchi M, Miura M, Gaozza E, Li SW, Baserga R (1993) Basic fibroblast growth factor stimulates DNA synthesis in cells overexpressing the insulin-like growth factor-I receptor. Mol Endocrinol 7: 1161-1168.
59. Kardami E, Pearson TW, Beecroft RP, Fandrich RR (1992) Identification of basic fibroblast growth factor-like proteins in African trypanosomes and Leishmania. Mol Biochem Parasitol 51: 171-181.
60. Baxter RC (2000) Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278: E967-976.
61. Biswas A, Bhattacharya A, Kar S, Das PK (2011) Expression of IL-10-triggered STAT3-dependent IL-4Ralpha is required for induction of arginase 1 in

visceral leishmaniasis. Eur J Immunol 41: 992-1003.
62. Wynes MW, Riches DW (2003) Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13. J Immunol 171: 3550-3559.
63. Chen F, Liu Z, Wu W, Rozo C, Bowdridge S, et al. (2012) An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. Nat Med 18: 260-266.
64. Myers MG, Jr., Grammer TC, Wang LM, Sun XJ, Pierce JH, et al. (1994) Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. J Biol Chem 269: 28783-28789.
65. Heller NM, Qi X, Junttila IS, Shirey KA, Vogel SN, et al. (2008) Type I IL-4Rs selectively activate IRS-2 to induce target gene expression in macrophages. Sci Signal 1: ra17.
66. Kim JH, Park HH, Lee CE (2003) IGF-1 potentiation of IL-4-induced CD23/Fc(epsilon)RII expression in human B cells. Mol Cells 15: 307-312.
67. Patel BK, Wang LM, Lee CC, Taylor WG, Pierce JH, et al. (1996) Stat6 and Jak1 are common elements in platelet-derived growth factor and interleukin-4 signal transduction pathways in NIH 3T3 fibroblasts. J Biol Chem 271: 22175-22182.
68. Zuluaga S, Alvarez-Barrientos A, Gutierrez-Uzquiza A, Benito M, Nebreda AR, et al. (2007) Negative regulation of Akt activity by p38alpha MAP kinase in cardiomyocytes involves membrane localization of PP2A through interaction with caveolin-1. Cell Signal 19: 62-74.
69. Cheekatla SS, Aggarwal A, Naik S (2012) mTOR signaling pathway regulates the IL-12/IL-10 axis in Leishmania donovani infection. Med Microbiol Immunol 201: 37-46.
70. Ruhland A, Kima PE (2009) Activation of PI3K/Akt signaling has a dominant negative effect on IL-12 production by macrophages infected with Leishmania amazonensis promastigotes. Exp Parasitol 122: 28-36.
71. Ruhland A, Leal N, Kima PE (2007) Leishmania promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. Cell Microbiol 9: 84-96.
72. Nandan D, Camargo de Oliveira C, Moeenrezakhanlou A, Lopez M, Silverman JM, et al. (2012) Myeloid cell IL-10 production in response to leishmania involves inactivation of glycogen synthase kinase-3beta downstream of phosphatidylinositol-3 kinase. J Immunol 188: 367-378.
73. Chaves J, Saif MW (2011) IGF system in cancer: from bench to clinic. Anticancer Drugs 22: 206-212.
74. Daniele G, Corral J, Molife LR, de Bono JS (2012) FGF receptor inhibitors: role in cancer therapy. Curr Oncol Rep 14: 111-119.
75. Dalton JE, Maroof A, Owens BM, Narang P, Johnson K, et al. (2010) Inhibition of receptor tyrosine kinases restores immunocompetence and improves immune-dependent chemotherapy against experimental leishmaniasis in mice. J Clin Invest 120: 1204-1216.
76. Nylen S, Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28: 378-384.
77. Wilson ME, Jeronimo SM, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing Leishmania species. Microb Pathog 38: 147-160.
78. Sacks DL, Melby PC (2001) Animal models for the analysis of immune responses to leishmaniasis. Curr Protoc Immunol Chapter 19: Unit 19 12.
79. van der Meide W, Guerra J, Schoone G, Farenhorst M, Coelho L, et al. (2008) Comparison between quantitative nucleic acid sequence-based amplification, real-time reverse transcriptase PCR, and real-time PCR for quantification of Leishmania parasites. J Clin Microbiol 46: 73-78.

Figure Legends

Figure 1. Growth factors upregulate arginase 1 in macrophages. A) Induction of arg1 mRNA expression in macrophages exposed to recombinant growth factors. Uninfected and *L. donovani* infected hamster BMDMs were stimulated with EGF (100 ng/mL), FGF-2 (20 ng/mL), IGF-1 (100 ng/mL), PDGF (100 ng/mL), or IL-4 (25 IU/mL) for 24 hrs and the expression of arg1 mRNA determined by qRT-PCR. Shown is the mean and standard error of the mean

(SEM; error bars) of 4 replicates from a single experiment that is representative of 2 independent experiments. **B)** Dose-dependent induction of arginase activity (urea production) in hamster BMDMs infected with *L. donovani* and exposed to 2-fold increasing concentrations of growth factors for 48h. The concentration of the growth factors was: EGF: 12.5-100 ng/mL; FGF-2: 6.25-50 ng/mL; IGF-1: 50-400 ng/mL; and PDGF: 25-100 ng/mL. Shown is the mean and SEM of 2 replicates per dose that is representative of 4 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Activation of signaling proteins in the FGFR canonical pathway in splenic macrophages from hamsters with VL. (A-L) Splenic macrophages were isolated by adherence from the spleens of uninfected hamsters (time 0) or hamsters infected for 7, 14, and 28 days and whole cell lysates probed with antibodies directed against arg1 (panel A, representative blot A) or members of the FGF signaling pathway (panels and representative blots B-L). Bars represent the fold change with reference to control cells of uninfected hamsters calculated by densitometry analysis of immunoblot bands from samples pooled from 1-4 hamsters per determination from 2-3 independent experiments. **M)** Simplified schematic of the canonical FGF signaling pathway for reference.

Figure 3. Activation of signaling proteins in the IGF-1R canonical pathway in splenic macrophages from hamsters with VL. (A-B, D-J) Immunoblot analysis of expression of proteins in the IGF-1R canonical signaling pathway in splenic macrophages was performed as described in Fig. 2. **C)** Detection of phospho-IGFR by immunoblot in BMDMs uninfected (Un) or infected *in vitro* with *L. donovani* for 20 min to 24 hrs. Shown is an immunoblot from a single experiment. **K)** Network analysis showing the activation of the FGF and IGF-1 canonical signaling pathways generated by comparing the fold change of 32 signaling proteins in splenic macrophages from uninfected and infected (7, 14, and 28 days) hamsters using Ingenuity Pathway Analysis software (Ingenuity Systems). The –log of the *p* value (vertical axis) represents the probability that the association of the data set in that pathway is due to chance. **L)** Simplified schematic of the canonical IGF-1 signaling pathway for reference.

Figure 4. Inhibition of FGFR signaling decreases arg1 expression and parasite burden in *L. donovani* infected macrophages. (A-C) Hamster BMDMs were treated with a FGFR-1 inhibitor (PD 166866) or an equivalent concentration of vehicle control (DMSO) and infected *in vitro* with *L. donovani* for 24 or 48 hrs. **A)** arg1 mRNA expression determined by qRT-PCR at 24 hrs post-treatment. **B)** Intracellular parasite burden determined by luminometry from luciferase-transfected *L. donovani* at 48 hrs post-treatment. **C)** Viability of BMDMs determined by luminometry (Cell titer Glo) at 48 hrs post-treatment. **D-G)** Splenic macrophages from *L. donovani* infected hamsters (21-28 days p.i.) were isolated by adherence and cultured *ex vivo* with an inhibitor of FGFR-1 (PD 166866) or an equivalent concentration of vehicle control (DMSO) for 24 or 48 hrs. **D)** arg1 mRNA expression determined by qRT-PCR at 24 hrs post-treatment. **E)** arg1 protein expression determined at 48 hrs post-treatment. Bars represent the percent of expression with reference to control (DMSO treated) cells calculated by densitometry analysis of immunoblot bands from 3 independent experiments. A representative immunoblot is also shown. **F)** Intracellular parasite burden determined by luminometry from luciferase-transfected *L. donovani* at 48 hrs post-treatment. **G)** Viability of splenic macrophages determined by luminometry (Cell titer Glo) at 48 hrs post-treatment. Shown is the mean and SEM of from a single experiment that was representative of 2-4 independent experiments. *p<0.05; **p<0.01.

Figure 5. Inhibition of IGF-1R signaling decreases arg1 expression and parasite burden in *L. donovani* infected macrophages. (A-C) Hamster BMDMs were treated with an IGF-1R inhibitor (Picropodophyllin, PPP) or an equivalent concentration of vehicle (DMSO) and infected *in vitro* with *L. donovani* for 24 or 48 hrs. **(A)** arg1 mRNA expression determined by qRT-PCR at 24 hrs post-treatment. **B)** Intracellular parasite burden determined by luminometry from luciferase-transfected *L. donovani* at 48 hrs post-treatment. **C)** Viability of BMDMs determined by luminometry (Cell titer Glo) at 48 hrs post-treatment. **D-J)** Splenic macrophages from *L. donovani* infected hamsters (21-28 days p.i.) were isolated by adherence and treated with an IGF-1R inhibitor, AKT inhibitor, or JAK inhibitor, or an equivalent concentration of vehicle control (DMSO) for 24 of 48 hrs. **D)** arg1 mRNA expression determined by qRT-PCR at

24 hrs post-treatment. **E)** arg1 protein expression determined at 48 hrs post-treatment. Bars represent the percent of expression with reference to control (DMSO treated) cells calculated by densitometry analysis of immunoblot bands from 3 independent experiments. A representative immunoblot is also shown. **F)** Intracellular parasite burden determined by luminometry from luciferase-transfected *L. donovani* at 48 hrs post-treatment. **G)** Viability of splenic macrophages determined by luminometry (Cell titer Glo) at 48 hrs post-treatment. **H)** arg1 mRNA expression determined by qRT-PCR at 24 hrs post-treatment with AKT inhibitor (AKTi; CAS# 612847-09-3, Calbiochem), JAK inhibitor (JAKi; CAS# 457081-03-07) or DMSO control. **I-J)** Intracellular parasite burden determined by luminometry from luciferase-transfected *L. donovani* at 48 hrs post-treatment with AKT inhibitor **(I)** or JAK inhibitor **(J)**, compared to DMSO treated controls. In each of the panels the mean and SEM from a single experiment that was representative of 2-3 independent experiments is shown. *p<0.05; ***p<0.001.

Figure 6. IL-4 enhances growth factor-induced arg1 in *L. donovani* infected macrophages. Infected hamster BMDM were exposed or not to hamster IL-4 (25 IU/mL), recombinant human IL-10 (100 ng/mL), recombinant human FGF-2 (20 ng/mL) and/or recombinant human IGF-1 (100 ng/mL) for 24 or 48 hrs. **A, C, E)** Arg1 mRNA expression determined by qRT-PCR at 24 hrs post-treatment. Shown is the mean and SEM of 6 replicates from a single experiment that was representative of 2 independent experiments. **B, D, F)** Arg1 protein expression determined in *L. donovani* infected BMDM exposed to IL-4, IL-10, and growth factors, alone or in combination, for 48 hrs. The membranes were stripped and stained with antibody against GAPDH to confirm equivalent protein loading. Bars represent the fold change with reference to control cells of uninfected hamsters calculated by densitometry analysis of immunoblot bands from 3 independent experiments. Also shown is a representative individual immunoblot. **G)** Arg1 protein expression determined in splenic macrophages from uninfected and *L. donovani* infected hamsters exposed *ex vivo* to IL-4 and growth factors, alone or in combination, for 48 hrs. The membranes were stripped and stained with antibody against GAPDH to confirm equivalent protein loading. Bars represent the fold change with reference to control cells of uninfected hamsters calculated by densitometry analysis of immunoblot bands from 3 independent experiments. Also shown is a representative individual immunoblot. **H)** Expression of IL-13Rα1 and IL-4Rα mRNA in splenic macrophages from uninfected (0) or 18-day infected hamsters determined by qRT-PCR. **I)** Expression of IL-13Rα1 and IL-4Rα mRNA in BMDM from uninfected (Un) and *L. donovani* infected (Inf) BMDMs (24 hrs p.i.) stimulated or not with IGF-1 or FGF-2. Shown is mean and SEM of the fold increase of receptor expression over uninfected, unstimulated controls from a single experiment representative of 2 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 7. Parasite-induced arg1 expression in macrophages is dependent on STAT6. Expression of **A)** STAT6 mRNA and **B)** arg1 mRNA in BMDMs that were uninfected (Un) or infected *in vitro* with *L. donovani* (Inf) for 24h after transfection with STAT6-specific knockdown siRNA (STAT6 KD) or scrambled siRNA (Control). Shown is the mean and SEM of the fold-change in mRNA compared to unstimulated controls as determined by qRT-PCR in 6 replicates from 2 independent experiments. **C)** Parasite burden at 24h post-infection of STAT6 KD BMDMs or control. Shown is the mean and SEM of the parasite burden with reference to control (uninfected) cells in 4 replicates determined by qRT-PCR. **D)** STAT6 and arg1 mRNA expression in splenic macrophages from *L. donovani* infected hamsters 48 hrs after *ex vivo* transfection with STAT6-specific siRNA (STAT6 KD) or scrambled siRNA (Control). Data are shown as the mean and SEM of the percent of maximal mRNA expression in the control samples. *p<0.05; ***p<0.001.

Figure 8. Growth factors and cytokines converge at STAT6 to induce arg1 expression in *L. donovani* infected macrophages. **A)** Growth factors activate STAT6. Hamster BHK fibroblasts transfected with a STAT6 luciferase reporter were uninfected (Un) or infected (Inf) with *L. donovani* and stimulated for 24h with FGF-2 (20ng/mL) or IGF-1 (100ng/mL) in the absence or presence (+i) of 250 nM of an inhibitor of FGFR-1 (PD166866) or IGF-1R (PPP). Shown is the mean and SEM of luciferase activity from

3 replicates from a single experiment that was representative of 2 independent experiments. **B)** phospho-STAT6 expression in BMDMs stimulated with IGF-1 and FGF-2. BMDMs were uninfected (Un) or infected *in vitro* with *L. donovani* (Inf) and stimulated or not with FGF-2 (20 ng/mL) or IGF-1 (100 ng/mL) for 20 min. STAT6 protein was immunoprecipitated in cell lysates and phosphorylated STAT6 determined by immunoblot. GAPDH was used to confirm that equivalent amounts of protein were subjected to the immunoprecipitation. Bars represent the mean and SEM of fold change with reference to the uninfected controls calculated by densitometry analysis of immunoblot bands from 6 independent experiments. Also shown is a representative individual immunoblot. **C)** Blockade of STAT6 activation by IGF-1R and FGFR inhibitors. Splenic macrophages from uninfected hamsters were pre-treated with IGF-1R inhibitor (100 nM PPP) or FGFR inhibitor (300 nM PD166866) and infected *in vitro* with *L. donovani* for 20 min in absence of the inhibitor. STAT6 protein was immunoprecipitated in cell lysates and the level of phosphorylated STAT6 determined by immunoblot. Data shown is from 3 independent experiments, with a representative individual immunoblot, as described for Fig. 8B. **D)** Expression of STAT6 mRNA in BMDMs that were uninfected-unstimulated (Un), infected (Inf), or infected and stimulated with FGF-2 after transfection with STAT6-specific siRNA (STAT6 KD) or scrambled siRNA (Control). Shown is the mean and SEM of the fold-increase in STAT6 mRNA with reference to uninfected control as determined by qRT-PCR in 4-10 replicates from a single experiment that was representative of 3 independent experiments. **E)** Abrogation of arg1 mRNA expression by knockdown of STAT6 in BMDMs infected *in vitro* with *L. donovani* and stimulated with FGF-2. BMDMs were transfected with the siRNA as described above and then infected and stimulated with FGF-2 (20 ng/mL) for 24 hrs. The data are shown as the mean and SEM of the fold-increase in arg1 mRNA relative to negative (uninfected) control cells from a single experiment that was representative of 3 independent experiments. **F)** Immunoblot showing efficiency of siRNAi-mediated knockdown of STAT6 protein in hamster BMDMs and the requirement of STAT6 in the FGF-2-induced arg1 expression in *L. donovani* infected cells. Following transfection with the STAT6-specific (STAT6 KD) or control siRNAi the BMDMs were uninfected (Un), infected with *L. donovani* (Inf), or infected and treated with FGF-2 (20 ng/mL) for 48 hrs. **G)** Expression of STAT6 mRNA in BMDMs that were uninfected and stimulated with IGF-1 (200 ng/mL) or infected (Inf) and stimulated with IGF-1 after transfection with STAT6-specific siRNA (STAT6 KD) or scrambled siRNA (Control). Shown is the mean and SEM of the fold-increase in STAT6 mRNA compared to IGF-1-treated STAT6 KD cells as determined by qRT-PCR in 3 replicates from a single experiment that was representative of 2 independent experiments. **H)** Abrogation of arg1 mRNA expression by knockdown of STAT6 in BMDMs infected *in vitro* with *L. donovani* and stimulated with IGF-1. BMDMs were transfected with the siRNA as described above and then infected and stimulated with IGF-1 (200 ng/mL) for 24 hrs. The data are shown as the mean and SEM of the fold-increase in arg1 mRNA as determined by qRT-PCR relative to nonstimulated cells from a single experiment that was representative of 2 independent experiments. **I)** Immunoblot showing efficiency of siRNAi-mediated knockdown of STAT6 protein in hamster BMDMs and the partial requirement of STAT6 in the IGF-1-induced arg1 expression in *L. donovani* infected cells. Experiment was designed and data presented as described for Fig. 8F. IGF-1 was used at 200ng/mL. Shown is an immunoblot from a single experiment that was representative of 2 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Figure 9. IL-4 and growth factors amplify STAT6 activation. A, B) STAT6 activation measured in a luciferase reporter assay in BHK cells stimulated for 24h with growth factors and a sub-maximal concentration of IL-4. **A)** IL-4 (3 IU/mL) and/or FGF-2 (20 ng/mL) and **B)** IL-4 (6 IU/mL) and/or IGF-1 (100 ng/mL). Shown is the mean and SEM of STAT6 activity determined by luminometry from a single experiment that was representative of 3 independent experiments. **C, D)** Immunoblots of cell lysates (not immunoprecipitation as shown in Fig. 8B) showing phospho-STAT6 in BMDMs stimulated for 20 min with *L. donovani* promastigotes and **C)** IL-4 (8 IU/mL) and/or FGF-2 (20 ng/mL) or **D)** IL-4 (20 IU/mL) and/or IGF-1 (100 ng/mL). Bars represent the mean and SEM of fold change with reference to the uninfected, unstimulated (Uns) controls calculated by densitometry analysis of immunoblot bands

from 3 independent experiments. Also shown is a representative individual immunoblot. **E)** IL-4-mediated activation of STAT6 in infected BHK fibroblasts is reduced by inhibition of FGFR and IGF-1R but not ERK. The cells were exposed to the control (DMSO) or FGFR inhibitor (FGFRi; PD166866; 10µM), IGFR inhibitor (IGFRi; PPP; 5µM), or ERK inhibitor (ERKi; PD98059; 5 µM) for 1 hr and then stimulated for another 24 hrs with IL-4 (25 IU/mL) in the presence or absence of inhibitor. Shown is the mean and SEM of STAT6 activity determined by luminometry from a single experiment that was representative of 2 independent experiments.

Figure 10. Working model for convergent signaling of growth factors and cytokines in the induction of arg1 in VL. *L. donovani* infection induces the production of IL-4, IL-10 and FGF-2 in the spleen. FGFR is activated, as is IGF-IR by a yet to be identified host or parasite ligand. JAK kinases are phosphorylated through the activated cytokine or growth factor receptors, which lead to IRS-1/2, AKT, ERK, and STAT activation. Translocated STAT6, and possibly STAT3 lead to the transcriptional activation of arginase, which generates polyamines from arginine and leads to parasite growth. These transcription factors also contribute to the polarization macrophages toward an M2 phenotype, which is more permissive to *L. donovani* survival and growth. The collective effect of AKT, ERK, and STAT3 activation, and the generation of polyamines, are likely to lead to growth, proliferation and survival of arginase expressing cells, but this needs experimental confirmation in VL. Solid lines indicate known mechanistic interactions; dashed lines represent suppositional interactions. Only key shared signaling proteins are included in the model.

SUPPORTING INFORMATION LEGENDS

Table S1. Antibodies used to study signaling pathways in the hamster model of visceral leishmaniasis.

Figure S1. IGF-1 and PDGF-β production in hamsters with VL. IGF-1 and PDGF- β proteins were measured by ELISA using anti-mouse/rat antibodies that are broadly cross-reactive across species (IGF-1 and PDGF-β generally have highly conserved sequences across species). We found no increase in their expression in serum (panels A and D), plasma (panels B and E), or spleen tissue homogenates (panels C and F) from hamsters infected with *L. donovani*. At day 7 post-infection both serum and splenic IGF-1 were significantly decreased relative to uninfected controls. By immunoprecipitation and immunoblot we were unable to detect the ligands of EGFR (EGF, HB-EGF, Epiregulin and Amphiregulin), or VEGF. These negative immunoblots are not shown. Antibodies used for these experiments were broadly reacting across multiple species, however, we cannot exclude the possibility that the lack of detection was due to an antibody that had low affinity to the hamster protein. *p<0.05; ***p<0.001.

Figure S2. Expression of PDGF-β and EGFR in hamsters with VL. Splenic macrophages were isolated by adherence from the spleens of uninfected hamsters (time 0) or hamsters infected for 7, 14, 28 or 45 days and lysates probed with antibodies directed against PDGF-β, p-PDGF-β, EGFR, p-EGFR and GAPDH (loading control). An immunoblot representative of 2-4 independent experiments is shown. Phosphorylated EGFR could not be detected with any of 3 different anti-Phospho-EGFR antibodies (Tyr1068, Tyr992, Tyr 1045; Cell Signaling); those negative immunoblots are not shown.

Figure S3. Inhibitors of FGFR and IGF-1R do not affect *Leishmania donovani* viability. Cultured *L. donovani* promastigotes were seeded in 96-well white-bottom luminometry plates at 10,000 parasites per well in DMEM with 2% heat-inactivated fetal bovine serum. The parasites were incubated at 26°C in the presence of increasing concentrations of (A) FGFR inhibitor (PD166866) or (B) IGFR inhibitor (PPP) or with vehicle control (DMSO). After 48 hours the number of viable promastigotes was determined by luminometry (cell titer Glo, Promega). Data represent the percent of viable parasites in 4 different replicates of each concentration of inhibitor compared to the control with the corresponding DMSO dilution.

