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Imunoregulação na infecção experimental por *C. albicans* induzida por uma proteína p43, imunossupressora/mitogénica linfocitária, excretada pelo fungo.

Perspectivas inovadoras de imunoprotecção.

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Dissertação da candidatura ao grau de Doutor em Ciências Biomédicas, apresentada no Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, sob a orientação do Senhor Prof. Doutor Mário Arala-Chaves.

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CAPÍTULO 1. Introdução

1. Os fungos do género *Candida* e as candidíases

Algumas espécies do Género *Candida,* particularmente a espécie *Candida albicans,* têm vindo a tornar-se cada vez mais importantes como patogénios para o Homem. De facto, nas últimas três décadas, a incidência de candidíases tem aumentado dramaticamente (1). Na última década, as infecções sistémicas por *C. albicans* aumentaram mais de 500% (2). Para este fenómeno relativamente recente, contribuem múltiplos factores de que destacamos os seguintes: 1) vulgarização de terapêuticas com antibióticos de largo espectro que, alterando a flora normal do hospedeiro, aumentam o risco de infecções por fungos oportunistas como é o caso da *C. albicans*; 2) uso de estratégias terapêuticas que interferem com os mecanismos de defesa do hospedeiro, de que é exemplo mais corrente a administração de corticoides, assim como a quimioterapia e a radioterapia usadas no tratamento de doentes cancerosos e terapêuticas imunossupressoras utilizadas em doentes com transplantes de orgãos; e, 3) aparecimento da pandemia do Síndroma de Imunodeficiência Adquirida (SIDA) que tem levado à expansão do número de doentes com imunodeficiência.

As candidíases e as dermatomicoses são referidas na literatura científica desde os primórdios da Microbiologia. Assim, em 1842, Gruby descreve o fungo causador de «le vrai muguet des enfants», que corresponde a uma situação clínica que hoje se poderia descrever como candidíase da mucosa oral. Em 1853, Robin classifica o fungo como *Oidium albicans* e, no início do século vinte, Berkout cria o Género *Candida.* Durante as duas

primeiras décadas deste século Castellani estuda o papel da *C. albicans* como agente etiológico das bronquiomonilíases e Ashford documenta a sua observação sistemática da presença de *C. albicans* em doentes com diarreia tropical.

São hoje conhecidas mais de 100 espécies do Género *Candida.* Contudo, apenas 7 destas espécies são isoladas com alguma frequência de material biológico de doentes suspeitos de sofrerem de infecção. Entre estas espécies de *Candida* potencialmente patogénicas para o Homem, as espécies *C. albicans* e *C. tropicalis* representam mais de 80% de todas as leveduras isoladas a partir de amostras clínicas (3). As espécies de *Candida* saprófitas podem ser isoladas de vários ambientes, nomeadamente do solo, da água doce e salgada, de plantas, de insectos, etc. Pelo contrário, as espécies patogénicas são frequentemente isoladas apenas do Homem e de outros mamíferos (4,5).

A *C. albicans* é considerada como fazendo parte da flora normal do Homem. É isolada, frequentemente, das superfícies mucosas e nas transições mucocutâneas, especificamente nas regiões urinária, genital, gastrointestinal e oral. A percentagem de isolamentos de *C. albicans* de secreções de indivíduos assintomáticos varia de 10 a 30% no tracto gastrointestinal, é de cerca de 50% na cavidade oral de adultos jovens e de cerca de 80% na superfície cutânea em recém-nascidos que contactem com o microrganismo durante o parto natural, ao atravessarem o canal vaginal. É frequente o isolamento do microrganismo a partir das secreções vaginais das grávidas. O isolamento de

C. albicans a partir da superfície cutânea é raro em indivíduos jovens saudáveis; sobe, no entanto, com a idade sendo observado em 30% das pessoas idosas (6). Assim, a distinção entre o carácter patogénico ou não patogénico de um isolado de *C. albicans* é frequentemente obtida tendo em conta parâmetros quantitativos: a *C. albicans* será considerada parte da flora normal do hospedeiro, a não ser que o microrganismo seja isolado em grande número e/ou em cultura pura. De facto, o isolamento e identificação de *C. albicans* em material biológico como fezes, urina, expectoração etc. indica colonização mas não, necessariamente, infecção patogénica.

1.1. Morfologia da *C. albicans* e sua implicação na invasão do hospedeiro.

O fungo *C. albicans* é um microrganismo interessante sob vários aspectos. A sua relação com o hospedeiro apresenta vários enigmas, particularmente no que respeita à sua patogenicidade para o Homem e os mamíferos em geral, à transição dimórfica que este microrganismo expressa e aos mecanismos de resposta imunológica que desencadeia no hospedeiro a colonização e/ou invasão dos seus tecidos. Estudos morfogenéticos têm documentado as propriedades dimórficas das células de *C. albicans* que se caracterizam pela transição da forma de levedura (forma-Y) para a forma de hifa (forma-H) (7,8,9). Esta transição morfológica é acompanhada por acentuadas alterações na expressão das estruturas da superfície destas células. Assim, sabe-se que existem determinantes antigénicos exclusivos ou predominantes numa ou outra das duas formas de *C. albicans;* estes

determinantes fazem parte sobretudo de manoproteínas de alto peso molecular (10,11,12). O significado da variação antigénica na patogenicidade do fungo não é ainda claro. Durante os primeiros estadios de infecção, a fagocitose das formas-Y remove e destrói a maioria das células Y presentes no hospedeiro. Todavia, as células de *Candida* que escapam à fagocitose, rapidamente passam à forma-H e ganham a capacidade de invadir tecidos do hospedeiro, onde produzem granulomas e causam lesões tecidulares crónicas (13). Embora as duas morfologias Y e H sejam encontradas nos tecidos afectados com candidíase, a capacidade de formar filamentos, ou seja de passar para a forma H, parece ser um factor importante de virulência do fungo (14,15).

A parede celular é a estrutura que, não só dá a forma característica da *C. albicans* em cada ciclo de crescimento como constitui, também, a interface entre o organismo e o meio ambiente. Assim, é plausível considerar que informação acerca da identidade e distribuição dos componentes da parede celular possam vir a ser relacionados com factores de virulência. Características da parede celular relacionadas com a apresentação de antigénios, a expressão de adesinas e receptores para proteínas dos tecidos do hospedeiro, ou substâncias com actividade proteolítica (proteases) exocelulares, etc., são importantes na patogenia do fungo (16,17 ,18 ,19 ,20,21,22,23). A adesão das células de *Candida* a células do hospedeiro é a etapa inicial para o estabelecimento de infecção. Frequentemente, os organismos causadores de doenças infecciosas têm como alvo tecidos

específicos do hospedeiro. A identificação de ligandos nas células do hospedeiro e de moléculas de adesão no parasita, envolvidos na especificidade tecidular do agente infeccioso, explicará as características particulares do modo como cada agente infeccioso se dissemina no hospedeiro.

Nos últimos anos, 4 categorias de moléculas de adesão foram descritas na superfície da *C. albicans* (24,25,26,27): 1) componentes proteicos de *Candida* parecem ser adesinas para estruturas igualmente de natureza proteica, ligandos, das membranas das células do hospedeiro; 2) proteínas do tipo das lectinas do fungo reagem com resíduos de açúcar das glicoproteínas das membranas celulares do hospedeiro; 3) resíduos glucídicos superficiais da *Candida* ligam-se a proteínas das membranas celulares do hospedeiro; 4) moléculas da superfície de *Candida* levam a interacções hidrofóbicas com células do hospedeiro. Estas últimas interacções hidrofóbicas são, geralmente, consideradas como tendo um papel particularmente importante na aderência do microrganismo à superfície das células do hospedeiro (28,29). De facto, a hidrofobicidade tem sido implicada na ligação da *C. albicans* a células epiteliais humanas, a catéteres plásticos e a próteses (22 ,30 ,31). Por outro lado, tem sido sugerido que as interacções hidrofóbicas contribuem para a aderência ao manterem ou aumentarem o número de receptores de adesinas no contacto entre o parasita e células do hospedeiro. Tem sido também referido que a hidrofobicidade da superfície celular ajuda a impedir a morte das células

fúngicas pelos leucócitos polimorfonucleares e aumenta a capacidade das células de *Candida* de se multiplicarem no hospedeiro (21 ,32).

A capacidade de aderência às células das mucosas é uma propriedade própria da *C. albicans* que está ausente em outras espécies de *Candida* menos patogénicas (33). A formação do tubo germinativo em *C. albicans* está associada ao aumento de aderência às células epiteliais da mucosa bucal (34) e também à patogenicidade do microrganismo (35). Alterações químicas e microbiológicas da superfície das mucosas podem influenciar a susceptibilidade às infecções por *C. albicans* por afectarem o processo de aderência intercelular. É clássico o conhecimento clínico de que a aderência de *C. albicans* às células do epitélio vaginal é mínima a pH ácido e máxima a pH neutro sendo este fenómeno igualmente influenciado pela flora bacteriana normal, tal como os *Lactobacilli,* os quais podem competir pelos sítios de ligação celular da *Candida* ao epitélio (36).

A invasão das células epiteliais é a segunda etapa importante na patogenia da *C. albicans* no homem e nos mamíferos em geral (37). O tubo germinativo penetra a membrana da célula epitelial, possívelmente ajudado por enzimas hidrolíticas (38,39). Dentro da célula epitelial o fungo continua o seu processo de crescimento (40). O mecanismo de passagem através das células epiteliais pode ser um método importante para a invasão dos tecidos pelo microorganismo. As células epiteliais do hospedeiro podem tolerar a presença do fungo no seu citoplasma sem lesões graves da sua fisiologia ou, pelo contrário, podem apresentar evidências histológicas de alterações profundas

que põem em causa a sua viabilidade (40, 41). A persistência de células de *Candida* no interior de células epiteliais pode ser um mecanismo para a resistência à quimioterapia e uma explicação para infecções recorrentes (42).

2. Candidíase Humana

2.1. Aspectos Clínicos da Doença

Para que a *C. albicans* provoque doença grave no Homem é necessário, quase sempre, que haja uma quebra na defesa imunológica do hospedeiro. No entanto, infecções locais pelo fungo, como as candidíases vaginais comuns, não requerem alterações sistémicas dos sistemas de defesa e são resultado apenas de modificações locais como do pH das secreções vaginais. Assim, este microrganismo, que é um comensal das mucosas, é classificado, enquanto agente infeccioso, como patogénio oportunista e, como tal, o estado imunofisiológico do hospedeiro é de importância primordial para que a patogenicidade do fungo se expresse.

Abordaremos de seguida os principais tipos de infecção induzidas pelo fungo, quais as condições que predispõem à infecção por *C. albicans,* e as defesas naturais do hospedeiro que impedem a infecção pelo microrganismo.

2. 1. 1. Tipos de Infecção, Factores que Predispõem ao Aparecimento da Doença e Defesas do Hospedeiro na Infecção.

Vários tipos de infecção clínica por *C. albicans* são habitualmente distinguidos. Passo a descrever de seguida as principais características das entidades clínicas denominadas de candidíase mucocutânea crónica (CMC), candidíase do tubo digestivo (CTD), candidíase vaginal (CV) e candidíase sistémica (CS), bem como os factores que predispõem ao seu aparecimento e as defesas do hospedeiro envolvidas na infecção.

Candidíase mucocutânea crónica (CMC). Esta designação engloba vários síndromas caracterizados por infecções recorrentes das membranas mucosas, pele e unhas por *C. albicans.* Embora a doença seja frequentemente associada a endocrinopatias, é geralmente aceite que o principal factor que predispõe ao aparecimento da doença é uma imunidade celular deficiente, sendo uma infecção frequente em doentes com SIDA (43). De facto, agentes supostamente capazes de aumentar a imunidade celular do doente parecem levar à remissão da doença (44). Os doentes com CMC podem apresentar, no entanto, um leque variado de anomalias da resposta imune, mas é importante notar que não há uma anomalia que seja comum a todos os grupos de doentes com afecções deste tipo.

Na maioria dos casos de CMC a linfoproliferação específica para antigénios de *C.albicans* está diminuída, enquanto que anticorpos específicos anti-*Candida* estão aumentados e parecem facilitar a infecção fúngica. Embora

o aumento de anticorpos específicos possa reflectir uma tentativa de combater a infecção em doentes com CMC, a incapacidade apresentada pelos neutrófilos de causarem a morte celular de *Candida* tem sido atribuída a anticorpos séricos específicos para o fungo (45, 46). Igualmente, a alta incidência de infecções com *Candida* observada em doentes com hiperimunoglobulinemia E é atribuída a anticorpos da classe IgE específicos contra o fungo e que inibem a função neutrofílica (47). Estes resultados foram obtidos *in vitro* tendo-se verificado que o soro de doentes com candidíase e com título de anticorpos contra *C. albicans* elevado, reduzia a capacidade fungicida dos neutrófilos. Todavia, a reduzida eficácia dos neutrófilos na eliminação do fungo poderá não ser uma consequência directa do aumento dos anticorpos específicos. A presença no soro de factores imunomodeladores como a IL-10, que é, como se sabe, responsável pela diminuição do metabolismo oxidativo e da citotoxicidade celular mediada por anticorpos nos neutrófilos (48), pode ser responsável por aquele fenómeno. De qualquer modo, estas observações sugerem que elevados títulos de anticorpos contra a *Candida* nas superfícies das mucosas não são eficazes na protecção do hospedeiro contra o fungo. Por outro lado, em modelos animais, foi já demonstrado em infecções por bactérias (49), vírus (50, 51, 52) e protozoários (53) que os anticorpos específicos podem facilitar a infecção, possívelmente pela produção de anticorpos anti-idiotipo (54). Outra explicação que tem sido dada é a de que os anticorpos específicos facilitam demasiadamente a

fagocitose dos microrganismos, sobrecarregando as células fagocíticas que seriam incapazes de matar todas as partículas fagocitadas.

Em conclusão, uma imunidade celular deficiente será determinante para o aparecimento das CMC. Os anticorpos específicos contra o fungo não parecem eficazes na protecção contra este tipo de infecção.

Candidíase do tubo digestivo (CTD). Esta forma de candidíase é referida na literatura médica desde o século XIX em que se descreve um caso fatal de perfuração do esófago num recém-nascido (55). *C. albicans* é o fungo mais frequente em todo o tubo digestivo nos individuos adultos normais, aumentando a frequência de isolamentos e a concentração do fungo progressivamente desde a orofaringe até ao cólon (55). Apesar da elevada colonização por *C. albicans,* em individuos normais e em circunstâncias normais não ocorre infecção devido aos mecanismos de defesa do tubo digestivo. Assim, a acidez gástrica, a flora bacteriana endógena, a produção local de anticorpos, particularmente da classe IgA, são factores que mantêm um equilíbrio microbiano. Quando este equilíbrio é quebrado, por exemplo pela administração de antibióticos, esteróides ou antiácidos, pode haver proliferação do fungo e invasão dos tecidos do hospedeiro.

Doentes com diabetes mellitus são mais susceptiveis à infecção por *C. albicans* uma vez que apresentam níveis de colonização da mucosa oral superiores aos indivíduos normais o que pode ser devido ao aumento de glucose (fonte particularmente nutritiva do fungo) na saliva (56,57). Também os

doentes cancerosos, particularmente os doentes com leucemia, têm frequentemente CTD o que parece estar relacionado com a resposta imunológica diminuída nestes doentes. Por outro lado, os tratamentos imunossupressores a que os doentes cancerosos são sujeitos aumentam o risco de candidíase. A candidíase sistémica, que é uma das principais causas de morte nestes doentes (58,59) parece ter a sua origem primária no aumento da colonização do tubo digestivo pela *C. albicans* e na passagem das células fúngicas através da camada epitelial da mucosa do intestino. Vários estudos clínicos sugerem uma correlação entre os factores que favorecem o aumento da colonização com a adesão às células epiteliais e subsequente invasão. A adesão das células fúngicas ao epitélio do TD é uma etapa crítica na patogenia da CTD. As células fúngicas atravessam as mucosas atingindo o espaço vascular e originam a disseminação da infecção.

Anticorpos da classe IgA na saliva podem inibir a adesão das células de *Candida* às células do revestimento epitelial da boca (60, 61) dificultando a colonização da mucosa. Todavia, e no mesmo estudo foi verificado que níveis de IgA e de outros anticorpos anti-*Candida* na saliva são mais elevados em indivíduos com candidíase do que em indivíduos sãos o que não favorece a hipótese de um papel protector destes anticorpos nas CTD, tal como na CMC. No entanto, em doentes com SIDA que são susceptíveis a infecções da orofaringe e do esófago (62) nem sempre a infecção evolui para uma forma disseminada de candidíase (63) e pensa-se que anticorpos específicos contra proteínas de choque térmico do fungo, contribuirão para evitar a disseminação

da infecção (64, 65). Nestes doentes a imunodeficiência mais evidente é nas células T CD4+ (66, 67) e é, assim, que normalmente se associa a ocorrência de CTD à deficiência naquele tipo de células. O facto de a população de linfócitos T CD8+ não estar severamente afectada nos doentes com SIDA tem sugerido que estas células possam estar envolvidas na resistência daqueles doentes à CS de origem endógena. Em doentes neutropénicos, a CTD é, simultâneamente, uma causa de morbilidade e uma via importante para o estabelecimento de CS (68).

Em conclusão, os dados clínicos sugerem que a CTD é uma infecção micótica relacionada com deficiência nas células T CD4+ e com alterações fisiológicas do hospedeiro. Os neutrófilos parecem ter um importante papel no controlo das CS de origem endógena, uma vez que nos doentes neutropénicos há, muitas vezes, invasão sistémica a partir do TD. Como na CMC, o papel protector dos anticorpos específicos para a *Candida* é posto em causa.

Candidíase vaginal (CV). Calcula-se que 3 em cada 4 mulheres tiveram, pelo menos uma vez, um episódio de candidíase vaginal durante a idade fértil (69). A prevalência de *Candida* como comensal da vagina varia nas várias populações humanas e nas diversas áreas geográficas, atingindo desde 20% das mulheres na Nova Zelândia (70) até 40% da população feminina da Nigéria (71). Algumas mulheres referem apenas episódios esporádicos de candidíase. No entanto, cerca de 5% das mulheres adultas referem infecções recorrentes de que resultam estados de morbilidade genital que podem

conduzir a desequilíbrios emocionais (69). Alguns factores têm sido considerados factores de risco para a CV uma vez que vão permitir a proliferação do fungo. É o caso do aumento de nutrientes favoráveis ao fungo nas células do hospedeiro, como a glucose nas mucosas nos diabéticos e do glicogénio das células epiteliais da vagina das grávidas. É também o que sucede quando há diminuição da flora bacteriana normal, impedindo a competição com o fungo. É o que acontece com a administração de antibióticos de largo espectro e na gravidez, devido ao baixo valor do pH vaginal que é inibitório do crescimento bacteriano e favorece a *C. albicans*. As CV, particularmente as suas recorrências, podem também ser resultado de transmissão sexual a partir de parceiro contaminado. Também o uso de vestuário íntimo que cause lesões das mucosas pode favorecer a infecção micótica vaginal.

Esta forma de candidíase, pode também ser a expressão de várias anomalias imunológicas. Por exemplo, está documentado que vaginites por *Candida* estão associadas a: 1) variações na fisiologia dos linfócitos T durante o ciclo menstrual (72); 2) alterações de respostas imunológicas específicas (73) e não específicas (74) ao fungo, relacionadas com os níveis de progesterona ou estradiol, evidenciando a influência de hormonas esteróides, particularmente a progesterona, em fenómenos de transição da fase de levedura para micélio da *C. albicans* e na destruição das células fúngicas pelos neutrófilos (75); 3) diferenças claras na imunidade celular em mulheres com CV em relação a grupos de controlo (76). A resposta linfoproliferativa a

antigénios de *Candida* feita por linfócitos de doentes com CV encontra-se reduzida comparativamente aos linfócitos de populações de controlo e, a resposta dos linfócitos de mulheres sem doença pode ser bloqueada em co-culturas de linfócitos e *C. albicans* quer por adição de soro ou de linfócitos de doentes (77). Anticorpos específicos contra antigénios de *C. albicans* diminuem a proliferação de linfócitos, inibindo a fagocitose e/ou o processamento de antigénios de *C. albicans* pelos macrófagos e, também, o reconhecimento pelos linfócitos de antigénios de *C. albicans* apresentados à superfície de macrófagos patogénicos (78). O efeito dos macrófagos na linfoproliferação específica induzida pela *C. albicans* mostrou que esta resposta linfócitária era restabelecida para valores normais quando as células eram incubadas em presença de macrófagos normais (79). Pelo contrário, macrófagos de doentes inibiam a resposta dos linfócitos de controlo. A inibição pode ser revertida pela adição de indometacina às culturas, mostrando que o efeito é dependente da secreção pelos macrófagos de prostaglandina E2, a qual bloqueia a proliferação dos linfócitos.

Diferentes quadros de alteração de imunidade mediada por células têm sido referidos em associação a micoses por *C. albicans*. Sabe-se que vaginites recorrentes causadas pelo fungo ocorrem em mulheres imunocompetentes que não são susceptíveis a outras infecções e apresentam imunidade humoral e celular contra o fungo. Todavia, o facto de a comparação do perfil de anticorpos de mulheres com CV com o de mulheres de grupos de controlo não revelar qualquer diferença nem no soro (80), nem nas secreções vaginais (81),

tem levado a interpretações que consideram que a susceptibilidade à infecção fúngica estará associada à imunidade celular. De facto, doentes com CV recorrente apresentam um baixo número de linfócitos T, aproximadamente 2 vezes inferior ao observado em populações de controlo (82). Em doentes com episódios de vaginite sintomática, é observada uma perda transitória da resposta celular específica à *C. albicans* avaliada por ensaios de reacção cutânea aos seus antigénios (83). Em alguns doentes com CV, o tratamento antifúngico não alivia os sintomas. Anticorpos específicos contra o fungo, nomeadamente imunoglobulina E (IgE), bem como protaglandina E2, foram encontrados no fluido vaginal de um grande número de indivíduos com candidíase sujeitos a tratamentos com agentes antifúngicos (84), sugerindo que uma resposta vaginal pode induzir a produção de prostaglandina E2, suprimindo a resposta celular e predispondo a infecções recorrentes. Em estudo ulterior (85), foi documentada uma significativa redução na frequência de infecções recorrentes após dessensibilização para antigénios de *C. albicans* num grupo de doentes que não respondia a outras formas de terapia.

Dado o número e complexidade de factores que podem influenciar a apresentação clínica das candidíases, é natural que haja pouca concordância nos vários estudos sobre as modificações de funções imunes que ocorrem nestes doentes. É importante notar que mesmo em doentes com estudos detalhados da sua resposta imunológica e com infecções por *C. albicans* provadas por exames culturais, o processamento dos antigénios e as respostas dos hospedeiros são variáveis. Exemplo disto é a detecção de

anticorpos contra a enolase, uma proteína de *C. albicans* que actua como um antigénio importante tanto em humanos (86, 87) como em murganhos (88,89), em apenas alguns dos doentes com vaginite por *C. albicans* (90).

Vários modelos de infecção das mucosas têm sido estudados numa tentativa de identificar uma resposta que, por ser deficiente ou anormal em mulheres, explicaria a sua susceptibilidade à *C. albicans.* Estes estudos têm tido pouco sucesso e parece claro que o progresso científico nesta área da infecção dependerá de uma mais completa compreensão da relação biológica entre fungo e hospedeiro.

Em conclusão, dos resultados apresentados pode dizer-se que esta forma de candidíase pode ser a expressão de anomalias imunológicas. Estas anomalias podem estar relacionadas com o ciclo menstrual e, particularmente, com as hormonas esteróides. Essas hormonas poderão ter uma influência na patogenia do fungo e na diminuição da capacidade fungicida dos neutrófilos. As anomalias imunológicas poderão estar também relacionadas com o baixo número de linfócitos T apresentado por alguns doentes ou com a diminuição da resposta linfoproliferativa a antigénios de *Candida* que parece ser mediada por anticorpos específicos contra o fungo. De qualquer modo, todas estas alterações não explicam que, na grande maioria das mulheres, com este tipo de infecção, haja apenas alterações fisiológicas não sendo possível identificar factores imunológicos predisponentes ao aparecimento da doença.

Candidíase sistémica (CS). Esta forma de candidíase ocorre em doentes imunocomprometidos cujas defesas estão debilitadas por situações várias, nomeadamente em doentes com diabetes mellitus, indivíduos sujeitos a tratamentos com antibióticos de largo espectro, com drogas citotóxicas ou por radiações ou ainda com drogas supressoras, e, pacientes submetidos a grande cirurgia envolvendo materiais como catéteres ou outro tipo de próteses plásticas. Na realidade, o aumento da tecnologia médico-cirúrgica tem estado associado a um aumento da infecção por *Candida* no ambiente hospitalar (91), sendo responsável por 7% das infecções contraídas nos hospitais (92) e pelo excesso de mortalidade dos doentes naquelas condições (93). Nos indíviduos sofrendo de SIDA ocorrem também episódios de CS de difícil tratamento.

As formas sistémicas de candidíase estão, geralmente, associadas a disfunções dos neutrófilos ou a neutropenias (44, 94 ,95). A importância das imunoglobulinas anti-*Candida* como opsoninas que favorecem a ingestão das células fúngicas pelos fagócitos parece ainda controverso. Estão publicados estudos referindo que baixos níveis de anticorpos da classe IgG, específicos contra *Candida,* no soro humano, aumentam a eficácia da fagocitose das hifas pelos neutrófilos (42). Pelo contrário, o soro de doentes com candidíase disseminada tem a capacidade de inibir a fagocitose das células de *Candida* pelos neutrófilos humanos (42), sugerindo, à semelhança do que é referido na CMC, que este efeito é devido à presença de títulos séricos elevados de anticorpos específicos contra o fungo. Por outro lado, alguns estudos (96,97,98) mostraram que anticorpos específicos contra a proteína de choque

térmico de 90 kDa (hsp 90) da *C. albicans* podem ter acção protectora do hospedeiro, pois doentes que recuperam de candidíase disseminada têm, no soro, títulos elevados destes anticorpos. Reforçando esta hipótese, esses anticorpos têm a capacidade de proteger murganhos contra a CS. A protecção induzida por estes anticorpos deve estar relacionada com a sua função como opsoninas aumentando a eficácia da fagocitose das células fúngicas pelos neutrófilos.

Em conclusão podemos dizer que a CS ocorre em doentes imunocomprometidos cujas defesas estão debilitadas por situações várias, estando geralmente associada a disfunções dos neutrófilos ou a neutropenias. O papel relativo de anticorpos específicos contra antigénios da *Candida* na protecção do hospedeiro é, ainda, um assunto que não está completamente esclarecido. Por um lado, anticorpos contra hsp 90 do fungo parecem ter um efeito protector, por outro, anticorpos específicos contra o fungo, em altas doses, são referidos como tendo um papel negativo no controlo desta infecção.

2.2. Resultados *in vitro* em Humanos

Os dados obtidos *in vitro* não permitem relacionar a resposta dos diversos tipos de células do hospedeiro aos antigénios de *Candida* com as diferentes patologias. Assim, passamos a referir os resultados obtidos de acordo com os mecanismos de defesa envolvidos.

In vitro, os leucócitos polimorfonucleares (PMN) têm a capacidade de ingerir grande número de células de *Candida* (99) e uma percentagem destes microrganismos é morta dentro do fagócito. As restantes células de *C. albicans* germinam (100) e crescem para fora das células, atravessando a sua membrana celular. A relativa baixa eficiência de morte celular das células fúngicas no interior dos neutrófilos humanos pode estar associada com diferenças funcionais nos fagosomas do granulócito (101).

Assim, os PMN representam a primeira linha de defesa inespecífica no controlo das infecções por *Candida.* Como se sabe, a acção do neutrófilo contra os microrganismos beneficia com a activação do sistema do complemento. Soro humano deficiente em C2 ou em C5, opsoniza eficientemente as células de *C. albicans* possibilitando a sua fagocitose e morte pelos neutrófilos através de C3b e/ou C4b. Pelo contrário, na ausência de C3, e portanto da opsonina C3b, as células do fungo que são fagocitadas não são mortas pelo neutrófilo (102,103,104). A activação de C3 pela presença de células de *Candida* é também favorável ao hospedeiro porque gera o factor quimiotáctico C3a que vai actuar no recrutamento de leucócitos para os locais de infecção (105). Por outro lado, a levedura pode diferir na susceptibilidade à morte intracelular. Blastósporos de estirpes virulentas para murganhos, sobrevivem intracelularmente, em PMN humanos e de murganho, melhor do que os de estirpes atenuadas (104). Além disso, descendentes de blastósporos de estirpes atenuadas que tenham germinado no interior de PMN, ganham virulência para o murganho e comportam-se como os das estirpes

virulentas, tanto em fagocitos humanos como de murganho. Outros elementos celulares fúngicos, como as hifas, são também susceptíveis ao ataque por PMN (106). A morte celular da *C. albicans* é mediada pela activação de mecanismos de explosão respiratória com libertação de radicais de oxigénio tóxicos. No entanto, a produção de moléculas oxidantes pode não ser suficiente para mediar a morte celular das hifas, na ausência de mecanismos complementares não oxidativos (107). A actividade antifúngica dos PMN está também claramente relacionada com a libertação da proteína lactoferrina que, pela sua forte avidez pelos iões de ferro, impede que estes iões sejam utilizados no metabolismo das células fúngicas (107).

Adicionalmente à morte da levedura ou da hifa por acção dos PMN, a própria morte de PMN na lesão micótica pode também contribuir para controlar a infecção pela libertação de pelo menos uma substância que tem um forte efeito inibidor sobre as células livres do fungo (108); essa substância foi identificada como uma proteína, com actividade antimicrobiana generalizada, designada de calprotectina (109). A activação não específica de PMN por exemplo pelo tratamento com lipopolissacarídeo de bactérias Gram negativas (LPS), que leva à produção de IL-1, IL-6, TNF-α e de lactoferrina, inibe o crescimento de *C. albicans,* aumentando o número de células ingeridas pelos neutrófilos, bem como o número de neutrófilos que fagocitam células fúngicas (110).

Células humanas do sistema monocítico/macrofágico também representam um importante componente na defesa do hospedeiro contra infecções por *C. albicans*. É interessante referir que células de *Candida* opsonizadas são ingeridas tanto por monócitos como por macrófagos, mas, células de *Candida* não opsonizadas são fagocitadas sómente pelas últimas destas células, fazendo-o principalmente pela ligação do fungo ao receptor da manose existente à superfície do macrófago (111,112). Monócitos do sangue periférico são capazes de diminuir a viabilidade de hifas e pseudo-hifas de *Candida* sem que haja completa ingestão destas (113). Nestas situações, o mecanismo de morte celular dominante parece envolver a via dependente da mieloperoxidase, embora, paradoxalmente, monócitos de doentes com deficiente produção de mieloperoxidase sejam igualmente capazes de lesar as hifas. Monócitos e macrófagos alveolares humanos são altamente eficientes na destruição de blastósporos de *Candida* (114 ,115) através de mecanismos oxidativos. Em contraste, os macrófagos peritoneais são pouco eficientes na destruição das células de *Candida* que ingerem (116). Assim, a actividade efectora inata contra a *C. albicans,* das células macrofágicas, parece depender da sua origem anatómica.

A levedura tem também mecanismos protectores contra a actividade efectora dos monócitos/macrófagos. De facto, está relatado que uma hora após incubação com blastoconídios de *C. albicans* um número significativo de monócitos do sangue periférico humano que fagocitava leveduras tinha morrido, o que foi constatado pela coloração com brometo de etídio, embora as

membranas celulares não apresentassem sinais de penetração pelo fungo (117). O mecanismo de morte dos monócitos devido à interacção com *C. albicans,* bem como a importância desta interacção no processo de colonização e invasão *in vivo* não está clarificado. Embora PMN, monócitos e macrófagos isolados expressem claramente uma actividade anti-*Candida,* numerosos estudos demonstram que a expressão das funções fungicidas por aqueles tipos celulares requer a activação quer pelos «colony stimulation factor» (CSF) de granulócitos (G-CSF) e de macrófagos (M-CSF), quer por outras citocinas produzidas pelos linfócitos T.

Os linfócitos humanos mostram uma forte resposta proliferativa após estimulação *in vitro* com antigénios de *C. albicans,* fenómeno este que não resulta do contacto directo entre as moléculas de *C. albicans* e linfócitos já que está dependente de células apresentadoras de antigénio (118).

Linfoproliferações podem ser induzidas *in vitro* por cultura dos linfócitos quer com blastósporos inactivados pelo glutaraldeído, quer com um complexo fosforilado glucomanana-proteína da parede celular (119); é interessante referir que um extracto de manana com pouca proteína tem muito menor actividade indutora de linfoproliferação. Adição de anticorpo monoclonal específico para epítopes de moléculas de HLA Classe II inibe a linfoproliferação, independentemente do antigénio de *Candida* presente na cultura (118). Este fenómeno indica que a apresentação dos antigénios fúngicos aos linfócitos T

em cultura tem lugar através do Complexo Maior de Histocompatibilidade (MHC) Classe II.

Como se sabe, as citocinas podem ser produzidas por um conjunto alargado de células. Nas infecções por *C. albicans* parecem ter importância as citocinas produzidas pelos linfócitos T específicos contra antigénios fúngicos, mas também as que são produzidas por células NK e pelas células fagocíticas. No Homem, as células NK constituem uma população de grandes linfócitos granulares. Esta população não é capaz de matar directamente as células de *Candida* (120) mas, quando activada com antigénios *in vitro*, liberta TNF-α (121) e GM-CSF (122) os quais estimulam os neutrófilos conferindo-lhes capacidades inibidoras do crescimento do fungo (123, 124,125, 126). A estimulação de células mononucleares do sangue periférico (PBMC) humano com *C. albicans* ou seus antigénios resulta na produção de citocinas várias. Assim, em culturas de PBMC humanas estimuladas com antigénios de *Candida* (127) ou com manoproteínas constituintes do fungo (128), a análise da expressão de genes das citocinas revelou a síntese de mRNAs para IL-1β, TNF-α e IL-6, bem como apreciáveis níveis de mRNAs para IL-2, GM-CSF e IFN-γ. A IL-2 liga-se à *C. albicans* (129) e quando complexada desta forma, aumenta a resposta linfoproliferativa dos linfócitos do sangue periférico humano normal, contra a levedura. O significado *in vivo* deste fenómeno não foi ainda estabelecido, mas, pode ser reflexo de aumento da eficiência da apresentação desta citocina aos PMN. Há também a assinalar diferenças na

cinética de produção das citocinas (130), por exemplo, IFN-γ é produzido habitualmente a partir de um dia de estimulação antigénica, enquanto que o TNF-α ocorre mais tardiamente.

PMN humanos expressam constitutivamente níveis detectáveis da cadeia β, do receptor da interleucina (IL)-2 e respondem funcionalmente a esta interleucina, aumentando a actividade antifungica mediada pela lactoferrina (131). Outras citocinas como IL-8, derivada dos monócitos, com funções quimiotácticas para os neutrófilos, também aumentam as capacidades antifúngicas dos polimorfonucleares *in vitro.* A IL-8 estimula actividade antifúngica de PMN mas não de monócitos (132). Em contraste com o IFN-γ e o TNF-α, não estimula a produção de superóxido nas células o que sugere que o mecanismo de morte celular do fungo pode ocorrer por uma via independente do oxigénio. Sabe-se que, após exposição às células leveduriformes, os PMN produzem TNF-α (133) o qual é um factor quimiotáctico para os PMN e monócitos (134). Assim, a produção autócrina desta citocina pode aumentar o recrutamento de células inflamatórias para o local da infecção. Os PMN podem ser estimulados a produzir TNF-α pelas células do fungo, pelo GM-CSF e também pela IL-2, e ainda pela ligação à cadeia β do receptor da IL-2 expresso à superfície das células (135). A citocina IFN-γ actua tanto isoladamente (124 ,136) como em sinergia com TNF-α para aumentar a actividade fungicida dos PMN *in vitro,* embora, outros estudos (137) demonstrem que as duas citocinas têm efeitos diferentes na função neutrofílica. Neste último estudo, refere-se que a activação de neutrófilos com

IFN-γ ou com TNF-α aumenta rápidamente a produção de superóxido; mas, embora o TNF-α aumente a produção de superóxido de uma forma mais acentuada do que o faz o IFN-γ, esta citocina é mais eficiente a aumentar a actividade fungicida dos PMN. Estes resultados sugerem que o aumento da explosão respiratória mediada por citocinas pode não corresponder a um incremento da actividade fungicida dos fagócitos. Por outro lado, estudos realizados sobre os efeitos da molécula G-CSF na explosão oxidativa e na actividade microbicida dos neutrófilos, mostraram que G-CSF aumentando a produção de superóxido em resposta à fagocitose de blastoconídios e a pseudo-hifas opsonizadas (138) aumenta também a actividade microbicida dos PMN.

A produção de IFN-γ por linfócitos T activados é ainda um dos principais factores que aumentam a actividade fagocítica e fungicida dos macrófagos humanos (139). De facto, o tratamento com esta citocina aumenta a capacidade tanto dos macrófagos como dos neutrófilos para ingerirem e destruirem as células de *Candida,* opsonizadas ou não (140, 111).

Os macrófagos alveolares humanos representam uma eficiente barreira à infecção respiratória com *C. albicans* (141). Adição *in vitro* de IFN-γ, IL-1α ou LPS, a macrófagos alveolares, aumenta significativamente a morte intracelular do fungo e restaura a sua actividade fungicida quando as células são mantidas em cultura. Tratamento de macrófagos alveolares com ionóforo de cálcio mostrou que a fagocitose e a destruição do fungo estão dependentes do incremento da concentração intracelular de ião Ca^{++} (142). Por outro lado,

verificou-se que o aumento da actividade fungicida intracelular está relacionada com actividade de proteases anti-*Candida,* a qual está dependente da presença do catião.

A relevância das diferentes vias efectoras, e da sua modelação, contra as várias manifestações da candidíase humana estão longe de estarem esclarecidas. De igual modo, a eficácia da terapêutica com citocinas na alteração do curso e no prognóstico da infecção ainda não está claramente definida. Se, por um lado, a administração local da proteína recombinante humana G-CSF a doentes neutropénicos com SIDA resultou num aumento transitório de neutrófilos associado a uma significativa elevação da sua actividade fungicida (143). Por outro lado, investigações clínicas do efeito terapêutico do M-CSF em infecções fúngicas em doentes com transplantes de medula óssea ainda não forneceu evidência definitiva de que tenha um efeito benéfico (144).

Em conclusão, os dados obtidos *in vitro* permitem-nos dizer, que os leucócitos polimorfonucleares representam a primeira linha de defesa inespecífica no controlo das infecções por *Candida.* Como se sabe, a acção dos PMN contra os microrganismos beneficia com a activação do sistema do complemento e a activação dos factores do complemento pelas células de *Candida* é de importância fundamental para o hospedeiro por gerar factores que funcionam como opsoninas e/ou como factores quimiotácticos para os leucócitos. Por sua vez os fagocitos humanos podem ser eficientes na eliminação de células de *Candida,* ainda que estas não estejam

completamente fagocitadas, e essa actividade fungicida é mediada pela mieloperoxidase.

A morte intracelular da *C. albicans* ocorre em PMN, monócitos e macrófagos. Nos fagocitos, a morte intracelular é mediada pela activação de mecanismos de explosão respiratória com libertação de radicais de oxigénio tóxicos. No entanto, a produção de moléculas oxidantes pode não ser suficiente para mediar a morte celular das hifas, estando a actividade antifúngica dos PMN também relacionada com a libertação da lactoferrina. A produção da citocina TNF-α que é um factor quimiotáctico para os PMN e monócitos, pode aumentar o recrutamento de células inflamatórias para o local da infecção. A actividade fungicida dos PMN *in vitro,* beneficia com a produção das citocinas IFN-γ, IL-2 e IL-8.

As experiências *in vitro* com células humanas revelaram ainda que as funções fungicidas dos fagocitos humanos podem ser modeladas pelo tratamento com diferentes citocinas. De qualquer forma não é explicito o papel directo ou indirecto dos linfócitos T na eficiência dos mecanismos de fagocitose. No entanto, tendo em conta a origem destas citocinas, estes resultados apontam para uma regulação por células linfóides das funções efectoras contra *C. albicans* que, como se sabe são exercidas pelos fagocitos.

