UNIVERSIDADE DE BRASÍLIA
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA MOLECULAR

DIANE STHEFANY LIMA DE OLIVEIRA

IMUNOGLOBULINA GAMA 3 DE CAMUNDONGO (mIgG3): ESTUDO DA INTERAÇÃO COM ANTÍGENOS E RECEPTORES DE MACRÓFAGOS

BRASÍLIA
2020

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Tese apresentada ao Programa de Pós-Graduação em Biologia Molecular da Universidade de Brasília como requisito parcial para a obtenção do título de Doutora em Biologia Molecular.

Orientador: Dr. André Moraes Nicola

Coorientadora: Dra. Maria Sueli Soares Felipe

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Aprovada em: ____ de _____ de 2020.

Banca Examinadora

_____

Prof. Dr. André Moraes Nicola (Presidente)
Programa de Pós-Graduação em Biologia Molecular – UnB

_____

Profa. Dra. Anamélia Lorenzetti Bocca (Membro interno)
Programa de Pós-Graduação em Biologia Molecular – UnB

_____

Prof. Dr. Aldo Henrique Fonseca Pacheco Tavares (Membro externo)
Programa de Pós-Graduação em Biologia Microbiana – UnB

_____

Prof. Dr. Sandro Rogério de Almeida (Membro externo)
Universidade de São Paulo (USP)

_____

Profa. Dra. Camila Guimarães de Freitas (Suplente)
Instituto Federal de Brasília – IFB

Agradecimentos

Acima de tudo e de todos, preciso começar agradecendo a Deus por ter me concedido o dom da vida e a Jesus Cristo por ter me ensinado os princípios necessários para encarar com ânimo essa experiência incrível e ao mesmo tempo desafiadora de se fazer pesquisa em plena crise da ciência brasileira. Sempre apostei num caminho onde a ciência e a religião convergessem e posso confessar como "religiosa" que a ciência aumentou muito a minha fé. Como cientista, posso dizer que a religião me ajudou, e muito, a ser perseverante.

Muito provavelmente esse trabalho não teria acontecido sem o prof. André Nicola. É notável que ele ama o que faz e, com muita empolgação, contagia as pessoas ao seu redor. Além disso, ele sempre se mostrou determinado em conseguir as ferramentas necessárias para que pudéssemos realizar um bom trabalho, mesmo com todos os empecilhos possíveis. Ele instigou meu senso crítico, desafiou-me a ir além dos limites criados por mim mesma, a ser independente e me ajudou a ver o lado positivo de cada situação, mesmo quando ele não existia (rs). Com as inúmeras confraternizações promovidas por ele, mostrou que a socialização e o *networking* são importantíssimos. Sou aluna do Prof. André desde o mestrado e mesmo sem ter conseguido se tornar meu orientador oficial durante esse período, ele nunca hesitou em me orientar, provando que acreditava em meu comprometimento com o trabalho. Ao André, concedo o título de orientador e amigo.

Profa. Sueli, agradeço por toda dedicação no projeto e por sempre se mostrar disponível, mesmo com suas inúmeras ocupações. Aprendi muito com sua objetividade, determinação, simplicidade. Com certeza é um grande exemplo a ser seguido!

Não poderia deixar de agradecer aos amigos que fiz ao longo desses anos. Em especial à Dra. Paredes! Ela é um grande exemplo de pesquisadora e, além disso, uma pessoa com um coração enorme. Devo a ela grande parte dos meus conhecimentos em anticorpos, o dia a dia de superação e trabalho duro no laboratório, discussões de horas sobre planejamento de experimentos e toda a paciência. Agradeço também pela amizade, por rir comigo mesmo quando os experimentos davam errado e por tornar os meus dias mais divertidos.

Outras amigas e colaboradoras que gostaria de agradecer com peso especial são Adrielle e Nicole. Meninas, muito obrigada por toda dedicação, resiliência e parceria. Com vocês aprendi a orientar, delegar tarefas, ser paciente, melhor comunicadora e com certeza a ser uma pessoa melhor. Sou grata pela paciência, por dramatizarem comigo quando os experimentos falhavam, pelos nossos encontrinhos e filosofias de vida.

Agradeço às colaboradoras e amigas Patrícia Albuquerque, Ágata e Fabiana. Patty, sou grata por sua disposição em ajudar em tudo, desde insumos a questões pessoais. Aprecio muito sua sinceridade, simplicidade, objetividade, gentileza e profissionalismo. Ágata, agradeço por sua companhia desde que o grupo do André surgiu e é um prazer estar colaborando contigo. Fabi, nos aproximamos em Baltimore e desde então nossa parceria só aumentou e é sempre bom poder contar contigo.

Agradeço às minhas duas famílias, Oliveira e Vieira, pela compreensão e apoio. Em especial à Mamis e "D. Jane" por todo amor! Agradeço muitíssimo, a minha grande fonte de inspiração: Lucas Vieira. Você foi meu colega de graduação e laboratório, amigo e hoje meu

marido. Você foi quem chorou e sorriu comigo, fez-me sempre extrair o melhor de todas as situações, serviu de exemplo muitas vezes e foi essencial para conclusão deste trabalho. Muito obrigada, Vidinha!

Provavelmente vou esquecer de mencionar alguém, mas quero ainda demonstrar minha gratidão aos demais colegas do LabMIB, ao prof. Casadevall e seu grupo - agradeço pela colaboração e por me receberem tão bem em seu laboratório. Agradeço ainda ao prof. Hugo, profa. Ildinete e demais professores que fizeram parte da minha formação. Agradeço também às instituições de fomento à pesquisa (CAPES, CNPq e FAP/DF) e à Universidade de Brasília.

Resumo

A imunoglobulina gama 3 de camundongo (mIgG3) apresenta papel dual na saúde e na doença, mas ainda é o menos estudado entre os isotipos IgG. Apesar de mIgG3 ser o principal anticorpo produzido contra carboidratos e epítopos em repetição, sendo importante no combate a patógenos, ele também é capaz de causar doenças autoimunes e foi encontrado agravando a criptococose. Tendo em vista que o mecanismo do papel dual de mIgG3 permanece desconhecido, este trabalho pretende contribuir para o seu entendimento através do estudo de interação do anticorpo com antígeno e receptores de macrófagos murinos. Para isso, duas linhas de pesquisa foram criadas: uma que estudou a influência da região constante do anticorpo na ligação ao antígeno capsular de *C. neoformans*, e outra que investigou qual seria o receptor de mIgG3. Os ensaios foram feitos utilizando anticorpos IgG comerciais, dois conjuntos de anticorpos IgG contra diferentes epítopos de Glucuronoxilomanana denominados “3E5” e “2H1”; e VLA-4 - receptor recombinante solúvel composto por cadeia α4/β1. Dessa forma, os principais resultados levaram as seguintes conclusões: 1) o par de anticorpos “2H1” utilizado nesse estudo demonstrou que a região constante pode desempenhar papel na ligação de IgG ao antígeno, e 2) a região de dobradiça de mIgG3 pode ser necessária nesta função; 3) integrina beta 1, assim como FcγRI, FcγRIII e complemento, provavelmente faz parte de um complexo de receptores de mIgG3; 4) a interação entre mIgG3 e Itgb1 solúvel revela a integrina como importante ligante do anticorpo; 5) IgGs produzidos de diferentes lotes ou métodos variam sua predileção ao receptor e isso talvez seja devido a uma mistura de isoformas que varia de lote para lote. As conclusões de como a região constante afeta a ligação de mIgG3 ao antígeno e de como o isotipo interage aos receptores celulares podem ter implicações no entendimento da evolução da imunidade mediada por anticorpo, na função de mIgG3 na imunidade à microorganismos, patogênese de doenças autoimunes e criptococose, e na engenharia de anticorpos.

Abstract

The mouse immunoglobulin gamma 3 (mIgG3) has a dual role in health and disease, but it is still one of the least studied among IgG isotopes. Although mIgG3 is the main antibody produced against carbohydrates and repeating epitopes, which is important against pathogens, it is also able to cause autoimmune diseases and disease enhancement. Considering that the mIgG3 dual role mechanism remains unknown, this work aims to contribute to its understanding by studying the antibody interaction with antigen and murine macrophage receptors. For this, two research lines were developed: the first was focused on the influence of the constant region in antibody binding to *C. neoformans* capsule, and the second aimed to investigate which is the mIgG3 receptor. The assays were performed using commercially available IgG antibodies; two sets of IgGs against different GXM epitopes called "3E5" and "2H1"; and also VLA-4, a soluble recombinant receptor composed of α4 and β1 subunits. Thus, the main results led us to the following conclusions: 1) the "2H1" antibodies used on this study demonstrated that the constant region can play a role in IgG binding to the antigen, and 2) the mIgG3 hinge may be necessary for this function; 3) beta 1 integrin is probably a part of a mIgG3 receptors complex, as well as FcγRI, FcγRIII and complement; 4) the interaction between mIgG3 and soluble Itgb1 reveals integrin as an important antibody ligand; 5) IgGs produced from different batches or methods vary their predilection for the receptor and this may be due to a mixture of isoforms that varies from batch to batch. The conclusions of how the constant region affects the binding of mIgG3 to the antigen and how the isotype interacts with cellular receptors may have implications in understanding the evolution of antibody-mediated immunity, in the role of mIgG3 in immunity against microorganisms, in the pathogenesis of autoimmune diseases and cryptococcosis, and antibody engineering.

Lista de abreviações

Ab	Antibody (anticorpo)
C	Constant (constante)
CD	Circular Dichroism (dicroísmo circular)
CH	Constant heavy chain (região constante da cadeia pesada)
CHO	Chinese Hamster Ovary cell (célula de ovário de hamster chinês)
CL	Constant light chain (região constante da cadeia leve)
CR	Complement Receptor (receptor de complemento)
DHFR	Dihydrofolate reductase (diidrofolato redutase)
DMEM	Dulbecco's Modified Eagle's Medium (meio de Eagle modificado por Dubecco)
ELISA	Enzyme-linked immunosorbent assay (ensaio de imunoabsorção enzimático)
EPS	Extracellular polysaccharide (polissacarídeo extracelular)
Fab	Fragment antigen-binding (fragmento de ligação a antígeno)
Fc	Fragment crystallizable region (fragmento de região cristalizável)
FcγR	Fc gamma receptor (receptor Fc gama)
FITC	Fluorescein isothiocyanate (isotiocianato de fluoresceína)
GXM	Glucuronoxylomannan (glucuronoxilomanana)
H	Heavy chain (cadeia pesada)
IF	Immunofluorescence (imunofluorescência)
IgG	Immunoglobulin γ (imunoglobulina γ)
IgM	Immunoglobulin μ (imunoglobulina μ)
ITC	Isothermal Titration Calorimetry (calorimetria de titulação isotérmica)
Itgb1	Integrin beta 1 (integrina beta 1)
L	Light chain (cadeia leve)
LIBS	Ligand Induced Binding Site (sítio de ligação induzido por ligante)
mAbs	Monoclonal antibodies (anticorpos monoclonais)
mIgG	Murine immunoglobulin gamma (imunoglobulina γ de murino)
NMR	Nuclear Magnetic Resonance (ressonância magnética nuclear)
NS0	Non-Ig secreting murine myeloma cell line (célula de mieloma murino não secretora de imunoglobulina)
RGD	Arginine-glycine-aspartic acid (arginina-glicina-ácido aspártico)
SAXS	Small-angle X-ray scattering (espalhamento de raios X a baixo ângulo)
shRNA	Short hairpin RNA
sRBC	Sheep red blood cells (hemácias de carneiro)
SPR	Surface plasmon ressonance (Ressonância plasmônica de superfície)
V	Variable chain (cadeia variável)
VH	Variable heavy chain (região variável da cadeia pesada)
VK	Variable kappa immunoglobulin light chain (região variável da cadeia leve kappa de imunoglobulinas)
VL	Variable light chain (região variável da cadeia leve)
VLA-4	Very late antigen-4 ou α4/β1integrin

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Sumário

I- Introdução

Conceitos básicos

Após sua descoberta em 1890, anticorpos passaram a ser definidos como glicoproteínas complexas produzidas por plasmócitos de vertebrados contra uma infinidade de antígenos. Após a descoberta da soroterapia (1), os anticorpos passaram a ser caracterizados como imunoglobulinas (2), tiveram sua estrutura molecular definida (3), diferentes tipos classificados e passaram a ser produzidos *in vitro* com o advento da ferramenta de hibridoma (4). A partir disso, anticorpos originados de um único clone de linfócitos B imortal e específicos contra o mesmo epítopo surgiram no mercado farmacêutico e estes foram denominados "anticorpos monoclonais" (mAbs) (5). Recentemente, a anticorpoterapia foi potencializada pela aplicação da tecnologia de DNA recombinante em células de mamíferos imortalizadas, que permitiu criar anticorpos mais específicos, humanizados e eficazes (6). Hoje, mais de 58 mAbs já foram aprovados para uso humano, em um mercado que vale bilhões de dólares (5).

Entre as cinco classes de anticorpos existentes, certamente, a mais comercializada e estudada é a IgG. E entre os quatro isotipos IgG humanos, o mais produzido é o IgG1 (7). No entanto, foi demonstrado que os outros isotipos também apresentam papéis importantes. O IgG3 de camundongo e seu correspondente em humanos (IgG2) é o principal anticorpo produzido contra carboidratos (8) e epítopos em repetição, sendo importante no combate a patógenos (9-11). Curiosamente, foi reportado que esse anticorpo faz parte do mecanismo patogênico de doenças autoimunes (12, 13). Além disso, IgG3 de murino (mIgG3) foi caracterizado por apresentar um papel agravante de doença quando administrado em camundongos infectados com *Cryptococcus neoformans* (14-16). A "resposta agravante de doença" pode ser também denominada Toxicidade letal aguda que é caracterizada por: hemoconcentração, hipotensão, letargia, dificuldade respiratória, colapso circulatório e morte dentro de 20 a 60 minutos após injecção do anticorpo (14).

Para compreender o mecanismo pelo qual o anticorpo trata ou agrava doenças é necessário contextualizar a estrutura de IgG. Ele é uma imunoglobulina heterodimérica composta por duas cadeias pesadas (H) e duas cadeias leves (L) que são unidas entre si por ponte dissulfeto (Figura 1). O anticorpo apresenta também uma região variável leve (VL) e pesada (VH), e uma região constante leve (CL) e pesada (CH). Além disso, um domínio variável e três domínios constantes compõem a cadeia pesada (VH, CH1, CH2 e CH3); ao

passo que um domínio variável e um constante compõem a cadeia leve (VL e CL). Ainda, segundo sua função, o anticorpo pode ser classificado em dois fragmentos que são unidos pela região de dobradiça: fragmento de ligação ao antígeno (Fab) e fragmento cristalizável (Fc) (17, 18).

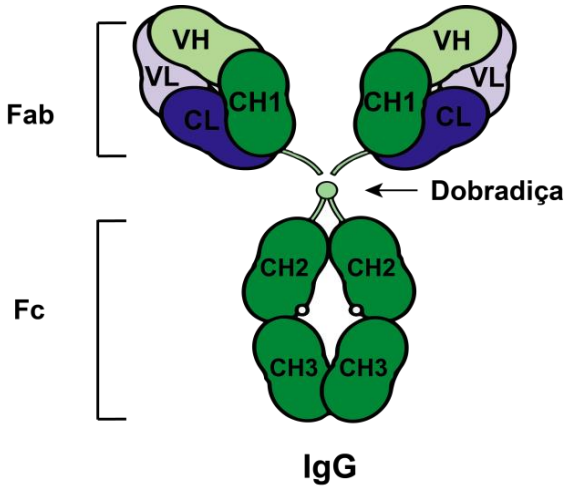
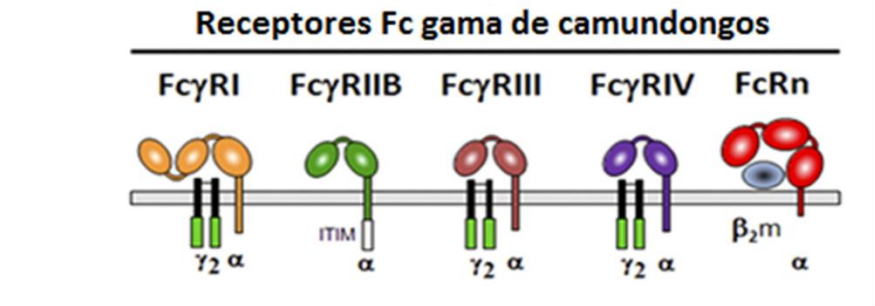


Figura 1- Representação estrutural de IgG. A cadeia pesada está destacada em verde e a cadeia leve, em azul. As regiões variáveis estão representadas em cores claras. A seta aponta a região de dobradiça e as chaves evidenciam as regiões Fab e Fc. Figura elaborada pela autora.

Os anticorpos desempenham suas respostas efetoras mediadas por receptor celular. A principal classe de receptores pelos quais os anticorpos IgGs interagem e medeiam suas funções é a de receptores da fração cristalizável gama (FcγR). Entre os cinco tipos de FcγR presentes em camundongos, FcγRI, FcγRIII e FcγRIV são importantes na resposta inflamatória e se encontram expressos em monócitos e macrófagos (Figura 2). Os isotipos de anticorpos apresentam diferentes afinidades aos receptores: FcγRI liga-se com alta afinidade a mIgG2a e baixa afinidade a mIgG2b; FcγRIII liga-se com baixa afinidade a mIgG1, mIgG2a e mIgG2b; FcγRIV interage com alta afinidade a mIgG2a e mIgG2b e não apresenta nenhuma afinidade a mIgG1 e mIgG3 (19-21).



Função	Ativação	Inibição	Ativação	Ativação	Reciclagem Transporte Apres. Ag
Isotipos IgGs	-	1	1	-	1
	2a	2a	2a	2a	2a
	2b	2b	2b	2b	2b
	(3)	-	-	-	3

Adaptado de Mukherjee, Casadevall e Scharff, 1993.

Figura 2– Receptores FcγR e sua afinidade aos diferentes isotipos IgG de camundongo. Os receptores FcγRI, FcγRIIB, FcγRIII, FcγRIV e FcRn murinos estão representados nas figuras abaixo de cada identificação. A função de cada um e a afinidade aos IgGs estão descritas na tabela.

Influência da região constante do anticorpo na ligação ao antígeno

Segundo o dogma da Imunologia Molecular, o Fab tem a função clássica de se ligar ao antígeno e gerar neutralização e o Fc apresenta a função de se ligar aos receptores celulares e mediar resposta efetora. No entanto, foi descrito que a região constante do anticorpo também apresenta influência na ligação ao antígeno e esse parece ser o mecanismo pelo qual mIgG3 atua como agravante de doença (22-25).

A fim de estudar o papel agravante de doença mediado por mIgG3, anticorpos de diferentes classes e isotipos tiveram suas funções estudadas pelo grupo do Dr. Arturo Casadevall (26-28), incluindo o anticorpo monoclonal IgG 3E5, que apresenta região variável idêntica e região constante diferente (de diferentes isotipos). Em ensaio de sobrevivência, ao administrar anticorpos 3E5 em camundongos previamente infectados com *C. neoformans*, pôde-se observar que os animais tratados com o anticorpo mIgG3 morreram antes mesmo dos animais que não receberam tratamento algum. Esse e outros experimentos revelaram que o anticorpo, ao invés de ser protetor, pode atuar como agravante de doença e esse tipo de resposta não foi observada em outros isotipos IgG (29).

Surpreendentemente, ao realizar experimentos de imunofluorescência indireta com esses anticorpos, incubando-os com *C. neoformans*, foi possível observar que mIgG3 se ligou ao fungo de forma diferente dos outros isotipos IgG (29). Mais especificamente, mIgG3

apresentou fluorescência em forma de pontos ao redor da cápsula do fungo, enquanto mIgG1, mIgG2a e mIgG2b apresentaram fluorescência em formato de anel. Esses tipos de ligações foram observadas de forma reprodutível por anticorpos 3E5 (23), também por dois outros conjuntos de anticorpos IgG (18B7 quiméricos e 4H3 murinos) específicos contra outros epítopos de *C. neformans* (30, 31) e ainda, por anticorpos de outras classes (13F1 IgM, por exemplo) (31). Por tal reprodutibilidade, as ligações em formas de pontos foram nomeadas como "padrão puntiforme" e as ligações em formas de anel, como "padrão anular" (32-34).

Experimentos mais precisos para avaliação de parâmetros termodinâmicos e cinéticos, como ressonância plasmônica de superfície - SPR e calorimetria de titulação isotérmica – ITC demonstraram que a afinidade e especificidade ao antígeno capsular de *C. neoformans* (Glucuronoxilomanana ou GXM) foram alteradas em ensaios realizados com três conjuntos de anticorpos murinos IgG de diferentes isotipos (23, 30, 31). Além disso, experimentos de ELISA de competição revelaram que, ao ser alterada a região constante dos anticorpos, eles passaram a competir por sítios diferentes de ligação. Em ensaio de ELISA com GXM de-O-acetilado, mIgG3 foi o único a se ligar com alta afinidade ao carboidrato modificado (30).

Dessa forma, um importante dogma da imunologia, que define que a especificidade e afinidade do anticorpo são determinadas exclusivamente pela região variável, pode ser questionado. Além de experimentos utilizando diferentes conjuntos de anticorpos contra *C. neoformans* demonstrarem que a região constante dos anticorpos influencia a região variável, pelo menos outros doze diferentes sistemas antígeno-anticorpo reportaram o mesmo (35-44). Uma possível interpretação dessas observações é que talvez a CH seja capaz de afetar a estrutura secundária do sítio de ligação ao antígeno presente no paratopo, contribuindo assim para variações na interação anticorpo-antígeno (23).

Para compreender o mecanismo pelo qual diferentes isotipos IgG com região variável idêntica alteram a ligação ao antígeno, os anticorpos 2H1-mIgG1 (PDB: 2H1P) (45) e 3E5 mIgG3 (PDB: 4HDI) (46) tiveram sua estrutura cristalográfica Fab resolvida. Estudo utilizando espalhamento de raios X a baixo ângulo (SAXS) para análise conformacional de 2H1 e 3E5 postulou que o padrão de ligação entre os isotipos pode ser alterado devido a diferenças na angulação da cadeia constante geradas pela região de dobradiça e pela região de junção entre o CH1 e a região variável (23, 46, 47). Além disso, modelos estruturais dos anticorpos 3E5 sugerem que as diferenças na ligação entre os isotipos sejam resultantes de polimorfismos na sequência de resíduos de aminoácidos da região constante (13).

Receptor de mIgG3

Embora mIgG3 tenha sido descoberto há décadas, até o momento, não há consenso na literatura sobre qual é o receptor que interage esse anticorpo (48-50). Em 1981, Diamond e Yelton sugeriram pela primeira vez que mIgG3 teria um receptor diferente dos demais isotipos IgG (50). No entanto, em 1998, Gavin et al. publicaram um estudo que demonstrava a interação de IgG3 com o receptor FcγRI, porém com fraca intensidade (49). Então, estudo publicado em 2010 por Saylor et al. afirmou que mIgG3 tem um receptor independente de FcγR, ou seja, que o receptor do anticorpo não é FcγRI. Além disso, Saylor também evidenciou que a fagocitose mediada por mIgG3 é independente de receptor de complemento (CR) (48). Recentemente, estudo de citometria de fluxo e fagocitose envolvendo macrófagos silenciados com sequências de short hairpin RNA (shRNA) revelou outra molécula, a integrina beta 1(Itgb1), como um importante efetor na resposta contra o patógeno *C. neoformans* mediada por mIgG3 (51).

Para compreender melhor o papel das integrinas na interação com mIgG3, é necessário avaliar a complexidade dessas proteínas. As integrinas fazem parte de uma família de receptores heterodiméricos α/β de 90 a 160 kDa que se apresentam na superfície da maioria das células metazoárias. Em mamíferos existem 24 subunidades α e 9 subunidades β, entre elas, a composição de interesse neste trabalho é a α4 (CD49d) e β1 (CD29), também conhecida como integrina α4/β1 ou *very late antigen-4* (VLA-4). Devido a sua diversidade, as integrinas medeiam muitas funções celulares, sendo a maioria delas relacionada à adesão (52-54). Além disso, as integrinas β1 e β2 atuam como mediadores de fagocitose por receptores de complemento (55, 56). A integrina β1, particularmente, tem sido associada a fagocitose de patógenos microbianos por macrófagos (57) e a eficiência de seu papel fagocítico requer o papel de um correceptor (58). A proteína possui estrutura cristalográfica resolvida (PFB:5XQ0 e P13612) e sítio de ligação às proteínas da matriz celular conhecido como arginina-glicina-ácido aspártico (RGD). Além disso, há uma região à qual anticorpos interagem e modificam o sítio de ligação da integrina, modulando a resposta da proteína, denominado sítio de ligação induzido por ligante (LIBS), entretanto o mecanismo pelo qual essa modificação ocorre é desconhecido. Ainda, as integrinas podem ser moduladas por íons divalentes que atuam como inibidores (Ca^{2+}) ou ativadores (Mg^{2+}, Mn^{2+}) em diferentes sítios da cadeia α/β (54, 59).

