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Efeito da desnutrição experimental na resposta imune induzida por BCG e DNAhsp65

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"Cada dia a natureza produz o suficiente para nossa carência. Se cada um tomasse o que lhe fosse necessário, não haveria pobreza no mundo e ninguém morreria de fome."

Mahatma Gandhi

"O que há de maravilhoso numa casa não é ela abrigar-nos, nem aquecer-nos, nem nós possuirmos as suas paredes; o que é maravilhoso é ela ter depositado em nós estas provisões de doçura, é ela formar, no fundo do nosso coração, este maciço obscuro, donde brotam, como águas de uma fonte, os sonhos…"

Saint-Exupéry, Terra dos Homens

Esta dissertação é dedicada aos meus pais, Kenji e Tomy, e à minha querida irmã Mari, que, mesmo distantes, são a fonte dos meus sonhos e minha força interior. Obrigada por todo amor, carinho, força e oração, e por acreditarem em mim. Dedico a vocês não só este trabalho, mas a minha vida.

"Entrega os teus caminhos ao Senhor, confia nele, e o mais ele fará."

Salmo 37:5

Primeiramente, a Deus, por cada amanhecer.

"Ser professor é importar-se com o outro numa dimensão de quem cultiva uma planta muito rara que necessita de atenção, amor e cuidado; é ter a capacidade de 'sair de cena, sem sair do espetáculo'; é apontar caminhos, mas deixar que o aluno caminhe com seus próprios pés..."

Autor desconhecido

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"A família não nasce pronta; constrói-se aos poucos, e é o melhor laboratório do amor. Em casa, entre pais e filhos, pode-se aprender a amar, pode-se experimentar com profundidade a grande aventura de amar sem medo."

Autor desconhecido

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Autor desconhecido

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"Cada pessoa que passa em nossa vida, passa sozinha. É porque cada pessoa é única e nenhuma substiui a outra. Cada pessoa que passa em nossa vida passa sozinha e não nos deixa só porque deixa um pouco de si e leva um pouco de nós. Essa é a mais bela responsabilidade da vida e a prova de que as pessoas não se encontram por acaso."

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"Unir-se é um bom começo, manter a união é um progresso, e trabalhar em conjunto é a vitória."

Henry Ford

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ÍNDICE

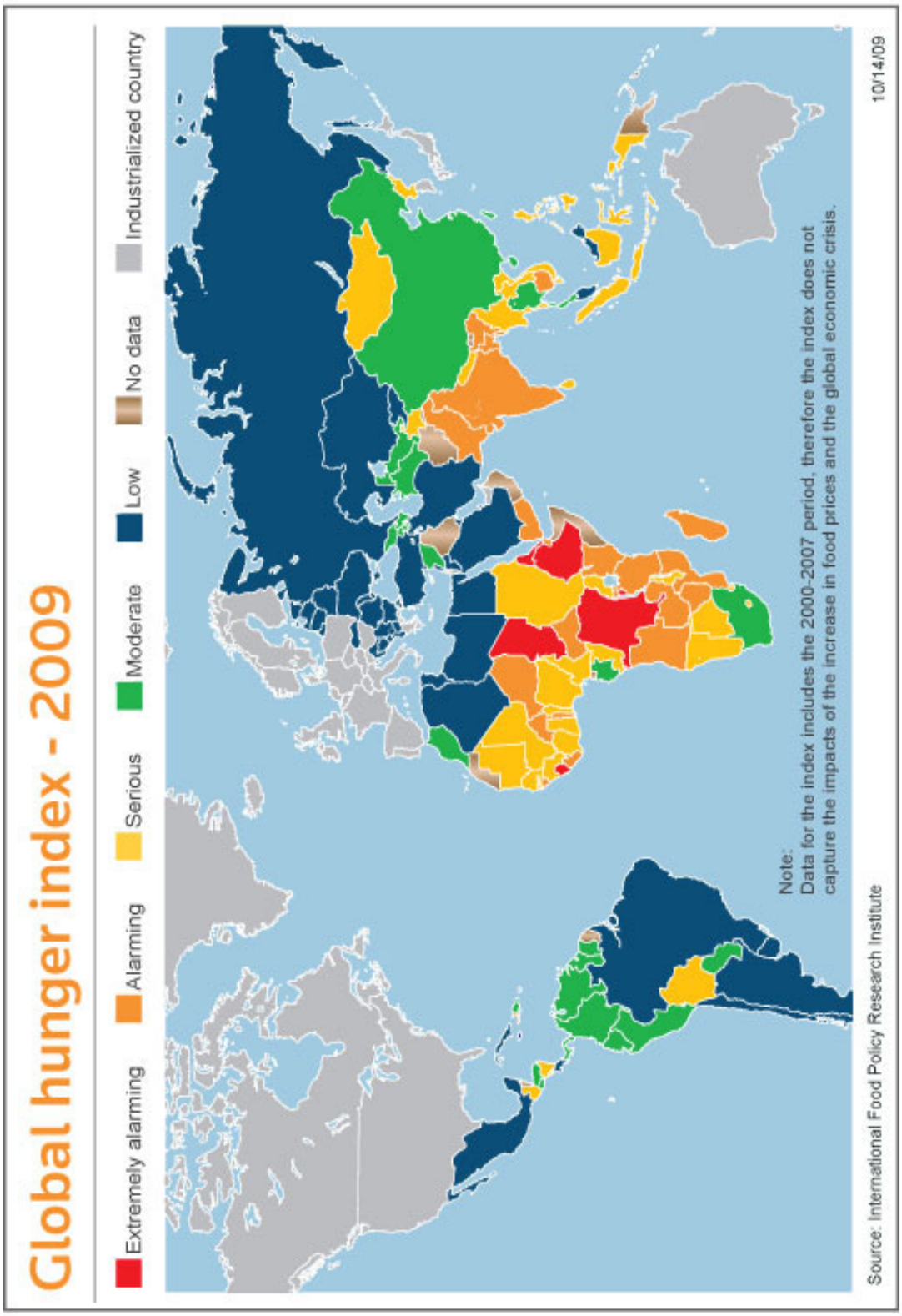
1. INTRODUÇÃO

1.1. Aspectos gerais da desnutrição

A desnutrição é uma das principais causas de morbidade e mortalidade no mundo e acomete cerca de 800 milhões de pessoas, sendo 20% destas, pertencentes a países em desenvolvimento. No Brasil, embora a prevalência da desnutrição infantil tenha caído nas últimas décadas, o percentual de óbitos por desnutrição grave em nível hospitalar, se mantém em torno de 20%, muito acima dos valores recomendados pela OMS (inferiores a 5%) (Monteiro et al., 2009). Diversos fatores sócio-econômicos contribuem para o aumento da desnutrição. A pobreza e a falta de condições sanitárias adequadas são sem dúvida os principais fatores, contribuindo para a maior prevalência da doença em países menos desenvolvidos (Schaible & Kaufmann, 2007). A figura 1 mostra os dados referentes à situação atual da desnutrição no mundo, publicados pela Thomson-Reuters Foundation.

Nos países bem desenvolvidos, os indivíduos desnutridos consomem cerca de 130 Kcal a menos por dia do que os bem nutridos. Já em países subdesenvolvidos, esse déficit pode chegar a 450 Kcal por dia. Nos lugares onde o déficit calórico é extremamente alto, a deficiência nutricional inclui todo tipo de alimento, enquanto que nos países onde a desnutrição é mais moderada, a deficiência nutricional se deve, principalmente, à falta de alguns alimentos que fornecem energia (FAO, 2004).

A desnutrição protéico-calórica (DPC) é o principal tipo de desnutrição no mundo. É usualmente complexa, envolvendo tanto uma restrição protéico-calórica quanto graus variados de deficiências em macro e micronutrientes tais como vitamina A, E, B_6, fosfato, zinco, ferro, cobre e selênio (Chandra, 1986; Thakur et al, 2004). Nos humanos, a DPC pode ser definida como marasmo ou kwashiorkor. O marasmo ocorre quando há insuficiência de todos os tipos alimentares, enquanto o kwashiorkor se desenvolve a partir de uma dieta deficiente em proteínas, podendo ser de elevado conteúdo calórico (Cuningham-Rundles et al., 2005).


Global hunger index - 2009
Extremely alarming
Alarming
Serious
Moderate
Low
No data
Industrialized country
Note:
Data for the index includes the 2000-2007 period, therefore the index does not capture the impacts of the increase in food prices and the global economic crisis.
Source: International Food Policy Research Institute
10/14/09

1.2. Desnutrição e resposta imune

As imunodeficiências decorrentes de defeitos genéticos ou ocorridos durante o desenvolvimento ontogenético são denominadas primárias. As secundárias ou adquiridas se referem àquelas em que a perda da função imunológica resulta de uma variedade de fatores extrínsecos. A causa mais conhecida de imunodeficiência secundária é a infecção pelo vírus da imunodeficiência humana (HIV), porém, a causa mais prevalente de imunodeficiência no mundo é a desnutrição grave a qual chega a afetar até 50% da população em países pobres. As alterações imunológicas resultantes da desnutrição afetam tanto a imunidade inata quanto a específica. Por exemplo, a disponibilidade de componentes do sistema complemento e a função fagocítica estão comprometidas na subnutrição e isto afeta diretamente a eliminação dos patógenos. Isto ocorre porque o sistema complemento pode destruir diretamente bactérias e vírus e também porque os receptores para fragmentos de componentes deste sistema, os quais se encontram na superfície das células fagocíticas, auxiliam na captura de patógenos. Tanto o nível de C3, que é o principal componente opsônico, quanto à capacidade dos fagócitos de internalizar e destruir patógenos encontram-se reduzidos em estados de desnutrição (Sakamoto et al., 1998; Chandra, 2002).

A desnutrição protéica grave está claramente associada com atrofia nos órgãos linfóides primários (medula óssea e timo) em recém-nascidos e crianças pequenas. As consequências são devastadoras porque estes órgãos são justamente os geradores do repertório de linfócitos B e T. A desnutrição afeta a hematopoiese, determinando anemia, leucopenia, diminuição acentuada da medula óssea e também redução na síntese de IL-6 e TNF-α por células da medula óssea (Fock et al., 2007). A atrofia do timo está associada com redução nítida no número de células presentes neste órgão e também afeta o desenvolvimento dos órgãos linfóides periféricos (Savino, 2002). A conseqüência imediata desta atrofia é a leucopenia, relação CD4/CD8 diminuída e aumento no número de células T imaturas no sangue periférico. A alteração ocorrida no timo tem sido estudada com maior detalhamento em modelos experimentais. Por exemplo, tem sido demonstrado que a atrofia é, em parte, causada pela depleção por apoptose que afeta principalmente células TCD4+ e TCD8+ imaturas (Ahima et al., 1996). Alterações morfológicas nas células epiteliais tímicas também têm sido descritas e associadas com menor produção de hormônios tímicos. A desnutrição também afeta de forma acentuada a resposta imune

associada com as barreiras epiteliais determinando, principalmente, achatamento e hipotrofia das vilosidades intestinais, redução no número de linfócitos presentes nas placas de Peyer e redução na secreção de IgA (Beisel, 1996).

A apresentação de antígenos aos linfócitos T é uma etapa fundamental durante a indução e regulação tanto da resposta imune humoral quanto celular (Mellmam & Steinman, 2001). Os três tipos celulares principais envolvidos neste processo de apresentação antigênica são os linfócitos B, os macrófagos e as células dendríticas (CD) sendo que as últimas são estudadas de forma mais sistemática durante a desnutrição. Vários estudos mostram que, de forma geral, a atividade biológica das diferentes células apresentadoras de antígenos está claramente comprometida durante as deficiências nutricionais (Redmond et al., 1991; Petro et al., 1994; Honda et al., 1995; Staplenton et al., 2001). Existe na literatura um consenso de que diferentes aspectos, tais como número de CD, produção de citocinas e capacidade de desencadear proliferação de linfócitos T de memória estejam significativamente afetadas durante a subnutrição (Abe et al., 2003). Recentemente, foi demonstrado que a transferência adotiva de CD restaurou a resposta imune celular em camundongos desnutridos (Hillyer et al., 2008).