3. Candidíase experimental

3.1. Modelos experimentais em murganhos.

Os factores cruciais envolvidos na resistência ou recuperação à infecção fúngica têm sido, prácticamente, impossíveis de identificar no homem devido à heterogeneidade genética assim como a diversos factores de outra ordem, tais como condições e estilos de vida e infecções concomitantes. Uma das formas de eliminar algumas destas variáveis, ou de identificá-las avaliando os seus efeitos, é o uso de modelos animais da infecção. Não é nossa intenção fazer aqui uma revisão sobre todos os modelos animais de infecção com *Candida,* porque para isso existem disponíveis na literatura excelentes artigos de revisão. Centraremos a nossa atenção particularmente nos estudos imunológicos recentes sobre a infecção no murganho. Os mecanismos de defesa do hospedeiro podem ser melhor compreendidos se compararmos as respostas inflamatórias e imunes entre estirpes de murganhos genéticamente iguais («inbred») que apresentem grau diferente de susceptibilidade à infecção por *C. albicans*.

3.2. Tipos de infecção.

Candidíase do tubo digestivo (CTD). Estudos experimentais com o objectivo de establecer a colonização gastrointestinal de murganhos com *C. albicans* revelaram que murganhos adultos normais são resistentes à

colonização por esta via, não mantendo níveis de colonização apreciáveis, ou seja, a colonização não evolui para uma situação crónica, mesmo usando inóculos com um número elevado de células viáveis de *C. albicans* (145). Assim, com o objectivo de predispor o hospedeiro à CTD tem-se recorrido ao uso de antibióticos, ou a drogas imunossupressoras e/ou citotóxicas ou ainda a traumatismos das mucosas do TD. Também a utilização de inóculos com um grande número de células viáveis de *C. albicans* facilita a CTD.

Vários modelos murinos têm sido utilizados para o estudo das candidíases das mucosas. Os resultados experimentais permitiram verificar que murganhos BALB/c convencionais, duas semanas após inoculação por via oral com *Candida* não apresentam culturas de fezes positivas para o fungo, enquanto que 30 a 40% dos animais sujeitos a tratamento com tetraciclina, apresentam exames de fezes positivos até 32 dias após a administração do fungo (146). Animais congenitamente imunodeficientes como os murganhos atímicos (*nu/nu*) e/ou "beige" (*bg/bg*), estes últimos animais têm uma diminuição da actividade quimiotáctica e microbicida dos granulócitos (147, 148) e uma deficiente actividade citolítica das células NK, têm sido usados como modelos de infecção das mucosas. Também murganhos com síndroma de imunodeficiências combinadas como os murganhos *scid*, que não têm células T e B funcionais, são modelos adequados ao estudo das candidíases das mucosas. Animais criados sem contacto com microrganismos («germfree»), são igualmente bons modelos de infecção das mucosas. Estas estirpes de murganhos são modelos adequados ao estudo das candidíases

das mucosas já que estas podem ser colonizadas de uma forma natural por *C. albicans*, sem necessidade de terapêuticas ou traumatismos especiais (149,150,151). Além disso, a colonização das mucosas do TD dos animais previamente "germfree" persiste durante toda a vida do animal (151).

Os murganhos *nu/nu* e/ou *bg/bg,* os murganhos *scid*, e os murganhos "germfree" apresentam culturas positivas até 8 semanas após colonização por via oral. No caso dos murganhos *nu/nu bg/bg* há mesmo invasão sistémica a partir das mucosas gastrointestinais.

A imunossupressão do hospedeiro resulta, muitas vezes, num crescimento excessivo da população de *C. albicans* endógena podendo causar CTD e, mesmo, CS (148, 149, 152). A infecção da mucosa oral com *Candida* desencadeia resposta inflamatória e estimula a imunidade celular do hospedeiro (153).

A principal função dos linfócitos nas CTD parece ser a produção de citocinas que aumentam a actividade fungicida das células efectoras, ou seja, dos fagocitos mononucleares e polimorfonucleares. Como se sabe, as células T periféricas podem ser divididas em duas subpopulações principais com base na expressão dos antigénios CD4 e CD8 na superfície linfocitária. Nos murganhos, pelo menos, as células T $CD4^+$ podem ser separadas em subgrupos funcionais de acordo com os padrões de citocinas que produzem. Originalmente, duas subpopulações principais foram definidas: 1) células Th1 que produzem IL-2 e IFN-γ mediando reacções de hipersensibilidade retardada e 2) células Th2 que segregam IL-4 e IL-5 e activas na indução de

respostas humorais (154). Grupos possívelmente precursores dos dois anteriores, incluem as células Th0 que podem produzir citocinas características tanto das células Th1 como das Th2, e as células Thp que produzem apenas IL-2, sendo, provávelmente, as mais primitivas em termos de estimulação imunológica das células Th (155,156). Os linfócitos Th1 e Th2 têm, por acção das suas citocinas, efeitos mutuamente inibitórios tanto funcionais como de expansão (154, 157). As citocinas produzidas pelas células Th2 inibem a secreção de citocinas pelas células Th1 e vice-versa. Por exemplo, os linfócitos Th2, através da secreção de IL-10 inibem a expansão de células Th1 e favorecem o seu próprio crescimento pela produção de IL-4, enquanto que as células Th1 inibem a proliferação das células Th2 e promovem o seu próprio crescimento pela produção de IFN-γ. A participação de um ou de outro tipo de células Th na resistência ou susceptibilidade à infecção foi demonstrada claramente em modelos murinos de leishmaníase cutânea (158,159,160). Sabe-se que o modo de apresentação do antigénio influencia o desenvolvimento de cada uma das subpopulações de linfócitos Th. Assim, antigénios apresentados por células B esplénicas parecem favorecer o desenvolvimento da subpopulação Th2, enquanto que a apresentação pelos macrófagos favorece o desenvolvimento das células Th1 (161).

A indução pelo parasita de uma resposta imune do tipo Th1 ou Th2, de acordo com as citocinas produzidas, aumentando ou diminuindo as funções das células fagocíticas, com implicação no desenvolvimento da infecção, tem merecido particular atenção nas infecções com *Candida*. Alguns modelos

experimentais de infecção intragástrica têm dado grande relevo à função das citocinas associadas às subclasses Th1 e Th2 na resistência/susceptibilidade a infecções micóticas. Assim, foi demonstrado que murganhos DBA/2 que desenvolvem candidíases disseminadas e fatais após infecção intravenosa com a estirpe avirulenta PCA-2 (162), quando colonizados, por via intragástrica com a estirpe virulenta CA-2, desenvolvem uma resposta celular do tipo Th1 e eliminam o agente infeccioso (163). O tratamento de murganhos com as interleucinas recombinantes IL-4 ou IL-10 tornou claro que estas moléculas causam exacerbação das candidíases das mucosas (164).

A influência de linfócitos T γ/δ no desenvolvimento da infecção das mucosas foi investigada em murganhos das estirpes BALB/c e DBA/2 que têm o mesmo haplotipo de MHC (H-2d). Após a infecção da mucosa oral com *C. albicans,* as duas estirpes apresentaram nas mucosas um aumento de células MAC-1^+ e de células T $CD4^+$ e $CD8^+$ idêntico (165). A infecção induziu um influxo de células T γ/δ no dia 3 na estirpe BALB/c e no dia 5 na estirpe DBA/2, o que coincidiu com a diminuição do número de microrganismos viáveis colhidos na mucosa oral. As duas estirpes murinas diferem na resposta de imunidade celular, observada pela reacção de hipersensibilidade retardada específica contra *Candida* que é desenvolvida pela estirpe DBA/2 mas não pela estirpe BALB/c. As células T γ/δ produzem IFN-γ que aumenta a capacidade fungicida dos macrófagos pelo aumento da produção de óxido nítrico. Esta subclasse de células T representa um elemento importante na resistência do hospedeiro já que a sua depleção específica, aumenta a

susceptibilidade à candidíase das mucosas, tanto em hospedeiros imunocompetentes como naqueles que se apresentam deficientes em células T, como é o caso dos murganhos atímicos (166).

Os dados experimentais mais convincentes que apoiam um papel importante dos linfócitos T nas candidíases murinas têm sido obtidos em estudos da infecção do TD em murganhos «germfree» e em murganhos congénitamente atímicos (*nu/nu*). Após colonização das mucosas, os murganhos de controlo *nu/+* eliminam as células de *Candida* da cavidade oral e do estômago, contrariamente ao que sucede com os murganhos *nu/nu* que apresentam candidíase das mucosas (167). De qualquer modo, estes murganhos não desenvolvem CS, indicando que outros mecanismos de defesa serão suficientes para impedir a disseminação da infecção a partir do TD. Quando os murganhos *nu/nu* têm associada a mutação «beige» (*bg/bg*) e são colonizados por via oral, verifica-se que desenvolvem infecções graves das mucosas e, subsequentemente, infecções sistémicas (149, 168), sugerindo que uma combinação de alterações funcionais das células fagocíticas e da imunidade celular é necessária para que os murganhos desenvolvam formas graves de candidíase tanto das mucosas como sistémicas a partir de infecção por via oral.

Em conclusão podemos dizer que os estudos experimentais com o objectivo de estabelecer a colonização do TD de murganhos com *C. albicans* revelaram que os murganhos adultos normais, à semelhança dos humanos, são resistentes à colonização por esta via, mesmo usando inóculos com um

número elevado de células viáveis de *C. albicans*. Modelos murinos de murganhos congenitamente imunodeficientes são adequados ao estudo das candidíases das mucosas, no entanto, verifica-se que apesar de colonizados por via oral, os animais só desenvolvem infecções graves das mucosas e, subsequentemente, infecções sistémicas se tiverem associadas alterações funcionais das células fagocíticas e da imunidade celular. A utilização destes modelos murinos permite concluir que, à semelhança do que sucede no Homem, a imunossupressão do hospedeiro resulta, muitas vezes, num crescimento excessivo da população de *C. albicans* endógena podendo causar CTD e, mesmo, candidíases sistémicas.

As células T γ/δ representam um elemento importante na resistência do hospedeiro à candidíase das mucosas, já que a sua deplecção específica, aumenta a susceptibilidade tanto em hospedeiros imunocompetentes como naqueles que se apresentam deficientes em células T, como é o caso dos murganhos atímicos.

Estes resultados, de uma forma geral, evidenciam que a resistência a infecções das mucosas por *Candida* é determinada por mecanismos de fagocitose cuja eficiência será aumentada ou reforçada pela produção de citocinas como IL-2 e IFN-γ que são produzidas pelas células Th1. Pelo contrário, as citocinas produzidas pelas células Th2 inibem a produção de citocinas pelas células Th1 e, em consequência, a activação de macrófagos.

Candidíase vaginal (CV). A infecção experimental intravaginal por *C. albicans* é conseguida apenas se os animais são mantidos no estado de pseudestros quer por ooforectomia ou pelo uso de hormonas, como estrogénios (169, 170). É possível manter por 10 semanas a infecção intravaginal em murganhos CBA/J, de resistência moderada à infecção, mantendo os animais em condições de pseudestros (171). Todavia, o distúrbio hormonal causado tem implicações negativas no estado de saúde do hospedeiro (172, 173). Estirpes de murganhos congenitamente imunodeficientes como os murganhos *nu/nu* e *bg/bg* ou com as duas mutações associadas *nu/nu bg/bg* têm sido utilizados nas infecções experimentais intravaginais. Nos três modelos de mutantes o número de células de *Candida* é máximo 1 dia após inoculação, decaindo ao longo do tempo e, por volta dos 14 dias, a infecção é debelada nas estirpes com uma das mutações e apenas a estirpe com as duas mutações *nu/nu bg/bg* desenvolvem infecções graves da mucosa vaginal (174), até 21 dias após inoculação. Estes resultados sugerem que uma combinação de alterações funcionais das células fagocíticas e da imunidade celular é necessária para que os murganhos desenvolvam formas graves de candidíase vaginal. A predominância de neutrófilos e de linfócitos T no epitélio da vagina (175) sugere que estes dois tipos celulares têm um papel importante na protecção do epitélio da mucosa da infecção com o fungo.

A *C. albicans* tem particular preferência pelas porções queratinizadas das mucosas e apenas quando as células epiteliais da vagina estão bastante queratinizadas, durante a fase de estros, é possível infectar os murganhos

intravaginalmente (171). A fase de estros dura em média 1 dia nos murganhos após o que há uma chamada de PMN (171) que são células eficientes na eliminação do fungo. Assim, a curta duração da fase de estros em combinação com os PMN tornam a colonização da vagina por *C. albicans* difícil, mesmo nos murganhos imunodeficientes. A predominância de neutrófilos e de linfócitos T no epitélio da vagina põe em destaque estes dois tipos celulares como elementos importantes na protecção do epitélio da mucosa da infecção com o fungo. Apenas nas situações em que se conjugam a ausência de timo e de células fagocíticas funcionais, se aumenta a susceptibilidade da vagina à colonização pelo fungo e, mesmo nestes casos, a mucosa vaginal mostra-se menos susceptível à infecção do que as mucosas oral, do esófago e do estômago (149).

A CV em ratinhos CBA/J induz imunidade celular (171, 176). A análise da produção de citocinas pelos gânglios linfáticos destes animais infectados revelou predominância de produção de IL-2 e de IFN-γ e ausência de secreção de IL-10 e apenas um pequeno aumento dos níveis de IL-4 nos estadios mais tardios da infecção. Murganhos que desenvolvam uma resposta imunológica do tipo Th1 em resposta a infecção vaginal primária por *Candida* apresentam, numa infecção vaginal secundária, uma colonização considerávelmente menor da vagina, pelo fungo, em comparação com os resultados observados na infecção primária (177). Embora, como já referimos, formas graves de candidíase oral possam ocorrer em associação com imunodeficiência envolvendo a redução do número de células T $CD4^+$ (178, 179) a depleção de

linfócitos $CD4^+$ e $CD8^+$ com anticorpos monoclonais não tem efeito na gravidade das candidíases vaginais primárias ou secundárias induzidas em murganhos (180). No entanto, vários estudos apontam para um papel importante tanto das células T $CD4^+$ como das células T $CD8^+$ na resposta do hospedeiro à infecção com *Candida* (181,182), sugerindo que a produção de citocinas pelas células T activadas será um elemento central na resistência à infecção pelo fungo.

Em conclusão, a infecção intravaginal por *C. albicans* em modelos murinos permitem concluir que a infecção é conseguida apenas se os animais são mantidos no estado de pseudestros. Quando os murganhos apresentam alterações funcionais das células fagocíticas e da imunidade celular, verifica-se que desenvolvem infecções graves das mucosas, podendo desenvolver infecções sistémicas, à semelhança do que sucede nas CTD. O desenvolvimento de uma resposta imunológica do tipo Th1, em resposta a infecção vaginal primária induz imunidade celular e protege contra uma infecção secundária. Parece evidente que, à semelhança do que foi referido nas CTD, nas CV uma resposta imunológica do tipo Th1 está relacionada com resistência à infecção.

Candidíase sistémica (CS). A candidíase disseminada em estirpes «inbred» assemelha-se muito à doença no homem, sob o ponto de vista da patologia que induz no hospedeiro. O cérebro e os rins estão normalmente envolvidos nas infecções disseminadas no homem (183) e no murganho

(184,185). O rim possui um sistema fagocítico que é capaz de eliminar os microrganismos tão eficientemente como o fígado ou o baço (186), podendo a alta susceptibilidade do rim no murganho estar relacionada com as elevadas doses de inóculo frequentemente usadas nos sistemas experimentais. De facto, quando o número de células de *Candida* usadas no inóculo é diminuído, as maiores infecções ocorrem no cérebro (185). Observam-se abcessos consistindo de uma mistura de células inflamatórias e células do fungo, blastoconídios e hifas, vindo, eventualmente, a infecção a ser debelada. Aumentando a dose de inóculo, consegue-se provocar a morte de murganhos de todas as estirpes. A morte dos animais ocorre pelos 5 a 7 dias. A causa próxima desta morte rápida dos murganhos pela Candida não está inequívocamente estabelecida mas, os rins dos ratinhos moribundos apresentam-se distendidos, com abundantes células fúngicas, e têm uma aparência microscópica anormal, sendo de supor que a mortalidade precoce devida à infecção se deva a pielonefrite aguda causada pelo fungo (187).

A susceptibilidade à CS é, normalmente, avaliada pela mortalidade induzida no hospedeiro, pela contagem de colónias microbianas em tecidos infectados e pela avaliação histológica da gravidade das lesões provocadas nos tecidos.

A mortalidade nas infecções por *Candida* não parece estar tão directamente relacionada com a susceptibilidade à infecção como nos casos de infecções por vírus ou bactérias. Sabe-se que a opsonização pelo complemento é um fenómeno fundamental para a redução do número de

células fúngicas no período inicial da infecção. Também as propriedades quimiotácticas de factores do complemento são importantes no combate às CS. Quando as estirpes de murganhos são seriadas de acordo com a mortalidade causada pela *Candida* ou pela contagem de colónias microbianas no rim, as estirpes que são deficientes em C5 morrem com doses de *Candida* substancialmente menores do que as estirpes murinas sem alterações do sistema do complemento (188, 189). Murganhos genéticamente deficientes em C5 são extremamente susceptíveis a infecção com doses elevadas de *C. albicans* (189, 190). Nas estirpes murinas deficientes em C5 o grau de infecção varia nos tecidos e nos orgãos, sendo a infecção mais marcada no rim (191). A capacidade de células fagocíticas de murganhos normais e de murganhos deficientes em C5 de destruirem células de *Candida* é, no entanto, equivalente (191) o que sugere que as propriedades quimiotácticas do componente C5a poderão ser importantes factores no processo de eliminação do fungo pelo hospedeiro.

Os exames histopatológicos mostram dois tipos diferentes de destruição do tecido cerebral do murganho (192). Assim, as lesões podem ser classificadas de severas, ou lesões do tipo I, caracterizando-se por numerosos abcessos contendo leveduras e micélio dentro dos restos necróticos, bem como infiltrados inflamatórios consistindo de células mononucleares e polimorfonucleares e infecções de severidade média, ou do tipo II, as quais mostram características qualitativamente similares às do tipo I mas são mais pequenas e menos frequentes. Estes dois tipos de lesões também podem ser

observados noutros orgãos, mas de forma menos evidente do que no cérebro. Todavia, estes tipos de lesão, de tipo I ou de tipo II, não parecem estar relacionados com a mortalidade ou com o número de organismos viáveis detectados no cérebro (189). Embora o número de organismos viáveis seja, geralmente, maior nas lesões do tipo I, que ocorrem, por exemplo, nas estirpes murinas CBA/CaH e AKR do que nas lesões do tipo II, que aparecem nos murganhos BALB/c e DBA/1. As cinéticas de crescimento do fungo e a eliminação do parasita nos murganhos dos dois grupos é semelhante (193), apresentando um pico ao dia 4 da infecção e caindo rápidamente daí para diante. A propensão para o desenvolvimento de lesões de um tipo ou de outro é hereditária (194). As variáveis que determinam a expressão dos dois tipos de lesão não foram ainda definidas, mas podem estar relacionadas, por exemplo, com diferenças qualitativas ou quantitativas nas funções efectoras das células fagocíticas do hospedeiro ou com diferenças no desenvolvimento ou evolução da sua imunidade celular.

A expressão de respostas protectoras após a infecção também está relacionada com os padrões de lesão tecidular induzida no hospedeiro. Quando murganhos que fazem lesões do tipo I em resposta à Candida, como é o caso dos murganhos CBA/CaH, são infectados com uma dose subletal do fungo observa-se que após recuperarem da infecção, a sua reinfecção leva ao aparecimento de lesões cerebrais muito mais pequenas e menos numerosas do que os animais de controlo não sujeitos a infecção prévia. Pelo contrário, em estirpes murinas como BALB/c que fazem respostas do tipo II, o efeito

protector da primeira infecção é menos aparente (195). A protecção contra a *Candida* pode ser transferida pelo soro de murganhos imunes (196) podendo admitir-se que possa ser mediada por factores séricos inibidores de virulência.

A investigação do papel dos linfócitos T nas infecções por *Candida* tem levado à obtenção de resultados difíceis de harmonizar entre si. Por exemplo, em murganhos timectomizados na idade neonatal, foi detectado um aumento da susceptibilidade à infecção sistémica por *C. albicans*, todavia, em murganhos timectomizados na idade adulta, irradiados e reconstituídos com medula óssea (197), ou ainda em murganhos congénitamente atímicos (198,199), a deficiência em células T parece contribuir para um aumento da resistência à infecção sistémica pelo fungo. A resistência destes últimos murganhos poderá ser explicada pela activação do sistema monócito/macrofágico (200, 201). Os macrófagos têm uma contribuição importante na resolução de infecções sistémicas com *Candida.* Exames histológicos mostram que orgãos com abundante número de macrófagos apresentam resistência acrescida à infecção. Por exemplo, após a infecção endovenosa do murganho a maioria do inóculo aloja-se no fígado e nos pulmões (185, 193) embora estes orgãos raramente apresentem danos histológicos, provávelmente devido à abundante população de fagócitos residentes. Na candidíase pulmonar, uma rápida acumulação de neutrófilos nos pulmões é essencial para a eliminação completa do fungo (202, 203). Em murganhos imunossuprimidos, contudo, os macrófagos alveolares são necessários para a eliminação das células de *Candida* impedindo a sua

disseminação (204). Em comparação com as candidíases humanas, a infecção sistémica de ratinhos granulocitopénicos e monocitopénicos permite concluir que os granulócitos são mais importantes que os monócitos ou macrófagos nas primeiras fases da resposta do hospedeiro contra *C. albicans* (205). Células peritoneais de murganhos infectados por via endovenosa com *C. albicans*, apresentam um aumento da síntese de óxido nítrico que está associado a aumento da capacidade de eliminarem as células fúngicas (206). Os murganhos «*scid*» convencionais (207) e os animais «*scid*» «germ-free» (208) não apresentam aumento da susceptibilidade à infecção sistémica por *C. albicans* apesar de serem mais fácilmente colonizáveis por via gastrointestinal do que os animais de controlo (150). Quando estes animais são injectados com um anticorpo monoclonal depletante para os granulócitos (anti-Gr-1), ou com sílica (que destrói um número elevado de macrófagos), apresentam aumento da susceptibilidade à CS de origem endógena e à CTD (209). Estas observações estão de acordo com estudos clínicos que põem em evidência o papel dos granulócitos na defesa natural do hospedeiro contra infecções por *C. albicans* e, por outro lado, o papel dos macrófagos (210) no controlo das candidíases sistémicas de origem endógena, em animais granulocitopénicos.

As células NK são capazes de matar células do fungo *Cryptococcus neoformans* (211) e de outros fungos (212). Todavia, há ainda pouca evidência do seu envolvimento na resolução das candidíases, nomeadamente a partir de estudos realizados em hospedeiros com deficiências funcionais destas células, como é o caso dos murganhos mutantes conhecidos como «beige». Após

inoculação com Candida, estes murganhos desenvolvem lesões no cérebro e noutros orgãos, mais graves do que em animais normais; a severidade das lesões parece estar mais relacionada com alterações funcionais dos PMN (213). Além disso, os murganhos «*scid*», não apresentam aumento da susceptibilidade à infecção fúngica após deplecção das células NK por tratamento com anticorpo especifico anti-asialo-GM1 (214). Também os murganhos convencionais se forem infectados com uma estirpe atenuada de *C. albicans* e depletados de células NK, por tratamento com anticorpo monoclonal, não apresentam alterações no desenvolvimento de resistência à reinfecção (215). Sabe-se, no entanto, que a infecção com *C albicans* estimula a actividade das células NK já que a administração por via intraperitoneal de células de *Candida* inactivadas induz uma subpopulação linfocitária que é sensível a tratamento com o anticorpo anti-asialo-GM1 (216).

Infecção de murganhos com uma estirpe atenuada de *C. albicans* (PCA-2), incapaz de fazer a transição de levedura para micélio, induz um aumento da resistência contra posterior infecção com a estirpe virulenta (CA-2) (217); esta protecção secundária depende da inoculação de um número de organismos suficiente para estabelecer uma infecção crónica prolongada, sem, no entanto, causar a morte do hospedeiro (218). Nos animais normais, a infecção com a estirpe PCA-2 induz uma forte reacção de hipersensibilidade retardada (DTH) à *C. albicans* e são detectáveis no soro e nos sobrenadantes de culturas de células esplénicas altos níveis de GM-CSF, TNF-α, IL-1 e IFN-γ (219). A DTH é atribuída aos linfócitos T $CD4^+$ Th1, que produzem IFN-γ e GM-CSF *in vitro,*

dado que a sua eliminação por tratamento com um anticorpo monoclonal, inibe a DTH e aumenta a colonização renal pelas leveduras PCA-2 (182), mas, não altera o tempo de sobrevivência dos hospedeiros infectados secundáriamente com a estirpe CA-2. Este resultado está de acordo com outros (220) que sugerem que a depleção dos linfócitos T, de murganhos infectados com doses subletais do fungo, diminui a capacidade de eliminar as leveduras mas não diminui o tempo de sobrevida do animal, pondo em relevo o papel dos macrófagos activados pela infecção primária, impedindo a morte do hospedeiro após infecção secundária com dose letal de *Candida* (217, 221).

Num estudo envolvendo a infecção de murganhos com uma de duas estirpes mutantes de *C. albicans*, que não tinham capacidade germinativa, para indução de protecção contra a infecção com uma estirpe virulenta, obtiveram-se os seguintes resultados: uma das estirpes mutantes, que induziu uma protecção significativa, induziu também a estimulação de células T $CD4^+$ produtoras de IFN-γ e de IL-2, enquanto que a estirpe mutante que não levou a protecção, estimulou células T $CD4^+$ produtoras de IL-4 e de IL-6 (222). A administração de um anticorpo monoclonal contra IFN-γ, simultâneamente com a infecção com a estirpe PCA-2, induz uma alteração no perfil das citocinas produzidas, do tipo Th1, associado a protecção, para o tipo Th2, associado a progressão da doença (223), estabelecendo-se assim uma relação entre os dois tipos de populações celulares, Th1 e Th2, e a evolução da infecção no hospedeiro.

Uma correlação semelhante, entre a produção de citocinas por células Th1 e Th2 e indução de resistência ou susceptibilidade à infecção foi observada em murganhos das estirpes BALB/c e DBA/2, após infecção com a estirpe PCA-2 de *C. albicans*. Os murganhos BALB/c desenvolveram resistência à infecção que estava associada a resposta do tipo Th1, enquanto que os murganhos DBA/2 desenvolveram uma infecção de progressiva gravidade e apresentavam produção de citocinas do tipo Th2. Além disso, quando os murganhos DBA/2 foram tratados com anticorpo específico para a IL-10, simultâneamente com o decurso da infecção, os animais sobreviveram (224).

A comparação da produção de citocinas em situações de resistência (infecções limitadas e sobrevivência) ou de susceptibilidade (infecções crónicas e morte) mostra que a produção de IL-4 e de IL-10 se relaciona com a susceptibilidade. Tanto nos casos de resistência como nos de susceptibilidade do hospedeiro à *Candida*, a administração de IL-4 ou de IL-10 acentuam a severidade da infecção e levam a um progressivo domínio da resposta Th2 (164). Pelo contrário, a produção de IFN-γ pelos linfócitos T $CD4^+$ e $CD8^+$ ocorre apenas em hospedeiros com infecções controladas (225). No entanto, elevados níveis de IFN-γ podem ser detectados no soro de murganhos antes de morrerem de candidíase. Em murganhos que recuperam da infecção, há, invariávelmente, expressão de mRNA para IL-12 nos macrófagos esplénicos (226). Sabe-se hoje que esta citocina é dos factores humorais mais potentes para induzir uma resposta do hospedeiro do tipo Th1 e é fundamental para a

resistência do murganho a infecções por parasitas intracelulares, como micobactérias (227). A importância desta citocina para a diferenciação Th1 *in vivo* em infecções por *Candida* foi avaliada ou pela sua depleção com anticorpos anti-IL-12 ou através da administração da citocina recombinante (228). Os murganhos capazes de controlar naturalmente a infecção com *Candida,* deixam de o fazer após a neutralização da IL-12, apresentando então respostas do tipo Th2. Todavia, murganhos susceptíveis à infecção, quando tratados com IL-12 recombinante, não são capazes de aumentar a sua resistência à infecção nem apresentam alteração do tipo de resposta Th. Isto pode ser explicado porque, embora a resistência e a susceptibilidade a infecção por *Candida* estejam associadas a outras citocinas que reflectem a expansão de subpopulações linfocitárias Th1 ou Th2, outras citocinas desempenham um papel importante na resistência do hospedeiro e na evolução da sua resposta imune contra o microrganismo. Assim, sabe-se que a citocina TGF-β regula a função dos linfócitos T e B e encontra-se aumentada nos murganhos capazes de controlar a infecção e diminuída naqueles que sucumbem à infecção sistémica (229). A neutralização de TGF-β *in vivo*, não afecta a resistência a uma CS primária, numa combinação de estirpes hospedeiro/parasita com características de resistência, mas compromete a resistência destes murganhos contra uma posterior infecção com estirpe virulenta. Vários estudos apontam para que o equilíbrio entre a resposta Th1 e Th2 na CS seja influenciado por vários factores, nomeadamente a virulência da estirpe de *Candida* e a constituição genética do hospedeiro. Outros factores

fisiopatológicos do hospedeiro são também importantes como se depreende da constatação de que murganhos tornados diabéticos desenvolvem CS aguda e letal após inoculação com a estirpe PCA-2 de *C. albicans* (230).

A maioria dos estudos que se encontram na literatura refere que as células T $CD4^+$ são de importância fundamental na resposta imune celular do hospedeiro contra *C. albicans*. No entanto, a avaliação da participação das células $CD4^+$ ou $CD8^+$ na CS de murganhos BALB/c (183), revelou que a depleção de células $CD8^+$, obtida com anticorpos monoclonais, não influencia nem o tempo médio de sobrevida nem o número de animais que sobrevive à infecção, enquanto que a depleção das células $CD4^+$ aumenta significativamente os dois parâmetros. Interessante é que a depleção simultânea dos dois tipos celulares, aumenta o tempo médio de sobrevida, mas não o número de animais que sobrevive. Estes dados indicam que as células $CD8^+$ podem exercer um efeito positivo na recuperação das infecções primárias, mas que esse efeito é neutralizado por uma forte resposta das células $CD4^+$. O aumento da resistência do hospedeiro após a depleção das células $CD4^+$ pode ser explicado porque, como se sabe, a subpopulação Th2 é a preferencialmente estimulada nas CS pelo que a depleção das células $CD4^+$ terá efeito mais notório nesta subpopulação. Assim, a infecção com o fungo, nos murganhos tratados com anticorpos anti-$CD4^+$ não originará um aumento das citocinas produzidas por aquela população celular, nomeadamente da IL-10, que aumentam a susceptibilidade à infecção. Também nas infecções com *Leishmania major,* em murganhos susceptiveis, foi claramente demonstrado

que o tratamento com anticorpos anti-$CD4^+$, simultâneamente com a infecção, induz um aumento da resistência dos hospedeiros (231). Além disso, os murganhos tratados com anti-$CD4^+$, desenvolvem, subsequentemente, uma forte resposta Th1 contra o parasita, resolvendo a infecção (231, 160).

A infecção intraperitoneal com *C. albicans* origina aumento dos níveis de TNF-α (232) e causa um aumento crónico de fibrinogénio no plasma, sem alteração de outros parâmetros bioquímicos do sangue. A possível contribuição de TNF-α na morte precoce que ocorre na infecção aguda por *C. albicans* foi avaliada em murganhos BALB/c infectados i.p. com 10^8 blastoconídios (233). Foi observado que os murganhos morrem 12 horas após a infecção e que a concentração de TNF-α no soro dos animais era considerávelmente superior aos murganhos de controlo. No entanto, o pré-tratamento dos animais com anticorpos específicos contra TNF-α não reduz a mortalidade, enquanto que a administração de TNF-α tornava os murganhos mais resistentes à infecção (233). O efeito protector do TNF-α foi também demonstrado na infecção sistémica com *C.albicans* em murganhos normais e granulocitopénicos (234). Durante a infecção fúngica, TNF-α e IL-6 são excretados para o soro. A neutralização de TNF-α, por tratamento com anticorpo monoclonal, aumentava significativamente a colonização renal dos murganhos normais por *Candida* mas não altera a dos murganhos granulocitopénicos. Estes resultados sugerem que o TNF-α é essencial para a actividade antifúngica dos granulócitos *in vivo*.

Em conclusão, dos resultados apresentados podemos afirmar que a CS em murganhos «inbred» se assemelha muito à doença no Homem, sob o ponto de vista da patologia que induz no hospedeiro. Como no Homem, a activação dos factores do complemento é um fenómeno fundamental para a redução do número de células fúngicas no período inicial da infecção, os macrófagos têm uma contribuição importante na resolução de infecções com *Candida* o que é demonstrado nos orgãos com abundante número de macrófagos residentes.

Comparativamente às candidíases humanas, a infecção sistémica de murganhos granulocitopénicos e monocitopénicos permite concluir que os granulócitos são mais importantes que os monócitos ou macrófagos nas primeiras fases da resposta do hospedeiro contra *C. albicans.*

A deficiência em células T implica um aumento da resistência à CS nos murganhos *nude* e não implica um aumento da susceptibilidade nos murganhos «*scid*». A depleção de granulócitos nestes animais leva a um aumento da susceptibilidade à CS o que está de acordo com estudos clínicos que põem em evidência o papel dos granulócitos na defesa natural do hospedeiro contra infecções sistémicas por *C. albicans.* No entanto, a função granulocítica pode ser afectada por acção da IL-10 libertada por linfócitos Th2 activados. De facto, a comparação da produção de citocinas em várias combinações de estirpes hospedeiro/parasita, que levam a resistência ou a susceptibilidade à infecção, permite concluir que a produção de interleucinas do tipo Th2, como IL-4 e/ou IL-10 se relacionam com o desenvolvimento de

infecções de gravidade progressiva que conduzem à morte do hospedeiro. Além disso, a administração de IL-4 e/ou de IL-10 acentuam a severidade da infecção e levam a um progressivo domínio da resposta Th2. A produção de IFN-γ ocorre em hospedeiros com infecções controladas, no entanto, elevados níveis de IFN-γ podem ser detectados no soro de murganhos que morrem de candidíase. Pelo contrário, a expressão de IL-12 está invariávelmente associada a recuperação da infecção.

De ressaltar ainda que vários estudos apontam para que o equilíbrio entre a resposta Th1 e Th2 seja influenciado por vários factores, nomeadamente a virulência da estirpe de *Candida,* a constituição genética do hospedeiro, assim como vários factores fisiopatológicos do hospedeiro.

3.3. Resultados *in vitro* em murganhos

Quando incubados em soro normal, PMNs do sangue periférico e macrófagos peritoneais ingerem células de *Candida* viáveis com a mesma eficácia, mas, os PMN destroem as leveduras fagocitadas mais eficazmente do que os macrófagos (235). Tal como nos PMN humanos, nos PMN de murganho, o sistema mais eficiente na destruição do fungo parece ser a via da mieloperoxidase-peróxido de hidrogénio. A quantidade de peróxido de hidrogénio libertado pelos PMN de murganho, em cultura com blastósporos de *Candida* mortos, opsonizados ou não, relaciona-se com o número de leveduras (236), o que não acontece quando os leucócitos são incubados com

blastósporos viáveis. Assim, parece que os blastósporos viáveis são capazes de alterar ou de diminuir a libertação de peróxido de hidrogénio pelas células fagocíticas do hospedeiro. A manana, constituinte da parede celular da *C. albicans,* parece ser o responsável pelo início do metabolismo oxidativo e pela produção de peróxido de hidrogénio pelos PMN (237). Por outro lado, PMN activados *in vivo* por *Blastomyces dermatitides* respondem não só a este fungo mas também a células de *C. albicans,* aumentando a resposta respiratória com consequente aumento da perda de viabilidade das células fúngicas (238), sugerindo que a optimização da fagocitose está associada com a reactividade imunológica ao antigénio. Sabe-se que esta activação das células *in vivo* é devida a citocinas produzidas por macrófagos e outros leucócitos.

Os macrófagos alveolares de murganho são altamente eficientes na eliminação de blastósporos de *Candida, in vitro* (239), embora essa eficiência esteja relacionada com a estirpe murina, sendo maior na estirpe C57BL/6.

Há uma considerável heterogeneidade na função efectora dos macrófagos de acordo com a sua proveniência anatómica. A capacidade fungicida é maior em macrófagos isolados do baço, em células de Kupffer ou em macrófagos alveolares do que em macrófagos peritoneais (240). Quando as populações celulares destes orgãos sofrem maturação *in vitro,* na presença de M-CSF, sómente os macrófagos do fígado e do baço apresentam significativa actividade fungicida. Por outro lado, a morte das células fúngicas e a produção de óxido nítrico pelos macrófagos activados são inibidas, de uma

forma dependente da dose, pela IL-4 e pela IL-10, separadamente ou em combinação (241).

Em conclusão, a partir dos resultados obtidos *in vitro* podemos dizer que os PMN de murganho eliminam as leveduras fagocitadas mais eficazmente do que os macrófagos e que à semelhança dos PMN humanos o sistema mais eficiente na destruição do fungo parece ser a via da mieloperoxidase-peróxido de hidrogénio.

A quantidade de peróxido de hidrogénio libertado pelos PMN de murganho é dependente da viabilidade das células fúngicas o que põe em evidência a capacidade modeladora da *C. albicans* sobre o hospedeiro. A manana, um polissacárido constituinte da parede celular da *C. albicans,* parece ser o responsável pelo início do metabolismo oxidativo e pela produção de peróxido de hidrogénio pelos PMN.

Os macrófagos alveolares de murganho são altamente eficientes na eliminação de blastósporos de *Candida, in vitro,* embora essa eficiência esteja relacionada com a estirpe murina, sendo maior nas estirpes mais resistentes à infecção, podendo a capacidade fungicida dos macrófagos ser aumentada pelo tratamento prévio com IFN-γ. É interessante referir que a morte das células fúngicas e a produção de óxido nítrico pelos macrófagos activados, são inibidas, de uma forma dependente da dose, pelas interleucinas IL-4 e IL-10, separadamente ou em conjunto. Este fenómeno explica o papel negativo destas interleucinas na resolução das diversas formas de candidíase uma vez

que, como se sabe, o óxido nítrico é uma molécula efectora directamente envolvida na actividade microbicida e citotóxica do macrófago.

4. Conclusões gerais

Do somatório dos dados apresentados pode dizer-se, que a levedura *C. albicans* faz parte da microflora normal do Homem e de outros animais e que uma diminuicão, mesmo ligeira, dos sistemas de defesa do hospedeiro ou alterações da homeostasia fisiológica pode levar a uma proliferação do fungo, originando uma resposta inflamatória, significativa de comportamento patogénio da *Candida.* Esta modificação do comportamento do fungo é acompanhada de variações fenotípicas como o desenvolvimento de formas filamentosas e reestruturação da parede celular, nomeadamente, apresentação de antigénios, ou a expressão de adesinas e receptores para proteínas dos tecidos do hospedeiro, ou substâncias com actividade proteolítica, variação da hidrofobicidade e das cargas electrostácticas da superfície, etc.

Por seu lado o hospedeiro tem mecanismos de defesa numerosos e complexos que intervêm em diferentes estruturas. Na camada tegumentar as células queratinizadas constituem uma barreira eficaz contra a penetração do fungo. Também o muco que recobre os epitélios pelas suas características fisico-químicas e a sua permanente renovação constitui uma barreira eficaz contra a instalação do fungo. A par da barreira cutâneo-mucosa a flora bacteriana da superfície, que entra em competição com o fungo, constitui um factor de defesa do hospedeiro.

Para que a *C. albicans* provoque doença grave no Homem é necessário, quase sempre, que haja uma quebra nas defesas imunológicas do hospedeiro.

No entanto, infecções locais do fungo, como as candidíases vaginais comuns, não requerem alterações sistémicas do sistema de defesa e são resultado apenas de modificações locais como, por exemplo, do pH das secreções vaginais. Assim, este microrganismo é classificado, enquanto agente infeccioso, como patogénio oportunista sendo o estado fisioógico do hospedeiro de importância primordial para que o fungo se torne patogénico.