Figure S4. IL-10 and IL-10Rα are increased in *L. donovani* infected macrophages but are not induced by growth factors. A) Expression of IL-10 and IL-10Rα mRNA was determined by qRT-PCR in splenic

macrophages from uninfected (Un) and 18-day *L. donovani* infected hamsters (Inf). **B)** IL-10 and IL-10Rα mRNA in BMDMs infected 1:2 with *L. donovani* was not amplified by exposure to IGF-1 (200 ng/mL) or FGF-2 (20 ng/mL) for 24 hrs. In fact, FGF-2 significantly decreased the expression of IL-10 and IL-10Rα mRNA in infected macrophages. Shown is mean and SEM of the fold increase of expression over uninfected, unstimulated controls from a single experiment representative of 2 independent experiments. **C)** STAT-3 reporter activity in hamster BMDM compared to human U-937 cells. Cells (10,000) were in plated in Opti-Mem 10% HIFBS and 2 µg/mL polybrene and transiently transfected with a lentiviral vector containing a STAT-3 luciferase reporter construct (20 MOI, Cignal lenti, Qiagen). 48 hrs after transfection the cells were serum starved for 24 hrs and then stimulated for 24 hrs with human IL-10 (100 ng/mL). Shown is the mean and SEM of the fold-increase of luciferase reporter activity in stimulated compared to unstimulated cells. Data are from a single experiment representative of 2 independent experiments. D) p-STAT3 detected by immunoblotting of whole cell lysates of hamster BMDM infected *in vitro* with *L. donovani* and exposed to IGF-1 (200 ng/mL) or FGF-2 (20 ng/mL) for 20 min to 48 hrs hrs. Bars represent the mean and SEM of fold change with reference to the unstimulated (Un) controls calculated by densitometry analysis of immunoblot bands from 1-3 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Table 1. Inhibitors of Receptor Tyrosine Kinases reduce Arg1 transcription.

Target	Inhibitor	Concentration (µM) [1]	Arg1 Inhibition (%) [2]
EGFRK	Tyrphostin 25	6	64.6
EGFRK	Tyrphostin23	70	84.5
PDGFRK	Tyrphostin 9	2.4	78.5
EGFRK, PDGFRK	AG-494	46	62
MEK	U-0126	0.13	73.5
MEK	PD-98059	4	52.2
MAPK-p38, PKA, GSK-3-beta	Rottlerin	100	52.4
PI 3-K	Quercetin	7.6	83.5

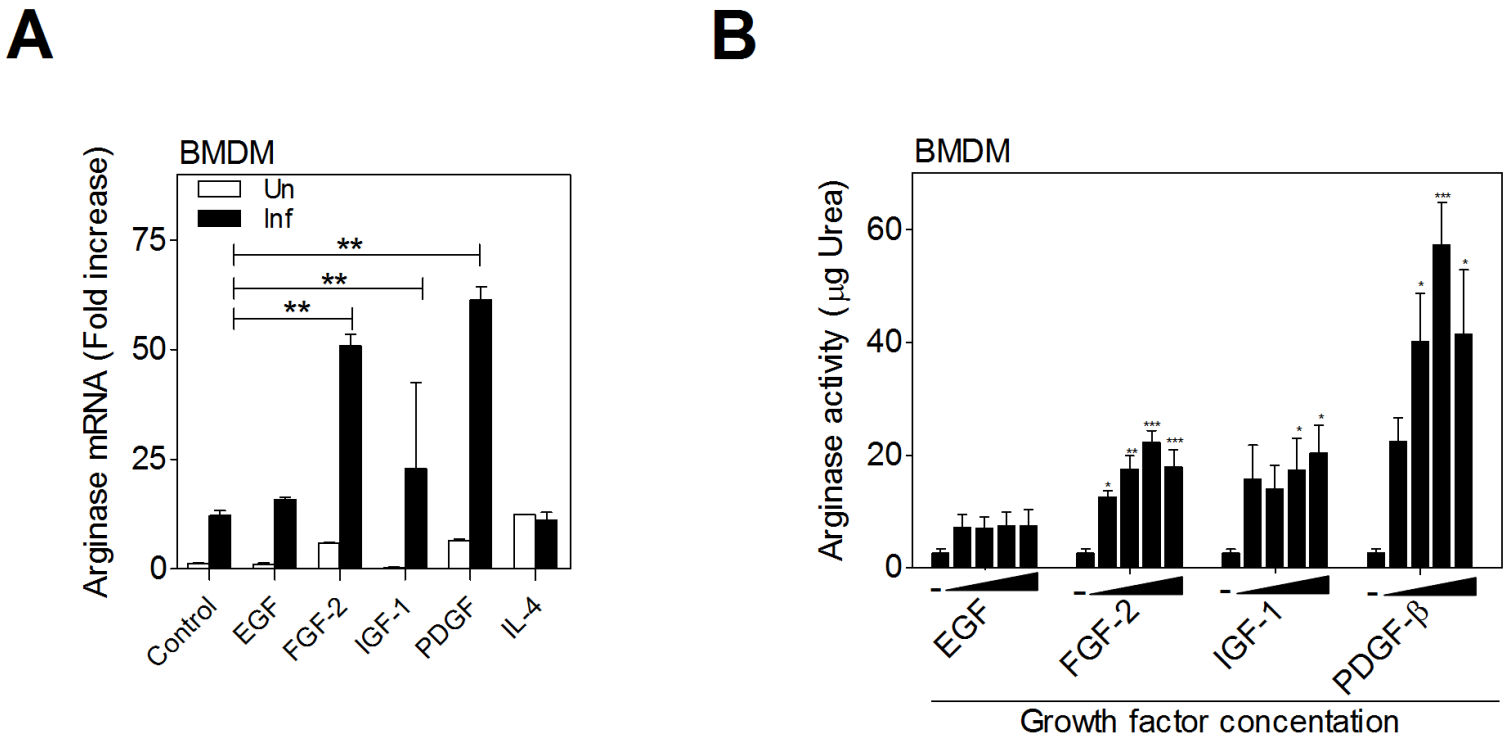
[1] Inhibitors (dissolved in DMSO) were used at twice the concentration reported to cause 50% inhibition of kinase activity. No attempt was made to maximize concentration for complete kinase inhibition.
[2] Arg1 mRNA expression was determined in ex vivo cultured spleen cells from hamsters infected with *L. donovani* exposed to the RTK inhibitor for 24 hrs. The percent inhibition of arg1 transcription was calculated with reference to untreated control cells exposed to DMSO and determined by qPCR.

Table 2. Activation of Receptor Tyrosine Kinases and downstream signaling proteins in splenic macrophages from hamsters infected with *L. donovani*. [1]

Phosphoprotein (Entrez Gene Name)	Symbol	Fold Increase [2]
v-akt murine thymoma viral oncogene homolog 1	AKT1	20.7 ± 12.1
v-akt murine thymoma viral oncogene homolog 2	AKT2	159.9 ± 22.15
EPH receptor A1	EPHA1	7 ± 3.95
EPH receptor A3	EPHA3	5.7 ± 5.25
EPH receptor B4	EPHB4	4.9 ± 0.3
Fibroblast growth factor receptor 1	FGFR1	2.1 ± 0.35
Fibroblast growth factor receptor 3	FGFR3	2.7 ± 0.3
Insulin receptor substrate 1	IRS1	40.2 ± 16.3
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	4.4 ± 3.55
Mitogen-activated protein kinase 3	MAPK3	2.1 ± 1.65
Neurotrophic tyrosine kinase, receptor, type 1	NTRK1	4.7 ± 2.15
Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	1.9 ± 0.95
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	152 ± 23.1
Signal transducer and activator of transcription 1	STAT1	5.7 ± 2.45
Signal transducer and activator of transcription 3	STAT3	2.4 ± 0.85
TEK tyrosine kinase, endothelial	TEK	7.3 ± 3.65
zeta-chain (TCR) associated protein kinase 70kDa	ZAP70	14 ± 11.2

[1] Determined using a PathScan Antibody Array (Chemiluniscent readout, Cell Signaling; 4 arrays per group) and analyzed with the IPA Software.
[2] Fold-change (mean ± SEM) of the phosphoprotein expressed in splenic macrophages isolated from 28-day infected hamsters compared to splenic macrophages from uninfected hamsters. RTKs and signaling proteins with a fold change > 1.5 are shown.

Figure1.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25
0
**
**
**
Control
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FGF-2
IGF-1
PDGF
IL-4
B
BMDM
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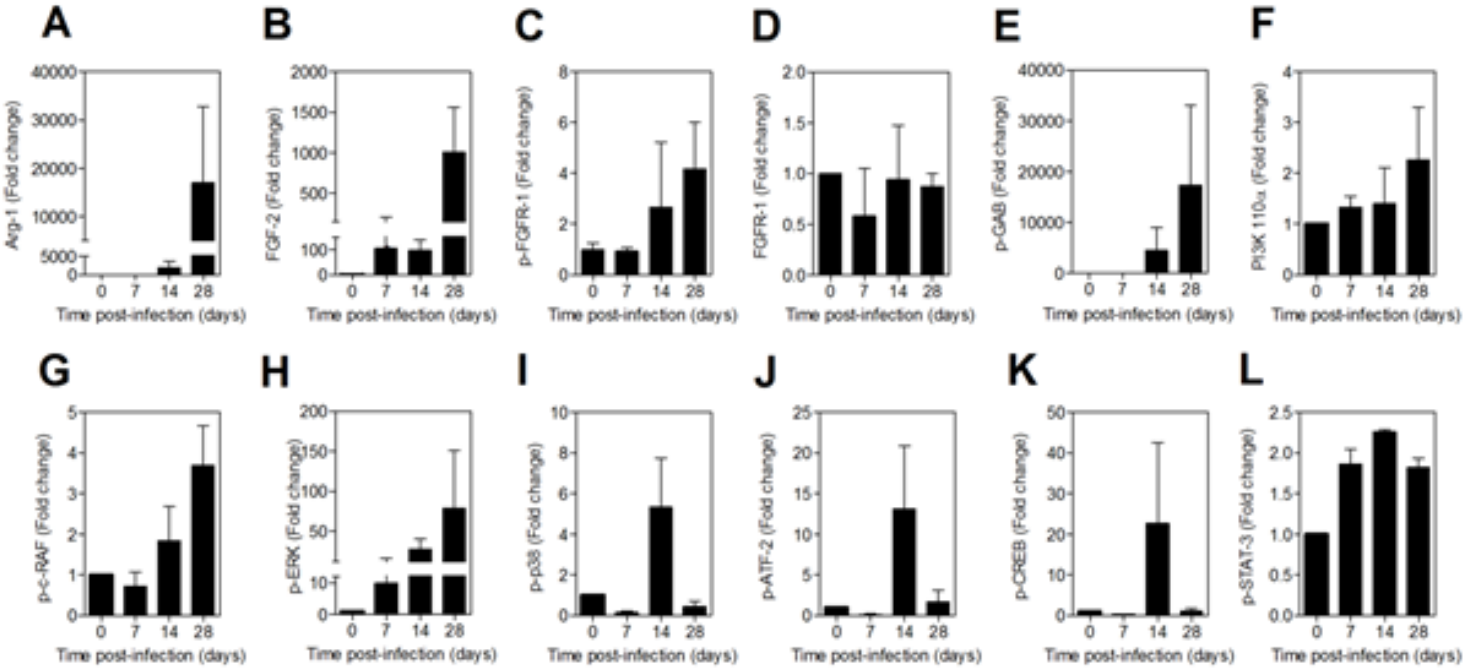
40
20
0
EGF
FGF-2
IGF-1
PDGF-β
Growth factor concentation

Figure 2.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B
FGF-2 (Fold change)
C
p-FGFR-1 (Fold change)
D
FGFR-1 (Fold change)
E
p-GAB (Fold change)
F
PI3K 110α (Fold change)
G
p-c-RAF (Fold change)
H
p-ERK (Fold change)
I
p-p38 (Fold change)
J
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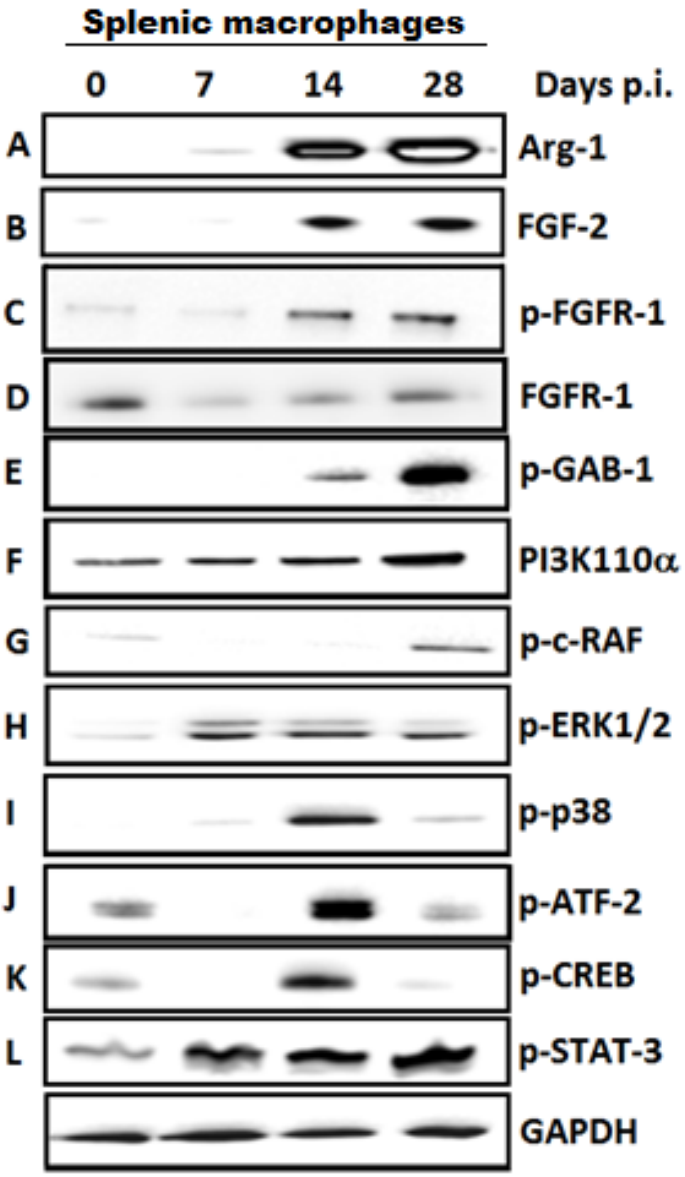
K
p-CREB (Fold change)
L
p-STAT-3 (Fold change)
Time post-infection (days)


Splenic macrophages
0
7
14
28
Days p.i.
A
Arg-1
B
FGF-2
C
p-FGFR-1
D
FGFR-1
E
p-GAB-1
F
PI3K110α
G
p-c-RAF
H
p-ERK1/2
I
p-p38
J
p-ATF-2
K
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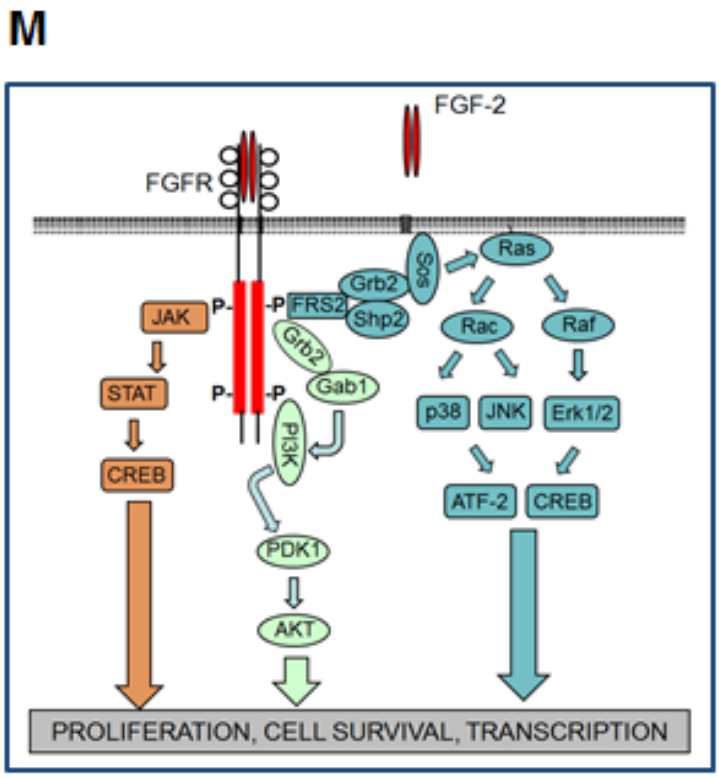
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CREB
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AKT
SOS
Ras
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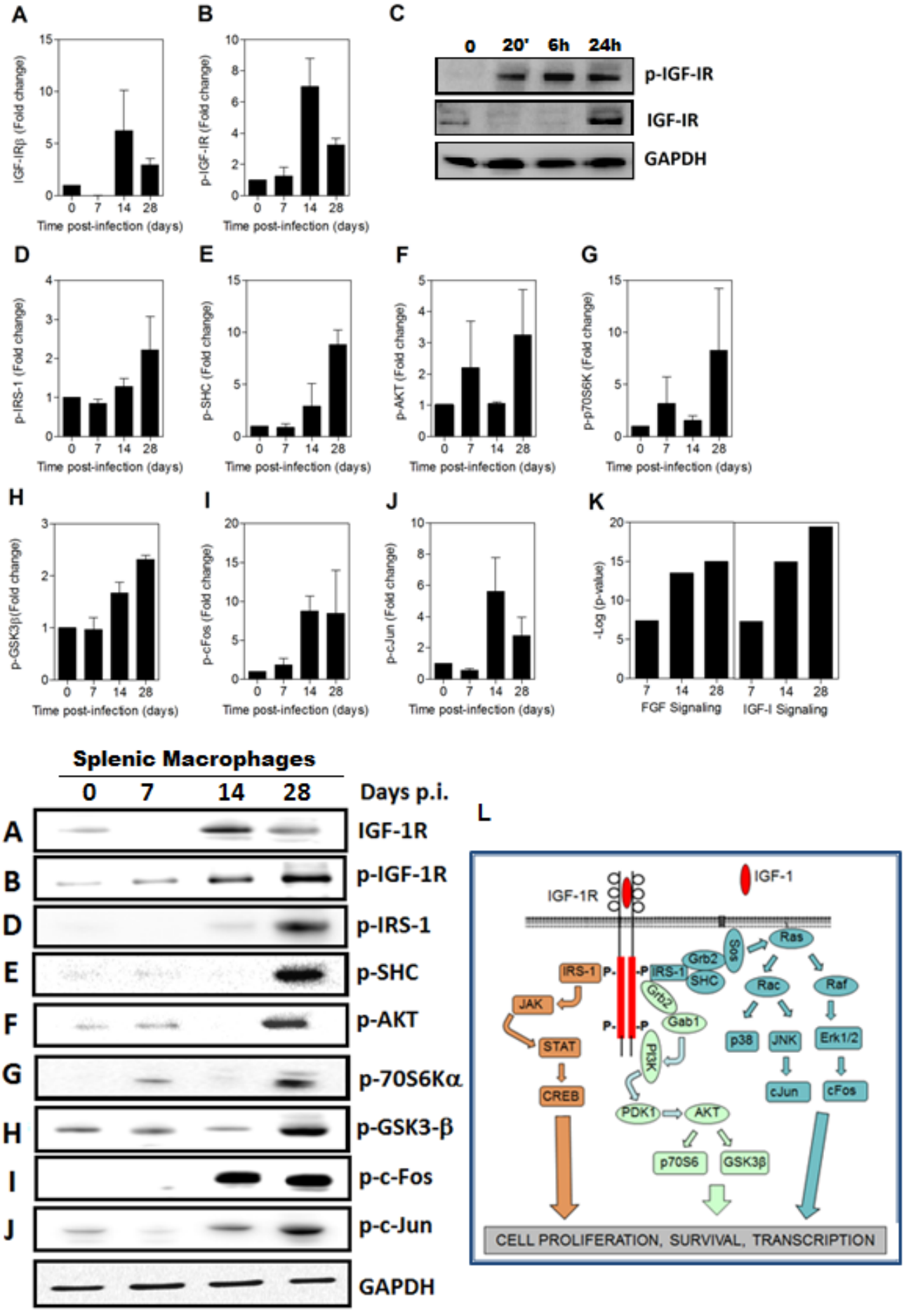
p38
JNK
Erk1/2
ATF-2
CREB
PROLIFERATION, CELL SURVIVAL, TRANSCRIPTION