Existe também uma concordância na literatura de que déficit protéico, energético ou ambos, determinam imunossupressão mais acentuada na resposta imune celular do que na humoral. Por exemplo, os níveis de imunoglobulinas (Igs) tipo Th2 (IgG1 e IgE) encontram-se elevados em camundongos submetidos à desnutrição aguda enquanto que os níveis das Igs do tipo Th1 (IgG2a e IgG3) não foram afetados (Neyestani & Woodward, 2005). Resultados similares foram descritos em modelo experimental de deficiência de vitamina A no qual foi constatado, após exposição inicial ao antígeno, aumento significativo de células T (Th2 ou T reguladoras) produtoras de IL-10 associado a uma queda no desenvolvimento de células Th1 de memória (Stephensen et al., 2004). Recentemente, Sakai et al. (2006), corroboraram com este tipo de resultado, demonstrando que a deficiência protéica diminuiu a proliferação de células T específicas mas não a ativação de células B específicas em camundongos inoculados com vacina gênica.

O processo de ativação dos linfócitos T parece ser um dos principais mecanismos que impedem a ativação completa da imunidade durante a desnutrição. Está bem estabelecido que os canais de potássio são vitais para a ativação de células T. Neste sentido, Fernández et al. (2005), relataram alterações significativas neste processo em linfócitos de ratos desnutridos. Também tem sido constatado que ratos desnutridos apresentam subpopulações reduzidas de linfócitos TCD3+ e TCD4+ juntamente com

redução na expressão de moléculas associadas com o processo de ativação e proliferação como é o caso de CD25 e CD71 (Cortés-Barberema et al., 2008). Esta ativação comprometida de células T tem sido claramente associada com baixa produção de citocinas, as quais são os principais mediadores moleculares da imunidade. Isto ficou claramente evidenciado em crianças desnutridas nas quais ocorreu redução acentuada na produção de citocinas do tipo Th1 (IL-2 e IFN-γ) (Rodríguez et al., 2005).

1.3. Desnutrição e Infecção

A relação entre desnutrição e infecção foi inicialmente descrita por Scrimshaw et al. (1990). A partir deste trabalho foram realizadas várias investigações que comprovaram de forma definitiva um maior índice de mortalidade ocasionada por infecções em crianças desnutridas. As doenças infecciosas de maior incidência em crianças subnutridas são tuberculose, sarampo, parasitoses intestinais e malária (Enwonwu et al., 2006). O estudo realizado por Man et al. (1998), o qual incluiu um número bastante elevado de crianças hospitalizadas, ilustra de forma evidente a incidência maior de mortalidade por doenças infecciosas em crianças com baixo peso.

Um terço da população mundial está infectada com o *Mycobacterium tuberculosis* e esta é considerada a causa mais importante de morte por agentes infecciosos (Young & Duncan, 1995; Flynn & Chan, 2001). Esta infecção é particularmente influenciada pela desnutrição e é a principal causa de morbidade e mortalidade nos países em desenvolvimento nos quais a DPC é também prevalente (Udani, 1994). Dados em modelos experimentais corroboram com este achado em populações humanas (Cegielski & McMurray, 2004). Um trabalho bastante recente indicou que baixos níveis séricos de vitamina D estavam associados com maiores riscos de desenvolvimento de tuberculose ativa (Nnoaham & Clarke, 2008). Um aspecto interessante no caso desta infecção é que a evolução da mesma é caracterizada por um processo inflamatório crônico que acentua a desnutrição causando um quadro típico de caquexia (Cegielski & McMurray, 2004).
Recentemente surgiu a hipótese de que a desnutrição contribui, juntamente com um esquema terapêutico mal implementado e co-infecção pelo HIV, para o aparecimento de cepas de *M. tuberculosis* multi-drogas resistentes (Beck, 2007).

O sarampo é outra infecção afetada pela desnutrição e em especial pela deficiência de vitamina A (Perry & Halsey, 2004). Evidências experimentais indicam que a

suplementação com vitamina A reduz de 23 a 30% a gravidade da doença e o risco de mortalidade. Por esta razão, a OMS recomenda administração de uma dose oral de vitamina A em crianças com sarampo que residam em áreas com deficiência desta vitamina (Huiming et al., 2005).

Desnutrição e parasitismo intestinal também apresentam distribuição geográfica similar, sendo que, os mesmos indivíduos apresentam as duas doenças simultaneamente (Pelletier et al., 1994). Neste caso a relação casual é claramente bi-direcional, ou seja, a desnutrição aumenta a suscetibilidade à infecção e a infecção determina aumento no grau de desnutrição (Scrimshaw & SanGiovanni, 1997). Nematódeos intestinais determinam desnutrição porque causam anorexia e uma variedade de alterações no trato gastrointestinal tais como vômitos, diarréia e má absorção. Em conjunto, estas alterações afetam de maneira deletéria a capacidade do hospedeiro de obter quantidades adequadas de nutrientes a partir da dieta (Koski & Scott, 2001).

Existe também um consenso de que a DPC esteja associada com maior morbidade e mortalidade nos casos de malária humana (Shankar, 2000). Por exemplo, tem sido demonstrado por ensaios clínicos que a suplementação com vitamina A ou zinco reduzem, de forma significativa, a reincidência de malária (Shankar, 2000; Caulfield et al., 2004). Por outro lado, o efeito de certos micronutrientes como é o caso do ferro, é ainda contraditório (Caulfield et al., 2004; Prentice et al., 2008).

1.4. Modelos experimentais de desnutrição

Os estudos sobre os tipos e graus das diferentes deficiências nutricionais foram realizados, de início, com populações humanas, especialmente aquelas residentes em países subdesenvolvidos ou em desenvolvimento. Estes estudos foram muito relevantes, pois permitiram demarcar as regiões mais gravemente afetadas e, desta forma, direcionar programas assistenciais definidos por organizações humanitárias e também pelos governantes locais (Millward & Jackson, 2004; Cunningham-Rundles et al., 2005; Grossniklaus et al., 2008).

Entretanto, animais de laboratório vêm sendo empregados de forma crescente para avaliar os efeitos dos graus variáveis de desnutrição e deficiência de nutrientes específicos na susceptibilidade às infecções e também nos diferentes parâmetros da resposta imunológica (Nodera et al, 2001; Niiya et al, 2007; Wintergest et al., 2007). A grande vantagem do uso destes modelos é permitir avaliação altamente controlada de cada parâmetro nutricional o que não é possível no caso das populações humanas. Camundongos e ratos adultos (isogênicos ou não), alimentados com quantidades reduzidas de proteínas, vitaminas ou micronutrientes, constituem os modelos mais utilizados. O percentual de restrição alimentar varia de 10 a 70% segundo diferentes autores. Camundongos jovens recém desmamados também são empregados como modelo de desnutrição pré-pubescente (Hillyer et al., 2008). Gatos e cachorros são eventualmente utilizados em estudos de desnutrição. Mais recentemente tem aumentado o uso de camundongos transgênicos e *knockouts* para elucidar os mecanismos envolvidos na maior suscetibilidade que a desnutrição determina aos agentes infecciosos (Freeman & Rush, 2007). A tabela 1 mostra alguns modelos experimentais empregados para avaliação dos efeitos da desnutrição no sistema imune e na suscetibilidade às infecções (França et al., 2009).

Tabela 1*: Modelos experimentais empregados para avaliação dos efeitos da desnutrição no sistema imune e na suscetibilidade às infecções.

Modelo experimental	**Tipo de restrição**	**Efeitos**	**Referência**
Camundongos SCID	Restrição total de vitamina A 7 dias durante a gestação	Comprometimento da produção de anticorpos após imunização com toxóide tetânico	Molrine et al., 1995
Camundongos BALB/c	Dietas com restrição parcial de proteína 6 semanas	Produção excessiva de PGE2 e decréscimo nos níveis de IL-10 e óxido nítrico	Anstead et al., 2001
Camundongos Swiss-Webster	Restrição total de proteína 1 semana	Diminuição da atividade da PKC e da expressão de Bcl-2, aumento da apoptose de macrófagos	Rivadeneira et al., 2001
Ratos Sgrague-Dawley	Restrição total de zinco 34 semanas	Atrofia tímica e testicular, oligoespermia e diminuição de espermatozóides	Nodera et al., 2001
Camundongos BALB/c	Restrição de micronutrientes (Fe e Zn) e proteínas 6 semanas	Perda de peso, diminuição da atividade do fator NFκ-B, diminuição da produção de TNF-α e NO pelos macrófagos	Anstead et al., 2003
Camundongos BALB/c	Restrição total de vitamina A 2-5 semanas	Aumento de células Th2 e Treg e diminuição de células Th1	Stephensen et al., 2004
Camundongos C57BL/6	Dieta com baixo teor de caseína 4 semanas	Comprometimento da resposta a uma vacina de DNA	Sakai et al., 2007
Camundongos Swiss	DPC (4% de proteína)	Perda de peso, anemia, leucopenia e baixa produção de TNF-α, IL1α e IL-6	Fock et al., 2007
Camundongos C57BL/6	Restrição alimentar total 52 semanas	Diminuição da resposta imune humoral ao vírus da hepatite B	Niiya et al., 2007

*Adaptada de França et al., 2009

1.5. Nossa experiência em desnutrição experimental

O modelo experimental utilizado para a realização deste trabalho de mestrado foi introduzido em nosso laboratório em 2006. Nesta ocasião, nosso objetivo era avaliar o efeito da desnutrição experimental na imunogenicidade de uma vacina gênica (pVAXhsp65) para tuberculose. Em uma primeira etapa, foi induzida a desnutrição em camundongos BALB/c, avaliada principalmente pela perda de peso. O status imunológico destes animais foi avaliado por hemograma, análise histopatológica de órgãos linfóides e produção de citocinas. Em uma segunda etapa, tanto animais normais quanto desnutridos foram imunizados com a vacina gênica e a resposta imune humoral e celular foi comparada. Obtivemos os seguintes resultados: 20% de restrição alimentar foi suficiente para desencadear uma desnutrição grave, com diminuição de peso corpóreo, esplênico e tímico dos camundongos, diminuição do número de leucócitos totais e linfócitos e déficit na produção de citocinas. Não houve produção de anticorpos específicos nos animais desnutridos imunizados com pVAXhsp65. Estes achados foram publicados na revista "Genetic Vaccines and Therapy".

Posteriormente, em 2007, utilizamos este mesmo modelo de desnutrição (20% de restrição alimentar) para avaliar seu efeito na suscetibilidade à infecção com S*taphylococcus aureus*. O número de unidades formadoras de colônias (UFC) indicou suscetibilidade similar nos grupos normal e subnutrido, enquanto que a análise histopatológica indicou maior quantidade de bactérias no pulmão dos animais normais. O processo de imunização controlou a multiplicação bacteriana no pulmão dos animais sadios, mas não dos animais subnutridos. A análise histopatológica também mostrou perfis inflamatórios distintos: camundongos normais apresentaram inflamação pulmonar somente após infecção com *S. aureus*, como esperado, e a imunização prévia com antígenos de *S. aureus* preveniu esta inflamação. Por outro lado, camundongos subnutridos já apresentavam inflamação pulmonar antes da infecção e o aspecto deste infiltrado inflamatório não foi alterado pela infecção com *S. aureus* ou pela imunização realizada antes desta infecção. Os resultados obtidos foram publicados na revista "BMC Microbiology".

Mais recentemente, empregamos este modelo para avaliar o efeito da desnutrição na resposta imune induzida por BCG. Os animais submetidos à restrição alimentar apresentaram alterações físicas, bioquímicas e imunológicas que caracterizaram uma desnutrição experimental. Além disso, foi observado um aumento do percentual de células

TCD4+ nos animais desnutridos em comparação com os animais sadios. Após a imunização com o BCG, um maior número de animais subnutridos apresentou carga bacteriana em diferentes órgãos em relação aos sadios e, somente o grupo desnutrido, apresentou disseminação para o timo. A avaliação das UFC nos permitiu inferir que os animais submetidos à restrição alimentar imunizados com BCG apresentaram, de maneira geral, maior carga bacteriana em relação ao grupo controle. Durante a realização deste experimento também foi possível avaliar a transmissão da infecção em animais desnutridos mantidos em contato domiciliar com sadios imunizados. Além disso, observamos um déficit na produção de IFN-γ e TNF-α nos animais com dieta restrita. Estes resultados se encontram em anexo, sob a forma de manuscrito, e serão em breve submetidos à publicação.