Como se pode depreender, os mecanismos de defesa do hospedeiro contra o fungo *C. albicans* não estão ainda completamente definidos, apesar dos vários estudos clínicos que têm relacionado disfunções imunes e endócrinas com susceptibilidade à infecção e dos resultados obtidos com modelos animais que simulam candidíases humanas.

No entanto, numa tentativa integratória aos diversos resultados julgamos poder concluir que:

1) Há diversas formas de candidíase que são: I) A candidíase vaginal, II) A candidíase mucocutânea, III) A candidíase do tubo digestivo e, IV) A candidíase sistémica. Estas diferentes formas de candidíase envolvem mecanismos fisiológicos gerais e imunes diversos.

2) Vários estudos mostram claramente que os neutrófilos e os macrófagos são importantes na eliminação das células do fungo. Deve no entanto ressalvar-se que a candidíase não é um problema apenas em indivíduos com deficiência de células fagocíticas. Resulta claro dos dados apresentados que a capacidade antifungica das células fagocíticas é beneficiada pela activação por citocinas como INF-γ, TNF-α ou IL-8.

3) Percebe-se assim que seja claramente aceite que nas candidíases envolvendo as zonas cutâneomucosas e nas candidíases sistémicas, o desenvolvimento da infecção é condicionado pela expansão das células Th1 ou Th2. A expansão da população Th1 com produção de citocinas como INF-γ e IL-2 resulta benéfica para o hospedeiro, uma vez que estas citocinas activam os fagócitos, aumentando a sua capacidade fagocítica e microbicida. A expansão das células Th2, com produção das interleucinas IL-4 e IL-10, resulta na evolução da doença, pela inibição das células Th1, e, em consequência, da capacidade efectora dos fagocitos.

4) Observações clínicas sugerem que a imunidade celular é de importância fundamental nas situações de candidíase sistémica, todavia, muitos indivíduos com esta forma de candidíase não apresentam defeitos conhecidos da sua imunidade celular o que é apoiado pelo facto de animais deficientes em células T não serem mais susceptíveis à candidíase sistémica do que animais normais.

5) A subclasse de linfócitos Tγ/δ é considerada elemento importante na resistência à infecção das zonas cutâneomucosas, já que, a sua deplecção específica aumenta a susceptibilidade dos hospedeiros. Todavia, esta subclasse de células T não parece importante nas candidíases sistémicas mesmo em animais congénitamente atímicos.

6) É de realçar que anticorpos específicos contra *C. albicans* estão habitualmente presentes no soro de doentes com candidíase, sem contribuirem

para a sua resolução, o que leva a pensar na sua fraca eficácia na protecção do hospedeiro contra a infecção.

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Dos dados clínicos e dos resultados experimentais apresentados na Introdução, parece claro que a resposta imune nas candidíases sistémicas apresenta várias contradições. Assim, o papel da resposta humoral nas CS é altamente controverso uma vez que anticorpos específicos contra antigénios do fungo podem ser protectores, não protectores ou mesmo facilitadores da infecção. Relativamente à resposta celular, os linfócitos T não têm sido considerados importantes para a protecção do hospedeiro uma vez que murganhos sem timo e murganhos eutímicos são igualmente susceptíveis à infecção. Além disso, murganhos atímicos têm sido considerados tão resistentes ou mesmo mais resistentes à infecção do que os murganhos eutímicos. Por outro lado, os neutrófilos são considerados elementos celulares cruciais para a resolução das infecções por *C. albicans*, como é claro nos estudos em murganhos deficientes nas funções neutrofilicas. Todavia, não se pode excluir a regulação destas células pelas células T, como é demonstrado pelo aumento da susceptibilidade à infecção após tratamento com as citocinas IL-4 e/ou IL-10. Parece-nos que, alguma da controvérsia existente nos resultados pode ser explicada porque se tem dado muita atenção à resposta do hospedeiro à infecção mas não à acção que o fungo pode exercer sobre o hospedeiro. Neste aspecto, é particularmente exígua a informação sobre factores de virulência fúngica e propriedades imunobiológicas. Assim, os objectivos do nosso trabalho experimental foram:

1. A execução de estudos experimentais que tiveram como objectivo principal a investigação da capacidade imunomodeladora que a *C. albicans* exerce sobre a resposta do hospedeiro. Assim, numa primeira fase, os nossos objectivos foram investigar se a *C. albicans*, à semelhança de bactérias como *S. intermedius* e *S. mutans,* vírus, como o da peste suína Africana, produz e excreta, para o meio de cultura liquído, uma proteína, ou uma substância de natureza proteica, com actividade imunobiológica, mitogénica e imunossupressora (ISM).

2. Purificar e caracterizar bioquímicamente a proteína ISM da *C. albicans*. Fazer comparação bioquímica com as proteínas ISM já anteriormente isoladas em outros microrganismos e com uma nova proteína ISM isolada a partir de culturas de *S. sobrinus*.

3. Definir a cinética das actividades mitogénica e imunossupressora da proteína.

4. Ensaiar a importância da proteína na protecção do microrganismo contra as acções antimicrobianas do hospedeiro.

5. Caracterizar os efeitos imunobiológicos da proteína ISM da *C. albicans* em hospedeiros imunizados e não imunizados, previamente, com esta proteína.

6. Avaliar o efeito da imunização de hospedeiros murinos com a proteína ISM na protecção contra a infecção sistémica por *C. albicans;* definir a especificidade dessa protecção e compará-la com o efeito protector obtido pela imunização de murganhos com proteínas totais do fungo.

7. Estudar os efeitos imunobiológicos da proteína em murganhos imunodeficientes: animais deplectados de linfócitos T $CD4^+$, timectomizados na idade adulta e congenitamente atímicos, comparando a evolução da infecção sistémica pela *C. albicans* nestes vários tipos de hospedeiros.

8. Determinação de níveis séricos de citocinas após administração da proteína ISM e após infecção por *C. albicans* de murganhos convencionais e de animais imunodeficientes: timectomizados na idade adulta e congenitamente atímicos.

9. Avaliar a contribuição das células do timo na produção de IL10 e, numa fase posterior tentar caracterizar as populações linfocitárias envolvidas na produção das principais citocinas produzidas após administração da ISM.

10. Avaliação da importância de algumas citocinas nos efeitos ISM da proteína e na evolução da infecção fúngica.

11. Elucidar o mecanismo de activação linfocitária pela proteína p43, estudando a expressão do antigénio CD69 e o influxo de Ca^{2+} em linfócitos B e

T estimulados com a proteína ISM. Estes dois eventos, expressão de CD69 e influxo de Ca^{2+}, estão associados a estadios precoces de activação linfocitária.

CAPÍTULO 3. Estudos experimentais

TRABALHO I

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Immunological Activities of a *Candida albicans* Protein Which Plays an Important Role in the Survival of the Microorganism in the Host

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A protein with an isoelectric point of 4.3 and a relative molecular mass of 43 kDa (p43) was purified from the supernatants of the cultures of pathogenic *Candida albicans* but could not be detected in the supernatants of cultures of this fungus with pathogenicity previously attenuated after being repeatedly subcultured in vitro. Treatment of BALB/c and C57BL/6 mice with p43 resulted in (i) marked increases in the numbers of splenic immunoglobulin-secreting plaque-forming cells (PFC) with peak responses of immunoglobulin M (IgM) PFC preceding those of IgG PFC, with an isotype restriction pattern of IgG2a>IgG2b>IgG3>IgG1>IgM, and (ii) specific immunosuppression of the murine primary immune response against sheep erythrocytes. Immunosuppressive and B-cell mitogenic properties of p43 were quantitatively associated and inversely correlated with susceptibility to *C. albicans* infection. C57BL/6 mice treated with p43 2 days before inoculation with *C. albicans* were considerably more susceptible to the fungal infection than untreated mice. The immunobiological and chemical properties of p43 are compared with previously described immunosuppressive and B-cell mitogenic proteins produced by bacteria and viruses, and strategies for immunointervention are discussed.

It is well-known that the commensal fungus *Candida albicans* is an opportunistic pathogen in the immunodeficient host. It has also been observed that sera of patients with chronic mucocutaneous candidiasis were able to suppress immune responses specific for *C. albicans* (4, 16). Furthermore, *C. albicans* or selected components thereof, such as mannan, other polysaccharides, cell wall glycoproteins, and fungal toxins or metabolites have been shown to induce immunosuppression (10, 21, 30, 32). On the other hand, increased resistance to challenge with *C. albicans* was achieved by treatment with anti-μ chain antibodies (23) and with immunosuppressive drugs such as cyclophosphamide (6, 9) or nitrogen mustard (20). Infection with *C. albicans* has also been shown to enhance the proliferative ability of lymphocytes from immunologically immature germ-free rats in response to mitogens (33) and to potentiate antibody responses in mice to antigens unrelated to the fungus as well (12). Finally, cell wall-derived mannoproteins or glucan fractions appear to activate natural killer and macrophage cell populations (36).

Similar contrasting observations have also been reported in other microbial infections. Thus, mice that are genetically B-cell deficient (Xid mice) or depleted of B cells by anti-μ treatment have increased resistance to *Trypanosoma cruzi* (28), *Leishmania donovani* (34), and *Plasmodium chabaudi adami* (19). Moreover, it is well-known that B-cell mitogenicity has been associated with numerous microorganisms and that a quantitative correlation appears to exist between immunosuppression and B-lymphocyte mitogenicity (1–3, 14). Some such activities have been ascribed to lipopolysaccharide (38, 40) and to proteins produced by *Streptococcus intermedius* (1, 2), *Streptococcus mutans* (14), and the African swine fever virus (3, 31).

Here we report a B-cell mitogenic and immunosuppressive protein produced by *C. albicans* which appears to play an important role in the protection of the microorganism from the defense mechanisms of the host.

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MATERIALS AND METHODS

Mice. Male BALB/c and C57BL/6 mice, 8 to 10 weeks old, were bred at the Gulbenkian Institute, Oeiras, Portugal.

C. albicans. *C. albicans*, identified according to the taxonomic criteria of Lodder (24), was isolated from a patient afflicted with generalized mucocutaneous candidiasis and maintained by passages every 3 weeks in Sabouraud dextrose agar medium (Difco Laboratories, Detroit, Mich.). The pathogenicity of the fungus was maintained by inoculations of *C. albicans* into C57BL/6 mice every 3 months and subsequent recovery of the fungus from the infected kidneys. An attenuated form of the same isolate of *C. albicans* was obtained after 60 passages in Sabouraud dextrose agar medium over 8 months.

Preparation of the product(s) secreted by *C. albicans*. *C. albicans* was cultured in Sabouraud dextrose agar medium for 24 h at 28°C and then transferred to a medium containing 0.3% (wt/vol) yeast extract (Difco) and 0.2% (wt/vol) glucose (Winge medium [WM]) for another 60 h. The last incubation was performed at 37°C with constant agitation at an initial concentration of 10^8 particles per liter of medium. The cultures were then centrifuged at 2500 × *g* for 30 min, and the supernatants were filtered through 0.45-μm-pore-size filters (Millipore) before being concentrated by vacuum dialysis through a Visking 100/8FT dialysis membrane with a 12,000 Da cutoff.

Fractionation by ion-exchange chromatography. Cu'ture supernatants from both the pathogenic and attenuated *C. albicans* cultures were fractionated by ion-exchange chromatography in DEAE-Sephadex A 50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) by using a discontinuous molarity gradient of 0.05, 0.5, 1, and 2 M NaCl with neutral

pH by a method previously described in detail (3, 31). The separated extracellular products of *C. albicans* (EP-Ca) were designated F0.05M to F2M EP-Ca path or inact, representing products from pathogenic or attenuated cultures, respectively. WM was fractionated by the same procedure, and the 0.05 and 0.5 M fractions were pooled together and designated F0.05+F0.5M WM for use as controls. All fractions were dialyzed against phosphate-buffered saline (PBS) and reconstituted by vacuum dialysis as culture supernatants to a volume equivalent to 400 μg of protein per ml. This was approximately the same proteinaceous concentration of the original EP-Ca and WM. Protein concentration was determined by the method of Lowry et al. (26).

Removal of mannoside constituents from the biologically active fractions. Because the immunosuppressive activity was detected in F0.05M and F0.5M EP-Ca path, 1 mg of F0.05+0.5M path protein was passed through a 5-ml concanavalin A-Sepharose column (Sigma Chemical Co, St. Louis, Mo.) previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) supplemented with 10 mM $MgCl_2$. The sugar-depleted subfraction was designated F'p EP-Ca path, as it was made up of proteinaceous material obtained by washing the column with the same Tris-HCl buffer. The sugar-enriched subfraction, F's EP-Ca path, was eluted with 15 ml of 0.5 M methyl-α-D-mannoside over 2 h. Both subfractions were dialyzed against PBS and concentrated by vacuum dialysis. F'p EP-Ca path was adjusted to 200 μg of protein per ml. F's EP-Ca path was concentrated to the same volume of F'p EP-Ca path and contained 340 μg of protein (glycoprotein) per ml.

Purification of the immunosuppressive factor. The immunosuppressive activity was detected exclusively in F'p EP-Ca path, and the biologically active protein was purified from this subfraction by polyacrylamide gel electrophoresis (PAGE) (13, 29) performed in 7.5% polyacrylamide gels with a prerun of 2 h to remove excess of *N,N,N',N'*-tetramethylenediamine (TEMED). After PAGE, the gels were cut longitudinally into two halves, one of which was stained with silver nitrate, methylene blue, or periodic acid-Schiff. Constituents in the unstained portion of the gel which corresponded to the silver nitrate-stained bands in the other half were eluted by electrophoresis (39). Each subfraction obtained by this method was dialyzed against PBS, concentrated by vacuum dialysis, and diluted to a proteinaceous concentration of 100 μg/ml.

Analysis of the purified biologically active protein. Analytical isoelectric focusing of the purified biologically active protein and of a mixture of proteins of known isoelectric point was performed with ampholytes of pH 3 to 9 in strips (Phastgel IEF 3–9; Pharmacia) by using computerized systems for the separation and staining (Phast system separation control and development units; Pharmacia). After a prerun of 10 min, 1 μl of each sample was applied, and analytical isoelectric focusing was performed over 30 min. These gel strips were also stained with silver nitrate, methylene blue, or periodic acid-Schiff. The relative mass of this protein was determined by sodium dodecyl sulfate (SDS; Sigma)-PAGE (41) with proteins of known relative masses as references. SDS-PAGE preparations were stained with silver nitrate.

Enzymatic treatment. Protease type XVII from *Staphylococcus aureus* V8 (Sigma) and endoglycosidase H (Boehringer Mannheim GmbH, Mannheim, Germany) were used for enzymatic treatment of samples. Samples were treated for 12 h at 37°C in PBS at pH 7.2 with enzymes at concentrations equivalent to 1/50 of the amount of the protein in the substrate.

Immunization, infection, and treatment protocols. BALB/C and C57BL/6 mice were immunized by injecting 5×10^7 sheep erythrocytes (SRBC) intraperitoneally (i.p.), and the direct and indirect splenic plaque-forming cell (PFC) responses were assayed before and after immunization. Mice were inoculated with pathogenic or nonpathogenic *C. albicans* by i.p. injection of either 1×10^7 or 5×10^7 microorganisms previously cultured in Sabouraud dextrose agar for 24 h at 28°C. Both kidneys were removed aseptically at selected times after inoculation, homogeneized in 5 ml of PBS, and diluted serially five times. CFU of *C. albicans* were counted 48 h after duplicated cultures of each serial dilution in Mycobiotic agar (Difco). Kidneys were also fixed in Bouin for 24 h and stained either by hematoxylin-eosin or by the method of Gridley (17). Mice were injected i.p. with 200 μg (each) of F0.05M, F0.5M, F1M, and F2M EP-Ca path or F0.05M, F0.5M, F1M, and F2M inact protein or with proportionally lower amounts of the subfractions, until 50 μg of the purified biologically active molecule was reached, 2 days before immunization with SRBC or fungal inoculation with *C. albicans*. As controls, mice were treated i.p. with a single injection of 200 μg of F0.05+F0.5M WM protein. In the case of enzyme treatment, control mice were also injected i.p. with the enzyme used (proteinase type VII or endoglycosidase H) at a concentration of 1/50 of the treated sample.

PFC assays. Production of specific anti-SRBC immunoglobulin M (IgM) and IgG antibodies was evaluated by direct and indirect hemolytic PFC assays, respectively (7). Nonspecific immunoglobulin production was assessed by the protein A PFC assay (18). Rabbit antisera against total immunoglobulin or specific for each isotype were used as developing antibodies and were previously characterized in detail (15).

RESULTS

Detection of immunosuppressive activities in fractions of culture supernatants derived from pathogenic and nonpathogenic *C. albicans*. The pathogenicity and attenuated character of the two *C. albicans* cultures were demonstrated by injecting 10^7 yeast cells i.p. into BALB/c mice and culturing their kidneys at selected intervals thereafter. As shown in Fig. 1, the attenuated culture did not become established in the kidneys over time, whereas the pathogenic culture did. When each of these cultures was employed to obtain and fractionate extracellular protein, none of the fractions from the attenuated culture was suppressive when compared with untreated mice or with mice treated with F0.05+0.5M WM as controls. The F0.05M and F0.5M fractions from the pathogenic culture markedly suppressed the primary immune response against SRBC in C57BL/6 mice (Table 1).

Characterization of the immunosuppressive protein. As shown in Table 1, after subfractionation of the mixture of F0.05+F0.5M EP-Ca path into sugar-enriched F's EP-Ca path and sugar-depleted F'p EP-Ca path subfractions, the immunosuppressive effect was detected only in F'p EP-Ca path. The analysis of F'p EP-Ca path by classical PAGE revealed two silver nitrate-stainable bands (Fig. 2). The immunosuppressive activity was detected in the material electroeluted from the more-anodal band (Table 1). This material was designated p43 for several reasons; though the immunoregulatory activity was retained after heating at 60°C for 60 min (Table 1), this band was degradated by protease

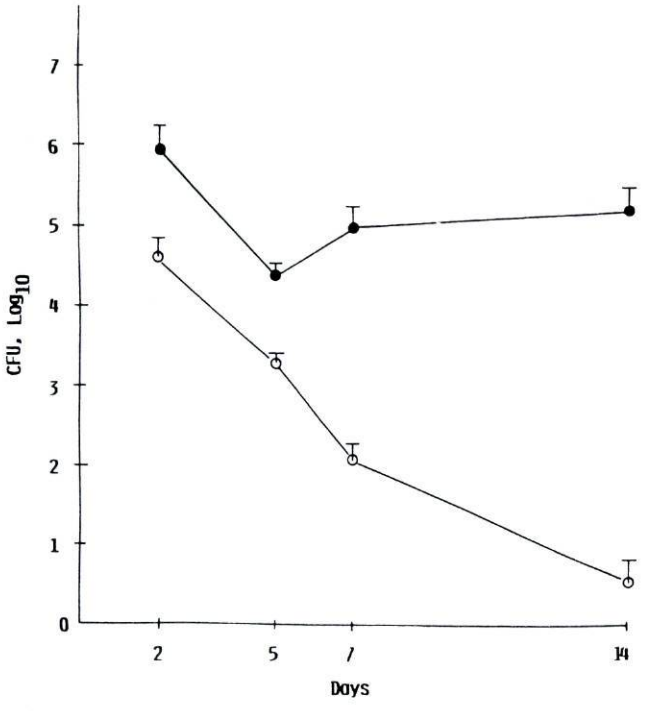

FIG. 1. Growth of pathogenic (●) and attenuated (○) *C. albicans* in the kidneys of BALB/c mice, expressed as CFU, at selected intervals after i.p. injection of 10^7 *Candida* particles. This is a representative experiment repeated three times with different inoculum sizes. In each experiment, the results are means of four mice and one standard deviation.

TABLE 1. Splenic anti-SRBC IgM-secreting PFC in mice[a]

Treatment or fraction	Protein (μg)	Log_{10} IgM-secreting PFC per spleen[b]
Untreated	0	6.00 ± 0.07
F0.05+F0.5M WM	200	5.95 ± 0.05
Protease type XVII B	1	5.88 ± 0.10
Endoglycosidase H	1	6.09 ± 0.20
F0.05M EP-Ca inact	200	5.97 ± 0.03
F0.5M EP-Ca inact	200	5.99 ± 0.07
F 1M EP-Ca inact	200	5.88 ± 0.08
F 2M EP-Ca inact	200	5.82 ± 0.15
F0.05M EP-Ca path	200	3.32 ± 0.58[c]
F0.5M EP-Ca path	200	3.72 ± 0.63[c]
F 1M EP-Ca path	200	5.95 ± 0.13
F 2M EP-Ca path	200	5.89 ± 0.14
F'p EP-Ca path	100	3.69 ± 0.27[c]
F's EP-Ca path	170	6.00 ± 0.09
p43	50	3.19 ± 0.86[c]
p43 heated for 60 min at 70°C	50	3.24 ± 0.57[c]
p43 treated with protease type XVII B	50	5.61 ± 0.21
p43 treated with endoglycosidase H	50	3.32 ± 0.66[c]

[a] Data are from 5 days after SRBC immunization of untreated C57BL/6 mice or mice treated 2 days before immunization with a single i.p. injection of the designated fractions.

[b] Results are from a representative experiment repeated three times. Results are means ± one standard deviation of three mice.

[c] Statistically significant; $P < 0.001$ compared with untreated or F0.05+0.5M WM controls.

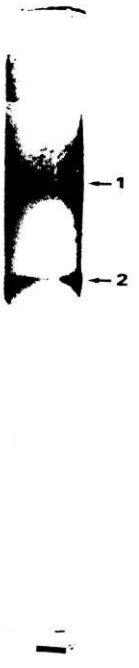

FIG. 2. Pattern of PAGE analysis of F'p EP-Ca showing two silver nitrate-stainable bands (bands 1 and 2). The immunosuppressive activity was detected in the material corresponding to the more anodal (−) band, band 2.

type XVII B but not by endoglycosidase H (Fig. 3), and the biological activity of the material was abrogated by treatment with the proteolytic enzyme but was resistant to treatment with the glycolytic enzyme (Table 1). No significant differences were observed in the numbers of splenic PFC in untreated or treated mice with proteolytic or glycolytic enzymes (Table 1). The material reacted with silver nitrate but not with periodic acid-Schiff or methylene blue, emphasizing its proteinaceous nature. The protein had an isoelectric point of 4.25 (Fig. 4A), as determined by analyt-

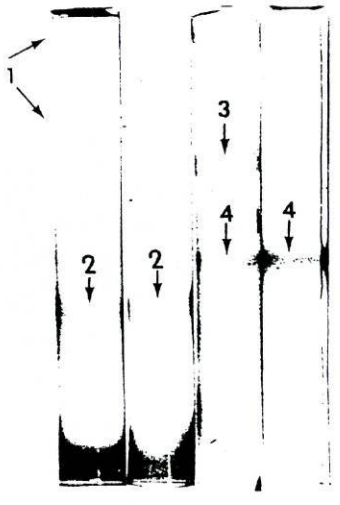

FIG. 3. Pattern of PAGE analysis of p43 after enzymatic treatment with protease B XVII (band 1) and after endoglycosidase (band 2). The bands observed after PAGE, corresponding to protease B XVII (band 3) and to endoglycosidase (band 4), are also shown after incubation with p43 or alone.

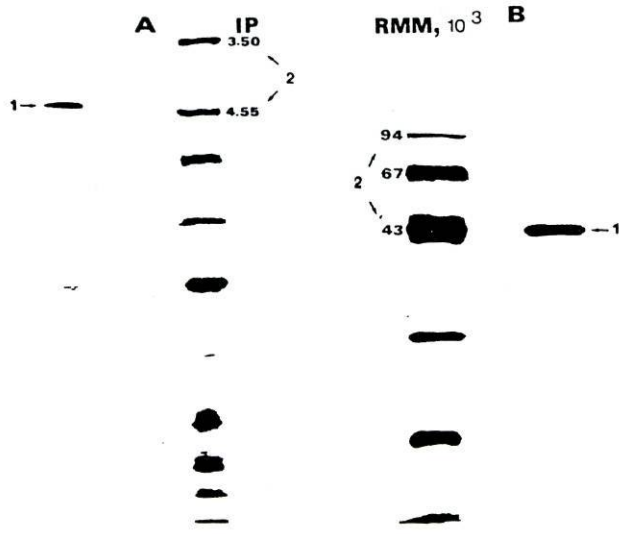

FIG. 4. Analytical comparison between the most purified protein (p43) obtained from the supernatants of *C. albicans* and a mixture of proteins of known isoelectric point (IP) and relative molecular mass (RMM). (A) Isoelectric focusing of p43 (band 1) and of standard proteins (band 2) with IP is indicated. (B) SDS-PAGE of p43 (band 1) and of standard proteins with indicated RMM (band 2).

ical isoelectric focusing, and a relative molecular mass of 43,000 Da (43 Kda), as determined by SDS-PAGE (Fig. 4B).

Immunosuppressive effects of p43 on nonspecific immunoglobulin production in SRBC-immunized and unimmunized mice. BALB/c and C57BL/6 mice, one-half of which had been treated with p43 2 days prior to immunization, were immunized with SRBC, and the production of nonspecific and SRBC-specific immunoglobulin was determined. As shown in Fig. 5, a marked increase in the numbers of splenic nonspecific IgG and IgM-secreting PFC was observed by protein A PFC assay, accompanying specific immunosuppression observed on day 5 in SRBC-immunized mice treated with p43. The increase in the numbers of nonspecific immunoglobulin-secreting PFC reached the highest values 3 days after immunization and preceded the specific immunosuppression which was also preceded by a slight enhancement of the specific responses. In general, the higher the increase in the numbers of a given nonspecific immunoglobulin-secreting PFC, the stronger was the suppression of specific anti-SRBC immunoglobulin-secreting PFC of the corresponding isotype observed in both strains of mice. Interestingly, as also shown in Fig. 5, both the nonspecific and specific immunosuppressive PFC responses to p43 were more pronounced in the strain of mice (BALB/c) that is more sensitive to *C. albicans* infection, i.e., the numbers of fungal CFU were 3 log units higher in the kidneys of BALB/c than of C57BL/6 mice 7 days after infection with 5×10^7 fungal particles.

As shown in Fig. 6 and Table 2, dramatic increases in the numbers of nonspecific protein A, splenic immunoglobulin-secreting PFC were also observed in both unprimed C57BL/6 and BALB/c mice after p43 treatment. As in the case of primed mice, these responses were, with the exception of IgG2b-secreting PFC, even higher (up to 320 times higher than controls in the case of IgG2a-secreting PFC) and more prolonged in time in BALB/c than in C57BL/6 mice. In both strains of mice, the nonspecific peak responses of IgM preceded those of IgG isotypes, and the isotype restriction pattern, taken on peak responses, was IgG2a>IgG2b>

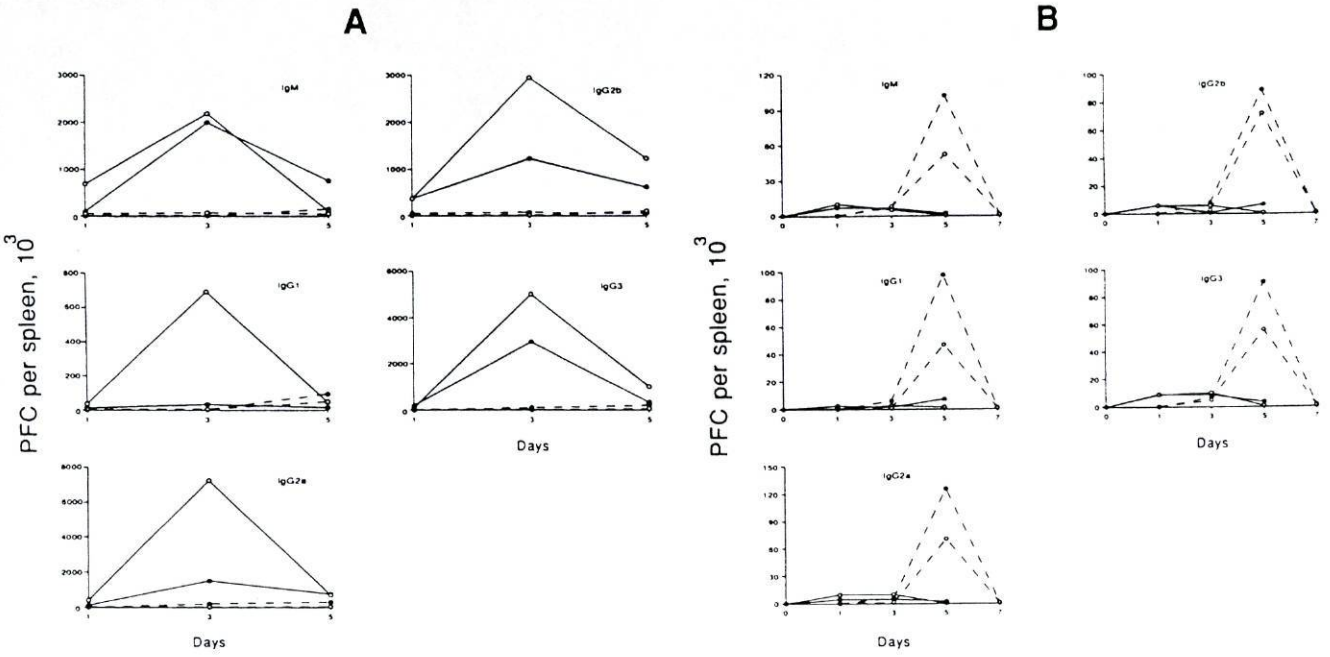

FIG. 5. Numbers per spleen of nonspecific (A) and specific (B) anti-SRBC immunoglobulin-secreting PFC of the indicated isotypes observed in SRBC-primed C57BL/6 (●) and BALB/c (○) mice either treated (——) or untreated (- - -) with p43 on the indicated days after immunization. Here and in Fig. 6, results are means of three mice and represent, as in Fig. 1, one typical experiment performed three times. Also, here and in Fig. 6, as the standard deviations never exceeded 20% of the mean of the PFC assays and in 90% of the cases were less than 10%, they were omitted for simplicity.

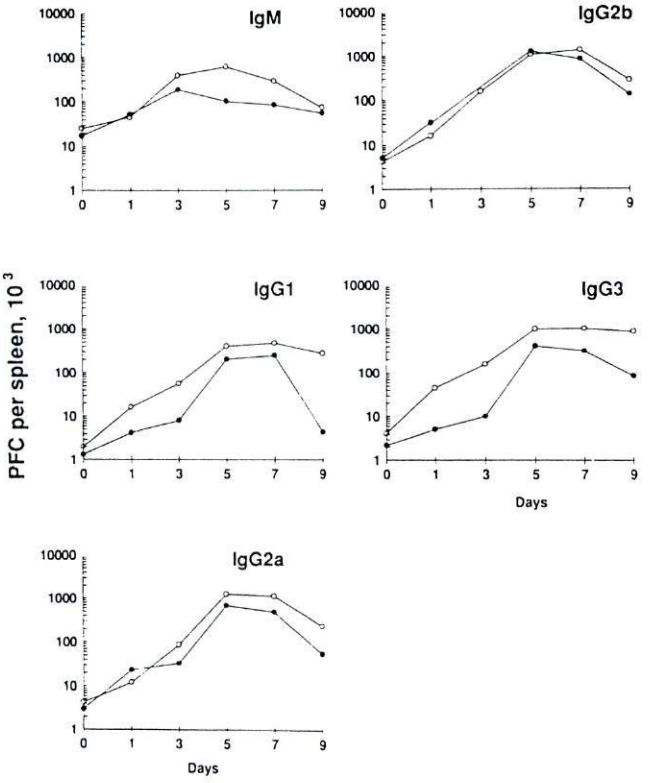

FIG. 6. Numbers per spleen of nonspecific immunoglobulin-secreting PFC of the indicated isotypes observed in SRBC-unprimed C57BL/6 (●) and BALB/c (○) mice, on the indicated days after p43 treatment. See legend to Fig. 5.

IgG3>IgG1>IgM (Fig. 6 and Table 2). No significant increases in IgA-secreting PFC were observed in either primed or unprimed mice after treatment with p43 (data not shown).

Role of p43 in the protection of the microorganism in the host. C57BL/6 mice, relatively resistant to *C. albicans*, were considerably more susceptible to fungus infection when treated 2 days before fungal challenge with 50 μg of p43 protein. This increase in susceptibility was specific, since it did not occur when mice were treated with up to 200 μg of F0.05+F0.5M WM nonmitogenic protein or when they were left untreated. Susceptibility was demonstrated by a significant increase in the numbers of CFU found in the kidney 2, 5, and 7 days after the infection (Fig. 7). Furthermore, the histopathological study of the kidneys of p43-treated C57BL/6 mice performed 5 days after *C. albicans* infection revealed a characteristic pattern of acute candidiasis, viz., the presence of *Candida* pseudohyphae and blastoconidia and hyphae in the kidney medulla and cortex, as well as necrotic regions (Fig. 8). In contrast, the fungal elements were confined to the medulla in mice treated with F0.05+F0.5M WM protein (Fig. 8). Moreover, no necrotic regions or fungal infiltrations were observed in the cortex of these mice. In contrast to the mice treated with F0.05+F0.5M WM, marked infiltration of mononuclear cells in the cortex and medulla was observed in the hematoxylin-eosin-stained preparations from mice treated with p43 (data not shown).

TABLE 2. Peak responses of nonspecific immunoglobulin-secreting PFC of the different isotypes observed in nonimmunized p43-treated BALB/c and C57BL/6 mice

Mouse line and treatment	Log_{10} nonspecific PFC per spleen[a]				
	IgM	IgG1	IgG2a	IgG2b	IgG3
BALB/c					
p43 treated	5.98	5.63	6.32	6.30	5.99
Control	4.58	3.25	3.82	3.89	3.60
C57BL/6					
p43 treated	5.32	5.47	5.75	6.26	5.60
Control	4.28	3.23	3.38	3.91	3.35

[a] Results were taken from Fig. 6. The significant higher differences between BALB/c and C57BL/6 mice in the numbers of nonspecific PFC observed on the peak responses are underlined ($P < 0.001$). The significance was calculated by the Student t test.

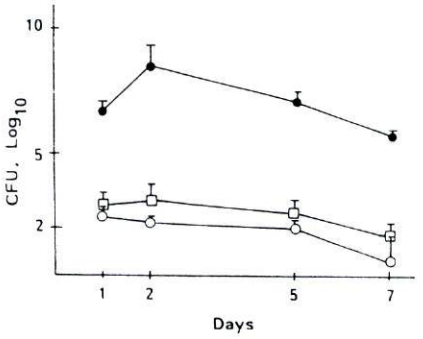

FIG. 7. Mean CFU of *C. albicans* observed, on the indicated days after i.p. infection with 5×10^7 particles of pathogenic *C. albicans*, in the kidneys of C57BL/6 mice either untreated (□) or treated with F0.05+0.5M WM protein (○) or with p43 (●) 2 days before the inoculation of *C. albicans*. Results are means and one standard deviation for four mice.

DISCUSSION

The present results indicate that, in analogy to other microorganisms (2, 3, 14), *C. albicans* produces an immunosuppressive and B-cell mitogenic protein. These p43 effects cannot be interpreted as mere antigenic competition, as control mice were treated with even larger amounts of protein from tissue culture medium of *C. albicans*. Indeed, treatment with this amount of WM protein did not induce significant differences from the unmanipulated mice in the numbers of both SRBC-specific and nonspecific PFC. Moreover, it was previously observed that no such immunosuppressive effects were observed when mice were treated with larger amounts of protein with similar relative molecular masses (31). The immunosuppressive and B-cell mitogenic effects of p43 were quantitatively associated. Similar quantitative association has also been ascribed to other proteins produced by other microorganisms (3, 14). These findings are compatible with the possibility that B-cell polyclonal activation is the cause of the specific immunosuppression induced by p43 and the other described immunosuppressive and B-cell mitogenic proteins. In fact, it was previously demonstrated that B-cell activation was a crucial step in the process of immunosuppression induced by such proteins (2, 14, 31). Therefore, overstimulation of the immune system seems to be the mechanism by which immunosuppression is induced by p43 and by the other mitogenic proteins. In agreement with this assumption is the observation that the strong immunosuppression of the peak responses against SRBC (day 5) induced by p43 is preceded by an increase on

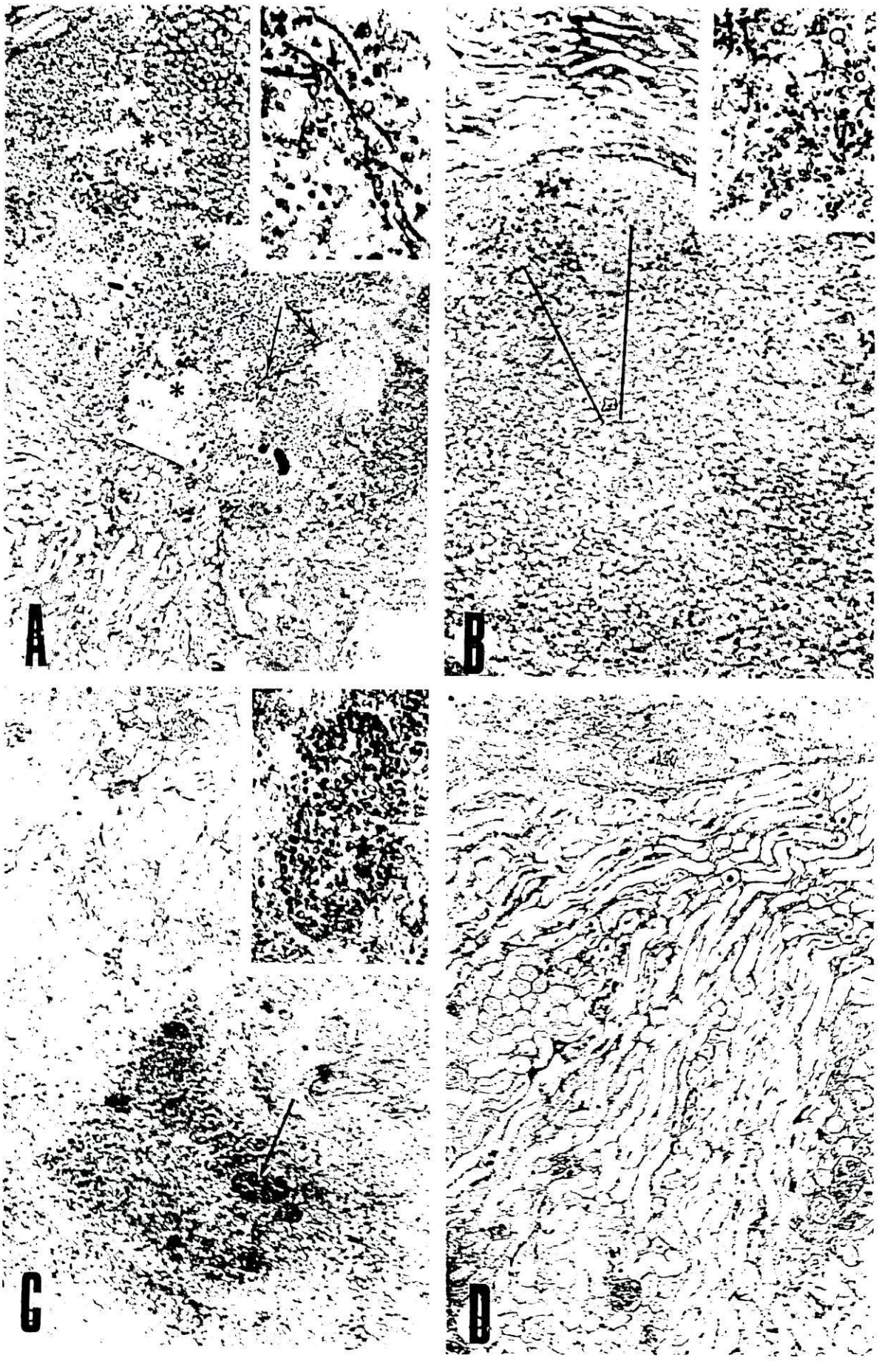
A
B
C
D

FIG. 8. Histopathological patterns of the kidney medulla of C57BL/6 mice treated 2 days before *C. albicans* infection with either p43 (A) or with F0.05+F0.5M WM protein (controls [B]). (C and D) Kidney cortex of the same infected mice treated (C) or untreated (D) with p43. The major histopathological preparations are amplified 450×, and the inserts were amplified 1,800×. The amplified inserts represent the tissue areas indicated by arrows in panels A and C and delimited by two convergent straight lines in panel B. The diffuse fungal infiltration areas consisting in blastoconidia and hyphae are shown in the insert of panel A. This diffuse fungal infiltration is accompanied by necrotic regions (*) (A), whereas it is delimited in the controls (B and respective insert). Extensive infiltration of *C. albicans* was observed in the kidney cortex of p43-treated mice (C and respective insert). In contrast, no fungal infiltration was observed in the kidney cortex of control mice (D).

day 1 in the numbers of splenic immunoglobulin-secreting PFC. The immunosuppressive effect of p43 plays an important role in the protection of the microorganism in the host. Indeed, p43 was undetectable in the supernatants of cultures of *C. albicans* with attenuated pathogenicity after being repeatedly subcultured. Moreover, both the immunosuppressive and B-cell mitogenic effects of p43 were more evident in BALB/c than in C57BL/6 mice, and the former strain of mice was more susceptible to our *C. albicans* inocula. Hector et al. (20) reported opposite results of mouse strain susceptibility to this fungus, but other reports (5, 9) are in agreement with our findings. Furthermore, susceptibility to the fungal infection of the strain found relatively resistant (C57BL/6 mice) was dramatically increased by treatment with p43 2 days before *C. albicans* infection. Indeed, a significant increase of CFU was observed in the kidneys of these mice accompanied by histopathological pattern of this organ characteristic of acute candidiasis which was undetectable in p43-untreated mice. Enhanced survival of *S. mutans* in the host was also achieved by an analogous immunosuppressive and B-cell mitogenic protein produced by that microorganism (35, 37). However, this protective effect is more clearly demonstrated in the *C. albicans* infection model because this fungus is distinctly more pathogenic in mice than *S. mutans* is.