Figure 3.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IGF-IRβ (Fold change)
Time post-infection (days)
B
p-IGF-IR (Fold change)
Time post-infection (days)
C
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20'
6h
24h
p-IGF-IR
IGF-IR
GAPDH
D
p-IRS-1 (Fold change)
Time post-infection (days)
E
p-SHC (Fold change)
Time post-infection (days)
F
p-AKT (Fold change)
Time post-infection (days)
G
p-p70S6K (Fold change)
Time post-infection (days)
H
p-GSK3β(Fold change)
Time post-infection (days)
I
p-cFos (Fold change)
Time post-infection (days)
J
p-cJun (Fold change)
Time post-infection (days)
K
-Log (p-value)
FGF Signaling
IGF-I Signaling
Splenic Macrophages
Days p.i.
A IGF-1R
B p-IGF-1R
D p-IRS-1
E p-SHC
F p-AKT
G p-70S6Kα
H p-GSK3-β
I p-c-Fos
J p-c-Jun
GAPDH
L
IGF-1R
IGF-1
IRS-1
SHC
Grb2
Sos
Ras
Rac
Raf
JAK
STAT
Gab1
PI3K
p38
JNK
Erk1/2
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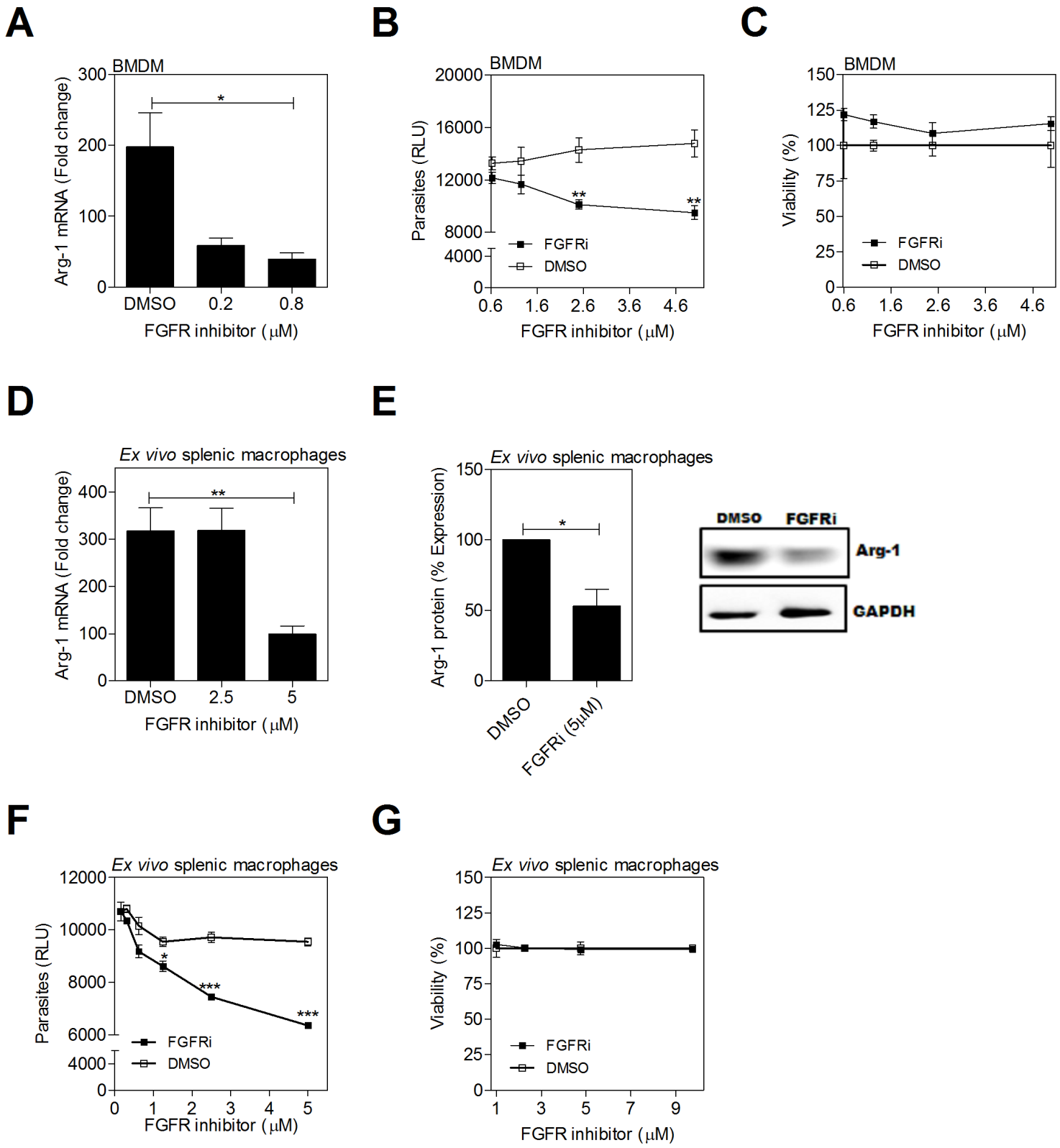
cFos
CREB
PDK1
AKT
p70S6
GSK3β
CELL PROLIFERATION, SURVIVAL, TRANSCRIPTION

Figure 4.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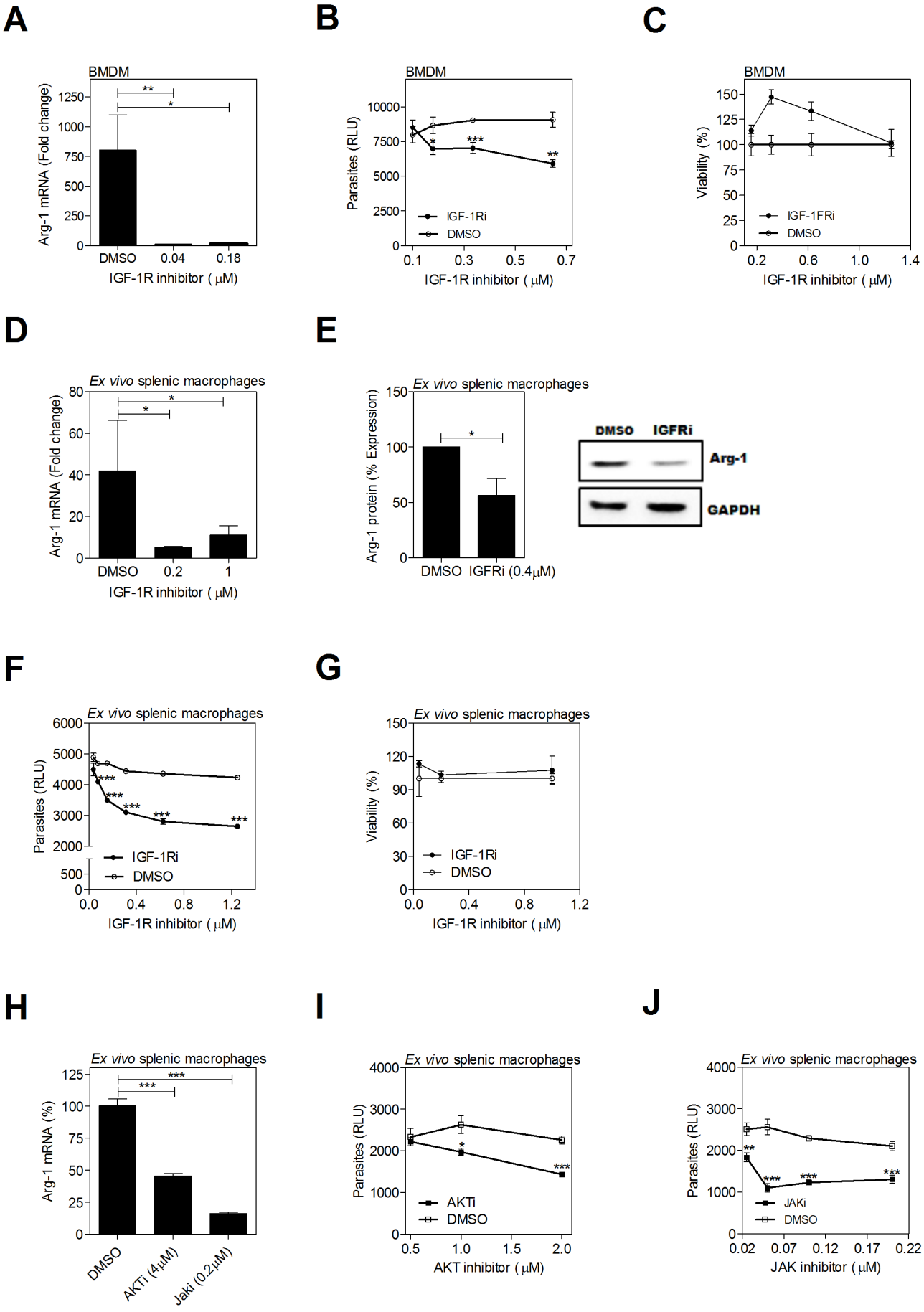
BMDM
Arg-1 mRNA (Fold change)
300
200
100
0
*
DMSO
0.2
0.8
FGFR inhibitor (μM)
B
BMDM
Parasites (RLU)
20000
16000
12000
8000
4000
0
**
**
FGFRi
DMSO
0.6
1.6
2.6
3.6
4.6
FGFR inhibitor (μM)
C
BMDM
Viability (%)
150
125
100
75
50
25
0
FGFRi
DMSO
0.6
1.6
2.6
3.6
4.6
FGFR inhibitor (μM)
D
Ex vivo splenic macrophages
Arg-1 mRNA (Fold change)
400
300
200
100
0
**
DMSO
2.5
5
FGFR inhibitor (μM)
E
Ex vivo splenic macrophages
Arg-1 protein (% Expression)
150
100
50
0
*
DMSO
FGFRi (5μM)
DMSO
FGFRi
Arg-1
GAPDH
F
Ex vivo splenic macrophages
Parasites (RLU)
12000
10000
8000
6000
4000
0
*
***
***
FGFRi
DMSO
0
1
2
3
4
5
FGFR inhibitor (μM)
G
Ex vivo splenic macrophages
Viability (%)
150
125
100
75
50
25
0
FGFRi
DMSO
1
3
5
7
9
FGFR inhibitor (μM)

Figure 5.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BMDM
Arg-1 mRNA (Fold change)
DMSO
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0.18
IGF-1R inhibitor (μM)
B
BMDM
Parasites (RLU)
IGF-1Ri
DMSO
IGF-1R inhibitor (μM)
C
BMDM
Viability (%)
IGF-1FRi
DMSO
IGF-1R inhibitor (μM)
D
Ex vivo splenic macrophages
Arg-1 mRNA (Fold change)
DMSO
0.2
1
IGF-1R inhibitor (μM)
E
Ex vivo splenic macrophages
Arg-1 protein (% Expression)
DMSO
IGFRi (0.4μM)
DMSO
IGFRi
Arg-1
GAPDH
F
Ex vivo splenic macrophages
Parasites (RLU)
IGF-1Ri
DMSO
IGF-1R inhibitor (μM)
G
Ex vivo splenic macrophages
Viability (%)
IGF-1Ri
DMSO
IGF-1R inhibitor (μM)
H
Ex vivo splenic macrophages
Arg-1 mRNA (%)
DMSO
AKTi (4μM)
Jaki (0.2μM)
I
Ex vivo splenic macrophages
Parasites (RLU)
AKTi
DMSO
AKT inhibitor (μM)
J
Ex vivo splenic macrophages
Parasites (RLU)
JAKi
DMSO
JAK inhibitor (μM)

Figure 6.

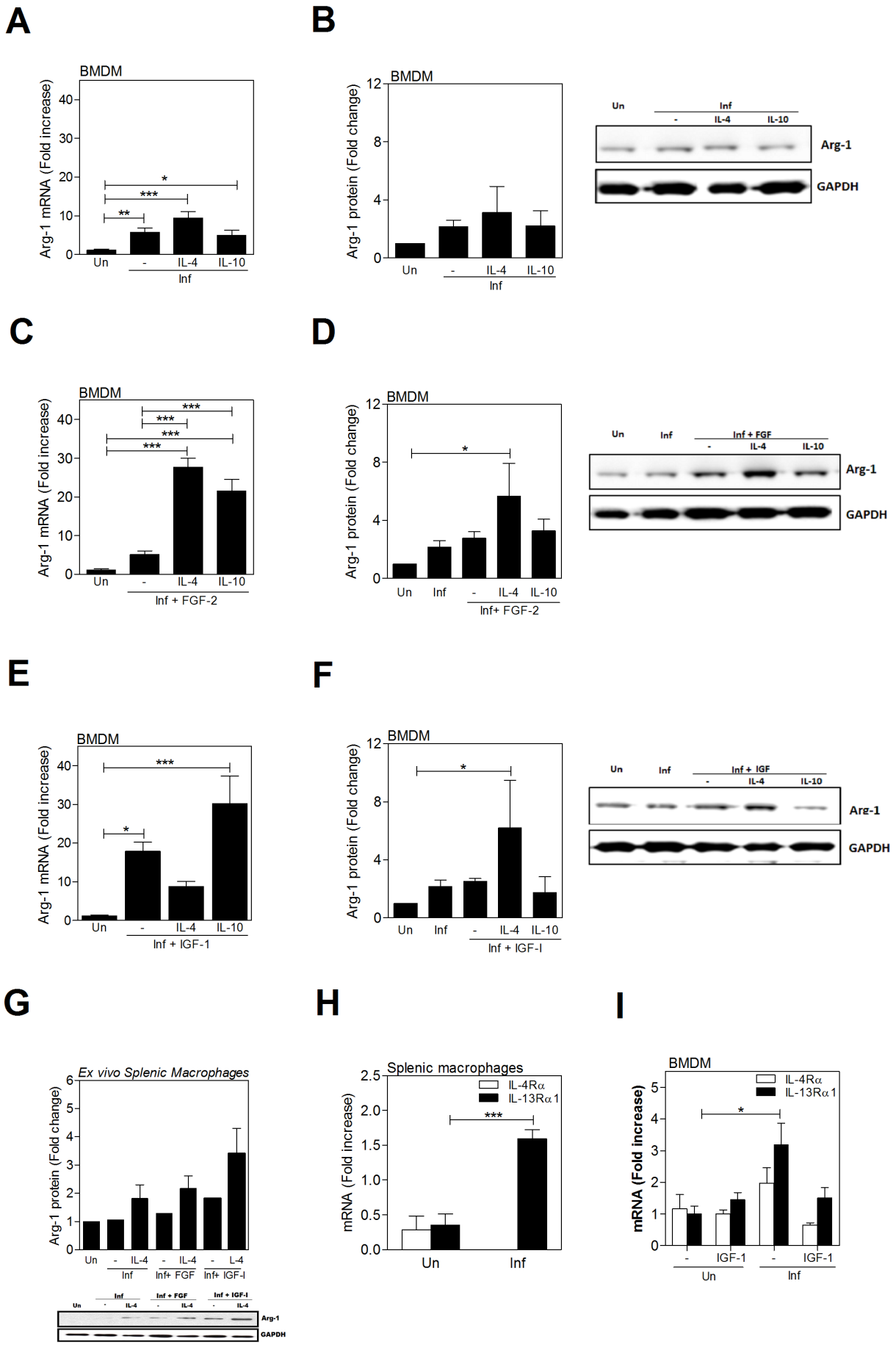

A
BMDM
Arg-1 mRNA (Fold increase)
Un
-
IL-4
IL-10
Inf
B
BMDM
Arg-1 protein (Fold change)
Un
Inf
Arg-1
GAPDH
C
BMDM
Arg-1 mRNA (Fold increase)
Inf + FGF-2
D
BMDM
Arg-1 protein (Fold change)
Inf+ FGF-2
Inf + FGF
E
BMDM
Arg-1 mRNA (Fold increase)
Inf + IGF-1
F
BMDM
Arg-1 protein (Fold change)
Inf + IGF-I
Inf + IGF
G
Ex vivo Splenic Macrophages
Arg-1 protein (Fold change)
L-4
Inf+ FGF
Inf+ IGF-I
H
Splenic macrophages
mRNA (Fold increase)
IL-4Rα
IL-13Rα1
I
BMDM
mRNA (Fold increase)
IGF-1

Figure 7.

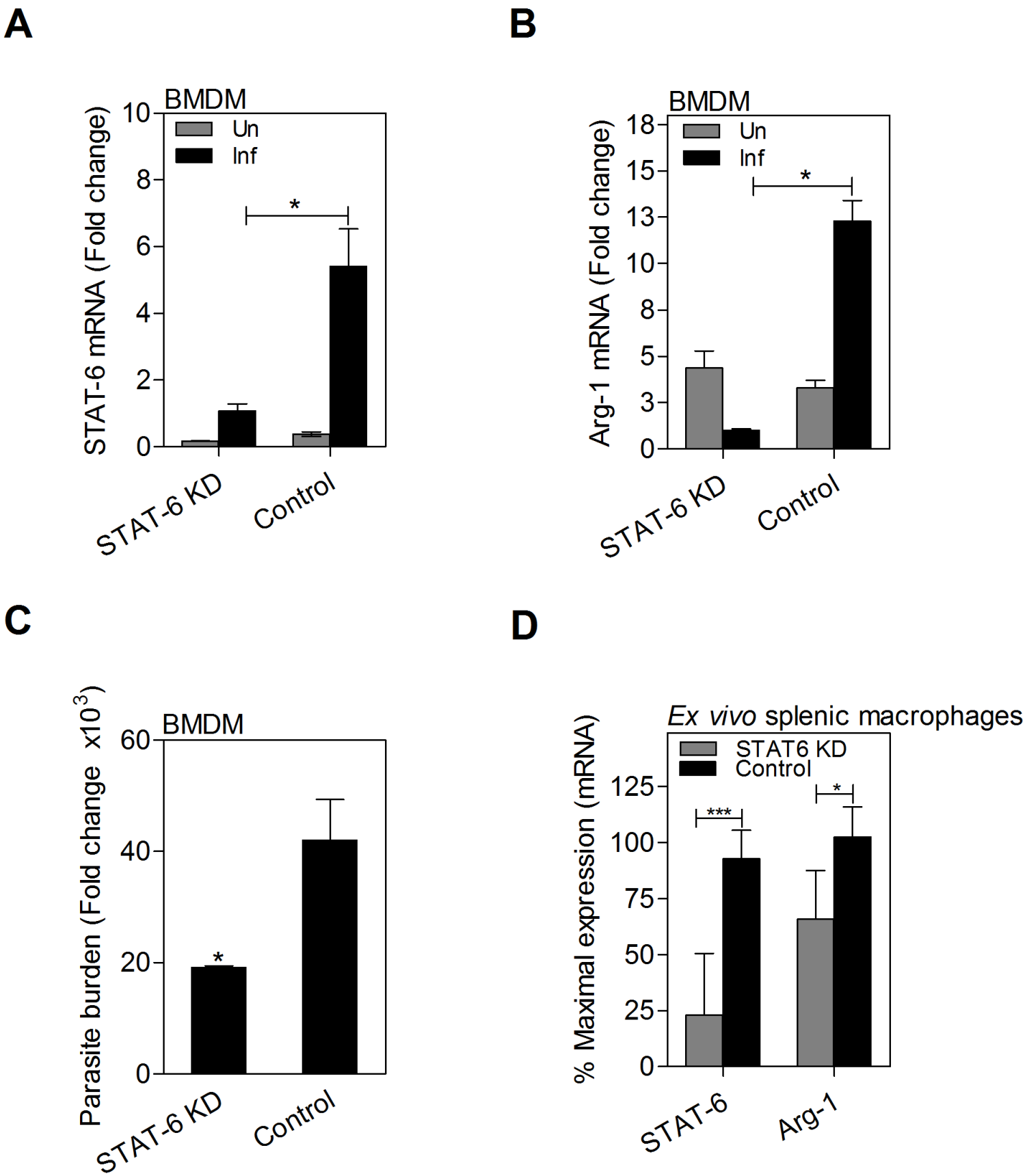

A
BMDM
Un
Inf
STAT-6 mRNA (Fold change)
10
8
6
4
2
0
*
STAT-6 KD
Control
B
BMDM
Un
Inf
Arg-1 mRNA (Fold change)
18
15
13
10
8
5
3
0
*
STAT-6 KD
Control
C
BMDM
Parasite burden (Fold change x10^3)
60
40
20
0
*
STAT-6 KD
Control
D
Ex vivo splenic macrophages
STAT6 KD
Control
% Maximal expression (mRNA)
125
100
75
50
25
0
***
*
STAT-6
Arg-1

Figure 8.

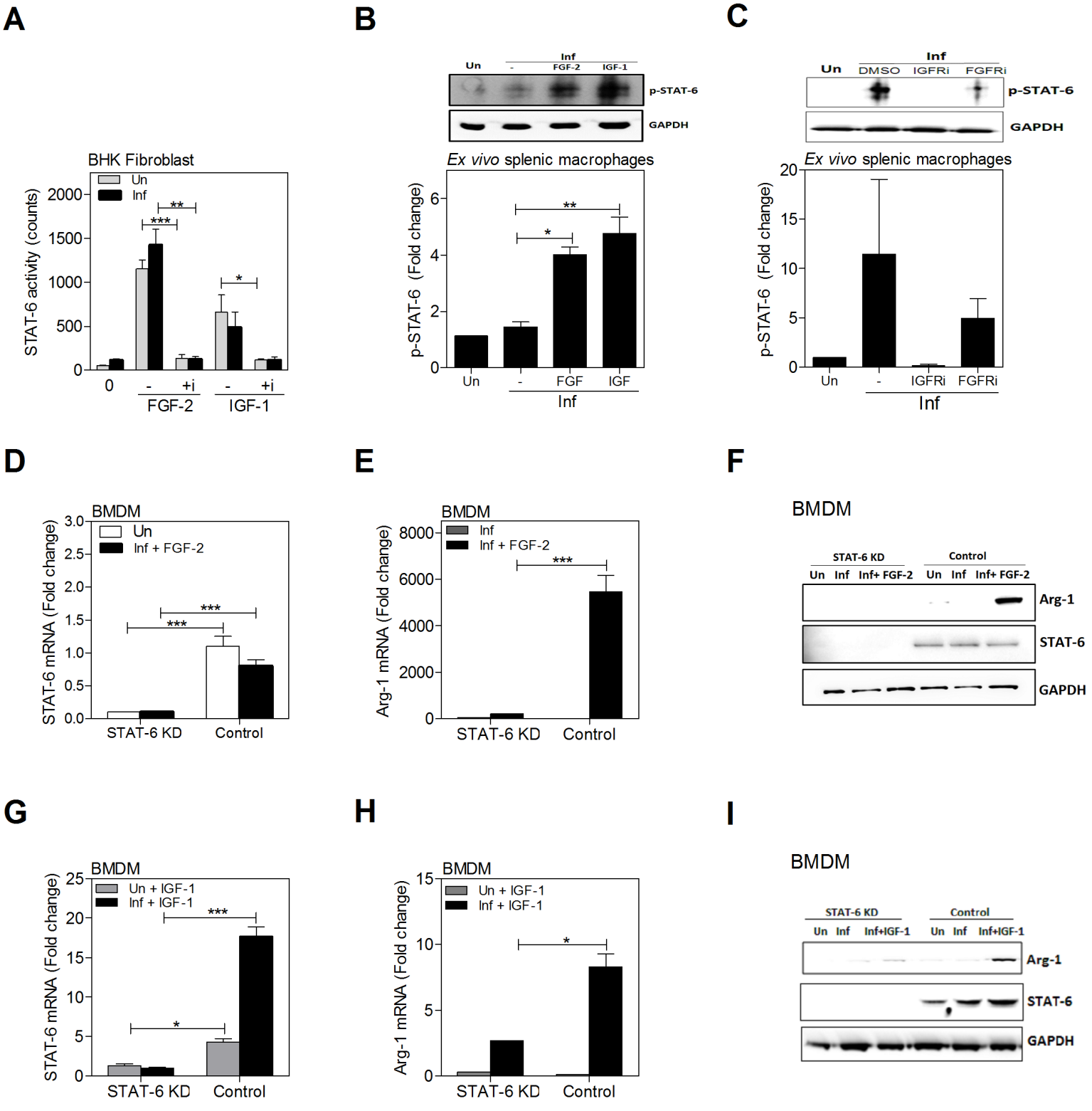

A
BHK Fibroblast
Un
Inf
STAT-6 activity (counts)
2000
1500
1000
500
0
**
***
*
0
-
+i
-
+i
FGF-2
IGF-1
B
Un
Inf
-
FGF-2
IGF-1
p-STAT-6
GAPDH
Ex vivo splenic macrophages
p-STAT-6 (Fold change)
6
4
2
0
**
*
Un
-
FGF
IGF
Inf
C
Un
Inf
DMSO
IGFRi
FGFRi
p-STAT-6
GAPDH
Ex vivo splenic macrophages
p-STAT-6 (Fold change)
20
15
10
5
0
Un
-
IGFRi
FGFRi
Inf
D
BMDM
Un
Inf + FGF-2
STAT-6 mRNA (Fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
STAT-6 KD
Control
E
BMDM
Inf
Inf + FGF-2
Arg-1 mRNA (Fold change)
8000
6000
4000
2000
0
***
STAT-6 KD
Control
F
BMDM
STAT-6 KD
Control
Un
Inf
Inf+ FGF-2
Un
Inf
Inf+ FGF-2
Arg-1
STAT-6
GAPDH
G
BMDM
Un + IGF-1
Inf + IGF-1
STAT-6 mRNA (Fold change)
25
20
15
10
5
0
***
*
STAT-6 KD
Control
H
BMDM
Un + IGF-1
Inf + IGF-1
Arg-1 mRNA (Fold change)
15
10
5
0
*
STAT-6 KD
Control
I
BMDM
STAT-6 KD
Control
Un
Inf
Inf+IGF-1
Un
Inf
Inf+IGF-1
Arg-1
STAT-6
GAPDH

Figure 9.