Fazendo uma análise retrospectiva destas investigações, acreditamos que os resultados obtidos foram relevantes no contexto da desnutrição. Entretanto, gostaríamos de destacar a maior dificuldade encontrada com o uso deste modelo e que se refere à reprodutibilidade do modelo em si.

O modelo de desnutrição empregado se baseia na restrição alimentar total, ou seja, uma restrição não específica, baseada no consumo diário de um grupo controle. A partir desta idéia, o nosso principal objetivo foi estabelecer o percentual de restrição adequado para que os animais ficassem desnutridos, porém não viessem a óbito durante as investigações. Dessa forma, avaliando alguns parâmetros físicos e imunológicos, chegamos à conclusão de que 20% de restrição seria o mais adequado. No entanto, durante a repetição de alguns dos protocolos experimentais, percebemos que diversos fatores extrínsecos podem influenciar o estado nutricional dos animais, como a idade e o peso inicial. Já é bem descrito na literatura que uma leve restrição alimentar, de cerca de 20 a 30%, pode melhorar o funcionamento do Sistema Imunológico de animais com idade avançada. No entanto, em camundongos recém-desmamados ou na puberdade, restrições alimentares com estes percentuais podem, inversamente, causar algum tipo de desnutrição. Assim, não foi possível estabelecer um modelo de desnutrição baseado em um único percentual de restrição, visto que os animais não estavam ficando desnutridos. Dessa maneira, o parâmetro utilizado para determinar a desnutrição experimental foi o percentual de perda de peso (cerca de 10% aos 10 dias) em relação aos seus pesos iniciais.

Apesar desta dificuldade, consideramos que este modelo de restrição alimentar reflete de forma fidedigna a maioria dos casos de desnutrição humana e que, por isso, deva ser utilizado para este tipo de investigação.

1.6. Aspectos gerais da tuberculose

A tuberculose (TB) é a doença infecciosa causada pelo *M. tuberculosis*. É responsável por cerca de 2 a 3 milhões de mortes por ano e permanece como a segunda maior causa de morte por doenças infecciosas no mundo. A associação com o vírus HIV e a situação de extrema pobreza de alguns países em desenvolvimento são condições que propiciam o aumento desses índices (Abolhassani et al., 2000). Dados da Organização Mundial da Saúde (OMS) indicam que 32% da população mundial está infectada com *M. tuberculosis* e o Brasil ocupa o 13º lugar entre os 22 países onde se estima que ocorram 80% dos casos de tuberculose no mundo (Dye et al.,1999).

A infecção se inicia com a entrada do *M. tuberculosis* no organismo através das vias aéreas superiores. Ao chegar nos pulmões, o bacilo interage com macrófagos alveolares e é fagocitado via receptores que reconhecem moléculas presentes na superfície da bactéria. Embora grande parte dos bacilos seja degradada no interior dos fagócitos, o *M. tuberculosis* é capaz de desenvolver mecanismos que lhe permitem não apenas sobreviver à atividade microbicida dos macrófagos como também replicar-se no interior dos fagócitos.

Está bem estabelecido que a resposta protetora na tuberculose está relacionada ao desenvolvimento da resposta de perfil Th1, com produção de IFN-γ, IL-2, IL-12, IL-18 e TNF-α (Cooper, 1997; Flynn, 1995). O IFN-γ é uma das principais citocinas associadas à resposta protetora durante a infecção por micobactérias, sendo produzido por células T CD4+, T CD8+, NK (Cooper, 1993; Yang & Mitsuyama, 1997). O principal papel atribuído ao IFN-γ e outras citocinas de padrão Th1 citadas acima, na resposta do hospedeiro contra a tuberculose consiste na ativação e recrutamento de macrófagos capazes de controlar a infecção por micobactérias (Bonecini Almeida et al., 1998). Além disso, Zhang et al., 1994 mostraram que a secreção de IL-12 por macrófagos pleurais humanos é também importante. Essa citocina induz a ativação de células *natural killers*, estimulando-as a produzir IFN-γ (D'Andrea et al., 1992) ou aumentando sua capacidade citotóxica diretamente sobre as células infectadas e, além disso, estimula a diferenciação de linfócitos T CD4+ para o padrão Th1 (Serbina et al., 2001).

Além das células T CD4+ e macrófagos, um importante papel na resposta protetora contra tuberculose tem sido atribuído às células T CD8+ citotóxicas. Esses linfócitos medeiam a lise de células infectadas por meio da formação de poros pela perforina e pela ação das moléculas efetoras granulisinas e granzimas, ou, ainda, por mecanismo Fas-FasL

(revisado por Silva et al., 2001). Células T CD8+ de camundongos infectados produzem IFN-γ em resposta ao reconhecimento de antígenos de *M. tuberculosis* apresentados por CD ou macrófagos (Serbina & Flynn, 1999). Portanto, as principais células envolvidas na resposta imune contra TB, são os linfócitos T CD4+ e TCD8+ citotóxicos produtores de IFN-γ.

Atualmente, existem vários medicamentos disponíveis para o tratamento da infecção, mas a prevenção continua sendo o método mais eficaz, já que a quimioterapia é de alto custo, o que dificulta o tratamento em países em desenvolvimento (Jordan & Davies, 2010). Além disso, existem algumas cepas do bacilo que são resistentes aos medicamentos (Agger & Andersen, 2002). Por isso, é fundamental que sejam investigadas várias estratégias para o controle da TB, as quais incluem novos fármacos e também vacinas mais eficazes.

1.7. Profilaxia na tuberculose

A única vacina disponível contra a tuberculose é o BCG (Bacilo de Calmette-Guérin), que é constituída por uma cepa atenuada de *Mycobacterium bovis*. Entretanto, estudos clínicos mostram que o nível de proteção dessa vacina alcançado em diferentes populações varia de 0 a 80% (Fine et al.,1999). Além disso, o BCG interfere nos testes de sensibilidade para detecção da doença e não é totalmente seguro para indivíduos com Síndrome da Imunodeficiência Humana, por ser uma vacina constituída de microorganismos vivos (Britton & Palendira, 2003).

Atualmente, mais de 80% das crianças de todo o mundo receberam o bacilo de Calmette-Guérin (BCG). A vacinação com BCG é considerada segura, sendo apropriada para administração no período neonatal. Entretanto, sua eficácia de proteção contra a tuberculose é controversa. O BCG é considerado eficiente na prevenção de formas graves de TB em crianças, entretanto, o mesmo parece não conferir boa proteção no adulto. Uma das razões para esta variabilidade parece estar relacionada com as diferentes cepas de BCG utilizadas nas diferentes populações.

Em função desta ineficácia do BCG e do aumento do número de casos de TB no mundo, uma nova vacina é necessária para evitar esta doença. Dentre as novas formulações profiláticas em teste para tuberculose destacam-se as vacinas gênicas. Este tipo de vacina baseia-se no uso de seqüências do material genético do agente infeccioso que codifiquem

antígenos imunodominantes. Estas seqüências são inseridas em vetores (plasmídeos ou vetores virais). Quando administrado ao indivíduo, esse DNA permite a produção da proteína antigênica pelas próprias células do indivíduo vacinado e é capaz de induzir resposta imune específica celular e humoral, e também memória imunológica. Além disso, as vacinas de DNA mimetizam os efeitos das vacinas vivas por possibilitarem a geração de antígenos endógenos, e conseqüentemente, a ativação dos linfócitos T CD8+, e também atuam como adjuvante da resposta celular devido a presença de seqüências CpG, que são determinadas sequências de DNA dotadas de capacidade imunoestimulatória (Gurunathan et al., 2000). Uma vez dentro do citoplasma, o DNA inserido se desloca até o núcleo, onde o gene de interesse é transcrito, produzindo o mRNA, que migra para o citoplasma, onde ocorre a síntese da proteína antigênica (Liu, 2003).

Uma das vacinas gênicas que vem sendo testada para tuberculose é baseada no gene que codifica a proteína de choque térmico de 65kDa (hsp65) de *Mycobacterium leprae*. A hsp65 de *M. tuberculosis* é um antígeno imunodominante, pois camundongos imunizados com esta micobactéria apresentam de 10 a 20% de células específicas para a hsp65 entre todas as células responsivas ao bacilo (Kauffman et al, 1987). Foi observado que camundongos BALB/c imunizados com esta vacina, por via intramuscular, apresentaram número elevado de células produtoras de IFNγ se comparado à produção de IL-4, indicando uma estimulação preferencial de células tipo Th1 (Bonato et al., 1998). Observou-se também produção de anticorpos específicos anti-hsp65; intensa resposta linfoproliferativa de células de baço estimuladas com hsp65 recombinante (Lowrie et al., 1997), aumento na freqüência de células hsp65 reativas nos linfonodos e produção de células de memória (Bonato et al., 1998, Silva et al., 1999). Além disso, a imunização intramuscular com DNAhsp65 protegeu camundongos contra posterior infecção com *M. tuberculosis* (Lowrie et al., 1997). Todos os estudos feitos até o momento foram realizados com animais submetidos à nutrição adequada.

2. RACIONAL DO PROJETO

Como descrevemos acima, apesar da eficácia variada do BCG, o mesmo continua como única medida profilática disponível para a tuberculose. Entre as várias alternativas em estudo, destacam-se as vacinas gênicas, incluindo a DNAhsp65. Em termos teóricos, o efeito dessas duas vacinas poderia ser significativamente afetado pela subnutrição. No caso do BCG existe a possibilidade deste bacilo atenuado se comportar de forma virulenta e causar infecção no hospedeiro. No caso da vacina gênica, também é razoável supor que sua capacidade imunogênica e, consequentemente protetora, esteja diminuída. Este pressuposto se baseia no princípio deste tipo vacinal que necessita do hospedeiro tanto para síntese do antígeno quanto para a montagem da resposta imune.

Nesse contexto, nossa hipótese de trabalho prevê que animais subnutridos apresentem baixa resposta imune após vacinação com DNAhsp65. Além disso, o BCG pode apresentar maior grau de replicação e disseminação nestes animais. Para testar essa hipótese avaliaremos o efeito da restrição alimentar na imunogenicidade destas duas vacinas para tuberculose e na patogenicidade do BCG.

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4. TRABALHO PUBLICADO

4.1. Dietary restriction abrogates antibody production induced by a DNA vaccine encoding the mycobacterial 65 kDa heat shock protein.

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Dietary restriction abrogates antibody production induced by a DNA vaccine encoding the mycobacterial 65 kDa heat shock protein

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Abstract

Background: Protein-calorie malnutrition (PCM) is the most common type of malnutrition. PCM leads to immunodeficiency and consequent increased susceptibility to infectious agents. In addition, responses to prophylactic vaccines depend on nutritional status. This study aims to evaluate the ability of undernourished mice to mount an immune response to a genetic vaccine (pVAXhsp65) against tuberculosis, containing the gene coding for the heat shock protein 65 from mycobacteria.

Methods: Young adult female BALB/c mice were fed *ad libitum* or with 80% of the amount of food consumed by a normal diet group. We initially characterized a mice model of dietary restriction by determining body and spleen weights, hematological parameters and histopathological changes in lymphoid organs. The ability of splenic cells to produce IFN-gamma and IL-4 upon *in vitro* stimulation with LPS or *S. aureus* and the serum titer of specific IgG1 and IgG2a anti-hsp65 antibodies after intramuscular immunization with pVAXhsp65 was then tested.

Results: Dietary restriction significantly decreased body and spleen weights and also the total lymphocyte count in blood. This restriction also determined a striking atrophy in lymphoid organs as spleen, thymus and lymphoid tissue associated with the small intestine. Specific antibodies were not detected in mice submitted to dietary restriction whereas the well nourished animals produced significant levels of both, IgG1 and IgG2a anti-hsp65.

Conclusion: 20% restriction in food intake deeply compromised humoral immunity induced by a genetic vaccine, alerting, therefore, for the relevance of the nutritional condition in vaccination programs based on these kinds of constructs.