Justificativa

O estudo de mIgG3 sobre sua influência na ligação ao antígeno e em receptores celulares é justificado pelas possíveis implicações no entendimento da função de mIgG3 na imunidade frente à microorganismos, na patogênese de doenças e na engenharia de anticorpos com diferentes aplicações biotecnológicas.

mIgG3 é o principal isotipo produzido contra carboidratos e epítopos em repetição, sendo importante na fagocitose de patógenos e ativação da via clássica do sistema complemento. O anticorpo é efetivo na resposta frente à infeções microbianas importantes em humanos, tais como as causadas por: *C. neoformans*, *Neisseria meningitidis (11)*, *Bacillus anthracis (60)*, *Streptococcus* do grupo A, *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, *Plasmodium yoelii* (35-44, 61), entre outros. Como respostas importantes mediadas por anticorpos acontecem dependentemente de receptores associados à membrana de leucócitos, estudar à qual receptor mIgG3 interage e caracterizar essa interação é importante para o entendimento de sua resposta efetora.

Por outro lado, tanto IgG3 de camundongos quanto de humanos têm sido associados à glomerulonefrite e lesões de pele, presentes em doenças autoimunes, tais como lúpus eritematoso sistêmico e artrite rematóide (62-64). O isotipo pode causar nefrite lúpica, visto que demonstra reatividade contra DNA e cromatina renais. Esse mecanismo foi investigado produzindo anticorpos cuja região variável anti-DNA era conservada e as regiões constantes eram alteradas para γ1, γ2a, γ2b e γ3. Com isso, diferentes reatividades ao DNA e cromatina foram encontradas (63). Esse resultado nos leva a interpretar que a região constante pode apresentar papel importante no mecanismo da nefrite lúpica e de outras doenças autoimunes. Provavelmente esse mecanismo de autorreatividade ocorre durante a mudança de classe das imunoglobulinas relizada por linfócitos B.

Ainda referente à patogênese mediada por mIgG3, o anticorpo foi encontrado também agravando a criptococose. Estudos realizados por Casadevall et al. com diferentes conjuntos de anticorpos (26-29) demonstraram que a forma que o anticorpo se liga ao antígeno pode estar associada à sua protetividade.

Referente às aplicações biotecnológicas, a caracterização da integrina como receptor de IgG possibilitaria a geração de uma tecnologia exclusiva, podendo apresentar aplicações interessantes. Com base no entendimento do mecanismo protetor desempenhado pela interação das proteínas, seria possível desenhar anticorpos com paratopo contra um antígeno alvo e a fração Fc com afinidade à integrina e assim, potencializar a atividade terapêutica dessas moléculas. Conhecendo bem as interações entre as proteínas, seria possível investigar

se esse seria o mecanismo pelo qual mIgG3 causa doença, possibilitando o desenvolvimento de uma terapia adequada. Tendo em vista que a alteração da cadeia constante é comum no desenvolvimento de anticorpos terapêuticos, conhecer a região da cadeia constante capaz de alterar a ligação ao antígeno é importante para evitar que um anticorpo se torne não protetor ou reativo após sua administração.

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II- Objetivos

O objetivo geral deste trabalho é compreender o papel de mIgG3 em sua interação com o antígeno e receptores celulares. Dessa forma, os objetivos específicos estão detalhados a seguir:

- Elucidar o mecanismo pelo qual a região constante dos anticorpos IgGs influencia a ligação da região variável ao antígeno (Capítulo I).

- Investigar se Itgb1 é o receptor de mIgG3 (Capítulo II).

- Caracterizar interação entre IgG3 e integrina (Capítulo III).

Capítulo I- Estudo de interação entre IgG3 e antígenos

Descrição

Durante o projeto de mestrado realizado pela autora deste trabalho, foi desenhado e produzido um par de anticorpos IgG recombinante, denominado 2H1. Os anticorpos foram desenhados com uma região constante diferente (γ1 e γ3) e com uma região variável idêntica, específica à GXM de *C. neoformans*, assim como os anticorpos 3E5, 18B7 e 4H3 mencionados anteriormente. Interessantemente, os anticorpos 2H1 geraram um padrão de ligação imunofluorescente semelhante ao da literatura, em que mIgG1 se ligou de forma anular e mIgG3 se ligou de forma puntiforme à cápsula do fungo. Durante o doutorado, esses anticorpos passaram a ter seu mecanismo de ligação investigado. Dessa forma, a especificidade dos anticorpos passou a ser avaliada, assim como a importância da região de dobradiça e CH1 para ligação ao antígeno.

O trabalho deste capítulo é fruto de uma colaboração com o prof. Dr. Arturo Casadevall, que há mais de três décadas trabalha produzindo diferentes anticorpos para tratamento da criptococose. A partir dos trabalhos do Dr. Casadevall surgiram anticorpos que estão presentes em muitos dos estudos que investigam a diferença da especificidade e afinidade de diferentes classes e isotipos. Os anticorpos 2H1, 18B7, 3E5, 12A1 e 13F1 (Quadro 1), por exemplo, estão envolvidos em várias publicações, inclusive foram importantes neste projeto. A razão de estudar anticorpos que tenham *C. neoformans* como alvo é que o fungo serve como um modelo de ligante de fácil avaliação a partir de experimentos de imunofluorescência e fagocitose e ainda, é muito bem estabelecido na literatura e em nosso grupo de pesquisa.

A investigação da influência da região constante na ligação ao antígeno gerou o trabalho de primeira autoria intitulado: “Hinge Influences in Murine IgG Binding to *Cryptococcus neoformans* Capsule”, que está em submissão em revista Qualis A1 e aguarda o aceite.

Anticorpo	Alvo antigênico	Isotipo	Padrão de ligação	Protetor	Método de produção	Célula
2H1	GXM	mIgG1	Anular	Sim	Recombinante	NS0, CHO DHFR-/-
		mIgG3	Puntiforme	Não testado		NS0, CHO DHFR-/-
		2H1-mIgG3-CH1a-1	Puntiforme			CHO DHFR-/-
		2H1-mIgG3-CH1b-1	Puntiforme			
		2H1-mIgG3-CH1c-1	Puntiforme			
		2H1-mIgG1-h-3	Puntiforme			
		2H1-mIgG3-h-1	Anular			
3E5	GXM	IgG1	Anular	Sim	Hibridoma por ascite	NS0
		IgG2a	Anular	Sim		
		IgG3	Puntiforme	Não		
18B7		IgG1	Anular	Sim	Hibridoma por ascite	
12A1		IgM	Anular	Sim	Hibridoma por ascite ou cultivo *in vitro*	
13F1		IgM	Puntiforme	Não		
MOPC	-	IgG1	-	-	Hibridoma por ascite	
FLOPC		IgG3				

Quadro 1 - Anticorpos utilizados nos experimentos do capítulo I

Hinge Influences in Murine IgG Binding to *Cryptococcus neoformans* Capsule.

Abstract

Decades of studies on antibody structure led to the tenet that the V region binds antigens while the C region interacts with immune effectors. Several studies, however, have highlighted antibodies in which the C region affects affinity and/or specificity for the antigen. One such case is that of the 3E5 antibodies, a family of monoclonal murine IgGs (mIgGs) in which the IgG3 isotype has different fine specificity to the *Cryptococcus neoformans* capsule polysaccharide than the other IgG isotypes. During studies with recombinant antibodies, our group serendipitously found another pair of mIgG1/mIgG3 antibodies based on the 2H1 hybridoma sequences to the *C. neoformans* capsule that recapitulated the differences observed with 3E5. In this work, we report localization of the molecular basis of the constant domain effects on antigen binding using recombinant antibodies. As with 3E5, immunofluorescence experiments show a punctate pattern for 2H1-mIgG3 and an annular pattern for 2H1-mIgG1, which is associated with non-protection and protection, respectively. Also as observed with 3E5, 2H1-mIgG3 bound on ELISA to both acetylated and non-acetylated capsular polysaccharide, whereas 2H1-mIgG1 only bound well to the acetylated form, consistent with differences in fine specificity. Engineering hybrid mIgG1/mIgG3 antibodies, we found that switching the 2H1-mIgG3 hinge for its mIgG1 counterpart changed the immunofluorescence pattern to annular, but a 2H1-mIgG1 antibody with a mIgG3 hinge still had an annular pattern. The hinge is thus necessary but not sufficient for these changes in binding to the antigen. This important role for the constant region in binding of antibodies to the antigen could affect the design of therapeutic antibodies and our understanding of their function in immunity.

Introduction

Since their discovery in 1890 (1), antibodies have been one of the most studied and most useful biomolecules. Their enormous versatility for both the immune response in animals and for biotechnology is in great part due to the fact that they are bifunctional, with a highly variable end that binds to antigens with exquisite specificity and a constant end that is bound by receptors in immune effector cells. More than half a century of studies on the structure and function of antibodies, starting with seminal work by Porter and Edelman (2-4), have shown that the prototypical IgG molecule is a glycosylated heterotetramer composed of

two Fab domains connected to an Fc domain by a flexible hinge (5). The heavy and light chain monomers form complete immunoglobulin (Ig) molecules that can be classified into five types: IgG, IgM, IgA, IgE, and IgD (6-8). Among the classes, the IgG isotypes are the most studied because they engage specific Fc receptors and their effectiveness in neutralizing pathogens and toxins, opsonizing targets for phagocytosis and antibody-dependent cellular cytotoxicity, activating complement and modulating the inflammatory response. Due to their numerous functions, IgG antibodies naturally play crucial roles in both health and in disease (9), are the basis for dozens of drugs used to treat illnesses (10, 11) and are widely used as diagnostic and biotechnological tools.

Until recently, the most widely accepted model for antibody structure is one in which specificity and affinity for the antigen is determined solely by the V region, whereas the C region determines antibody effector functions and class/isotype. This concept emerged from the understanding that V and C regions functioned independently without affecting each other's activity. Since the 1980's, a series of antibody studies have questioned this classical definition, instead suggesting intramolecular or allosteric cooperativity between antibody C and V regions (12-20). Evidence for this comes mostly from experiments in which different IgG isotypes with identical variable region had differences in antigen binding. Such evidence suggests that the structure-function model of the antibody molecule needs to revision.

Studies from our group have demonstrated the impact of the C region on antigen affinity and specificity using *Cryptococcus neoformans* as a model with different antibody sets (14-17, 21). The most studied set of identical V region antibodies is the 3E5 IgG1, IgG2a, IgG2b and IgG3 hybridoma mAbs. The specificity, affinity and structure of these antibodies has been extensively studied by immunofluorescence, ELISA, phagocytosis assay, ITC, SPR biosensor, molecular modeling, CD, NMR, fluorescence emission spectroscopy, molecular dynamics simulations, SAXS and X-ray crystallography. Immunofluorescence experiments using 3E5 have demonstrated that 3E5-IgG3 binds to the *C. neoformans* capsule in a punctate pattern, which differs from the annular pattern observed with other IgG isotypes. ELISA with regular (acetylated) and de-O-acetylated capsular polysaccharide demonstrated that 3E5-IgG3 had a much higher affinity for the de-O-acetylated polysaccharide when compared with the other IgG isotypes, whereas all bound acetylated GXM with similar affinities. Both observations indicate an influence of the C region in antigen binding in 3E5. Strikingly, this paralleled a difference in passive immunization experiments with mice: IgG3 is non-protective or disease enhancing, whereas the other IgG isotypes protected mice infected with

C. neoformans (17, 22). Circular dichroism further demonstrated that IgG3 antibodies allosteric changes to their structure upon exposure to antigen. These and other results suggest that there is a strong correlation between the protective mechanism of antibodies and antigen binding. However, the strength of coupling between V and C regions appears to vary for different combinations such that some are more conducive to structural communication than others (PMID: 26870003).

Changing the C region while maintaining the same V region is a crucial step in the biotechnological development of therapeutic and diagnostic antibodies. It is also very important for an effective immune response in animals, which switch antibody isotypes depending on the type of antigen present. Given that C region changes can not only affect the paratope, but transform a protective antibody into a disease-enhancing one, clarifying these effects is important both for our understanding of the immune system and also for the safe and effective development of therapeutic antibodies. While carrying out studies on the mIgG3 receptor we generated recombinant IgG1/IgG3 antibodies to the *C. neoformans* capsule that serendipitously showed the same difference in immunofluorescence pattern as the 3E5 antibodies. These recombinant antibodies are based on the V region sequences of the 2H1 hybridoma, originally an IgG1 mAb against the *C. neoformans* capsule that differs from 3E5 in four positions on the VK sequence and eight residues on the VH domain. In this work, we followed up that observation by engineering these IgG1/IgG3 antibodies to switch CH1 and hinge regions between the mIgG1 and mIgG3 isotypes to understand the structural mechanism that leads to their differences in specificity and affinity to their antigen.

Materials and Methods

Fungal strains and culture

We used the wild type H99 (serotype A, ATCC® 208821™) and the Cas1 knockout mutant *(cas1Δ*) strains of *C. neoformans*. The Cas1 gene encodes an O-acetyltransferase that is necessary for acetylation of capsular polysaccharides (23). Yeast cells were maintained in Sabouraud agar plates and grown in Sabouraud dextrose broth (Difco) or minimal medium (0.3% glucose, 13 mM glycine, 29.4 mM KH_2PO_4, 10 mM $MgSO_4$, 3 μM thiamine, pH 5.5) at 30°C with agitation for 2 days.

Cell lines

The following cell lines were used: J774.16 – murine macrophage-like cells; CHO dhFr-/- (ATCC® CRL-9076) – Chinese hamster ovary cells lacking dihydrofolate reductase; NS0 (ATCC® PTA-3570) – murine myeloma. J774 and CHO dhFr-/- cells were grown at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Media (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (from South American origin), 10% NCTC-109 Media (Thermo Fisher) and 1% non-essential amino acids (Thermo Fisher). NS0 cells were maintained in the same supplemented medium and were grown in CD Hybridoma chemically defined medium (Thermo Fisher) in a 37°C incubator with 125 rpm shaking and 5% CO_2. Adherent cells were grown on tissue culture treated plates (BD Falcon) and removed by treatment with 3-5 mL trypsin (Gibco) then pelleted by centrifugation at 300 x g for 10 min, and finally re-suspended at the appropriate concentration in DMEM.

Exopolysaccharide (EPS) purification

H99 and *cas1Δ* cells were grown in minimal media for five days. Cells were removed by centrifugation and filtration, and the supernatants concentrated by ultrafiltration with a 100k Da membrane (Millipore) (24). The viscous layer containing EPS was harvested and dialyzed against distilled water. The EPS was then lyophilized, weighted and dissolved in ultrapure water. H99 EPS was de-O-acetylated by alkaline hydrolysis in ammonium hydroxide (pH 11.25–11.50) overnight (17, 25).

Nuclear magnetic resonance (NMR)

C. neoformans EPS was prepared as indicated above. After lyophilization, the < 100 kDa fraction was solubilized in deuterated water (D_2O). 1D [^{1}H] NMR (600 MHz) spectra were recorded on Bruker spectrometers equipped with Avance II console and triple resonance, TCI cryogenic probe with z-axis pulsed-field gradients at 30 °C in water-d2 (D_2O). [^{1}H] NMR spectra were standardized against the residual solvent peak (internal D_6DSS, δ = 0.00 ppm). All experiments were conducted with 64, 128, or 256 scans and an FID size of 16384 points. Standard Bruker pulse sequences were used to collect the 1D data (p3919gp and zggpw5). Data were processed in Topspin (Bruker version 3.5) by truncating the FID to 8192 points, using a squared cosine bell window function, and zero filling to 65536 points.

Hybridoma antibodies

The *C. neoformans*-specific monoclonal antibodies used in these studies were originally isolated following immunization of mice with GXM conjugated to tetanus toxoid (26). mAb 3E5 are mIgG1, mIgG2a, mIgG2b or mIgG3 (27), whereas mAbs 12A1 and 13F1 are IgM (26-29).

Recombinant antibodies - Synthetic genes and cloning

Due to 2H1 antibody (mIgG1) being the only antibody to the *C. neoformans* besides 3E5 to have a crystallographic structure (30, 31), we decided to create a recombinant mIgG1 and mIgG3 mAbs with identical 2H1 V regions. To produce 2H1 and 2H1-hybrid mIgG1/mIgG3 recombinant antibodies, the heavy (VH) and light (VL) variable regions of 2H1 and 2H1-hybrid antibodies were codon-optimized using a proprietary algorithm and synthetized by GenScript and then cloned into the commercially available pFUSE vectors (InVivoGen). These vectors contain the C region sequences for murine (strain 129S) IgG1 and IgG3. The 2H1 VH sequences were inserted into the pFUSE-CHIg-mG1 and pFUSE-CHIg-mG3 vectors to generate the pFUSE-CHIg-2H1-mIgG1, pFUSE-CHIg-2H1-mIgG3. The 2H1-hybrid antibodies were created by changing the CH1 or hinge sequences between 2H1-VH_pFUSE-CHIg-mG1and pFUSE-CHIg-2H1-mIgG3, resulting in these five vectors: 2H1-VH_pFUSE-CHIg-mG3-CH1a-1, 2H1-VH_pFUSE-CHIg-mG3-CH1b-1, 2H1-VH_pFUSE-CHIg-mG3-CH1c-1, 2H1-VH_pFUSE-CHIg-mG1-h-3 and 2H1-VH_pFUSE-CHIg-mG3-h-1. Briefly, “a-1” (KTTPPSVYPLAPGSAAQTNSM), “b-1” (TWNSGSLSSGVHTFPAVLQSD) and “c-1” (LYTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKK) are fragments present in the vectors above which compose CH1 sequence originated from mG1; and “h” is the hinge sequence that is originated from mG1 (VPRDCGCKPCICT) or from mG3 (EPRIPKPSTPPGSSCP). The VL sequences for 2H1 were inserted into the pFUSE2-CLIg-mK vector to generate the 2H1-VL_pFUSE2-CLIg-mKvector. All constructions were confirmed by digestion, PCR and Sanger sequencing before transfection.

Production of recombinant antibodies

NS0 cells were seeded at 8 x 10^5 cells/mL in 24-well plates 24 h before transfection and then co-transfected with the following combination of vectors: 1) 2H1-VH_pFUSE-CHIg-mG1 + 2H1-VL_pFUSE2-CLIg-mK to produce 2H1-mIgG1; 2) 2H1-VH_pFUSE-CHIg-mG3 + 2H1-VL_pFUSE2-CLIg-mK to produce 2H1-mIgG3 (Supplemental material). Similarly, CHO dhFr-/- cells were cultured and co-transfected with 2H1 heavy and light chain and also with DHFR vector to produce 2H1-mIgG3-CH1a-1, 2H1-mIgG3-CH1b-1, 2H1-mIgG3-CH1c-1, 2H1-mIgG1-h-3 and 2H1-mIgG3-h-1 antibodies. Transfections were made using Lipofectamine 2000 (Invitrogen), following manufacturer instructions. Approximately 72 hours post-transfection, the cells were transferred to 6-well plates and selection started by addition of Zeocin (Invitrogen) at 2 mg/mL and Blasticidin (Gibco) at 10 µg/ml. After three weeks of selection, stable antibody producing cells were obtained. Antibody producing NS0 were adapted to the serum free medium CD Hybridoma AGT (ThermoFisher), supplemented with 1x cholesterol (Gibco) and 8 mM L-Glutamine (Sigma Aldrich). Transfected CHO dhFr-/- cells were kept in negative selection of HT and the gene amplification was done using MTX at 10 µM/well. The culture volume was then increased to 1 L to produce the first lot of recombinant antibodies. Subsequent lots of the recombinant antibodies were produced by thawing and expanding frozen antibody producing cells adapted to serum-free medium, obtained as described above.

Purification and concentration of recombinant antibodies

The purification of 2H1 from NS0 cells consisted of affinity chromatography using rProtein A/Protein G GraviTrap (GE Healthcare). The antibodies were eluted with a 0.1 M glycine-HCL pH 2.7 solution and immediately neutralized with a 1 M Tris-HCl 1 M NaCl pH 9.0 buffer to maintain their stability in solution, then concentrated by ultrafiltration. The 2H1 antibodies from CHO cells supernatant were concentrated in Amicon (Millipore, Danvers, MA) ultrafiltration cells (cutoff 30 kDa).

Enzyme-linked immunosorbent assay (ELISA)

In solutions with low immunoglobulin amounts the concentration of recombinant antibodies was measured by direct ELISA with antigen. For the antigen-specific ELISA,

serial dilutions of IgG1 (18B7) or IgG3 (3E5) purified antibodies that had been previously quantified by direct ELISA were used as standards. Plates were coated with 10 µg/mL GXM purified from *C. neoformans* H99 cultures dissolved in PBS. Dilutions of the standards and recombinant antibodies were added and detected with isotype-specific goat anti-mouse polyclonal antibody. For the direct ELISA, plates were coated with serial dilutions of purified myeloma IgG1/k (MOPC 21) and IgG3/k (FLOPC 21) standards (Cappel) and dilutions of the recombinant antibodies. After blocking with bovine serum albumin (BSA), the bound antibodies were detected with alkaline phosphatase-conjugated isotype-specific goat anti-mouse polyclonal serum (Southern Biotech).

Competition assays between the 2H1 and 12A1/13F1 were performed by adding a constant amount of one mAb (0,2 to 5 µg/ml) and varying the concentration of another antibody of a different isotype (0 to 100 µg/ml) in an antigen-specific ELISA assay. Ab binding to de-O-acetylated GXM was measured by antigen-specific ELISA, as described above.

C. neoformans phagocytosis assay

Phagocytosis assays were performed in 96 well tissue-culture treated plates (BD Falcon) containing J774.16 cells plated at least 2 h before the experiment. Then, the *C. neoformans* suspension with opsonizing Ab was added at 10 µg/mL, with a macrophage to *C. neoformans* ratio ranging from 1:1 to 1:2. Phagocytosis was allowed to proceed for 2 h at 37°C in 5% CO_2. Cells were then washed, fixed with methanol at -20°C for 30 minutes and finally stained with Giemsa. Cells were then analyzed under an inverted microscope, counting three fields/well, with at least 100 cells/field. Percent phagocytosis was calculated as the number of macrophages containing one or more internalized *C. neoformans* divided by the total number of macrophages visible in one field. Each experimental condition was done in triplicate.

Indirect immunofluorescence

The pattern of antibody binding to the *C. neoformans* capsule was evaluated by epifluorescence microscopy. H99 cells were cultivated overnight, washed and diluted to 10^6 - 10^7/mL concentration. The cell suspensions were then incubated with antibodies at 10 µg/mL for 1 h at 37°C. After washing with PBS, bound antibodies were detected with an Alexa

Fluor® 488-conjugated isotype-specific goat anti-mouse polyclonal serum (*Thermo Fisher Scientific*) for 1 h at 37°C. After washing, stained fungi were mounted on slides with ProLong® Gold Antifade Mounting medium (*ThermoFisher*) and viewed with Zeiss Axio Observer Z1 microscope. For some cells, Z-stacks were collected and subjected to a constrained iterative deconvolution algorithm on Zeiss ZEN software, followed by processing and 3D reconstruction on ImageJ and VOXX2 softwares (17).

Statistical analysis

Phagocytosis assays were analyzed with Fisher's exact test with GraphPad Prism 6,0 software (CA, USA).

Results

Production and validation of recombinant antibodies

In order to understand the structural basis of the differences in IgG1 and IgG3 immunofluorescence pattern we had previously observed, we produced more of these two recombinant antibodies (2H1-mIgG1 and 2H1-mIgG3) and five new hybrid antibodies (Figure 1A). We hypothesized that the CH1 domain and the hinge are the C region domains most likely to affect the paratope structure and antigen binding, due to the IgG protein structure (31). Two hybrid antibodies were made to study the role of the hinge domain: 2H1-mIgG1-h-3 is an IgG1 whose hinge has been substituted by the IgG3 hinge and 2H1-mIgG3-h-1 is an IgG3 whose hinge has been swapped with the corresponding IgG1 sequence. The other three antibodies were used to study the role of the CH1 domain. 2H1-mIgG3-CH1a-1, 2H1-mIgG3-CH1b-1, 2H1-mIgG3-CH1c-1 are all IgG3 antibodies in which three different portions of the CH1 sequence were swapped by the corresponding IgG1 aminoacids. These portions a, b and c correspond to three different regions of the CH1 domain that differ between the isotypes (Figure 1A). Following the IMGT nomenclature, fragment a corresponds to beta-sheets A and B; fragment b includes beta-sheets C and D, loops BC and CD and part of loop DE; fragment c is composed of beta-sheets E and F and the remaining CH1 domain loops.

Following production and concentration, we confirmed that the recombinant antibodies bound to GXM by ELISA (Figure 1B). To determine if the antibodies could bind

to the capsule and mediate phagocytosis, we coated *C. neoformans* with 2H1 (mIgG1 and mIgG3) and hybrid 2H1 antibodies (2H1-mIgG3-CH1a-1, 2H1-mIgG3-CH1b-1, 2H1-mIgG1-h-3 and 2H1-mIgG3-h-1). The coated *C. neoformans* cells were then exposed to J774 cells, which phagocytosed fungi coated with 2H1-mIgG1 (74%), 2H1-mIgG3 (42%), 2H1-mIgG1-h-3 (40%) and 2H1-mIgG3-h-1 (39%) to a similar rate as a 3E5 hybridoma mAb positive control (Figure 1C and D). The CH1 hybrid recombinant antibodies were also functional, but mediated phagocytosis to a significant ($p<0.0001$) lower rate: 2H1-mIgG3-CH1a-1 (10%), 2H1-mIgG3-CH1b-1 (17%). We did not obtain 2H1-mIgG3-CH1c-1 in concentrations that were necessary for phagocytosis tests.