Several observations are in agreement with the role of B-cell mitogenicity in the immunosuppression induced by microorganisms which subsequently increase their survival in the host. Indeed, impaired infections to protozoa, plasmodia, and helminths were seen in mice depleted of B cells by anti-μ chain or in animals genetically B-cell deficient, such as Xid mice (19, 28, 34). The predominant splenic nonspecific synthesis of IgG2a and IgG2b isotypes induced by p43 is also observed after murine treatment with the B-cell mitogenic proteins produced by streptococci (2, 14) and viruses (3). Moreover, a similar isotype restriction pattern is also observed after viral (11), protozoan (28), or helminth (25) infections. Interestingly, p43 is biochemically rather similar to the immunosuppressive and B-cell mitogenic proteins produced by bacteria and African swine fever virus. Indeed, the isoelectric point of p43 is identical to the corresponding proteins secreted by *S. intermedius* (4, 25) and *S. mutans* (eluted after preparative isoelectric focusing between the isoelectric points of 3.9 and 4.2). The immunosuppressive and B-cell mitogenic protein produced by African swine fever virus has a relative molecular mass of 36 kDa and an isoelectric point of 3.89 (1, 3, 14). Although the basic structure of these proteins cannot be inferred from isoelectric points and/or mass data, it should not be very surprising that different microorganisms survive in the host through the production of B-cell mitogenic proteins with a common similar biological structure. Experiments are now in progress in order to test this possibility. In any case, neutralization, by previous elective immunization of these microbial immunosuppressive and B-cell mitogenic substances, seems an appropriate strategy in immunointervention. Indeed, this strategy proved to be appropriate in the protection of mice against *S. mutans* infection (37). Although it is well-known that humoral immunity against unfractionated fungal particles of *C. albicans* has not been shown to provide a protection against infection, we are also now testing the possibility of inducing immunoprotection against this fungus by elective immunization with p43, because this protein seems to be a crucial fungal compound.

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TRABALHO II

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Immunoprotection against systemic candidiasis in mice

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Key words: activation, *Candida albicans*, immunoprotection, p43, protection

Abstract

We have previously described an immunosuppressive B cell mitogenic (ISM) protein, p43, produced by *Candida albicans*, which plays an important role in the survival of the microorganism in the host. The N-terminal amino acid sequence of p43 was found to be different from all amino acid sequences registered in updated protein databanks. Immunization of BALB/c mice with p43 partially neutralized the biological effects of this protein, namely depletion of bone marrow pre-B and B cells, the increased numbers of total and large B and $CD4^+$ lymphocytes, and the non-specific polyclonal response of splenic IgG2a-, IgG2b- and IgM-secreting plaque forming cells. Immunization of BALB/c mice with p43 fully protected the mice against the fungal infection. In contrast, immunization with *C. albicans* sonicates (Cs) was not protective. Our data indicated that specific antibodies against p43 protected, whereas those against Cs facilitated *C. albicans* infection. Thus, the ratio between anti-p43 and anti-Cs antibody titres was much lower in the non-protected mice (Cs-immunized and control non-immunized) than in p43-immunized mice. Moreover, passive administration of specific anti-p43 antibodies significantly protected against fungal infection, whereas passive administration of specific anti-Cs antibodies markedly increased the susceptibility to *C. albicans* infection. These observations are discussed on the basis of alternative approaches of immunointervention.

Introduction

Classical approaches of vaccination are based on the assumption that unlimited potentiation of the host immune system with microbial epitopes will confer immunoprotection against infection. However, in spite of the world-wide effort on vaccine development, immunoprotection has only been achieved against a few pathogenic bacteria and viruses (1), and efficient vaccines are simply not available against fungi or parasitic protozoa, plasmodia and helminths. These observations suggest that current strategies of vaccine development are not necessarily correct and, as recently pointed out (2), previous immunopotentiation of the host with microbial epitopes may even facilitate microbial growth. Therefore, an alternative strategy may be the neutralization of microbial substances which are virulence factors. Immunosuppressive B cell mitogenic (ISM) proteins produced by bacteria and viruses have previously been described (3–5). These ISM proteins are virulence factors because the immunosuppression, a consequence of B cell overstimulation induced by these proteins, is crucial for the survival of the producing microorganisms in the host (6,7).

Candidal infections frequently occur in immunodeficient patients: mucocutaneous candidiasis is prominent in AIDS individuals and systemic candidiasis in cancerous patients or in humans afflicted with impaired phagocytic function. However, immunosuppression observed during the clinical course of severe systemic mycotic diseases also appears to be caused by the infectious fungi (8–10). In particular, fungal constituents or products have been described as responsible for the immunosuppression observed in candidiasis (11–20). Moreover, experimental models of candidiasis have provided evidence of a suppressive effect of *Candida* blastoconidia (21–24) in previously healthy hosts.

We have recently found (25) that *Candida albicans* produces an ISM protein, designated as p43, which plays an important role in the survival of the microorganism in the host. In particular, we have shown that: (i) the immunosuppressive

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and B cell mitogenic properties of p43 were quantitatively associated with the host susceptibility to *C. albicans* infection, (ii) treatment of C57BL/6 mice with p43 decreases the resistance of the host and subsequently facilitates the fungal growth, and (iii) the loss of capacity to produce p43 by *C. albicans* correlates with the loss of the fungal virulence. We decided, therefore, to investigate whether neutralization of p43 by previous elective immunization of the host with this protein may protect against *C. albicans* infection.

Methods

Mice

Male BALB/c and C57BL/6 mice (6–8 weeks old) were bred in the Gulbenkian Institute of Science, Oeiras, Lisbon.

C. albicans

C. albicans was obtained as previously described (25). Briefly, *C. albicans*, identified according to the taxonomic criteria of Lodder (26), was isolated from a patient with generalized mucocutaneous candidiasis and maintained by passages every 3 weeks in Sabouraud dextrose agar medium (Difco, Detroit, MI). The pathogenicity of the fungus was assured by inoculations of *C. albicans* in C57BL/6 mice every 3 months.

Listeria monocytogenes

L. monocytogenes was a kind gift of Dr R. Appelberg, Center for Experimental Cytology, Porto. *L. monocytogenes* strain EGD was grown in Antibiotic 3 Broth (Difco), split into aliquots and kept frozen at −70°C.

Candida sonicates (Cs)

Candida blastoconidia were disrupted by sonication (10 cycles of 30 s at 100 W) with a Branson cell disrupter, model W 185 D, on ice. The sonicates were successively filtered through 0.45 and 0.2 μm Millipore filters, and stored in small aliquots at −70°C. The protein content was determined by the method of Lowry *et al.* (27).

Protein isolation and sequence analysis

Purified p43, as described in detail before (25), was used for mice treatment. For amino acid analysis p43 was run in SDS (Sigma, St Louis, MO)–PAGE, electroblotted to an Immobilon P membrane in 10% methanol, 10 mM CAPS buffer, and analysed in an Applied Biosystems model 470A gas-phase sequencer equipped with a 120A PTH analyser. The determined sequence was compared with those contained in updated versions of the SwissProt and NBRF/PIR protein databases using the FASTA program (28).

In vivo treatment for lymphocyte depletion

Depletion of $CD4^+$ cells was accomplished in BALB/c mice by using rat anti-$CD4^+$ (GK1.5) (29) mAb (a kind gift of Dr P. Minoprio, Pasteur Institute, Paris, France). Anti-$CD4^+$ mAb was administered i.p. on the previous day (day −1) and at the same time (day 0) of either infection with *C. albicans* or p43 treatment in two doses of 100 μg. This schedule of antibody treatment has previously been shown and confirmed by us to result in functional inactivation and removal of >90% of $CD4^+$ cells (30,31).

Challenge infections

C. albicans infection was evaluated in both kidneys of all groups of mice, 5 days after i.p. infection with 10^6 viable blastoconidia in 0.5 ml of PBS. The blastoconidia were previously incubated in Sabouraud glucose broth (Difco) for 18 h at 37°C. The two kidneys were removed aseptically, homogenized in PBS and diluted serially five times. The c.f.u. values of *C. albicans* were counted 48 h after duplicated cultures of each serial dilution in Mycobiotic agar (Difco).

L. monocytogenes infection was evaluated in the spleens of the mice, 3 days following i.v. infection with 10^4 viable bacteria in 0.2 ml of PBS. The spleens were removed aseptically, homogenized in PBS and diluted serially. The c.f.u. values of *L. monocytogenes* were counted 24 h after duplicated cultures of each serial dilution in Antibiotic 3 (Difco).

Immunizations with p43/ISM or Cs protocols

BALB/c mice were immunized twice with intradermical (i.d.) injections of 10 μg of p43 or 10 μg of Cs in 0.2 ml of PBS. C57BL/6 mice were immunized i.d. four times with 10 μg of Cs. Immunizations were repeated with 3 week intervals.

Systemic treatment with purified p43/ISM

p43-immunized, non-immunized and depleted of $CD4^+$ cells BALB/c mice, were treated i.p. with 50 μg of the purified p43, and the mitogenic effect of the ISM protein was evaluated 5 days later.

Antibody detection

A standard ELISA was done to quantify specific antibodies in mice sera. Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml of p43 or Cs overnight at 4°C. THe wells were then saturated during 30 min with 1% BSA in PBS at room temperature. Appropriate serial dilutions of the serum samples were then incubated in the plates for 2 h at room temperature. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat antibodies anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. *o*-Phenylenediamine (Sigma) and H_2O_2 were used to develop the reaction. The reaction was stopped by the addition of SDS. The colorimetric change was measured with a Biotek Chromoscan at 450 nm. The ELISA antibody titre was expressed as the reciprocal of the highest dilution giving an A_{450} of 0.1 above that of the control (no serum added) (32).

Passive serum administration

Non-immunized BALB/c mice were treated i.p., 1 day before (day −1), at the same time (day 0) and 1 day after (day +1) infection, with sterile Ig enriched fraction (IgF) (ammonium sulphate precipitation), obtained from 0.3 ml of mice sera, of the same strain, immunized with p43 or with Cs. C57BL/6 mice were treated using the same protocol with only the IgF of the mice of the same strain immunized with Cs because, in opposition to Hector *et al.* (33) but in agreement with other authors (34,35), we observed that BALB/c mice were susceptible, whereas C57BL/6 mice were relatively resistant, to *C. albicans* infection. Therefore, C57BL/6 mice were only used for the assays of susceptibility. The same protocol was

also used for the treatment of control BALB/c and C57BL/6 mice with IgF obtained from sera of non-immunized mice of the same strain.

Plaque forming cell (PFC) assays

Splenic non-specific Ig production was assessed by the Protein A PFC assay in agar (36,37). Rabbit antisera against total Ig or specific for each isotype were used as developing antibodies and were previously characterized in detail (38). Indirect measurement of specific anti-p43 PFC was accomplished by adding different concentrations of the mitogenic protein to the bacto-agar medium.

Cell suspensions

Splenic cell suspensions were prepared by gently teasing the spleen in cold BSS. Bone marrow cell suspensions were obtained from one femur by flushing the femoral shaft, after cutting the extremities of the bones, with 2.5 ml of BSS. Cell suspensions were made in BSS for the purpose of PFC assays and in BSS supplemented with 3% of FCS (Gibco Biocult, Glasgow, UK) for cytometric analysis.

Immunofluorescence and flow cytometry analysis

Dual-parameter analysis of forward light scatter for cell size and surface markers of lymphoid populations of the bone marrow and spleen was performed in a FACScan analyser (Becton Dickinson, Mountain View, CA). The mAb were: goat anti-mouse IgM FITC conjugate (Southern Biotechnology), rat anti-mouse CD4 phycoerythrin conjugate (Coulter Immunology, Coulter, Hialeah, FL), rat anti-mouse CD8 FITC conjugate (Coulter Immunology) and rat anti-mouse Ly5 (B220) phycoerythrin conjugate (Coulter Immunology). After staining, the cells were washed twice and suspended in FACS medium containing propidium iodide (Sigma), for exclusion of dead cells. The list mode data, collected as 10^4 gated lymphoid cells, were analysed and statistically treated with the PC-Lysys software (Becton Dickinson).

Results

N-terminal sequence of p43/ISM protein of C. albicans

The N-terminal amino acid sequence of p43 was found to be GVVEQXAXSYXNLVA. This sequence is not represented in updated versions of the SwissProt and PIR/NBRF protein databanks.

Systemic administration of p43/ISM in mice: a $CD4^+$-dependent process which affects the B cell compartments in both bone marrow and spleen

At day 5, following i.p. inoculation of 50 μg p43/ISM, BALB/c mice showed a marked increase in the number of total and large B lymphocytes and also of large $CD4^+$ cells (Table 1). No significant differences were observed in the numbers of total or large $CD8^+$ cells (data not shown). The increase in the number of cells observed upon p43 treatment was significantly reduced with mice previously immunized with the ISM protein for up to 30 days following immunization with p43/ISM. In this case, the numbers of large $CD4^+$ cells were approximately reduced by half, whereas the numbers of total and large B cells were only slightly reduced. However, the numbers of B lymphocytes were already significantly higher in p43-immunized than in non-immunized mice before treatment. As shown in Fig. 1, cytofluorimetric analysis indicates that the relative frequency and the absolute number of both bone marrow pre-B and B lymphoid cells markedly decreased (by a factor of 4) in non-immunized mice after i.p. treatment with the ISM protein. This depletion was abrogated if mice were previously immunized with p43.

Local immunization with p43/ISM partially abrogates the biological activity of systemically administered p43

As shown in Fig. 2, only splenic IgG1- and IgM-secreting PFC were increased in p43-immunized BALB/c mice. Enhancement in IgG1-secreting PFC was more evident at 15 and 30 days than at day 7 of immunization, whereas the increase in IgM-secreting PFC became less evident from day 7 to 30. In contrast, a marked reduction in the numbers of IgG2b- and IgG3-secreting PFC was observed 7, 15 and 30 days following immunization. No significant differences induced by the immunization were observed in the number of Ig-secreting PFC of IgG2a isotype. Systemic treatment of non-immunized mice with p43 resulted, after 5 days, in a marked increase in the numbers of splenic non-specific IgG- and IgM-secreting PFC, isotype restricted as IgG2a > IgG2b > IgG3 > IgG1 > IgM. This mitogenic effect of p43 was

Table 1. Absolute numbers ($\times 10^6$) of total and large splenic IgM^+ and $CD4^+$ lymphocytes observed on the indicated groups of BALB/c mice (results are means ± SD of six mice representing a demonstrative experiment out of three)

	IgM^+		$CD4^+$	
	Total	Large	Total	Large
Control (non-immunized)	73.2 ± 7.0	7.0 ± 1.3	44.0 ± 7.0	0.9 ± 0.4
p43-Immunized	93.0 ± 10.0[a]	13.8 ± 1.3	39.0 ± 6.0	1.0 ± 0.2
Non-immunized, p43 treated	150.0 ± 11.0 (2.04)[b]	39.7 ± 5.0 (5.7)[b]	40.0 ± 7.0	4.7 ± 0.8 (5.22)[b]
p43-Immunized, p43 treated	130.0 ± 7.0 (1.4)[c]	28.0 ± 5.0 (2.03)[c]	43.0 ± 6.0	2.3 ± 0.6 (2.3)[c]

[a]Significantly different values (all corresponding to $P < 0.01$) calculated by the *t*-test are underlined with dashes in the cases of differences between the ISM-untreated mice, p43-immunized and control, and with solid lines in the case of differences between the ISM-treated mice, non-immunized or p43-immunized.

[b,c]Times increase (ratios) of the numbers of total and large cells observed in p43-treated compared with p43-untreated mice. Ratios were calculated between [a]non-immunized, p43-treated and controls, and [b]p43-immunized, p43-treated and p43-untreated mice.

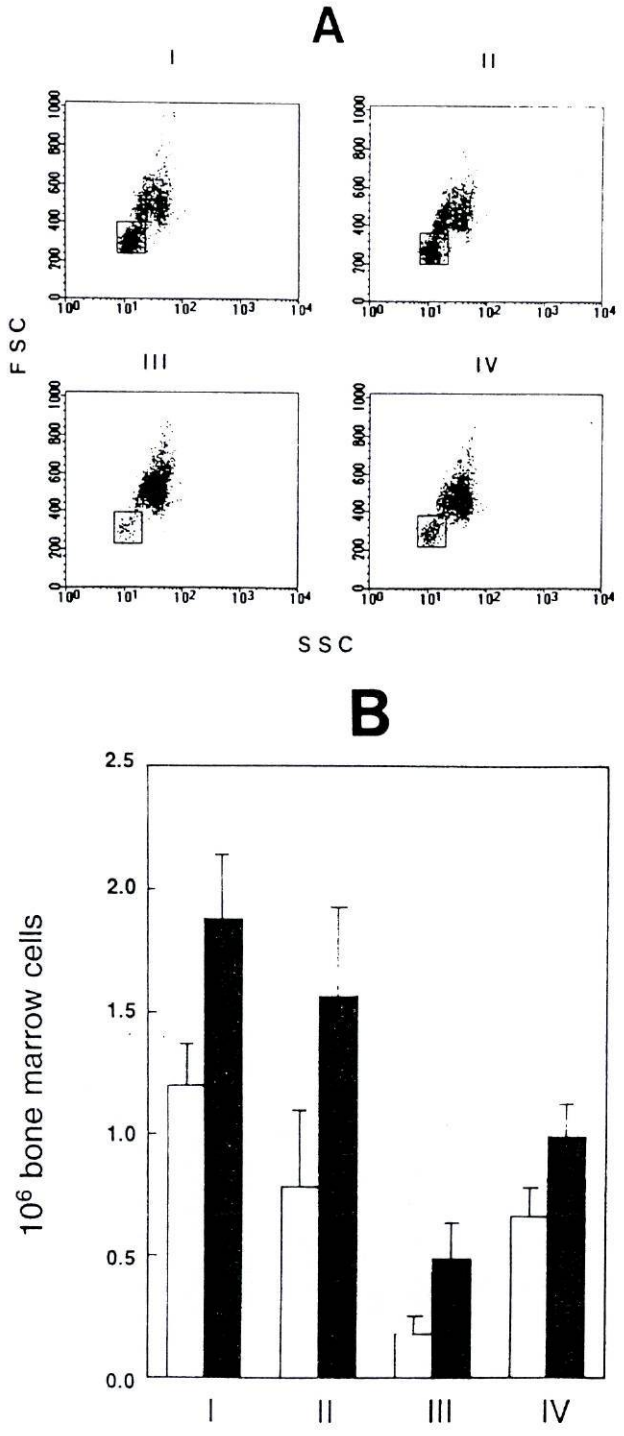

Fig. 1. (A) Demonstrative example of cytofluorimetric analysis using the forward scatter (FSC) and side scatter (SSC) light parameters of bone marrow lymphoid population of unmanipulated (control) (I), p43-immunized (II), p43 non-immunized after p43 systemic treatment (III) and p43-immunized after p43 systemic treatment (IV) BALB/c mice. (B) Absolute numbers of bone marrow B (open bars) and pre-B (closed bars) lymphoid cell populations of unmanipulated (control) (I), p43-immunized (II), p43 non-immunized and p43 systemically treated (III) and p43 systemically treated, previously immunized with the ISM (IV) BALB/c mice. In p43-immunized mice observations were taken 30 days after the last immunization. In this and in Figs 2–4 and 6 the results are means of quadruplicated samples +1 SD. In this and in the following figures the results represent a demonstrative experiment from a minimum of three different experiments.

partially abrogated in p43-immunized mice for the isotypes IgG2a, IgG2b and IgM over days 7, 15 and 30 after p43 immunization, and for isotype IgG3 7 and 15 days after local immunization. No differences were observed in the numbers of IgG3-secreting PFC between the two groups 30 days after the last immunization. In contrast, IgG1-secreting PFC markedly increased in immunized mice treated systemically with p43. This enhancement was progressive from day 7 to 30 after the last immunization with protein and was due to a higher production of specific anti-p43 IgG1. Indeed, 30 days after the last immunization there was an ~60% reduction in the numbers of splenic IgG1-secreting PFC in all groups of mice treated i.p. and/or immunized i.d. with p43 when the PFC assays were performed by adding this protein to the agar (Fig. 3). More than 90% of IgG1 secreted by the PFC of controls (p43 non-immunized and p43 untreated mice) were non-specific for anti-p43 by these criteria (data not shown). The peak reduction of the IgG1-secreting PFC was observed with a concentration of 2.5 μg/ml of protein in p43-immunized mice (treated or untreated with p43). In contrast, such a peak reduction was observed with a concentration of 10 μg/ml of the mitogenic protein in mice treated with p43 but not previously immunized with this protein (Fig. 3). These observations suggest that immunization of mice with p43 increases antibody affinity to this protein. No reduction in the numbers of the other isotype-secreting PFC was observed when the agar was supplemented with p43 (data not shown), indicating that the other secreted Igs are non-specific for p43. As shown in Fig. 4, anti-$CD4^+$ mAb treatment of BALB/c mice abrogrates all isotype production induced by p43.

Local immunization with p43/ISM induces protective immunity against C. albicans

As shown in Fig. 5, protection against *C. albicans* infection was dramatic in BALB/c mice immunized with two i.d. injections of ISM protein 7, 15 and 30 days after immunization. Surprisingly, fungal growth was detected only in the kidneys of one mouse out of the 25 immunized as late as 30 days after the last p43 immunization. The numbers of c.f.u. observed in this mouse were remarkably lower than those observed in control non-immunized mice. In contrast, no protection against *C. albicans* infection was observed in Cs-immunized BALB/c mice 30 days after immunization. Figure 6 shows that the ELISA titres of IgG anti-p43 and anti-Cs observed 30 days after immunization increased in all groups of mice from day 0 to day 5 after *C. albicans* infection; the ratio between anti-p43 and anti-Cs titres kept constant or very similar. Interestingly, although the titres of antibodies against p43 were higher in the protected mice (immunized against p43) than in the non-protected groups of mice (controls and immunized against Cs), immunoprotection does not seem to correlate strictly with the levels of serum antibodies against the mitogenic protein. Indeed, as depicted in Fig. 6, the ELISA titres of serum IgG anti-p43 were higher in BALB/c mice immunized with Cs than in controls before and after *C. albicans* infection, and the susceptibility to fungal infection was identical in the two groups of mice. The relative production of antibodies anti-p43 and anti-Cs seems to account for this discrepancy. Thus, the ratio between the antibody titres of anti-p43 and anti-Cs was 2 before and 2.7 at 5 days after *C. albicans* infection

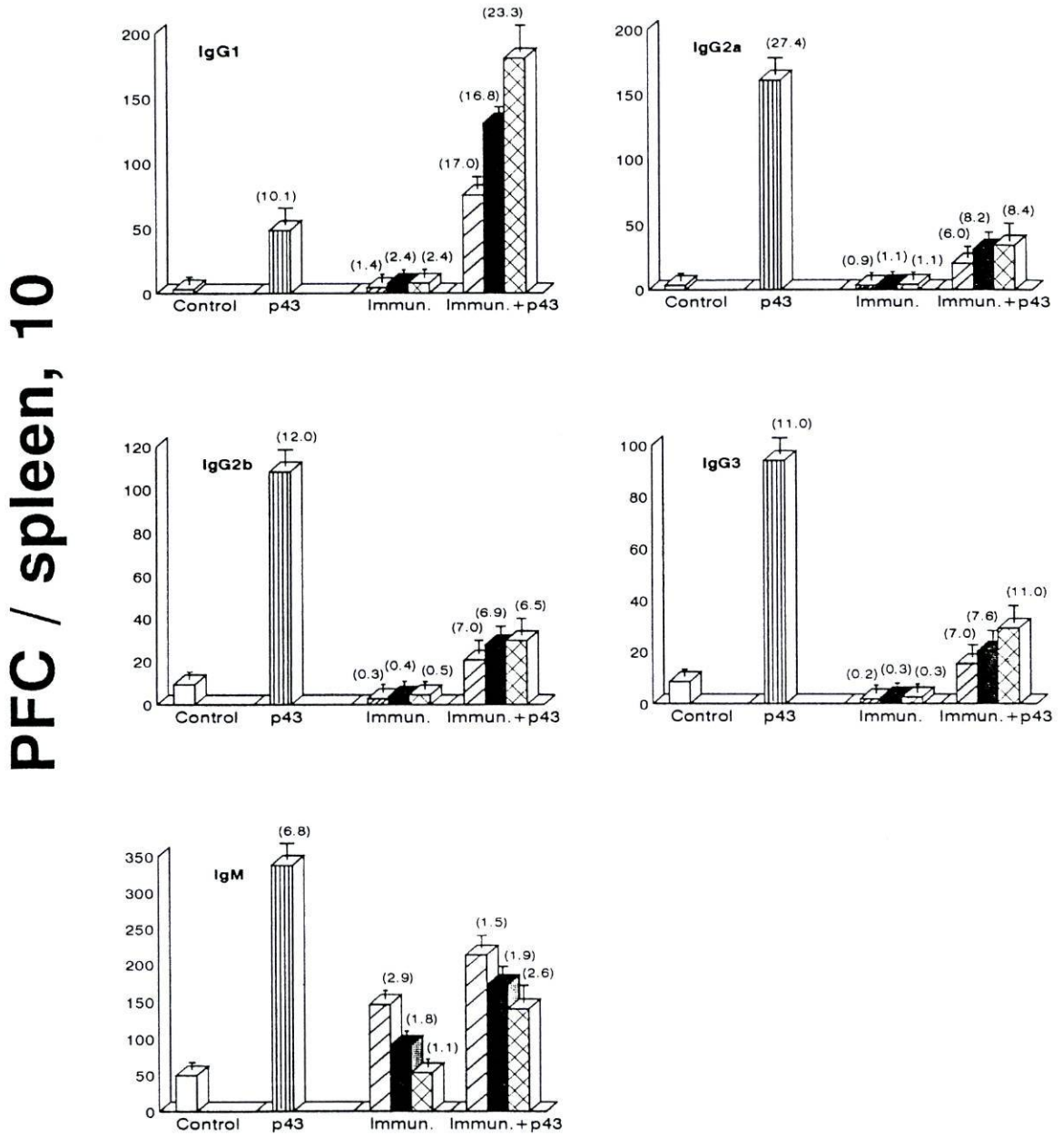

Fig. 2. Numbers of splenic non-specific Ig-secreting PFC of the indicated isotypes, in naive non-immunized (□), non-immunized systemically treated with p43 (▥), p43-immunized (immun.), and p43-immunized and subsequently treated systemically with p43 (immun. + p43) 7 (▩), 15 (■) and 30 (⊠) days after the last immunization with p43/ISM, BALB/c mice. The following ratios of the numbers of splenic Ig-secreting PFC between systemically treated with ISM and unmanipulated control mice, p43-locally immunized and unmanipulated control mice, p43 locally immunized and systemically treated with ISM and p43 locally immunized are indicated above the dividend bar numbers.

respectively in the gorup of mice protected against the fungal infection, contrasting with 0.33 and 0.15 in controls and in Cs-immunized mice respectively before and after *C. albicans* infection (Fig. 6). Therefore, it seems that development of anti-p43 and of anti-Cs antibodies results in opposite effects: the former prevents fungal growth whereas the latter facilitates it. Reinforcing this assumption is the observation that BALB/c mice treated i.p. with IgF of serum containing 2450 ELISA titres of specific anti-p43 IgG antibodies, obtained from p43-immunized BALB/c mice 30 days following immunization, became markedly more resistant to the fungal infection. Indeed, as shown in Fig. 7, no *C. albicans* c.f.u. were observed 5 days after infection with 10^6 blastoconidia in the kidneys of four out of six BALB/c mice treated with IgF containing antibodies anti-p43. Moreover, this kidney fungal load of the two mice that were not fully protected was very reduced in comparison with controls treated with IgF obtained from normal serum of the same strain, all heavily fungal loaded. In contrast, the kidneys of BALB/c mice treated with the IgF of serum obtained from BALB/c mice 30 days after the last

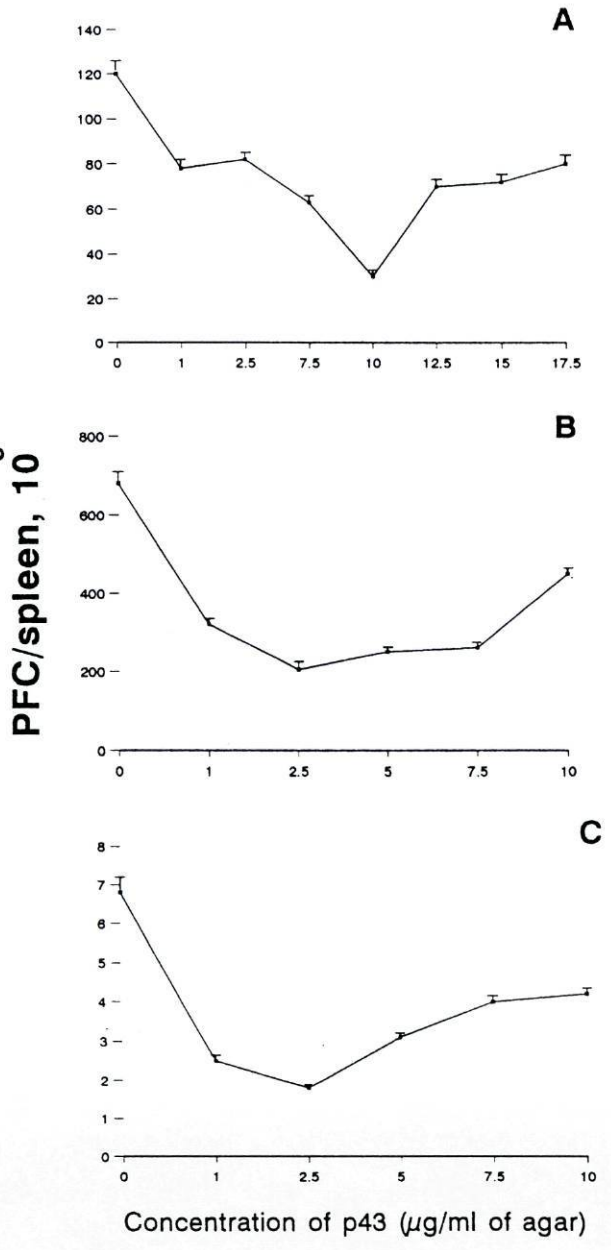

Fig. 3. Numbers of splenic anti-p43 IgG1-secreting PFC according to the indicated concentrations of p43 added to the agar of the PFC assays, observed after p43 treatment of naive (A) and p43-immunized (B), and in p43-untreated ISM-immunized (C) BALB/c mice. In p43 locally immunized mice observations were taken 30 days after immunization.

immunization with Cs containing 3620 ELISA titres of antibodies against Cs were markedly more fungal infected than controls. Furthermore, even C57BL/6 mice, the relatively resistant strain to the fungal infections treated with the IgF of serum containing 13,000 ELISA titres of specific anti-Cs IgG antibodies obtained from Cs-immunized C57BL/6 mice, were strikingly more susceptible to infection than C57BL/6 mice treated with IgF obtained from normal serum of the same strain.

Impaired C. albicans infection is also observed in mice depleted of CD4$^+$ cells

As shown in Fig. 8, BALB/c mice depleted of CD4$^+$ cells became dramatically more resistant to fungal infection. Indeed, no fungal colonization was observed in the kidneys of two out of seven mice depleted of CD4$^+$ T lymphocytes 5 days after infection with 10^6 *Candida* blastoconidia. Moreover, low levels of infection load were observed in the remaining five animals of the same group. In contrast, heavy fungal load was observed in the kidneys of four control BALB/c mice, similar to that seen in all other control experiments.

Local immunization with p43/ISM does not induce protective immunity against unrelated infection by L. monocytogenes

In contrast with the immunoprotection against *C. albicans* achieved by immunization with p43, BALB/c mice immunized with the ISM were not protected against *L. monocytogenes* infection 30 days after immunization against the ISM protein (Fig. 9).

Discussion

The present results, which indicate almost complete protection from *C. albicans* infection of BALB/c mice immunized with p43 up to 30 days after immunization, are in agreement with our previous proposal that immune neutralization of microbial ISM proteins is an adequate strategy to induce immunoprotection in the host against infection. This protection seems to be somewhat specific as p43-immunized mice were not protected from *L. monocytogenes* infection. However, it cannot be excluded that the protective effect of p43 on *C. albicans* growth cannot be shared by other microorganisms since the immunosuppressive protein may occasionally facilitate other microbial loading. Immunoprotection from candidiasis was also obtained previously, by immunization with a 47 kDa heat shock protein of *C. albicans* (39). However, this protein does not seem to be related to p43, which appears to be a new protein since its N-terminal amino acid sequence was not found among the proteins contained in updated versions of protein databanks.

Mice only immunized twice with p43 seem to have the capability of neutralizing crucial effects of this protein up to 30 days following immunization for several reasons. After p43 treatment the numbers of non-specific IgG2a-, IgG2b- and IgM-secreting PFC were lower in p43-immunized than in naive mice. The reduction in the numbers of bone marrow B and pre-B cells induced by p43 treatment was abrogated when the mice were immunized with this protein. Reduction in the numbers of bone marrow B and pre-B cells may reflect an accelerative B cell maturation with subsequent migration to the peripheral organs. Such a maturative process induced by p43 may be due to a direct effect of this ISM protein on the bone marrow B and pre-B cells or alternatively to the effects of Igs oversynthesized upon the B cell mitogenic effect of p43. Supporting this last hypothesis is the fact that similar effects are observed when mice are treated with i.v. Ig (40). Furthermore, as with other ISM proteins (6,31), p43 activates T and B cell compartments, and this activation was reduced in p43 immunized mice.

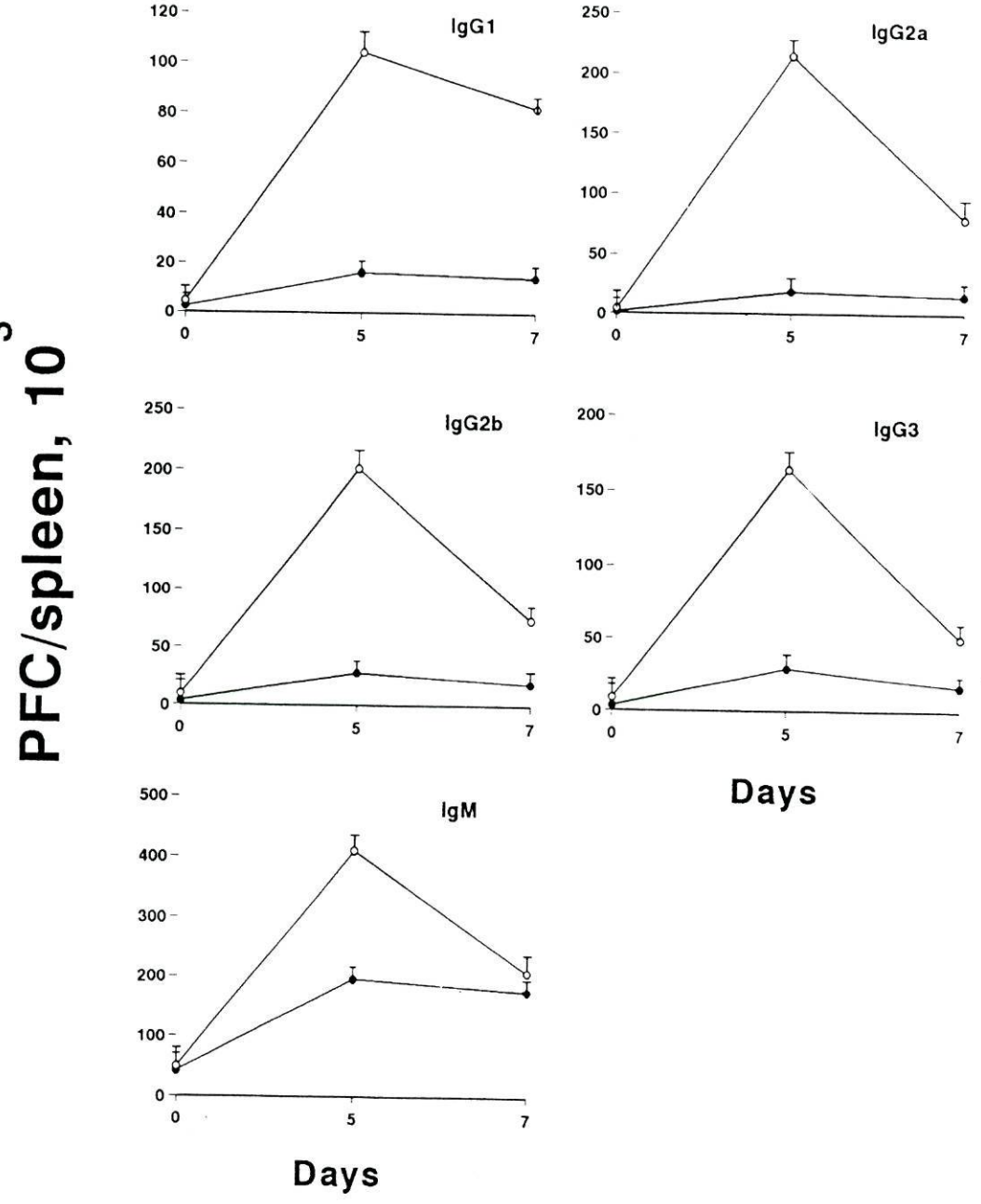

Fig. 4. Numbers of splenic non-specific Ig-secreting PFC of the indicated isotypes observed in normal (○) and anti-CD4$^+$-treated (●) BALB/c mice, before (day 0) and on the indicated days after p43 systemic treatment.

It can be argued that there is some discrepancy between the abrogation of p43 effects and the protection against *C. albicans* in mice immunized against this ISM protein. Indeed, immunization against p43 almost fully protected against infection whereas it only partially abrogates the biological effects of the ISM protein. However, 3×10^8 *Candida* blastoconidia are required for the production of 50 µg p43 (25) and mice were infected, for infection challenging purposes, with 10^6 blastoconidia with the same degree of virulence.

High serum titres of antibodies anti-p43 were seen in immunized mice. It is concluded, based on PFC assays performed adding p43 to the agar, that these antibodies are high-affinity IgG1. The numbers of specific high-affinity IgG1-secreting PFC increased dramatically in p43-immunized mice after treatment with this ISM protein.