Background

Protein-calorie malnutrition (PCM) is still the most common type of undernutrition and approximately 800 million people in the world present some kind of malnutrition [1]. This deficiency is usually complex, frequently involving both protein calorie and varying degrees of micronutrient deficiency of vitamin A, vitamin E, vitamin B6, folate, zinc, iron, copper, and selenium. PCM leads to atrophy of the lymphoid organs, profound T-lymphocyte deficiency, and increased susceptibility to pathogens, reactivation of viral infections, and development of opportunistic infections [2]. The immune response to infection involves a complex process, including synthesis of acute-phase proteins, cytokines and immunoglobulins and also clonal expansion and cellular differentiation [3]. Clearly this requires an appropriate supply of nutrients to optimize the response and consequently the nutritive status of the host critically determines the outcome of infection.

Effects of nutritional depletion can be found in the innate immune system, for example, lysozyme production by monocytes and polymorphonuclear cells is decreased, complement factors are diminished in both concentration and activity and macrophage functions are also impaired [4]. Multiple abnormalities in specific immunity have also been frequently described in connection with malnutrition. These studies indicate decrease in T-cell function, cytokine production and also in the ability of lymphocytes to respond appropriately to cytokines [5]. T cells have been characterized as Th1 and Th2, depending on their cytokine profile. Th1-type responses are dominated by the production of IFN-γ and are associated with cell-mediated immunity, whereas Th2-type responses are characterized by IL-4 production and more related to humoral responses [6]. In general, innate and cell-mediated immunity are more sensitive to undernutrition than humoral immunity [7]. Nevertheless, more recent investigations also indicate a reduced Th2 activity [8].

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* that is historically known to be particularly influenced by undernutrition. It is a major cause of morbidity and mortality in developing countries where PCM is also prevalent [9]. Even though some reports suggest contribution of humoral immunity against *M. tuberculosis*, it is believe that celular immune response is much more relevant [10-12]. Therefore, the design of all the new vaccines to control TB is based on induction of a predominant cellular immune response. The attenuated BCG strain of *Mycobacterium bovis* has been extensively used as a vaccine against tuberculosis. However, well documented trials showed that the protective efficacy of BCG varies from 0 to 80%. This highly variable and poorly protective efficacy in certain countries has been attributed to the various BCG strains used as vaccines, environmental factors as well as host genetic characteristics [13]. In addition, experimental studies showed that animals were adequately protected by BCG vaccine when properly nourished but exhibited significant weight loss and tuberculin anergy when maintained on a protein-deficient diet [9]. Despite BCG vaccination, malnourished children developed serious and often fatal types of tuberculosis such as miliary, meningitic and disseminated tuberculosis [14].

DNA vaccines represent a promising new approach to vaccination in which the gene for a foreign antigen is expressed within the host's cells. These vaccines generated humoral and cell-mediated immune responses followed by protective efficacy in different experimental models of infectious diseases including tuberculosis. DNA vaccination has been proposed as a hope for better vaccination programs in developing countries [15].

Our group has been working with DNA vaccines constructed by inserting the heat shock protein 65 gene from *Mycobacterium leprae* (hsp65) into plasmid vectors (DNAhsp65). Theoretically, this construction could protect against TB because hsp65 family is one of the most conserved families of proteins presenting more than 97% homology among prokaryotes [16]. In addition, hsp65 and other molecular chaperones are highly immunogenic. Around 10 to 20% of all T cells specifically stimulated are reactive with hsp65 in mice immunized with *M. tuberculosis* [17]. Indeed, this construction displayed both, prophylactic and therapeutic effect in experimental tuberculosis [18,19]. These evaluations were done with mice or guinea pigs submitted to normal chow. Malnutrition could affect both, antigen synthesis and the immune response itself, as they rely on the host's metabolism. Based on this scenario, we hypothesized that immune response induced by a genetic vaccine (pVAXhsp65) could be jeopardized in malnourished mice.

Materials and methods

Mice and diets

Isogenic female BALB/c mice, 5–6 weeks old, were housed in plastic cages with white wood chips for bedding and with free access to filtered drinking water, and under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature (23 ± 2°C). After weaning, mice received a 10 day acclimation on a standard chow (Labina, São Paulo, SP, Brazil). This animal chow is considered adequate for mice and is approved by the Brazilian Ministry of Agriculture (n° SP-0311730758). These mice were initially distributed into two groups including a control experimental group (normal), fed *ad libitum* and an undernourished group (restricted) that received 80% of the amount of food consumed by the normal group. Later, they were further allocated to three groups and inoc-

ulated with saline solution (vaccine diluent), empty vector (pVAX) or DNA vaccine (pVAXhsp65). Each experimental group included 4 to 8 animals and all evaluations were done at the 40th day after the beginning of dietary restriction.

Animals were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation (COBEA), being the experimental protocol approved by the local Ethics Committee.

Hematological parameters

Blood samples were collected by cardiac puncture and total leukocyte number was counted after blood dilution in Turk's solution. Differential leukocyte count was performed by blood smear stained with eosin/methylene blue (Leishman's stain).

Histopathological analysis

The whole thymus and a transversal section from small intestine were fixed in formalin (10%), embedded in Paraplast plus (McCormick), prepared routinely and then sectioned for light microscopy. Sections (5 μm each) were stained with haematoxylin and eosin (HE), analyzed in an optical microscope and the images acquired with a digital camera coupled to the microscope.

Plasmid DNA construction and purification

The vaccine pVAXhsp65 was derived from the pVAX vector that uses the CMV intron (Invitrogen, Carlsbad, CA, USA), previously digested with BamH I and Not I (Gibco BRL, Gaithersburg, MD, USA) to insert a 3.3 kb fragment corresponding to the *M. leprae* hsp65 gene. The empty pVAX vector was used as a control. DH5α *E. coli* transformed with plasmid pVAX or the plasmid carrying the hsp65 gene (pVAXhsp65) were cultured in LB liquid medium (Gibco BRL, Gaithersburg, MD, USA) containing kanamicin (50 μg/ml). The plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL, Gaithersburg, MD, USA). Plasmid concentrations were determined by spectrophotometry at λ = 260 and 280 nm by using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK).

Immunization procedures

Normal and restricted groups were immunized by intramuscular route with three doses of pVAXhsp65 (100 μg/100 μl) plus 25% of sucrose (with 10 days interval), being the first dose delivered 10 days after the beginning of dietary restriction. Saline solution or pVAX were also injected in groups submitted to normal or restricted diet.

Quantification of anti-hsp65 antibodies

Serum samples were obtained by blood centrifugation and anti-hsp65 specific antibody levels were evaluated by enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc, Life Tech. Inc., USA) were coated with 5 μg/ml of purified recombinant hsp65 in coating solution (Na_2CO_3/$NaHCO_3$, pH 9.6), at 4°C, overnight. Non-specific protein binding was blocked by incubation with 0.05% Tween 20, 10% fetal calf serum (FCS) in phosphate buffered saline (PBS, 200 μl per well) for 1 h at 37°C. Subsequently, plates were incubated with serum diluted 1:10 (1 h, 37°C). For the detection of specific serum IgG1 and IgG2a, the plates were incubated with biotinylated anti-mouse antibodies (PharMingen, BD Biosciences, USA) for 1 h at 37°C. Plates were then incubated for 30 min at room temperature with Strept AB (kit from Dako, Carpinteria), and revealed by adding H_2O_2 with ortho-phenylenediamine (OPD) (Sigma, USA). Color development was stopped with H_2SO_4 and optical density was measured at 490 nm.

Evaluation of cytokine production

Splenic cells were obtained at the 40th day after the beginning of dietary restriction. Cell suspensions were adjusted to 5 × 10^6 cells/ml in RPMI 1640 medium, supplemented with 10% FCS, 2 mM L-glutamine and 40 mg/L of gentamicin. The cells were cultured in 48-well flat-bottomed culture plates (Nunc) in the presence of concanavalin A (ConA), 10 μg/ml, type IV-S (Sigma Chemicals, USA), lipopolysaccharide (LPS), 10 μg/ml, *E. coli*, sorotype 055:B5 (Sigma) or fixed *Staphylococcus aureus* Cowan 1 strain (SAC), final diluition 1:2500 (Calbiochem, Behring Co., USA). Cytokine levels were evaluated 48 hours later by ELISA in culture supernatants using anti-IFN-γ and anti-IL-4 as capture antibodies.

Statistical analysis

Results were expressed as the mean ± SD for each variable. Statistical analysis was performed using Minitab Version 1996 (Minitab Inc, State College, PA, USA). One-way ANOVA and comparative Fisher test were used to analyze the results of antibody production. The other results were analyzed by unpaired t test. Values of $p < 0.05$ were considered statistically significant.

Results

Dietary restriction decreased body and spleen weight

Body weight was daily recorded and losses were already observed 24 h after the beginning of dietary restriction. However, a significant weight loss was detected only from day 4 on. Weight values referring to day 1, before dietary restriction, and days 10, 20, 30 and 40 after dietary restriction are documented in figure 1a. Spleen weight, that was assessed at the 40th day, after animal's euthanasia, was significantly lower in comparison to the control group and it is shown in figure 1b.

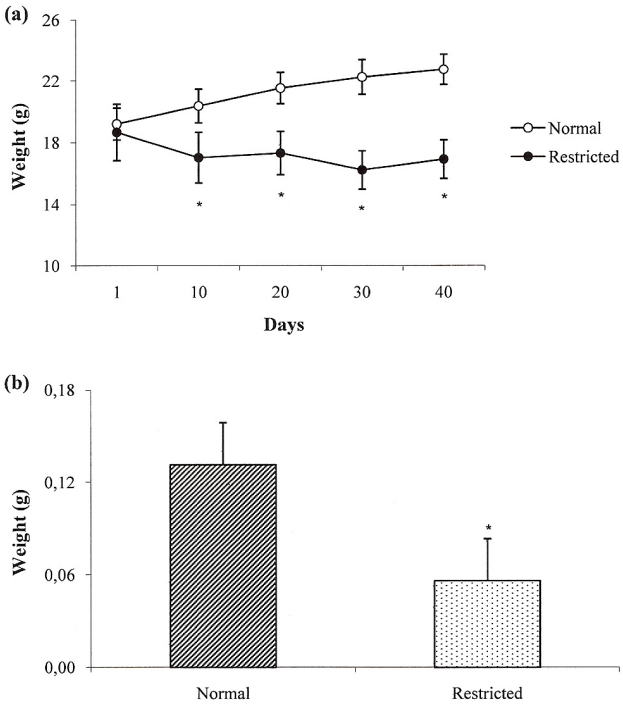

Figure 1
Effect of dietary restriction on body (a) and spleen (b) weights. Weight values refer to day 1 (before dietary restriction) and days 10, 20, 30 and 40 after dietary restriction. Spleen weight refers to the 40thday of dietary restriction. *Mean value was significantly different from that of the normal group ($p < 0.05$).

Lymphoid organs were selectively affected during dietary restriction

By comparison to the normal thymus showed in figure 2a, a severe atrophy is observed in this organ in malnourished animals. Weight evaluation indicated a 52% reduction in comparison to the normal control group (data not shown). In addition to atrophy, the distinction between cortical and medullar areas was also not evident in the group with dietary restriction (figure 2b).

The most striking changes observed in undernourished mice, at the mucous membrane associated with the small intestine, was a villous atrophy. In addition of being smaller and irregular, these intestinal villosities lost their brush borders. Alterations can be observed in figure 2d, comparing to normal structures shown in figure 2c.

Dietary restriction decreased lymphocytes but not PMN cell number

Total leucocyte number was significantly decreased in undernourished mice comparing to the control group. This reduction coincided with an also significant diminished lymphocyte number. No alteration was detected in the total PMN cell count. These results can be observed in figure 2e.

Production of IFN-γ and IL-4 was affected by dietary restriction

Production of IFN-γ, that is documented in figure 3a, varied according to the stimulus. In ConA stimulated cultures there was no difference between control and the experimental group under dietary restriction. However, IFN-γ production was significantly reduced in cultures stimulated with LPS or SAC. IL-4 levels are shown in figure 3b. As can be observed, only ConA addition was able to induce detectable IL-4 levels. The group submitted to dietary restriction showed reduced levels of this cytokine, even though this reduction was not statistically significant.