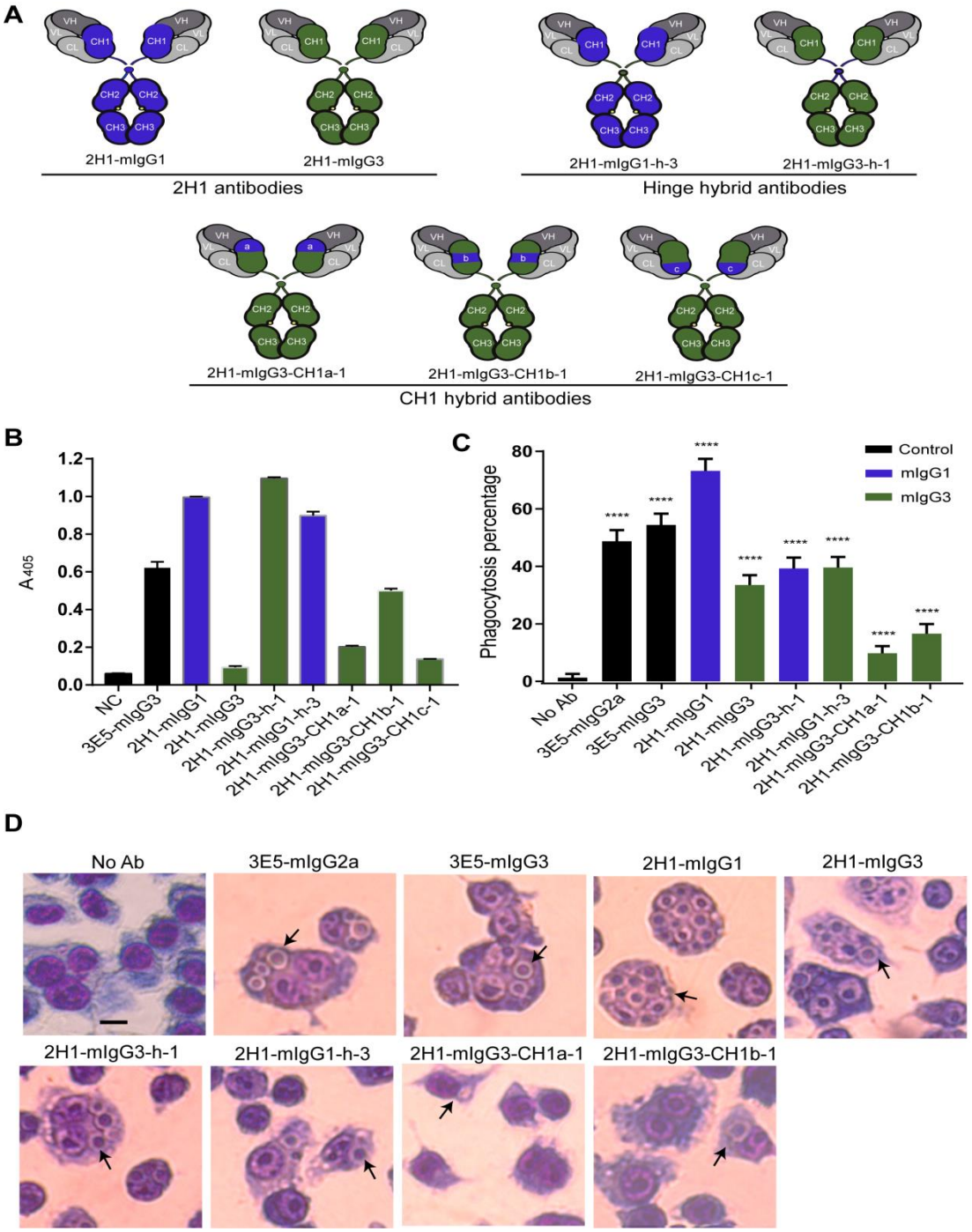

Figure 1– Production and validation of the recombinant antibodies. (A) Schematic representation of the recombinant antibodies. 2H1-mIgG1 sequences are represented in blue, whereas 2H1-mIgG3 are represented in green. The three different regions of the CH1 domain that were swapped in the CH1 hybrid antibodies are labeled as "a", "b" and "c". (B) ELISA experiment with *C. neoformans* capsular polysaccharide and the recombinant antibodies. Bars represent the mean absorbance from a duplicate experiment. (C) Phagocytosis assay with J774 cells and *C. neoformans* opsonized with 10 µg/mL of each recombinant antibody. Cells were co-incubated at a 1:2 (macrophage: yeast) ratio, stained and imaged. Bars represent the percentage of macrophages with at least one internalized fungal cell and the 95% confidence interval (Wilson/Brown). **** $p<0.0001$. (D) Representative images from the phagocytosis assay. Arrows point to macrophages with internalized yeast.

Hinge is necessary but not sufficient to change V binding to antigen

Having validated the recombinant antibodies, we next evaluated their binding pattern to the *C. neoformans* capsule. Immunofluorescence (IF) staining patterns have been considered good indicators of antibody-mediated protection, such that annular staining correlates with protection in animal models and punctate staining correlates with lack of protection or even disease enhancement (16, 32). As positive controls, we used two hybridoma-derived antibodies, 3E5-IgG2a and 3E5 IgG3, know to bind with annular and punctate patterns respectively (17). Our results matched these expected patterns (Figure 2A). The IF patterns for 2H1-IgG1 (annular) and 2H1-IgG3 (punctate) also matched those previously described for hybridoma-derived 2H1 (32) and for the recombinant antibodies. All three hybrid antibodies in which CH1 fragments were swapped by their IgG1 counterparts (2H1-mIgG3-CH1a-1, 2H1-mIgG3-CH1b-1 and 2H1-mIgG3-CH1c-1) bound with the punctate pattern expected for IgG3 antibodies. In contrast, the IgG3 antibody with an IgG1 hinge (2H1-mIgG3-h-1) bound with an annular pattern, demonstrating that the hinge is necessary for the punctate binding. The converse antibody, 2H1-mIgG1-h-3, presented the same annular pattern observed for 2H1-IgG1, which suggests that the IgG3 hinge is not sufficient for the punctate pattern. To confirm our observations, we collected Z-stacks that were deconvolved and 3D-reconstructed (Figure 2B).

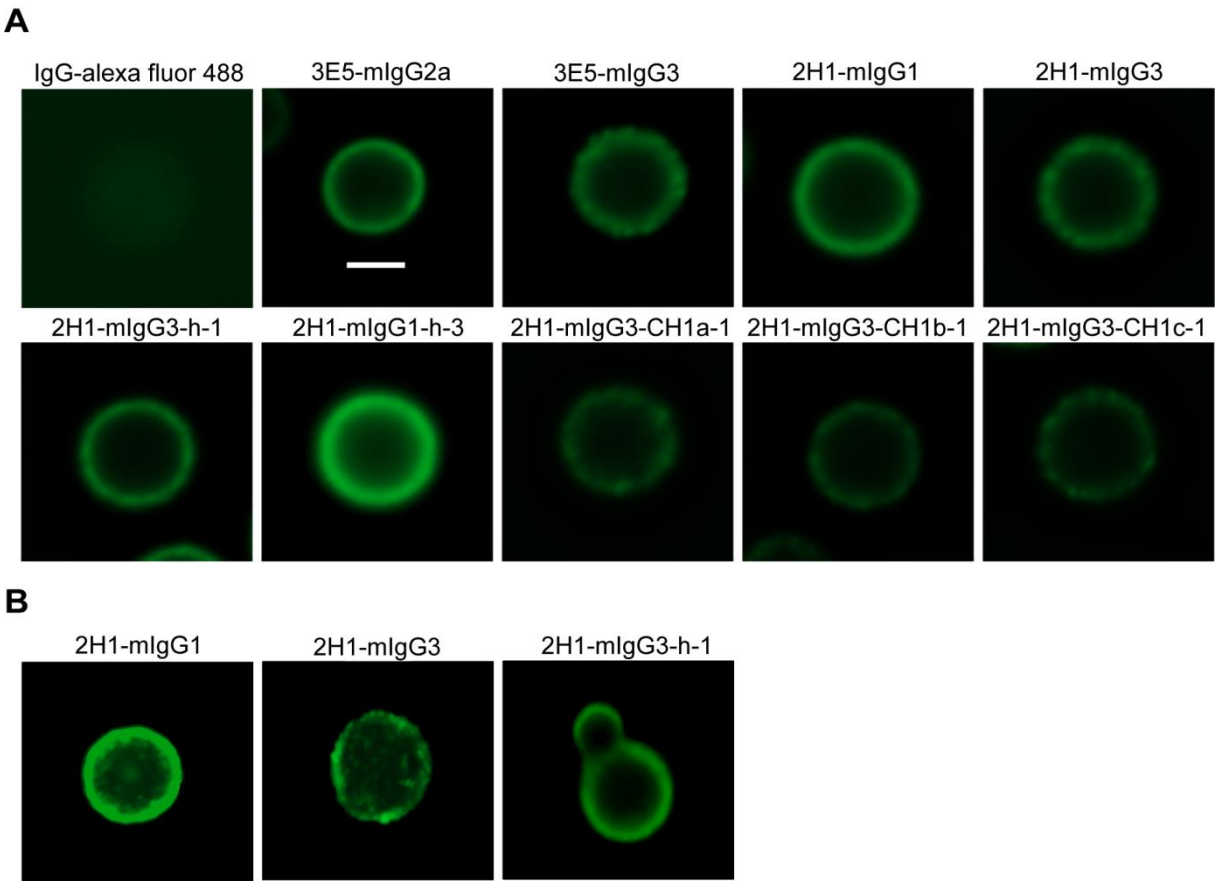

Figure 2 – Immunofluorescence pattern of 2H1 antibodies. A) Indirect immunofluorescence of 2H1 antibodies. 2H1-mIgG1 showed annular fluorescence pattern while 2H1-mIgG3 showed punctate pattern, similar to 3E5-mIgG2a and 3e5-mIgG3 patterns respectively. Antibody 2H1-mIgG1-CH1-h-3 showed annular pattern, while antibodies 2H1-mIgG3-CH1a-1, 2H1-mIgG3-CH1b-1 and 2H1-mIgG3-CH1c-1 were punctate. Only 2H1-mIgG3-h-1 changed its punctate pattern to annular. The control, which the fungus was incubated with secondary antibody, did not reveal significant fluorescent signal. Scale bar used :10 micrometers. (B) 3D representation of 2H1 immunofluorescence pattern. It can be clearly observed that immunofluorescence pattern of 2H1-mIgG3-h-1 was annular, which is similarly to the found with 2H1-mIgG1, but different from 2H1-mIgG3 punctate pattern.

2H1 antibodies bind to different epitope than 13F1

The competition assay to GXM among 2H1, 12A1 and 13F1 showed that 2H1-IgG1 and IgG3 antibody were able to recognize a similar epitope that 18B7 and 12A1 (Figure 3A); but they were not able to recognize the same epitope that 13F1 (Figure 3B).

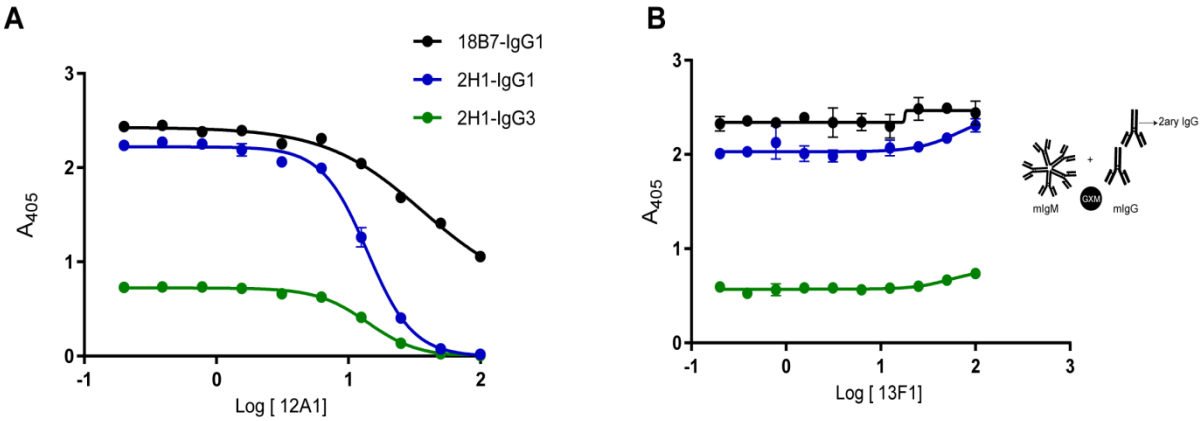

Figure 3 – 2H1 antibodies binding to GXM epitope. (A) Competitive GXM-ELISA with 2H1 and 12A1 antibodies. 2H1-mIgG1 (blue line), 2H1-mIgG3 (green line) and control antibody (black line) competed for same epitope of 12A1 antibody, an IgM that presents an annular fluorescence pattern. (B) Competitive GXM-ELISA with 2H1 and 13F1 antibodies. 2H1-mIgG1, 2H1-mIgG3 and control antibody did not compete for the same epitope of 13F1, an IgM that presents a punctate fluorescence pattern. The scheme in the figure represents the ELISA assay conditions. The graphs represent mean absorbance of duplicate wells.

2H1-mIgG3 binding to GXM is different than 2H1-mIgG1 binding

To better understand the differences between 2H1-mIgG1 and 2H1-mIgG3 binding to antigen, we tested their reactivity to native and de-O-acetylated EPS obtained from *cas1Δ* or chemical reaction (Figure 4A). We found that the relative strength of 2H1 binding to de-O-acetylated GXM was IgG3>IgG1 (Figure 4B). Consistent with this observation the 3E5 reactivity to de-O-acetylated GXM was similar(17). The antibody reactivity results reinforces IF patterns found to 2H1, 3E5 and 18B7. Because the switch variants have identical variable region the stronger binding by IgG3 isotypes suggests that C region may affect the V region specificity.

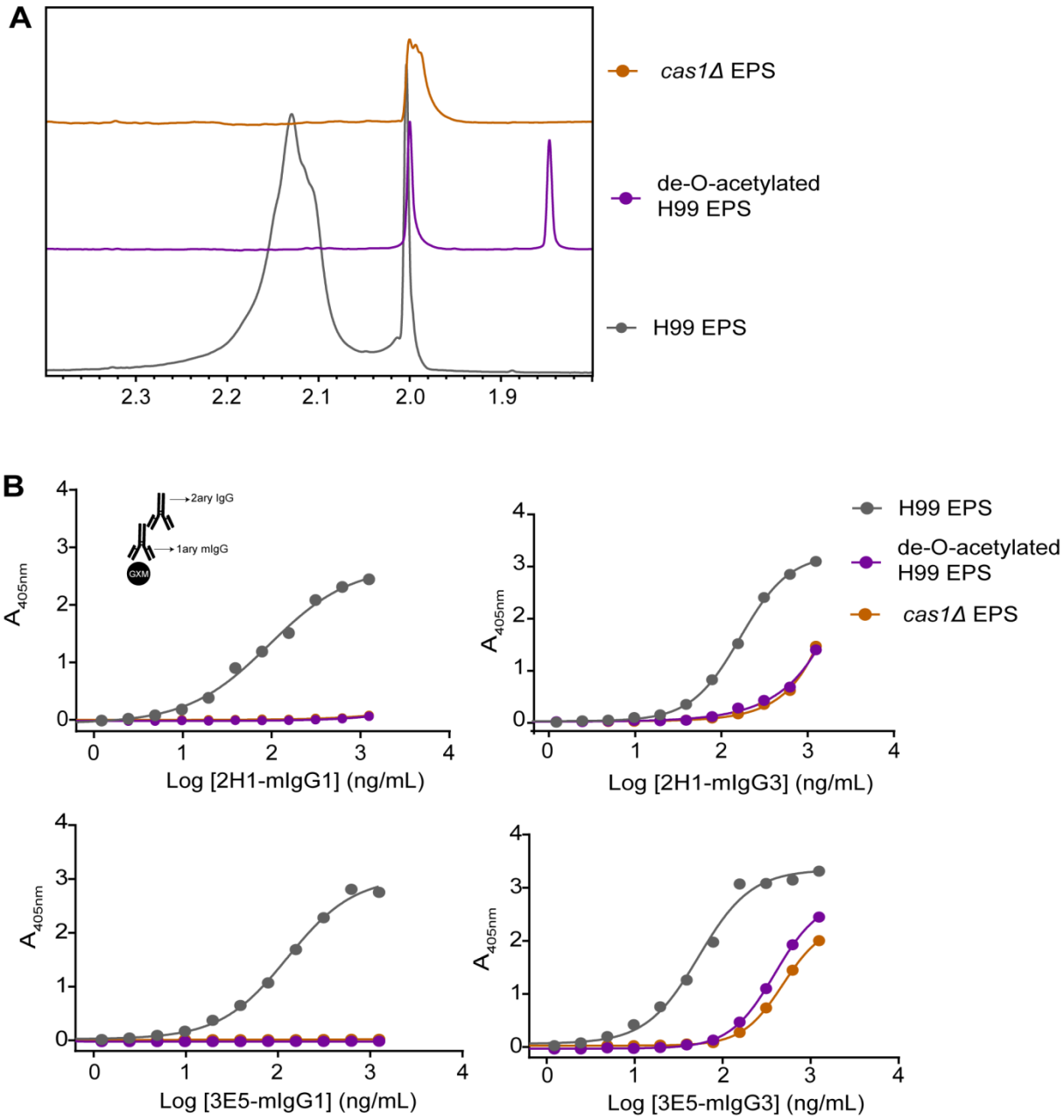

Figure 4 – 2H1 antibodies binding to de-O-acetylated GXM. (A) Analysis of EPS de-O-acetylation by NMR. The peak in range 2.0 represents de-O-acetylation fraction of *cas1Δ* (orange line), chemically modified EPS (purple line) and non-de-O-acetylated EPS (gray line). (B) Interaction ELISA of 2H1 antibodies to de-O-acetylated GXM. 2H1-mIgG3 antibody binds to de-O-acetylated GXM, while 2H1-IgG1 antibody does not bind. The graphs represent the mean absorbance of three technical replicate.

Discussion

The classical view is that variable and constant domains of immunoglobulin molecules function independently, providing antigen binding and effector functions, respectively (12, 13). However, for several antibodies changing the C region domains can cause differences in

specificity and affinity GXM (14, 15). Based in that, some IgGs (3E5, 18B7 and 4H3) which express different constant region but identical V regions have been extensively studied in the last 30 years (14, 16, 17, 21).

Indirect immunofluorescence of 3E5 and chimeric 18B7 isotypes revealed differences in binding to *C. neoformans* capsule when IgG3 and IgG1 coat the fungus, resulting in a punctate and annular pattern respectively (16, 17). The immunofluorescence pattern was also determined to 2H1 (mIgG1) which showed to be annular, as described before (35). Then, we created by genetic engineering a pair of 2H1 (mIgG1 and mIgG3) which corroborated the results obtained with 3E5 and 18B7, reinforcing the notion that the C region can play a role in antibody specificity. We specifically focused on the 2H1 antibody because it structure had been solved by crystallography (30).

GXM is the main component of *C. neoformans* capsule (36) and the original mAbs used in this study were generated from mice immunized with a protein-GXM conjugate vaccine (21). This and prior studies had shown that O-acetyl groups are required for the binding of protective antibodies to GXM (29). Our results revealed that recombinant 2H1-IgG3 still bound to GXM in absence of acetylation, as well 3E5-IgG3; whereas the binding of both 2H1-IgG1 and 18B7 decreased drastically. The fact that IgG3 is non protective antibody and still bound to GXM even when it was de-O-acetylated and that IgG1 almost lost GXM binding after GXM modification, led to interpret the antibodies isotypes recognizes different epitopes.

The competition assay was done to evaluate difference on specificity of 2H1-IgG1 and IgG3 antibodies. Our expectation about the experiment was if there is competition in binding between antibodies that they probably were able to recognize the same epitope or a spatially close one that interfered with binding through steric hindrance. However, absence of competition implies that the antibodies recognize different epitopes. When 2H1 was tested in competition assays with mAbs 12A1 and 13F1, we found that both 2H1-IgG1 and 2H1-IgG3 isotype competed with 12A1 but not with 13F1. Thus, 2H1 showed a variation in its ability to compete with 12A1: IgG1>IgG3, maybe because the influence of their different C regions. Because 12A1 and 2H1 use the same VH (IGHV5-6-2) and JH (IGHJ2) genes and they are classified as members of class II mAbs (21), the competition for the same epitope was expected (17). Although 13F1 also belong to the same family its VH mutations (34) can reveal residues important in binding to GXM that probably affected the competition with the other antibodies. As for the 2H1-IgG3 competition result, was found that 3E5-IgG3 – the

most 2H1 identical antibody – competed with 12A1 but not with 13F1 (34). Furthermore, there is evidence that IgM is more flexible than IgG and this can result in formation of Ag-Ab complexes with a particular geometry (16, 37).

Although several experiments have had done before to study the influences of C region on antibody binding (14-17), none have experimentally probed the mechanisms involved. Comparison of 2H1-IgG1 and 3E5-IgG3 crystal structures revealed the influence of flexible sequences between VH and CH1 domains (the elbow region). Also hinge was again described being important in Fab-Fc flexibility, as evaluated by SAXS and modeling experiments of 3E5 isotypes (31). Based on this information and on amino acid alignment of CH domains from mIgG isotypes we decided to evaluate the CH1 and hinge region influences on the V region by creating 2H1-mIgG1 and 2H1-mIgG3 recombinant antibodies with exchange between their C region. When hinge was exchanged in mIgG3 antibody, immunofluorescence resulted in conversion from a punctate to an annular pattern. We interpreted this result as indicating that the hinge probably affects the secondary structure of the antigen-binding site leading to changes in antibody specificity. However, when hinge was exchanged in mIgG1 antibody we did not observe a conversion from an annular to a punctate pattern. Thus it rises the hypothesis the hinge is important on antigen binding, but not essential. Also when the CH1 was exchanged we observed no effect on antigen binding, maybe because this region is not necessary or even not sufficient for causing changing in paratope specificity.

mIgG3 was a non-protective or disease enhancing antibody in mice infected with *C. neoformans* (38-40). Among IgG described to aggravate infection were 3E5-IgG3 and 4H3-IgG3 because when they were administrated on infected mice, the animals died even before the one who received saline buffer as treatment (22). Furthermore, the IgG3 isotype is reported to play a role on autoimmune diseases causing immunoprecipitation/autoreactivity as in glomerunonephritis and vasculitis (41-43). Once, just a little is known about the C effects in V antibody region; due to the importance that IgG has in several disease treatments (44); and also because IgG plays malefic role on cryptococosis and autoimmune diseases is relevant to understand the mechanism which the antibody uses to alter the paratope specificity and affinity. Following the advance in antibody engineering (44) we expected that the information about the hinge influences on paratope can be useful to improve Ab design, aiming to avoid creation of drugs potentially dangerous. Also, creation another set of IgG for recombinant

DNA and achievement of same immunofluorescence and Elisa results provided strong evidence about C region effects.

Acknowledgements

We would like to acknowledge Dr. Ildinete Silva Pereira from the University of Brasilia of Molecular Biology, the Genomic Sciences and Biotechnology facility at Catholic University of Brasilia and the Immunology Core at Johns Hopkins University for the technical support.

Disclosures

The authors have no financial conflicts of interest.