The ISM proteins induce specific suppression against allo- or heteroantigens (4,7,25,41) through an immunopathway which meets the broad concept of overstimulation of the immune system as a causal effect of immunosuppression

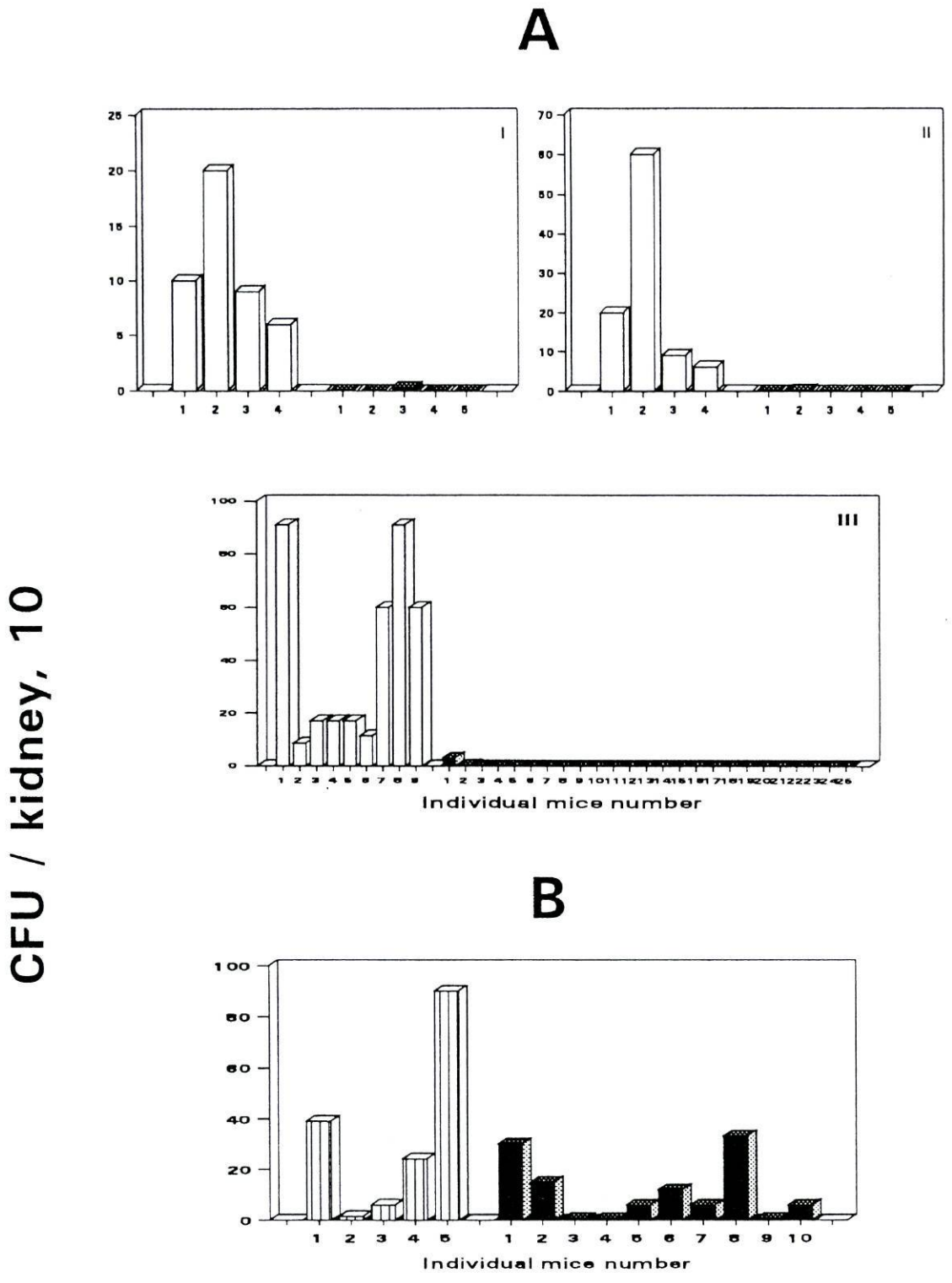

Fig. 5. Numbers of c.f.u. of *C. albicans* recovered from both kidneys of BALB/c mice infected with *C. albicans*, observed in control (open bars) and in immunized (closed bars) mice. The results were obtained 7 (I), 15 (II) and 30 (III) days after immunization with p43 (A) or 30 days after immunization with Cs (B). In three other additional experiments using quadruplicated mouse samples all control mice were highly colonized whereas no colonization was observed in p43-immunized mice.

(3–5,31,42). Thus, both immunosuppressive and mitogenic effects of ISM protein are abrogated in mice thymectomized as adults or depleted of $CD4^+$ T cells (6,43). Moreover, the observation of impaired growth of *C. albicans*, *Plasmodium chabaudi*, *Leishmania donovani* and *Trypanosoma cruzi* in naive mice depleted of B cells by anti-μ treatment or in

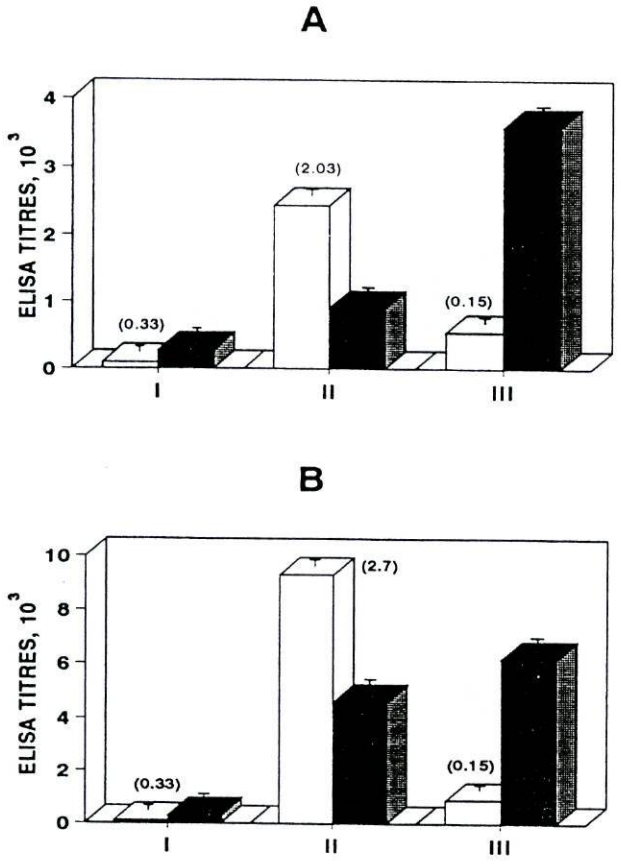

Fig. 6. Comparison between the serum levels, expressed as ELISA titres, of anti-p43 (open bars) and anti-Cs (closed bars) specific IgG, observed in control (I), p43-immunized (II) and Cs-immunized (III) BALB/c mice, before infection (A) and 5 days after infection (B) with *C. albicans*. The ratios between the ELISA titres of anti-p43 and anti-Cs specific IgG are indicated between parentheses.

mice genetically deficient in B cells (44–47) suggests that overproduction of Ig as a consequence of B cell mitogenicity may be crucial for the survival of these microorganisms in the host. Susceptibility of different strains of mice to *Mycobacterium avium* infection also seems to correlate with the B cell mitogenicity induced by this microorganism (48). Furthermore, susceptibility to the infection with *Leishmania major*, *T. cruzi* and *Schistosoma mansoni* has also been ascribed to a preferential stimulation of the T_h2 subpopulation of $CD4^+$ cells (49–52). Increased resistance to *S. mutans* infection was also observed in mice thymectomized as adults, a procedure that depletes T_h2 cells (53), and in mice depleted of $CD4^+$ lymphocytes (43). In the present study we also demonstrate that the mitogenic effect of p43 is markedly abrogated in mice depleted of $CD4^+$ cells and that these mice become more resistant to infection by *C. albicans*. Furthermore, reinforcing the role of B cell polyclonal activation in this endeavour, the increased resistance to *C. albicans* infection in these mice is accompanied by a decrease of the B cell mitogenic effect of p43. These results are in apparent opposition to the report of Cenci *et al.* (54) stating that fungal colonization was lower in the group of control mice treated with media (hybridoma culture supernatants) than in the group

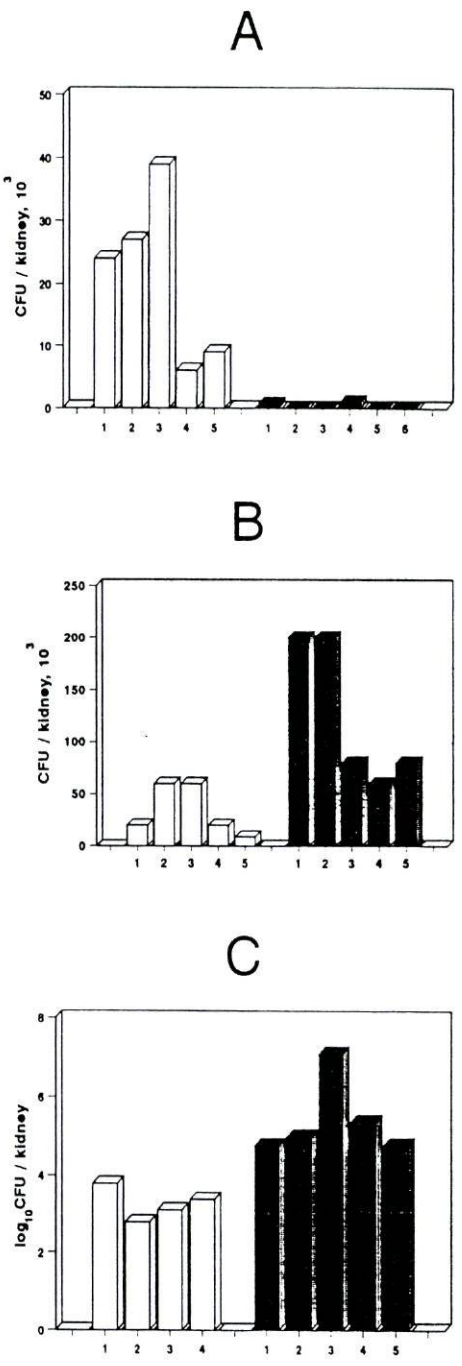

Fig. 7. Infection of mice with *C. albicans* after adoptive transfer with different IgF. Open bars represent mice treated with IgF of normal serum and closed bars represent mice treated with IgF of serum containing anti-p43 or anti-Cs IgG antibodies. (A) Comparison between the number of c.f.u. of *C. albicans* found in the kidneys of BALB/c mice passively transferred with IgF of normal serum or with IgF of serum containing an ELISA titre of 2450 anti-p43 IgG antibodies. (B) Comparison between the number of c.f.u. of *C. albicans* found in the kidneys of BALB/c mice passively transferred with IgF of normal serum or with IgF of serum containing an ELISA titre of 3620 anti-Cs IgG antibodies. (C) Comparison between the numbers of c.f.u. of *C. albicans* found in the kidneys of C57BL/6 mice passively transferred with IgF of normal serum or with IgF of serum containing an ELISA titre of 13,000 anti-Cs IgG antibodies.

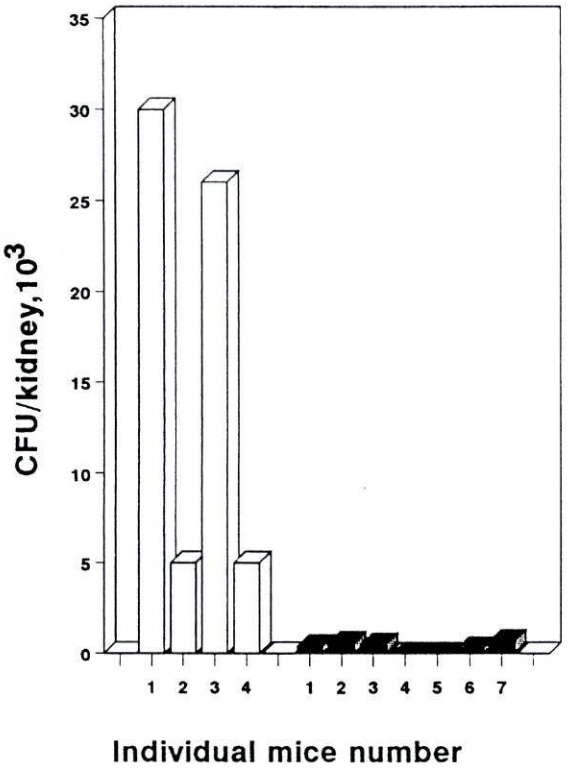

Fig. 8. Number of c.f.u. of *C. albicans* observed in seven CD4$^+$ cells depleted (closed bars) and in four control (open bars) BALB/c mice.

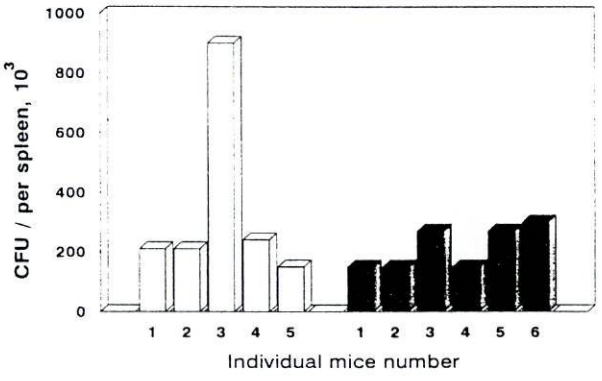

Fig. 9. Number of c.f.u. of *L. monocytogenes* recovered from the spleens of infected BALB/c observed in naive non-immunized (open bars) and in p43-immunized (closed bars) mice 30 days after immunization.

treated with anti-CD4$^+$ mAb. However, this media apparently protects the mice against candidiasis because the anti-CD4$^+$ mAb treated mice were actually more resistant than naive medium untreated mice (54). Moreover, we have used roughly eight times more CD4$^+$ cell depleting mAb than Cenci *et al.* (54) (200 and 25 μg respectively). Another report indicates that mucosal but not systemic candidiasis was increased upon CD4$^+$ cell depletion of *bg/bg nu/+* mice (55). However, these mice are congenitally immunodeficient and results cannot be compared with normal mice.

In contrast with p43 immunization and in agreement with the assumption that immunopotentiation of the host with microbial antigens may not be an appropriate strategy of vaccination is the observation that mice immunized against Cs were not protected against *C. albicans* infection and that anti-Cs antibodies facilitate fungal load. Thus, protection against fungi directly correlates with the ratio between anti-p43 and anti-Cs antibodies. Furthermore, passive administration of high titres of anti-Cs antibodies markedly increased the susceptibility to *C. albicans* infection, whereas passive administration of anti-p43 significantly decreased the fungal load. Similarly, it was observed that infection with *M. avium* and with various viruses is increased in relatively resistant strains of mice, if these animals were treated with specific antimicrobial antibodies (46,56–59). Moreover, mice developing high antibody titres of anti-*Toxoplasma gondii* antigens upon immunization with the microorganism epitopes (60) and mice immunized with a soluble antigen fraction from *L. major* (49) are more susceptible to infection by these parasites than non-vaccinated animals. These antibodies can be designated as facilitating antibodies. However, this designation is merely functional because this kind of antibody is not characterized biochemically. Also, high levels of antibody anti-microorganism epitopes facilitating microbial growth may reflect, in some cases, easier opsonization of the microorganism in the target phagocytic cells. However, suppression achieved by the administraton of antigen together with specific antibodies is a very well known event and may also reflect overstimulation of the immune system.

The present results clearly demonstrate that neutralization of the mitogenic effect of p43 has a protective value in a murine model of systemic candidiasis and offer the basis for attempting new general strategies of immunoprotection.

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Abbreviations

Cs	*Candida* sonicates
IgF	Ig enriched fraction
ISM	immunosuppressive mitogenic
PFC	plaque forming cell

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TRABALHO III

Scand. J. Immunol. **43**, 155–163, 1996

Role of Monocytes in the Up-Regulation of the Early Activation Marker CD69 on B and T Murine Lymphocytes Induced by Microbial Mitogens

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Vilanova M, Tavares D, Ferreira P, Oliveira L, Nóbrega A, Appelberg R, Arala-Chaves M. Role of Monocytes in the Up-Regulation of the Early Activation Marker CD69 on B and T Murine Lymphocytes Induced by Microbial Mitogens. Scand J Immunol 1996;43:155–163

CD69 is an early marker of lymphoid cell activation. The authors report on an up-regulation of CD69 in splenic B and T cells of C57Bl/6 mice after administration of lipopolysaccharide (LPS) or microbial immunosuppressive/mitogenic (ISM) proteins produced by *C. albicans* (p43) and African Swine Fever Virus (p36). This up-regulation of CD69 was observed 6 and 24 h after mitogenic treatments. The same pattern of increased CD69 expression was observed in the lymph nodes of mice treated with p43 or LPS, whereas p36 treatment failed to induce increased CD69 expression in this organ. Intracellular calcium mobilization was induced in splenic B and T lymphocytes after incubation of total spleen cells with LPS, p43 or p36. This increase was higher in B than in T cells. Increased calcium mobilization was also seen in lymph node B cells after incubation with p43 or p36 and in lymph node T cells after p43 stimulation. Up-regulation of CD69 expression on B and T cells was also observed after *in vitro* stimulation of spleen cells with the three mitogens used. Similar results were obtained with culture supernatants of macrophage/monocyte (Mϕ) cells activated with LPS (LPS MϕCS). Stimulation of Mϕ cells with LPS or with the ISM proteins is demonstrated by the increased production of nitrites by these cells. The increased *in vitro* expression of CD69 was, however, not abolished by monoclonal antibodies to Mϕ cytokines such as IL-6, IL-1,0 or TNFα. No increased expression of CD69 was found *in vitro* on purified B or T cells, even when mixed upon stimulation with p43, p36, LPS or with LPS/MϕCS. However, an increase in the expression of CD69 was observed on B cells co-cultured with Mϕ cells after treatment with LPS or p36. All three mitogens failed to induce increased CD69 expression on cultured T cells mixed with Mϕ cells.

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INTRODUCTION

We have already described two immunosuppressive/lymphocyte mitogenic (ISM) proteins of microbial origin: p36, isolated from culture supernatants of porcine monocytes infected with African swine fever virus (ASFV) [1] and p43 produced by *Candida albicans* [2]. *In vivo* proliferation of splenic B and T cells was observed in mice treated with p36 or p43 [1–4]. This mitogenic effect correlates to the suppression of specific immune responses to allo or heteroantigens [1, 2, 4] and seems to play an important role in the survival of the micro-organisms in the host [2, 3]. We have previously hypothesized that these kind of proteins were B-cell mitogens and that T-lymphocyte proliferation was secondary to B-cell activation [4]. However, this hypothesis was based only on two observations. One was concerned with earlier B- than T-cell proliferation observed *in vivo* after p36 treatment [4]; the other with *in vitro* proliferation of T-cell-depleted murine spleen cells, but not of purified T cells, upon stimulation with p90, an ISM produced by *S. intermedius* [5].

CD69 is a protein that is expressed early on the surface of activated murine B and T lymphocytes after mitogenic stimulation [6, 7]. Surface expression of CD69 on T cells can be detected as early as 2–3 h after stimulation, declining with a

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half-life of 24 h [8]. It has also been shown that intracellular signals can be induced by CD69 triggering. Lymphocyte proliferation can be obtained with crosslinking of CD69 by monoclonal antibodies in the presence of phorbol miristate acetate (PMA) [6, 9]. Moreover, crosslinking of CD69 induces the expression of genes for IL-2, IFN-γ and TNF-α [10]. Thus, the early expression of CD69 upon stimulation makes this molecule a good marker for the assessment of initial stages of lymphocyte activation.

Therefore, aiming at further elucidating the mechanism of lymphocyte activation by the ISM proteins, we decided to study CD69 expression on murine B and T cells stimulated by p36 and p43. Similar studies were performed with Ca^{2+} intake which is also an event associated with early stages of lymphocyte activation. The results were compared with those obtained with lipopolysaccharide (LPS) treatments.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice, 6–8-weeks-old were bred at the Gulbenkian Institute of Science (Oeiras, Portugal). C57BL/6 × 129Sv IL-6^{-}/$^{-}$ (5th generation) and C57BL/6 × 129 Sv IL-6^{+}/$^{+}$ mice [11], a kind gift of Dr Manfred Kopf, were bred at the Center for Experimental Cytology of Porto University (Porto, Portugal).

Lymphocyte cell suspensions. Splenic and lymph node cell suspensions were obtained after gentle teasing of the organs in balanced salt solution supplemented with 3% fetal calf serum (BSS/FCS). Spleen cells not adhering to nylon wool after two serial passages through columns of this material were used as a source of enriched T-cell populations [12]. Splenic B cells were sorted in a FACSort (Becton Dickinson, San José, CA, USA) using the Lysys II Software. The purified T and B lymphocyte populations consisted of 98% of CD3^{+} and 98% of IgM^{+} cells, respectively, as determined by flow cytometry analysis.

Macrophage cell cultures. Cell cultures of bone marrow-derived monocyte/macrophage cells (Mϕ) were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% of L929 cell-conditioned medium (LCCM, also in DMEM), in 24-well tissue culture plates (0.6×10^6 cells in 1 ml/well). The cells were incubated for 10 days at 37°C in a 7% CO_2 93% humidified air atmosphere. On day 4, an additional volume of 0.1 ml of LCCM was added and on day 7 the medium was removed and replaced again by fresh DMEM with 10% of LCCM.

In vivo mitogen treatments. LPS from *Brucella abortus equi* (Sigma, Chemical Co, St Louis, MO, USA) and the ISM proteins p36, and p43 purified as previously described in detail [1–4] were used as lymphocyte mitogens. Mice were injected i.p. with 50 μg of p43 or p36 and with 10 μg of LPS in 0.5 ml of PBS. Mice untreated or injected i.p. with 0.5 ml of PBS were used as controls.

In vitro mitogenic treatments. Total spleen cells, T- or B-enriched cell populations (0.2×10^6 cells/well) or a mixture of these two populations (0.1×10^6 cells / well each) were resuspended in 0.2 ml of RPMI/FCS and plated in 96-well tissue culture plates. In some of the cultures, T- or B-enriched cell populations were incubated in the presence of 20% of bone marrow Mϕ cells. All cultures were incubated for 6 h at 37°C in a 7% CO_2 93% humidified air atmosphere and stimulated with 10 μg of p43, 10 μg of p36, or 2 μg of LPS/well. Alternatively, some of the cultures were performed in supernatants of Mϕ cells (MϕCS). MϕCS were obtained as follows. On day 10 of culture of the Mϕ cell monolayers the medium was removed and 1 ml of DMEM containing 50 μg of p43 or p36, or 10 μg of LPS was added. The new cultures were incubated for 15 min as described above and washed twice to remove the mitogens. Finally, 1 ml of fresh DMEM was added and the cultures were incubated for other 15 min. Supernatants of the last cultures were designated LPS/MϕCS, p43/MϕCS and p36/MϕCS according to the stimulants initially used.

Flow cytometry. For cytometric analysis all cells were resuspended in BSS/FCS supplemented with 10 mM of sodium azide. The following MoAb were used for immunofluorescence cytometric analysis in a FACScan (Becton Dickinson, San Jose, CA, USA) with the Lysys II software (Becton Dickinson): goat anti-mouse IgM FITC conjugate (Southern Biotechnology Associates, Birmingham, ALA, USA); rat anti-mouse CD4 (GK 1.5; 25) FITC conjugate [13]; rat anti-mouse CD8 FITC conjugate (Coulter Immunology, Hialea, FL, USA); rat anti-mouse B220 PE conjugate (Coulter Immunology); hamster anti-mouse CD3 PE conjugate (PharMingen, San Diego, CA, USA); hamster anti-mouse early activation marker (CD69) Biotin conjugate (PharMingen). Biotin conjugated antibodies were revealed by PE conjugated avidin (Southern Biotechnology Associates). Dead cells were excluded by propidium iodide incorporation. Changes in intracellular calcium were measured using dual-colour analysis [14]. Fluo-3 loaded cells were washed twice and stained with PE-conjugated hamster anti-mouse CD3 (PharMingen) and goat anti-mouse Ly-5 (B220) (Coulter immunology) MoAb. Before each assay, cells were resuspended in RPMI at a concentration of 0.5×10^6/ml and incubated for 10 min in a 37°C water bath. Temperature was strictly maintained at 37°C throughout the experiments.

Nitrite production measurements after LPS or ISM treatments. Nitrite production by bone marrow-derived Mϕ cells, cultured as described above, was quantified with a colorimetric assay using the Griess reagent as reported elsewhere [15]. Quantification of nitrite production was performed after 48 h incubation in RPMI, or after the addition of 50 μg of p43 or p36, or 10 μg of LPS per ml. The protein content of the Mϕcell monolayer was determined by the method of Lowry *et al.* [16].

Cytokine-neutralizing treatments. Cytokine neutralizing MoAb were obtained from hybridomas MP6-XT22 (anti-TNFα IgG1), MP5-20F3 (anti-IL-6 IgG1) and JES5-2A5 (anti- interleukin 10 IgG1) (kind gifts from Drs Paulo Vieira and Anne O'Garra, DNAX, Palo Alto, CA, USA) and were grown in ascites in HSD nude mice primed with incomplete Freund's adjuvant and were purified by affinity chromatography. An IgG1 anti - β-galactosidase antibody obtained from GL113 hybridoma was used as isotype control. Cytokine neutralizing MoAb were added to total spleen cell cultures to a final concentration of 0.1 mg/ml simultaneously with the mitogens or LPS/MϕCS addition.

Statistical analysis. Data were statistically analysed with the Student's *t*-test.

RESULTS

In vivo CD69 expression induced by treatment with p43, p36 or LPS

As shown in Table 1, the percentage of CD69^{+} B and T

Table 1. Proportion of T ($CD4^+$ or $CD8^+$ cells) and B (IgM^+) lymphocytes from spleen or lymph nodes expressing CD69 at baseline and 6, 24 or 48h after injecting C57BL/6 mice i.p. with p43 (50μg), p36 (50μg) or LPS (10μg)

	Treatments									
		p43			p36			LPS		
Cell populations	None	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Spleen										
T cells	8.23 ± 1.20	78.3 ± 7.60[a]	45.5 ± 27.1	7.98 ± 0.54	48.0 ± 33.1	22.8 ± 10.1	6.77 ± 1.23	82.2 ± 20.2	36.4 ± 6.20	13.9 ± 5.02
B cells	3.20 ± 1.42	83.9 ± 7.70	47.8 ± 35.0	4.21 ± 0.98	60.0 ± 35.0	29.3 ± 13.0	2.88 ± 0.78	94.4 ± 1.0	58.9 ± 6.51	5.33 ± 1.09
Lymph nodes										
T cells	12.5 ± 2.52	22.0 ± 1.6	22.5 ± 12.6	10.5 ± 2.00	12.8 ± 0.62	15.4 ± 4.30	11.5 ± 2.58	48.9 ± 6.40	11.8 ± 4.10	14.3 ± 5.05
B cells	12.2 ± 5.18	60.4 ± 23.8	25.9 ± 17.4	14.7 ± 3.28	9.94 ± 1.44	12.3 ± 2.4	11.89 ± 3.02	61.3 ± 14.9	15.3 ± 9.45	16.3 ± 3.09

[a]In this and in the following tables significant differences from control values are underlined ($P < 0.05$) or double underlined ($P < 0.01$ or $P < 0.001$).

splenocytes was markedly increased in C57BL 6 mice after i.p. treatment either with p43, p36, or LPS, as compared with unmanipulated controls. Normal unmanipulated mice had no significantly different expression of CD69 from mice treated i.p. 6, 24, or 48 h before with PBS (data not shown) and were used as controls. No significant differences were observed between $CD4^+$ and $CD8^+$ T lymphocytes on the expression of CD69 (data not shown). Peak responses of CD69 expression in both T- and B-lymphocyte populations were observed at 6 h after *in vivo* treatment with the three mitogens used. An increased CD69 expression was still observed 24 h after mitogen treatment, but no significant differences from controls were observed at 48 h after stimulation (Table 1).

In agreement with what has been described previously [6] we found a higher expression of CD69 on the lymphocyte populations of lymph nodes than on the spleen of unmanipulated mice (Table 1). As also shown in Table 1, a significant up-regulation of CD69 was observed in mesenteric lymph node B and T lymphocytes 6 h after treatment i.p. with p43 or LPS. This increase in CD69 expression was no longer observed 24 h or 48 h after treatment with these two mitogens. The expression of CD69 on this organ was percentually higher on B than on T cells. Similar to the spleen, no preferential CD69 expression was observed between lymph nodes $CD4^+$ and $CD8^+$ T lymphocytes (data not shown). No increased CD69 expression on the surface of the different lymph node lymphocyte populations was observed after p36 treatment (Table 1).

In vitro CD69 expression induced by treatment with p43, p36 or LPS

Increased CD69 expression on the surface of B, $CD8^+$ or $CD4^+$ lymphocytes, was observed *in vitro* 6 h after stimulation of total spleen cells with the two ISM proteins or LPS (Fig. 1). An up-regulation of CD69 expression on the surface of B and T lymphocytes was also observed in total spleen cell cultures performed in LPS/MϕCS (Fig. 2). The increase in CD69 expression was more marked on the B- than on the T-cell populations. MϕCS from the two ISM had no effect on CD69 expression (Fig. 2). Increased up-regulation of CD69 was also observed 6 h after stimulation with p36 or LPS in B but not in T cells co-cultured with Mϕ cells. CD69 up-regulation in B cells was more evident after LPS than after p36 stimulation (Table 2). This increase was, however, not observed if T cells were used instead of B cells. No up-regulation of CD69 was observed either in purified B or T cells stimulated by the ISM proteins, LPS, or LPS MϕCS (Table 2).

Calcium mobilization in spleen and lymph nodes lymphocytes after treatment with p43, p36 and LPS

Treatment of total spleen cells with p43, p36 or LPS induced calcium mobilization in either $B220^+$ or $CD3^+$ cells. Nearly

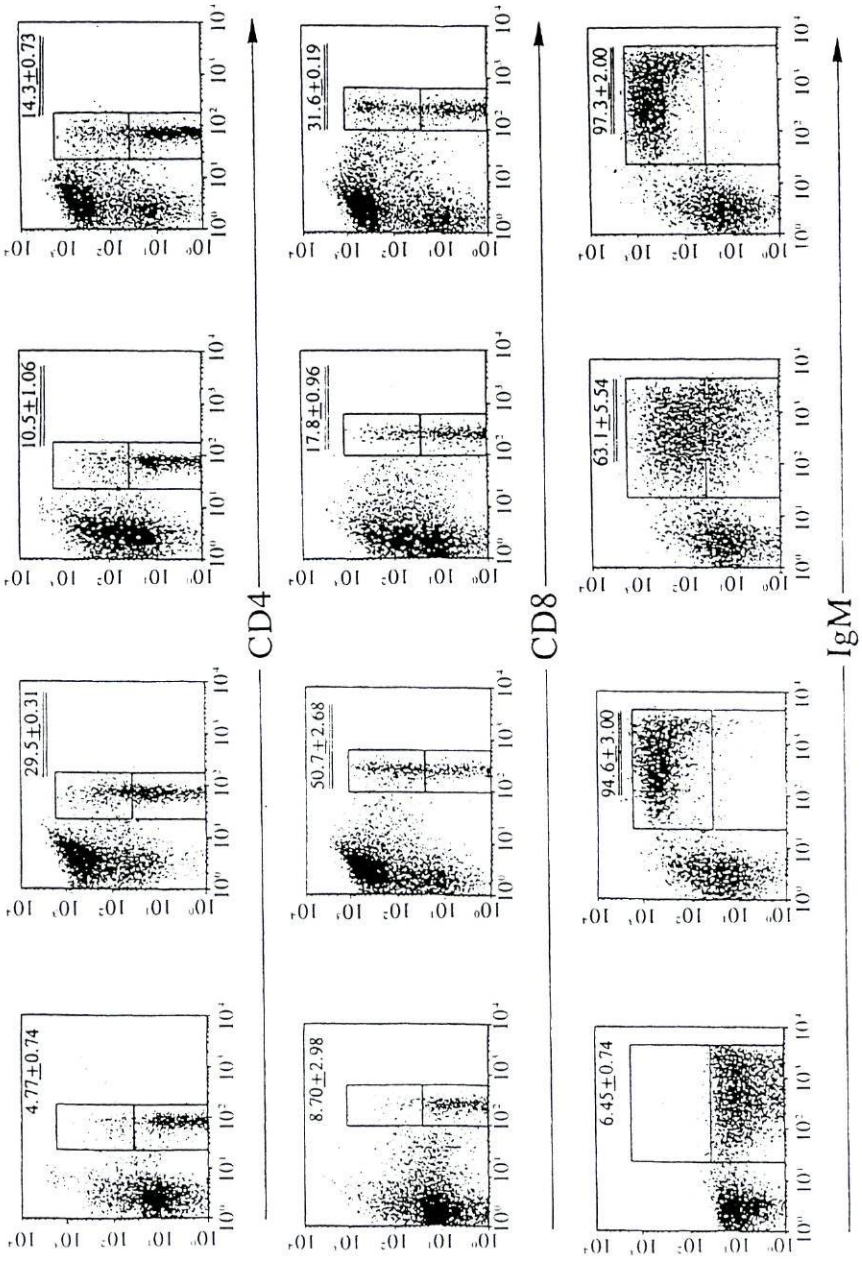

Fig. 1. Typical example of the expression of CD69 on the surface of splenic IgM$^+$, CD4$^+$ or CD8$^+$ lymphocytes after incubation of total spleen cells with RPMI after treatments with 10 μg of either p43 or p36, with 2 μg of LPS and after incubation with supernatants of previously activated Mϕ cells by LPS treatment (LPS/MϕCS). Results in this and in Fig. 2 are means ± one S.D. of triplicated samples, repeated three times. Significance of the results is shown in underlining ($P < 0.05$) or double underlining ($P < 001$ or $P < 0.0011$).

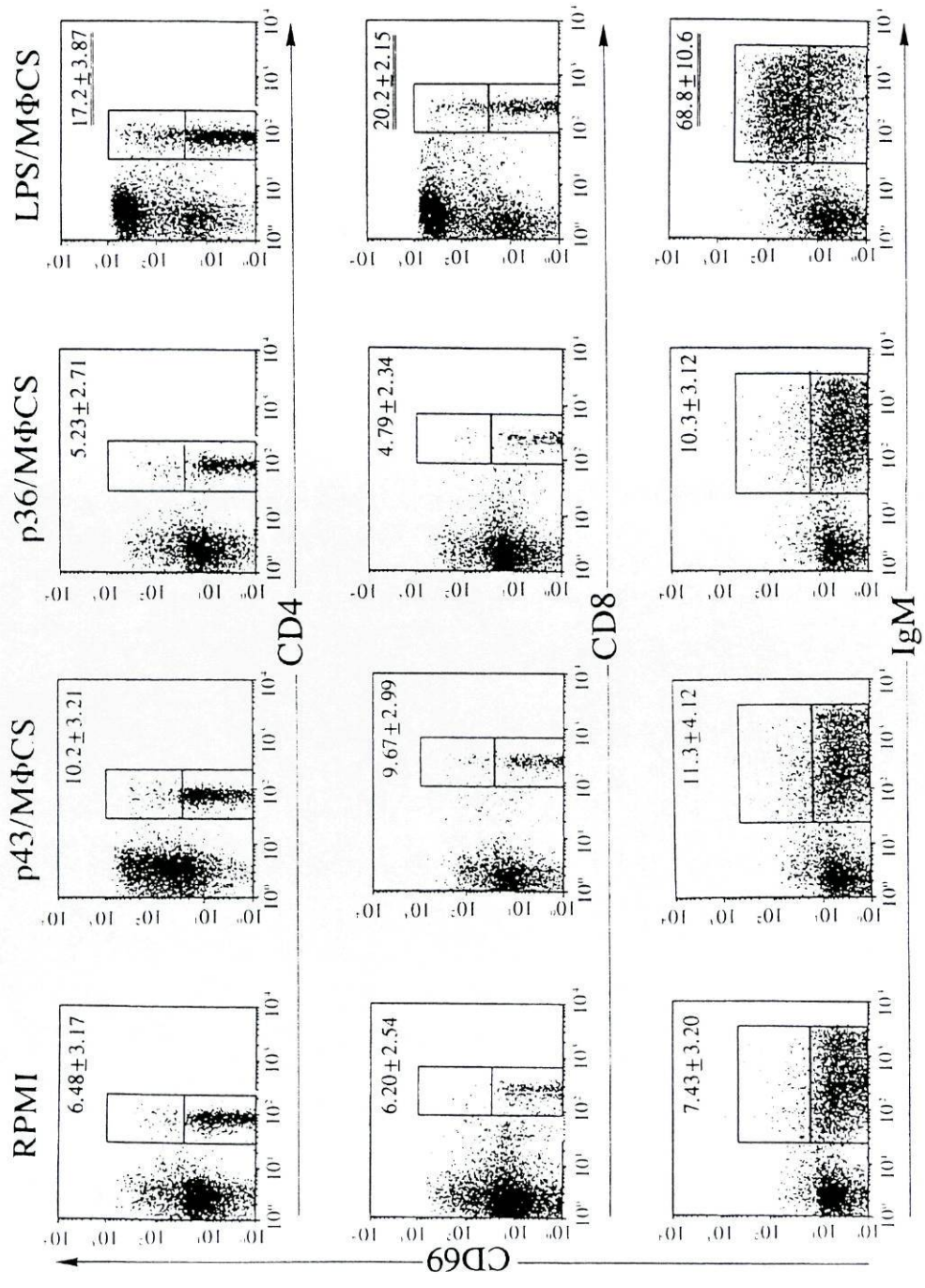

Fig. 2. Typical example of CD69 expression on the surface of splenic IgM$^+$, CD4$^+$ or CD8$^+$ lymphocytes after incubation of total spleen cells with RPMI and after incubation with supernatants of Mϕ cells previously treated with LPS (LPS/MϕCS), p43 (p43/MϕCS) or p36 (p36/MϕCS).

Table 2. Proportion of $CD4^+$, $CD8^+$, IgM^- or $CD3^+$ splenic lymphocytes expressing CD69 6 h after culture of different cell populations without stimulation or stimulated with 50 μg/ml of p43 or p36 or 10 μg/ml of LPS or with a supernatant of Mϕ cells incubated with the same amount of LPS (LPS/MϕCS)

		Stimulants				
Cells cultured	$CD69^+$ cells within	None (control)	p43	p36	LPS	LPS MϕCS
B + Mϕ	IgM^+	1.70 ± 0.33	ND	10.2 ± 1.54	20.5 ± 3.50	N.D.
T + Mϕ	$CD4^+$	0.90 ± 0.11	1.02 ± 0.12	0.51 ± 0.28	0.92 ± 0.21	N.D.
	$CD8^+$	0.20 ± 0.06	0.20 ± 0.06	0.35 ± 0.10	0.28 ± 0.08	N.D.
B + T	$CD3^+$	7.72 ± 1.20	6.00 ± 1.45	5.72 ± 1.09	9.45 ± 1.40	8.68 ± 0.94
	IgM^+	2.20 ± 0.52	2.42 ± 0.36	2.47 ± 0.32	3.05 ± 0.76	2.21 ± 0.20
B	IgM^+	9.89 ± 0.84	8.12 ± 1.06	8.84 ± 0.72	10.7 ± 0.53	10.0 ± 0.82
T	$CD4^+$	2.49 ± 1.86	1.75 ± 0.44	1.49 ± 0.97	1.65 ± 1.02	1.10 ± 0.32
	$CD8^+$	1.95 ± 1.23	3.21 ± 1.79	3.04 ± 1.54	3.29 ± 2.64	2.69 ± 0.38

Results are means ± one S.D. of triplicated samples repeated three times.