Dietary restriction abrogated humoral immune response induced by a DNA vaccine

Immunization of BALB/c mice with pVAXhsp65 vaccine by intramuscular route induced high levels of both, IgG2a and IgG1 specific antibody levels. As expected, no antibodies were induced by inoculation of the empty vector (pVAX). Diet restriction deeply affected the immune response induced by this vaccine, none of these specific isotypes was detected in their serum (figure 4).

Discussion

Experimental dietary restriction by deprivation of variable percentages of food intake is being used to explore effects of PCM on immunity and susceptibility to infectious agents [20]. In this study, we first characterized the immunological status of mice submitted to a dietary restriction protocol for 40 days and then evaluated the effect of this restriction on their ability to mount an immune response against a DNA vaccine containing the mycobacterial hsp65 gene.

A significant weight loss was already observed at the fourth day of diet and this was maintained until the end of the experiment that was at the 40th day. Weight losses are described in many studies with undernourished animals and used as a criteria to characterize undernutrition. A striking decrease in leucocyte number that selectively affected lymphocytes was also observed.

Alterations in body and spleen weights were compatible with the findings from the histopathological analysis that showed evident alterations in lymphoid organs. Thymus sections from dietary restricted group revealed severe atrophy that was reinforced by a 52% reduction in their weights (not shown). These findings are highly supported by the literature in both, experimental and human malnutrition [21]. Peyer's patches and inguinal lymph nodes were clearly atrophic (not shown). The deleterious effect

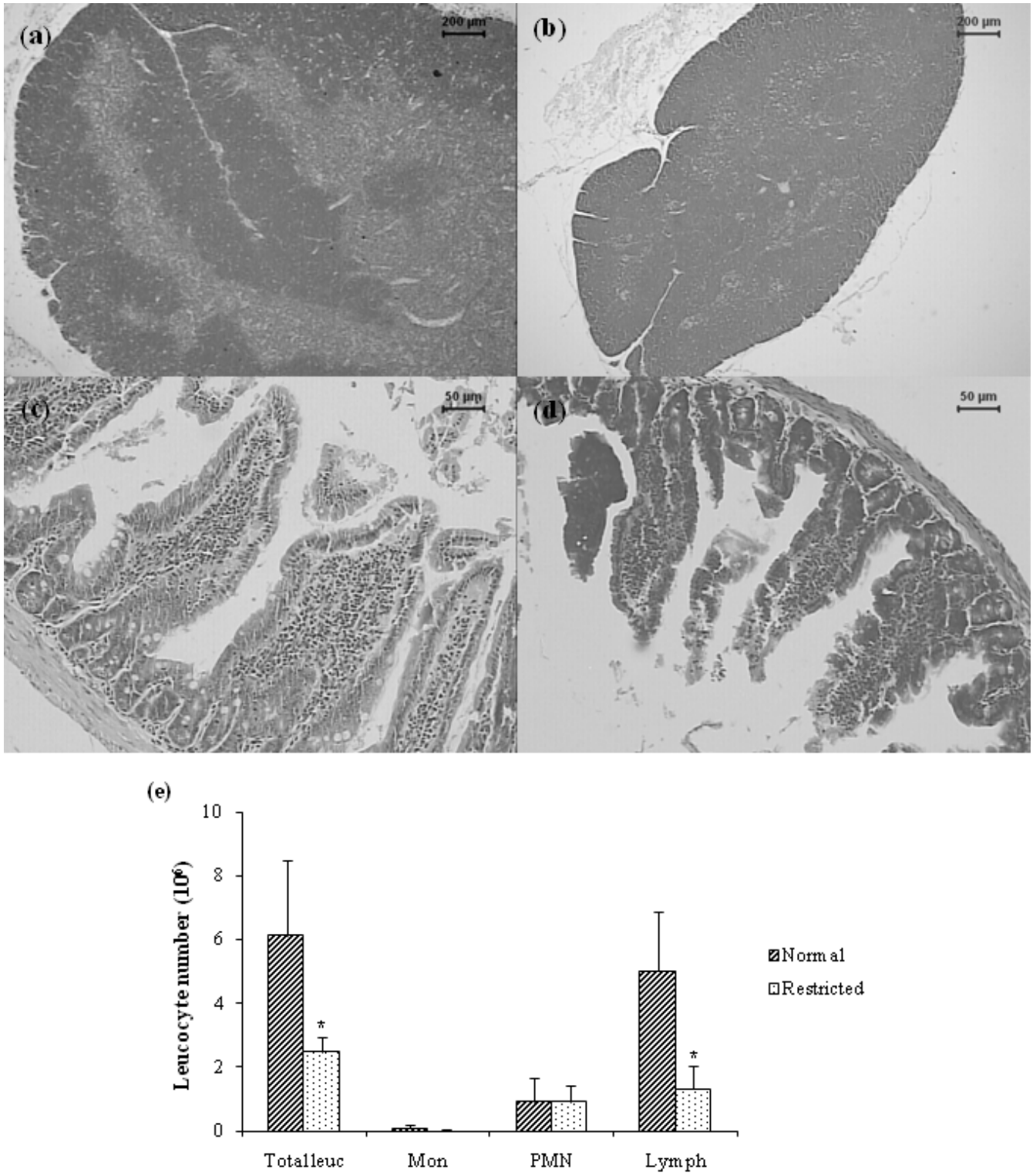

Figure 2

Effect of dietary restriction on lymphoid organs architecture and on hematological parameters. Thymus (a, b) and small intestine (c, d) sections stained with HE from BALB/c mice fed with normal diet (left column) or 80% of normal diet (right column). Total and differential number of monocytes, PMN cells and lymphocytes (e). *Mean value was significantly different from that of the normal group ($p < 0.05$).

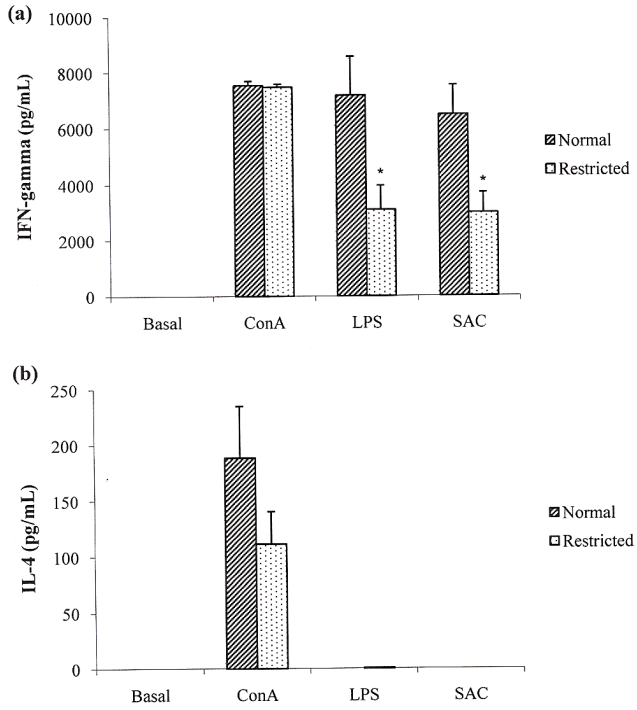

Figure 3
Effect of dietary restriction on cytokine production by spleen cell cultures. IFN-γ (a) and IL-4 (b) levels were determined by ELISA in supernatants from cultures stimulated with Concanavalin A (ConA), lipopolysaccharide (LPS) and *S. aureus* (SAC) and non-stimulated cultures (basal). *Mean value was significantly different from that of the normal group ($p < 0.05$).

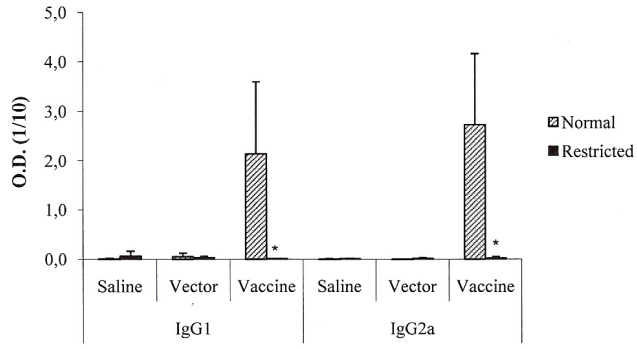

Figure 4
Effect of dietary restriction on antibody production induced by pVAXhsp65. Anti-hsp65 antibody production (IgG1 and IgG2a) was tested by ELISA in serum samples from BALB/c mice fed with normal diet (normal) or 80% of normal diet (restricted) groups. *Mean value was significantly different from that of the normal group ($p < 0.05$).

over mucosal immune system was attested by the evident villous atrophy observed in the small intestine. Sulivan *et al*. [22] have shown that poor dietary protein has a direct effect on mucosal IgA, secretory component, number of IgA-containing cells and IgG levels in rats.

As cytokines are the major effectors and regulators of the immune response, we next evaluated the ability of spleen cells to produce IFN-γ and IL-4 that are considered key cytokines in the development of Th1 and Th2 cells, respectively [23]. As IFN-γ can be directly induced by polyclonal activation of T cells, the spleen cells were stimulated with ConA, LPS and SAC were additionally used because they indirectly induce IFN-γ production by NK cells, i.e, via IL-12 production [24,25]. In ConA stimulated cultures there was no difference between normal and dietary restricted groups. However, IFN-γ production was significantly compromised in cultures stimulated with LPS or *S. aureus* (SAC). This decreased IFN-γ production is consistently described in humans and experimental models with malnutrition [26,27].

The mechanism involved in this differential IFN-γ response associated with distinct stimuli was not investigated. However, we could think that the decreased T cell number was associated with a higher degree of apoptosis as was clearly demonstrated by Pires *et al*. [28]. In this context, the remaining T cells, i.e., the ones spared from apoptosis, could still be able to produce this cytokine if adequately stimulated. This was hypothesized from the additional fact that ConA is a strong stimulus that directly and strongly interacts with glycoproteins from T cell surface [29]. On the other hand, the reduced IFN-γ levels induced by LPS and SAC could indicate that other cell functions or cytokine synthesis are compromised by dietary restriction. IL-12 availability is considered the dominant factor in driving the development of Th1 cells that are characterized by IFN-γ synthesis [30]. Therefore, lower levels of this cytokine could profoundly impair IFN-γ production. It is also well described that IL-12 is involved in IFN-γ production in protocols where LPS and SAC are used to stimulate human cells [31]. The possibility that reduced IFN-γ production is associated with a deficit in IL-12 supply is reinforced by a recent publication in which the authors demonstrated a significant reduction in both, IL-12p70 and IFN-γ synthesis in mice whose diet was reduced to 70% of the amount of food consumed by the corresponding control group [20].

The effect of these alterations on the immune response induced by the pVAXhsp65 vaccine was devastating. In comparison to the control group that produced significant amounts of both, IgG1 and IgG2a anti-hsp65 antibodies, undernourished mice did not produce even basal levels of these antibodies. As Th1 cells are characterized by IFN-γ

production and, in mice, the selective switching to IgG2a whereas Th2 cells produce IL-4 and trigger switch to IgG1 and IgE [6] these results indicate that this degree of diet restriction is highly deleterious for both, cellular and humoral components of the immune response.

The effect of the nutritional status during conventional vaccination has been investigated. Measles vaccines did not show efficacy in undernourished children in Africa and India [32]. On the other hand, Moore *et al*. [33] studying the immune response to different vaccines in undernourished children in Gambia, concluded that the secretion of antibodies was not altered even by different degrees of nutritional deficiencies. Only a few reports addressed the consequences of a nutritional deficiency on DNA vaccines. Recently, Sakai *et al*. [34] found a selective impairment of T cells with no effect over B lymphocytes, in a protein deficiency model.

This complete abrogation of the immune response towards a DNA vaccine in undernourished mice could be explained by the double role of the host submitted to this kind of vaccination. In this case, in addition of cellular interactions that are necessary to mount the immune response, the host cells also need to synthesize the antigen. Therefore, it is expected that the immunity to DNA vaccines is even more compromised than the response to conventional vaccines.

Further investigations will be necessary to answer very relevant questions in this area. It will be important to establish if this finding will apply to other plasmids, if other delivery vectors will behave the same way and also if the immunization route can affect the final immune response.