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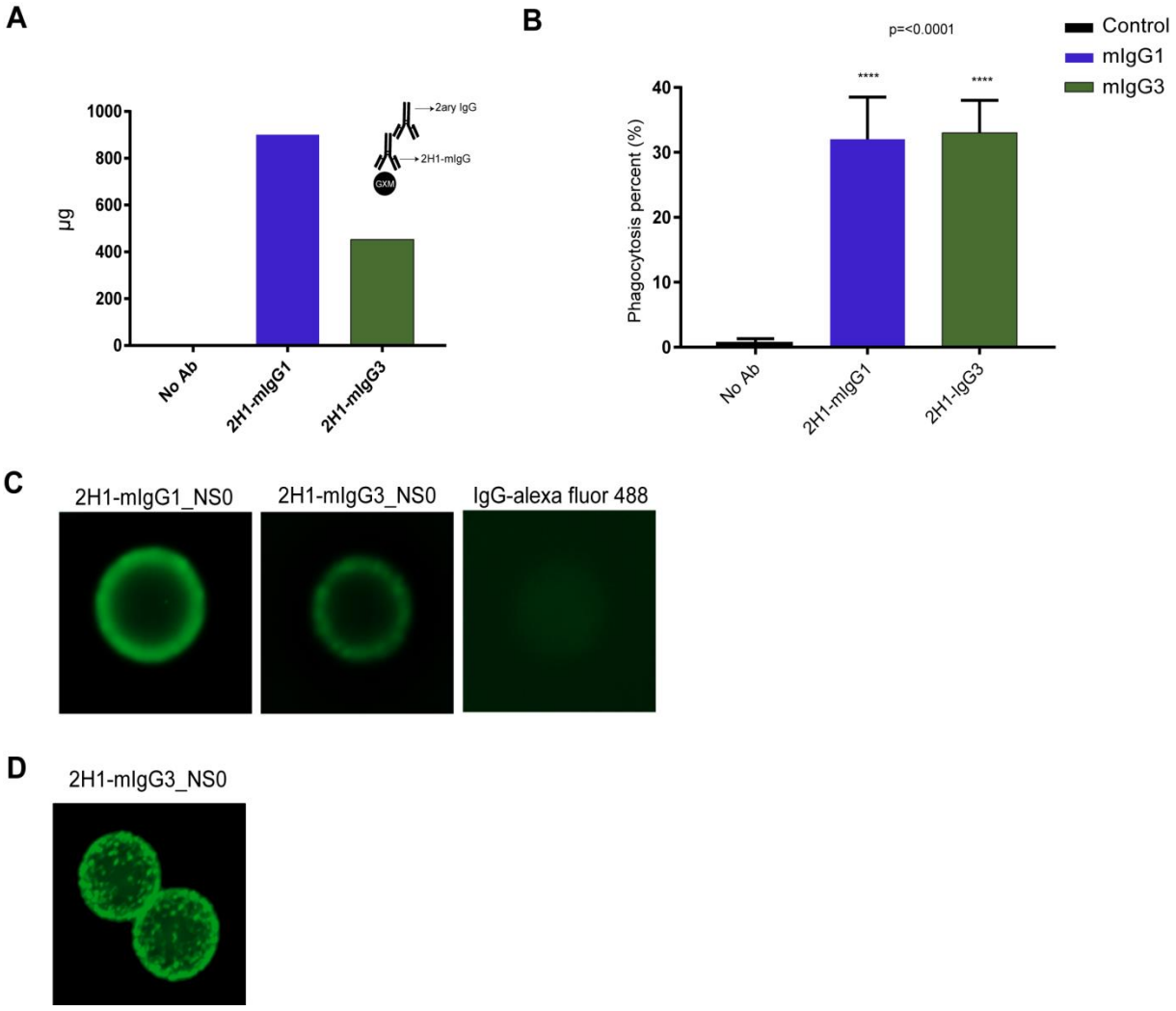

Supplementary figure – 2H1 antibodies from NS0 cells. (A) ELISA experiment with *C. neoformans* capsular polysaccharide and the recombinant antibodies. Bars represent the mean absorbance from a duplicate experiment. (B) Phagocytosis assay with J774 cells and *C. neoformans* opsonized with 10 µg/mL of each recombinant antibody. Cells were co-incubated at a 1:2 (macrophage: yeast) ratio, stained and imaged. Bars represent the percentage of macrophages with at least one internalized fungal cell and the 95% confidence interval (Wilson/Brown). **** p<0.0001. (C) Representative images from indirect immunofluorescence assay. (D) 3D reconstruction of 2H1-mIgG3_NS0 immunofluorescence pattern.

Capítulo II e III- Estudo da interação entre IgG3 e receptores

Descrição

O estudo da interação entre IgG3 e integrina surgiu do trabalho de doutorado de Carolyn Saylor, do qual o prof. Dr. André foi colaborador. Em 2011, os resultados do estudo foram submetidos para publicação, mas voltaram com uma lista de sugestões feitas pelos revisores. Entre elas estavam: a necessidade de caracterizar a interação bioquímica entre IgG e a integrina; a ausência de testes realizados com um par de anticorpo distinto do 3E5; a ausência de testes realizados com anticorpos que tivessem como alvo um antígeno que não fosse o de *C. neoformans*; e a carência de experimentos de ganho de função da integrina que reforçassem os resultados anteriores.

A autora do presente trabalho iniciou o mestrado nesta área com o objetivo de responder às perguntas descritas. Durante o projeto de mestrado, a aluna desenvolveu, por expressão heteróloga, um novo par de anticorpos contra *C. neoformans* (2H1) e também um novo par de anticorpos contra um hapteno, nesse caso o isotiocianato de fluoresceína (FITC). Também foi testada a interação desses anticorpos a receptores convencionais FcγR, sendo observado que mIgG3 apresentou uma dependência parcial deles. Dessa forma, o projeto de doutorado seguiu com o intuito de realizar experimentos que caracterizassem a interação entre o anticorpo e a integrina e que, por meio de outras estratégias, fortalecessem o achado anterior.

A fim de responder se Itgb1 é de fato o receptor de mIgG3, foi publicado em coautoria o trabalho que consta no Capítulo II deste trabalho: "Integrin β1 Promotes the Interaction of Murine IgG3 with Effector Cells". Nesse artigo, a autora desta tese colaborou com a produção, quantificação e validação dos anticorpos 2H1 e 4-4-20; experimentos de fagocitose com bloqueio de receptoroes de macrófagos (Figura 3D e F); e experimento de ganho de função (Figura 5). Após a publicação, a caracterização da interação entre mIgG3 e itgb1 continuava em questão, e a tentativa responder essa pergunta conduziu o trabalho presente no Capítulo III: "Binding Characterization between Murine Immunoglobulin γ 3 and Integrin α4β1". Algumas das especificações dos anticorpos utilizados em ambos os capítulos estão descritas no quadro a seguir:

Anticorpos monoclonais	Alvo antigênico	Isotipo	Método de Produção
4-4-20	FITC	IgG1	Mieloma em cultivo *in vitro*
		IgG3	
MOPC21	-	IgG1	Mieloma por ascite
UPC10	-	IgG2a	
UPC120	-	IgG2b	
FLOPC21	-	IgG3	
3E5	GXM	IgG1	Mieloma por cultivo in vitro
		IgG2a	Mieloma por ascite
		IgG3	Mieloma por ascite e cultivo *in vitro*
Bt1- Bt8		IgG3	Mieloma por ascite
Hmβ1.1	Murino Itgb1	-	-
2.4G2	Murino FcγRIII/FcγRII	-	
GAME-46	Murino CD18	-	
M1/70	Murino CD11b	-	
HL3	Murino CD11c	-	

Quadro 2 - Anticorpos utilizados em experimentos do capítulo II e III.

Capítulo II- Integrin β1 Promotes the Interaction of Murine IgG3 with Effector Cells

Abstract

Abs exert several of their effector functions by binding to cell surface receptors. For murine IgG3 (mIgG3), the identity of its receptors (and the very existence of a receptor) is still under debate, as not all mIgG3 functions can be explained by interaction with FcγRI. This implies the existence of an alternate receptor, whose identity we sought to pinpoint. We found that blockage of integrin β1 selectively hampered binding of mIgG3 to macrophages and mIgG3-mediated phagocytosis. Manganese, an integrin activator, increased mIgG3 binding to macrophages. Blockage of FcγRI or Itgb1 inhibited binding of different mIgG3 Abs to variable extents. Our results are consistent with the notion that Itgb1 functions as part of an IgG receptor complex. Given the more ancient origin of integrins in comparison with FcγR, this observation could have far-ranging implications for our understanding of the evolution of Ab-mediated immunity as well as in immunity to microorganisms, pathogenesis of autoimmune diseases, and Ab engineering.

Introduction

Antibodies are among the most-studied biomolecules in history. Starting with late 19th century studies on serum therapy that gave Emil von Behring the first Nobel Prize in Medicine, a steady stream of important discoveries clarified how B cells make Abs, B cell structure, and functions in both health and disease. This body of knowledge allowed harnessing of Abs as biotechnological tools with major applications in diagnosis and therapy of human and animal diseases. Behind their success, both as immune effectors and as biotechnological tools, is the fact that Abs are bifunctional molecules with a variable domain that recognizes the Ag and a constant domain that mediates effector functions. For the IgG Abs, many of these functions depend upon binding of the Ab Fcγ domain to host proteins such as complement or cell surface receptors, which in turn activate (or inhibit) immune cells and regulate immunity (1, 2). Thus, binding of IgG to host receptors is essential to such varied phenomena as phagocytosis of a pathogen by macrophages, tissue damage resulting from deposited immune complexes, and destruction of tumor cells by therapeutic anticancer Abs.

The detection, cloning, and sequencing of most cell surface IgG receptors date from research in the 1970s and 1980s (3). This family of proteins, called the "classical" FcγR, was completed by the discovery of FcγRIV, which was found by sequence homology with known FcγR a subunit (4). More recently, "nonclassical" Fc receptors have been added to this list; these known FcγR unambiguously explain functions of all human IgG isotypes and murine IgG1, IgG2a, and IgG2b (5, 6). Despite several decades of research, one major riddle remains in this field. In 1981, Diamond and Yelton posited that murine IgG3 (mIgG3) had its own receptor based on the observation that a clone of the macrophage-like J774 cell line lost the ability to phagocytose mIgG3-opsonized particles while retaining functionality with all other mIgG isotypes, indicating the existence of a distinct receptor for mIgG3 (7). In the late 1990s, another group reported that murine FcγRI, the high affinity FcγR, was responsible for mIgG3 function, based on the failure of FcγRI deficient murine bone marrow–derived macrophages (BMM) to internalize mIgG3-coated erythrocytes (8). However, subsequent studies have established that mIgG3 does not have measurable affinity for the classical FcγR (6). Furthermore, mIgG3 was found to be opsonic even when all the classic FcγR were absent or blocked (9). In other models, mIgG3 Abs mediated its function solely by activating complement, without evidence of binding to any cell surface receptor (10–12). Thus, the receptor whose function was missing in Diamond's 1981 subclone of the J774 cell line remains obscure, and the very existence of a mIgG3 receptor is uncertain.

The mIgG3 isotype is enriched during murine humoral response to carbohydrate Ags (13, 14). Additionally, mIgG3 frequently behaves as a cryoglobulin, being involved in autoimmune diseases such as glomerulonephritis or lupus-like skin lesions in mice (15–17). Technological interest in the mIgG3 isotype is marginal because mIgG3 mAbs are prone to aggregation as a result of in termolecular C region interactions (18), which decreases production efficiency, stability, and safety. However, engineering Abs to modify their binding to different FcγR and thus modulate pharmacological effects is an important strategy in mAb drug development (19). The existence and identity of this yet unknown non- FcγR IgG receptor could thus be relevant in immunology, immunopathology, and production of therapeutic Abs.

In this study, we show the results of a loss-of-function screening (followed by experimental validation with further loss-of-function and gain-of-function assays for several mIgG3 Abs), which indicates that integrin β1 (Itgb1) is either a receptor, or functions as part of a receptor complex, for mIgG3 in mouse macrophages. The finding of a non- FcγR for IgG

Abs could have a large impact on our understanding of the humoral immune response and on Ab engineering.

Materials and Methods

Mice

Wild-type C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), from colonies maintained at the Animal Facility of the Albert Einstein College of Medicine, were used to obtain the peritoneal macrophages. To obtain Itgb1-deficient (Itgb1$^{-/-}$) macrophages, a conditional knockout model had to be used because the Itgb1 knockout is embryonically lethal. Mice with loxP sites flanking exon 3 of the Itgb1 gene (20) were crossed with mice expressing the Cre recombinase under control of the lysozyme promoter (21). In all experiments, mice were treated in accordance with institutional guidelines, and the animal protocols were accepted by the Institutional Review Committee of the Albert Einstein College of Medicine, where the experiments were made.

Microbe strains

Cryptococcus neoformans strains 24067 (serotype D) and H99 (serotype A) were used. Yeast cells were grown overnight in Sabouraud dextrose broth (Difco) at 30˚C with agitation.

Cell lines

For experiments, the following cell lines were used: J774.16 (J774), murine macrophage-like cells; L929, murine fibroblast, producer of M-CSF; CHO-K1, Chinese hamster ovary (CHO) cells; and NS0, murine myeloma, used to produce rAbs. J774, L929, and CHO-K1 cells were grown at 37˚C and 5–10% CO_2 and maintained in DMEM supplemented with 10% FBS (from North American or South American origin) that was heat inactivated by treatment at 56˚C for 30 min, 10% NCTC-109 media (Thermo Fisher Scientific), 1% nonessential amino acids (Mediatech or Thermo Fisher Scientific), and 1% penicillin streptomycin solution (Mediatech or Thermo Fisher Scientific). NS0 cells were maintained in the same supplemented DMEM and were grown in CD Hybridoma chemically

defined medium (Thermo Fisher Scientific) in a 37˚C incubator with 125 rpm shaking and 5% CO_2

Primary cell cultures

BMM were differentiated from the marrow of femoral and tibial bones of donor mice. Briefly, cells were obtained by flushing the marrow and then growing in differentiation media for 6 d (DMEM with 20% conditioned medium from a confluent culture of L929 fibroblasts as a source of M-CSF, 10% FCS, 10 mM HEPES, 2.0 mM L-glutamine, 0.05 mM 2-ME, 1% nonessential amino acids, 1% penicillin, and 100 µg/ml streptomycin). Nonadherent cells were removed and adherent macrophages were recovered from plates using Cellstripper. These BMM were then maintained in DMEM with 10% L929 conditioned medium, 10% FCS, 10 mM HEPES, 2.0 mM L-glutamine, 0.05 mM 2-ME, 1% nonessential amino acids, 1% penicillin, and 100 µg/ml streptomycin, and used within 3 d.

Generation of genetically modified cell lines

We initially amplified murine Itgb1 and Itga4 from J774 total RNA by RT-PCR. These genes were cloned respectively in the pIRES2-EGFP and pIRES2-DsRed2 vectors (Clontech, Mountain View, CA). All sequences in these vectors were confirmed by Sanger sequencing before transfection. To allow simultaneous selection of cotransfected cells, the Neo^R sequence in the pIRES2-DsRed2 vector was swapped with the Bsr blasticidin resistance marker from the pFUSE2-CLIg-mK vector (InvivoGen, San Diego, CA) using In-Fusion Cloning (Clontech). The two vectors, as well as the empty vectors without the Itgb1 and Itga4 genes, were transfected into CHO cells using Lipofectamine 3000 (Thermo Fisher Scientific, Carlsbad, CA). Stable transfectants were selected by treating with 1 mg/ml G418 and/or 10 µg/ml blasticidin. Populations with stable, high expression of the respective transgene(s) and reporter genes were selected by two consecutive rounds of flow cytometry sorting.

Western blot

For Western blot, cell pellets were lysed in RIPA buffer for extraction of total proteins. Protein extracts were quantified, and Western blot was performed with NuPAGE electrophoresis system (Thermo Fisher Scientific), following manufacturer instructions.

Proteins were detected with Itgb1 (8798; Santa Cruz Biotechnology) or Itga4 (4600; Cell signaling) and actin (47778; Santa Cruz Biotechnology) Abs, followed by species-specific secondary Abs labeled with HRP (Southern Biotech) detected with ECL (SuperSignal WestPico Plus from Thermo Fisher Scientific).

Hybridoma Abs

The *C. neoformans*–specific mAbs, 3E5 IgG1 and IgG3, have been described previously (22). Briefly, mAb 3E5 was originally isolated as an IgG3 mAb following immunization of mice with GXM conjugated to tetanus toxoid (23), and the other isotypes were later generated via in vitro isotype switching (22). Ascites was generated by injecting hybridoma cells into the peritoneal cavity of pristane-primed BALB/c mice (National Cancer Institute, Frederick, MD) and harvesting the fluid. Abs were purified from ascites using a protein G column, following the manufacturer's instructions (Pierce, Rockford, IL), then dialyzed in PBS and quantified by ELISA with an isotype-matched standard to determine concentration. For quantification, ELISA plates were coated with serial dilutions of purified myeloma IgG1/k (MOPC 21) and IgG3/k (FLOPC 21) standards (Cappel) and dilutions of the purified Abs. After blocking with BSA, bound Abs were detected with alkaline phosphatase–conjugated isotype-specific goat anti-mouse polyclonal serum (Southern Biotech).

rAbs

To produce 2H1 and 4-4-20 mIgG1/mIgG3 rAbs, the V region from the H chain (V_H) and V region from the L chain (V_L) of 2H1 (24) and 4-4-20 (25) Abs were codon optimized and synthetized by GenScript and then cloned into the commercially available pFUSE vectors, the V_Hs into the pFUSEss-CHIg-mG1 and pFUSEss-CHIg-mG3 vectors and the V_Ls into the pFUSEs-CLIg-mk vector (InvivoGen). All constructions were confirmed by Sanger sequencing before transfection. NS0 cells were seeded at 8 3 105 cells/ml in 24-well plates 24 h before transfection and then cotransfected with the pFUSEss-CHIg-mG1 or pFUSEss-CHIg-mG3 and the pFUSEs-CLIg-mk vectors containing the V_H and V_L sequences for 2H1 or 4-4-20 Ab expression. Transfections were made using Lipofectamine2000 (Thermo Fisher Scientific), following manufacturer instructions. Seventy-two hours posttransfection, cells were set to selection by addition of Zeocin (Thermo Fisher Scientific) at 1 mg/ml and blasticidin (Thermo Fisher Scientific) at 5 μg/ml. After 3 wk of selection, stable Ab-

producing cells were obtained. These cells were adapted to serum-free medium, CD Hybridoma AGT (Thermo Fisher Scientific), supplemented with cholesterol (Thermo Fisher Scientific) and 8 mM L-Glutamine (Sigma-Aldrich). The culture volume was then escalated to 1 l to produce the first lots of 2H1 and 4-4-20 mIgG1/mIgG3 rAbs. Subsequent lots of the rAbs were produced by thawing and expansion of frozen Ab-producing cells adapted to serum-free medium, obtained as described above. rAbs were purified by anionic exchange chromatography using a HiScreen DEAE FF column (GE Healthcare) in a nonbinding mode, followed by a two-step ammonium sulfate precipitation at 64% saturation; size-exclusion chromatography with PBS buffer using a HiLoad 16/600 Superdex 75 pg column (GE Healthcare); or by affinity chromatography using Protein G GraviTrap columns (GE Healthcare), following instructions in manual, and then concentrated by ultrafiltration. Quantification was made by direct ELISA as described above for hybridoma Abs or by an Ag-specific ELISA for preparations with low concentration. For the Ag-specific ELISA, plates were coated with 10 µg/ml of GXM purified from *C. neoformans* H99 cultures. After blocking with BSA, serial dilutions of isotype-specific Abs IgG1 (18B7) or IgG3 (3E5) and purified rAbs were added to wells and detected with alkaline phosphatase–conjugated isotype-specific goat anti-mouse polyclonal serum (Southern Biotech).

Immunofluorescence microscopy

Recombinant 2H1 Abs were functionally validated by indirect immunofluorescence with *C. neoformans* cells (Supplemental Fig. 1). Briefly, 24067 (serotype D) and H99 (serotype A) were incubated for 1 h with 1 µg/ml of each Ab. After washing, the cells were incubated for another 1 h with secondary goat anti-mouse IgG labeled with Alexa Fluor 488. After washing, labeled cells were mounted in Prolong Gold (Thermo Fisher Scientific) and imaged on a Zeiss Axio Observer Z1 inverted microscope equipped with 63x, numerical aperture 1.4 oil immersion objective. Z-stacks were collected, deconvolved with a constrained iterative algorithm, and used for the maximum intensity projections on Zeiss ZEN.

C. neoformans phagocytosis assay

Phagocytosis assays were performed in 96-well tissue culture–treated plates (BD Falcon) containing either primary peritoneal cells isolated 1 d prior to the experiment, BMM cultured to maturity, or J774 cells plated at least 2 h or up to 1 d before the experiment. In

some experiments, various receptors were blocked using receptor-specific Abs prior to phagocytosis. For complement receptor (CR) block, a mixture containing Abs to CR3 and CR4 (GAME-46, a rat IgG1 to mouse CD18; M1/70, a rat IgG2b to mouse CD11b; HL3, an Armenian hamster IgG1 to CD11c; all Abs from BD Pharmingen) is known to be effective at blocking CR function (26). For FcγRII and FcγRIII block, the rat IgG2b Ab 2.4G2 (BD Pharmingen) was used. For FcγRI block, monomeric IgG2a (Cappel) was used as a competitive inhibitor. For Itgb1 block, the unlabeled mAb HMβ1-1 (produced at the Yagita laboratory or purchased from Abcam) was used, which has been shown to be effective at binding and inhibiting adhesion of tumor cell lines to extracellular matrix via Itgb1 (27). Blocking Abs were added at concentrations ranging from 10 to 50 μg/ml 30 min before addition of the fungi and opsonizing Ab. Then, the *C. neoformans* suspension with opsonizing Ab (IgG1, IgG3, or 18B7, as indicated) was added, with blocking Abs at 10 μg/ml, opsonizing Abs at 10 μg/ml, and the macrophage/*C. neoformans* ratio ranging from 1:1 to 1:2. Phagocytosis was allowed to proceed for 2 h, at 37˚C in 5–10% CO_2. Cells were then washed, fixed with methanol at -20˚C for 30 min, and finally stained with Giemsa. Cells were then analyzed under an inverted microscope with a 20x, numerical aperture 0.30 objective, counting three fields per well, with at least 100 cells per field. Macrophages with internalized *C. neoformans* were readily distinguishable from cells that had taken up no fungi, or from those in which *C. neoformans* was simply attached to the outside, because of the visible vacuole containing an engulfed cell. Percentage of phagocytosis is calculated as the number of macrophages containing one or more internalized *C. neoformans* divided by the total number of macrophages visible in one field. At least 100 macrophages were counted in each condition (27).

Phagocytosis experiments shown in Fig. 4 were performed using an automated microscopy platform, as described previously (28). Briefly, macrophages were seeded in a glass-bottom 96-well plate (MGB096-1-2- HG-L; Matrical Bioscience, Spokane, WA) at a density of 2.4 x 10^4 cells per well, and phagocytosis of *C. neoformans* proceeded as described above. After a 2-h period to allow phagocytosis, cells were fixed with methanol and stained consecutively with wheat germ agglutinin conjugated to Alexa Fluor 633 (Thermo Fisher Scientific) for detection of macrophage cytoplasmic membrane, Uvitex 2B (Polysciences, Warrington, PA) at 0.1 μg/ml to stain *C. neoformans* cells, and finally propidium iodide (Sigma-Aldrich) at 5 μg/ml. Images were acquired and analyzed in iCys Compucyte

(CompuCyte Corp., Westwood, MA) with a 40x objective with at least 15 images per field averaging around 700 cells per well in triplicate wells.

Pooled short hairpin RNA library transduction and sorting

A pool of lentiviral vectors containing ~80,000 different short hairpin RNA (shRNA) sequences targeting ~15,000 different mouse genes was obtained from Sigma-Aldrich, the distributor for the product developed by the RNAi Consortium (29, 30). J774 cells were maintained as indicated above and were plated in 10-cm tissue culture–treated plates (BD Falcon) 1 d prior to transduction to generate 10 plates the next day, each at a density of 2 x 10^6 J774 cells per plate. Hexadimethrine bromide (Sigma-Aldrich) was added at 8 µg/ml to facilitate transduction, and 2 x 10^5 viruses were added to each plate of J774 cells to maintain a multiplicity of infection of 0.1. Cells were incubated with virus for 24 h, and the media was then changed, and the cells were incubated an additional day. At this point, 5 µg/ml of puromycin (Sigma-Aldrich) was added, and cells were grown under selection for 2 d. After selection, dead cells were removed, and cells were replated under continued puromycin selection and allowed to recover overnight. The next day, the 4.6 x 10^7 total cells were stained with two labeled Abs: mIgG3- dichlorotriazinylaminofluorescein (DTAF) and mIgG1-allophycocyanin. The mIgG3 was the 3E5 mAb featured in other experiments, generated as indicated earlier in this section, and labeled with DTAF (Sigma-Aldrich) according to the protocol for labeling amine-reactive probes, following provided instructions (Thermo Fisher Scientific). Mouse IgG1 isotype control Ab conjugated to allophycocyanin was purchased (Southern Biotech). For the sorting of transduced cells, staining was carried out in Cellstripper for 1 h at 4˚C. Afterwards, the cells were collected by centrifugation, washed twice, and then filtered in a 40-µm cell strainer and analyzed on the MoFlo XDP sorter (Albert Einstein College of Medicine Flow Cytometry Core Facility). Sorting gates were generated for cells with high IgG1 signal and low IgG3 signal, and this first sort was run in "yield" mode to get the highest return. We recovered 1.5 x 10^6 cells that were then grown under continued puromycin selection for 1 wk, after which we performed a second sort under similar conditions as above except in "purity" mode to increase the selectivity of the method and recover only those cells in the sorting gate population. We recovered ~50,000 cells in the postsort population and maintained them in culture under puromycin selection. A portion of J774 cells not subjected to sorting was also maintained as the presort population. These cell populations were maintained under puromycin selection and used for DNA extraction and

sequencing. Following bioinformatics analysis, 17 specific shRNA lentiviral vectors were purchased, corresponding to our candidate selection criteria. These individual shRNAs were transduced into J774 cells following a similar protocol, maintained as separate transduced cell lines, and analyzed as indicated.