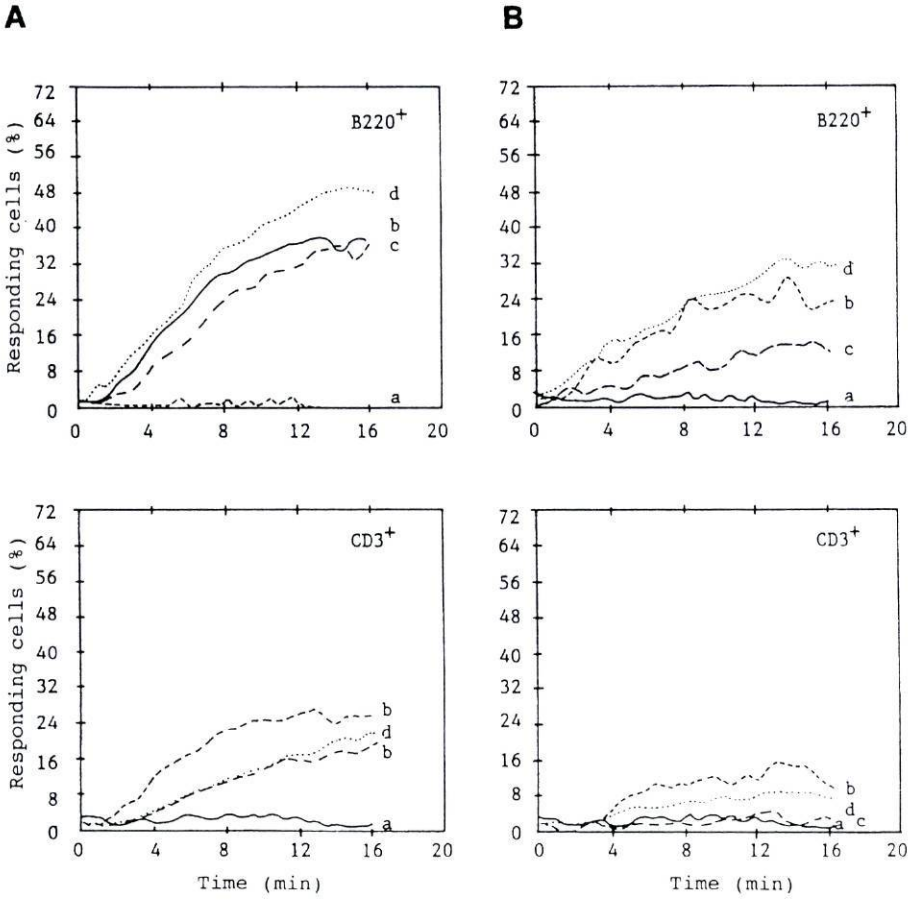

Fig. 3. Percentage of $CD3^+$ and $B220^+$ (A) spleen cells and (B) lymph node cells from C57Bl.6 mice responding with a calcium influx in (a) unstimulated lymphocytes or (b) after stimulation with p43 (c) p36 or (d) LPS. The data are representative of three independent experiments.

Table 3. Nitrite production by bone marrow-derived Mφ cells after 48 h incubation in RPMI (control), or 50 μg ml of p43 or p36, or 10 μg ml of LPS. The results are given in mM of $NO_2 \times 10^4$/mg of protein

Control	p43	p36	LPS
0.52 ± 0.09[a]	6.25 + 0.64	7.28 + 1.22	14.81 + 3.96

[a]Results are means ± one S.D. of three replicate wells from a typical experiment repeated twice.

40% and 50% of $B220^+$ cells responded with an increase in intracellular free Ca^{2+} 14 min after treatment with each one of the ISM proteins and LPS, respectively (Fig. 3). As also shown in Fig. 3, the percentage of $CD3^+$ responding cells was lower than the B cells after stimulation with the three mitogens. The highest response on T cells was observed with p43 stimulation (approximately 25% of responding cells).

Increased free Ca^{2+} was seen in B but not in T lymph node cells after stimulation with p36 (12% of B responding cells). In contrast, p43 stimulation induced an increased calcium flux in both B and T lymphocytes in a cell percentage of 29% and 15%, respectively (Fig. 3).

Mφ cell stimulation by ISM proteins and LPS

A stimulatory effect on bone marrow-derived Mφ cells was observed after 48 h incubation with p43, p36 or LPS as detected by increased nitrite production (Table 3).

Effect of TNFα, IL-6 or IL-10 neutralization on in vitro CD69 expression

The Mφ cell activation was not correlated with the release of TNFα, IL-6 or IL-10. Indeed, increased expression of CD69 in total spleen cells stimulated by the ISM, LPS or LPS/MφCS was observed after neutralization of these Ils by monoclonal antibodies (data not shown). Similarly, no inhibitory effect on CD69 up-regulation was obtained in cultures of total spleen cells from IL-6 'knock-out' mice, stimulated with each one of the three mitogens or LPS/MφCS (data also not shown).

DISCUSSION

In the present paper we have studied the effect of several microbial mitogens on the expression of the early activation marker CD69 by lymphocytes. We have observed that CD69 is up-regulated in splenic and lymph node lymphocytes in mice treated with both LPS and p43. Similar results were obtained in spleen but not in lymph nodes following administration of p36. Up-regulation of CD69 is observed in both B and T lymphocytes. Such up-regulation precedes the proliferative/differentiative responses that were described previously for p36 and p43 [1–4], which is well established for LPS.

We have also shown that CD69 up-regulation induced by the three mitogens in B and T lymphocytes can be reproduced in cultures of total spleen cells. The correlation between the *in vivo* and the *in vitro* observations provided us with a simple experimental setting where the mitogen effect could be easily studied. B and T lymphocytes respond readily to any of the mitogens as revealed by the imediate increase in calcium influx and CD69 up-regulation. The percentage of cells that respond with Ca^{2+} influx is much smaller than that of cells responding with CD69 expression. However, the proportionality of the two responses is kept constant in the organs as well as in the cell populations studied.

These results suggest that the mitogens would act directly on the two lymphocyte types rather than being B-cell specific as it was ascribed to the two ISM proteins [1–4] and particularly to LPS [17]. However, this hypothesis seems to be unlikely since purified B or T cells, even when co-cultured, do not respond to the mitogens by CD69 up-regulation. This result clearly indicates that this up-regulation of CD69 on lymphocytes depends on the presence of other cell populations, namely Mφ cells. Indeed, both ISM proteins and LPS-stimulated nitrite production in Mφ cells suggest the presence of receptors for these molecules in these cells. Furthermore, supernatants of LPS-stimulated Mφ cells were able to induce increased CD69 expression by lymphocytes on cultures of total spleen cells. The response to these Mφ supernatants is abrogated when purified B or T lymphocytes are cultured separately or together. This observation suggests that CD69 expression on these lymphocytes is likely to involve a cell interaction with Mφ cells. Such interaction may be triggered and/or enhanced by soluble factors, namely cytokines, present in the supernatant. The stimulatory effect either of LPS/MφCS or of the three mitogens used on total spleen cells was not abolished, however, by the addition of anti-IL 6, anti-IL-10 or TNF-α neutralizing monoclonal antibodies. Furthermore, CD69 expression was not impaired in Il-6 knock-out mice treated with LPS or with the ISM proteins. These three cytokines produced by Mφ cells seem not to be responsible, therefore, for the Mφ–lymphoid stimulatory effect. Unfortunately, neutralizing monoclonal antibodies anti-Il-1 were not available since it is very well known that this monokyne has a stimulatory effect on T and B lymphocytes. Moreover, Il-1 receptor engagement raises the intracellular content of ceramide, and ceramide production can lead to cellular oncongene Ras activation [18]. CD69 expression on T cells is apparently dependent of the activation of Ras since expression of a Ras gene coding for an active $p21^{ras}$ protein leads to CD69 expression on Jurkat T cells. Moreover, dominant negative Ras mutant prevents expression of CD69 following TCR/CD3 stimulation [19]. We have previously observed that the lymphocyte proliferative effect ascribed to the ISM proteins was due to intrinsic immunogenicity, particularly in the case of p43 [3]. However, Ras activation is usually

associated with cell growth and proliferation [20, 21] through a pathway independent of lymphocyte receptors for antigen engagement. It is possible, therefore, that CD69 expression induced by the three mitogens may also reflect some independence of lymphocyte receptors for antigen engagement on the lymphocyte mitogenicity induced by the ISM proteins.

Up-regulation of CD69 induced by Mϕ cells appears to be more biased towards B than to T cells. Indeed, increased CD69 expression was observed on B lymphocytes when cultured in the presence of bone marrow Mϕ cells upon p36 or LPS stimulation, but not on T cells cultured under the same conditions. Moreover, the percentage of B cells responding with intracellular calcium influx was greater than the one observed on T cells. Therefore, it seems that T-cell activation induced by p36 or LPS is dependent on the presence of B cells or of B-cell stimulation. This may account for earlier B- than T-cell proliferation and activation that was observed in kinetic studies performed after *in vivo* treatment with these mitogens [3, 4]. Late reciprocal effects of stimulated T or B cells can also occur as postulated before for the ISM proteins [4, 22] and observed for LPS [23]. Indeed, mitogen-induced up-regulation of CD69 observed in cultures of purified B cells co-cultured with Mϕ cells was markedly lower than the one observed in cultures of total spleen cells. These results taken together indicate that the increase of CD69 expression on T and B lymphocytes, induced by p36, p43 or LPS is due to Mϕ cell activation. This activation involves cell interactions between Mϕ cells and lymphocytes, as well as Mϕ produced soluble factors. It was reported that the immunosuppressive effect of the ISM p90 could be observed even in 95% Mϕ depleted human peripheral mononuclear cells [24]. Since the immunosuppressive and immunostimulatory effects of the ISM correlate [1, 2, 24] this observation seems contradictory with the important role of Mϕ cells in the ISM effects reported here. However, it is possible that just a few numbers of Mϕ cells are necessary to trigger ISM lymphocyte activation.

Different patterns of CD69 expression were observed in mice treated with all mitogens. Some of these differences can be explained by separate degrees of mitogenicity. This may be the case of lower CD69 up-regulation and Ca^{2+} influx induced by p36 comparatively to p43 or LPS. However, the observation of greater *in vitro* up-regulation of CD69 and Ca^{2+} influx in T cells induced by p43 than by LPS may indicate different target cells and/or mitogen activation pathways.

The model of lymphocyte activation described in the present report can be useful for further studies on T or B cell intracellular events that mediate early stages of lymphocyte activation.

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TRABALHO IV

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THE PRODUCTION OR RELEASE OF IL10 INDUCED BY P43 A *C. ALBICANS* PROTEIN IS CRUCIAL FOR THE DEVELOPMENT OF SYSTEMIC CANDIDIASIS

by

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[1] The two authors shared equal tasks and, therefore, should be both granted as first authors.

Running title: p43 effects and *Candida albicans* infection depend on IL10.

Number of page:29

Number of figures:5

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Key words: *Candida albicans*, p43, IL10, IL4.

Abbreviations: **Cp:** *C. albicans* particles **ISM:** Immunosuppressive and mitogenic

Abstract

An immunosuppressive/mitogenic protein (p43) produced by *C. albicans* facilitates infection in a resistant mouse strain (C57BL/6). We show here that these effects are mediated by IL 10. Thus: 1) depletion of IL10 in p43 treated animals impairs the increased growth of *C. albicans,* and its mitogenic and immunosuppressive effects; 2) The biological effects of p43 are abrogated and *C. albicans* infection is impaired in thymectomized and in nude C57BL/6 mice; 3) IL10 is readily detected in the serum of normal C57BL/6 mice after either p43 treatment or *C. albicans* infection but much less so in the serum of thymectomized and nude C57BL/6 mice.

Taken together, these observations show that the biological effects of p43 and *C. albicans* are tightly correlated and both p43 and *C. albicans* infection induce an almost immediate release of IL10 in C57BL/6 mice, leading to facilitation of fungal load.

Introduction

The role of the humoral immune response in systemic candidiasis is highly controversial. Specific antibodies against *C. albicans* antigens were thought to be either protective (1), non protective (2-5) or even facilitating (6) the systemic candidiasis in experimental models. In contrast, it is generally accepted that systemic fungal growth is ascribed to impaired neutrophil function (7). For instance, mice with deficient neutrophil function (bg/bg) are very susceptible to this form of candidal infection. T lymphocytes were not considered to be important in systemic candidiasis because mice without thymus and deficient in neutrophil function (bg/bg nu/nu) are not more susceptible to systemic candida infection than mice equally neutrophil-deficient but euthymic (bg/bg nu/nu$^+$) (8). Also, it was reported that T cell-deficient mice are equally (9,10) or even more resistant (11) to systemic candidiasis than normal mice. However, even if the adequate neutrophil function is crucial to the clearance of *C. albicans* as the bg/bg mice experiments suggest, that does not exclude the possible down-regulation of normal neutrophils by T cells. In agreement with this possibility are the recent observations pointing to the role of Th2 responses on favouring systemic fungal load in BALB/c mice because *C. albicans* infection is abrogated after depletion of IL4 and/or IL10 (12). At least IL10 has been claimed to be a powerful down-regulator of the oxidative metabolism and antibody-dependent cytotoxicity of neutrophils (13).

We have observed that *Streptococcus mutans* (14-16), *Streptococcus intermedius* (17), African swine fever virus (18,19) and *C. albicans* (20) produce immunosuppressive and lymphocyte mitogenic proteins which were designated ISM(s) (6). Such as the other ISM proteins, p43 produced by *C. albicans* is crucial to the systemic growth specifically of the producing microorganism (6). We have shown that: a) treatment with p43 facilitates microbial growth and diminishes host resistance (6,20), b) selective loss of the ISM production by *C. albicans* correlates with "attenuation" (subsequent to repeated subcultures *in vitro*) and increased host resistance (20) and c) specific immunization against ISM increases host resistance and results in complete protection (6).

The role of Th2 cells in the effects of p43 and in the fungal growth in BALB/c mice is also compatible with the observation that both are abrogated in mice depleted of $CD4^+$ lymphocytes (6). We have now investigated the differential effect of IL4 and IL10 on systemic *C. albicans* infection and also on the biological effects of p43, using C57BL/6 mice which were reported to be higher fungal-infection resistant than BALB/c mice (21,22). Such higher resistance to fungal infection fit with our observation because approximately 35% of BALB/c mice die whereas all C57BL/6 remain alive 15 days after i. p. infection with $5x10^7$ fungal cells. Moreover, the numbers of *C. albicans* colony forming units (CFU) were several hundred fold higher in the kidneys of BALB/c than of C57BL/6 mice on days 11 and 15 after infection.

Since we have observed that the biological effects of the ISM proteins (16,17,19) were abrogated after adult thymectomy, we also paid particular

attention to the role of the thymus which has already been described previously as a mouse reservoir of Th2 lymphocytes (23).

Materials and methods

Mice. Male, C57BL/6, C57BL/6 nu/nu mice purchased from the Gulbenkian Institute of Science, Oeiras, Portugal, were used. All mice were 6-8 weeks old at the time of the experiments. Some of the mice were thymectomized (Tx) at 15 days before (4-6 weeks old).

***Candida albicans* infection protocols**. *C. albicans* was isolated and maintained as previously described (6,20). Experimental animals were infected by i.p. injection of $5x10^7$ blastoconidia isolated from fungi cultures made in Sabouraud broth (Difco, Detroit, MI) for 24h at 37°C. The two kidneys were removed asseptically at selected times after inoculation. Both organs were homogenized in 5ml of PBS, and the homogenizates were serially diluted five times. CFU of *C. albicans* were counted after 48h of incubation at 37°C in Mycobiotic agar (Difco) in duplicated cultures of each serial dilution.

Enzyme linked immunospot (ELISPOT) assay. The enumeration of splenic cells secreting specific antibodies was evaluated as previously described (24). Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 10μg/ml of Candida sonicates overnight at 4ºC. The wells were then saturated during 30 min. with 1% BSA in PBS at 37ºC. Appropriate serial suspensions of splenic cells in RPMI 1640 (Sigma, St. Louis, Mo)

supplemented with 3% of FCS (Gibco Biocult, Glasgow, UK) were incubated in the plates for 5 hours at 37°C in a humidified atmosphere of 5% CO2 in air. The plates were then rinsed with distilled water with 0,05% of Tween 20 (Sigma) and washed 4 times with PBS-0,05% Tween 20. The specific antibody-secreting cells were revealed by the addition of phosphatase alkaline-labelled goat antibodies anti-mouse IgM (Southern Biotechnology Associates, Birmingham, ALA, USA), overnight at 4°C. After washing, 5-bromo 4-chloro 3-indolyl phosphate (5-BCIP) (Sigma) in 2-amino 2-methyl 1-propanol (AMP) (Sigma) buffer were used as substrate for 2 hours at 37°C. After washing 4 times with distilled water, the number of spots was quantified, in triplicate, using a dissecting microscope and the data are driven from wells with more than ten spots.

Plaque forming cell (PFC) assays. Non-specific Ig production was assessed by protein A PFC assay (25) in nonimmunized mice. Isotype-specific and anti-total Ig rabbit antiserum, previously characterized in detail (26) were used as developing antibodies.

Preparation and evaluation of the biological activity of p43. Isolation and purification of p43 was previously described in detail (6,20). All preparations of p43 were LPS-free as assessed by the *Limulus* amebocyte lysate (LAL) test (E-Toxate®, Sigma). The biological activity of p43 was evaluated in mice after i.p. administration of 50μg of the ISM protein. The B cell mitogenic effect of p43 was evaluated 5 days after treatment by the numbers of splenic non-

specific Ig-secreting PFC of the IgM and IgG isotypes. The immunosuppressive and the fungal growth facilitating properties of the ISM were evaluated by injecting the protein two days before i. p. immunization with $5x10^7$ heat killed *C. albicans* particles (Cp) in PBS or before *C. albicans* infection, respectively.

Cell and cytokine depletion protocols. C57BL/6 mice were Tx by suction of the thymic lobes under ether anaesthesia. These mice were kept for 15 days before experimental procedure. Some C57BL/6 nu/nu mice were depleted of more than 98% of intestinal $\gamma\delta$ T cells suspensions as assessed by flow cytometric analysis (Fig. 1) after administration of 200 μg at two days interval of an hamster IgG1 mAb anti-δ chain. Depletion of IL4, TNFα and IL10 was accomplished by injecting C57BL/6 mice i.p. with 1mg of rat IgG1 mAb against each IL two hours before p43 treatment, or two days before Cp immunization or *C. albicans* infection. These mAb were obtained by growing hybridomas: A403A.10.2 (anti δ-chain) 11-B-11 (anti-IL4), MP6-XT22 (anti-TNFα), and JES5-2A5 (anti-IL10) (kindly supplied by Dr. Anne O'Garra, DNAX Institute, Palo Alto, California) in the peritoneal cavity of C57BL/6 nu/nu mice. All mAb were purified from ascitic fluids by ammonium sulphate precipitation and by protein A affinity chromatography (27). One mg of rat IgG1 (Zymed Laboratories, San Francisco, CA) with unrelated specificity was used to treat control mice following the same protocol of the specific mAb.

Serum cytokine determination. The mouse serum cytokine titers of IFN γ, IL4 and IL10 was determined with the Intertest-10x ^{TM} ELISA kit (Genzyme Corp, Cambridge, MA).

Cell suspensions for cytometry analysis. Intestinal lymphoid populations were prepared according to the methods of Lick (28) and Heijden (29), washed and suspended in PBS supplemented with 3% of FCS (Gibco Biocult) and with 10mM of sodium azide (Sigma) and incubated with the specific mAb in the same buffer.

Immunofluorescence and flow cytometry analysis. Analysis of surface markers of the intestinal cell populations was performed in a FACScan (Becton Dickinson, San Jose, Ca, USA) with the LYSYS II software (Becton Dickinson). The following mAb were used for the surface markers: hamster anti-mouse γ/δ-TCR-FITC conjugate (PharMingen, San Diego, CA), and rat anti-mouse CD3 PE conjugate (PharMingen).

Results

The biological effects of p43 and *C. albicans* infection depend on IL10. As shown in Fig.2 *Candida* infection either when unpotentiated or potentiated by p43 are abrogated by depletion of IL10. As expected since pathogenic *C. albicans* produces p43, the relative reduction in the numbers of CFU after depletion of IL10 is similar in p43 treated or untreated mice (Fig.2). In contrast, as also depicted in Fig.2 depletion of IL4 failed to change *C. albicans* load potentiated or unpotentiated by p43. The specific immunosuppression induced by p43 of the primary immune response, evaluated by the numbers of IgM-secreting cells, was also abrogated after IL10 depletion (Fig.2). Likewise the nonspecific effect of p43 evaluated by the numbers of IgM- and IgG isotype- secreting cells (Fig. 2) was reduced after IL10 depletion. However, depletion of IL10 in nonimmunized p43 treated mice induced roughly 50% of mortality (8 out of 18 mice died 3 to 4 days after IL10 depletion combined with p43 treatment) that can be prevented if the mice were also depleted of TNFα.

The primary immune responses to Cp remained unchanged after depletion of IL10 or IL4 in p43 untreated mice.

The ISM biological effects of p43 are impaired in Tx and in nude C57BL/6 mice. As depicted in Fig.3 and in contrast with the strong suppressive effect of p43 observed in normal Cp-immunized C57BL/6 mice

the primary immune response to Cp was similar in p43 treated or untreated Tx mice. Moreover, instead of being suppressed the specific anti-Cp response was about 16 times higher in congenitally athymic mice treated with p43 before immunization with Cp than in p43-untreated control mice. Furthermore, a markedly lower B cell polyclonal response of the IgG1, IgG2a and IgG2b thymic dependent isotype-secreting cells was observed in Tx, 5 days after p43 treatment, comparatively to normal mice. The IgM and IgG3 responses were kept or even slightly enhanced in Tx animals. As it is also shown in Fig.3, a decrease in the B cell mitogenicity induced by p43 was observed in nude mice as well. In these mice, however, not only the thymic-dependent isotype secreting cells were refractory to the mitogenic effect of p43 but also the IgG3-secreting cells. The non-specific IgG1, IgG2a, IgG2b and IgG3 PFC responses of the nude mice were: 1, 4.5, 0.03 and 3 percent, respectively, of the responses observed in euthymic mice 5 days after p43 treatment (Fig. 3). Only the IgM secreting PFC responses were similar in nude and euthymic mice after the ISM treatment.

Systemic candidiasis is also impaired in Tx and in C57BL/6 nu/nu mice. As shown in Fig.4, all C57BL/6 normal mice were heavily infected after being inoculated i. p. with $5x10^7$ candida blastoconidia. In contrast, Tx C57BL/6 mice controlled parasitemia over the course of infection. Similarly, the kidneys of euthymic controls were more fungal infected than those of C57BL/6 nu/nu mice 5 days after i.p. candida infection. Moreover, no candida CFU were found in the kidneys of the nude mice whereas the kidney infection was still

very pronounced in euthymic mice 14 days after infection. This increased resistance of C57BL/6 nu/nu mice to candida infection does not seem to be due to γδ T cells because resistance to infection is maintained in mice 98% depleted of this cell population at the same extent.

A rapid increase in the serum levels of IL10 was observed in normal C57BL/6 mice treated with p43 or infected with *C. albicans*. As shown in Fig. 5, the serum levels of IL4 and IL10 were detectable shortly after treatment with p43 or after infection with *C. albicans*, as opposed to uninfected and p43 untreated controls. However, the serum increase in the levels of IL10 was considerably higher than of IL4 which can be hardly explained by differences in sensitivity of the cytokines assays. The peak responses of the two cytokines were seen two hours later and were identical to p43 untreated control level 24 hours after p43 treatment (Fig. 5). The serum levels of IL4 and mainly of IL10 were considerably higher in mice treated with the ISM protein than in mice infected with candida blastoconidia. This observation is not surprising since the 50μg of p43 used in the experiments was obtained from $3x10^8$ candida blastoconidia whereas the infecting inoculum was 6 times smaller. Therefore, it is expected that only afterwords over the kinetic growth of *C. albicans* infection similar levels of p43 would be produced by the fungal inoculum. After *C. albicans* infection the serum levels of IL10 were still increasing as expected, due to the progressive fungal proliferation up to at least 24 hours. The serum levels of IL4 reached a plateau 12 hours after infection. As it was also shown in Fig.5, *C. albicans*

infection did not elicit detectable serum levels of IFN γ whereas the serum levels of this cytokine were substantially increased after p43 treatment although at a less extent than IL10. This observation may also be explained by the larger amount of candida particles used to obtain 50 μg of p43 as compared to those used to infect the mice. Peak responses of IFN γ were observed four hours later (6 hours after the ISM protein treatment) than the peak responses observed with IL10 and IL4. As in the case of IL10 and IL4 the serum levels of IFN γ were no longer detectable 24 hours after p43 treatment. Serum IL2 was never observed after p43 treatment or *C. albicans* infection (data not shown). Since Tx or nude mice are refractory to p43 treatment or *C. albicans* infection, as compared to normal C57BL/6 mice, it is interesting to observe that the increase in serum IL10 levels in the former two groups was markedly lower than in latter group of mice after the fungal infection or the ISM protein treatment (Fig. 5).

Discussion

It has been shown that IL4 or IL10 responses facilitate the growth of *C. albicans* (12,30,31) and also of several different microorganisms (32-38) in mice. However, IL4 alone is unable to modify the course of infection of highly resistant mice to *Leishmania major* (38) whereas administration of IL10 abolishes innate resistance to *Listeria monocytogenes* (35). The present results are in agreement with these reports because *C. albicans* load was abrogated in highly resistant C57BL/6 mice depleted of IL10 but not of IL4 and serum levels of IL10 were considerably higher than of IL4 in these mice after *C. albicans* infection. The pathogenic role of p43 previously shown (6, 20) is now reinforced by its specific immunosuppressive effect on the primary immune response to *Candida* antigens (*Candida* sonicates). Therefore, it is not surprising that in a close correlation between *C. albicans* infection and p43 treatments, the enhancing of fungal growth and the immunosuppressive effects of p43 are also abrogated after depletion of IL10 but not of IL4. The mitogenic effect of p43 is abrogated as well in IL10-depleted but not in IL4-depleted C57BL/6 mice. This abrogation seems to be due at least partially to cell death caused by upregulation of TNFα because, although depletion of TNFα alone does not modify the mitogenic effects of p43, combined depletion of this cytokine and of IL10 allows higher expression of the B cell mitogenicity induced by the ISM protein. Upregulation of TNFα may also explain the 50% mortality seen in p43 treated and IL10-depleted mice since depletion of TNFα

prevents such mortality. Higher mortality of mice depleted of IL10 due to higher production of toxic cytokines such as TNFα was also observed after *Toxoplasma gondii* infection (39). Moreover, the TNF-α mediated shock induced by LPS and/or Staphylococcal enterotoxin B treatments can be prevented by the administration of IL10 (40). Furthermore, neutralization of endogenous IL10 led to the appearance of high levels of serum TNFα (41). Interestingly, no mortality was seen in p43 treated and IL10-depleted mice when immunized with *Candida* antigen or infected with *C. albicans*. It is possible that combined antigenic stimulation and p43 treatment trigger additional control mechanisms leading to suppression and preventing excessive TNFα upregulation.

Also, such as in the case of *C. albicans* infection much higher levels of serum IL10 than of IL4 were observed after p43 treatment and, in both cases, serum detection of IL10 were early observed. This observation is in agreement with previously reported data (12) indicating that IL10 depletion should be performed shortly after *C. albicans* infection in order to achieve impaired fungal growth.

It was shown several years ago that adult thymectomy impairs the course of primary infection by bacteria (16,17), fungi (42, 43) and protozoa (44,45). Our results are in agreement with these reports and confirm others indicating that increased resistance to systemic *C. albicans* infection was observed in nude mice (11,46). It was reported that nude mice develop γδT cells that are responsible for such resistance at the early stages of infection with *L.*

monocytogenes (47). However, these cells do not seem to be responsible for the increased resistance to the systemic *C. albicans* infection in these mice, because almost full depletion of γδ T cells does not facilitate Candida colonization. Reinforcing the close dependence of *C. albicans* infection on the production of p43, both the specific immunosuppressive and the nonspecific B cell mitogenic effects of this ISM are also abrogated in Tx and in nude mice. Interestingly, both B cell polyclonal activation induced by *T. cruzi* and the parasite infection are also impaired in Tx mice (45,48). Since the serum levels of IL10 of Tx were considerably lower than those of normal mice after *C. albicans* infection or p43 treatment, thymocytes or thymus recent emigrant cells seem to be the main source of IL10. This is in agreement with the observation that serum levels of IL10 were even lower in nude mice than in Tx mice after p43 treatment. These results match with the observation that development of regulatory/immunosuppressor cells are dependent on the existence of an intact thymus (49). Furthermore, abnormal thymus dependent colitis (50) can be controlled by the administration of IL10 (51). Finally, it was described that thymic recent emigrant cells are keen cytokine producers (52).

Experiments are now in progress in order to determine if IL10, crucial to *C. albicans* infection and to the biological effects of p43, is produced/released by thymocytes or by the thymic recent emigrant cells.

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Figure Legends

Fig.1. Flow cytometric analysis of TCR-γδ expressed by intestinal cells from C57BL/6 mice either normal (Panel A) or 10 days after treatment with mAb anti-TCR δ chain (Panel B).

Fig.2. Effects of depletion on normal (non Tx, euthymic) C57BL/6 mice of the indicated ILs by i.p. injection of 1 mg of specific mAb two days before *C.* albicans infection or Cp immunization and two hours before p43 treatment. In this and in the following Figs, p43 treatment and *C. albicans* infection were performed by an i.p. injection of 50 μg of ISM protein and $5x10^7$ candida blastoconidia, respectively.

Panel A:

Numbers of CFU of *C. albicans* observed in the kidneys of mice 5 days after the fungal infection. These mice were distributed to the following groups: nonmanipulated (control), treated with p43 two days before infection and depleted of IL10 or IL4 as indicated.

Panel B:

Numbers of specific anti-Cp IgM-secreting splenic cells observed in mice, five days after immunization with Cp, either untreated or treated with p43 two days before immunization and depleted or nondepleted of the indicated ILs.

Panel C:

Numbers of splenic non-specific Ig-secreting PFC observed in non-immunized mice, untreated or five days after i.p. p43 treatment and depleted or nondepleted of the indicated ILs.

The results represent arithmetic means of 8 mice and one SD per group analysed individually and are representative of three experiments.

Fig. 3. Comparison between the biological effects of p43 in normal, Tx and nude C57BL/6 mice.

Panel A:

Numbers of splenic non-specific Ig-secreting PFC of the indicated isotypes in normal (□) Tx (▨) and nude (■) C57BL/6 mice observed 5 days after p43 treatment. Between parentheses are indicated the time increase in the numbers of Ig-secreting PFC for each isotype comparatively to the respective p43 untreated controls.

Panel B:

Numbers of splenic specific anti Cs IgM-secreting splenic cells in normal (□) Tx (▨) and nude (■) C57BL/6 mice observed five days after Cp-immunization, either untreated (a) or treated with p43 two days before immunization (b).

Fig. 4. Comparison between the growth of *C. albicans* in normal, Tx and nude (depleted or nondepleted of γδ T cells) C57BL/6 mice.

Panel A:

Course of infection in the kidneys of normal (□) and Tx (□) C57BL/6 mice expressed as CFU, on the indicated days after i.p. infection with *C. albicans* blastoconidia.

Panel B:

Numbers of CFU of *C. albicans* recovered from kidneys of C57BL/6 (□) C57BL/6 nu/nu (□) and C57BL/6 nu/nu depleted of γδ T cells (■) 5 days (I) and 14 days (II) after infection.

Fig. 5. Comparison between serum levels of IFN γ, IL10 and IL4 observed in normal (solid lines) and of IL10 in Tx (dashed lines) and nude (dotted lines) C57BL/6 mice before (control, 0 hours) and on the indicated hours after i.p. p43 treatment (A) or *C. albicans* infection (B).

In this Fig., all the results represent the arithmetic mean of 4 to 6 mice per group analysed individually and one SD and are representative of 3 experiments.

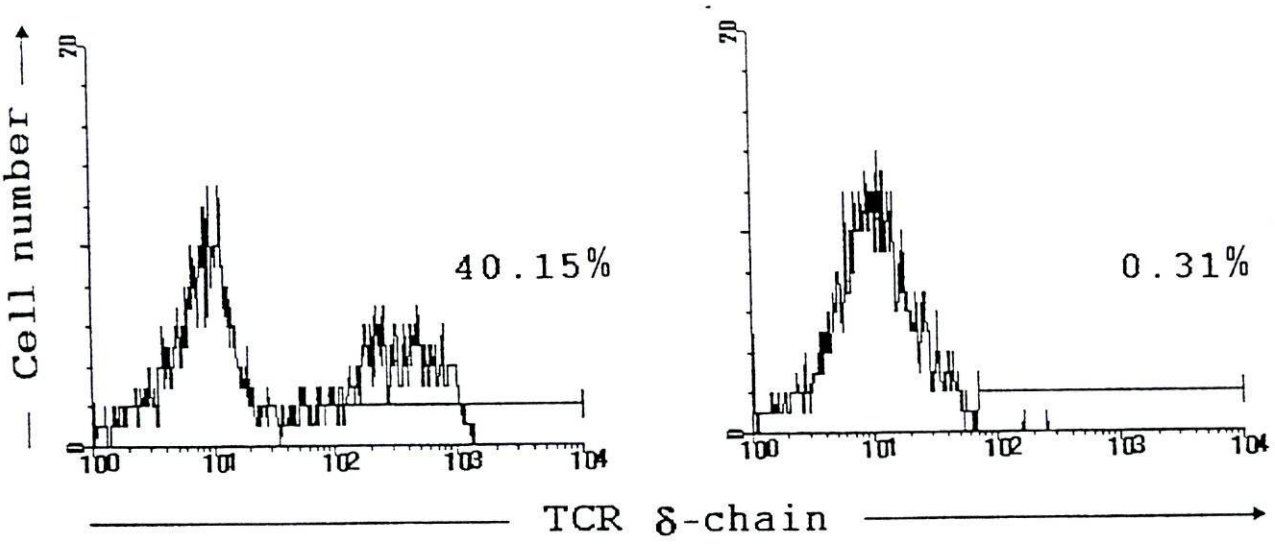
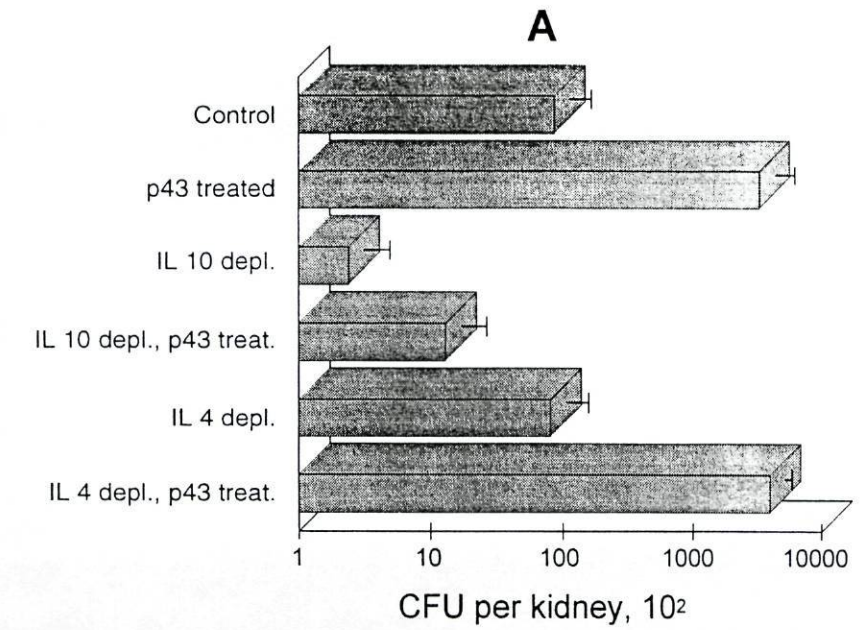
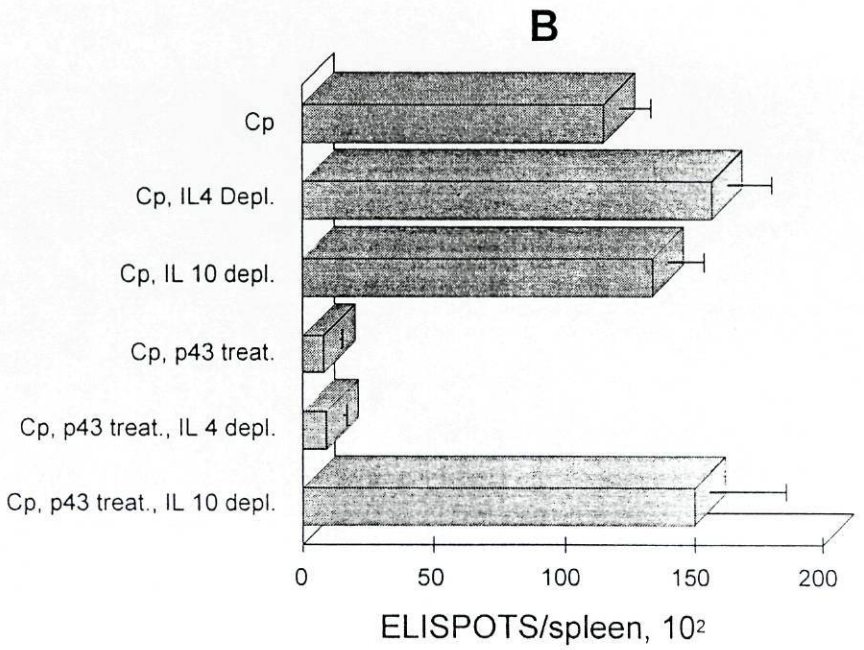


Fig. 1


A
Control
p43 treated
IL 10 depl.
IL 10 depl., p43 treat.
IL 4 depl.
IL 4 depl., p43 treat.
1
10
100
1000
10000
CFU per kidney, 10²


B
Cp
Cp, IL4 Depl.
Cp, IL 10 depl.
Cp, p43 treat.
Cp, p43 treat., IL 4 depl.
Cp, p43 treat., IL 10 depl.
0
50
100
150
200
ELISPOTS/spleen, 10²

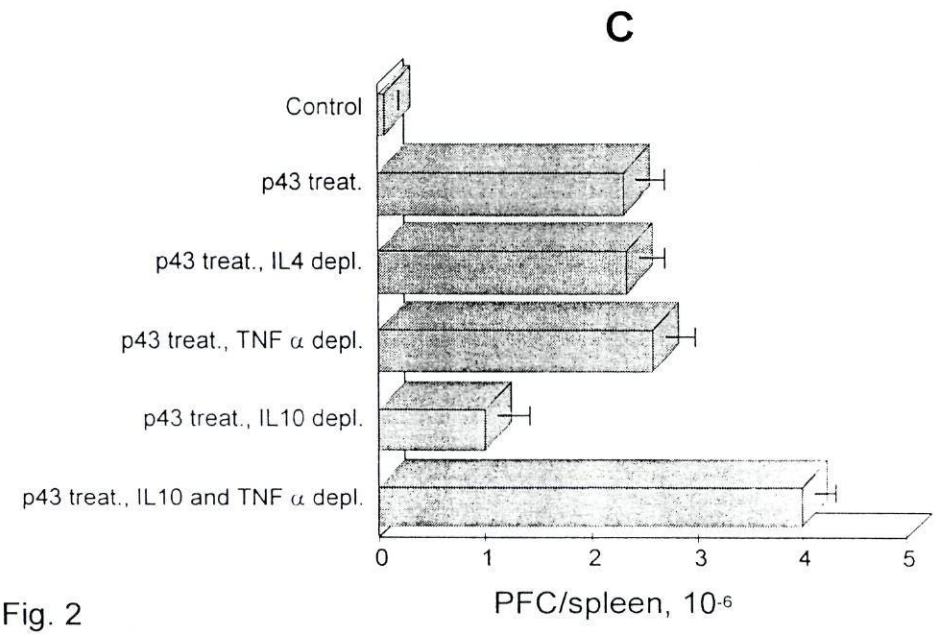
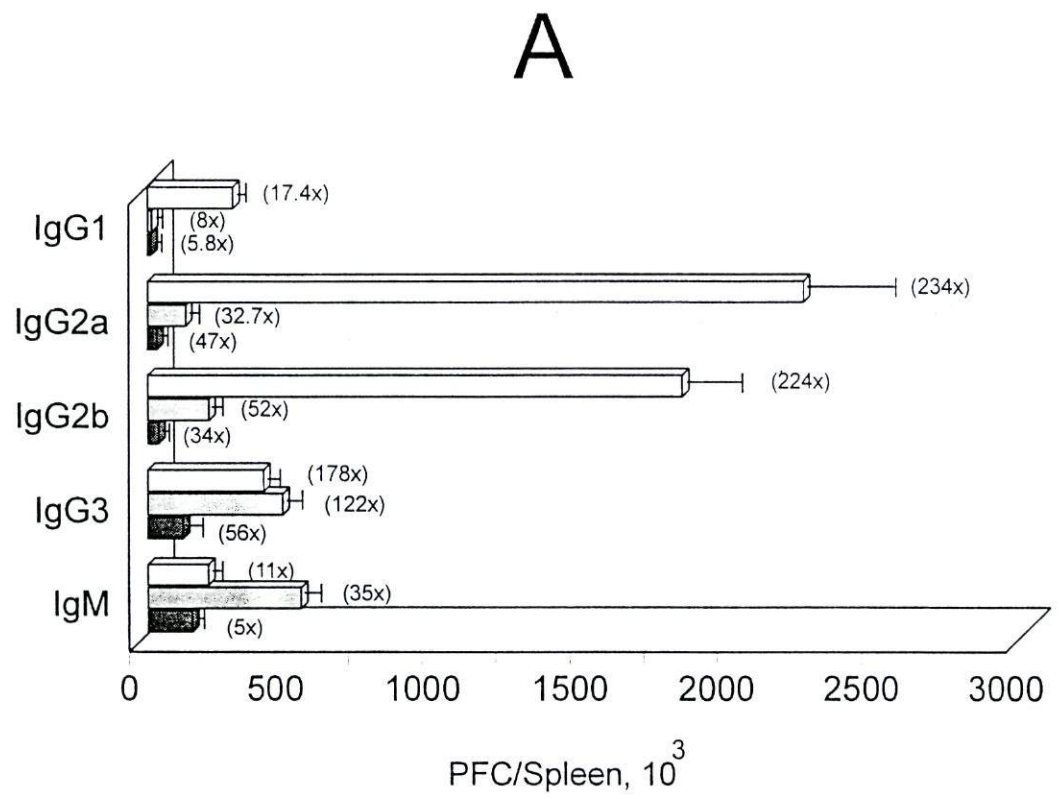