Conclusion

Together these results demonstrate that a 20% reduction in the amount of food intake was able to significantly alter the immune system. The physiological relevance of these alterations was demonstrated by the abrogation of the immune response induced by a DNA vaccine against tuberculosis. These results alert for the fundamental role of the nutritional state, which is frequently affected in developing countries, in vaccine programs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LLWI, TGDF and AS are the main investigators in this study. FCM, SFGZP and NMM largely contributed with the immunological experiments. PCMP and CLS provided critical input.

Acknowledgements

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5. MANUSCRITO A SER SUBMETIDO

5.1. Is BCG indicated for undernourished?

Is BCG indicated for undernourished?

Abstract

It is widely accepted that cellular immune, whose integrity is compromised during undernutrition, is fundamental to avoid tuberculosis. The present study was designed to evaluate if BCG, that is an attenuated mycobacteria, is a safe vaccine for undernourished individuals.

A mice model of undenutrition was initially established by submitting BALB/c mice to a dietary restriction. These animals received 65-75% of the amount of food consumed by the control healthy group and present alteration compatible with a malnutrition status. They showed significant body, spleen and thymus weight losses. They also presented reduced levels of triglycerides, cholesterol and glucose associated with reduced lymphocyte number. A much higher proportion of undernourished mice presented bacteria dissemination to lymph nodes, spleen and liver. In addition, only undernourished animals showed bacteria at the lungs and thymus. High loads and wider BCG spread in undernourished mice was concomitant to a decreased production of TNF-α, IFN-γ and IL-10.

Together these results indicate that BCG causes a stronger infection in undernourished mice comparing to healthy ones. Occurrence of a similar phenomenon in undernourished children must be thoroughly investigated.

Introduction

Undernutrition is one of the major causes of mortality and morbidity worldwide. Latest estimates indicate that over 800 million people in the world remained undernourished (FAO, 2009). The lack of adequated sanitary conditions and poverty contribute to a higher prevalence of malnutrition in underdeveloped countries. Varying degrees of protein calorie restriction or macro/micronutrient deficiencies such as vitamin A, vitamin E, vitamin B6, folate, zinc, iron, copper, and selenium determine a protein-calorie malnutrition (PCM) that is the most common type of undernutrition (Cuningham-Rundles et al, 2005). Inadequate energy intake leads to various physiologic

adaptations, including growth restriction, loss of fat, muscle, and visceral mass and determines deleterious effects on the immunity (Grover & Ee, 2009). Decrease in lysozyme production by monocytes and polymorphonuclear cells, depletion of complement factors and impairment of macrophage functions in the innate immune system are observed during undernutrition (Chandra, 1992). Adaptative immunity is also compromised during nutritional depletion. PCM impairs cytokine production, T-cell function, and also the ability of lymphocytes to appropriately respond to these mediators (Dai & McMurray, 1998).

The most immediate consequence of undernutrition is the increased susceptibility to infections. Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* that is historically known to be particularly influenced by undernutrition. It is the major cause of morbidity and mortality in developing countries where malnutrition is also prevalent (Udani, 1994). It is also well established that the cellular immune response, whose efficacy is compromised during undernutrition, is required to control tuberculosis. The only available vaccine against TB is the attenuated *M. bovis* Bacillus Calmette-Guérin (BCG) that is recommended by the World Health Organization for all infants under 1 year of age. Around 100 million newborn children are given this vaccine and the global vaccine coverage is estimated to be 80% (Andersen & Doherty, 2005). In spite of this extensive use, large numbers of well documented trials showed expressive variation, from 0 to 80%, in BCG protective efficacy (Colditz et al., 1994). Although the overall efficacy is low, most studies agree that BCG protects against disseminated disease in newborns and children but that this immunity wanes with age, resulting in any or insufficient protection against adult pulmonary TB (Fine, 1995; Skeiky & Sadoff, 2006). In this context, there is a great interest in the development of new vaccines to avoid this infection. A number of alternative living and non-living putative TB vaccines, including genetic vaccines, are being studied and discussed by many authors (Orme, 1998; Andersen, 2001; Pelizon et al., 2007). DNAhsp65, for example, was engineered by insertion of hsp65 mycobacterial gene in a bacterial plasmid. As the coded antigen is also found in BCG, this construction has been used in prime-boost protocols. Safe and effective heterologous prime-boost regimen, which augments BCG or recombinant BCG, is being considered the most realistic strategy for future TB control through immunization (Skeiky & Sadoff, 2006). In this context, we observed that both, DNAhsp65 and BCG,

similarly primed neonate mice for a strong immune response at the adult stage (Pelizon et al., 2007). We also found that prime-boost strategies combining these two vaccines were able to protect mice and guinea pig against TB infection (de Paula et al., 2007; Gonçalves et al., 2007). More recently, we demonstrated that undernutrition was highly deleterious for immunization against mycobacteria and *S. aureus* (Ishikawa et al., 2008; França et al., 2009).

In this context, the present study was designed to compare BCG spread and load between well nourished and undernourished mice.

Materials and Methods

Animals

Isogenic female BALB/c mice, 4-5 weeks old, were housed in plastic cages with white wood chips for bedding and with free access to filtered water, and under controlled conditions of lighting (12h light/12h dark cycle) and temperature (23 ± 2 ºC). After weaning, mice received a 10 day acclimation on a standard chow (Labina, SP, Brazil). This animal chow is considered adequate for mice and is approved by the Brazilian Ministry of Agriculture (nº SP-0311730758). Animals were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation (COBEA), being the experimental protocol approved by the local Ethics Committee (19/08-CEEA).

Experimental design

The animals were initially distributed into two groups including a control experimental group (normal), fed *ad libitum* and an undernourished group (restricted) that received 65-75% of the amount of food consumed by the normal group. Physical, hematological and immunological parameters were analyzed at 10 and 40 days after the beginning of dietary restriction. In a second experiment, mice were first allocated into two groups: normal and restricted diet. After 10 days of dietary restriction, they were inoculated intradermically with saline solution (vaccine diluent) or BCG vaccine at the base of the tail. All evaluations were done 25 days after the beginning of dietary

restriction. In all these experiments, the different animal groups were maintained in separated isolated cages. In parallel, normal and undernourished groups were housed in conventional conditions (not isolated), i.e., with normal and BCG immunized groups in the same room and housed in conventional cages. Each experimental group included 4 to 7 animals.

Hematological and biochemical parameters

Blood samples were collected by retro-orbital puncture and the sera were separated by centrifugation. Total leukocyte number was counted after blood dilution in Turk's solution and differential leukocyte count was performed by blood smear stained with eosin/methylene blue (Leishman's stain). Blood glucose was measured with Prestige Smart System (Home Diagnostics, FL, USA). Total proteins, albumin, triglycerides and cholesterol were measured in sera by biuret, bromocresol green and enzymatic Trinder standard methods, respectively (Labtest, SP, Brazil).

Splenocytes preparation

Murine spleens were removed, disrupted and filtered through a nylon mesh (Beckton Dickinson, USA). Splenocytes were washed in RPMI-1640 medium (Cultilab, Brazil), centrifuged (10 min, 1500 rpm, 4ºC) and resuspended in specific solution or medium depending on the analysis. Cell viability was assessed with trypan blue 0,2%.

Flow cytometry

Splenocytes were resuspended in 1 mL of buffer solution without azide and adjusted to $1x10^7$ cells/mL. Commercially conjugated antibodies to fluorescein isothiocianate (FITC), phycoerythrin (PE) and phycoerythrin-cyanine 5 (PE-Cy5) labeling were used (eBioscience, CA, USA). Hundred microliters of cell suspensions were incubated with 0,05 μg of anti-CD3 PE-Cy5 (145-2C11), anti-CD4 FITC (GK1.5) and anti-CD8 PE (CT-CD8b) for 30 min at room temperature, sheltered from light. After incubation, the cells were fixed in paraformaldehyde 1%. The analysis was performed with a FACSCalibur flow cytometer and CellQuest software (Beckton Dickinson, CA, USA).

Tissue bacterial loads

Thymus, inguinal and popliteal lymph nodes, spleen, lungs and liver from each mouse were collected and homogenized in 1 mL of saline. Then, hundred microliters of organ homogenates were plated in 7H11 mycobacteria agar and incubated at 37°C. After 30 days, *Mycobacterium bovis* colony forming units (CFU) were counted.

Cytokine production

Splenocytes were resuspended in 1 mL of RPMI medium supplemented with 10% of fetal calf serum, 2mM of L-glutamine and 40 mg/L of gentamicin, adjusted to $5x10^6$ cells/mL, cultured in 48-well flat-bottomed culture plates (Nunc) and stimulated with 10 μg/ml of concanavalin A (ConA) – type IV-S (Sigma, USA) or 10 μg/ml of recombinant heat shock protein 65kD (rhsp65). Cytokine levels were evaluated 48 hours later by enzyme linked immunosorbent assay (ELISA) in culture supernatants by using IFN-γ and IL-10 BD Opteia Sets (Beckton Dickinson, CA, USA) and TNF-α Duoset (R&D Systems, MN, USA). The assays were performed according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed using SigmaPlot (Systat Software, CA, USA). Body weight was analyzed by paired t test. All experiments related to BCG immunization were analyzed by One-way ANOVA parametric test or Kruskal-Wallis nonparametric test followed by comparative Dunn's test. The other results were analyzed by unpaired t test and all data were expressed as mean ± standard deviation. Values of $p<0,05$ were considered statistically significant.

Results

1. Alterations determined by experimental undernutrition

Effects on physical, biochemical and hematological parameters were initially determined twice, at the 10^{th} and the 40^{th} day after the beginning of dietary restriction.

Body weight was already significantly lower 10 days after the beginning of dietary restriction. More accentuated body weight loss was detected at the 40^{th} day. Thymus and spleen weights were also significantly lower in undernourished animals at the 40^{th} day. Triglycerides, cholesterol and glucose were all significantly below the normal levels at the 40^{th} day but only triglycerides and glucose already presented low levels at the 10^{th} day. Total leukocyte number was significantly decreased at 10 days in undernourished mice comparing to the control group. At 40 days this decrease was even more striking. This reduction was coincident with diminished lymphocyte number. These results can be observed at table 1.

2. Immunological alterations during undernutrition

The total leukocyte and lymphocyte numbers were already significantly decreased 10 days after the beginning of dietary restriction (table 1). However, the total number of splenic cells and the percentage of TCD4+ and TCD8+ peripheral blood cells were very similar in both experimental groups. However, at the 40^{th} day of dietary restriction, the percentage of CD4+ T cells, but not of CD8+ T cells, was significantly increased in the undernourished group. Contrasting with this high number of CD4+ T cells, the production of IFN-γ was significantly below the normal levels found in healthy animals. The ability to produce TNF-α and IL-10 was preserved in the undernourished group. These results can be all observed at table 2. Examples of the FACS analysis are documented at figure 1.

3. BCG spread and load in undernourished mice

Fifteen days after subcutaneous immunization with BCG, normal and undernourished mice were euthanized and the amount of bacteria was determined in secondary lymphoid organs, liver, lungs and thymus. As expected, bacteria were not recovered from non immunize animals. However, striking differences were observed

between normal and undernourished mice. A much higher proportion of undernourished mice presented bacteria dissemination to lymph nodes, spleen and liver. Only undernourished animals showed bacteria at the lungs and thymus. Results from each group can be observed at table 3. In addition, a much higher amount of bacilli was recovered from undernourished animals as can be observed at figure 1.

4. Effect of BCG on cytokine production

BCG inoculation in the well nourished mice, but not in undernourished ones, determined increased production of IFN-γ, TNF-α and IL-10 induced by the specific stimulus (rhsp65). BCG was also able to increase IL-10 production induced by ConA in healthy mice but not in the undernourished ones.

Table 1: Physical, biochemical and hematological parameters from BALB/c mice submitted to normal and restricted diets 10 and 40 days after the beginning of dietary restriction.