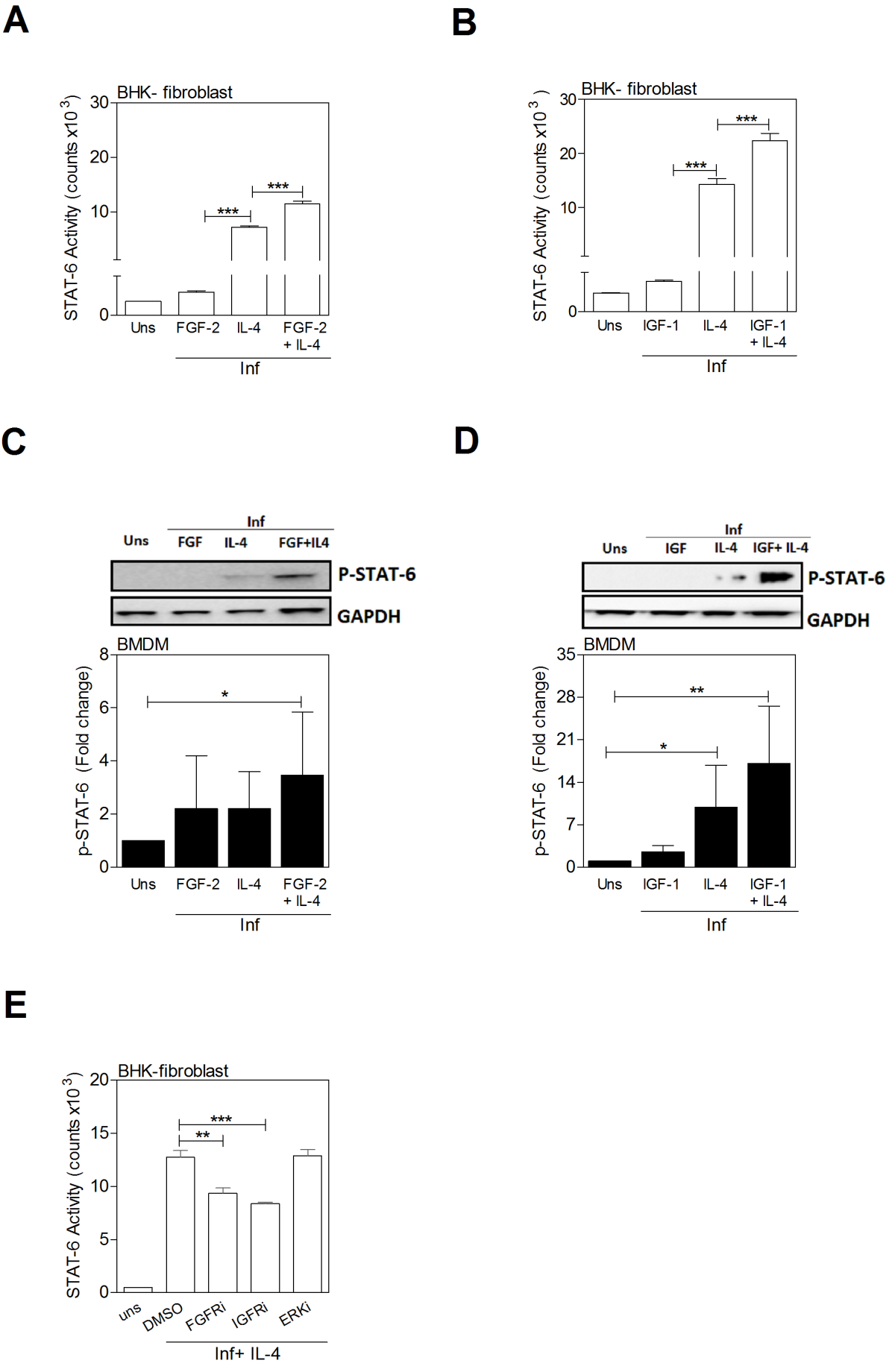
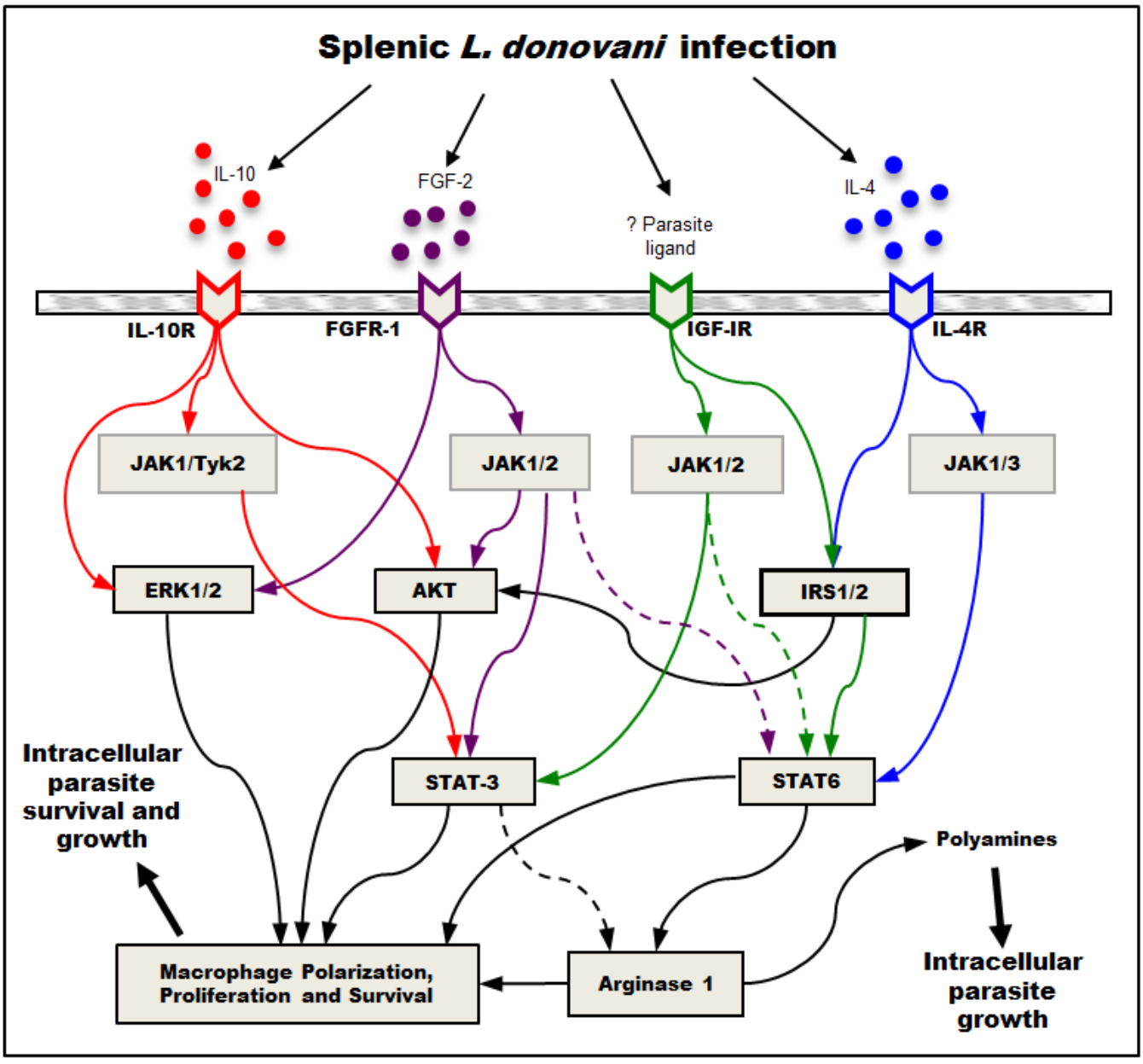
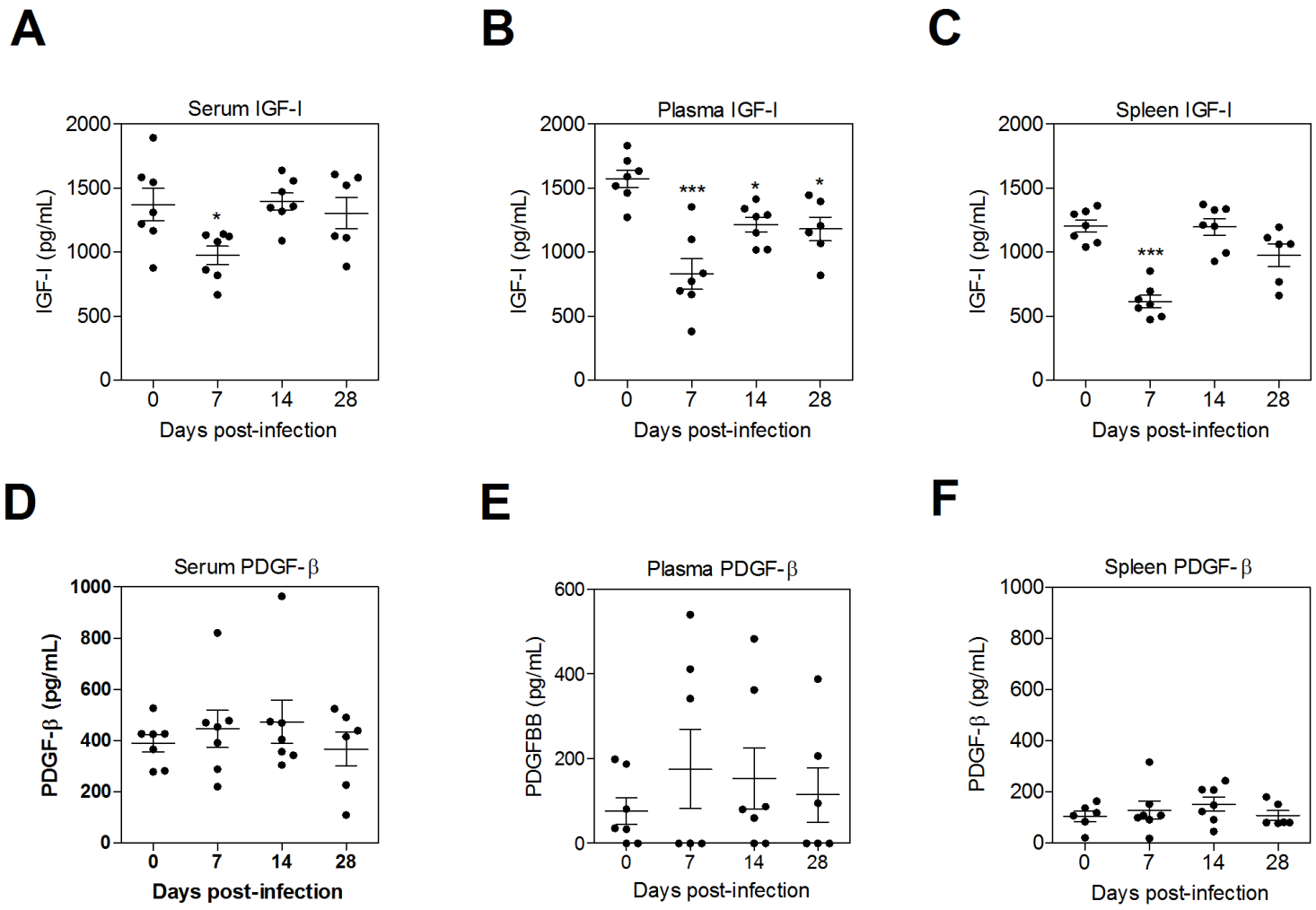

A
BHK- fibroblast
STAT-6 Activity (counts x10 3)
30
20
10
0
***
***
Uns
FGF-2
IL-4
FGF-2 + IL-4
Inf
B
BHK- fibroblast
STAT-6 Activity (counts x10 3)
30
20
10
0
***
***
Uns
IGF-1
IL-4
IGF-1 + IL-4
Inf
C
Inf
Uns
FGF
IL-4
FGF+IL4
P-STAT-6
GAPDH
BMDM
p-STAT-6 (Fold change)
8
6
4
2
0
*
Uns
FGF-2
IL-4
FGF-2 + IL-4
Inf
D
Inf
Uns
IGF
IL-4
IGF+ IL-4
P-STAT-6
GAPDH
BMDM
p-STAT-6 (Fold change)
35
28
21
14
7
0
**
*
Uns
IGF-1
IL-4
IGF-1 + IL-4
Inf
E
BHK-fibroblast
STAT-6 Activity (counts x10 3)
20
15
10
5
0
***
**
uns
DMSO
FGFRi
IGFRi
ERKi
Inf+ IL-4

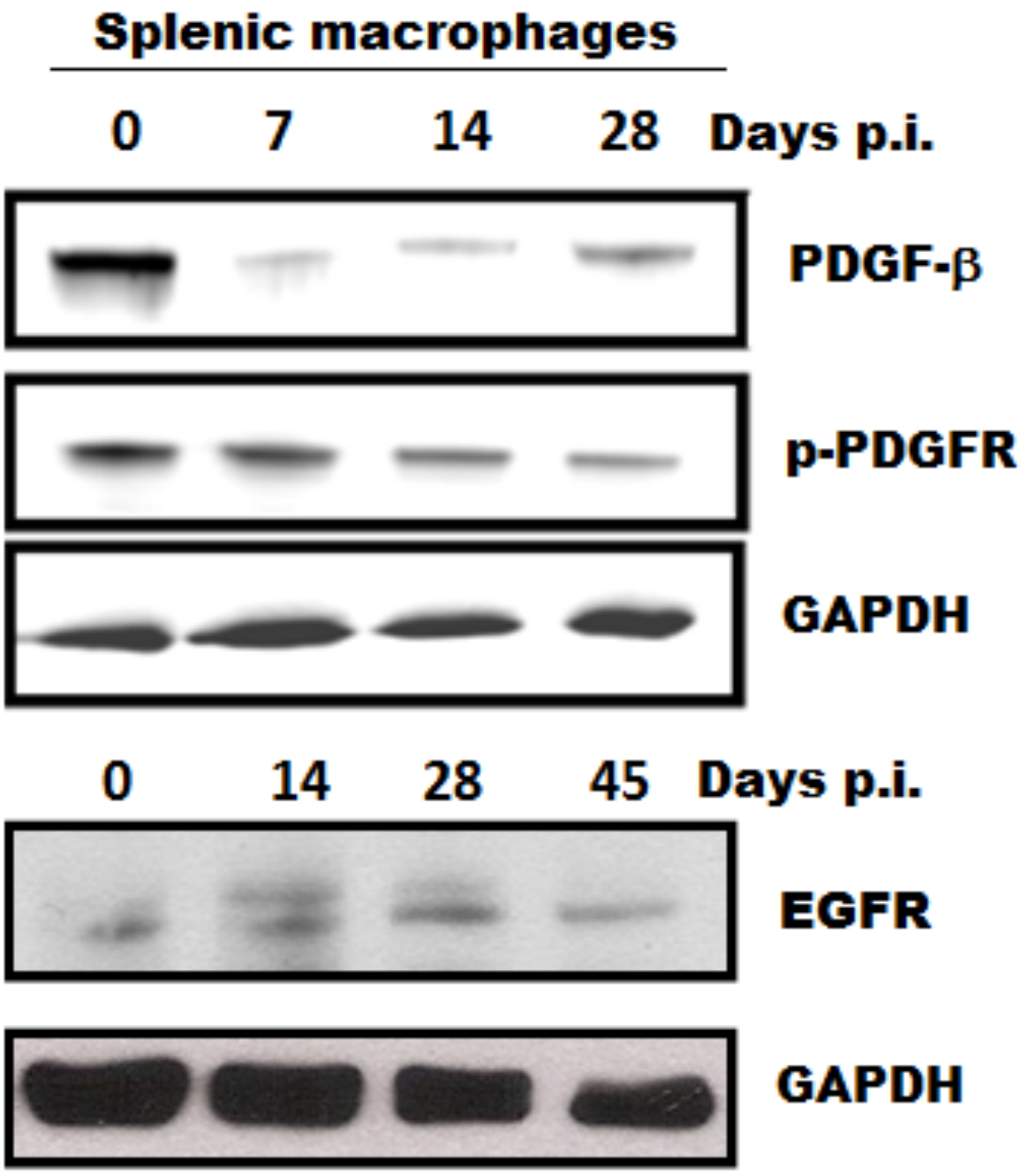
Figure 10.


Splenic L. donovani infection
IL-10
FGF-2
? Parasite ligand
IL-4
IL-10R
FGFR-1
IGF-IR
IL-4R
JAK1/Tyk2
JAK1/2
JAK1/2
JAK1/3
ERK1/2
AKT
IRS1/2
STAT-3
STAT6
Intracellular parasite survival and growth
Macrophage Polarization, Proliferation and Survival
Arginase 1
Polyamines
Intracellular parasite growth

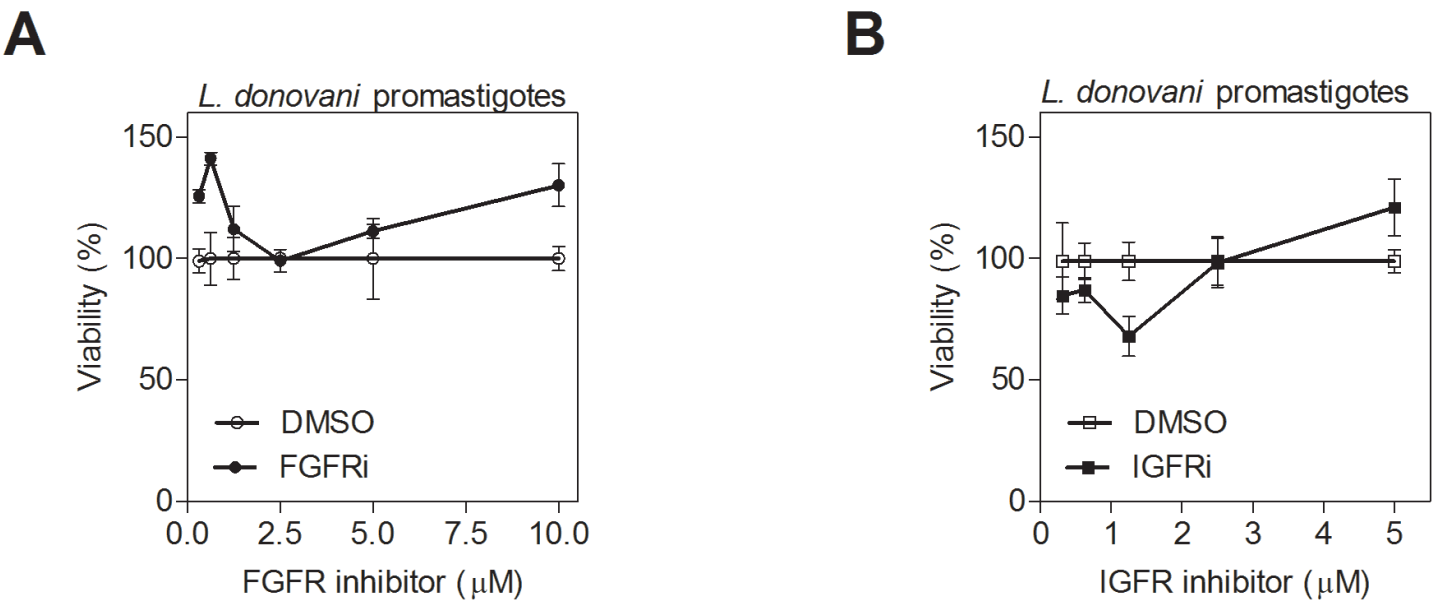
SFigure1.