Flow cytometry and sorting

J774 and CHO cells were detached from tissue culture plates using non-enzymatic solution (Cellstripper; Corning, Corning, NY). Approximately 1 x 10^6 cells were stained in each experiment. To test mIgG1 and mIgG3 binding, 10 µl of IgG1-Alexa568 and IgG3-Alexa488 were used per sample. These Abs were made by conjugating the 3E5 IgG1 and IgG3 mAbs with their respective Alexa fluorophore, following the kit instructions (Molecular Probes, Eugene, OR). For staining Itgb1, PE-labeled HMβ1-1 was used at 1:100 dilution. After staining for 1 h at 4˚C, cells were centrifuged and washed twice, then analyzed on BD LSR II, BD LSR Fortessa, or BD FACS Aria cytometers. Flow cytometry data were analyzed with FlowJo (Tree Star) and presented as the median fluorescence intensity, histograms, or density plots. A FACS Aria cytometer was used to sort J774 cells according to Itgb1 surface expression following shRNA transduction using a PE-labeled HMβ1-1. For transfected CHO cells, Alexa Fluor 647–conjugated HMβ1-1 and Brilliant Violet 421–conjugated rat anti-mouse Itga4 (BD Biosciences, East Rutherford, NJ) were used to stain cells that were separated on a MoFlo XDP sorter.

DNA extraction and sequencing

Genomic DNA was extracted from the pre- and postsorted J774 cell populations using a commercial kit (QIAGEN, Valencia, CA). Primers flanking the shRNA region were used to amplify the shRNA sequences from 1.5 µg of DNA. The amplified library was then analyzed with a Solexa sequencer (Illumina, San Diego, CA). Partners HealthCare (Cambridge, MA) performed PCR amplification and high-throughput sequencing on pre- and postsort DNA samples. SeqWise LLC (Boston, MA) performed bioinformatics on the sequencing data to generate actual counts.

Quantification and statistical analysis

Bioinformatics data from the shRNA high-throughput sequencing was analyzed with the Audic and Claverie method for pairwise testing (31, 32). Phagocytosis assays were analyzed on GraphPad Prism 7.0; pairwise comparisons were made with the Fisher exact test to compare the proportions of macrophages that internalized *C. neoformans* cells. Confidence intervals for the proportions were calculated using the Wilson/Brown method.

Results

Screening for the IgG3 receptor using pooled shRNA libraries

We designed and conducted a screen for the mIgG3 receptor in mice with a pooled shRNA library from the RNAi Consortium (33). Using engineered lentiviral vectors targeting 15,000 different mouse genes, we transduced J774 cells to generate a pool of macrophage-like cells, each with a single gene knocked down. We screened the knockdown library by sorting twice for cells that showed decreased binding to mIgG3 but had normal levels of mIgG1 binding (Fig. 1A, 1B). Sequencing the shRNA sequences of presort and postsort J774 pools determined that 8145 of the 8937 shRNA sequences detected were differentially represented in the separated populations. Of these, 2794 had higher representation in the postsort population (i.e., low binding to mIgG3). We narrowed our search to select for plasma membrane proteins, which were targeted by 266 of these shRNAs. Among these 266, we chose five candidate genes based on known macrophage expression, known phagocytic activity, and/or immunomodulatory function: Lrig2 (leucine-rich repeats and Ig-like domains 2), Itgb2l (integrin β2–like or Pactolus), Itgb1, TLR2, and Fasl (Fas ligand).

To confirm their involvement in mIgG3 binding, we transduced individual shRNA sequences targeting these candidates (five each for Lrig2, Itgb2l, and Itgb1; two for Fasl; and one for TLR2) into J774 cells. We then performed phagocytosis assays to detect decreased uptake of *C. neoformans* after opsonization with 3E5-IgG3, a hybridoma-derived mAb that binds to the fungal capsule (22). The experiment was performed in the presence of 2.4G2 Ab (Fc block), which blocks CD16 and CD32, the α subunits of FcγRIII and FcγRII, but does not block FcγRI and FcγRIV. We also used Abs to CD18, CD11b, and CD11c (CR block) to inhibit CR3 and CR4, which can directly bind to *C. neoformans* capsule carbohydrate upon

Ab binding to the capsule and promote complement-independent phagocytosis (26). Although all candidates showed uptake of mIgG3–*C. neoformans*, two clones transduced with Itgb1 shRNAs showed lower (albeit not statistically significant) phagocytic efficacy (Fig. 1C). However, the microscopic observation of this phagocytosis experiment suggested that the number of internalized *C. neoformans* cells was decreased (data not shown). These two clones, Itgb1 c. and e., were also the ones that showed the greatest reduction in surface expression of Itgb1 (Supplemental Fig. 2).

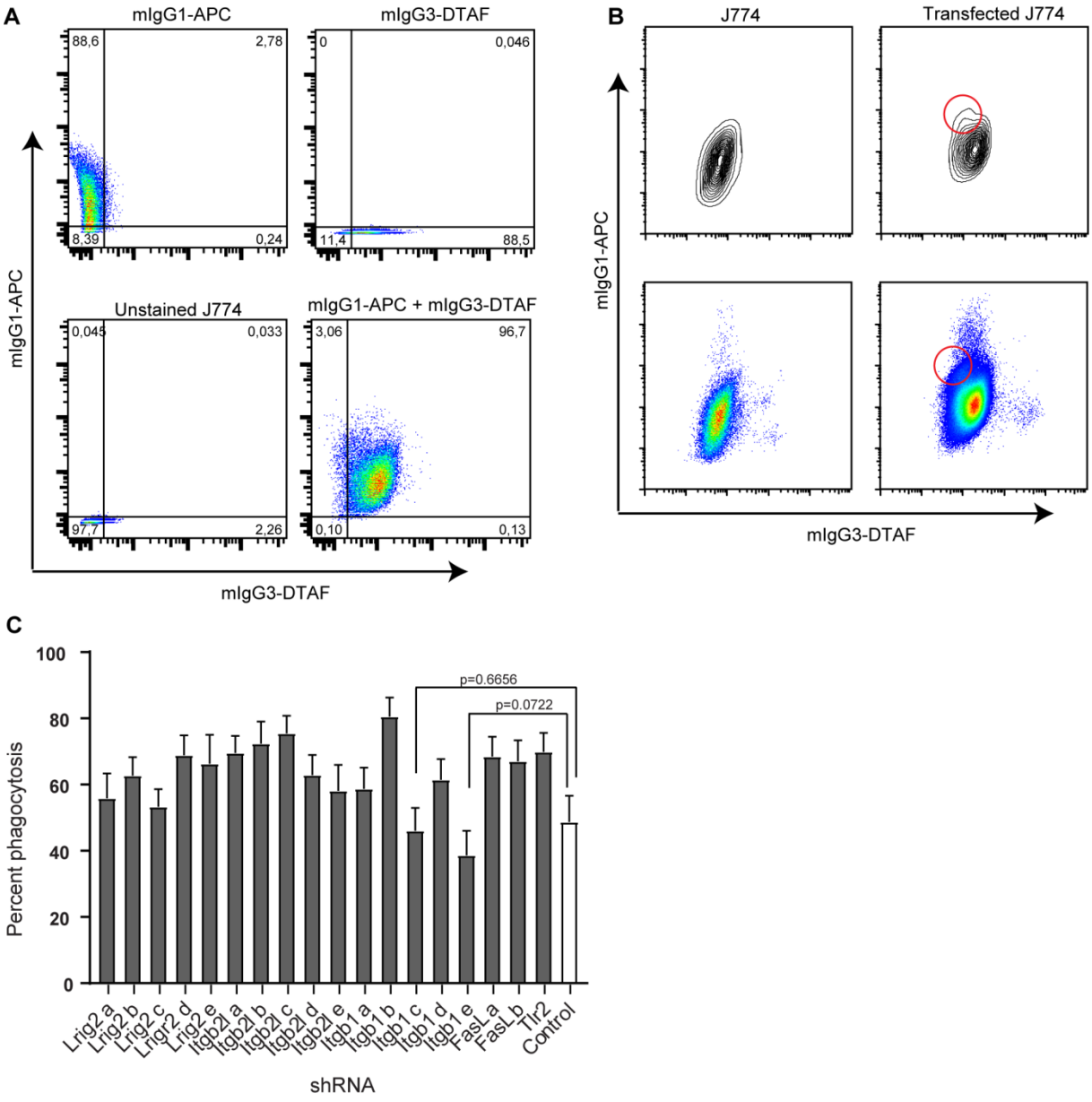

Figure 1-High-throughput screening for an mIgG3 receptor (A and B) show J774 cells transduced with the entire library and stained with fluorescent mIgG1 and mIgG3, whereas (C) shows a secondary screen with individual shRNA clones to test some candidates. (A) Untransduced J774 cells stained with mIgG1-allophycocyanin and/or mIgG3-DTAF. (B) Comparison of untransduced J774 cells and J774 cells transduced

with the shRNA library, stained with both mIgG1-allophycocyanin and mIgG3-DTAF. Data from the bottom two panels are the same as the top two panels, represented differently to better visualize the population of interest (approximated by red circle). (C) Phagocytosis assay using *C. neoformans* opsonized with 3E5-mIgG3 and J774 cells transduced with 17 individual shRNA-encoding lentiviruses targeting five candidates from the screening. All cells were preincubated with CR block (mAbs specific for CD18, CD11b, and CD11c) and Fc block (mAb 2.4G2, specific for FcγRII and FcγRIII) prior to addition of *C. neoformans*. Control cells were transduced with a nontarget shRNA. Four fields were analyzed per well, with one well for each condition. Bars indicate percentage of phagocytosis (number of macrophages containing ingested *C. neoformans* divided by the total number of macrophages) with 95% confidence interval.

mIgG3 binding to macrophages depends on Itgb1 expression and divalent cations

Given the previous screening results, we tested the influence of integrin expression on binding of fluorescently labeled mIgG3 and mIgG1 Abs to macrophages. First, we took advantage of the fact that integrin activity is modulated by divalent cations (34) to test Ab binding to macrophages in the presence or absence of Mg^{2+}, Mn^{2+}, and Ca^{2+}. Consistent with an integrin type of interaction, 1 mM Mn2+ increased the binding of mIgG3 to J774 cells. In the presence of 1 mM Ca^{2+} and of 1 mM Mg^{2+}, mIgG3 binding was increased and unchanged, respectively (Fig. 2A). We next tested a range of concentrations of each cation, which showed that the increased mIgG3 binding resulting from supplementation with divalent cations was dose dependent and that the divalent cations had little effect on mIgG1 binding to cells (Supplemental Fig. 3). Finally, when these cations were combined, the increased mIgG3 binding in the presence of Mn^{2+} and Ca^{2+} was additive, whereas addition of Mg^{2+} had no effect on Ca^{2+}; the Mg^{2+} combination with Mn^{2+} abolished mIgG3 binding (Supplemental Fig. 4A).

Next, we evaluated the binding of IgG3 to J774 cells under *Itgb1* interference by shRNA and/or HMβ1-1, a hamster mAb that inhibits murine Itgb1 binding to collagen, laminin, and fibronectin (27). To increase the efficiency of *Itgb1* knockdown, we sorted the population of J774 cells transduced with Itgb1 c. and e. and selected for cells with lowest expression of Itgb1 (data not shown). To activate the integrins, we added Mn^{2+} and Ca^{2+} (as determined above). *Itgb1* knockdown decreased 3E5-mIgG3 binding by 12.6 and 14.7% in comparison with control cells (Fig. 2B). In the presence of HMβ1-1, 3E5-mIgG3 binding to the control cells also decreased by 30.9%. When the effects of the shRNAs and HMβ1-1 were combined in the presence of divalent cations, the median 3E5-mIgG3 fluorescence decreased by roughly 50%. We next performed another experiment with J774 cells incubated with an Alexa Fluor 647–labeled J606-mIgG3. HMβ1-1 decreased the median J606-mIgG3 fluorescence by more than 20%, reproducing the effect observed with 3E5-mIgG3 with a different mIgG3, whereas an isotype control hamster IgG reduced J606-mIgG3 binding by

<5% (Fig. 2C). This result confirmed that HMβ1-1 decreased mIgG3 binding by blocking Itgb1 specifically.

To confirm the results obtained with macrophage-like J774 cells, we used primary macrophages. Because *Itgb1* is essential in many cellular functions, genetically deficient (knockout) mice are not viable. To overcome this, we used BMM from LysM-Cre, $Itgb1^{flox/flox}$ ($Itgb1^{f/f}$) ($Itgb1^{-/-}$) mice, in which *Itgb1* is conditionally knocked out in monocytes/macrophages and other myeloid cells. Flow cytometry with PE-labeled HMβ1-1 showed Itgb1 reduction in ∼50% of the BMM $Itgb1^{-/-}$ mice in comparison with a 95% Itgb1-positive population derived from the Cre negative, $Itgb1^{f/f}$ control littermate mice (Fig. 2D). Flow cytometric mIgG3 binding assays showed that like J774 cells, BMM bound monomeric mIgG3 and that binding was increased in the presence of Ca^{2+} and Mn^{2+}. Binding of mIgG3 to the cells was similar between $Itgb1^{-/-}$ and $Itgb1^{f/f}$ cells, except for a population with high mIgG3 binding that is severely reduced by *Itgb1* conditional knockout (Fig. 2E).

We made an interesting observation in one of the flow cytometry binding experiments. Because BMM had been used by Gavin et al. (8) to conclude that FcγRI is an mIgG3 receptor, we repeated the mIgG3 binding to BMM in the presence of excess monomeric mIgG2a, which binds to FcγRI with high affinity (2), as well as 2.4G2 to block FcγRII and FcγRIII. This resulted in reduced mIgG3 binding (Supplemental Fig. 4B), suggesting partial competition between mIgG3 and mIgG2a Abs.

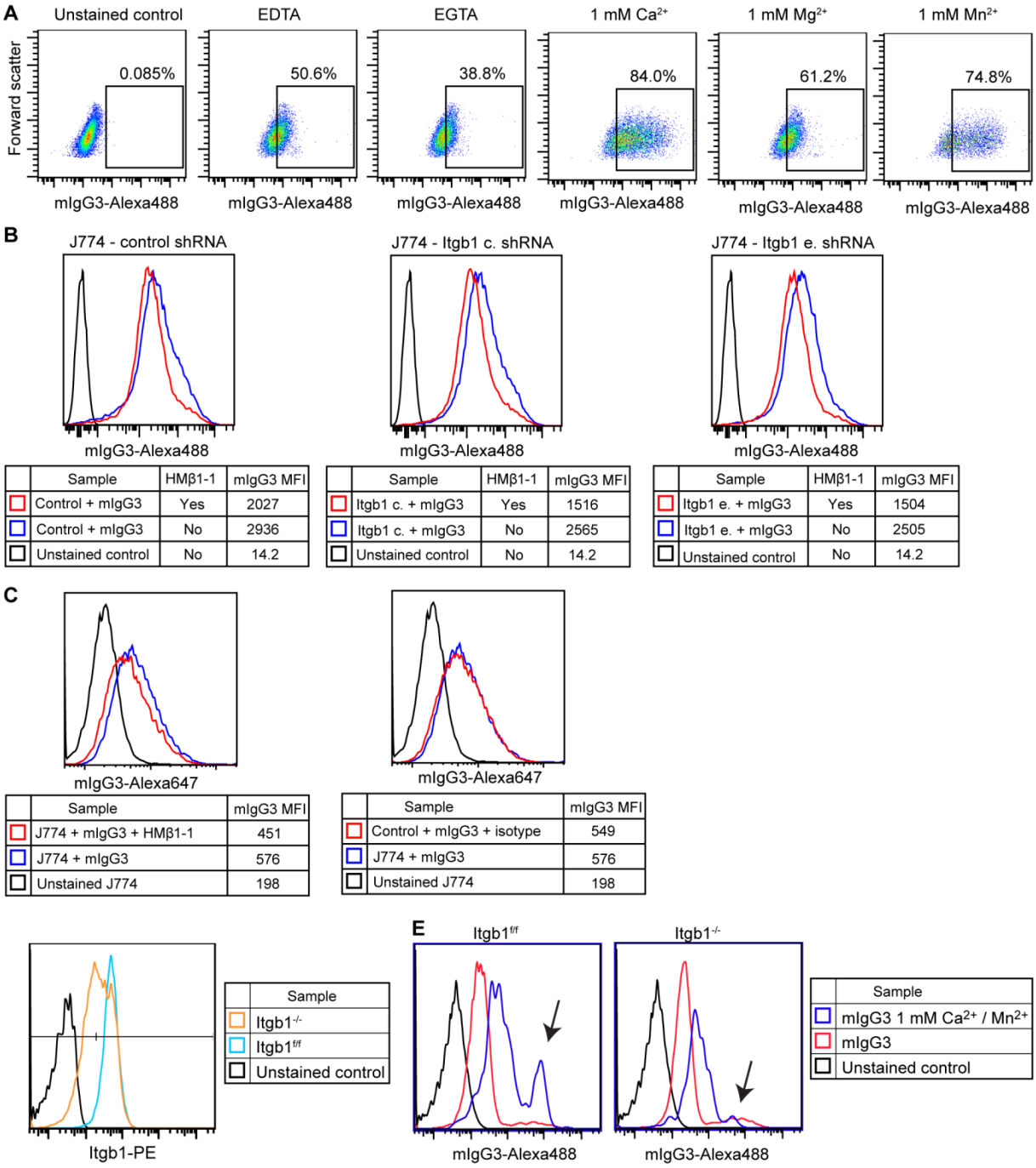

Figure 2 – Divalent cations and Itgb1 expression affect mIgG3 binding to macrophages. Divalent cations and Itgb1 expression affect mIgG3 binding to macrophages. (A) Fluorescently labeled 3E5-mIgG3 was used to measure, by flow cytometry, binding of mIgG3 to J774 cells in the presence of the divalent cation chelator EDTA, the Ca^{2+}-specific chelator EGTA, and the cations Ca^{2+}, Mg^{2+}, and Mn^{2+}. All chelators and salts were present at 1 mM in PBS. (B) Fluorescently labeled 3E5-mIgG3 was used to measure, by flow cytometry, binding of mIgG3 to J774 cells transduced with shRNAs targeting *Itgb1* and control nontarget shRNA in the presence or absence of HMβ1-1. All cells were incubated with Abs in the presence of 1 mM Ca^{2+}/Mn^{2+}. (C) Alexa Fluor 647–labeled commercial J606-mIgG3 mAb was incubated with J774 cells in the absence of additional divalent cations to test the specificity of the HMβ1-1Ab in comparison with a hamster IgG isotype control. (D) Itgb1 expression on *Itgb1*$^{-/-}$ or *Itgb1*$^{f/f}$ BMM, detected by flow cytometry with fluorescently labeled HMβ1-1. (E) Binding of fluorescently labeled 3E5-mIgG3 to BMM in the presence of 1 mM Ca^{2+}/Mn^{2+}. The arrows indicate a population that is reduced by *Itgb1* conditional knockout. MFI, median fluorescence intensity.

Loss of Itgb1 function decreases mIgG3-mediated phagocytosis

Next, we measured the effect of blocking Itgb1 function with several different strategies on mIgG3-mediated phagocytosis. We first repeated the phagocytosis assay with the sorted Itgb1 c. and Itgb1 e. knockdowns as well as cells transduced with a control shRNA. As shown in Fig. 3A, only one of the cell lines with shRNA-mediated knockdown showed a decrease in phagocytosis in comparison with the rate observed in control cells, from 59.6 to 43.3%. We also interfered with Itgb1 function using HMβ1-1, which decreased phagocytosis in control cells with no Itgb1 knockdown from 59.6 to 27.8%. The combination of these two strategies had pronounced effects on IgG3-mediated phagocytosis, which decreased to 38.4% in one of the cell lines and 4.6% in the other. In addition to decreasing the percentage of phagocytosis, *Itgb1* shRNA and HMβ1-1 resulted in a noticeable decrease in the number of *C. neoformans* cells internalized per macrophage (Fig. 3B).

To confirm the results obtained with macrophage-like J774 cells, we used primary macrophages. BMM from both Itgb1$^{f/f}$ and the Itgb1$^{-/-}$ mice showed high levels of phagocytosis of *C. neoformans* cells coated with both 3E5-mIgG1 and 3E5-mIgG3. We had previously shown that BMM phagocytosis of 3E5-mIgG3–coated *C. neoformans* was not affected by Fc block and was only partially reduced by CR block, an indication of the existence of a non-FcγR (9). In the present experiments, BMM from *Itgb1*$^{-/-}$ mice phagocytosed *C. neoformans* to the same extent as those from control Itgb1$^{f/f}$ mice, presumably because of the redundancy between Itgb1 and CR (Fig. 3C). In presence of CR block, however, 3E5-mIgG3–mediated phagocytosis was drastically reduced in *Itgb1*$^{-/-}$cells in comparison with control *Itgb1*$^{f/f}$ BMM; integrin knockout had very little effect on 3E5-mIgG1 opsonization.

To test how generalizable these findings are, we used genetic engineering to produce two pairs of recombinant mIgG1 and mIgG3 Abs. For the first pair, 2H1-mIgG1 and 2H1-mIgG3, we have used the V region sequences of another Ab to *C. neoformans* capsule whose Fab structure has been solved by x-ray crystallography (24). The other pair, 4-4-20-mIgG1 and 4-4-20-mIgG3, has the variable domains of a high-affinity Ab to the hapten FITC. Detailed structural information is also available for the 4-4-20 Fab structure (25). rAbs were produced by cotransfecting mammalian cells with vectors expressing 2H1 and 4-4-20 H and L chains. Comparing the J774 phagocytosis assays performed with *C. neoformans* cells coated with 3E5 hybridoma Abs with the ones performed with 2H1 Abs, we have observed the same pattern of almost complete abrogation of mIgG1-mediated phagocytosis

upon CR and Fc block (Fig. 3D). However, 2.4G2 did significantly reduce the phagocytosis of *C. neoformans* coated with 2H1-mIgG3 from 38 to 16% ($p < 0.0001$) when compared with mIgG3-coated *C. neoformans* in the presence of CR block. This already low figure was further reduced to 8.4% upon addition of HMβ1-1 ($p < 0.0001$).

With the 4-4-20 Ab pair, we made a phagocytosis assay using a different particle, FITC-labeled sheep RBCs (sRBC). The background in this experiment was higher than that in experiments with *C. neoformans*, as we observed 8.7% phagocytosis with non-opsonized FITC-labeled sRBC (Fig. 3E). Addition of 2.4G2 reduced 4-4-20-IgG1 phagocytosis to the same level observed with nonopsonized sRBC. It also reduced 4-4-20-IgG3 phagocytosis but to a level (19%) that was still significantly above background ($p < 0.0001$). HMβ1-1 alone had no effect on the phagocytosis rates for this Ab pair. When 2.4G2 and HMβ1-1 were combined, the phagocytosis rate was decreased to 12.1% ($p = 0.0087$). The results from all phagocytosis experiments are summarized in Fig. 3F.