	10 days		*40 days*	
Parameters	***Normal n=6***	***Restricted n=6***	***Normal n=6***	***Restricted n=6***
Initial body weight (g)	20.66 ± 1.23	20.65 ± 1.15	20.66 ± 1.23	20.65 ± 1.15
Final body weight (g)	21.21 ± 1.16	18.68 ± 0.87 ♦*	23.47 ± 1.67 ♦	16.67 ± 1.39 ♦*
Spleen weight (g)	0.096 ± 0.027	0.096 ± 0.023	0.111 ± 0.016	0.071 ± 0.020 *
Thymus weight (g)	0.130 ± 0.025	0.127 ± 0.032	0.061 ± 0.014	0.028 ± 0.012 *
Total leucocyte number (10^6 cells/mL)	7.70 ± 2.54	3.95 ± 0.56 *	5.97 ± 1.35	1.65 ± 1.13 *
Lymphocyte number	6.89 ± 2.51	3.43 ± 0.56 *	5.04 ± 1.20	1.28 ± 0.97 *
PMN number	0.78 ± 0.30	0.51 ± 0.19 *	0.87 ± 0.38 l	0.37 ± 0.21 l *
Monocyte number	0.03 ± 0.01	0.01 ± 0.03 *	0.02 ± 0.05	0
Glucose (dL/mL)	68.25 ± 9.75	58.27 ± 7.23 *	71.42 ± 10.95	49.67 ± 9.47 *
Total proteins (g/dL)	6.16 ± 0.39	6.21 ± 0.19	4.91 ± 0.28	4.93 ± 1.03
Albumin (g/dL)	2.52 ± 0.18	2.62 ± 0.08	2.30 ± 0.19	2.98 ± 0.25
Triglycerides (mg/dL)	198 ± 54	65 ± 8 *	171 ± 32	66 ± 21 *
Cholesterol (mg/dL)	87 ± 9	82 ± 5	78 ± 7	66 ± 7 *

♦ $p \leq 0.05$ compared to initial weight.

* $p \leq 0.05$ compared to normal counterpart.

Table 2: Spleen T cell subsets determined by FACS and cytokine production determined by ELISA in splenic cell cultures stimulated with ConA during 10 and 40 days of dietary restriction.

	10 days		*40 days*	
Immunological parameters	***Normal n=6***	***Restricted n=6***	***Normal n=6***	***Restricted n=6***
Number of splenic cells	37.0 ± 10.6	40.5 ± 9.4	57.8 ± 9.9	26.9 ± 12.0 *
% of TCD4+ cells	24.04 ± 4.13	26.28 ± 4.64	24.58 ± 0.90	37.58 ± 11.88 *
% of TCD8+ cells	10.98 ± 2.86	12.62 ± 2.04	11.35 ± 0.83	12.00 ± 3.50 *
INF-γ (pg/mL)	26181 ± 6589	22076 ± 3783	31597 ± 6154	99 ± 48 *
TNF-α (pg/mL)	153 ± 23	127 ± 26	202 ± 12	238 ± 30
IL-10 (pg/mL)	506 ± 96	487 ± 95	1181 ± 150	1072 ± 142

* $p \leq 0.05$ compared to normal counterpart.

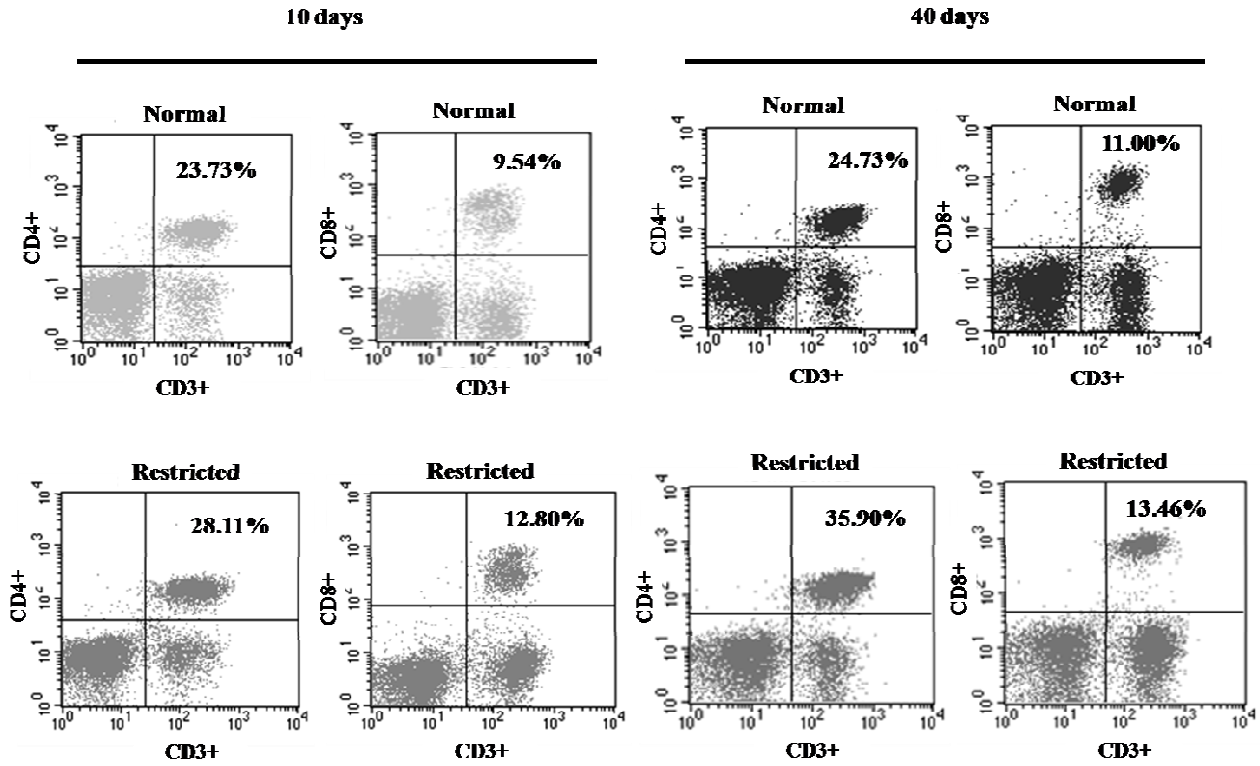

Figure 1: TCD4+ and TCD8+ cells percentages in BALB/c mice submitted to normal and restricted diets during 10 and 40 days.

Table 3: Number of colonized BCG immunized BALB/c mice submitted to normal and restricted diets 25 days after the beginning of dietary restriction.

Groups	*Lymph nodes*	*%*	*Spleen*	*%*	*Liver*	*%*	*Lung*	*%*	*Thymus*	*%*
Normal + BCG	7/13	53,8	2/6	33,3	1/12	8,3	0/7	0	0/12	0
Restricted + BCG	10/11	90,9	4/5	80,0	5/12	41,7	3/6	50,0	9/11	81,8
Household contacts	2/4	50	1/4	25	1/4	25	-	-	0/4	0

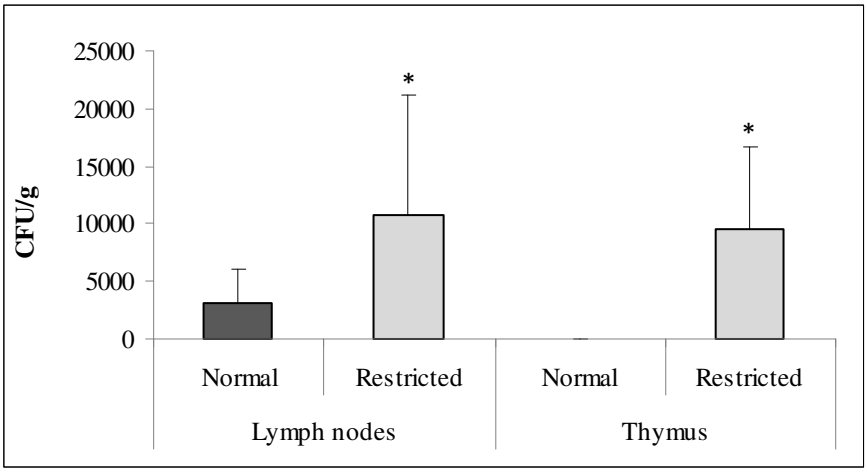

Figure 2: Number of *M. bovis* CFU recovered from lymph nodes and thymus of BALB/c mice submitted to normal and restricted diets 25 days after the beginning of dietary restriction. * $p \leq 0.05$ compared to normal counterpart.

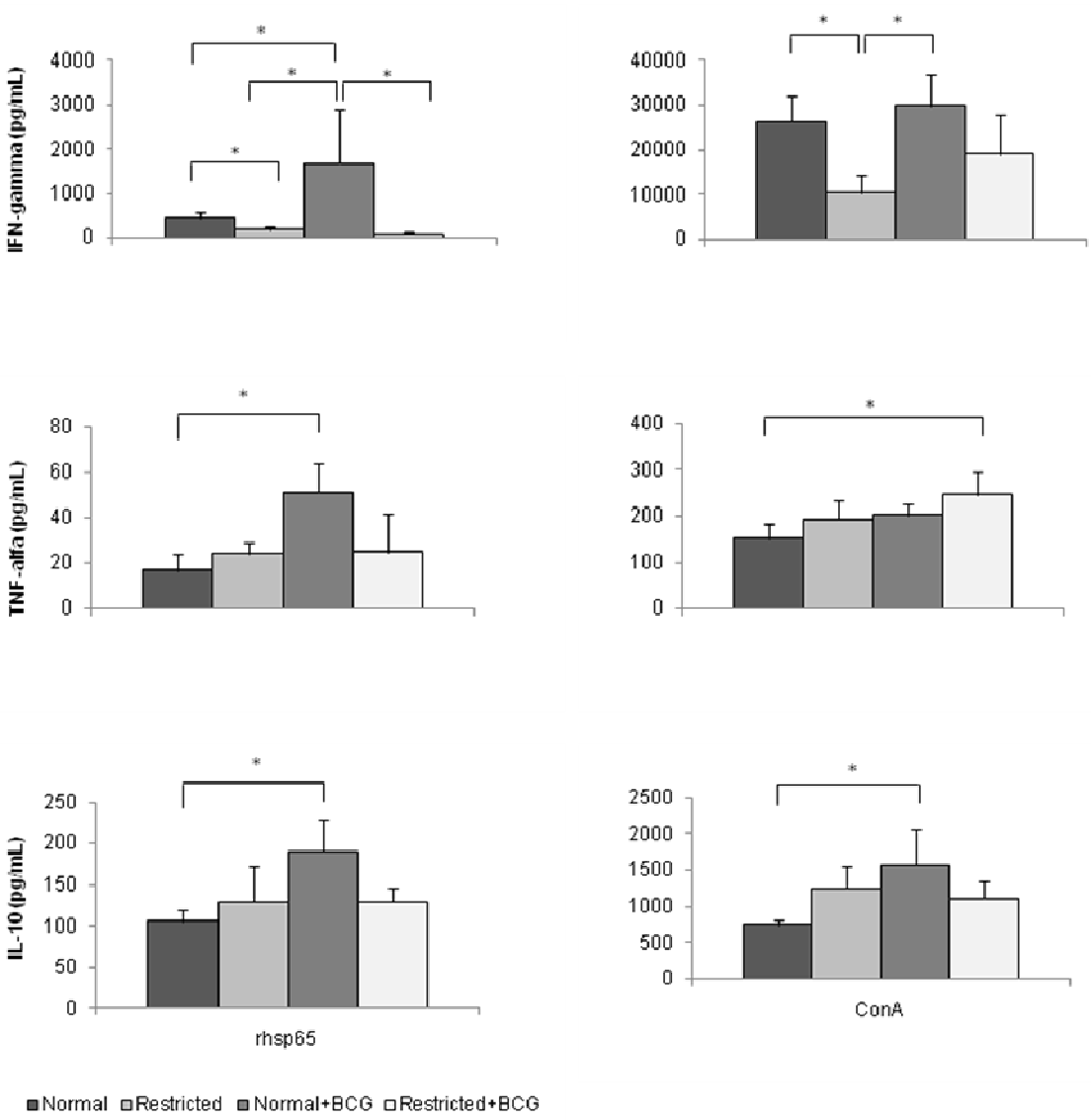

Figure 3: Cytokine production in BCG immunized BALB/c mice submitted to normal and restricted diets during 25 days. * $p \leq 0.05$.