A
Serum IGF-I
IGF-I (pg/mL)
2000
1500
1000
500
0
*
0
7
14
28
Days post-infection
B
Plasma IGF-I
IGF-I (pg/mL)
***
*
*
Days post-infection
C
Spleen IGF-I
IGF-I (pg/mL)
***
Days post-infection
D
Serum PDGF-β
PDGF-β (pg/mL)
1000
800
600
400
200
0
Days post-infection
E
Plasma PDGF-β
PDGFBB (pg/mL)
600
400
200
0
Days post-infection
F
Spleen PDGF-β
PDGF-β (pg/mL)
Days post-infection

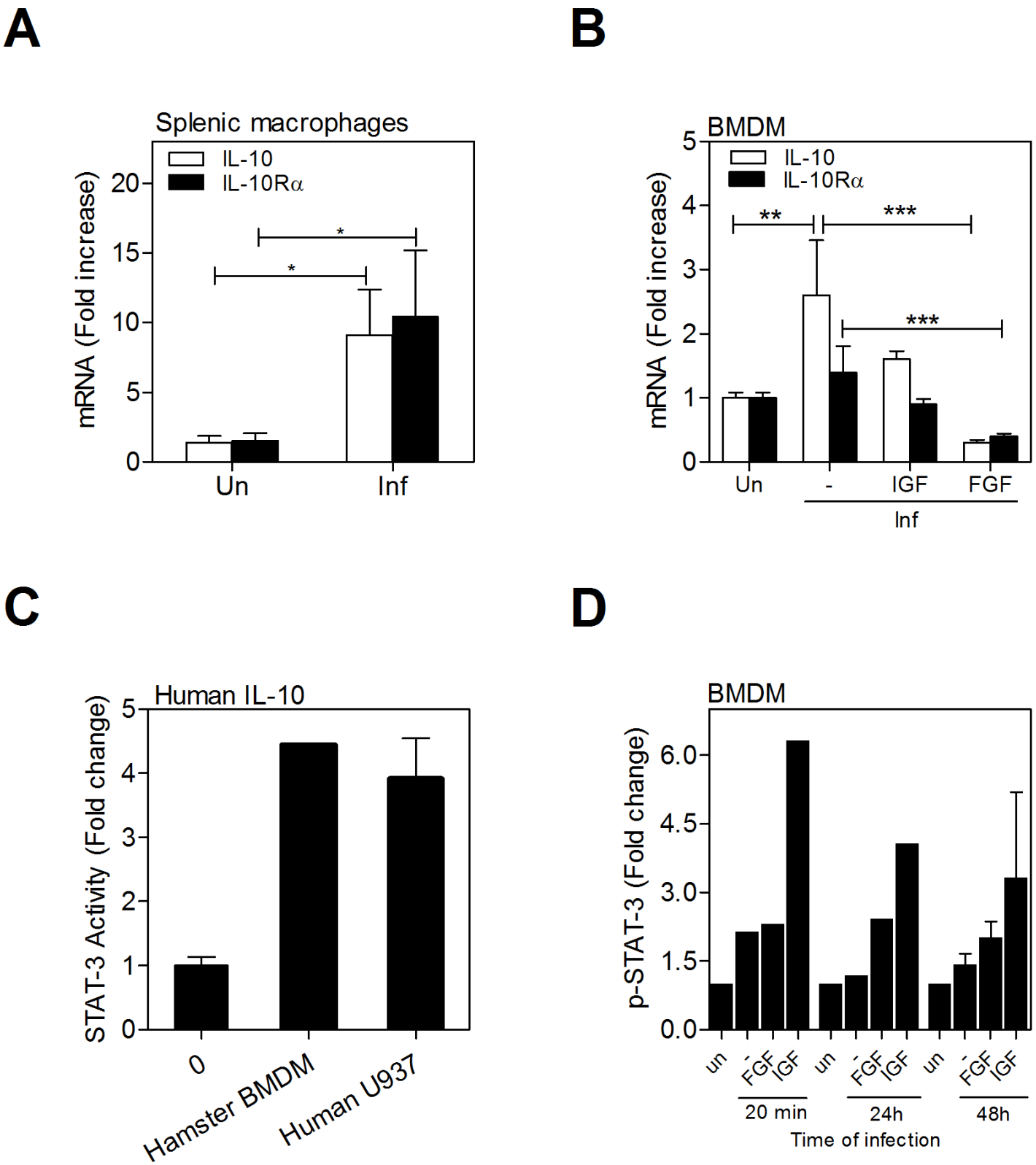
SFigrue2.


Splenic macrophages
0
7
14
28
Days p.i.
PDGF-β
p-PDGFR
GAPDH
0
14
28
45
Days p.i.
EGFR
GAPDH

SFigure 3.


A
L. donovani promastigotes
Viability (%)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
FGFR inhibitor (μM)
DMSO
FGFRi
B
L. donovani promastigotes
Viability (%)
150
100
50
0
0
1
2
3
4
5
IGFR inhibitor (μM)
DMSO
IGFRi

SFigure 4.


A
Splenic macrophages
IL-10
IL-10Rα
mRNA (Fold increase)
20
15
10
5
0
*
*
Un
Inf
B
BMDM
IL-10
IL-10Rα
mRNA (Fold increase)
5
4
3
2
1
0
**
***
***
Un
-
IGF
FGF
Inf
C
Human IL-10
STAT-3 Activity (Fold change)
5
4
3
2
1
0
0
Hamster BMDM
Human U937
D
BMDM
p-STAT-3 (Fold change)
6.0
4.5
3.0
1.5
0.0
un
-
FGF
IGF
20 min
24h
48h
Time of infection

3.3. DOCUMENTO 3:

Role of arginase and fibroblast growth factor in human visceral leishmaniasis (manuscrito em preparação)

A infecção por protozoários das espécies *Leishmania (Leishmania) infantum* e *L. (L.) donovani* causam a leishmaniose visceral (LV) que é uma doença sistêmica progressiva que leva a morte em 10% dos casos (Alvar *et al*. 2012). Por esta razão, a identificação dos fatores associados com a patogênese, e o estabelecimento de medidas de controle e tratamento da doença tem impacto importante na saúde da população. Achados experimentais e em humanos sugeriram que a arginase (Arg)-1 estava envolvida no aumento da carga parasitária na LV e com os mecanismos de imunossupressão. Nossos resultados anteriores mostraram que o STAT-6 foi o fator chave na regulação da Arg-1 e susceptibilidade no modelo hamster com LV crônica. Embora a ativação deste fator tenha sido dirigida pelo parasito e amplificada por citocinas Th2, também foi ativado por outras vias não canônicas como as vias dos receptores tirosina quinase (RTK). Porém, neste estudo avaliou-se se os mesmos fatores identificados no modelo experimental hamster (FGF-2 e IGF-I) regularam Arginase na infecção experimental do sangue no homem. Por outro lado, também se estudou a evolução dos níveis plasmáticos da Arg-1 e FGF-2 em pacientes na doença ativa e resolutiva, a fim de estabelecer se havia a mesma relação de causalidade observada nos modelos *in vitro* e, portanto, confrontar os resultados experimentais com aqueles observados na doença humana.

3.3. RESULTADOS PRELIMINARES (ROLE OF ARGINASE AND FIBROBLAST GROWTH FACTOR IN HUMAN VISCERAL LEISHMANIASIS)

PUBLICACÃO COLABORATIVA UFMS (MS, BRASIL) - LIPMED (FIOCRUZ, RJ, BRAZIL)- UTMB (GALVESTON,TX,USA)

Arginase-1 e Arginase-2 são moduladas diferencialmente na estimulação do sangue total de doadores sadios.

Com o fim de estabelecer a participação dos fatores de crescimento na regulação da Arg-1 humana, foram realizados experimentos de estimulação *in vitro*. Encontrou-se que a IL-4 *per se* não aumentou a expressão da Arg-1 nos cultivos de sangue periférico de humanos sadios infectados (n=4). Mas, observou-se um incremento na expressão de Arg-1 pela combinação da infeção, e IL-4 com os fatores de crescimento FGF-2 e IGF (Figura 1A).

A isoforma de arginase expressa nestas condições foi variável, Arg-1 foi induzida em 3 de 4 doadores, enquanto Arg-2 foi induzida em 1 de 4 doadores (Figura 1B). A expressão da Arg-2 foi associada com a expressão do oxido nítrico (ON) do doador, enquanto os que expressaram Arg-1 não expressaram ON (Figura 1B). A expressão de Arg-2 por monócitos humanos foi comprovada mediante a estimulação de celulas monociticas THP1 estimuladas com *L.donovani* e FGF-2 (Figura 1C). Este conjunto de resultados sugere que Arg-1 e Arg-2 estão sendo reguladas diferencialmente na infecção com *L. donovani*, e implica Arg-2, mas não Arg-1 associada com a produção do oxido nítrico.

Arginase-1 não foi modulada diferencialmente no plasma de pacientes com leishmaniose visceral em diferentes fases evolutivas da doença.

Os níveis plasmáticos da Arg-1 não foram modulados positivamente nas crianças com LV durante a doença ativa, quando comparados com os níveis de doadores sadios da mesma área endêmica (Figura 2A). Os níveis da Arg-1 nos pacientes foram variáveis, por em foi feita uma comparação dos níveis plasmáticos no mesmo paciente ao longo do tempo de evolução após do tratamento. O Analise dos níveis plasmáticos da Arg-1 na doença ativa e após

do tratamento, não indicaram uma mudança significativa nos níveis de Arg-1 quando comparados aos tempos de 1 mês, 6 meses e 1 ano após tratamento (Figura 2B). O análise da resposta individual, não permitiu identificar um padrão de resposta 1 mês após do tratamento, pois 9 pacientes apresentaram aumento enquanto outros 8 evoluíram com diminuição de Arg-1 nestas condições (Figura 2C).

O aumento nos níveis plasmáticos do fator de crescimento do fibroblasto associou-se com o período de recuperação 1 ano após o tratamento.

Na tentativa de estabelecer uma relação de causalidade entre o FGF-2 e a patogênese da LV, foram comparados os níveis plasmáticos de doadores sadios e de pacientes com doença ativa. Não foi observada um aumento significativo no FGF-2 do plasma dos pacientes com LV ativa (Figura 3A). Mais ainda, o seguimento dos níveis do FGF-2 1 mês, 6 meses e 1 ano após tratamento mostraram que houve tendência ao aumento do FGF-2 no plasma dos pacientes analisados 1 ano após o término do tratamento (Figura 3B). Os níveis individuais calculados como diferenças dos valores observados na doença ativa e 1 mês após do tratamento, mostraram padrões de variação bastante diversos (Figura 3C), havendo um aumento em 8 dos casos testados. Correlações dos níveis de Arg-1 e FGF-2 em cada paciente ao longo do tempo, não mostraram associação nas distintas fases (doença ativa, 1 mês e 6 meses após tratamento). Mas ainda, em contrário ao esperado, a Arg-1 e o FGF-2 se correlacionaram negativamente no tempo de 1 ano após o tratamento ($R2=0.7$, $p=0.05$, Figura 3D), indicando o balanço da ação anti-inflamatória da arginase e inflamatória do FGF-2 nos processos de reparação tecidual.

PONTOS DA DISCUSSÃO.

O resultado sugere que Arg-1 e Arg-2 pode ser modulado por *L. donovani*, onde a expressão da a expressão da Arg-1 é compatível com a resposta susceptível tipo th2 e a expressão de Arg-2 e compatível com resposta protetora tipo Th1 e produtora de Oxido nitrico. Não obstante, é necessário fazer uma identificação precisa das células produtoras da Arg1 o Arg2, com respeito ao fenótipo, capacidade de produzir oxido nítrico e

capacidade anti-leishmania. Isto permitirá estabelecer se existe uma resposta diferencial de Arg-1 e Arg-2 dependendo da susceptibilidade o resistência do hospedeiro.

A regulação positiva de Arg-1 mediante STAT-6/IL-4 e fatores de crescimento encontrada no modelo hámster (Osorio et al., 2014, aceito) também ocorreu numa proporção dos doadores sadios, implicando que existe a mesma via de regulação na LV humana.

Os níveis plasmáticos de Arg-1 e FGF-2 em crianças com LV não implicaram estes fatores como marcadores da doença ativa quando dosados neste compartimento.

O nível plasmático de FGF-2 e Arg-1 estiveram inversamente correlacionados 1 ano após do tratamento, quando existe uma maior recuperação da resposta imune.

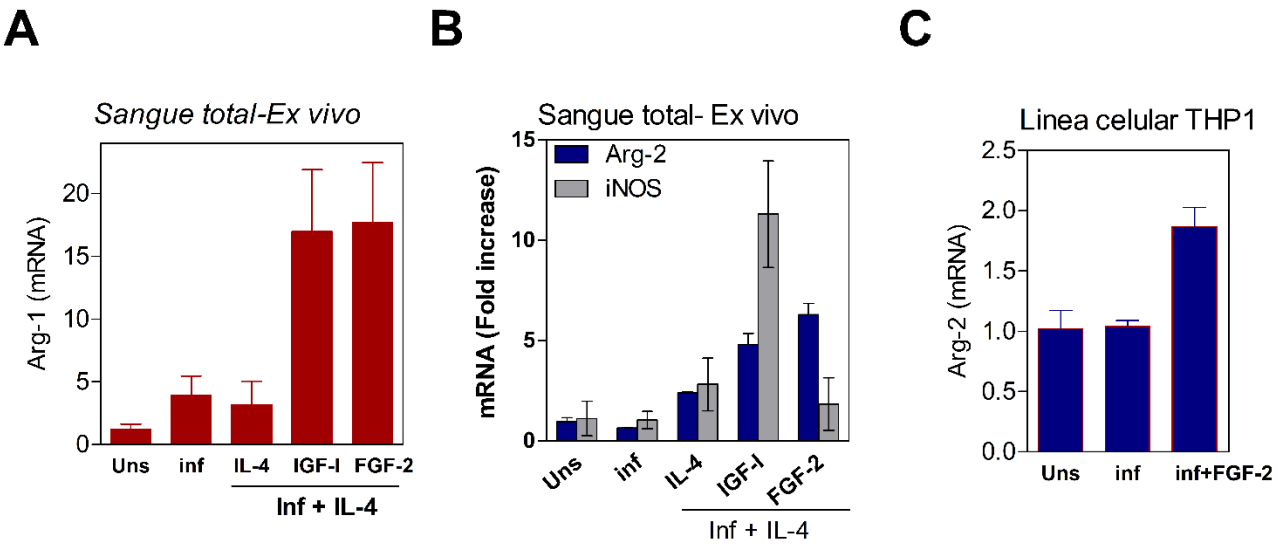


Figura 1. Arginase (Arg)-1 e Arg-2 expressas em células humanas infectadas com *Leishmania donovani*. (A, B) Expressão de Arg-1 e Arg-2 por células de sangue periférico de humanos sadios estimuladas com promastigotas de *L. donovani*. Os doadores que expressaram Arg-1 (A) não expressaram Arg-2 (B) nem oxido nitrico sintetase induzível (iNOS) nas condições de estimulação (infecção com *L. donovani*, e estimulação simultânea com a interleucina (IL)-4 e fatores de crescimento IGF-I (fator de crescimento semelhante a insulina) ou FGF-2 (fator de crescimento de fibrobroblastos), comparada com células sem estimulo (Uns).(C) Arg-2 no Linhagem celular THP1 após 24h de estimulação (infecção com *L. donovani*, e estimulação simultânea com FGF-2. O RNA foi extraído (Tempus RNA isolation reagent kit, ife Technologies), transcrito reversamente (High capacity reverse transcription kit, Applied Biosystems)e amplificado com *primers* e sondas comerciais (Arg-1 humana, ID Hs00968978_m9; Arg-2 humana, ID Hs00265750_m1; iNOS humano, ID Hs01075521_m1; Taqman MGB probes, Applied Biosystems). A expressão relativa do gene foi estimada mediante o método delta delta CT.

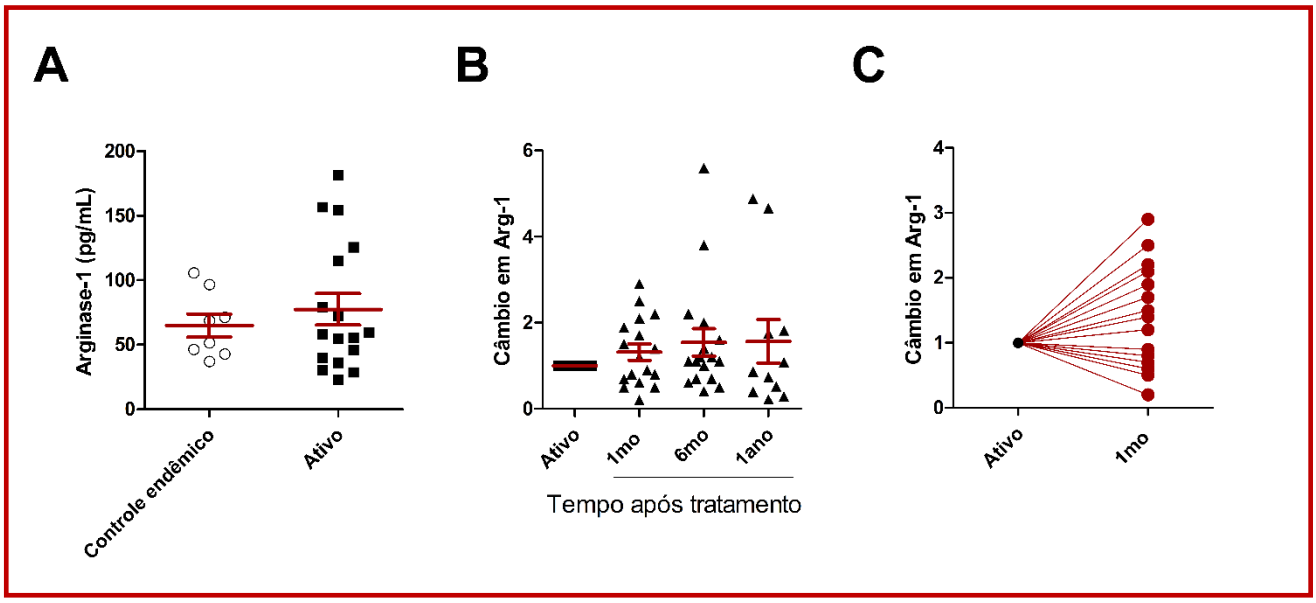


Figura 2. O nível plasmático de arginase (Arg)-1 não é um marcador de doença ativa nem de controle de remissão clínica. (A) Arg-1 no plasma de crianças com LV (n=17) comparadas com controles sadios da mesma região (n=8). (B) Avaliação dos níveis de Arg-1 por individuo após tratamento não indicou aumento ou diminuição significativa ao longo da resolução da doença (1 mes até 1 ano Após do tratamento). Variação = níveis no tempo avaliado – níveis antes do tratamento; (C) A discriminação individual do cambio nos níveis plasmáticos de Arg-1 na doença ativa comparada com os níveis 1 mês após o tratamento não obedeceu um padrão de resposta. Crianças com LV, provenientes do Estado do Matogrosso do Sul (MS), Brazil. As amostras de plasma foram testadas pelo ensaio da ELISA com o kit comercial Arg-1 (Arginase I, Human, ELISA kit, Hycult Biotech). A concentração plasmática foi calculada com referência à curva padrão em pg/mL (limite mínimo de detecção 1pg/mL).

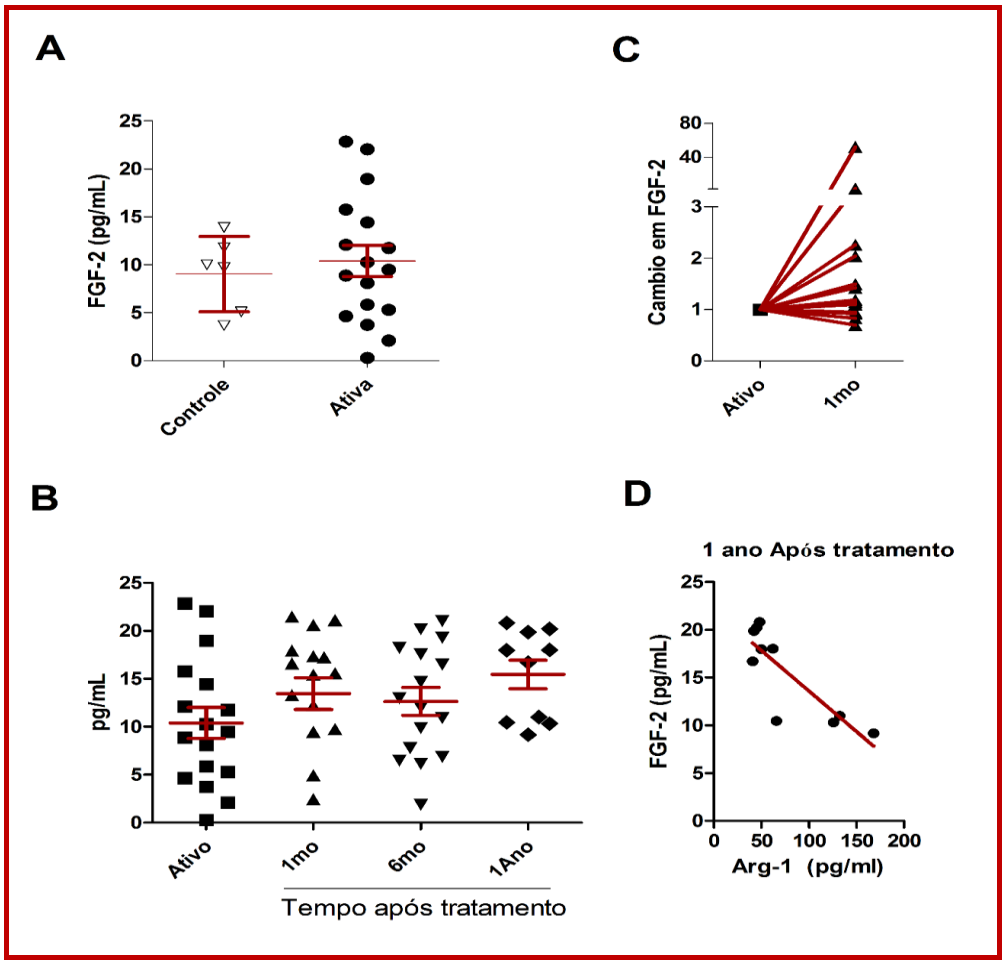


Figura 3. Os níveis plasmáticos de fator de crescimento de fibroblasto(FGF)-2 não estão associados com doença ativa, mas pode influenciar a reparação do tecido 1 ano após o tratamento. (A) Os níveis plasmáticos de FGF-2 em crianças com LV ativa (n=17) são similares aos níveis em crianças sadias da mesma área endêmica (n=6). (B) A evolução nos níveis plasmáticos de FGF-2 após do tratamento das crianças, não mostrou cambio significativo ao longo da resolução após tratamento (1mes até 1 ano Após do tratamento). (C) A avaliação dos níveis de FGF-2 por individuo antes do tratamento o 1 mês após tratamento não indicou um padrão de resposta. Cambio= níveis 1 mês após tratamento – níveis antes do tratamento. (D) A Correlação dos níveis de FGF-2 e Arg-1 1 ano Após do tratamento foi significativa e inversa (n=9 crianças). As amostras de plasma foram testadas pelo ensaio da ELISA com o kit comercial (Human FGF basic Quantikine HS ELISA Kit, R&D SYSTEMS, INC). A concentração plasmática foi calculada com referência à curva padrão em pg/mL (limite de detecção 0.3 pg/mL).

4. DISCUSSÃO E PERSPECTIVAS

4.1. Efeitos da ativação da arginase-1 e do STAT-6 na leishmaniose visceral

Os resultados apresentados nesta tese identificaram que hamsters com LV apresentaram um programa de ativação de macrófagos alternativo. Este fenótipo caracterizou-se pela expressão dominante da Arg-1 sobre o óxido nítrico em tecido de baço do hamster e nos macrófagos infectados *com L. donovani*. O aumento da atividade enzimática e a transcrição de Arg1 ocorreram em paralelo ao aumento da carga parasitária visceral. A importância de poliaminas no crescimento parasito é enfatizada pelo seu papel crítico no crescimento já descrito em outros protozoários, incluindo *Trypanosoma brucei* e *T. cruzi* (Stempin *et al*. 2010) e está de acordo com o que foi observado no modelo murino de infecção de *L. major* (Iniesta *et al*. 2005).