C
Control
p43 treat.
p43 treat., IL4 depl.
p43 treat., TNF α depl.
p43 treat., IL10 depl.
p43 treat., IL10 and TNF α depl.
0
1
2
3
4
5
PFC/spleen, 10⁻⁶

Fig. 2


A
IgG1
IgG2a
IgG2b
IgG3
IgM
(17.4x)
(8x)
(5.8x)
(234x)
(32.7x)
(47x)
(224x)
(52x)
(34x)
(178x)
(122x)
(56x)
(11x)
(35x)
(5x)
0
500
1000
1500
2000
2500
3000
PFC/Spleen, 10^3

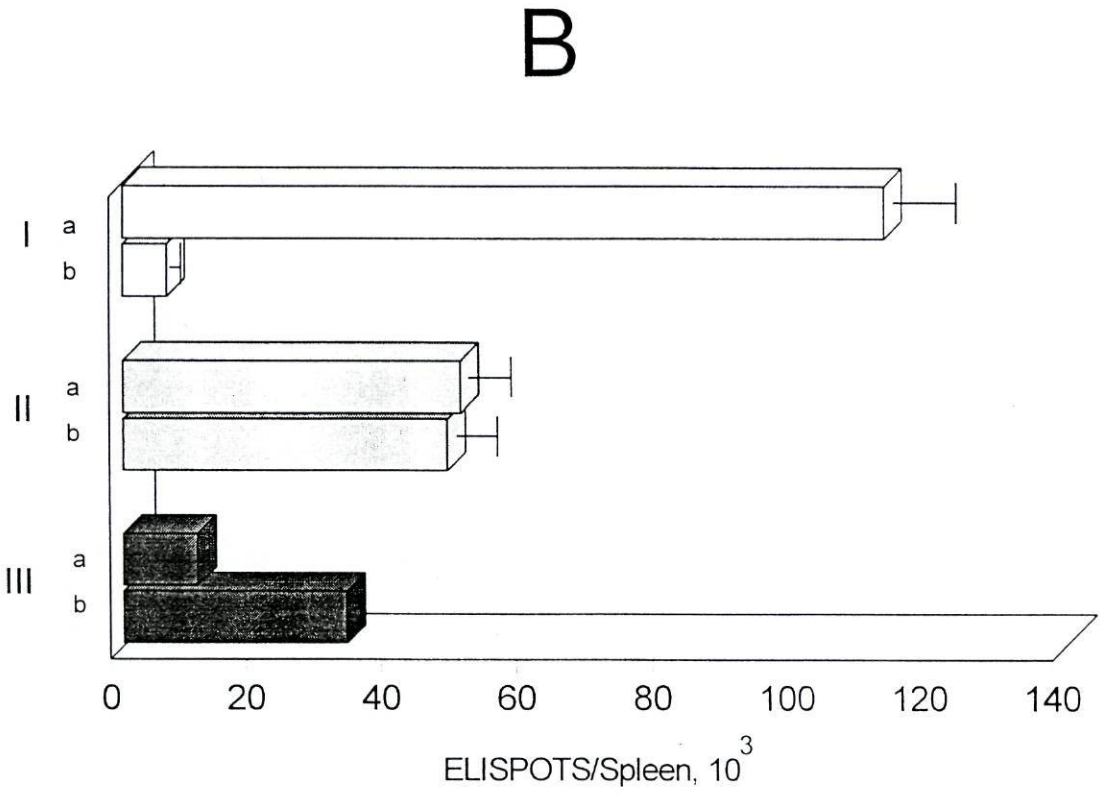
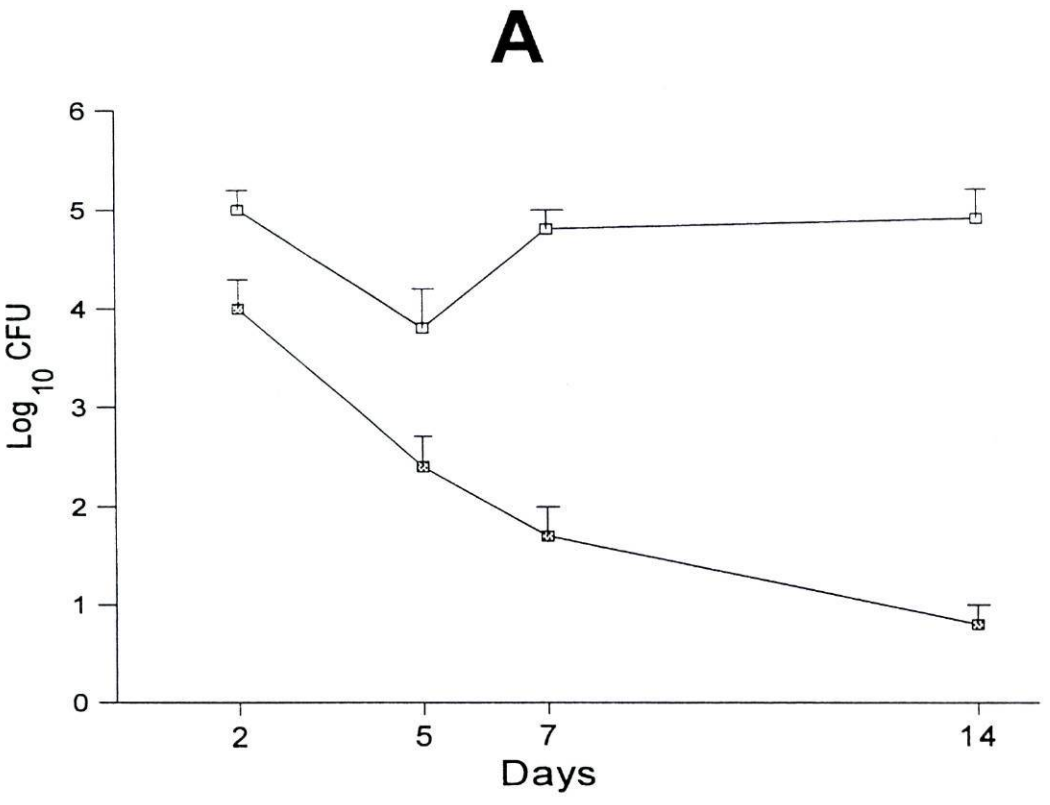

B
I
II
III
a
b
0
20
40
60
80
100
120
140
ELISPOTS/Spleen, 10^3

Fig. 3


A
Log10 CFU
0
1
2
3
4
5
6
2
5
7
14
Days

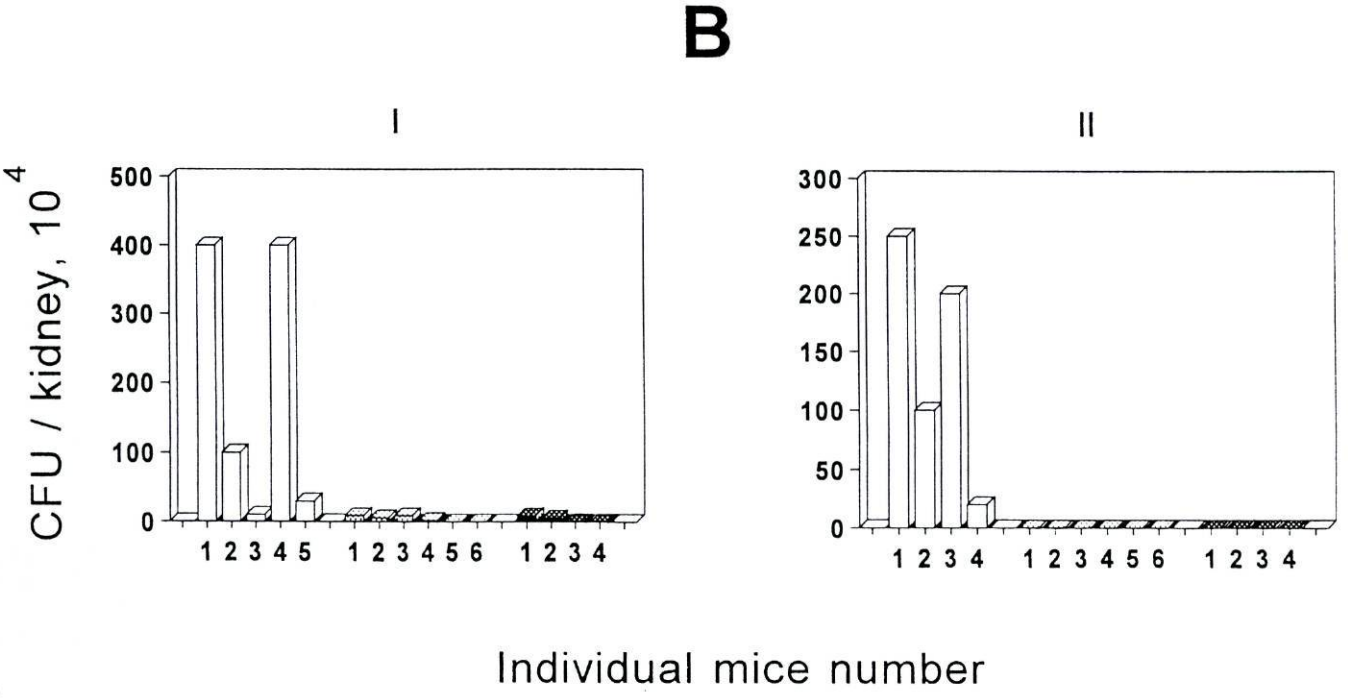

B
I
II
CFU / kidney, 10^4
500
400
300
200
100
0
300
250
200
150
100
50
0
1 2 3 4 5
1 2 3 4 5 6
1 2 3 4
1 2 3 4
1 2 3 4 5 6
1 2 3 4
Individual mice number

Fig. 4

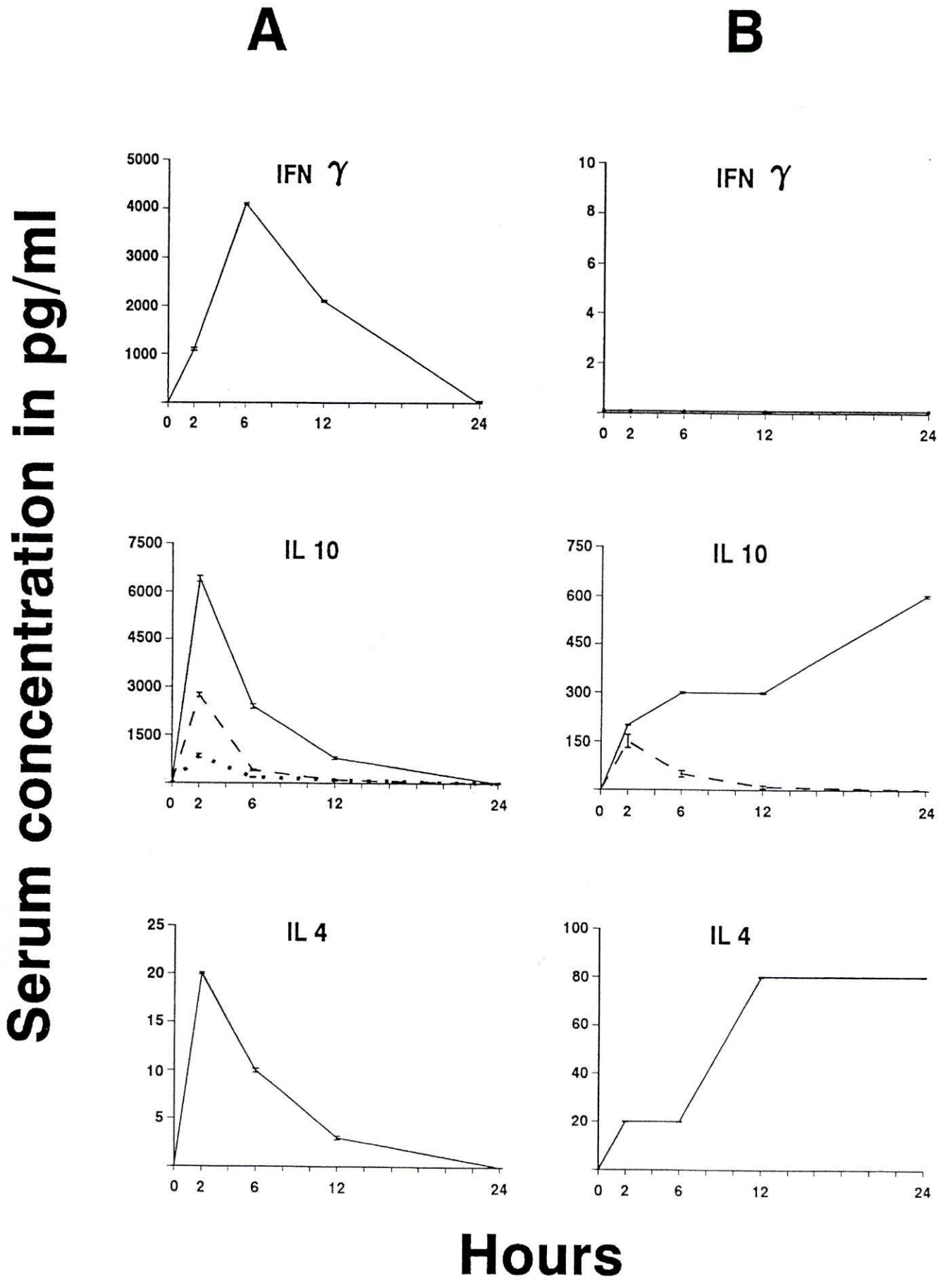

A
B
Serum concentration in pg/ml
IFN γ
5000
4000
3000
2000
1000
0 2 6 12 24
IFN γ
10
8
6
4
2
0 2 6 12 24
IL 10
7500
6000
4500
3000
1500
0 2 6 12 24
IL 10
750
600
450
300
150
0 2 6 12 24
IL 4
25
20
15
10
5
0 2 6 12 24
IL 4
100
80
60
40
20
0 2 6 12 24
Hours

Fig. 5

TRABALHO V

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Purification, biochemical and biological characterization of an immunosuppressive and lymphocyte mitogenic protein secreted by *Streptococcus sobrinus*

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Keywords: activation, IL-10, p38, suppression

Abstract

An immunosuppressive/mitogenic (ISM) protein was purified from the supernatants of cultures of *Streptococcus sobrinus* with an isoelectric point of 4.75 and a relative molecular mass of 38 kDa (p38). Treatment of C57BL/6 mice with p38 induced an increase in the numbers of non-specific splenic Ig-secreting plaque-forming cells (PFC) with peak responses on day 3 for IgM-secreting PFC and on day 5 for IgG-secreting PFC, with an isotype pattern consisting predominantly of IgG2a and IgG2b. This increase was accompanied by a lymphocyte blastogenic response of both T and B lymphocytes. The *in vitro* effects of p38 on pure B, T and total splenic lymphocytes indicated that this ISM protein was primarily a B cell mitogen, being T cells activated subsequently by the generation of B blasts. Suppression of the murine primary immune response against sheep red blood cells was observed in C57BL/6 mice treated 4 days before with p38. The amino acid sequence of the N-terminus of p38 has a significant similarity with several enolases, particularly with rabbit enolase. However, the biological effects ascribed to p38 have not been detected after *in vivo* treatment with that enolase. The immunosuppressive effect of p38 was abrogated by depletion of IL-10 but not of IL-4. In agreement with this observation IL-10 was the only cytokine detected in serum of C57BL/6 mice after p38 treatment and the peak of serum levels was observed as soon as 2 h after treatment.

Introduction

Combined immunosuppression and polyclonal lymphocyte mitogenicity is a common feature in microbial infection (1–9). Both immunosuppression and lymphocyte mitogenicity effects were ascribed to immunosuppressive/mitogenic (ISM) microbial proteins (1,2,4,8,10–12). Among these ISM proteins we have particularly studied those proteins produced by *Streptococcus intermedius* (1), *Streptococcus mutans* (4), African swine fever virus (ASFV) (2) and *Candida albicans* (8). We have shown that these ISM proteins seem to play an important role in the pathogenicity and survival of the microorganism in the host because: (i) treatment of the host with ISM proteins facilitates microbial growth and diminishes host resistance (8,13,14), (ii) selective loss of ISM protein production by microbial strains correlates with 'attenuation', loss of virulence (8,13,14), and (iii) specific immunization against ISM proteins increases host resistance (15) and often results in a complete protection (16). On the basis of these observations it was postulated (17) that the adequate strategy of immunoprotection will be the immunization against the secreted ISM proteins rather than against the microbial constitutive epitopes.

Streptococcus sobrinus is a particularly important bacterium since it is considered one of the principal agents of dental caries (18–21). Treatment of dental caries is probably the world costliest due to its wide distribution (20,22) and does not include a viable preventive approach based upon the control of bacterial factors involved in this pathology (22,23).

In the present report we decided to search for an ISM protein produced by *S. sobrinus*, and to define the biochemical and

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biological characteristics of such protein. Since host T_h1 and T_h2 responses were recently considered as neutralizing and favouring respectively microbial growth (24–33), particular attention was paid to the involvement of T_h2 ILs in the analysis of the immunobiological effects of p38.

Methods

Mice

Male C57BL/6 mice, 8 to 10 weeks old, were bred at the Gulbenkian Institute (Oeiras, Portugal).

Bacteria

S. sobrinus strain 6715, able to induce dental caries in rats (34,35) (a kind gift of Dr Klausen, Department of Oral Diagnosis and Microbiology, Royal Dental College, University of Copenhagen, Denmark), was stored at –70°C in Todd–Hewitt broth medium (Difco, Detroit, MI) with 10% (v/v) glycerol. The pathogenicity of the bacterium was maintained every 3 months by i.p. inoculations of *S. sobrinus* into C57BL/6 mice and subsequent recovery of the bacterium from the peritoneal exudates. An attenuated form of the same strain of *S. sobrinus*, unable to induce dental caries, was obtained after 60 passages in Todd–Hewitt agar medium.

Preparation of the crude extracellular products of S. sobrinus (CEP-Ss)

Pathogenic or attenuated *S. sobrinus* was cultured anaerobically in Todd–Hewitt broth medium for 3 days at 37°C at an initial concentration of 10^8 microorganisms/l of medium. The cultures were then centrifuged at 29,000 *g* for 30 min, and the supernatants were filtered through a 0.22 μm pore size filters (Millipore, Bedford, MA) and concentrated by vacuum dialysis through a Visking 100/8FT dialysis membrane with a 12,000 Da cut-off. This product was designated crude extracellular product of *S. sobrinus* being CEP-Ss path or CEP-Ss inact when obtained from pathogenic or attenuated *S. sobrinus* cultures respectively.

Immunization and immunosuppression protocols

Immunization of C57BL/6 mice with sheep red blood cells (SRBC), as previously described exhaustively (1,2,4,8), was observed in mice treated 4 days before immunization with CEP-Ss path or inact, the fractions thereof, the purified protein and rabbit enolase (Sigma, St Louis, MO). Control mice were injected i.p. at the same time with PBS.

Plaque-forming cells (PFC) assays

Evaluations of the immune responses by the numbers of splenic IgM-secreting PFC and of non-specific Ig production by the Protein A assay were performed as described in detail elsewhere (1,2,4,8).

Fractionation of CEP-Ss path and inact

Ion-exchange chromatography of CEP-Ss path and inact was performed in DEAE–cellulose (DE52; Pharmacia, Piscataway, NJ), using a continuous molarity gradient of 0.05–1.0 M NaCl in 0.05M Tris–HCl, pH 7.0. The fractions were concentrated by vacuum dialysis. Protein concentration was determined by the method of Lowry *et al.* (36).

Subfractionation of CEP-Ss path

The material eluted from ion-exchange chromatography with immunosuppressive activity was subfractionated by preparative isoelectric focusing (PIEF) in sucrose and a pH gradient of 2.5–10 using a mixture of ampholytes of pH 2.5–5.0 and 3.0–10.0 (Pharmacia) as previously described in detail (14).

Purification of the immunosuppressive protein

The ISM protein was then purified from the biologically active material obtained after PIEF by preparative PAGE (37,38), performed in 5% polyacrylamide gels according to methodology also previously described in detail (8).

Electrophoretic analysis of the purified biologically active protein

Analytical isoelectric focusing of the purified biologically active protein and of a mixture of proteins of known isoelectric point was performed with ampholytes of pH 3–9 in strips (Phastgel IEF 3–9; Pharmacia) by using computerized systems for the separation and staining (Phast system separation control and development units; Pharmacia). After a prerun of 10 min, 1 μl of each sample was applied and analytical isoelectric focusing was performed over 30 min. These gel strips were also stained with silver nitrate, methylene blue or periodic acid–Schiff. The relative molecular mass of this protein was determined by SDS (Sigma)–PAGE (39). SDS–PAGE preparations were stained with silver nitrate.

N-terminal amino acid sequencing

The purified protein was separated by SDS–PAGE and transferred to an Imobilon P membrane in 10% methanol, 10 mM CAPS buffer. The N-terminal amino acid sequence of the protein was then analyzed on an Applied Biosystems model 479A gas-phase sequencer equipped with a 120 A PH analyser. The determined sequence was compared with those contained in updated versions of the Swiss Prot and NBRF/PIR protein databanks using the FASTA program (40).

Splenic lymphocyte purifications and in vitro assays

T and B cell-enriched splenic populations, cultures of total, B or T splenic lymphocytes and evaluation of the proliferative responses by incorporation of [^{3}H]thymidine were performed as previously described in detail (1,13,41,42). The degree of purification of T and B cells populations was evaluated by flow cytometry analysis. Lipopolysacharide (LPS) obtained from *Brucella abortus equi* (Difco) and p38 were used as stimulants at final concentrations of 12.5 μg/ml of LPS or of 6.25, 12.5 or 25 of the ISM protein. Alternatively, some cultures of unfractionated and T splenic lymphocytes were carried out over 5 days. In this particular case a final concentration of 6.25 μg/ml of p38 was used as a single stimulant. Stimulation of the cultures was also evaluated by Protein A PFC and the flow cytometry analysis was determined as below.

Serum IL determination

The mouse serum cytokine titers of IFN-γ, IL-2, IL-4 and IL-10 were determined with the Intertest-10×TM ELISA kit (Genzyme, Cambridge, MA).

IL depletion protocols

Depletion of IL-4 and IL-10 was accomplished by injecting C57BL/6 mice i.p. with 1 mg of rat IgG1 mAb against each IL 2 h before p38 treatment or 2 days before bacterial particle immunization. These mAb were obtained by growing hybridomas 11-B-11 (anti-IL-4) and JES5-2A5 (anti-IL-10) (kindly supplied by Dr Anne O'Garra, DNAX Institute, Palo Alto, CA) in the peritoneal cavity of C57BL/6 *nu/nu* mice. All mAb were purified from ascitic fluids by ammonium sulphate precipitation and by Protein A-affinity chromatography (43). The amount of the mAb used has proven to be effective in the depletion of each IL (Dr Anne O'Garra, pers. commun.). One milligram of rat IgG1 (Zymed, San Francisco, CA) with unrelated specificity was used to treat control mice following the same protocol of the specific mAb.

Immunofluorescence and flow cytometry analysis

The spleen cells suspension was prepared by teasing the organ in cold BSS supplemented with 3% of FCS (Gibco Biocult, Glasgow, UK). The cell suspension were washed twice in the same buffer and then suspended in PBS supplemented with 3% of FCS and with 10 mM of sodium azide (Sigma). Dead cells were excluded by propidium iodide incorporation. Analysis of surface markers of the splenic cell populations was performed in a FACScan (Becton Dickinson, San Jose, CA) with the Lysys II software (Becton Dickinson). The following mAb were used for the surface markers: rat anti-mouse CD4 phycoerythrin-conjugate (Coulter Immunology, Hialeah, FL), rat anti-mouse CD8 FITC-conjugate (Coulter Immunology) and goat anti-mouse IgM FITC-conjugate (Southern Biotechnology Associates, Birmingham, AL).

Statistical analyses

Comparisons between p38-treated and p38-untreated mice were analysed by Student's *t*-test.

Results

Fractionation of CEP-Ss path by DEAE–cellulose and semi-purification by preparative isoelectric focusing

Figure 1 shows the fractionation of CEP-Ss path by ion-exchange chromatography with continuous increasing NaCl gradient from 0.05 to 1.0 M at neutral pH. Four fractions (F1–F4 DEAE) were obtained based on optical readings at 280 nm. This pattern was found to be reproducible in several preparations. The immunosuppressive effect on the primary immune response against SRBC of C57BL/6 mice evaluated by the numbers of splenic anti-SRBC IgM-secreting PFC of CEP-Ss was mainly detected in the F2 and F3 DEAE path (Table 1). None of the fractions obtained from the attenuated culture, F1–F4 DEAE inact, was suppressive when compared to the PBS-treated mice (Table 1). Figure 1 also shows that the subfractionation of a pool of several (F2 + F3) DEAE path by PIEF resulted in five subfractions, F'1–F'5 PIEF, identified by

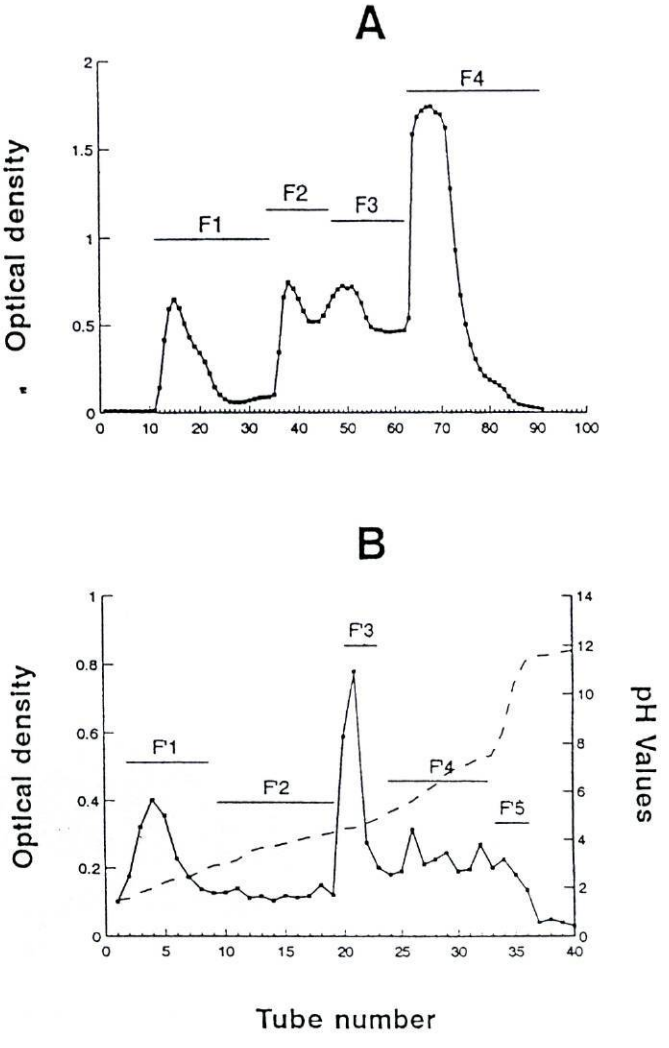

Fig. 1. Semi-purification of CEP-Ss by ion-exchange chromatography and PIEF. (A) Diagram of fractionation of CEP-Ss by ion-exchange chromatography in a DEAE–cellulose column. The fractions F1–F4 were pooled as shown. (B) Diagram of the fractionation of (F2 + F3) DEAE by PIEF in a sucrose gradient with an ampholyte range of pH 2.5 to 10. The fractions F'1 to F'5 were pooled as shown. The optical density measured at 280 nm (solid line) and the pH values (dashed line) of the eluted fractions are indicated. The results in this and in Fig. 2 are a representative example of several assays.

optical density at 280 nm and by the pH values. This pattern was also found to be reproducible in several preparations. The biological activity was detected mainly in F'3 PIEF (Table 1), with an isoelectric point between 4.45 and 4.87 (Fig. 1). The immunosuppressive activity increased considerably per proteinaceous concentration over the purification procedure. Thus, although the immunosuppressive effect was distributed in two and one fractions after ion-exchange chromatography and PIEF respectively, the numbers of splenic IgM-secreting PFC of C57BL/6 mice treated with 200 μg of F2 or F3 DEAE were ~25% of the control values, whereas the suppressive effect of 100 μg of F'3 PIEF equalled 7% of the control values (Table 1).

Table 1. Splenic anti-SRBC IgM-secreting PFC in mice[a]

Fractions	Protein (μg)	PFC per spleen ($\times 10^{-2}$)
PBS	0	1320 ± 95
CEP-Ss	200	**600 ± 75**[b] (49%)[c]
F1 DEAE path	200	**850 ± 100** (64%)
F2 DEAE path	200	**400 ± 58** (30%)
F3 DEAE path	200	**330 ± 60** (25%)
F4 DEAE path	200	1256 ± 110 (95%)
F1 DEAE inact	200	1430 ± 103 (108%)
F2 DEAE inact	200	1300 ± 90 (98%)
F3 DEAE inact	200	1280 ± 230 (97%)
F4 DEAE inact	200	1520 ± 130 (115%)
F'1 PIEF	100	1458 ± 150 (110%)
F'2 PIEF	100	*1082 ± 150* (82%)
F'3 PIEF	100	*97 ± 14* (7%)
F'4 PIEF	100	*858 ± 130* (65%)
F'5 PIEF	100	1256 ± 170 (95%)
Band 1 PAGE	50	**93 ± 15** (7%)
Band 2 PAGE	50	1580 ± 197 (120%)
Rabbit enolase	50	1380 ± 100 (104%)

[a]Data are from 5 days after SRBC immunization of untreated C57BL/6 mice or mice treated 4 days before immunization with a single i.p. injection of the designated fraction or rabbit enolase.
[b]The results are means ± SD of four samples and are a representative experiment repeated three times. The significant values are given in italic ($P < 0.05$) or bold ($P < 0.01$) or $P < 0.001$).
[c]Percentage of values found in PBS-treated mice (control).

Purification and characterization of the immmunosuppressive protein

As shown in Fig. 2, two silver stained bands were detected in F'3 PIEF after PAGE. These bands were not stainable by methylene blue or periodic acid–Schiff. The immunosuppressive activity was detected in the material electroeluted from the more-cathodal band (Table 1). After electroelution this band clearly showed a more anodal behaviour (Fig. 2) without losing the immunosuppressive activity. The material contained in this band represented a pure protein because it was revealed as a single silver nitrate band in SDS–PAGE (Fig. 2). Likewise, the immunosuppressive effect per microgram of protein increased from F'3 PIEF to the pure ISM; the numbers of IgM-secreting PFC of C57BL/6 mice treated with 100 μg of the former fraction or 50 μg of the pure ISM protein in both cases equalled 7% of the control values. Comparison with standard proteins of known relative masses indicate that the protein has a relative molecular mass of 38 kDa (Fig. 2). Therefore, this protein was designated as p38. After analytical isoelectric focusing and comparison with proteins of known isoelectric point, it was seen that the isoelectric point of p38 equalled 4.75 (Fig. 2).

Structural analysis of the N-terminal amino acid sequence of p38

The sequence of the first 18 N-terminal amino acids of the p38 is shown in Fig. 3. Although the N-terminus of p38 is not represented in an updated version of protein databanks,

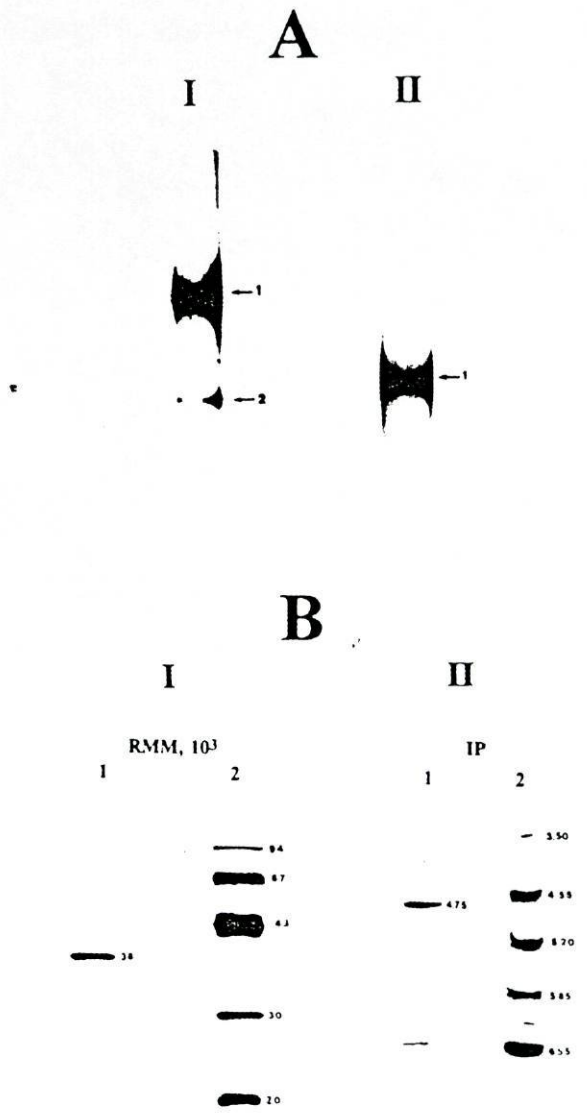

Fig. 2. PAGE analysis of F'3 PIEF and of the purified protein obtained from this fraction, and analytical comparison between the purified protein and a mixture of proteins of known relative molecular mass and isoelectric point (IP). (A) Pattern of PAGE analysis of (I) F'3 PIEF showing two silver nitrate-stainable bands (band 1 and band 2) and (II) of the fraction eluted with immunosuppressive activity (band 1 on I) showing one silver nitrate-stainable band. (B) Analytical comparison by SDS–PAGE (I) and by isoelectric focusing (II), the most purified protein (p38) (1), and a mixture of proteins of known relative molecular mass (2) and IP (2) respectively.

comparison with other protein sequences revealed significant similarity with enolases from bacterium (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*), mouse, rat, rabbit and humans (Fig. 3). However, as opposed to p38, 50 μg of rabbit enolase was unable to suppress the murine primary response against SRBC (Table 1).

In vivo proliferative and blastogenic response to p38

A considerable increase in the numbers of splenic non-specific IgM- and IgG-secreting PFC was observed, in com-

parison with controls, when C57BL/6 mice were treated with p38 (Fig. 4). The increase in the numbers of non-specific IG-secreting PFC reached the highest value on day 3 for the IgM and on day 5 for the IgG isotypes after treatment with p38. The isotype restriction pattern, of the ratios between p38-treated and p38-untreated C57BL/6 mice taken on day 5 after treatment, was: IgG2a > IgG2b > IgG1 > IgG3 > IgM. As also depicted in Fig. 4, the numbers of total and large B (IgM^+) and $CD4^+$ lymphocytes increased in the spleen of C57BL/6 mice 5 days after treatment with p38. No significant differences were observed between p38-treated and control mice in the numbers of total and large $CD8^+$ cells (data not shown).

```
p38      S I I T D V Y A R E V L D S R G N P
EnoE   M.S.K.I.V.K.I.I.G.R E.I.I.D.S.R.G.N.P.T V.E.-
         *   *           * *     * * * * * * .

p38      S I I T D V Y A R E V L D S R G N P
EnoY1      A V S K V Y A R S V Y D S R G N P T V E -
EnoY2      A V S K V Y A R S V Y D S R G N P T V E -
                 * * * *   *   * * * * * * *

p38      S I I T D V Y A R E V L D S R G N P
EnogR      S I Q K I W A R E I L D S R G N P T V E -
EnogH      S I Q K I W A R E I L D S R G N P T V E -
EnogM      S I E K I W A R E I L D S R G N P T V E -
EnobR      A M Q K I F A R E I L D S R G N P T V E -
EnobRab    A M Q K I F A R E I L D S R G N P T V E -
                     * * *   * * * * * * * *
```

Fig. 3. Comparison between the N-terminal amino acid sequence of p38 and several enolases. Enolase from *E. coli* (EnoE), 1 and 2 enolase from yeast *S. cerevisiae* (EnoY1 and EnoY2), β- and γ-enolase from rat (EnobR and EnogR), γ-enolase from mouse (EnogM), β-enolase from rabbit (EnobRab), and β-enolase from humans (EnobH). Conserved amino acid residues are indicated by asterisks.