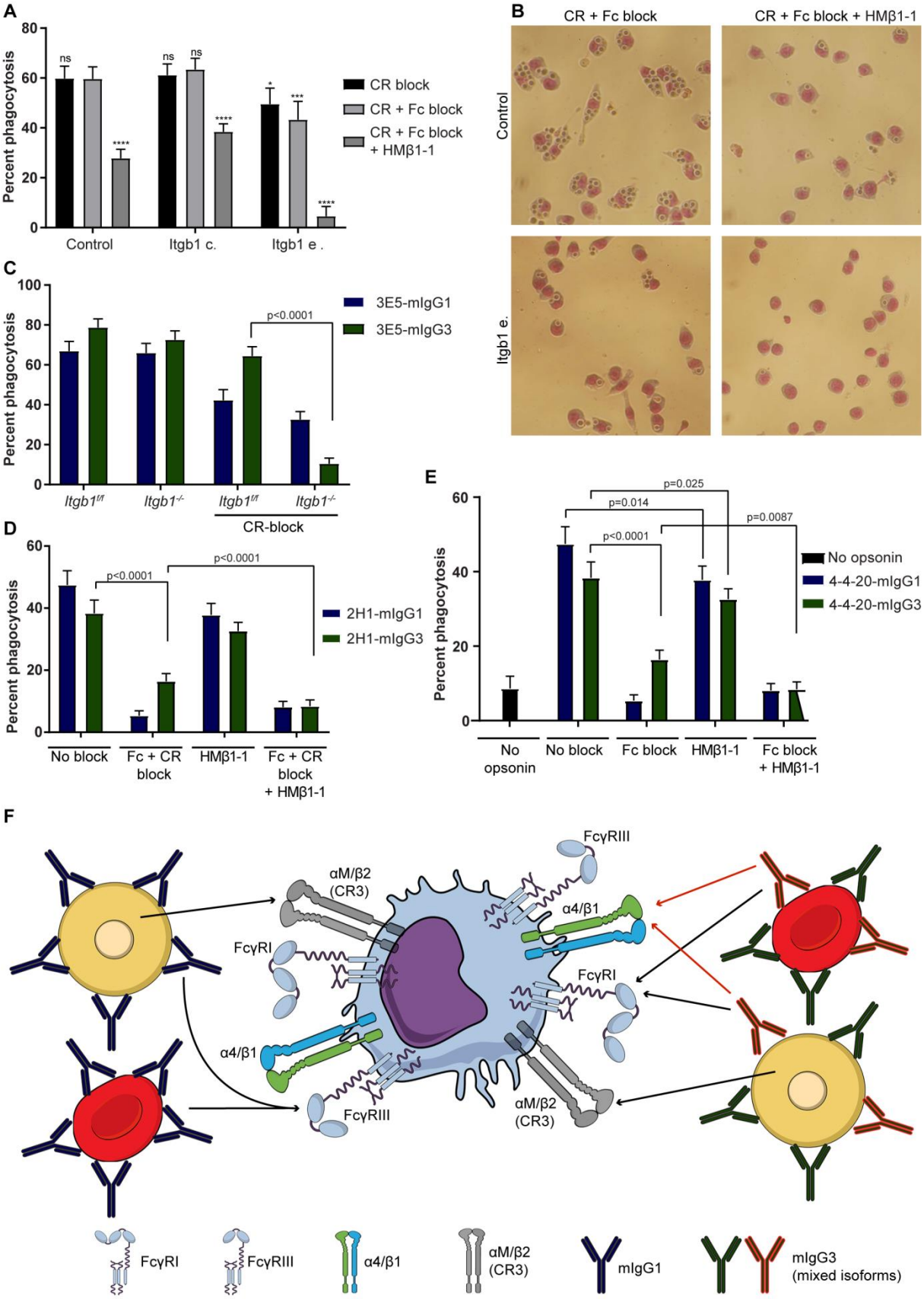

Figure 3– **Itgb1 silencing, knockout, or blockage with HMβ1-1 decreases phagocytosis in macrophages**. (A) Phagocytosis assay using *C. neoformans* cells opsonized with 3E5-IgG3 and Itgb1 c. and e. transduced J774 cell lines. Macrophages were preincubated with Abs to block FcγRII/III (2.4G2), CR (CD11b, CD11c,

and CD18), and Itgb1 (HMβ1-1), as indicated, prior to addition of fungi. (B) Images showing representative fields of the phagocytosis assay shown in (A). Top two panels represent control cells (non target shRNA), and bottom two panels represent the Itgb1 e. transduced cell line, with blocking conditions denoted at top. Giemsa stain. Scale bar, 20 μm. (C) Phagocytosis assay with BMM derived from *Itgb1*$^{-/-}$ mice or their littermate Itgb1$^{f/f}$ controls in the presence or absence of CR-blocking Abs and *C. neoformans* cells opsonized with 3E5-mIgG3 or 3E5-mIgG1. Bars indicate the percentage of phagocytosis and the 95% confidence interval. (D) Phagocytosis assay with recombinant 2H1-mIgG1 and 2H1-mIgG3 Abs and *C. neoformans*. Bars indicate the percentage of phagocytosis and the 95% confidence interval. (E) Phagocytosis of FITC-labeled sRBC coated with recombinant 4-4-20-mIgG1 and 4-4-20-mIgG3 Abs. In (A) and (C)–(E), the *p* values were calculated with Fisher exact test: *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. Bars indicate the percentage of phagocytosis and the 95% confidence interval. At least three fields were analyzed per well, with at least two wells per condition. (F) Model summarizing the data obtained with different phagocytosis assays. The black arrows indicate results that reproduced what had been previously known about the interaction of mIgG isotypes and FcγR (53, 54) and between the *C. neoformans* capsule and CR3 (26). The red arrows indicate the Itga4/Itgb1 role in mIgG3-mediated phagocytosis demonstrated in the other panels.

Integrin α 4 knockdown decreases mIgG3 function

Integrins are heterodimeric complexes, composed of β and α subunits. Using shRNAs to decrease surface expression of five α integrins that can pair with *Itgb1* and are known to be expressed in macrophages (*Itga3*, *Itga4*, *Itga5*, *Itga9*, and *Itgav*), we tested which ones decreased phagocytosis of mIgG3-coated *C. neoformans.* As shown in Fig. 4A, two shRNAs targeting *Itga4* reduced 3E5-mIgG3 phagocytosis, whereas the knockdown of any of the other α subunits had no effect on fungal internalization. Given that this experiment was conducted in the absence of CR blockage, the redundancy of phagocytic pathways could have masked the Itga4 knockdown effect on mIgG3-mediated phagocytosis. We confirmed this by repeating the phagocytosis assay using CR block and cells that were transduced with shRNAs to *Itgb1* and *Itga4*, which showed more intense reductions in phagocytosis (Fig. 4B). An immunoblot with Abs to Itgb1, Itga4, and β actin confirmed that the knockdown was effective (Fig. 4C, 4D). We note that Itga4 knockdown had significant effects in cell health, an unavoidable confounding factor. However, we did detect loss of 3E5-mIgG3– mediated phagocytosis without loss of mIgG1 function for one of the shRNA clones targeting Itga4. Therefore, mIgG3 is dependent to a certain extent on Itga4 surface expression, presumably because of formation of a heterodimer with Itgb1.

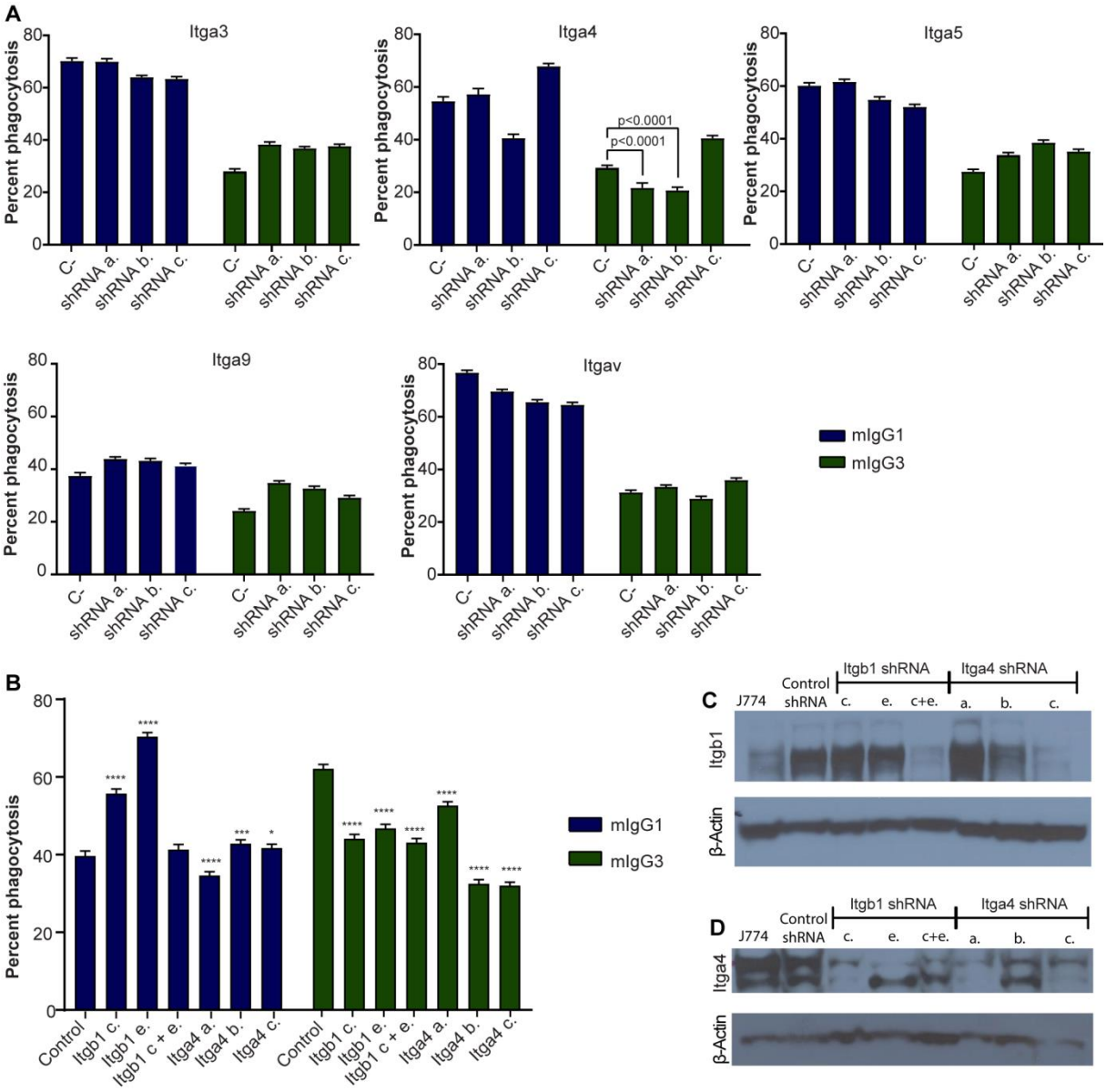

Figure 4– **Phagocytosis with J774 cells transduced with shRNAs to integrin *α*-chains.** (A) J774 cells were transduced with shRNAs targeting five integrin *α*-chains that pair with Itgb1 and stably transfected cells selected with puromycin. A phagocytosis assay was then performed with 3E5-IgG1 or 3E5-IgG3 Abs, in the presence of CR block, and fluorescently labeled *C. neoformans* using a scanning cytometer. Bars represent the percentage of phagocytosis and 95% confidence interval from one to two independent experiments, each containing three individual wells from which ~2000–18,000 macrophages were evaluated. (B) J774 cells were transduced with individual shRNAs targeting *Itgb1* or *Itga4* or a combination of two shRNAs targeting *Itgb1*. A scanning cytometer was then used to make a phagocytosis assay with *C. neoformans* cells opsonized with 3E5-mIgG1 or 3E5-mIgG3. Bars indicate the percentage of phagocytosis and 95% confidence interval of one experiment with three wells per condition. The *p* values were calculated by Fisher exact test for both (A) and (B): **p* < 0.05, ****p* < 0.001, *****p* < 0.0001. (C) Western blot quantification of *Itgb1* knockdown efficiency. (D) Western blot quantification of *Itga4* knockdown efficiency.

Gain-of-function assays with Itgb1

We transfected CHO cells so they would express murine *Itgb1* and/or murine *Itga4* to perform gain-of-function assays. Stable transfected cells were selected with drug resistance markers and high-expressing cells obtained by two rounds of flow cytometry cell sorting. As shown by flow cytometry, these cells adequately express both the cell surface integrins (Fig. 5A) and the vector-encoded reporter proteins (Fig. 5B). Four additional stable cells lines were created by cotransfection of vectors to express each integrin individually, both integrins simultaneously, or none of them (Fig. 5C). We measured binding of two different fluorescently labeled mIgG3 Abs to these cells, showing that expression of the murine integrins (especially Itga4) increased mIgG3 binding (Fig. 5D). Blocking the expressed murine Itgb1 with HMβ1-1 prior to the addition of the mIgG3 Abs decreased binding in comparison with samples that were preincubated with a hamster IgG isotype control (Fig. 5E). To confirm the specificity of the blockage with HMβ1-1, we showed that it did not bind to native hamster Itgb1 on CHO cells (Fig. 5F). We summarized our findings in a new model for binding of mIgG Abs to their receptors (Fig. 6).

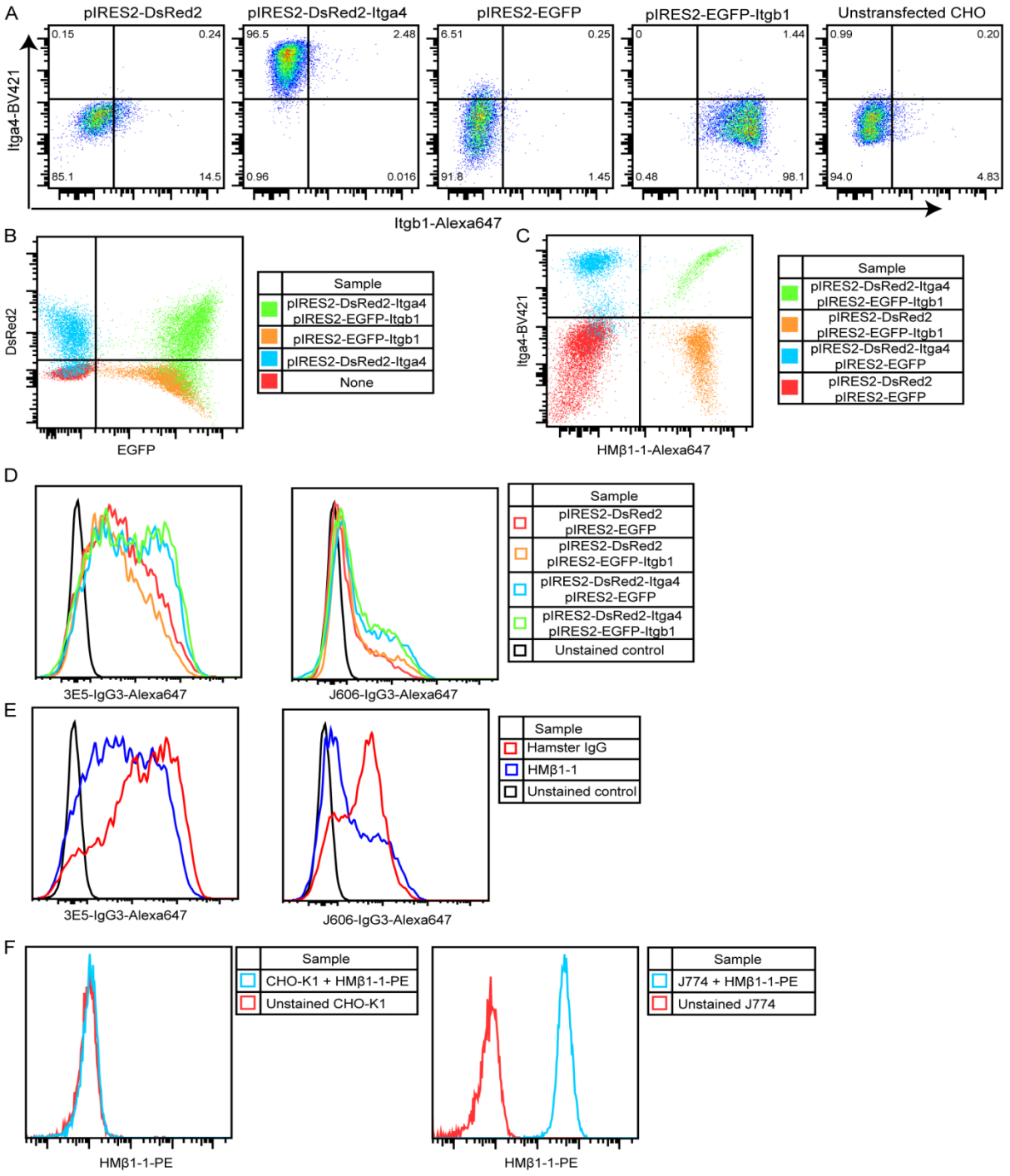

Figure 5– Expression of Itgb1 in knockout cells increases binding to mIgG3. (A) Flow cytometry detection of Itga4 and Itgb1 expression on the surface of CHO cells that were transfected with pIRES2 plasmids. (B) Expression of the EGFP and DsRed2 reporter fluorescent proteins in transfected cells. (C) Expression of Itgb1 and/or Itga4 in CHO cells that were cotransfected with pairs of pIRES2 vectors. (D) Binding of Alexa Fluor 647–labeled 3E5-mIgG3 and J606-mIgG3 Abs to the four cotransfected cell lines in the presence of 1 mM Ca^{2+}/Mn^{2+}. (E) Binding of mIgG3 Abs to the CHO cell that had been cotransfected to overexpress murine Itga4 and Itgb1 in the presence of 1 mM Ca^{2+}/Mn^{2+} and HMβ1-1 or control hamster IgG. (F) Binding of HMβ1-1to CHO and J774 cells, showing that it does not bind to CHO cell integrins.

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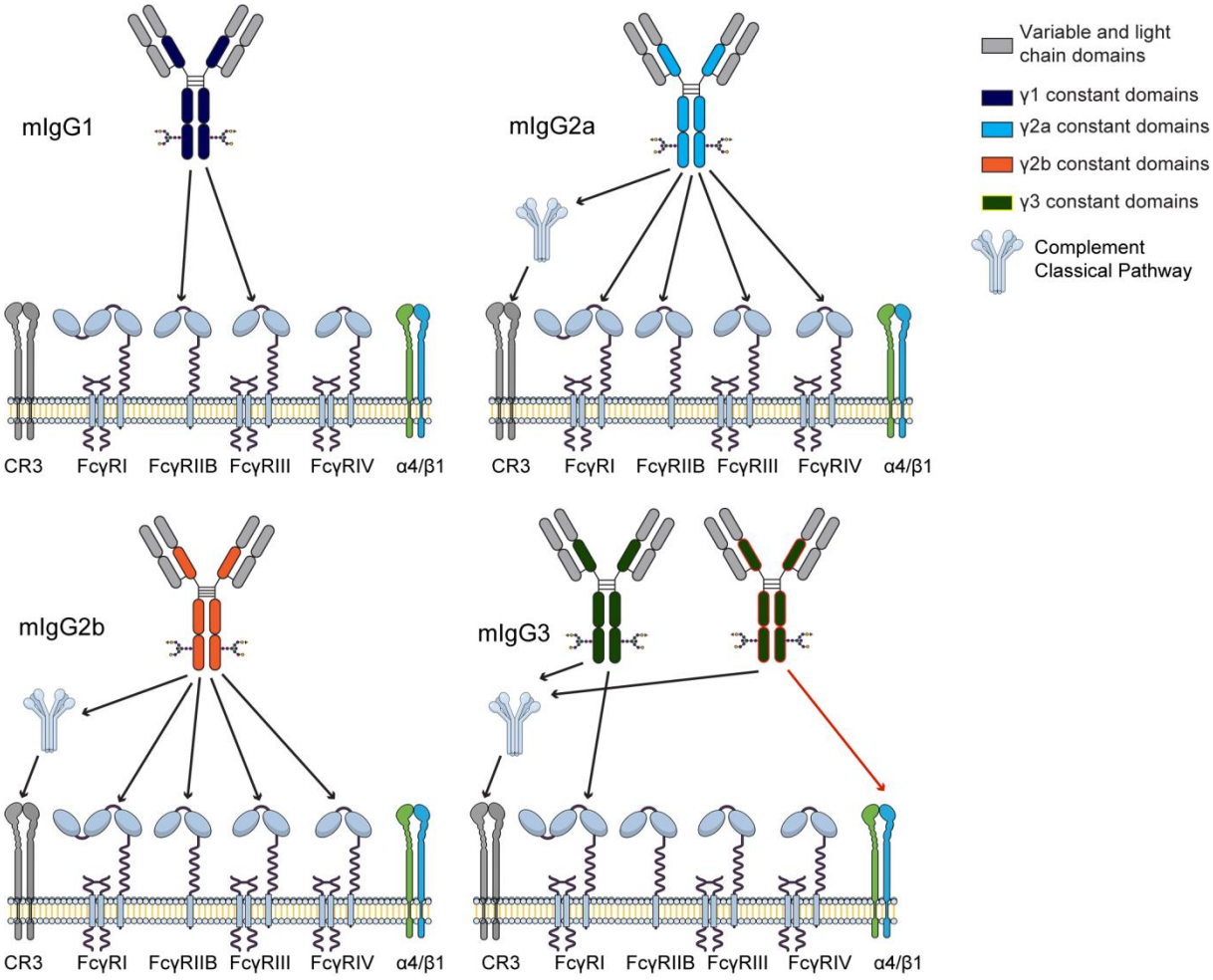

Figure 6– Model for mIgG interaction with cell surface receptors. Model summarizing the cell surface receptors that mediate mIgG functions in macrophages. Black arrows indicate known functions, including the direct interaction between mIgG Abs and FcγR (53, 54) and the indirect interaction with CR, mediated by classical pathway activation. Red arrows indicate the Itga4/Itgb1 role in mIgG3 function described in this article. The two different colors used for mIgG3 represent different isoforms, which we hypothesize are mixed in mIgG3 preparations and bind to different receptors.

Discussion

Ab isotype switching in response to Ags of different compositions or origins is conserved in many animals, probably because different Ab isotypes have different effector functions and thus allow a host to tailor the immune response to each particular threat. Ab engineers also change the isotype of the Abs they work with to obtain biotechnological or pharmaceutical tools with different effector functions. In both cases, most of the functional differences between isotypes can be ascribed to their differential interactions with cell surface receptors.

Almost four decades ago Diamond and Yelton (7) proposed the existence of another Ab receptor. Since that time, several research groups have delved into the issue of the mIgG3 receptor. Three different hypotheses surfaced from these studies.

Hypothesis 1—interactions between complement and mIgG3 explain function of mIgG3

In a model of anemia induced in mice by injection of high-affinity anti-RBC Abs, Fcγ knockout reduced mIgG2a-, mIgG2b-, and mIgG1-induced anemia but had no effect on mIgG3-induced anemia. However, the mIgG3 anemia was abrogated in C3-deficient animals, suggesting that it functions simply by activating complement (10). A monoclonal mIgG3 to *Candida albicans* was also only protective when complement C3 was available (12). Hjelm et al. (11) also found that mIgG3 enhancement of the Ab response was normal in FcγRI-deficient mice but that this enhancement was abolished in the absence of complement. Earlier, Díaz de Ståhl et al. (35) had also found that knocking out FcγR made no difference in mIgG3 enhancement and that C3 was important for IgG3-mediated Ab enhancement; however, they were not able to completely abrogate the enhancement and found lower but nonnegligible enhancement with mIgG3 in animals depleted from C3 with cobra venom or in CR2 knockout mice.

Hypothesis 2— FcγRI is the mIgG3 receptor

Experiments made with macrophages from transgenic mice showed that FcγRI knockout abolished mIgG3-mediated phagocytosis of erythrocytes (8). This hypothesis can be further supported by earlier studies with murine mAbs to human CD3, which showed that human monocytes bound mIgG3 via the high-affinity FcγRI (36, 37).

Hypothesis 3—there is an unknown dedicated receptor for mIgG3

In a murine model of malaria, an mIgG3 mAb remained protective in animals lacking the common Fcγ-chain (38) and the FcγRI α-chain (39). Our group has shown evidence of functional differences between a monoclonal mIgG3 and its isotype switches in a murine model of acute lethal toxicity (40). Moreover, we have observed mIgG3-mediated phagocytosis in macrophages derived from animals lacking several genes encoding Fcγ and CR subunits (9).

These three hypotheses appear at first glance to be mutually exclusive: the experiments leading to conclusion 1 contradict 2 and 3. In some reports, mIgG3 mediated function even when complement was absent or CR blocked, contradicting 1; in others, blocking or knocking out FcγR had no effect on mIgG3 functions, contradicting 2. However, our results suggest a model that can reconcile all three hypotheses (Fig. 6). We propose that integrins play a part in mIgG3 binding and phagocytosis, as do FcγR and complement. In some of our experiments, (e.g., Fig. 3A), Itgb1 blockage drastically reduced but did not abolish mIgG3 function, whereas FcγRII/III blockage had no effect; the same Ab batch was used for the flow cytometry binding assay shown in Supplemental Fig. 4B, which shows excess soluble mIgG2a (a competitive FcγRI block) partially decreasing mIgG3 binding. Other Ab batches, such as the recombinant 2H1 Abs used in the phagocytosis assay shown in Fig. 3D, do show a similar pattern of mIgG3-mediated phagocytosis reduction upon Itgb1 blockage; the extent of the reduction, however, is not as large as what we observed with 3E5- mIgG3. Moreover, FcγRII/III blockage did affect 2H1-mIgG3– mediated phagocytosis, whereas it did not affect 3E5-mIgG3. We have also observed variability in the extent to which Itgb1 blockage decreases mIgG3 binding to macrophages, with HMβ1-1 decreasing binding of 3E5-mIgG3 to a higher extent than binding of J606-mIgG3. The observations that 1) different mIgG3 batches produced variable results with regards to phagocytosis, 2) HMβ1-1 blocking of mIgG3 binding was variable, 3) monomeric mIgG2a partially inhibited mIgG3 binding, and 4) integrin blockage never completely abolished mIgG3 function or binding suggest that monoclonal mIgG3 preparations are a mixture of isoforms that mediate function through both FcγR and Itgb1. The existence of a mixture of isoforms that varies from batch to batch could explain the seemingly disparate data suggesting that FcγRI (8) or an unknown protein (7) was the mIgG3 cell surface receptor. Such heterogeneity in mAb production is very common and has been extensively studied in industrial mAb production settings, in which variations in glycosylation have significant impact on biologic drug pharmacokinetics and pharmacodynamics (41). We plan to formally test this hypothesis in the future.

Integrins are transmembrane receptors present as α + β heterodimers that regulate cell–cell and cell–matrix interactions (42). Several integrins also have an intrinsic ability to mediate phagocytosis as well (43). Probably the most studied ones are the β2 integrin phagocytic CR3 (αM + β2) and CR4 (αX + β2). In the *C. neoformans* system, Itgb2 was shown to mediate complemente independent phagocytosis as a result of direct interactions with the capsular polysaccharide component of Ab-coated cryptococcal cells (26). Itgb1 can

also mediate particle internalization: 1) macrophages lacking Itgb1 expression manifest reduced phagocytic capacity for some types of bacteria (44), 2) microglia are able to phagocytose fibrillar β-amyloid in vitro in a mechanism that does not use FcγR or CR and is blocked by an antagonist to Itgb1 (45), and 3) Itgb1 pairs with α2 integrin to form a receptor that binds complement component C1q (46). The interaction between IgG molecules and the non-FcγR cell surface protein DC-SIGN has also been reported before (47).