Neste trabalho demonstrou-se que a infecção por *L. donovani* ocasiona a expressão da Arg-1 através de um mecanismo que envolve a ativação STAT6. Mas essa ativação difere do paradigma conhecido, no qual a ativação alternativa é dependente da resposta de citocinas Th2 (Iniesta *et al*.2002; Kropf *et al*. 2004). Foi demonstrado que a infecção pelo parasito *L. donovani* poderia ativar o STAT-6 e dirigir a transcrição da Arg-1 na célula hospede. A importância crítica da via STAT6-Arg1 induzida pelo parasito em células infectadas foi demonstrada pelo fato de fazer parte do controle da infecção e da Arg-1 após a interferência da transcripção do STAT6. Demostrou-se uma redução de 92% na expressão de RNAm de Arg1, e de 45% da carga parasitária, quando a transcrição do STAT6 foi eliminada, em comparação com o controle em células BHK na ausência da citocina IL-4 e IL-13.

No entanto, a expressão dominante da Arg-1 no hamster com LV progressiva sugeriu que IL-4, IL-10, IL-13 ou IL-21, ou uma combinação dessas citocinas, contribuíram para a amplificação da expressão de Arg1 na fase crônica da doença. Recentemente, foi descrita uma sinergia entre STAT6/IL-4 e IL-10/STAT3 no modelo murino de infecção de *L. donovani* (Biswas *et al*. 2011), a qual sugere que outras citocinas e a IL-10 poderiam aumentar a expressão de Arg1-STAT6 no modelo de hamster.

O STAT6 pode ser ativado por outros estímulos, tais como: IL-15, fator de crescimento derivado de plaquetas, ligante kit e leptina, mas a via canônica para ativação deste fator de transcrição é através de IL-4 ou IL-13. O fato do aumento de citocinas Th2 coincidente com, mas não precedente, ao aumento dramático na carga parasitária e a atividade da Arg-1 aos 28 dias de infecção, indicaram que fatores independentes de citocinas podem dirigir a indução inicial do STAT-6 e de Arg-1 na LV. Portanto, fatores adicionais que poderiam ativar STAT-6 foram estudados na segunda parte da tese. Os resultados preliminares deste trabalho sugeriram que a expressão da Arg-1 em hamsters com LV crônica foi impedida por inibidores químicos dos RTK. Então, a ativação destes receptores e o seu efeito no STAT-6 foram estudados com a finalidade de entender a interação da via de citocinas e da via RTK na ativação do STAT-6 e na regulação da Arg-1 na LV.

Os resultados desta tese indicaram que, na infecção com *L. donovani,* a ativação do FGFR e IGF-1R apresentavam um efeito adicional na expressão de Arg-1 e de STAT-6, quando os macrófagos estavam expostos à IL-4. É sabido que a ativação

do macrófago mediada por IL-4/STAT-6 leva à ativação do macrófago pela via alternativa (Sica & Mantovani 2012), porém é factível que o efeito adicional na ativação do STAT-6, mediado por RTK, esteja contribuindo com a diferenciação para o fenótipo M2. Com o fim de apoiar esta hipótese, foram feitos testes de inibição química dos receptores. Encontramos uma diminuição da Arg-1 e da carga parasitária em macrófagos tratados com inibidores químicos dos RTK (FGFR-1 e IGF-IR) e em macrófagos de hamster infectados *in vitro* com *L. donovani* ou provenientes dos animais com a doença crônica. Embora, este estudo não tenha determinado se esta diminuição foi acompanhada por a elevação de iNOs, ele sugere que a diminuição na Arg-1 pode estar desviando o metabolismo para uma maior capacidade de produzir moléculas efetoras anti-leishmânia a partir do substrato comum L-arginina, e inibindo a produção de poliaminas que promovem o crescimento intracelular de *Leishmania* (Iniesta *et al*. 2001, 2002, 2005).

Por outro lado o encontro das isoformas de Arginasa (Arg-1 e Arg-2) no sangue periférico humana estimulada com *L. donovani,* IL-4 e fatores de crescimento, indicou que as duas isoformas podem exercer papéis regulatórios na infecção *in vitro*. A complexidade deste sistema aumenta com a infecção, pois a arginase do parasita tem sido demostrada como componente importante no modelo camundongo (Gomes *et al*., 1997). Embora nossos resultados indicaram que a expressão da arginase do hospede, Arg-1, predominou sobre a Arg-2 e a Arginase do parasita no baco de hamsteres com doença crônica (Osorio *et al*., 2012). Sabe-se que a indução de arginase tem um efeito temporal inibitório na produção do iNOS, direcionado pela

expressão de citocinas Th1 ou Th2 (Iniesta *et al*. 2001, 2002). Este fato contrasta com nossos resultados que mostraram que a Arg-2 foi associada à expressão de iNOS no humano. Embora os resultados sejam similares a de outros autores que indicam a expressão simultânea de iNOs e arginase *in vivo* e *in vitro*, foi relacionada à redução do estimulo do LPS, e de endotoxemia (Bansal & Ochoa 2003). Estes mecanismos de regulação das isoformas de arginases com relação ao iNOs podem ser relevantes no manejo da LV, pois estudos mostraram uma associação do estimulo bacteriano com a gravidade da doença (Santos-Oliveira *et al*. 2011, Santos-Oliveira & Da-Cruz 2012).

A arginase expressa no mesmo ambiente do iNOS implica também na possibilidade de coexistência dos fenótipos M1 e M2 no ambiente infeccioso e inflamatório da LV, como esta sendo sugerido por informação preliminar no modelo hamster (Saldarriaga *et al*. manuscrito em preparação), mostrando que existe uma dinâmica dos fenótipos do macrófago M1 e M2 na infecção.

Os efeitos da arginase sob o sistema imune são conhecidos. A depleção da L-arginina do meio de cultura inibe a resposta celular mediante a degradação da L-arginina (Chen & Broome 1980), que leva à inibição da cadeia ξ do receptor TCR e à subsequente perda da transdução de sinais do linfócito (Rodriguez *et al*. 2006, Zea *et al*. 2005). Um estudo recente demostrou este fenômeno na LV, pois a cadeia ξCD3 estava significativamente inibida nos linfócitos de pacientes com doença ativa (Abebe *et al*. 2013). Outros estudos sugerem que o IGF-I também participa na supressão da resposta imune neonatal mediante o apagamento da resposta do IFN-

ɣ nas células estimuladas com PMA e Ionomicina (Puzik *et al*. 2012). Estas informações suportam a proposta de que indução de Arg-1 nos humanos e nos hamsters infectados com *Leishmania spp.* participam na imunossupressão associada à LV.

Além disso, fica por ser determinado se as MDSC humanas, que expressam $CD13^+$, $CD33^+$, HLA-DR e variavelmente expressam marcadores de monócitos (CD14) ou do granulócito (CD15) tem uma participação na depressão da resposta imune na LV. É sabido que a função imunossupressora das células mielóides supressoras de camundongo está aumentada frente a IL-4 (Bronte *et al*. 2003), sugerindo uma possível relação direta ou indireta com a via de sinalização dos RTK. O conjunto de resultados anteriores sugere que a arginase na LV humana exerce um papel potencial na inativação do neutrófilo e do macrófago, com possíveis implicações no aumento da susceptibilidade ao parasito e imunossupressão.

Quando esta tese foi proposta não se conhecia se a Arg-1 tinha expressão aumentada nos humanos como consequência da leishmaniose visceral. A arginase humana é detectada nas células mononucleares do sangue periférico de pacientes com tuberculose (Ochoa *et al*. 2001; Zea *et al*. 2006), e em macrófagos inflamatórios de pacientes com artrites entre outros (Corraliza & Moncada 2002). Embora os estudos indiquem que macrófagos humanos ativados pela via alternativa não expressam Arg-1 como marcador (Martinez *et al*. 2009), sabe-se que a Arg-1 é expressa constitutivamente nos neutrófilos de sangue periférico de doadores sadios (Munder *et al*. 2005). No único estudo sobre o tema publicado no ano passado na

LV humana, os neutrófilos foram identificados como a fonte de Arg-1 (Abebe *et al*. 2013). Por conseguinte, até agora não é claro se a produção de arginase é relevante no macrófago humano na LV. A demonstração de que os neutrófilos são fonte de Arg-1 é relevante, pois sabe-se que eles fagocitam a Leishmânia e servem como reservatório seguros para a sobrevida do parasito (Peters *et al*. 2008). O neutrófilo infectado é logo fagocitado pelo macrófago, que não reconhece o parasito dentro do neutrófilo, e assim é estabelecida uma infecção silenciosa (Ribeiro-Gomes & Sacks 2012). Os mecanismos de subversão do neutrófilo pelo parasito estão relacionados com a inibição da fusão do lisossoma, a explosão respiratória, a produção de aníon superóxido e de outras moléculas secretadas pela leishmânia que inativam o macrófago, assim, é possível supor que a arginase expressa pelo neutrófilo esteja cooperando para o prejuízo da ação anti-leishmânia, tanto do neutrófilo portador quanto do macrófago que o fagocita. Por outro lado, demonstrou-se que macrófagos humanos infectados com *L. chagasi* (atual *L. infantum*), produziram IFN-ɣ, e que essa produção foi inibida drasticamente quando inibiu-se a produção de óxido nítrico com o l-NMMA (Gantt *et al*. 2001). Esta informação sugere que o sistema contra regulatório da arginase/óxido nítrico potencialmente ocorre no controle de Leishmânia na infecção humana.

Outra possibilidade que ilustra a relevância da Arg-1 em humanos, é que esta enzima também é expressa por células mielóides imaturas que tem ação imunossupressora, conhecida como células mielóides supressoras (MDSC). A relevância desta possibilidade fica mais marcante pelo fato de que o 98% das

células identificadas como produtoras de Arg-1 nos pacientes com LV tinham o fenótipo $CD15^{+}$ e foram identificadas como neutrófilos (Abebe *et al*. 2013). Entretanto, existem questionamentos quanto a estes resultados, já que os neutrófilos foram purificados da fração mononuclear, e argumentaram que estas células eram granulócitos que foram classificados como neutrófilos de baixa densidade (Abebe *et al*. 2013). O fenótipo dessas células merece ser melhor caracterizado, pois se sabe que as MDSC também expressam CD15 e Arg-1 em condições patológicas (Khaled *et al*. 2014). Esta possibilidade não tem sido explorada na LV. Na leishmaniose cutânea as MDSC produziram imunossupressão da resposta de linfócitos, mas estiveram envolvidas no controle parasitário mediado pelo óxido nítrico (Pereira *et al*. 2011). De fato, o novo conceito emergente considera que as células mielóides que respondem aos ambientes inflamatórios infecciosos estão em diversos estados transitórios de maturação (Schmid *et al*. 2012), o que dificulta o estabelecimento claro dos fenótipos.

Considerando que a indução de Arg-1 nos pacientes com LV já foi demonstrada (Abebe *et al*. 2013), sugere-se que a estimação do nível plasmático de Arg-1 realizado nesta tese provavelmente não reflete a atividade intracelular desta enzima. O conjunto de resultados acima indicam que Arg-1 e Arg-2 podem ser induzidas na LV humana, e que apesar do pequeno número de doadores avaliados (n=4), esses respondem diferencialmente à infecção dependendo do estímulo. Assim, uma proporção de indivíduos expressou Arg-1 sem óxido nítrico (3/4) e outra expressou Arg-2 acompanhada de óxido nítrico (1/4), como resposta ao estímulo combinado de

exposição ao parasito, IL-4 e fator de crescimento. Entretanto, a definição do fenótipo das células envolvidas na regulação de cada isoforma de arginase e a definição de sua contribuição para a susceptibilidade ou a resistência ao parasito, ainda requer estudos adicionais que, no entanto, estão fora do propósito desta tese.

4.2. Efeitos da ativação dos receptores de tirosino quinases (RTK) na leishmaniose visceral

Este é o primeiro estudo que avaliou a possível ativação dos receptores RTK na LV. Encontramos que o FGFR-1 e o seu ligante FGF-2 estavam aumentados em macrófagos esplênicos de hamster na fase crônica da infecção com *L. donovani*. Interessantemente, nem a expressão do RNAm do FGF-2 nem o FGFR-1 (quantificados por qPCR) tiveram um aumento significativo ao nível transcricional (não mostrado). Isto sugere que mecanismos de regulação pós-transcricional induzidos pela infecção estão controlando o aumento da fosforilação do receptor e a estabilidade do FGF-2. É conhecido que a estabilidade do RNAm do FGF-2 é controlada por outras proteínas que são expressas de uma forma restrita e temporal (Touriol *et al*. 1999). No caso do FGF2, a regulação ocorre em 90% dos casos ao nível pós-transcricional, pois a maior parte do RNAm consiste de regiões não traduzíveis com códons de iniciação alternativos, que são controlados mediante a interação com outras proteínas. Estudos da regulação do FGF-2 em retinoblastos humanos encontraram que a quantidade de proteína aumentada não se associava com aumento do RNAm transcrito. Ao contrário, os níveis de RNAm foram

diminuídos, evidenciando um mecanismo de *feedback* negativo, mediado pelo fator induzível da hipóxia alfa (HIF-α) (Conte *et al*. 2008). Chama a atenção que o HIF é regulado positivamente nos macrófagos de hamster infectados com *L. donovani* (Saldarriaga *et al*. manuscrito em preparação), pelo que poderíamos especular que também estão envolvidos na regulação positiva do FGF-2, sem afetar os níveis dos transcritos. A fosforilação do IGF-IR encontrada neste estudo apoia outros estudos que encontraram uma relação do IGF-1 com a susceptibilidade na infecção com *Leishmania spp.* e o favorecimento do crescimento do parasito (Gomes *et al*. 2000, Vendrame 2007, 2010).

A fosforilação do IGFR na infecção foi confirmada em macrófagos infectados *in vitro* com *L. donovani*, mas ela não foi acompanhada por aumento de IGF no baço, nem no soro ou no plasma. Uma explicação possível é que moléculas homólogas ao IGF-1 estejam induzindo a fosforilação do receptor. Assim, estudos mostraram que *Leishmania spp.* elicita a fosforilação de resíduos de tirosina nos macrófagos (Martini *et al*. 1996). As proteínas regulatórias do IGF-1 (IGFBP), principalmente o IGFBP-3, que regula o 90% da IGF, podem sequestrar o IGF, regulando a sua biodisponibilidade, vida média e distribuição (Yu & Rohan 2000). Por exemplo, os IGFBP podem concentrar espacialmente o IGF no receptor, aumentando a sua atividade ou, também podem sequestrá-lo e inibi-lo (Firth *et al*. 2002). Também existe uma produção autócrina do IGF, que não envolve a liberação parácrina nem endócrina do fator de crescimento, fenômeno que já foi observado nos macrófagos de camundongo infectado com *L. major* (Reis *et al*. 2013). Interessantemente,

estudos do mesmo grupo encontraram que os níveis de IGF-I no soro de cães com LV estiveram diminuídos comparados com os de cães controle sadios, enquanto os níveis hepáticos de RNAm do IGF-I foram maiores nos cães infectados (Pinho *et al*. 2013). Mostrando, mais uma vez, a falta de correlação entre o RNAm e a produção da proteína, e a diferença nas avaliações locais comparadas com as sistêmicas. Este fenômeno resulta em maior complexidade quando existem outros mecanismos regulatórios que impactam no destino final do transcrito. Apesar do RNAm de IGF-I não terem sido analisados neste estudo, os resultados sugerem a relevância da ativação e da sinalização do IGFR na patogênese da LV. O resultado indica que existe uma regulação diferencial dos fatores de crescimento ao nível sistêmico e local, onde são exercidos mecanismos de controle ao nível transcricional e pós-transcricional.

Por outro lado, o receptor do PDGF-β (PDGF-βR) encontrou-se regulado negativamente na infecção, e os níveis do ligante PDGF-β não apresentaram diferenças significativas no baço, no plasma ou no soro dos animais infectados. Interessantemente, a liberação de PDGF-β pelas plaquetas no sítio de infecção induz o recrutamento de monócitos inflamatórios (Gr-1+) do sangue periférico de camundongos C57BL/6 infectados com *L. major* que tem a como função eliminar o parasito (Gonçalves *et al*. 2011). Isto sugere que a inativação do PDGFR observado nos macrófagos dos hamsters na etapa crônica contribui para o incremento da carga parasitária.

Quanto ao receptor do EGFR, não encontramos evidências de fosforilação, nem aumento no ligante EGF ou outros ligantes que o ativam este receptor (HB-EGF, Epiregulin, Amphiregulin, TGF). Embora o anti-EGFR utilizado no teste da imunoprecipitação é específico para o hamster (Figure 2, suplementar) não podemos descartar a falta de reação cruzada dos anticorpos fosforilados anti-EGFR utilizados no Western blot subsequente. Também é possível que o ensaio não seja o suficientemente sensível para detectar estas proteínas fosforiladas ou detectar os ligantes do EGFR, pois são expressas em níveis muito baixos. Por conseguinte, a verificação do papel do EGFR na LV experimental dependerá da disponibilização futura de anticorpos fosforilados específicos para o hamster ou de métodos mais sensíveis que possam ser usados nesta espécie animal. Esclarecer o papel do EGFR torna-se importante uma vez que este receptor exerce uma regulação cruzada que envolve a interação, independente do ligante, com outros receptores RTK, como o PDGFR e IGF1R, chamada transativacão do EGFR (Berasain *et al*. 2011). Por exemplo, quando o EGFR é ativado pode se unir fisicamente ao IGF-IR e regular a expressão das proteínas (IGFBP) que ligam e regulam a biodisponibilidade do IGF-I (van der Veeken *et al*. 2009).

Este estudo é o primeiro a mostrar que os receptores RTK ativaram STAT-6 e induziram Arg-1 na LV experimental. A fosforilação direta do STAT-6 foi evidente rapidamente (20 min) após a infecção parasitária e estimulação com os fatores de crescimento. Esta fosforilação pode ser ocasionada mediante a ação de tirosina quinase nos receptores FGFR-1 após o ligamento do FGF-2. As JAK quinases são

então recrutadas pelo receptor e fosforilam as STATs, os quais formam dímeros que são translocados ao núcleo, ligam-se ao DNA e iniciam a transcrição (Krejci *et al*. 2009). A verificação da dinâmica de fosforilações prévias e seguintes à ativação do STAT-6 não foi estudada nesta tese, pois as metodologias necessárias para esses, tais como co-imunoprecipitação-Western blot, ensaios de ocupação do promotor e imunoprecipitação de cromatina-PCR (Chip-PCR) não foram inlcuídas na proposta original do projeto de tese.

Nossos resultados indicaram que, apesar o STAT-6 ser fosforilado rapidamente pela ação dos fatores de crescimento, foi necessário um tempo de incubação para o início da transcrição da Arg-1, que ocorreu após 16h. O retardo na expressão de Arg-1 já foi reportado (Osorio *et al*. 2012) e pode ser explicado pelo requerimento de cofatores e modificações pós-transcricionais que são necessárias para o início da transcrição do gene (Pourcet *et al*. 2013). Por exemplo, a ubiquitinação ou deacetilação do C/EBP-β (Serrat *et al*. 2012, Ye *et al*. 2012). Também é possivel que seja necessária à transcrição de outros componentes que amplifiquem a fosforilação do STAT-6, superando os efeitos de controle das fosfatasses como o SHP-1, que regulam negativamente a fosforilação dos resíduos de tirosina do STAT-6 (Hanson *et al*. 2003).

No estudo dos fatores que potencialmente contribuem para a ativação do STAT-6 na LV, encontramos aumento do receptor da IL-13 no baço dos animais infectados e em macrófagos tratados com IGF-I. É conhecido que a cadeia alfa do receptor da IL-13 (IL-13Rα1) associa-se com a cadeia alfa do receptor da IL-4 (IL-4rα), e ativa o

STAT-6 (Roy *et al*. 2002), porém, concluímos que a IL-13 e o receptor da IL-13 podem contribuir para a ativação do STAT-6 mediada pela infecção e os RTK na LV. Por outro lado, a infecção com *L. donovani* ativou PI3K/IRS/ AKT, que regula positivamente a IL-10 (Ruhland *et al*. 2007, Cheekatla *et al*. 2012,), e a Arg-1, também pode contribuir, mediante a indução da expressão do receptor da IL-4 (Biswas *et al*. 2011).

O STAT-3 ativado na infecção com *Leishmania spp.* faz parte da via de ativação canônica do FGFR e IL-10 (Bohrer *et al*. 2014). Embora nossos resultados tenham mostrado que IL-10 regula positivamente Arg-1 mediada pelos fatores de crescimento, o efeito não foi verificado no nível da proteína, sugerindo que a combinação IL-10 e fator de crescimento foram regulados negativamente no nível pós-transcricional. Apesar disso, observou-se um efeito de amplificação na Arg-1 quando a IL-4 foi adicionada a essa mistura de estímulos (Osorio *et al*. não publicado). Este resultado indicou que na infecção a combinação de citocinas IL-4 + IL-10 e fator de crescimento resultoram na amplificação máxima da Arg-1 na LV. Este efeito da máxima indução da Arg-1 na combinação de IL-4 e IL-10 na LV já foi reportado anteriormente (Biswas *et al*. 2011) e indica a importância das citosinas Th2 na regulação positiva da Arg-1.

O STAT-6 foi necessário para a expressão da Arg-1 mediada por o FGF-2, conforme demonstrado nos ensaios de inibição da expressão do STAT-6 mediante RNA de interferência. Isto indicou que a expressão da Arg-1 mediada pelo FGF-2 dependia dos mecanismos induzidos por STAT-6. Por outro lado, observou-se uma

dependência parcial mediada pelo IGF-I, sugerindo que outros mecanismos independentes do STAT-6 colaboram na indução da Arg-1 mediada pelos RTK na LV. Entre os candidatos plausíveis, podemos considerar os fatores c-Fos/c-Jun. Estes fatores de transcrição são ativados mediante os receptores tirosina quinase Ron (RonRTK) em macrófagos de camundongos C57BL/6 (Sharda *et al*. 2011). Eles demonstraram que a proteína estimuladora do macrófago (macrophage stimulating protein, MSP), ligante do receptor Ron, ativou a via das MAPK e Fos estimulando a ligação ao sítio de união ao promotor da Arg-1 (Sharda *et al*. 2011). Os receptores PPARɣ também podem induzir Arg-1 independente do STAT-6, assim, a atividade de Arg-1 estava reduzida em macrófagos de camundongo nulos para o gene PPARɣ quando estimulados com IL-4. Camundongos com macrófagos deletados do mesmo gene (Mac-PPARɣ KO) desenvolveram uma lesão menor que os controles, quando infectados com *L. major* (Odegaard *et al*. 2007). Pauleau (2004) mostrou que IL-10, IL-4, G-CSF, ativam o STAT-3 e ele ativa o fator de transcrição PU.1, que une-se diretamente ao promotor da Arg-1. O conjunto de resultados sugere que a ativação do gene da Arg-1, mediado pelos receptores RTK na infecção com *Leishmania* spp requereu mecanismos dependentes do STAT-6, embora mecanismos independentes também sejam relevantes na regulação do gene.