Predominant in vitro B cell mitogenicity of p38

Figure 5 shows that the *in vitro* mitogenic effects of p38 on pure B murine splenic lymphocytes evaluated at day 2 by proliferative responses and at day 3 by the generation of PFC were at the same level of LPS-stimulated B cells. In contrast, only a small proliferation was detected in cultures of almost pure T cells (contaminated with 2% of B lymphocytes) when stimulated with p38. Moreover, this proliferative effect seems

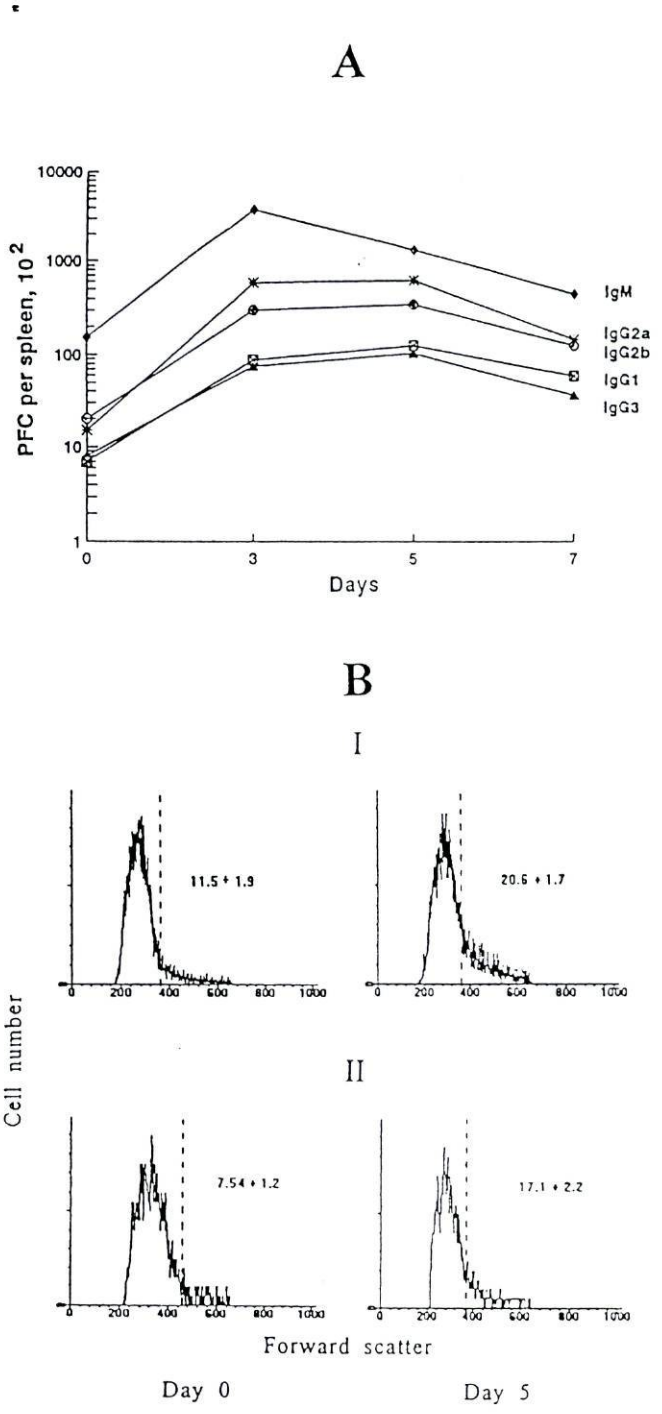

Fig. 4. *In vivo* lymphocyte mitogenic effect of p38. (A) Numbers per spleen of non-specific Ig-secreting PFC of the indicated isotypes observed in C57BL/6 mice, on indicated days after p38 treatment. The results are means of quadruplicated samples. As the SD were never >20% and in 90% of the cases were <10% of the mean of the PFC assays, they were omitted for simplicity. In this and Fig. 6, p38 treatment was performed by an i.p. injection of 50 μg of ISM protein. (B) Number of splenic non-specific IgM- and IgG-secreting PFC and cell size distribution, assessed by forward light scatter, of IgM^+(I) and $CD4^+$(II) lymphocytes in controls (Day 0) and 5 days after p38 treatment (Day 5). The absolute numbers were: (1) $65.6\times10^6 \pm 3.32\times10^6$ and $90.4\times10^6 \pm 5.6\times10^6$ for total numbers of IgM^+ cells observed on day 0 and on day 5 respectively (significant difference, $P < 0.01$), (2) $10.0\times10^6 \pm 1.84\times10^6$ and $27.1\times10^6 \pm 2.8\times10^6$ for the numbers of large IgM^+ cells observed on day 0 and on day 5 respectively (significant difference, $P < 0.01$), (3) $22.4\times10^6 \pm 0.57\times10^6$ and $25.0\times10^6 \pm 1.27\times10^6$ for total $CD4^+$ cells observed on day 0 and on day 5 respectively (significant difference, $P < 0.05$), and (4) $0.8\times10^6 \pm 0.2\times10^6$ and $2.0\times10^6 \pm 0.2\times10^6$ for the numbers of large $CD4^+$ cells observed on day 0 and on day 5 respectively (significant difference, $P < 0.01$). In this panel and in panel (B) of Fig. 5 large cell populations were delimited on the right side of the dashed line and the mean of the percentage of quadruplicated samples and SD of large cells are indicated. Also in this and in the following figures these are demonstrative experiments repeated at least three times.

to be due to the contaminating B cells since the amount of [^{3}H]thymidine uptake was proportional to that observed in p38-stimulated cultures of B lymphocytes. These results are in agreement with the flow cytometry observation of 3 day p38-stimulated cultures of unfractionated splenic cells. Although a large amount of B blasts was observed in these cultures, all T cells remained of small size (Fig. 5). However, late T cell stimulation, subsequent to B cell activation, also occurs since large (activated) CD4 cells were observed, also by flow cytometry, in cultures of unfractionated splenic cells 5 days after stimulation by p38 (Fig. 5). Subsequent T cell activation by B blasts is also reinforced by the lack of proliferation observed in cultures of T splenic lymphocytes 5 days after p38-stimulation (data not shown) and by the observation that cultures of unfractionated splenic cells (~50% of T and B lymphocytes) incorporated nearly the same amount of [^{3}H]thymidine as pure B cells cultures when stimulated with high amounts of p38 (Fig. 5).

The immunosuppressive effect of p38 is dependent on IL-10 production

As depicted in Fig. 6, a rapid increase in the serum levels of IL-10 was observed in C57BL/6 mice with peak responses as soon as 2 h after p38 treatment. The serum levels of this cytokine declined progressively, reaching the undetectable control levels 24 h after the ISM protein treatment (Fig. 6). In contrast, the serum levels of IL-2, IL-4 and IFN-γ were undetectable after p38 treatment, such as in the case of controls (data not shown). Figure 6 also shows that the immunosuppressive effect induced by p38 was abrogated in mice depleted of IL-10 but not of IL-4. No significant differences in the immune response to SRBC were observed in p38-untreated mice either depleted or not depleted of IL-10 or IL-4 (Fig. 6). IL-10 depletion did not induce any alteration in B lymphocyte polyclonal activation induced by p38 (Fig. 6).

Discussion

Herewith, we report that *S. sobrinus* produces p38, an ISM protein such as *S. intermedius*, *C. albicans* and ASFV (1,2,8). The heterogeneous group of microorganisms produces ISM proteins which are in biochemical and immunobiological terms relatively similar. The relative molecular masses of p38 and of the ISM proteins produced by ASFV (2), and by *C. albicans* (8) of 36 and 43 kDa respectively are rather close. Only the ISM protein produced by *S. intermedius* was reported to have a relative molecular mass of 90 kDa (p90) (44) and it cannot be excluded that this ISM protein is dimerized. Some degree of polymerization or adherence to irrelevant molecules cannot be excluded either with p38 since the purified protein is more anodal after two than after one PAGE. Similar protein complex

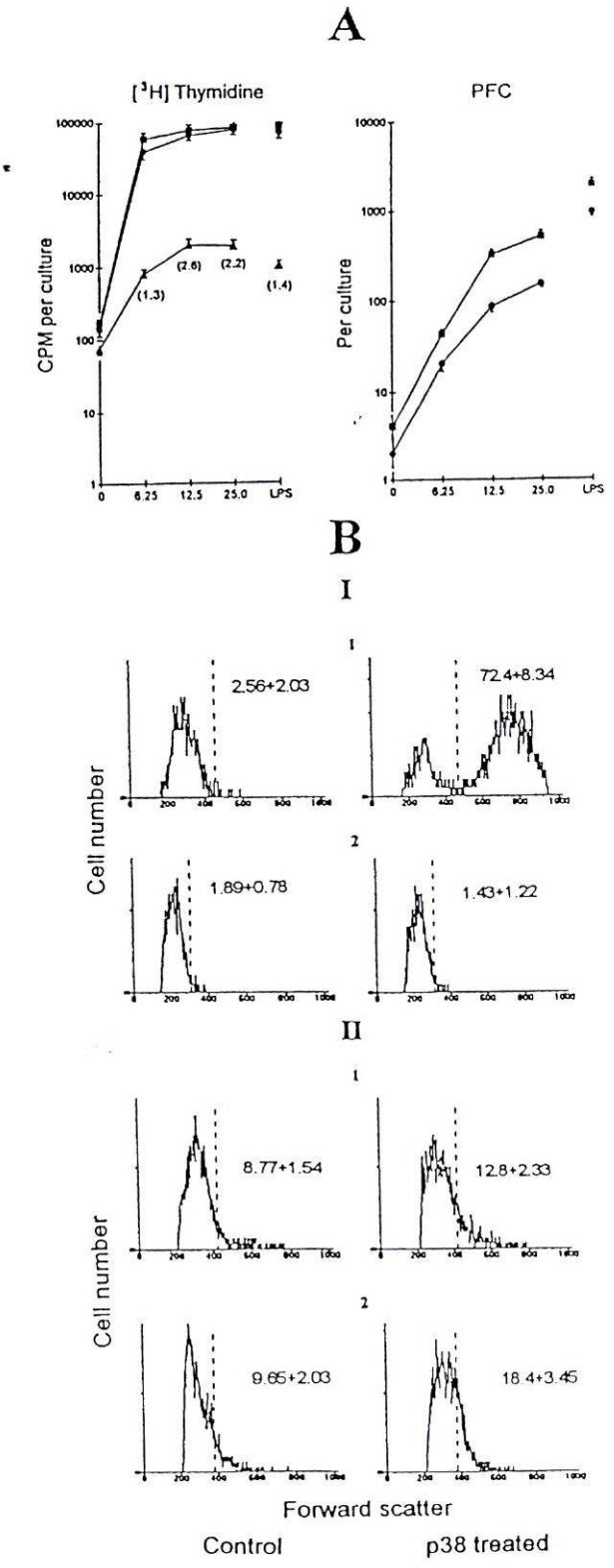

Fig. 5. Analysis of *in vitro* lymphocyte mitogenicity induced by p38. (A) Means and SD of [^{3}H]thymidine uptake observed after 2 days of culture of total (●), B (■) and T (▲) splenic lymphocytes and of the numbers of non-specific total Ig-secreting PFC observed after 3 days of culture of total (I) or B (n) splenic lymphocytes. Cultures were either unstimulated or stimulated by the indicated concentrations of LPS or p38 per mililitrel. The percentage of [^{3}H]thymidine uptake observed in cultures of enriched T lymphocytes population compared to that observed in B lymphocyte cultures is indicated between brackets. (B) Cell size distribution, assessed by forward light scatter of IgM$^+$ (1) and CD4$^+$ (2) cells observed 3 (I) and 5 (II) days after culture of total splenic lymphocytes unstimulated (control) or stimulated with 6.25 μg p38/ml.

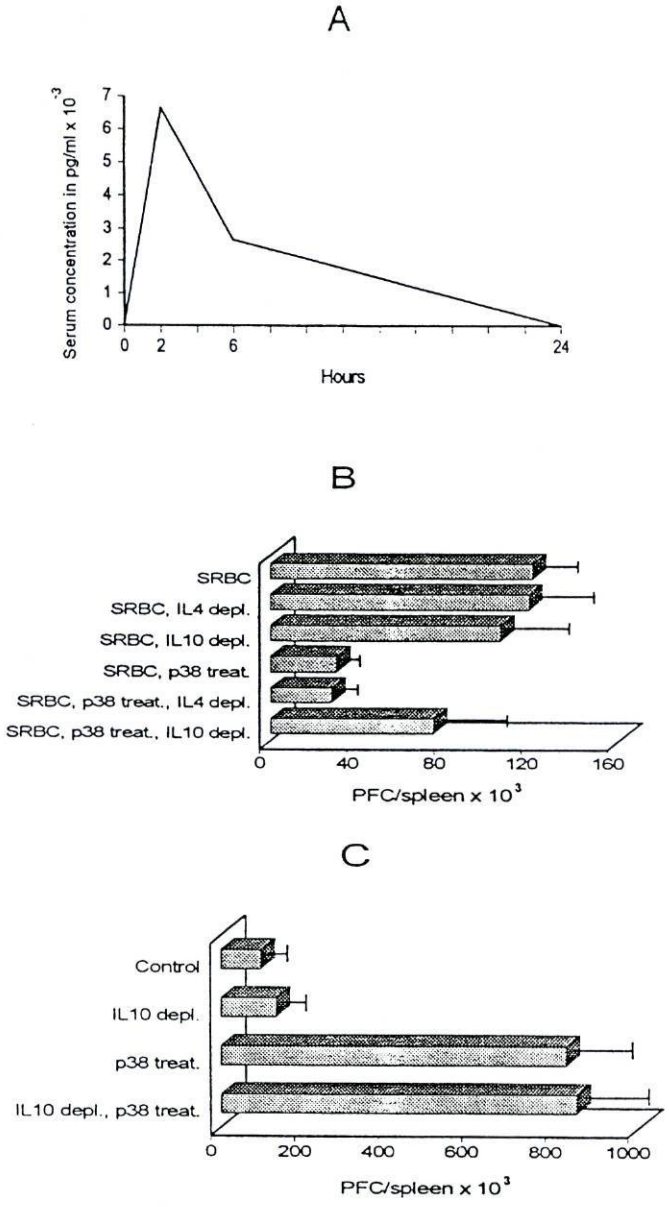

Fig. 6. Analysis of the serum levels and the effects of depletion of IL-10 and IL-4 upon p38 treatment. (A) The serum level of IL-10 observed in C57BL/6 mice before (control, 0 h) and on the indicated hours after i.p. p38 treatment. (B) Numbers of splenic specific anti-SRBC IgM-secreting PFC observed in mice 5 days after immunization with SRBC, either untreated or treated with p38 2 days before immunization and either non-depleted or depleted with anti-IL-10 and anti-IL-4 2 h before p38 treatment or 2 days before SRBC immunization. (C) Numbers of splenic non-specific Ig-secreting PFC observed in non-immunized mice untreated or 5 days after i.p. p38 treatment and depleted or undepleted with IL-10.

dissociation after electrophoresis was also ascribed to p90 (44). Also, all the reported ISM proteins have a low isoelectric point which is 4.25 for the ISM proteins produced by *S. intermedius* and *C. albicans* (8,44), 3.89 for the ISM protein produced by ASFV (2), and 4.75 for the now reported p38. Moreover, B cell polyclonal activation induced an isotype restriction pattern privileging IgG2a and IgG2b production, either after treatment with p38 or with the other ISM proteins described previously. However, similarity between the N-terminal sequence of p38 and the other ISM proteins has not been observed (16). Thus, the molecular structure responsible for the mitogenic effect on B cells of each ISM protein needs further clarification. The N-terminal sequence of p38 is quite similar to the N-terminus of various enolases, suggesting that they may belong to the same protein family. However, the immunobiological properties of this ISM protein are not due to mere enolase activity because no immunosuppressive activity was observed in mice treated with the rabbit enolase, which is one of those with a partially similar N-terminal sequence to p38.

An important increase in the survival of *C. albicans* (8) and of *S. intermedius* (13) was observed in mice when previously treated with the produced ISM protein, p43 and p90 respectively. These observations are probably due to the immunosuppressive effect of the ISM proteins which is quantitatively associated with lymphocyte polyclonal activation (2,4,8). An interplay between production of IL-10 and IL-4 or IL-10 alone, T_h2 cell immune response and progression of infectious diseases has been reported (24–33,45). As postulated before, (17,46) T_h2 responses, production of IL-10, subsequent immunosuppression and progression of infectious diseases may be elicited by B blasts induced by the ISM protein. The present report is in agreement with these assumptions.

The *in vitro* studies on T and B cell cultures stimulated by p38 indicate that this ISM protein is a direct B cell mitogen and does not display detectable direct stimulatory effects on T cells. However, as it was postulated before for other ISM proteins (17), T lymphocytes are subsequently stimulated by B blasts. Moreover, the suppressive effect of p38 was abrogated in IL-10-depleted mice. Furthermore, IL-10 was the single cytokine detected in serum of C57BL/6 mice after p38 treatment. The observation that increased levels of IL-10 in serum occurred very shortly after p38 treatment are in agreement with the need for depletion of this cytokine before or soon after infection in order to prevent microbial growth (29,33,46,47). As opposed to some reports indicating that IL-4 is also crucial for microbial invasion, p38 effects do not seem to be dependent on this cytokine. No increased serum levels of IL-4 were observed after treatment with the ISM protein and the immunosuppressive effect of p38 was not abrogated by IL-4 depletion.

In the near future we aim to identify the amino acid structure of p38 as well as of the other described microbial ISM proteins. We believe that the knowledge of these structures together with the investigation of other possibilities of preventing IL-10 release will provide us with the tools needed for an efficient immunointervention and immunoprotection.

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Abbreviations

ASFV	African swine fever virus
CEP-Ss	crude extracellular products of *S. sobrinus*
ISM	immunosuppressive and mitogenic
LPS	lipopolysaccharide
PIEF	preparative isoelectric focusing
PFC	plaque-forming cell
SRBC	sheep red blood cells

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CAPÍTULO 4. Discussão geral

A estrutura desta dissertação pelo facto de incluir trabalhos publicados ou submetidos para publicação, envolve a discussão em detalhe dos resultados experimentais feita em cada uma das publicações. Assim, penso ser redundante voltar a expor as interpretações que podem ser encontradas nas publicações apresentadas. Deste modo, o texto desta «Discussão» não pretende substituir-se aos componentes homónimos que fazem parte dos vários artigos originais incluídos nesta tese. Antes, pretendo aqui apresentar uma discussão geral integrando, de forma relativamente breve, o significado conjunto das conclusões que se encontram parcelarmente expressas em cada um dos artigos.

É clássicamente aceite que a imunoprotecção contra os microrganismos patogénicos se consegue por uma estimulação do sistema imune do hospedeiro induzida contra determinantes antigénicos (epítopes) microbianos. Assim, as tentativas de desenvolvimento de vacinas têm sido dirigidas para a imunização do hospedeiro com os microrganismos tornados atenuados ou, mais recentemente, a imunopotenciação do hospedeiro tem sido feita com antigénios microbianos purificados, por vezes expressos em vectores apropriados, com apresentação contínua detes epítopes ao sistema imune do hospedeiro. Contudo, várias observações indicam que esta estratégia clássica de vacinação não tem sido bem sucedida. Apenas um número muito limitado de vacinas antibacterianas ou antivirais tem-se revelado eficaz (1), comparativamente com o número elevado de microrganismos

reconhecidamente patogénicos. A acentuar este facto está a actual completa ausência de vacinas eficientes contra infecções por fungos, protozoários ou helmintas. Uma possível explicação para a falência das estratégias clássicas de vacinação é que, como se sabe, a hiperestimulação do sistema imune leva a um estadio de imunossupressão contrariando assim, a ideia militarista de resposta imune de que quanto mais células imunocompetentes e mais estimuladas mais eficaz será a resposta do hospedeiro contra os agentes infecciosos.

Os processos infecciosos no homem e nos modelos animais provocam vários efeitos imunológicos, dos quais apenas a resposta imune específica, para os antigénios dos parasitas, é normalmente considerada. Todavia, invariávelmente, a infecção é também acompanhada da resposta imune não específica e de imunossupressão. Estes dois efeitos, estimulação não específica e imunossupressão parecem estar relacionados. O estadio de imunossupressão do hospedeiro tem sido atribuído a excesso de antigénio, como foi proposto para as Micobacterioses (2) ou à hiperactivação de populações de monócitos/macrófagos, como foi indicado na Toxoplasmose (3,4,5), Leishmaníase (6,7) e Schistosomíase (8,9,10). Também o lipopolissacárido (LPS), existente na parede celular de bactérias Gram-negativas, induz uma estimulação linfocitária policlonal B, com subsequente imunossupressão (11,12). A estimulação linfocitária policlonal é observada em infecções por bactérias como *S. intermedius* (13,14,15,16), *S. mutans* (17), *S. sobrinus* (Trabalho 5), *M. avium* (18), por vírus (19,20,21,22), por protozoários

animais de controlo (Trabalho 1). Além disso, quando o *S. mutans* e a *C. albicans* são atenuados por repicagens sucessivas, tendo isto sido confirmado pela incapacidade de estabelecer infecção sistémica no hospedeiro, os microrganismos perderam a capacidade de segregar as respectivas proteínas ISM (30 e Trabalho 1). Mais importante ainda e confirmando que as proteínas microbianas ISM são factores de virulência, verificou-se que a imunização de murganhos com p43 conferia uma protecção total contra a infecção sistémica por *C. albicans* (Trabalho 2).

A estimulação policlonal e a imunossupressão são dois fenómenos imunológicos que, como se sabe, acompanham infecções por vários microrganismos e que parecem favorecer a sobrevivência dos microrganismos no hospedeiro. Assim, se a hiperestimulação do sistema imune pode facilitar a infecção, algumas alterações experimentais conducentes à sua diminuição devem contribuir para uma maior resistência dos hospedeiros às infecções. Efectivamente, com os nossos modelos de infecção experimental, verificámos que murganhos tratados com anticorpos monoclonais anti-$CD4^+$ ou ainda murganhos timectomizados na idade adulta, ou murganhos congenitamente atímicos apresentavam, quando infectados por via sistémica com *C. albicans* (Trabalhos 2 e 4), níveis de colonização consideravelmente inferiores comparativamente aos avaliados em animais de controlo. As células T γ/δ não parecem ser responsáveis pelo aumento da resistência à infecção sistémica com *C. albicans* observado em murganhos atímicos, uma vez que nestes animais a sua deplecção específica não aumenta a susceptibilidade à infecção

pelo fungo (Trabalho 4). Também na infecção sistémica por *C. albicans* em murganhos imunodeficientes com uma mutação *xid,* que são desprovidos de células B $CD5^+$, e que têm cerca de metade do número normal de células B com consequente diminuição do número de imunoglobulinas circulantes, observámos um aumento da resistência comparativamente a animais imunocompetentes (resultados não publicados). Muito interessante foi a verificação de que os efeitos imunobiológicos das proteínas ISM, nomeadamente a resposta linfocitária policlonal e a imunossupressão se encontravam diminuídas nos murganhos imunodeficientes (Trabalhos 2 e 4). Particularmente relevante foi o facto de nos murganhos congénitamente atímicos, o tratamento com a p43 induzir um aumento da resposta a antigénios T dependentes, como os SRBC (resultados não publicados) e a células de *Candida* mortas pelo calor (Trabalho 4), em vez de um estado de imunossupressão como se verificou nos animais controlo imunocompetentes. Estes resultados sugerem que a produção exagerada de imunoglobulinas, em consequência da estimulação policlonal das células B, é importante na interacção micróbio-hospedeiro pois está correlacionada com a sobrevivência dos microrganismos no hospedeiro e é dependente das células T. Como se sabe, uma das características da activação policlonal das células B com produção de imunoglobulinas na resposta às infecções, é a sua dependência das células T $CD4^+$ (31).

Com o objectivo de obter mais informação sobre os mecanismos moleculares de acção destas proteínas ISM estudámos a expressão de um

marcador precoce de activação celular a proteína CD69, em células T e B de murganhos estimulados com as proteínas ISM p36 e p43 e com o LPS (Trabalho 3). Os resultados sugerem que estes mitogénios actuam directamente nas duas sub-populações de células T, $CD4^+$ e $CD8^+$. Em estudos efectuados com as populações celulares T e B puras ou com as duas populações misturadas, verificámos que não havia expressão do marcador CD69 nestas populações. A expressão do marcador à superfície dos linfócitos depende da presença de macrófagos na cultura. A avaliação das citocinas possívelmente responsáveis pelo efeito estimulador dos macrófagos sobre os linfócitos, permitiu excluir as citocinas IL6, IL10 e TNF-α. Os estudos em curso no nosso grupo de trabalho sobre a pesquisa de receptores nas células do sistema imune para as proteínas ISM, e sobre a clonagem das proteínas p43 e p38 irão, certamente, elucidar os mecanismos moleculares de acção destas proteínas e permitirão avaliar as homologias existentes entre elas e que lhes conferem actividades biológicas semelhantes.

Foi demonstrado recentemente que as citocinas presentes durante a activação das células T podem determinar o fenótipo que estas células irão apresentar em resposta a um antigénio (32). Por exemplo, a presença de IL 12, produzida por macrófagos e células dendítricas (33,34,35) leva, normalmente, ao desenvolvimento de clones do tipo Th1, com grande produção de IFN-γ (36,37,38), enquanto que a presença *in vivo* de citocinas produzidas por clones do tipo Th2, antes ou durante a administração do antigénio, leva à expansão de linfócitos Th2 (39). Verificámos que o tratamento

sistémico de murganhos convencionais C57BL/6 com as proteínas ISM, p43 e p38 induz um pico sérico da citocina IL10 e no caso da p43 também de IL4, embora com valores menores, 2 horas após o tratamento (Trabalhos 4 e 5). Um aumento dos níveis séricos das duas citocinas foi também observado por nós, na infecção sistémica, dos murganhos referidos, com *C. albicans* (Trabalho 4). A produção destas citocinas quer pela administração das proteínas ISM quer pela infecção com os microrganismos vai, necessariamente, condicionar a regulação das células Th. Resultados semelhantes são descritos com o tratamento com LPS *in vivo* (40) em que se registam valores máximos de IL10 no soro, 90 minutos após o tratamento. Vários estudos *in vitro* e *in vivo* evidenciam a importância da produção precoce de citocinas na diferenciação das células Th «naive». Nas candidíases experimentais, o desenvolvimento da infecção é condicionado, de forma significativa, pela expansão das células Th1 protectoras ou das células Th2 que estão ligadas à progressão da doença. Assim, a população Th1 expande-se nos murganhos BALB/c (41), C57BL/6 (42) e CD2F1 (43) quando estes são infectados por via sistémica com uma estirpe de *C. albicans* atenuada, enquanto que há uma expansão da população Th2 quando os murganhos são infectados com uma estirpe virulenta (44). Mais especificamente, as citocinas libertadas em consequência do contacto inicial da estirpe patogénica do fungo com os macrófagos do hospedeiro, exercem uma função determinante na diferenciação e na selecção da subclasse de células Th. Assim, murganhos pouco resistentes à infecção pelo fungo tornam-se resistentes se forem

tratados com anticorpos anti-IL4 (44,45) ou anti-IL10 (46), simultâneamente com a infecção. Pelo contrário, a administração de anticorpos anti-IFN γ (43) ou anti-IL12 (47) simultâneamente com a infecção com uma estirpe atenuada, leva à diferenciação de células Th2 e a uma resposta não protectora para o hospedeiro. A importância das citocinas IL4 e IL10 na candidíase ficou claramente demonstrada quando a administração das citocinas a murganhos resistentes a infecção com estirpes virulentas os tornou susceptíveis à infecção com uma estirpe atenuada (48). O aumento dos níveis séricos de IL10 observados imediatamente após a administração das proteínas ISM p43 e p38 pode explicar o aumento da colonização observado quando os murganhos são tratados com a proteína p43 2 dias antes da infecção com o microrganismo que a produz (Trabalho 1).

Esta correlação entre proteínas ISM, produção de IL10 e aumento da susceptibilidade do hospedeiro aos microrganismos que as produzem é bem evidenciada nos murganhos imunodeficientes que se mostraram resistentes à infecção com *C. albicans*. Assim, após o tratamento com a p43, o doseamento sérico de IL10 nos murganhos congenitamente atímicos e nos murganhos timectomizados na idade adulta (Trabalho 4) e ainda, nos murganhos da estirpe C57BL/10 ScCr (B10) (Observações não publicadas) apresentou valores significativamente inferiores aos observados nos murganhos imunocompetentes. A deficiente produção de IL10 está relacionada com a diminuição dos efeitos imunobiológicos da p43 e com o aumento da resistência à infecção com *C. albicans* observada nos murganhos imunodeficientes

(Trabalho 4). Estes resultados foram confirmados pelos estudos de depleção da IL10, com anticorpos monoclonais, em murganhos convencionais. A depleção de IL10, simultâneamente com o tratamento com a p43, abolia os efeitos imunobiológicos da proteína p43, nomeadamente, o seu efeito imunossupressor e a capacidade de induzir susceptibilidade à infecção pelo fungo (Trabalho 4). Pelo contrário, a depleção de IL4 não alterou nem os efeitos imunobiológicos da p43 nem o efeito potenciador da infecção. Embora a IL4 seja detectável no soro após tratamento dos murganhos com p43 ou na infecção fúngica, os resultados sugerem que, pelo menos na estirpe de murganhos C57BL/6, altamente resistente à infecção pelo fungo, a IL10 é a citocina mais importante para o desenvolvimento da infecção. Os nossos resultados confirmam os de outros (46) ressaltando a ideia de um papel dominante da IL10 nas candidíases. Resultados semelhantes são referidos nas infecções murinas por *Leishmania major* (49) em que a IL4 não é suficiente para modificar o curso da infecção nos murganhos altamente resistentes ao agente infeccioso.

Os nossos resultados referentes ao papel da IL10 nas candidíases estão de acordo com o conceito prevalente na literatura científica que afirma que a produção precoce de IL10 nas infecções favorece a sobrevivência dos microrganismos ao suprimir a actividade microbicida dos fagócitos e a função dos macrófagos como células apresentadoras de antigénio. É também já clássico o conhecimento que a IL10 inibe a secreção de IFN γ e de IL2 pelas células Th1 e a produção de IL1α, IL1β, TNFα, IL6 e GM-CSF pelos

macrófagos e por outras células apresentadoras de antigénios (50,51,52,53,54,55,56). Além disso, esta citocina inibe a citoxicidade dos macrófagos e suprime a produção de óxido nítrico e a actividade antifúngica dos macrófagos murinos contra *C. albicans* (57,60). Todas estas funções associadas à IL10 podem estar, portanto, envolvidas na modelação da resposta do hospedeiro às micoses. Em estudos com *Aspergillus fumigatus*, fungo causador de aspergiloses sistémicas em doentes imunodeficientes, verificou-se que a IL10 aumenta a actividade fagocítica dos macrófagos, que ingerem os conídios do fungo mais rápidamente e em maior número do que os fagócitos dos animais de controlo, enquanto que suprime a actividade microbicida (58). Assim, os microrganismos que se multiplicam no interior do macrófago serão ajudados pela IL10 na sua entrada no macrófago o qual, por outro lado, está diminuído na sua actividade microbicida, nomeadamente no que se refere à produção do anião superóxido e de óxido nítrico (59,60,61,62). Esta situação permitirá a evolução da infecção para um estado crónico e, como se sabe, situações de infecção crónica progressiva devidas a *Aspergillus sp.* são frequentes em doentes infectados com HIV (63) em que há um aumento da produção de IL10 (64). A importância da citocina IL10, no desenvolvimento da infecção por microrganismos, foi também demonstrada nas infecções por *L. monocytogenes* em murganhos deficientes em IL10, em que se verificou um aumento da resistência à infecção, com aumento da resposta inflamatória (65).

Os resultados obtidos nos murganhos imunodeficientes (Trabalho 4), quer quanto aos efeitos imunobiológicos da proteína ISM, quer quanto à

infecção com o microrganismo que a produz põem em evidência o papel desempenhado pela IL10 na acção imunomodeladora da p43 e na infecção com *C. albicans*. Nos animais privados de células tímicas, murganhos timectomizados na idade adulta ou congénitamente atímicos, a estimulação policlonal induzida pela p43 estava considerávelmente diminuída, e não se verificou efeito imunossupressor induzido pela p43, havendo, pelo contrário, estimulação da resposta imune contra as células de *Candida* mortas pelo calor, ao invés da imunossupressão verificada nos animais controlo. Além disso, aqueles dois grupos de animais imunodeficientes mostraram-se considerávelmente mais resistentes à infecção por *C. albicans*. A acrescentar ainda que os níveis séricos da citocina IL10 após tratamento com p43 ou após infecção sistémica com o fungo, eram considerávelmente inferiores aos observados nos animais controlo. Estes resultados evidenciam uma correlação entre células tímicas ou células que emigraram recentemente do timo e a libertação/produção de IL10 o que está de acordo com observações que sugerem que o desenvolvimento de células reguladoras/imunossupressoras é dependente da existência de um timo intacto (66). Além disso, em animais que desenvolvem colite devido à existência de um timo anormal, a infecção pode ser controlada pela administração de IL10 (67), sendo atribuído às células que emigraram recentemente do timo um papel importante na produção de citocinas (68,69,70,71). Os nossos resultados evidenciam uma correlação entre células tímicas ou que migraram recentemente do timo, proteínas ISM, produção de IL10 e susceptibilidade à infecção.

Em estudos de infecção por *Toxoplasma gondii*, de murganhos «knock out» para a IL10, verificou-se que os animais imunodeficientes morriam mais rapidamente do que os animais de controlo devido a uma resposta exacerbada do tipo Th1, com níveis séricos de TNF-α, IFN-γ e IL12 muito elevados, atribuíndo-se à IL10 um papel regulador de resposta inflamatória exagerada de consequências nefastas para o hospedeiro (72). No Trabalho 4 verificámos que a deplecção de IL10, simultâneamente com o tratamento com a p43, originou a morte de cerca de 50% dos animais, o que não se verificou quando os animais foram depletados de IL10 e de TNF-α simultâneamente. No entanto, é de realçar que na infecção por *C. albicans* de murganhos deficientes de IL10 não será de esperar um resultado tão dramático, dado que a *Candida*, como as bactérias, por exemplo *L. monocytogenes* (65), fazem infecções localizadas, com formação de granulomas, não originando respostas inflamatórias exageradas como os protozoários *T. gondii* e *T. cruzi.*

Os nossos resultados demonstram de forma clara a importância das proteínas ISM no desenvolvimento da resposta imune do hospedeiro e, consequentemente, no desenvolvimento das infecções com os microrganismos que as produzem. Sendo assim, a imunização dos hospedeiros com estas proteínas deve induzir protecção contra os efeitos imunobiológicos por elas produzidos e aumentar a resistência à colonização com os microrganismos. Efectivamente, a imunização dos hospedeiros com a proteína ISM p43 reduziu de forma significativa o efeito mitogénico da p43, excepto para o isotipo IgG1, em que houve um aumento significativo, sendo estes anticorpos específicos

para a p43 (Trabalho 2) e conferiu uma protecção total contra a infecção sistémica por *C. albicans* (Trabalho 2). A imunização com a proteína ISM p38 conferiu uma protecção parcial contra a infecção sistémica por *S. mutans* (73). A protecção contra a infecção por *C. albicans* pode ser transferida pelo soro de murganhos imunes com título elevado de anticorpos anti-p43. Por outro lado, verificámos que a imunização contra antigénios totais do fungo (sonicado de células de *C. albicans*) não confere protecção contra a infecção e que a transferência do soro, o título elevado de anticorpos anti-sonicado, não induz protecção contra o fungo, nos animais recipientes (Trabalho 2). Além disso, a imunização com a p43 mostrou especificidade para a *C. albicans,* já que não resultou eficaz na protecção à infecção sistémica por *L. monocytogenes* (Trabalho 2). O nosso modelo de imunização vem sugerir fortemente que a imunopotenciação do hospedeiro com antigénios microbianos estruturais, uma forma clássica de imunizar, não parece ser uma boa estratégia de vacinação. Resultados semelhantes foram descritos em murganhos imunizados contra *T. gondii*, com títulos de anticorpos contra o parasita elevados, em que os animais se mostraram mais susceptíveis à infecção com o protozoário do que os animais não imunizados (74); ou na imunização com constituintes antigénicos de *L. major* em que se verificou um agravamento da doença nos murganhos imunizados (75).

Estratégias de vacinação idênticas à seguida por nós, ou seja, utilizando como agente imunogénico factores de virulência, começam a ser usadas com vários microrganismos como *Mycobacterium tuberculosis* com

proteínas segregadas pela bactéria (76,77,78), *Bordetella pertussis* com uma proteína de membrana, a p69 que só se encontra nas estirpes virulentas (79,80,81) e *S. pneumonia* com uma proteína de membrana, a qual é essencial para a virulência da bactéria (82,83,84).

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CAPÍTULO 5. *Conclusões finais*

Atendendo à forma como a tese se encontra organizada, parece-me importante fazer a apresentação das principais conclusões obtidas a partir da interpretação do trabalho experimental que foi desenvolvido neste programa de dissertação.

A capacidade imunomodeladora da *C. albicans* pela produção de uma proteína, p43, imunossupressora e mitogénica (ISM) do sistema imune do hospedeiro é um fenómeno importante da interacção hospedeiro-parasita que ocorre nas infecções por este fungo. O efeito imunossupressor induzido pela proteína ISM não é devido a citotoxicidade celular nem a indução de apoptose.

A produção de proteínas do tipo das ISM parece ser um mecanismo comum a vários microrganismos, nomeadamente a vírus como o da peste suína Africana que produz a p36, a bactérias como *S. sobrinus,* que produz a ISM p38, e a fungos como a *C. albicans* que produz a p43, o que poderá significar uma adaptação evolutiva dos microrganismos aos hospedeiros em que estão inseridos com vista a aumentar a sua sobrevivência no hospedeiro. É de realçar que as proteínas ISM tenham efeitos imunobiológicos identicos e sejam bioquímicamente muito semelhantes. Estas proteínas ISM podem ser consideradas como factores de virulência dos microrganismos que as produzem, uma vez que contribuem para a sua sobrevivência no hospedeiro.

A modelação do sistema imune do hospedeiro pela proteína p43 da *C. albicans* envolve a activação sequencial dos linfócitos B e T $CD4^+$ e $CD8^+$, dependendo da presença de macrófagos. Esta activação leva à produção exagerada de imunoglobulinas não específicas pelas células B e à produção de IL10 e de IL4, presumívelmente por linfócitos Th2, muito precocemente após a sua administração. Após a infecção com a estirpe de *C. albicans* produtora de p43, há igualmente um aumento da produção de IL10 que é detectado precocemente no soro dos murganhos infectados.

A produção precoce e exagerada de IL10 induzida pela p43, leva a um estado de supressão do hospedeiro no que diz respeito a antigénios T dependentes (ou seja antigénios que requerem uma resposta do tipo Th1) como é o caso dos antigénios de SRBC ou das células de *C. albicans* mortas pelo calor.

Os efeitos imunobiológicos da proteína p43 encontram-se consideravelmente diminuídos em murganhos timectomizados na idade adulta e em murganhos congenitamente atímicos que apresentam níveis séricos de IL10 significativamente menores do que nos murganhos eutímicos, o que poderá ser explicado pela ausência de timócitos naqueles dois grupos de animais. Os animais atímicos apresentam um aumento da resistência à infecção com *C. albicans*, uma diminuição dos níveis séricos de IL10 após a infecção, sugerindo uma correlação entre a produção de IL10, induzida pela p43, e a susceptibilidade à infecção por *C. albicans*.

As proteínas ISM podem ser utilizadas como potenciais vacinas uma vez que, a imunização do hospedeiro com estas proteínas induz um aumento da sua resistência a subsequentes infecções com os microrganismos que as produzem. A protecção induzida pela p43 da *C. albicans* traduz-se num aumento de anticorpos específicos contra a p43, numa diminuição dos efeitos mitogénico e imunosssupressor da p43 nos animais imunizados e tem alguma especificidade para *C. albicans*, uma vez que não se verificou eficaz na protecção contra infecção endovenosa por *Listeria monocytogenes*.

A interpretação mais sugestiva destes resultados reside na possibilidade de a *C. albicans* através da proteína ISM que produz e excreta é capaz de induzir uma resposta preferencialmente Th2 que, pelo menos nos murganhos altamente resistentes à infecção fúngica, leva à produção/libertação de IL10, de origem tímica ou de células tímicas emigrantes recentes. Esta libertação/produção de IL10 conduz a uma supressão de macrófagos, neutrófilos e linfócitos Th1.

RESUMOS

RESUMO

Em estudos utilizando sobrenadantes de culturas de uma estirpe virulenta de *C. albicans*, isolámos uma proteína de peso molecular de aproximadamente 43 kDa, com N-terminal sem homologia com qualquer proteína conhecida. A sua actividade imunomodeladora foi avaliada *in vivo*. O tratamento de murganhos com esta proteína induz um aumento do número de células B esplénicas produtoras de imunoglobulinas e suprime especificamente a resposta primária contra antigénios T dependentes. Verificámos que quando esta proteína é administrada ao hospedeiro antes da infecção sistémica com *C. albicans,* há um aumento significativo da susceptibilidade à infecção. As características mitogénicas e imunossupressoras da proteína p43, estão directamente relacionadas com a capacidade de induzir susceptibilidade às infecções pelo fungo. A imunização de murganhos BALB/c com a proteína p43, neutraliza parcialmente os efeitos imunomodeladores da p43 e protege o hospedeiro contra subsequente infecção sistémica com o fungo. Os efeitos imunobiológicos da p43, estão altamente correlacionados entre si e estão dependentes da produção de IL-10 que é libertada/produzida pelos timócitos, ou células emigrantes recentes do timo, imediatamente após o tratamento dos murganhos com a p43 ou após a infecção dos animais com o fungo. Em hospedeiros atímicos, congenitamente ou por timectomia, a capacidade infectante de *C. albicans* e os efeitos imunobiológicos da p43 encontram-se diminuídos, assim como a capacidade de induzir a libertação/produção de IL10.

ABSTRACT

We have isolated a protein of approximately 43kDa (p43) molecular weight out of supernatants of cultures of a virulent strain of *C. albicans* and evaluated its immunomodulatory effects *in vivo*. No homology of the N-terminal of p43 with other already known proteins was found. Increase in the numbers of immunoglobulin-producing B cells and specific suppression of primary response against T dependant antigens were observed in mice treated with p43. We also observed that p43 treatments before *C. albicans* infection markedly increased kidney fungal colonization. The mitogenic and immunosuppressive properties of p43, are closely related with its ability of inducing susceptibility to infection by the producing fungus. Immunization of BALB/c mice with p43 partially neutralizes the immunomodulatory effects of p43 and protects the host from any subsequent fungal systemic infection. All the immunobiological effects of p43 are highly correlated with each other and dependant on the IL-10 released/produced by the thymocytes or new emigrant cells from the thymus. The *C. albicans* virulence and the immunobiological effects of p43 are abrogated in congenitally athymic or thymectomized hosts as well as the release/production of IL-10.