Additional evidence for the conclusion that Itgb1 is involved in mIgG3 function comes from experiments using divalent cations. Integrins have many binding sites for these ions, each with distinct functional roles (48). For most integrins, Mn^{2+} binding to one of these motifs (the metal ion-dependent adhesion site [MIDAS]) changes the integrin conformation into an active open state. Consistent with this, Mn^{2+} increased binding of IgG3 to the surface of cells. Mg^{2+}, which also binds to MIDAS to activate integrins, resulted in more mIgG3 bound to J774 cells; however, the activation mediated by Mg^{2+} seemed to be different from the Mn^{2+} activation, as addition of Mg^{2+} competed with Mn^{2+} and completely abolished increased mIgG3 binding. In addition to the Mn^{2+}/Mg^{2+} MIDAS, integrins have different types of Ca^{2+} binding sites (34). One of them, with mM affinity, flanks MIDAS and, when occupied, inhibits Mg^{2+}/Mn^{2+} binding and thus integrin function. However, Ca^{2+} actually increased mIgG3 surface binding in our assays. This seemingly surprising result is actually very informative, as a detailed study on the effect of calcium in β1 integrins showed it decreased the activation of the heterodimers containing α2, α3, α5, and α6 but increased the activation of the α4/β1 integrin (49), further indication that Itga4 is the Itgb1 partner in the mIgG3 receptor.

Given that the problem of the mIgG3 receptor identity has vexed investigators and lingered unsolved for decades and that novel nonclassical FcγR have been discovered quite recently (50), we arrive at our conclusions humbly. The most straightforward interpretation of our findings is that Itgb1 (possibly as a heterodimer with Itga4) is at least part of an mIgG3 receptor complex. We acknowledge that we lack direct evidence for an Ig–integrin protein–protein interaction, which would provide unequivocal evidence for an mIgG3 integrin receptor. Nevertheless, the available data provide strong evidence associating integrin function with IgG3 phagocytosis, which leads to a tantalizing suggestion regarding the evolution of the humoral response. Abs appeared around half a billion years ago with cartilaginous fishes. Fc receptor–like sequences appeared soon after with the bony fishes. The classical FcγR, however, are much more recent: they first appeared with mammals (51).

The most accepted interpretation is that through most of its existence, Abs functioned solely by activating complement and neutralizing Ags. However, as integrins are far more ancient and are present in all metazoans, they could actually have functioned as early IgG receptors for non-mammalian vertebrates. In this regard, it is interesting to note that mIgG3 is the murine IgG isotype whose sequence differs the most from the others and which seems to have appeared first in the evolutionary history of the mouse constant γ locus (52).

Acknowledgments

We would like to acknowledge Dr. Jinghang Zhang from the Albert Einstein College of Medicine flow cytometry core facility and Dr. Hao Zhang from the Bloomberg Flow Cytometry and Immunology Core at Johns Hopkins University for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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Capítulo III- Binding Characterization between Murine Immunoglobulin γ 3 and Integrin α4β1

Abstract

Murine IgG3 (mIgG3) plays a dual role in health and disease but is still the least studied of IgG isotypes. The receptor by which the antibody exerts its effector response has not been determined. It was recently demonstrated that integrin beta 1 (Itgb1) interacts with mIgG3 and plays a role in phagocytosis of fungal cells by macrophages. Nonetheless, several questions still need to be clarified to define Itgb1 as a mIgG3 receptor or co-receptor. In order to answer some of these questions, we demonstrated the interaction between mIgG3 and soluble integrin by solid-phase and dot blot assays. However, besides the interaction with Itgb1, mIgG3 also interacted with FcγR (>>FcγRIII>FcγRI). Interestingly, we observed that other IgG isotypes (>>mIgG2a, mIgG2b, and >mIgG1) were able to interact with Itgb1, but with less affinity. There was a variation among the percentage of phagocytosis results using 3E5-mIgG3 from different batches when FcγR/CR and Itgb1 were blocked. Our results corroborate the hypothesis that mIgG3 interacts with more than one molecule to exert its effector function, reinforcing the idea that there is a variable mixture of isoforms generated by different production methods or different batches, which affects the antibody predilection to receptors. Moreover, we demonstrated the interaction between different IgGs and Itgb1, raising a new question: could Itgb1 exert its function with other IgG isotypes?

Introduction

IgG antibodies are one of the most studied proteins, both for their central role in immune response and for their several biotechnological applications (1,2). This versatility that immunoglobulins present is because they are bifunctional molecules, in which one portion interacts with antigens and another part interacts with cell receptors through which they exert an effective response. In the same way that antibodies play a bifunctional role related to Fab and Fc structures, they can also present dual function when acting on both health and disease (3). The murine IgG3 isotype (mIgG3), for example, is capable of performing a protective and non-protective role. Even though mIgG3 is the main antibody produced against carbohydrates and repetitive epitopes (4-6) - being important against a range of pathogens (7-9) - it is well-

known as an important cause of autoimmune diseases (10-12) and it has also been found as a disease enhancing factor for cryptococcosis (13, 14). Despite its intriguing role, the mIgG3 isotype is understudied, probably due to it is prone to behave as cryoglobulin and because it is produced in low concentrations (10, 12). Thus, owing to these and other issues, the mechanism by which the antibody exerts its effector response remains unknown.

The classic family of proteins to which the different mIgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) interact to fulfill their pro-inflammatory role is that of fraction crystallizable gamma receptors (FcγR) of the types FcγRI, FcγRIII and FcγRIV (3, 15, 16). Nevertheless, despite the fact that FcγRs were discovered almost 50 years ago, there is still no consensus in the literature to define the mIgG3 receptor. In 1981, Diamond and Yelton first suggested that mIgG3 would have a different receptor than other IgG isotypes (17). However, in 1998 Gavin et al. demonstrated the interaction of mIgG3 with the FcγRI (18), but this interaction was later characterized as weak. In 2010, Saylor et al. corroborated Diamond and Yelton's findings: that the mIgG3 receptor is not the FcγRI. In addition, Saylor et al. showed that mIgG3-mediated phagocytosis is independent of CR (19).

Recently, a gain- or loss-of-function study revealed that Integrin beta 1 (Itgb1) has an important role in the mIgG3-mediated response against the fungal pathogen *Cryptococcus neoformans*. For this purpose, a study in which ~80,000 different short hairpin RNA (shRNA) sequences were transduced into J774 macrophage-like cells targeting 15,000 mouse genes in order to identify the mIgG3 receptor. From these targets, they selected knocked down genes that resulted in macrophages less effective at binding to mIgG3 but with regular levels of mIgG1 binding. In summary, two clone cells transduced with individual shRNA sequences, which knocked down *Itgb1*, demonstrated a reduction in mIgG3 binding and in phagocytosis specifically mediated by mIgG3. A number of subsequent tests showed that the presence of Itgb1 is associated with an increase in mIgG3-mediated phagocytosis, but this relation was not observed when testing mIgG1 (20). It is worth emphasizing that although the interaction between mIgG3 and integrin had been reported, experiments with different antibody sets have demonstrated that this interaction did not occur exclusively depending on the receptor in question. Therefore, the study formulated the hypothesis that Itgb1 is one, albeit not the only one, of the mIgG3 receptors, as well as FcγRI.

Despite the important suggestion that Itgb1 may be the receptor/co-receptor or be a part of the mIgG3 receptor complex, some questions still need to be answered regarding the interaction between the molecules: 1) The experiments were carried out evaluating the

interaction between the antibody and receptors coupled to the complex cellular surface of murine macrophages. Thus, it is necessary to investigate whether the antibody continues to interact with Itgb1 when the molecule is individualized. 2) The interaction of mIgG1 and mIgG3 isotypes with Itgb1 was evaluated, but it has yet to confirm the interaction between integrin and other IgG isotypes. 3) Variation of isoforms could explain the literature controversy about what receptor binds to mIgG3, hence it is necessary to evaluate whether different production methods or lots affect the interaction between IgGs and macrophage receptors. 4) Mammalian Itgb1 is a heterodimer formed by alpha and beta chains that may be combined with twelve different alpha units. Although a previous study revealed the alpha chain as an important subunit that interferes with the binding to mIgG3, no study has been carried out to characterize this interaction. 5) It is acknowledged that the Ig CH2 domain interacts with FcγR, but it is not yet known which IgG region interacts with integrin. 6) The interaction between Itgb1 and mIgG3 has been demonstrated only by experiments carried out with murine antibodies. It would also be interesting to evaluate the interaction between Itgb1 and the human ortholog of murine IgG3.

Considering this discussion, this work aims to answer some of the research questions described above by characterizing the antibody-receptor interaction in order to enable future applications of basic and biotechnological science. A good number of the effector activities of antibodies used in technological applications depends on their interaction with cell surface receptors. For this reason, many research groups and companies have invested in engineering the Ig constant portion to improve or modify their action. The characterization of a supposed new receptor may impact the understanding and treatment of autoimmune diseases, as well as infectious diseases, especially in those in which mIgG3 plays a role in antibody-dependent enhancement, such as cryptococcal infection. Moreover, the identification of integrin as an antibody receptor may be useful in improving protective immune responses, such as phagocytosis of pathogens.

Materials and Methods

Antibodies

mIgG1 (MOPC21-3000), mIgG2a (UPC10-3000), mIgG2b (UPC120-3000), and mIgG3 (FLOPC21 or J606-3000) commercial antibodies were used (Mp Biomedicals and

Immunovision).Monoclonal IgG antibodies against the capsular component (glucuronoxylomannan-GXM) of *Cryptococcus neoformans*, called "3E5", were produced by hybridoma technique (21). Three murine antibody sets (3E5-mIgG1, 3E5-mIgG2a, and 3E5-mIgG3) were generated by ascites production in BALB/c mice (National Cancer Institute) primed with Pristane. A second antibody set (3E5-mIgG2a and 3E5-mIgG3) was produced exclusively by in-vitro hybridoma technique (21). In addition, 3E5-mIgG3 antibodies from eight different batches, called "Bt", were evaluated. In-house-produced antibodies were purified by affinity chromatography using rProtein A/Protein G GraviTrap Sepharose columns (GE Healthcare) according to the manufacturer's recommendations and quantified by ELISA (20).

Receptors

The soluble recombinant mouse proteins: integrin α4/β1 (VLA-4), FcγRIII (CD16) and FcγRI (CD64) were commercially available (R&D Systems.) Additionally, an in-house version of the integrin α4/β1 was produced, where its coding sequence was codon-optimized and synthesized (Genewiz). Each gene had the sequences encoding the transmembrane and intracellular domains removed and substituted for a coiled-coil domain sequence, to aid in integrin heterodimerization, and a sequence encoding an affinity tag (His-tag for Itgb1, Strep-tag for Itga4). The genes were cloned in pVITRO1-neo-mcs and the vector was transfected into FreeStyle™ 293-F cells using FreeStyle™ MAX reagent (Thermo Fisher Scientific) following the manufacturer's protocol. After five days of culture, transfected cells were centrifuged at 1,200 rpm for 5 min at 4 ºC. The supernatant was collected and concentrated using a Vivaspin® 20 ultrafiltration unit with a 100 kDa molecular weight cut-off (GE Healthcare). The expression of integrin was confirmed by Western blotting. The samples were subjected to 8% denaturing SDS-polyacrylamide gel electrophoresis and semi-dry transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The membrane was blocked for 1 h at room temperature in 5% skim milk diluted in Phosphate buffer saline containing 0.05% Tween 20 (PBST), washed thrice for 5 min with PBST and then incubated for 1 h with monoclonal alkaline phosphatase-conjugated anti-polyhistidine antibody (Sigma-Aldrich) at 1:1000 dilutions. After three 5 min washes with PBST, alkaline phosphatase was detected with nitro-blue tetrazolium/indolyl-phosphate (NBT/BCIP) chromogenic substrate (Thermo Fisher Scientific).

Solid-phase assay

Commercial available or in-house-produced antibodies (10 μg/mL) were incubated in a 96-well polystyrene plate at 4 ºC overnight. The plate was blocked for 1 h at 37 ºC using 1% bovine serum albumin (BSA) or Ovoalbumin in PBST and then washed thrice for 5 min with PBST. Integrin α4/β1, FcγRIII and FcγRI were added to the wells at four different concentrations (10, 5, 2.5 and 1.25 μg/mL) and incubated again at the same conditions. After washing again with PBST, monoclonal alkaline phosphatase-conjugated anti-polyhistidine antibody (Sigma-Aldrich) at 1 μg/mL was added and incubation and washing were repeated as in the last steps. The reaction was developed using p-nitrophenyl phosphate (pNPP) substrate from Sigma-Aldrich and the absorbance was measured at 405 nm using a microplate reader (Molecular Devices). The data were collected using SoftMax® pro software (Molecular Devices).

Dot blot immunoassay

The PVDF membrane (GE Healthcare) was treated according to the manufacturer's recommendations and 1 μg/mL of commercial murine IgG1/IgG2a/IgG2b/IgG3 antibodies (Mp Biomedicals) was individually dropped on the membrane and allowed to dry at room temperature. After drying, the membrane was blocked for 1 h in continuous shaking at room temperature in 5% skim milk diluted in PBST and then washed thrice for 5 min with PBST. The membrane was incubated under gentle agitation with 1 μg/mL of VLA-4 (integrin α4/β1) for 1 h at 37 ºC. After washing, rabbit anti-mouse β1 antibody at 1 μg/mL was added and incubated for 1 h at 37 ºC. After washing, the membrane was incubated under the same conditions as before with secondary HRP-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) at 1 μg/mL, followed by washing three times with PBST. Antibody binding detection was carried out using Novex™ ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific). Images were acquired using ChemiDoc XRS+ imaging system (Bio-Rad) and quantified using ImageJ software (NIH, USA).

Phagocytosis assay

The murine macrophage-like J774.16 cell line was plated in 96-well flat-bottomed tissue culture plates at 0.5 x 10^5 cells/well and incubated overnight at 37 °C and 5% CO_2. In order to block some receptors, the following antibodies at 10 μg/mL

were added: HMβ1-1 from Abcam (Itgb1 block), anti-CD18 + anti-CD11b + anti-CD11c from BD Pharmingen (complement receptor block) and 2.4G2 from BD Pharmingen (FcγRII and FcγRIII block). In addition, cells without blocking antibodies were maintained so as to control the experiment. Then, the plates were incubated for 20-30 min. Afterward, phagocytosis was carried out by adding 10 µg/mL of 3E5 from different batches and 2 x 10^5 cells/well of *C. neoformans* H99 strain (ATCC® 208821™), which resulted in an infection ratio of 1:2. Fungal cells incubated with macrophage-like cells without opsonizing antibodies were used as a negative control. The cells were incubated at 37 ºC and 5-10% CO_2 for 2 h and then they were washed twice with PBS, fixed with methanol and stained with panoptic dye. Cells were visualized using a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Inc), counting three fields per well with at least 100 cells/field. The percentage of phagocytosis was calculated from the number of macrophages that phagocyted *C. neoformans* divided by the total number of macrophages observed.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc.). Solid-phase assays were analyzed using Fisher's exact test or linear regression. Dot blot and phagocytosis assays were evaluated by one-way ANOVA with Tukey's post-hoc test.

Results

mIgG3 interacts with integrin α4β1

The solid-phase assay demonstrated a significant interaction between FLOPC-mIgG3 and VLA4. Also, various control conditions showed no significant signal when each one of the molecules was incubated individually or together with a detection antibody (Figure 1A). When carried out using in-house-produced IgGs and VLA4, the assay evidenced that the interaction between 3E5-mIgG3 and integrin resulted in higher signal levels than the control conditions. In addition, this experiment also demonstrated that 3E5-mIgG2a did not interact with VLA4 (Supplementary figure 1A-C).

As shown by dot blot assay, mIgG3 binds to VLA4 with strong signal intensity and the control conditions results were similar to those of solid-phase assay. It was also possible to

note that the signal relative to the interaction between the MOPC-mIgG1/UPC-mIgG2b isotypes and VLA4 was not significant (Figure 1B).

Interestingly, notwithstanding that the signal relative to the interaction between UPC-mIgG2b was not significant, it could be seen in a PVDF membrane image acquired after the revelation reaction (Figure 1C). A similar result had been found when the UPC10 (mIgG2a) isotype and VLA4 interaction was tested (Supplementary figure 1D). In an independent solid-phase assay, it was also obtained low interaction signal intensity when evaluating MOPC-mIgG1 and integrin interaction (Supplementary material 1E).

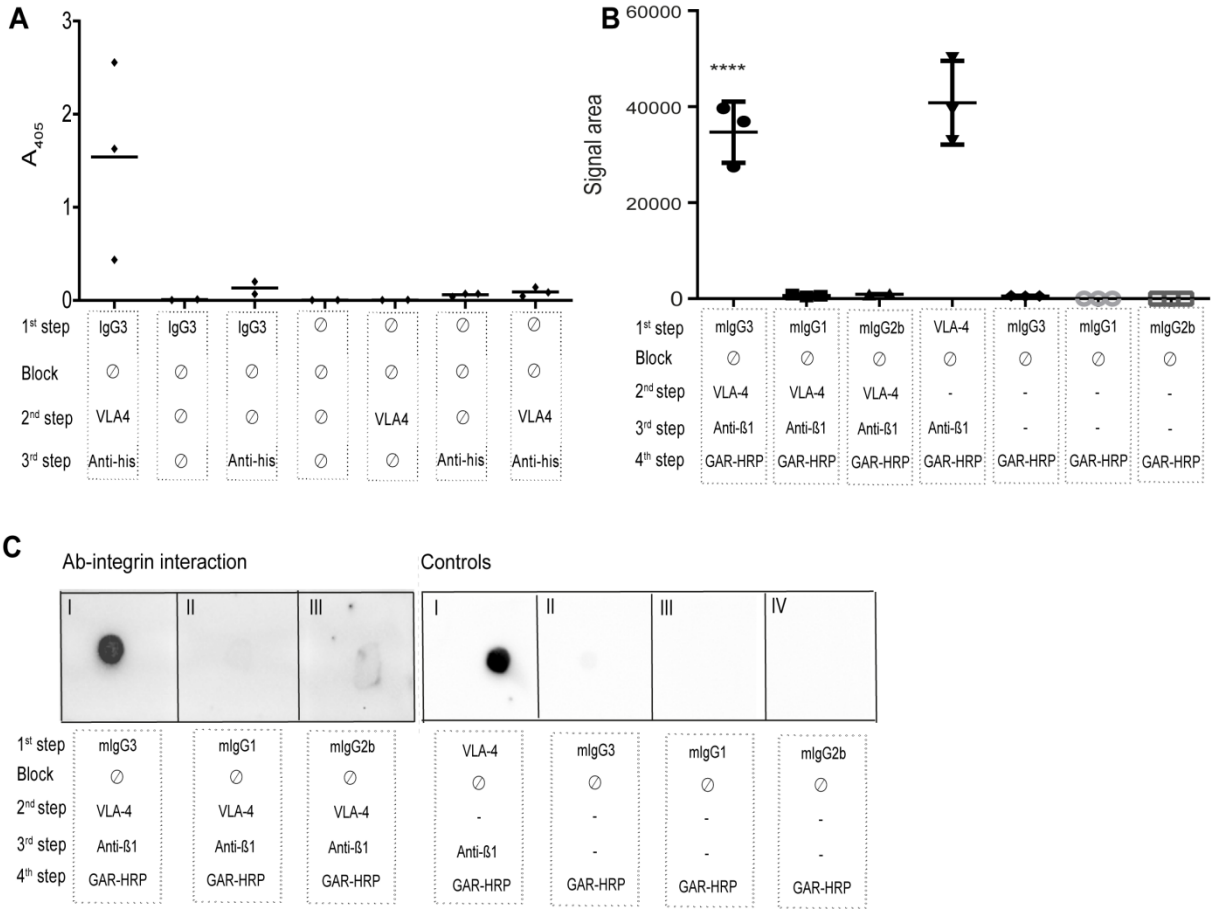

Figure 1 – Interaction assays between IgG isotypes and VLA4. A) Solid-phase interaction assay with FLOPC-mIgG3 and VLA.The first condition (on the left) represents the interaction test and the others represent the controls. Below the graph are listed the steps of the experiment and "Ø" represents the conditions that BSA 1% was used as a blocking agent. The bars represent the median absorbance of three independent experiments (p=0.0219). B) Dot blot assay to evaluate the interaction between FLOPC-mIgG3/MOPC-mIgG1/UPC-mIgG2b and VLA. The first three conditions (left) represent the interaction tests; the others are controls. Below the graph are listed the experiment steps and "-" represents the conditions that the indicated reagent was not used. The bars represent the average signal per membrane area of three independent experiments (**** p<0.0001). C) PVDF membrane from the dot blot assay. Conditions I to III (left) represent tests of interaction tests between IgG antibodies and VLA4; and the other conditions (right) are controls. The dark spots on the membrane represent the GAR-HRP signal after reaction development with substrate.

Distinct IgG isotypes interact differently with integrin α4β1, FcγRI and FcγRIII

The solid-phase assay was carried out to evaluate the interaction between different IgG isotypes and soluble receptors. When evaluating the interaction between FLOPC-mIgG3 and VLA4, it revealed a signal intensity enhancement as the receptor concentration was increased. Weak signal levels were observed when FLOPC-mIgG3 was incubated with FcγRIII and no interaction signal was showed when it was incubated with FcγRI (Figure 2A). MOPC-mIgG1 isotype presented a significant increase in interaction signal when incubated with FcγRIII, but a weak signal intensity was observed when the antibody was incubated with VLA4 and a near-zero signal level was demonstrated when incubated with FcγRI (Figure 2B). The UPC-mIgG2a isotype showed a significantly high interaction signal when incubated with FcγRI. Despite it demonstrated a weaker interaction signal when compared to UPC-mIgG2a and FcγRI interaction, it was possible to note UPC-mIgG2a interacts with FcγRIII and with VLA4 (Figure 2C).

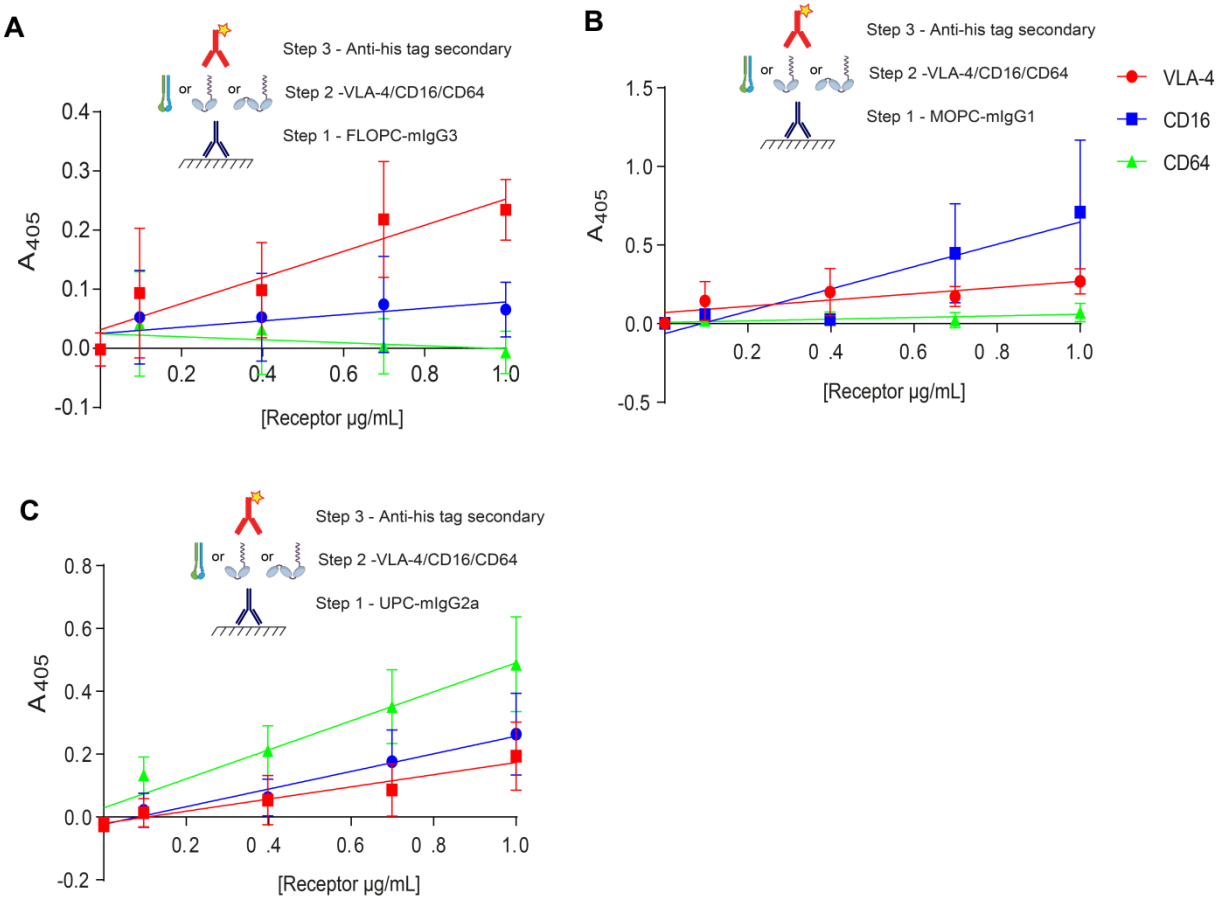

Figure 2 - Interaction assay between IgG isotypes and VLA4/FcγRI/FcγRIII. A) Solid-phase interaction assay with FLOPC-mIgG3 and VLA4/FcγRI/FcγRIII. Each point in the linear regression graph represents the average absorbance corresponding to the conditions in which FLOPC was incubated with receptors at different concentrations (1.25, 2.5, 5.0 and 10 µg/mL). The receptors shown in the graph are: VLA4 (red), FcγRI (green)

and FcγRIII (blue).The standard deviation represented by the vertical bar at each point on the graph refers to the dispersion around the average absorption of three independent experiments. *p* values: 0.0727 (VLA4), 0.2675 (FcγRIII) and 0.0219 (FcγRI). B) Solid-phase interaction assay between MOPC-mIgG1 and VLA4/FcγRI/FcγRIII. *p* values: 0.1611 (VLA4), 0.0647 (FcγRIII) and 0.2312 (FcγRI). C) Solid-phase interaction assay between UPC-mIgG2a and VLA4/FcγRI/FcγRIII. *p* values: 0.0420 (VLA4), 0.0144 (FcγRIII) and 0.0072 (FcγRI).