Os resultados desta tese indicaram que Arg-1 e Arg-2 estão expressos no RNA das células purificadas de sangue total de humanos sadios, estimuladas simultaneamente com promastigotas de *L. donovani*, IL-4 e o fator de crescimento

FGF-2 ou IGF-I. Além disso, a linhagem celular monocítica THP1, expressou Arg-2 nas condições de cultivo com *L. donovani* e estimulação com o FGF-2. A Arg-1 também foi detectada no plasma dos pacientes com LV pela *L. infantum*, embora não estivesse aumentada em relação aos voluntários sadios usados como controle, nem manifestou aumentos ou diminuição significativos nos níveis plasmáticos durante a resolução da doença pós tratamento (1 mês a 1 ano após tratamento). Em consequência, os resultados da estimulação *in vitro* com splenócitos de hamsters infectados e sangue total do humano diferiram dos achados no plasma dos pacientes. Embora Arg-1 e FGF-2 não se encontrem ativados no plasma na doença ativa nos pacientes com VL, os resultados experimentais indicaram que a interação da infecção com a via canônica da IL-4, e os fatores de crescimento regularam positivamente Arginase na LV.

Além da ativação do STAT-6, a sinalização dos RTK gera a transcrição de genes relacionada com o crescimento, diferenciação e proteção da apoptose nos macrófagos. Os resultados anteciparam então que existe uma via de causalidade na ativação dos RTK com o aumento nos macrófagos no baço e no fígado na leishmaniose visceral experimental e na humana (Osorio *et al*. 2012; Kaye *et al*. 2004). A investigação desta possibilidade não foi contemplada nos objetivos iniciais desta tese. No entanto, a ideia está sendo explorada e os resultados preliminares a este respeito já fizeram parte de dois resumos apresentados em congresso. Neles estudamos o papel potencial da ativação da IL-4, o STAT-6 e fatores de crescimento no aumento do número de precursores mielóides no baço de hamsters com LV

(Osorio *et al*. manuscrito em preparação). Parece contraditório um processo de incremento de mielopoiese local, pois a LV grave se manifesta com uma depressão geral das celularidade ao nível da medula óssea, timo e do sangue (Kaye *et al*. 2004). Um exemplo de mielopoiese extramedular ocorre na infecção experimental de camundongos com *Schistosoma mansoni*. O baço e fígado dos animais crescem em tamanho no curso da infecção, acompanhados de um aumento localizado de granulomas formadores de colônias mielóides, que tornam-se gradualmente maiores que o da medula óssea (Van Ginderachter *et al*. 2010). A proliferação local dos monócitos humanos já foi observada na leishmaniose. A infecção de monócitos de $CD14^{+}$ purificados de sangue periférico infectados com *L. major* induziu a maturação e aumento no número de macrófagos como o tempo (12-14 dias), comparado com células controle não infectadas (Mock *et al*. 2012).

Outras observações implicam os fatores de crescimento na elevação do número de células mielóides. O baço é um dos órgãos linfóides que possui células pluripotentes (*Stem Cells*), e o FGF-2 pode atuar em conjunto com o fator de crescimento estromal (SCF, *stem cell growth factor*), e citocinas levando a produção de células progenitoras das linhagens mielóides (Gabrilove *et al*. 1994, Itkin *et al*. 2012). O IGF-1 participa no desenvolvimento e expansão de células hematopoiéticas, induz a formação de colônias granulócito-monocíticas e protege da apoptose (Kurmasheva & Houghton 2006). O IGF-I sinergiza com a IL-4, elevando a síntese de DNA em progenitores hematopoiéticos num processo que aumenta a fosforilação dos resíduos de tirosina na cascata das MAPK (ERK-2) e o STAT-6 (Kurmasheva &

Houghton 2006). Os mecanismos descritos em outras doenças parasitárias onde há estimulo à proliferação local dos macrófagos, estão associados com o ambiente Th2 e a IL-4. Camundongos infectados com o nematódeo filária *Litomosoides sigmodontis* desenvolvem uma inflamação de tipo Th2 que leva à proliferação *in situ* de macrófagos no peritônio (Jenkins *et al*. 2011). O aumento da arginase em células aórticas altera a proliferação mediante mecanismos que envolvem o aumento na produção de poliaminas, sendo elas importantes no crescimento da célula que antecipa a divisão celular (Wei *et al*. 2001). Fatores de transcrição como o PU.1, KLF4, e STAT-6 interferem na ativação da transcrição da Arg-1 e estão envolvidos também na diferenciação e na divisão celular (Pourcet & Pineda-Torra 2013).

No estudo dos fenômenos que envolvem a divisão local, o modelo experimental camundongo infectado com o nematódeo *Heligmosomoides polygyrus bakeri* suporta a proliferação dos macrófagos nos sítios de produção de IL-4 mediante mecanismos que dependem parcialmente da sinalização do IL-4R. Os mecanismos independentes da IL-4 obedeceram ao aumento local do fator de crescimento CSF-1 que sinaliza através da via das MAPK e IRS (Jenkins *et al*. 2013). Estas observações vem nos conjecturar a relevância da sinalização dos RTK e IL-4 (dependente do STAT-6) como possível mecanismo implicado no aumento de macrófagos no baço na LV experimental e humana.

Embora os fatores citados acima mostrassem associação como o aumento das populações mielóides no baço, é possível que ao nível sistêmico ocorra o contrário,

e que estes fatores estejam envolvidos num mecanismo de *feedback* inibitório que ocasiona, como conseqüência, o detrimento das populações celulares na medula óssea e ao nível sistêmico, característico da LV. Por exemplo, o IGF-I exerce um papel regulatório negativo na liberação do hormônio de crescimento (GH) do hipotálamo e pituitária, através da inibição da transcrição da GH. Isto sucede mediante a desestabilização do complexo transcricional POU1F1/CBP, produzido pela fosforilação do CBP mediada pelo IGFR (Romero *et al*. 2012). É interessante especular que na LV ocorra um estado de ativação dos receptores RTK e a sua sinalização, o que poderia estabelecer um *feedback* regulatório negativo na produção de fatores de crescimento, que finalmente leva à depressão de populações sistêmicas. Estudos a este respeito, e a comparação de outros fatores de crescimento produzidos ao nível local e sistêmico poderiam trazer outra perspectiva sobre o papel destes fatores na LV humana. Estudos adicionais poderiam determinar se a ativação da IL-4 e os RTK são a causa ou consequência da "quebra" nas populações geradoras na medula e do aumento das células mielóides no baço.

O aumento dos níveis plasmáticos do FGF-2 nos pacientes com LV, 1 ano após o tratamento, sugeriu que este fator desempenha algum papel nos processos de regeneração tissular; embora o remodelamento do tecido, a acumulação de macrófagos e os depósitos de colágeno e fibroses no baço na LV experimental e humana indicaram a associação dos fatores de crescimento com fatores patológicos (Kaye *et al*. 2004). É contraditório que o aumento no nível plasmático de FGF-2 seja inversamente proporcional à Arg-1, no tempo de 1 ano após do tratamento dos

pacientes com LV, pois esta última também está envolvida no processo reparatório (Novak & Koh 2013). A regulação da resposta inflamatória mediada pela produção de ornitina favorece a produção de prolina, a regeneração celular e a cicatrização. Possivelmente a contribuição mais importante dos macrófagos ativados pela via alternativa durante a cicatrização envolva a expressão de fatores de crescimento, incluindo IGF-I, VEGF e TGF-ß, que diminuem o estado inflamatório (Jan & Nair 2013, Novak & Koh 2013). Também é possível que a diminuição sistêmica da arginase com relação ao FGF-2 excerça papel de evitar a ativação excessiva pela via alternativa dos macrófagos e a geração de fibrose. O fato que os resultados experimentais no modelo hamster associam o FGF-2 com patogênese da LV e no humano aos processos resolutivos, poderiam ser explicados pela diversidade de consequências funcionais destes fatores de crescimento, que podem exercer efeitos benéficos ou prejudiciais ao organismo, dependendo das condições do sistema imune em dado momento.

No caso do aumento do FGF-2 nos pacientes 1 ano após o tratamento se associar a processos reparatórios, implicaria em que seria necessário um longo período de tempo para ocorrer a resposta ao tratamento com reparação tecidual, o que seria bastante provável em se tratando de uma infecção que causa tantos distúrbios sistêmicos. Estudos sugerem que os processos de reparação tissular após tratamento de lesões de leishmaniose cutânea não correspondem com a cura clínica, pois ao nível histopatológico lesões cicatrizadas ainda apresentam algum grau de tecido com infiltrado inflamatório após tratamento (Viana *et al*. 2013). Este

tipo de informação não existe na leishmaniose visceral. Por outro lado, os níveis de IGF-I não foram analisados no presente trabalho, pois se conhece que este fator não é um marcador de doença ativa na leishmaniose visceral humana e que os níveis plasmáticos são controlados pelo fígado (Goto Hiro., Pessolani MC comunicação pessoal).

4.3. Inibidores de receptores de tirosina quinases no tratamento da leishmaniose visceral

A ativação do FGFR e IGF-IR encontrados neste estudo aumentaram a Arg-1, a carga parasitária e o potencialmente de gerar efeitos imunossupressores na LV. Porém, a inibição desses sinais surgem como uma possibilidade de estratégia terapêutico desejavel. De fato, nossos experimentos *ex vivo* com macrófagos esplênicos de hamsters cronicamente infectados demonstraram diminuição significativa na transcrição da arginase e da carga parasitária medida pelo efeito inibitório dos receptores. A terapêutica baseada na inibição dos receptores RTK tem tido muitas aplicações, em particular para o tratamento do câncer, que conta com inibidores potentes e de baixa toxicidade (Chaves *et al*. 2011). Ainda também a inibição dos receptores impacta na sinalização intracelular comum que é compartilhada com outros RTK. Por exemplo, quando o IGF-IR é inibido, evita processos de angiogênese tumoral ocasionada por outros receptores pois são inibidos o mTOR, fosfatidil inositol-3-quinase (PI3K) e AKT, que também sinalizam através do EGFR ou do VEGFR (Kurmasheva *et al*. 2006).

O único trabalho publicado no qual testaram um inibidor RTK na leishmaniose foi utilizado com objetivo de diminuir o processo angiogênico associado com a esplenomegalia em camundongos infectados com *L. donovani*. Foi observada uma inibição significativa na inflamação do baço no tratamento simples e da carga parasitária no tratamento combinado com antimônio (Dalton *et al*. 2010). Então, o uso dos inibidores RTK na LV humana poderia ter um racional associado na redução da excessiva inflamação sistêmica característica da LV (Nery Costa *et al*. 2010, Santos-Oliveira *et al*. 2011; 2012).

Nossos resultados nos levam a supor que a arginase nos macrófagos e neutrófilos infectados podem balancear o estimulo inflamatório causado por um excesso IFN-ɣ e citocinas pró-inflamatórias liberadas pelas células Th17 (IL-17, IL-6) (Kumar & Nylen 2012). Os macrófagos ativados pela via alternativa induzem IL-10 e IGF-I, que contrarregulam a resposta inflamatória das células Th17 e de neutrófilos (Kumar & Nylen 2012). O macrófago de camundongos infectados com *S. mansoni* apresenta uma resposta anti-inflamatória mediada pelo receptor de IL-4, o que protege o hospedeiro da inflamação induzida pelo parasito, e no caso de *Leishmania* spp., os macrófagos ativados por a via alternativa produzem IL-10 e TGF-B, que torna o macrófago susceptível devido a tentativa de contrarregular a excessiva resposta Th1. Esta informação sugere que o macrófago ativado pela via alternativa pode estar exercendo um papel regulatório. Assim, o controle da inflamação mediada pelos inibidores RTK parece ser relevante como alternativa terapêutica na LV.

A pesar disso, a sinalização cruzada entre os receptores RTK pode apresentar dificuldades na terapêutica e, tem sido reportada resistência aos inibidores do EGFR quando utilizados inibidores do IGFR, em estudos de fase clinica 1 no tratamento do câncer. Portanto, foi recomendado, como alternativa, o bloqueio simultâneo de múltiplos receptores com o fim de aumentar a eficácia terapêutica (Van der Veeken *et al*. 2009).

A utilização dos inibidores RTK na LV também tem racional no contexto de interferir com a atividade enzimática da arginase que leva à depleção da L-arginina e causa imunossupressão. O efeito benéfico da interferência farmacológica da arginase tem sido demonstrado no caso da imunossupressão causada pelas MDSC. Neste caso, a função do linfócito T foi restaurada mediante a inibição direita da Arginase, ou mediante a suplementação com L- arginina (Munder *et al*. 2009).

O uso dos inibidores RTK, pode exercer efeitos potenciais no controle do recrutamento de macrófagos, divisão local e angiogênese, sobretudo pelo fato que os mecanismos descritos como causa destes processos são dependentes da IL-4 e dos fatores de crescimento (Jenkis *et al*. 2011, 2013). Os resultados desta tese que demostraram a regulação cruzada dos RTK e da sinalização da IL-4 (dependente do STAT-6) sugere que são alternativas terapêuticas potencialmente úteis para controlar os efeitos patogênicos desta citosina. Embora, a utilidade dos inibidores RTKs no tratamento da LV humana precisa ser comprovada em outros sistemas de estudo pré-clínico, como cães infectados experimental ou naturalmente.

Interessantemente, um modelo matemático desenvolvido com o fim de predizer a resposta imune que desencadeia um efeito de resolução da leishmaniose implicou, como vias na patogênese, a sinalização cruzada do EGFR - CD14-TLR e TNF e a via das MAPK. Estas vias e os seus componentes de sinalização ERK1/2 e JNK estiveram associadas com uma resposta inflamatória elevada. Assim, o estudo concluiu que os objetivos terapêuticos desejados estiveram ligados à inibição de PI3K e EGFR (Mol *et al*. 2014). Em conclusão, a utilização de inibidores dos RTK com o fim de controlar arginase e a excessiva resposta do macrófago, inflamação e imunossupressão na LV parece promissora.

5. CONCLUSÕES

- Os RTKs ativam o STAT-6 e desencadeiam sinais comuns de ativação com a via das citocinas, tais como a via das MAPK, PI3K, e AKT, que potencialmente originam a expressão de IL-10. Estes sinais convergem com a ativação do STAT-6 causada por a infecão e a IL-4 e, em conjunto, regulam a Arg-1 na LV experimental.
- O incremento da Arg-1 como consequência da infecção, a ativação dos RTK e das citocinas Th2 orientam o macrófago ao fenótipo alternativamente ativado na LV, consequentemente, o crescimento do parasito é favorecido em detrimento da produção de proteínas potencialmente com atividade modulatória anti-*Leishmania* spp da via óxido nítrico sintase.
- A ativação alternativa do macrófago pode exercer o papel de limitar a excessiva resposta inflamatória (causada por IFN-ɣ e TNF) através da produção concomitante de citocinas anti-inflamatórias (IL-10, IL-4, L-13 e TGF-β), resultando na inativação do macrófago e no bloqueio da ação anti-leishmânia, o que possivelmente ocasiona a doença crônica não resolutiva.
- Tanto os níveis plasmáticos da Arg-1 quanto os do FGF-2 não foram correlacionados com doença ativa na LV humana. Embora, a associação negativa da Arg-1 e do FGF-2 1 ano após do tratamento sugeriu a necessidade de um longo período de tempo para desenvolver uma resposta resolutiva relacionada com o aumento do FGF-2.
- Arg-1 e Arg-2 foram reguladas positivamente em cultivos de sangue periférico humanos infectados *in vitro* com *L. donovani* e expostos à estimulação simultânea com IL-4 e fator de crescimento.
- Os inibidores químicos de RTK podem trazer efeitos regulatórios benefícios como a redução de Arg-1, carga parasitária, inflamação, além de controlar a imunossupressão. Os resultados sugerem que são alternativas terapêuticas promissórias da LV.

6. REFERÊNCIAS BIBLIOGRAFICAS

1. Abebe T, Hailu A, Woldeyes M, Mekonen W, Bilcha K, Cloke T, *et al*. Local increase of arginase activity in lesions of patients with cutaneous leishmaniasis in Ethiopia. PLoS Negl Trop Dis. 2012;6(6):e1684.
2. Abebe T, Takele Y, Weldegebreal T, Cloke T, Closs E, Corset C, *et al*. Arginase activity - a marker of disease status in patients with visceral leishmaniasis in ethiopia. PLoS Negl Trop Dis. 2013;7(3):e2134.
3. Aït-Oudhia K, Gazanion E, Vergnes B, Oury B, Sereno D. Leishmania antimony resistance: what we know what we can learn from the field. Parasitol Res. 2011;109(5):1225-32.
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, *et al*. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671.
5. Arkins S, Rebeiz N, Brunke-Reese DL, Biragyn A, Kelley KW. Interferon-gamma inhibits macrophage insulin-like growth factor-I synthesis at the transcriptional level. Mol Endocrinol. 1995;9(3):350-60.
6. Aslan H, Dey R, Meneses C, Castrovinci P, Jeronimo SM, Oliva G, *et al*. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. J Infect Dis. 2013;207(8):1328-38.
7. Babu S, Kumaraswami V, Nutman TB. Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis. 2009;199(12):1827-37.
8. Bansal V, Ochoa JB. Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care. 2003;6(2):223-8.
9. Bañuls AL, Hide M, Prugnolle F. Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol. 2007;64:1-109.
10. Berasain C, Ujue Latasa M, Urtasun R, Goñi S, Elizalde M, Garcia-Irigoyen O, *et al*. Epidermal Growth Factor Receptor (EGFR) Crosstalks in Liver Cancer. Cancers (Basel). 2011;3(2):2444-61.
11. Bermudez LE, Petrofsky M, Shelton K. Epidermal growth factor-binding protein in Mycobacterium avium and Mycobacterium tuberculosis: a possible role in the mechanism of infection. Infect Immun. 1996;64(8):2917-22.

12. Biswas A, Bhattacharya A, Kar S, Das PK. Expression of IL-10-triggered STAT3-dependent IL-4Rα is required for induction of arginase 1 in visceral leishmaniasis. Eur J Immunol. 2011;41(4):992-1003.
13. Bogdan C. [Leishmaniasis: principles of the immune response and function of nitric oxide]. Berl Munch Tierarztl Wochenschr. 1998;111(11-12):409-14.
14. Bogdan C. [Leishmaniasis: principles of the immune response and function of nitric oxide]. Berl Munch Tierarztl Wochenschr. 1998;111(11-12):409-14.
15. Bohrer LR, Chuntova P, Bade LK, Beadnell TC, Leon RP, Brady NJ, *et al*. Activation of the FGFR-STAT3 pathway in breast cancer cells induces a hyaluronan-rich microenvironment that licenses tumor formation. Cancer Res. 2014;74(1):374-86.
16. Bronte V, Serafini P, De Santo C, Marigo I, Tosello V, Mazzoni A, *et al*. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol. 2003;170(1):270-8.
17. Caldas A, Favali C, Aquino D, Vinhas V, van Weyenbergh J, Brodskyn C, *et al*. Balance of IL-10 and interferon-gamma plasma levels in human visceral leishmaniasis: implications in the pathogenesis. BMC Infect Dis. 2005;5:113.
18. Costa CHN, Werneck GL, Costa DL, Holanda TA, Aguiar GB, Carvalho AS, *et al*. Is severe visceral leishmaniasis a systemic inflammatory response syndrome? – A case control study. Revista da Sociedade Brasileira de Medicina Tropical. 2010;43(4):386-92.
19. Carrillo E, Jimenez MA, Sanchez C, Cunha J, Martins CM, da Paixão Sevá A, *et al*. Protein malnutrition impairs the immune response and influences the severity of infection in a hamster model of chronic visceral leishmaniasis. PLoS One. 2014;9(2):e89412.
20. Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, Reed SG. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol. 1994;152(12):5949-56.
21. Carvalho EM, Badaró R, Reed SG, Jones TC, Johnson WD. Absence of gamma interferon and interleukin 2 production during active visceral leishmaniasis. J Clin Invest. 1985;76(6):2066-9.

22. Chan MM, Adapala N, Chen C. Peroxisome Proliferator-Activated Receptor-γ-Mediated Polarization of Macrophages in Leishmania Infection. PPAR Res. 2012;2012:796235.
23. Chaves J, Saif MW. IGF system in cancer: from bench to clinic. Anticancer Drugs. 2011;22(3):206-12.
24. Chawla A. Control of macrophage activation and function by PPARs. Circ Res. 2010;106(10):1559-69.
25. Cheekatla SS, Aggarwal A, Naik S. mTOR signaling pathway regulates the IL-12/IL-10 axis in *Leishmania donovani infection*. Med Microbiol Immunol. 2012;201(1):37-46.
26. Chen PC, Broome JD. Mouse macrophage arginase. Proc Soc Exp Biol Med. 1980;163(3):354-9.
27. Conte C, Riant E, Toutain C, Pujol F, Arnal JF, Lenfant F, *et al*. FGF2 translationally induced by hypoxia is involved in negative and positive feedback loops with HIF-1alpha. PLoS One. 2008;3(8):e3078.
28. Corraliza I, Moncada S. Increased expression of arginase II in patients with different forms of arthritis. Implications of the regulation of nitric oxide. J Rheumatol. 2002;29(11):2261-5.
29. Costa AS, Costa GC, Aquino DM, Mendonça VR, Barral A, Barral-Netto M, *et al*. Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2012;107(6):735-9.
30. Costa CH, Werneck GL, Costa DL, Holanda TA, Aguiar GB, Carvalho AS, *et al*. Is severe visceral leishmaniasis a systemic inflammatory response syndrome? A case control study. Rev Soc Bras Med Trop. 2010;43(4):386-92.
31. Costa DL, Carregaro V, Lima-Júnior DS, Silva NM, Milanezi CM, Cardoso CR, *et al*. BALB/c mice infected with antimony treatment refractory isolate of *Leishmania braziliensis* present severe lesions due to IL-4 production. PLoS Negl Trop Dis. 2011;5(3):e965.
32. Costa DL, Rocha RL, Carvalho RM, Lima-Neto AS, Harhay MO, Costa CH, *et al*. Serum cytokines associated with severity and complications of kala-azar. Pathog Glob Health. 2013;107(2):78-87.

33. Dalton JE, Maroof A, Owens BM, Narang P, Johnson K, Brown N, *et al*. Inhibition of receptor tyrosine kinases restores immunocompetence and improves immune-dependent chemotherapy against experimental leishmaniasis in mice. J Clin Invest. 2010;120(4):1204-16.
34. de Medeiros IM, Castelo A, Salomão R. Presence of circulating levels of interferon-gamma, interleukin-10 and tumor necrosis factor-alpha in patients with visceral leishmaniasis. Rev Inst Med Trop Sao Paulo. 1998;40(1):31-4.
35. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004;27(5):305-18.
36. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, *et al*. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 2008;9(12):1399-406.
37. Engwerda CR, Ato M, Kaye PM. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. Trends Parasitol. 2004;20(11):524-30.
38. Erdely A, Kepka-Lenhart D, Clark M, Zeidler-Erdely P, Poljakovic M, Calhoun WJ, *et al*. Inhibition of phosphodiesterase 4 amplifies cytokine-dependent induction of arginase in macrophages. Am J Physiol Lung Cell Mol Physiol. 2006;290(3):L534-9.
39. Fakiola M, Strange A, Cordell HJ, Miller EN, Pirinen M, Su Z, *et al*. Common variants in the HLA-DRB1-HLA-DQA1 HLA class II region are associated with susceptibility to visceral leishmaniasis. Nat Genet. 2013;45(2):208-13.
40. Fazzani C, Guedes PA, Senna A, Souza EB, Goto H, Lindoso JA. Dynamics of immunosuppression in hamsters with experimental visceral leishmaniasis. Braz J Med Biol Res. 2011;44(7):666-70.
41. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. 2002;23(6):824-54.
42. Forget G, Gregory DJ, Olivier M. Proteasome-mediated degradation of STAT1alpha following infection of macrophages with *Leishmania donovani*. J Biol Chem. 2005;280(34):30542-9.
43. Gabrilove JL, White K, Rahman Z, Wilson EL. Stem cell factor and basic fibroblast growth factor are synergistic in augmenting committed myeloid progenitor cell growth. Blood. 1994;83(4):907-10.

44. Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, *et al*. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. J Immunol. 2001;167(2):893-901.
45. Gautam S, Kumar R, Maurya R, Nylén S, Ansari N, Rai M, *et al*. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis. 2011;204(7):1134-7.
46. Gomes CM, Goto H, Corbett CE, Gidlund M. Insulin-like growth factor-1 is a growth promoting factor for Leishmania promastigotes. Acta Trop. 1997;64(3-4):225-8.
47. Gomes CM, Goto H, Ribeiro Da Matta VL, Laurenti MD, Gidlund M, Corbett CE. Insulin-like growth factor (IGF)-I affects parasite growth and host cell migration in experimental cutaneous leishmaniasis. Int J Exp Pathol. 2000;81(4):249-55.
48. Goncalves R, Zhang X, Cohen H, Debrabant A, Mosser DM. Platelet activation attracts a subpopulation of effector monocytes to sites of *Leishmania major* infection. J Exp Med. 2011;208(6):1253-65.
49. Goto H, Prianti M. Immunoactivation and immunopathogeny during active visceral leishmaniasis. Rev Inst Med Trop Sao Paulo. 2009;51(5):241-6.
50. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene. 2005;353(1):98-106.
51. Hanson EM, Dickensheets H, Qu CK, Donnelly RP, Keegan AD. Regulation of the dephosphorylation of Stat6. Participation of Tyr-713 in the interleukin-4 receptor alpha, the tyrosine phosphatase SHP-1, and the proteasome. J Biol Chem. 2003;278(6):3903-11.
52. Harhay MO, Olliaro PL, Costa DL, Costa CH. Urban parasitology: visceral leishmaniasis in Brazil. Trends Parasitol. 2011;27(9):403-9.
53. Hölscher C, Arendse B, Schwegmann A, Myburgh E, Brombacher F. Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice. J Immunol. 2006;176(2):1115-21.
54. Hubbard SR, Miller WT. Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol. 2007;19(2):117-23.
55. Ibrahim MK, Barnes JL, Anstead GM, Jimenez F, Travi BL, Peniche AG, *et al*. The malnutrition-related increase in early visceralization of *Leishmania donovani* is

associated with a reduced number of lymph node phagocytes and altered conduit system flow. PLoS Negl Trop Dis. 2013;7(8):e2329.
56. Iniesta V, Carcelén J, Molano I, Peixoto PM, Redondo E, Parra P, *et al*. Arginase I induction during *Leishmania major* infection mediates the development of disease. Infect Immun. 2005;73(9):6085-90.
57. Iniesta V, Gómez-Nieto LC, Corraliza I. The inhibition of arginase by N(omega)-hydroxy-l-arginine controls the growth of Leishmania inside macrophages. J Exp Med. 2001;193(6):777-84.
58. Iniesta V, Gómez-Nieto LC, Molano I, Mohedano A, Carcelén J, Mirón C, *et al*. Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular Leishmania parasites. Parasite Immunol. 2002;24(3):113-8.
59. Itkin T, Ludin A, Gradus B, Gur-Cohen S, Kalinkovich A, Schajnovitz A, *et al*. FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 down-regulation. Blood. 2012;120(9):1843-55.
60. Jang JC, Nair MG. Alternatively Activated Macrophages Revisited: New Insights into the Regulation of Immunity, Inflammation and Metabolic Function following Parasite Infection. Curr Immunol Rev. 2013;9(3):147-56.
61. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, *et al*. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science. 2011;332(6035):1284-8.
62. Jenkins SJ, Ruckerl D, Thomas GD, Hewitson JP, Duncan S, Brombacher F, *et al*. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. J Exp Med. 2013;210(11):2477-91.
63. Kadl A, Meher AK, Sharma PR, Lee MY, Doran AC, Johnstone SR, *et al*. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. Circ Res. 2010;107(6):737-46.
64. Karp CL, el-Safi SH, Wynn TA, Satti MM, Kordofani AM, Hashim FA, *et al*. In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma. J Clin Invest. 1993;91(4):1644-8.
65. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, *et al*. The immunopathology of experimental visceral leishmaniasis. Immunol Rev. 2004;201:239-53.

66. Khaled YS, Ammori BJ, Elkord E. Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients. J Immunol Res. 2014;2014:879897.
67. Krejci P, Prochazkova J, Bryja V, Jelinkova P, Pejchalova K, Kozubik A, *et al*. Fibroblast growth factor inhibits interferon gamma-STAT1 and interleukin 6-STAT3 signaling in chondrocytes. Cell Signal. 2009;21(1):151-60.
68. Kropf P, Fuentes JM, Fähnrich E, Arpa L, Herath S, Weber V, *et al*. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. FASEB J. 2005;19(8):1000-2.
69. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. Front Immunol. 2012;3:251.
70. Kurmasheva RT, Houghton PJ. IGF-I mediated survival pathways in normal and malignant cells. Biochim Biophys Acta. 2006;1766(1):1-22.
71. Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, *et al*. Krüppel-like factor 4 regulates macrophage polarization. J Clin Invest. 2011;121(7):2736-49.
72. Malafaia G. Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review. Parasite Immunol. 2009;31(10):587-96.
73. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 2009;27:451-83.
74. Martins-Melo FR, Lima MaS, Ramos AN, Alencar CH, Heukelbach J. Mortality and case fatality due to visceral leishmaniasis in Brazil: a nationwide analysis of epidemiology, trends and spatial patterns. PLoS One. 2014;9(4):e93770.
75. Martiny A, Vannier-Santos MA, Borges VM, Meyer-Fernandes JR, Assreuy J, Cunha e Silva NL, *et al*. Leishmania-induced tyrosine phosphorylation in the host macrophage and its implication to infection. Eur J Cell Biol. 1996;71(2):206-15.
76. Melby PC, Tabares A, Restrepo BI, Cardona AE, McGuff HS, Teale JM. *Leishmania donovani*: evolution and architecture of the splenic cellular immune response related to control of infection. Exp Parasitol. 2001;99(1):17-25.
77. Ming XF, Rajapakse AG, Yepuri G, Xiong Y, Carvas JM, Ruffieux J, *et al*. Arginase II Promotes Macrophage Inflammatory Responses Through Mitochondrial Reactive Oxygen Species, Contributing to Insulin Resistance and Atherogenesis. J Am Heart Assoc. 2012;1(4):e000992.

78. Mock DJ, Hollenbaugh JA, Daddacha W, Overstreet MG, Lazarski CA, Fowell DJ, *et al*. Leishmania induces survival, proliferation and elevated cellular dNTP levels in human monocytes promoting acceleration of HIV co-infection. PLoS Pathog. 2012;8(4):e1002635.
79. Modolell M, Choi BS, Ryan RO, Hancock M, Titus RG, Abebe T, *et al*. Local suppression of T cell responses by arginase-induced L-arginine depletion in nonhealing leishmaniasis. PLoS Negl Trop Dis. 2009;3(7):e480.
80. Mohamed HS, Ibrahim ME, Miller EN, White JK, Cordell HJ, Howson JM, *et al*. SLC11A1 (formerly NRAMP1) and susceptibility to visceral leishmaniasis in The Sudan. Eur J Hum Genet. 2004;12(1):66-74.
81. Mol M, Patole MS, Singh S. Immune signal transduction in leishmaniasis from natural to artificial systems: role of feedback loop insertion. Biochim Biophys Acta. 2014;1840(1):71-9.
82. Muleme HM, Reguera RM, Berard A, Azinwi R, Jia P, Okwor IB, *et al*. Infection with arginase-deficient *Leishmania major* reveals a parasite number-dependent and cytokine-independent regulation of host cellular arginase activity and disease pathogenesis. J Immunol. 2009;183(12):8068-76.
83. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158(3):638-51.
84. Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol. 1999;163(7):3771-7.
85. Munder M, Mollinedo F, Calafat J, Canchado J, Gil-Lamaignere C, Fuentes JM, *et al*. Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. Blood. 2005;105(6):2549-56.
86. Murray HW, Cartelli DM. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. J Clin Invest. 1983;72(1):32-44.
87. Nelin LD, Chicoine LG, Reber KM, English BK, Young TL, Liu Y. Cytokine-induced endothelial arginase expression is dependent on epidermal growth factor receptor. Am J Respir Cell Mol Biol. 2005;33(4):394-401.

88. North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. Am J Physiol Lung Cell Mol Physiol. 2009;296(6):L911-20.
89. Novak ML, Koh TJ. Phenotypic transitions of macrophages orchestrate tissue repair. Am J Pathol. 2013;183(5):1352-63.
90. Nylén S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med. 2007;204(4):805-17.
91. Ochoa JB, Bernard AC, O'Brien WE, Griffen MM, Maley ME, Rockich AK, *et al*. Arginase I expression and activity in human mononuclear cells after injury. Ann Surg. 2001;233(3):393-9.
92. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, *et al*. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature. 2007;447(7148):1116-20.
93. Oliveira EF, Silva EA, Fernandes CE, Paranhos Filho AC, Gamarra RM, Ribeiro AA, *et al*. Biotic factors and occurrence of *Lutzomyia longipalpis* in endemic area of visceral leishmaniasis, Mato Grosso do Sul, Brazil. Mem Inst Oswaldo Cruz. 2012;107(3):396-401.
94. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev. 2005;18(2):293-305.
95. Osorio Y, Rodriguez LD, Bonilla DL, Peniche AG, Henao H, Saldarriaga O, *et al*. Congenital transmission of experimental leishmaniasis in a hamster model. Am J Trop Med Hyg. 2012;86(5):812-20.
96. Osorio Y, Travi BL, Renslo AR, Peniche AG, Melby PC. Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. PLoS Negl Trop Dis. 2011;5(2):e962.
97. Pauleau AL, Rutschman R, Lang R, Pernis A, Watowich SS, Murray PJ. Enhancer-mediated control of macrophage-specific arginase I expression. J Immunol. 2004;172(12):7565-73.
98. Pereira WF, Ribeiro-Gomes FL, Guillermo LV, Vellozo NS, Montalvão F, Dosreis GA, *et al*. Myeloid-derived suppressor cells help protective immunity to *Leishmania*

major infection despite suppressed T cell responses. J Leukoc Biol. 2011;90(6):1191-7.
99. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF, Cheever AW, *et al*. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest. 2006;116(7):2044-55.
100. Pessanha AP, Martins RA, Mattos-Guaraldi AL, Vianna A, Moreira LO. Arginase-1 expression in granulomas of tuberculosis patients. FEMS Immunol Med Microbiol. 2012;66(2):265-8.
101. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, *et al*. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science. 2008;321(5891):970-4.
102. Pinho FA, Magalhães NA, Silva KR, Carvalho AA, Oliveira FL, Ramos-Sanchez EM, *et al*. Divergence between hepatic insulin-like growth factor (IGF)-I mRNA expression and IGF-I serum levels in *Leishmania (Leishmania) infantum chagasi*-infected dogs. Vet Immunol Immunopathol. 2013;151(1-2):163-7.
103. Popovic PJ, Zeh HJ, Ochoa JB. Arginine and immunity. J Nutr. 2007;137(6 Suppl 2):1681S-6S.
104. Pourcet B, Feig JE, Vengrenyuk Y, Hobbs AJ, Kepka-Lenhart D, Garabedian MJ, *et al*. LXRα regulates macrophage arginase 1 through PU.1 and interferon regulatory factor 8. Circ Res. 2011;109(5):492-501.
105. Pourcet B, Pineda-Torra I. Transcriptional regulation of macrophage arginase 1 expression and its role in atherosclerosis. Trends Cardiovasc Med. 2013;23(5):143-52.
106. Presta M, Andrés G, Leali D, Dell'Era P, Ronca R. Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. Eur Cytokine Netw. 2009;20(2):39-50.
107. Puzik A, Rupp J, Tröger B, Göpel W, Herting E, Härtel C. Insulin-like growth factor-I regulates the neonatal immune response in infection and maturation by suppression of IFN-γ. Cytokine. 2012;60(2):369-76.
108. Qualls JE, Neale G, Smith AM, Koo MS, DeFreitas AA, Zhang H, *et al*. Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. Sci Signal. 2010;3(135):ra62.

109. Raes G, Brys L, Dahal BK, Brandt J, Grooten J, Brombacher F, *et al*. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. J Leukoc Biol. 2005;77(3):321-7.
110. Rajabi M, Mansourian A, Bazmani A. Susceptibility mapping of visceral leishmaniasis based on fuzzy modelling and group decision-making methods. Geospat Health. 2012;7(1):37-50.
111. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, *et al*. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res. 2010;20(10):1352-60.
112. Reis LC, Ramos-Sanchez EM, Goto H. The interactions and essential effects of intrinsic insulin-like growth factor-I *on Leishmania (Leishmania) major* growth within macrophages. Parasite Immunol. 2013;35(7-8):239-44.
113. Ribeiro-Gomes FL, Sacks D. The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front Cell Infect Microbiol. 2012;2:59.
114. Rodríguez PC, Ochoa AC. T cell dysfunction in cancer: role of myeloid cells and tumor cells regulating amino acid availability and oxidative stress. Semin Cancer Biol. 2006;16(1):66-72.
115. Rodríguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev. 2008;222:180-91.
116. Romero CJ, Pine-Twaddell E, Sima DI, Miller RS, He L, Wondisford F, *et al*. Insulin-like growth factor 1 mediates negative feedback to somatotroph GH expression via POU1F1/CREB binding protein interactions. Mol Cell Biol. 2012;32(21):4258-69.
117. Roy B, Bhattacharjee A, Xu B, Ford D, Maizel AL, Cathcart MK. IL-13 signal transduction in human monocytes: phosphorylation of receptor components, association with Jaks, and phosphorylation/activation of Stats. J Leukoc Biol. 2002;72(3):580-9.
118. Ruhland A, Leal N, Kima PE. Leishmania promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. Cell Microbiol. 2007;9(1):84-96.

119. Saha S, Mondal S, Ravindran R, Bhowmick S, Modak D, Mallick S, *et al*. IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of *Leishmania donovani* infection in India. J Immunol. 2007;179(8):5592-603.
120. Sampaio MJ, Cavalcanti NV, Alves JG, Filho MJ, Correia JB. Risk factors for death in children with visceral leishmaniasis. PLoS Negl Trop Dis. 2010;4(11):e877.
121. Santos-Oliveira JR, Da-Cruz AM. Lipopolysaccharide-Induced Cellular Activation May Participate in the Immunopathogenesis of Visceral Leishmaniasis Alone or in HIV Coinfection. Int J Microbiol. 2012;2012:364534.
122. Santos-Oliveira JR, Regis EG, Giacoia-Gripp CB, Valverde JG, Alexandrino-de-Oliveira P, Lindoso J, *et al*. Microbial translocation induces an intense proinflammatory response in patients with visceral leishmaniasis and HIV type 1 coinfection. J Infect Dis. 2013;208(1):57-66.
123. Santos-Oliveira JR, Regis EG, Leal CR, Cunha RV, Bozza PT, Da-Cruz AM. Evidence that lipopolisaccharide may contribute to the cytokine storm and cellular activation in patients with visceral leishmaniasis. PLoS Negl Trop Dis. 2011;5(7):e1198.
124. Schmid M, Wege AK, Ritter U. Characteristics of "Tip-DCs and MDSCs" and Their Potential Role in Leishmaniasis. Front Microbiol. 2012;3:74.
125. Schneider E, Ihle JN, Dy M. Homogeneous interleukin 3 enhances arginase activity in murine hematopoietic cells. Lymphokine Res. 1985;4(2):95-102.
126. Seixas Duarte MI, Tuon FF, Pagliari C, Kauffman MR, Brasil RA. Human visceral leishmaniasis expresses Th1 pattern in situ liver lesions. J Infect. 2008;57(4):332-7.
127. Serrat N, Pereira-Lopes S, Comalada M, Lloberas J, Celada A. Deacetylation of C/EBPβ is required for IL-4-induced arginase-1 expression in murine macrophages. Eur J Immunol. 2012;42(11):3028-37.
128. Sharda DR, Yu S, Ray M, Squadrito ML, De Palma M, Wynn TA, *et al*. Regulation of macrophage arginase expression and tumor growth by the Ron receptor tyrosine kinase. J Immunol. 2011;187(5):2181-92.
129. Sheldon KE, Shandilya H, Kepka-Lenhart D, Poljakovic M, Ghosh A, Morris SM. Shaping the murine macrophage phenotype: IL-4 and cyclic AMP synergistically activate the arginase I promoter. J Immunol. 2013;191(5):2290-8.

130. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 2012;122(3):787-95.
131. Stempin CC, Dulgerian LR, Garrido VV, Cerban FM. Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals. J Biomed Biotechnol. 2010;2010:683485.
132. Sundar S, Reed SG, Sharma S, Mehrotra A, Murray HW. Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. Am J Trop Med Hyg. 1997;56(5):522-5.
133. Sundar S, Rosenkaimer F, Lesser ML, Murray HW. Immunochemotherapy for a systemic intracellular infection: accelerated response using interferon-gamma in visceral leishmaniasis. J Infect Dis. 1995;171(4):992-6.
134. Takele Y, Abebe T, Weldegebreal T, Hailu A, Hailu W, Hurissa Z, *et al*. Arginase activity in the blood of patients with visceral leishmaniasis and HIV infection. PLoS Negl Trop Dis. 2013;7(1):e1977.
135. Toby IT, Chicoine LG, Cui H, Chen B, Nelin LD. Hypoxia-induced proliferation of human pulmonary microvascular endothelial cells depends on epidermal growth factor receptor tyrosine kinase activation. Am J Physiol Lung Cell Mol Physiol. 2010;298(4):L600-6.
136. Touriol C, Morillon A, Gensac MC, Prats H, Prats AC. Expression of human fibroblast growth factor 2 mRNA is post-transcriptionally controlled by a unique destabilizing element present in the 3'-untranslated region between alternative polyadenylation sites. J Biol Chem. 1999;274(30):21402-8.
137. van der Veeken J, Oliveira S, Schiffelers RM, Storm G, van Bergen En Henegouwen PM, Roovers RC. Crosstalk between epidermal growth factor receptor- and insulin-like growth factor-1 receptor signaling: implications for cancer therapy. Curr Cancer Drug Targets. 2009;9(6):748-60.
138. Van Ginderachter JA, Beschin A, De Baetselier P, Raes G. Myeloid-derived suppressor cells in parasitic infections. Eur J Immunol. 2010;40(11):2976-85.
139. van Griensven J, Diro E. Visceral leishmaniasis. Infect Dis Clin North Am. 2012;26(2):309-22.
140. Vendrame CM, Carvalho MD, Rios FJ, Manuli ER, Petitto-Assis F, Goto H. Effect of insulin-like growth factor-I on *Leishmania amazonensis* promastigote

arginase activation and reciprocal inhibition of NOS2 pathway in macrophage in vitro. Scand J Immunol. 2007;66(2-3):287-96.
141. Vendrame CM, Souza LD, Carvalho MD, Salgado K, Carvalho EM, Goto H. Insulin-like growth factor-I induced and constitutive arginase activity differs among isolates of Leishmania derived from patients with diverse clinical forms of *Leishmania braziliensis* infection. Trans R Soc Trop Med Hyg. 2010;104(8):566-8.
142. Viana AG, Mayrink W, Fraga CA, Silva LM, Domingos PL, Bonan PR, *et al*. Histopathological and immunohistochemical aspects of American cutaneous leishmaniasis before and after different treatments. An Bras Dermatol. 2013;88(1):32-40.
143. Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against Leishmania infection. Immunol Res. 2008;41(1):15-25.
144. Wei LH, Wu G, Morris SM, Ignarro LJ. Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. Proc Natl Acad Sci U S A. 2001;98(16):9260-4.
145. Weisser SB, McLarren KW, Kuroda E, Sly LM. Generation and characterization of murine alternatively activated macrophages. Methods Mol Biol. 2013;946:225-39.
146. Werneck GL. Geographic spread of visceral leishmaniasis in Brazil. Cad Saude Publica. 2010;26(4):644-5.
147. Yang Z, Mosser DM, Zhang X. Activation of the MAPK, ERK, following Leishmania amazonensis infection of macrophages. J Immunol. 2007;178(2):1077-85.
148. Ye S, Xu H, Jin J, Yang M, Wang C, Yu Y, *et al*. The E3 ubiquitin ligase neuregulin receptor degradation protein 1 (Nrdp1) promotes M2 macrophage polarization by ubiquitinating and activating transcription factor CCAAT/enhancer-binding Protein β (C/EBPβ). J Biol Chem. 2012;287(32):26740-8.
149. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst. 2000;92(18):1472-89.
150. Zea AH, Culotta KS, Ali J, Mason C, Park HJ, Zabaleta J, *et al*. Decreased expression of CD3zeta and nuclear transcription factor kappa B in patients with pulmonary tuberculosis: potential mechanisms and reversibility with treatment. J Infect Dis. 2006;194(10):1385-93.

151. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, *et al*. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res. 2005;65(8):3044-8.
152. Zhao A, Urban JF, Anthony RM, Sun R, Stiltz J, van Rooijen N, *et al*. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. Gastroenterology. 2008;135(1):217-25.e1.