An independent solid-phase assay carried out between 3E5-mIgG3 and soluble VLA4 showed a similar interaction signal (Supplementary material 1F) when compared to the result obtained with FLOPC-mIgG3 (Figure 1C). Likewise, although 3E5-mIgG3 did not show significant interaction with FcɣRI, it demonstrated a notable interaction with FcɣRIII, as well as with VLA4, even when compared to their signal intensities. 3E5-mIgG1 showed high interaction signal when incubated with FcɣRIII and a near-zero signal when incubated with FcɣRI, but conversely to what was found with MOPC-mIgG1 in the previous experiment, the 3E5 antibody demonstrated a predominant interaction with VLA4. Similarly to the UPC-mIgG2a antibody, the 3E5-mIgG2a isotype showed a high interaction signal when incubated with FcɣRI, but it decreased when analyzing antibody interaction to FcɣRIII and especially to VLA4, which showed no interaction signal (Supplementary material 1H).

IgGs from different productions interact differently to Itgb1 and FcγRI on the surface of macrophage-like cells

Phagocytosis assay carried out using *C. neoformans* and macrophage-like cells with receptors blockade revealed a different percentage of phagocytosis when incubated with 3E5 antibodies from different batches or production methods. Similarly to Saylor et al. results (19), 3E5-mIgG3 produced by ascites method in mice did not dependent on FcγR and CR to mediate fungal phagocytosis, whereas 3E5-mIgG2a was significantly dependent on receptors (Figure 3A). Nevertheless, 3E5-mIgG3 produced exclusively by hybridoma technique showed significant dependence on FcɣR and CR, but it was not dependent on Itgb1 for fungal internalization (Figure 3B). An assay with eight different batches of 3E5-mIgG3 antibodies produced by ascites demonstrated similar dependence on FcɣR/CR and independence of Itgb1 in most batches, except for "Bt-2" and "Bt-5" which were partially dependent on both FcɣR/CR and Itgb1, but it was only statistically significant for "Bt-5" (Figure 3C).

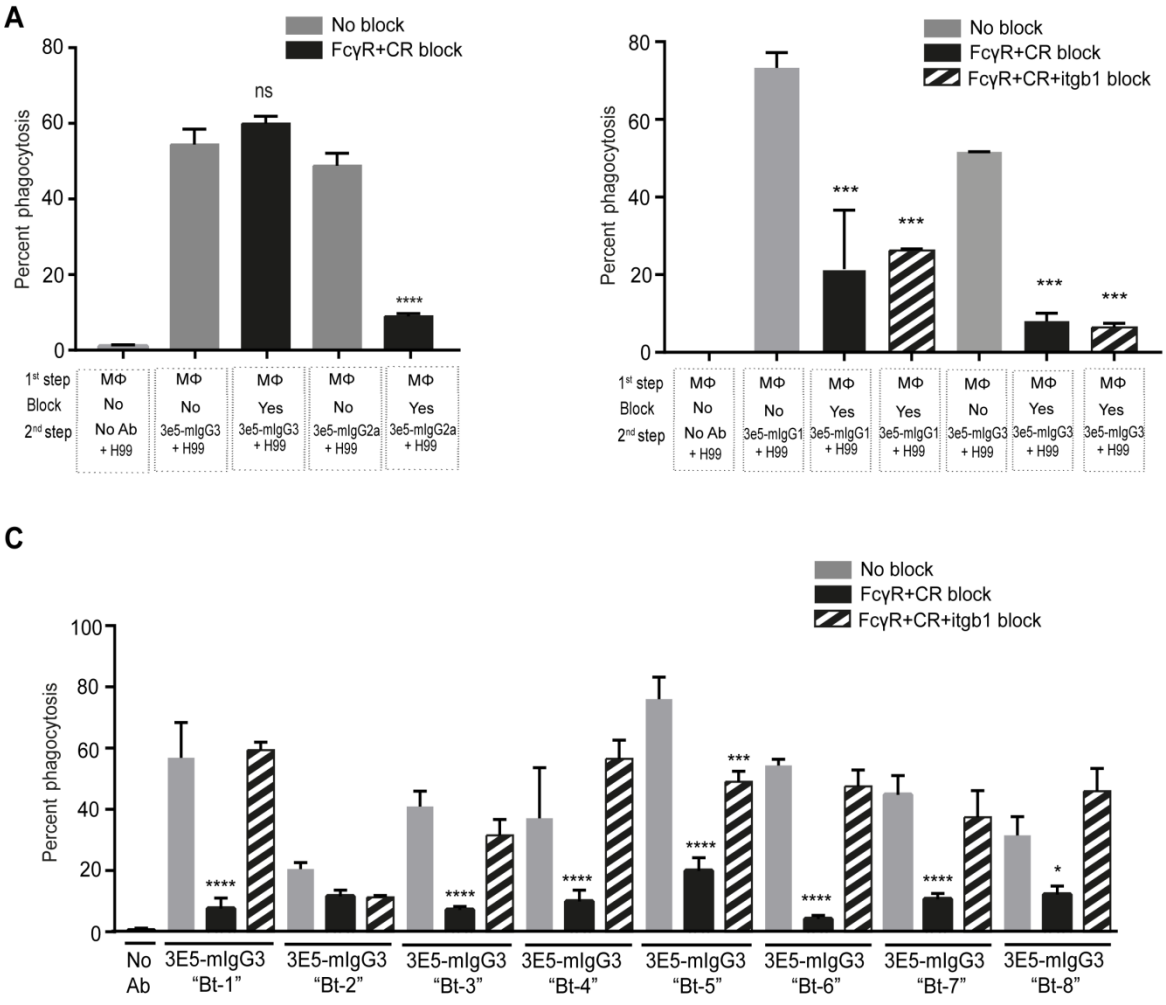

Figure 3 - Interaction assay between 3E5 antibodies and macrophage transmembrane receptors. A) Phagocytosis assay with J774 and *C. neoformans* cells opsonized with the ascites antibodies 3E5-mIgG3 and 3E5-mIgG2a. The macrophage cells were pre-incubated with blocking antibodies against α subunits of FcγRIII/FcγRII and complement receptors CR3 and CR4; then, the cells were incubated with a 1:2 ratio of macrophage:yeast. The bars represent the percentage of macrophages that internalized at least one yeast. The bars in black represent the conditions in which the macrophage (Mφ) receptors FcγR/CR were blocked; the bars in gray represent the conditions in which there was no receptor blockade. B) Phagocytosis assay with J774 and *C. neoformans* cells opsonized with 3E5-mIgG3 and 3E5-mIgG1 antibody from hybridoma. The cells were pre-incubated with FcγR/CR or Itgb1 blocking antibodies. The striped bars represent the conditions in which FcγR/CR and Itgb1 were blocked. C) Phagocytosis assay with J774 and *C. neoformans* cells opsonized with ascites 3E5-mIgG3 from different batches. The numbering after "Bt" is used to identify each antibody batch. **** $p<0.0001$.

Discussion

In spite of the importance of the hypothesis that Itgb1 may be the main interacting molecule of mIgG3, contributing to its effector response (20), many questions still need to be answered in order to corroborate whether Itgb1 can function as the mIgG3 receptor/co-receptor. One of these questions is: 1) Does mIgG3 interact with Itgb1 outside the membrane

complex? This question is important to be answered since the interaction between the molecules has been tested only using Itgb1 anchored to the macrophage membrane. One of the hypotheses that could be formulated from this condition is whether the interaction between Itgb1 and mIgG3 has occurred mediated by another molecule present on the cell surface, and not as Itgb1 as the sole or main ligand. To aid answering that question, solid-phase assays and dot blot immunoassay performed with both commercially available IgGs and soluble recombinant integrin (Figure 1A-C), as well as solid-phase assay performed with in-house-produced IgGs and soluble recombinant integrin (Supplementary figure 1A and B) demonstrated that mIgG3 interacts with Itgb1 outside the membrane complex. These results indicate that Itgb1 probably interacts independently of another membrane-associated molecule, or at least that it is important in the interaction with mIgG3.

Besides the significance of studying the interaction with soluble Itgb1, the solid-phase and dot blot results described above present quantitative information about integrin and mIgG3 interaction. It is important because flow cytometry was the only quantitative method previously used to evaluate the interaction between the molecules. Thus, the FLOPC-mIgG3 interaction results obtained in this study corroborate previous findings regarding 3E5-mIgG3 interaction (20).

Since only the interaction between mIgG1/mIgG3 isotypes and integrin was tested, a second question was raised: 2) Are there other IgG isotypes able to interact with Itgb1? Thus, solid-phase and dot blot interaction assays were performed to answer this question. It was possible to extract from the dot blot assay results that although the interaction between Itgb1 and UPC-mIgG2a (Supplementary Figure 1D) or UPC-mIgG2b (Figure 1C) produced low signal intensity, it was visible in the PVDF membranes. It was also possible to observe a significant signal related to the interaction between UPC-mIgG2a and Itgb1, and slightly lower signal intensity for the interaction between UPC-mIgG1 and integrin (Figure 3B and C). Furthermore, it was possible to note a low signal level related to the interaction between MOPC-mIgG1 and Itgb1 (Supplementary Figure 1E); and also between 3E5-mIgG1 and Itgb1 (Supplementary figure 1G).

In order to investigate whether mIgG3 interacts with FcγR, we tested the interaction between FcγRI and FLOPC-mIgG3 or 3E5-mIgG3, which showed a near-zero interaction signal (Figure 2A and Figure 1F). In contrast to the previous result that suggested FcγRI as a mIgG3 receptor, we demonstrated an interaction between FLOPC-mIgG3 and FcγRIII ($p = 0.2675$), and also between 3E5-mIgG3 and FcγRIII ($p = 0.0235$), which corroborates other

previous findings (17, 22, 23). Moreover, the mIgG1 and mIgG2a isotype interactions reflect what is described in the literature (3, 18). The interaction results between mIgG3 and FcγRIII are similar to those observed in the phagocytosis assay, in which the percentage of phagocytosis was drastically reduced when macrophage-like cell receptors were blocked (Figure 3B and C).

Experiments performed using different IgG antibodies sets demonstrated conflicting results regarding their interaction with cell receptors, thus it was proposed that production batches may be composed of a mixture of isoforms that varies from batch to batch (20). This would explain why studies are claiming that FcγRI is the mIgG3 receptor (18), while others claim that mIgG3-mediated phagocytosis is dependent on complement, and others disagree with both (17, 19). To investigate this hypothesis further, we decided to answer the following question: 3) Is there a variation in the macrophage receptors interaction using the same antibody from different production methods or different batches? For this purpose, we compared the percentage of phagocytosis between two different 3E5 productions (one was generated by ascites production in mice and the other was produced exclusively by in-vitro hybridoma technique). Thus, we identified two different mIgG3-mediated phagocytosis profiles: the former mediates phagocytosis completely independent of FcγR/CR, while the latter mediates phagocytosis with significant dependence on FcγR/CR (Figure 3A and B). We also found a difference between eight batches of ascites-produced 3E5 antibodies, in which two batches mediated phagocytosis with only partial dependence on FcγR/CR and Itgb1; and the other batches mediated phagocytosis dependent on FcγR/CR (Figure 3C). Furthermore, when comparing the solid-phase results of myeloma-produced antibodies (Figure 2A-C) and hybridoma-produced antibodies (Supplementary figures 1F-H), a variation in the interaction profile between the antibodies and receptors can be observed.

Besides the fact that the production method and different batches of the same antibody may induce isoform variation, it is possible that different integrin production methods could also change integrin, interfering with antibody interaction. This hypothesis could be formulated when comparing results represented in figure 1A, which shows evidence of interaction between FLOPC-mIgG3 and VLA4 from Chinese hamster ovary cells, with the results represented in supplementary figures 1A and B, in which no evidence of interaction between FLOPC-mIgG3 and VLA4 produced in human embryonic kidney cells is shown.

Given the discussion and regarding the research questions raised previously, we can draw some conclusions from our findings: 1) Itgb1 is the main ligand of mIgG3; 2) Other IgG

isotypes interact with Itgb1, but with less affinity; 3) mIgG3 interacts with FcγRIII; 4) mIgG3 from different production methods or batches alter their interaction to Itgb1 and FcγRII/ FcγRIII.

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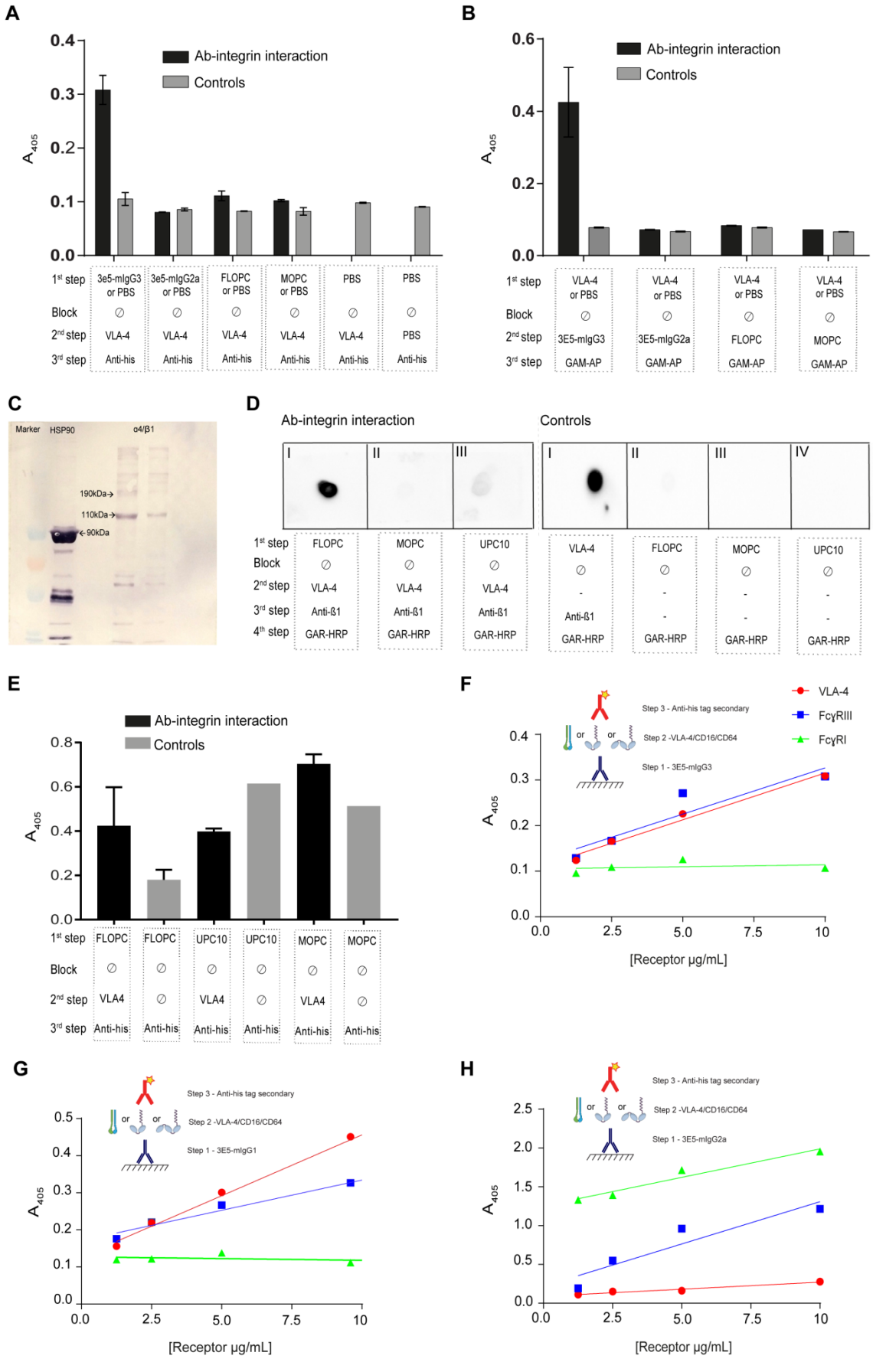
A
Ab-integrin interaction
Controls
A405
1st step
Block
2nd step
3rd step
3e5-mIgG3 or PBS
3e5-mIgG2a or PBS
FLOPC or PBS
MOPC or PBS
PBS
VLA-4
Anti-his
B
Ab-integrin interaction
Controls
VLA-4 or PBS
3E5-mIgG3
3E5-mIgG2a
FLOPC
MOPC
GAM-AP
C
Marker
HSP90
α4/β1
190kDa
110kDa
90kDa
D
Ab-integrin interaction
Controls
4th step
FLOPC
MOPC
UPC10
Anti-ß1
GAR-HRP
E
Ab-integrin interaction
Controls
F
VLA-4
FcγRIII
FcγRI
Step 3 - Anti-his tag secondary
Step 2 -VLA-4/CD16/CD64
Step 1 - 3E5-mIgG3
[Receptor µg/mL]
G
Step 1 - 3E5-mIgG1
H
Step 1 - 3E5-mIgG2a

Supplementary Figure - Pilot and control conditions of interaction tests. A e B) Solid-phase assay with mIgG3 and VLA4 from in-house production. Two different solid-phase interaction assays were performed on ELISA plates. The first was done with antibodies immobilized on the plate and incubated with a supernatant containing soluble integrin; the other was done with integrin immobilized on the plate and incubated with soluble antibodies. The bars represent the average absorbance obtained after a single test, in which each condition was performed in duplicate. (C) Western blot assay of the soluble α4/β1 integrin domain produced in 293F cells. The band with the highest signal intensity represents the positive control (HSP90) of approximately 90 KDa. Adjacent to HSP90, the 5x concentrated α4/β1 sample and non-concentrated α4/β1 sample were also transferred. The bands close to 190 KDa and 110 KDa had the expected molecular weight for α4/β1 and β1, respectively. (D) Dot blot assay presented significant signal when UPC-mIgG2a was incubated with VLA4 (condition III). (E) Solid-phase assay showed interaction signal when MOPC-mIgG1 was incubated with VLA4. (F-H) Interaction assay between 3E5 IgG isotypes and VLA4/FcγRI/FcγRIII. The bars represent the average absorbance of two wells in a single experiment. For these conditions the *p* values were, respectively: 0.0037 (VLA4), 0.0235 (FcγRIII) and 0.6738 (FcγRI); 0.0212 (VLA4), 0.0660 (FcγRIII) and 0.0241 (FcγRI); 0.0098 (VLA4), 0.0703 (FcγRIII) and 0.7264 (FcγRI).

IV. Conclusão

Capítulo I

- 2H1-mIgG3 apresentou padrão de imunofluorescência diferente de 2H1-mIgG1, reforçando que a região constante do anticorpo influencia a ligação ao antígeno e que provavelmente não se trata de um evento que ocorra ao acaso;
- 2H1-mIgG3 compete em baixa afinidade e 2H1-mIgG1 compete em alta afinidade ao epítopo de 12A1. Essa variação na afinidade levanta a hipótese de que talvez isso ocorra devido à diferença na região V dos anticorpos 2H1, a qual influencia a ligação a epítopos diferentes;
- 2H1-mIgG3 continuou a se ligar à GXM quando de-O-acetilada, enquanto que 2H1-mIgG1 teve ligação drasticamente reduzida. Isso sugere que os anticorpos são capazes de reconhecer epítopos de GXM diferentes;
- A região de dobradiça se mostrou necessária, mas insuficiente para alterar a ligação do anticorpo 2H1-mIgG3 ao antígeno.

Capítulo II e III

- Itgb1, assim como FcγRI e complemento, provavelmente faz parte de um complexo de receptores de mIgG3;
- Ca^{2+} aumenta a ligação de mIgG3 à Itgb1 na superfície de macrófagos, isso sugere que Itga4 seja o par alfa da integrina;
- A interação entre mIgG3 e Itgb1 solúvel revela a integrina como importante ligante do anticorpo;
- mIgG3 interage com diferentes moléculas (Itgb1 e FcγRIII, FcγRI) para exercer suas respostas efetoras. A predileção ao receptor pode variar dependendo do método ou lote de produção do anticorpo;
- Itgb1 interage predominantemente com mIgG3, mas também pode interagir com mIgG2a, mIgG2b e mIgG1.

Anexo I – Trabalhos desenvolvidos, apresentações e produções bibliográficas.

O estudo de IgG3 rendeu apresentações de pôsteres em eventos nacionais e internacionais; palestras em eventos nacionais; realização de visita técnica; treinamento de estudantes de Iniciação Científica, mestrado e intercambistas; coorientação em banca de graduação; e publicação de trabalhos e artigos científicos em primeira autoria ou por colaboração:

Palestras

- Métodos imunológicos aplicados à prática laboratorial. 2019. XIII Simpósio de Biomedicina da Universidade Católica de Brasília (DF, BR).

- Cientistas: o que são, o que comem e onde vivem? Saiba mais sobre essa misteriosa espécie. 2018. XIII Jornada da Saúde – Unieuro Asa Sul (DF, BR).

- O uso de anticorpos na terapia de doenças negligenciadas. 2017. I Congresso de Ciências Biomédicas de Brasília– a biomedicina vista por diferentes ângulos, ICESP (DF, BR).

- Imunoglobulina gama 3 de camundongo: estudo da interação com antígenos e com receptores de macrófagos. 2017. VII Simpósio do Programa de Pós-Graduação em Ciências Biológicas (Biologia Molecular) da Universidade de Brasília (DF, BR).

- Variable-region identical antibodies of different IgG isotypes show different binding patterns to *Cryptococcus neoformans*. 2014. XXXIX Congress of the Brazilian Society of Immunology. (RJ, BR).

Apresentaçãoes de pôsteres

- D. S. L. Oliveira, V. Paredes, A. V. Caixeta, P. Albuquerque, M. S. S. Felipe, A. M. Nicola, and A. Casadevall. Imunoglobulina gama 3 (IgG3) de camundongo: estudo da interação com antígenos e com receptores de macrófagos. 2014-2019. IV-IX Simpósio do Programa de Pós-

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- D. S. L. Oliveira, N. M. Henriques, V. Paredes, P. Albuquerque, M. S. S. Felipe, A. M. Nicola, and A. Casadevall. Murine immunoglobulin gamma 3: a study of its interaction with macrophage receptors. 2018. Protein and antibody engineering summit (LX, POR).

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Treinamento de estudantes

- Aluna de Iniciação Científica Nicole Henriques (graduação em Farmácia/Unieuro). 2017-2019. Tema do projeto: O papel das integrinas como receptores de IgG3.

- Aluna de Iniciação Científica Larissa Biângulo (graduação em Biomedicina/ ICESP). 2018. Tema do projeto: Estudo *in vitro* de anticorpos monoclonais contra *C. neoformans* e *Paracoccidioides sp*.

- Mestranda Adrielle Veloso (programa de Biologia Molecular/UnB). 2016-2017. Tema do projeto: Anticorpos quiméricos IgG e o papel da cadeia constante pesada na ligação à cápsula de *C. neoformans*.

- Aluno de Iniciação Científica Lucas Mendelson (graduação em Biotecnologia/UnB). 2016-2017. Tema do projeto: O papel das integrinas como receptores de IgG3.

- Biomédico bolsista DTI-C do CNPq Kaio de Melo Gorgonha (empresa *Karan biotechnologies* Pesquisa e Desenvolvimento). 2015. Desenvolvimento da fração Fc de anticorpos IgG3 para estudo de interação com integrina.

- Graduandas intercambistas do curso de Medicina Kasha (Polônia) e Alejandra (Espanha) em parceria com a Universidade Católica de Brasília. 2014. Ferramentas de Biologia Molecular para estudo de anticorpos monoclonais e doenças infecciosas.

Visita técnica

- Produção de anticorpos recombinantes e MS/MS. 2015. Johns Hopkins University (MD, EUA).

Coorientação

- Coorientadora de TCC da estudante Larissa Biângulo – ICESP. 2018. Estudo *in vitro* de anticorpos monoclonais contra *C. neoformans* e *Paracoccidioides sp*.

Artigos científicos e trabalho em finalização

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