Discussion

Experimental dietary restriction by deprivation of variable percentages of food intake is being used to explore the effects of PCM on immunity and susceptibility to infectious agents (Niiya et al., 2007). There is not, however, a systematic evaluation of the effect of undernutrition on susceptibility to attenuated vaccines that could, theoretically, behave as virulent pathogens. In this context, we first characterized the establishment of an undernutrition state in a mice model and then determined its effect in the load and spread of BCG. The immunological alterations triggered by undernutrition and the effect of BCG on cytokine production were also evaluated. BCG was intradermically injected at the 10^{th} day after the beginning of dietary restriction. Some parameters already presented alterations at this period. Body weight, total leukocyte, PMN and lymphocyte number, glucose and triglycerides serum levels were all below the normal levels in undernourished animals. At the 40^{th} day of food restriction, these alterations were, as expected, much more pronounced. Other changes as decreased spleen and thymus weight and cholesterol serum levels were additionally observed at this advanced period of food restriction.

Cellular immunity is vital for both, induction of protection by BCG and control of BCG multiplication in the vaccinated host, the percentage of CD4+ and CD8+ T cells was also evaluated after 10 and 40 days of dietary restriction. Even though the total number of lymphocytes was already significantly below normal levels after 10 days of dietary restriction, the percentage of TCD4+ and TCD8+ cells in the spleen was still in the normal range. However, at 40 days after food restriction, the number of TCD4+, but not of TCD8+, was significantly above the normal range. Even though these results seemed initially contradictory, a few literature reports described differential CD4/CD8 ratios in blood and secondary lymphoid organs (Manhart et al., 2000). Lee & Woodward, 1996, demonstrated an expected low CD4/CD8 ratio in the peripheral blood samples, but not in spleen, similarly to our result.

As some undernourished vaccinated mice begun to present a cachectic appearance before the end of the planned protocol all groups were submitted earlier (15 days after BCG immunization) to euthanasia. The presence and amount of bacilli was determined in lymph nodes, spleen, thymus and liver to compare the severity of BCG infection. The percentage of bacilli recovered from all evaluated organs was always clearly higher in undernourished mice. In addition, no bacteria spread to lung and

thymus was detected in well nourished mice, whereas in the undernourished animals there was bacilli recovery in 50 and 81.1% of the animals, respectively.

Another indication of more severe infections in undernourished mice was obtained by comparing the loads of BCG recovered from the analyzed organs. A significant higher amount of bacilli was detected in lymph nodes and thymus derived from undernourished immunized mice in comparison to well nourished immunized ones. No difference was detected when BCG loads in spleen and liver were compared (not shown).

An additional experiment was done to compare the effect of household contact between healthy and undernourished mice. No bacilli were recovered from well nourished non immunized mice maintained at the same environment as the well nourished BCG immunized mice. Inasmuch, some undernourished non BCG immunized house contacts present BCG in lymph nodes, spleen or liver as demonstrated at table 2. Even though this finding deserves a much deeper evaluation, it alerts for the relevance of undernourishment in household contacts, especially in the case of tuberculosis and attenuated vaccines. Severe malnutrition is an important risk factor for the transmission of infections to children in household contact with adults having pulmonary tuberculosis (Singh et al., 2005). In addition, the safety of live attenuated rotavirus vaccines in malnourished children has being discussed (Wood, 2005).

For the best of our knowledge, this is the first report showing the behavior of BCG in experimental undernutrition. Two aspects related to these findings deserve special attention and merit future evaluation. The first one relates to the efficacy of BCG and the potential triggering of disseminated BCG infection (BCGosis) in undernourished children. Severe combined immunodeficiency children can present BCGosis after BCG immunization due to compromised immunological system, clearly indicating that vaccination should be only performed in those with an intact immune system (Sadeghi-Shabestari & Rezaei, 2009). A second aspect relates to the immunological consequences of BCG presence in the thymus in undernourished mice. Only few reports analyzed mycobacterial colonization in mice thymus. According to Nobrega et al., 2007, BCG started to colonize the thymus at a later time, by the time bacterial load is already decreasing in other organs. Our results demonstrated that undernourished immunized mice presented early thymus colonization, i.e., 15 days after the inoculation of BCG whereas the well nourished group did not present the

mycobacteria. It should be evaluated at the future whether the presence of mycobacteria can influence the thymus selection and affect the course of this or other infectious diseases.

The comparative analysis of cytokine production evaluated 15 days after BCG immunization indicated that the decreased ability to produce IFN-γ in response to ConA in the restricted group was already present 25 days after the beginning of dietary restriction. In addition, healthy animals immunized with BCG, but not the undernourished immunized group, produced significant levels of IFN-γ, TNF-α and IL-10 in response to in vitro stimulation with rhsp65. As TNF-α and IFN-γ activate macrophages to restrain mycobacterial growth (Flynn et al., 1995; Flynn et al., 1993) this low specific cytokine induction could explain the higher BCG load in undernourished animals. In this same line of thought we could also think that this cytokine deficit is allowing BCG spread to thymus and lungs.

Even though the extension of the protection ability of BCG is highly debated, there is a general consensus that this vaccine is able to protect children against the more severe tuberculosis infection (Hussey et al., 2009), to determine some resistence to leprosy (Merle et al., 2010) and also to establish a non specific protection against other infectious diseases (Spencer et al., 1977). In this scenario, it is expected that these capabilities could be also detrimentally affected by undernutrition.

Differently from healthy mice that upregulated IL-10 production by polyclonal activation, this upregulation was not observed in undernourished mice. In this context, it is tempting to hypothesize that the immunoregulatory ability attributed to BCG in relation to autoimmunity and allergies is lost in undernourished individuals. Support for BCG immunoregulatory potential in healthy animals has been described in both cases. Sewell et al., 2002, for example, observed that *Schistosoma mansoni* OVA pretreatment, BCG infection and lyophilized *M. tuberculosis* all decreased clinical manifestations of experimental autoimmune encephalomyelitis in mice. Interestingly, this immunoregulatory activity has been associated with IL-10 production by Th17 cells (Nikoopour et al., 2008) and dendritic cells (Bilenki et al., 2010).

Together these results bring about much questioning related to BCG vaccination in undernourished children. One of these aspects is directly related to safety. It will be fundamental to analyze, for example, if they develop BCGosis. Studies of BCG

protective ability against leprosis and the more serious forms of tuberculosis is certainly also mandatory. The possibility that BCG loses its ability to down-modulate the deleterious immune response associated with autoimmunity and allergy also deserves investigation.

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6. ANEXO: TRABALHO PUBLICADO RELACIONADO AO PROJETO

6.1. Immunization protected well nourished mice but not undernourished ones from lung injury in Methicillin-resistant *Staphylococcus aureus* (MRSA) infection.

BMC Microbiology

Research article

Immunization protected well nourished mice but not undernourished ones from lung injury in Methicillin-resistant *Staphylococcus aureus* (MRSA) infection

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Abstract

Background: *Staphylococcus aureus* methicillin-resistant (MRSA) has been frequently isolated from endotracheal and lung puncture aspirates in malnourished children with pneumonia. In this work we evaluated the susceptibility of undernourished BALB/c mice and its ability to mount a protective immunity against MRSA with emphasis on the lung involvement.

Results: BALB/c mice submitted to a 20% dietary restriction during 20 days presented a significant decrease in body weight, lymphocyte number and also atrophy in thymus and intestinal epithelium. Determination of bacterial load by the number of colony forming units (CFU) indicated a similar susceptibility whereas the findings of Gram stain clearly suggested a higher amount of bacteria in the lungs of normal mice than in the undernourished ones. Immunization reduced bacterial growth in the lungs of normal mice but not in the undernourished ones. Histopathological analysis showed that inflammation appeared in the lungs from normal mice only after infection and that immunization prevented this pulmonary inflammatory process. On the other hand, undernourished mice presented lung inflammation even before infection. In addition, the degree of this inflammatory process did not change with infection or previous immunization.

Conclusion: Our results indicated that lung injury during MRSA infection is prevented by previous immunization in well nourished but not in undernourished mice.

Background

Protein energy malnutrition (PEM) is the most frequent type of malnutrition, affecting at least 800 million people worldwide [1]. It is especially prevalent in certain groups as children, elderly people, patients with chronic diseases or neoplasia, and also in 50 to 90% of hospitalized patients [2,3]. Malnutrition by itself can cause death [4] but epidemiological data reveals that it greatly increases susceptibility to and severity of infections, being a major cause of illness and death from infectious diseases [3,5]. A direct correlation between higher degrees of malnutrition and higher risk of death is supported by the observation that severely malnourished children experienced substantially higher mortality rates [6,7]. Increased morbidity and mortality in malnutrition is associated with decreased immunocompetence with particular involvement of cell-mediated immunity, antibody secretion and affinity and also complement components and cytokine production [8]. We recently demonstrated that diet restriction reduced IL-4 and IFN-γ and also abrogated specific antibody production in BALB/c mice immunized with a genetic vaccine containing the mycobacterial hsp65 gene [9]. As described above, a significant proportion of hospitalized patients are undernourished and at a greater danger to get severe hospital-infections. *Staphylococcus aureus* has been one of the most common bacterial causes of severe pneumonia in children with nosocomial infections [10]. Although previously considered as a purely nosocomial event, community-acquired methicillin-resistant *S. aureus* (MRSA) pneumonia is underestimated and is spreading worldwide [11]. In addition, leukocytopenia and malnutrition are described as high risk factors that lead to death by nosocomial *S. aureus* pulmonary infections [12]. In spite of its relevance, the behaviour of *S. aureus* in undernourished subjects has not been fully investigated.

In this context, we used a PEM murine model to evaluate both, the susceptibility and the ability to mount a protective immunity against a MRSA with emphasis on lung involvement.

Results

Alterations determined by undernutrition

We initially characterized a model of dietary restriction by determining body weight, triglyceride seric levels and leucogram. Effects of two percentages (10 and 20%) of dietary restriction were compared with parameters observed in a control group that received food *ad libitum*. Both levels of restriction determined a significant weight loss and decreased serum concentration of triglycerides (figure 1a and 1b, respectively). However, only the group submitted to 20% of dietary restriction presented alterations compatible with secondary immunodeficiency as decreased lymphocyte number (figure 1c).

Effect of dietary restriction and immunization on bacterial load

Twenty-four hours after intraperitoneal infection with 5×10^8 CFU/0.5 mL of *S. aureus*, all animals from the four experimental groups presented bacteria in the blood (figure 2a). Determination of CFU in the spleen did not show any significant difference among these groups (figure 2b). However, differences were observed in lung analysis. Well nourished mice immunized with formolized *S. aureus* presented a significant reduction in CFU in this organ. Interestingly, this effect was not triggered in undernourished mice. An even increased amount of bacteria was present in undernourished immunized animals (figure 2b). A reduced amount of bacteria was also observed in the liver of well nourished mice that were previously immunized with *S. aureus* (figure 2c). Injection of Complete Freund's Adjuvant alone did not reduce bacterial load (not shown).

Lung histopathological analysis

As expected the pulmonary parenchyma from well nourished and non infected mice showed a very well preserved alveolar structure without any inflammatory process (figure 3a). Infection of well nourished animals determined a clear inflammatory infiltration in the lungs (figure 3c). This inflammatory reaction clearly subsided if the animals were immunized before infection (figure 3e). However, undernourished mice presented a distinct lung involvement. They already presented a pulmonary disseminated inflammatory process before infection with *S. aureus*. This reaction was characterized by septal thickening and a clear mononuclear cell infiltration (figure 3b). Interestingly, the intensity and the quality of this inflammatory reaction were not altered by infection preceded or not by immunization with killed *S. aureus*, as documented at figure 3d and 3f, respectively.

Bacterial density evaluated by Gram stain

Staining of lung sections by Gram showed absence of the typical Gram positive cocci in non infected mice (figure 4a and 4b), independently of their nutritional status. A great amount of cocci was, as expected, present in infected well nourished mice (figure 4c). Immunization of these animals before infection visibly reduced the amount of these bacteria in lung parenchyma (figure 4e). Lung evaluation in undernourished mice indicated two striking differences. Comparing to well nourished group, the undernourished one presented a clear reduction in the amount of cocci in the lungs (figure 4d). In addition, previous immunization of these animals did not reduce lung colonization by the bacteria (figure 4f).

Discussion

Protein energy malnutrition (PEM) is the most common type of undernutrition. It leads to secondary immunode-

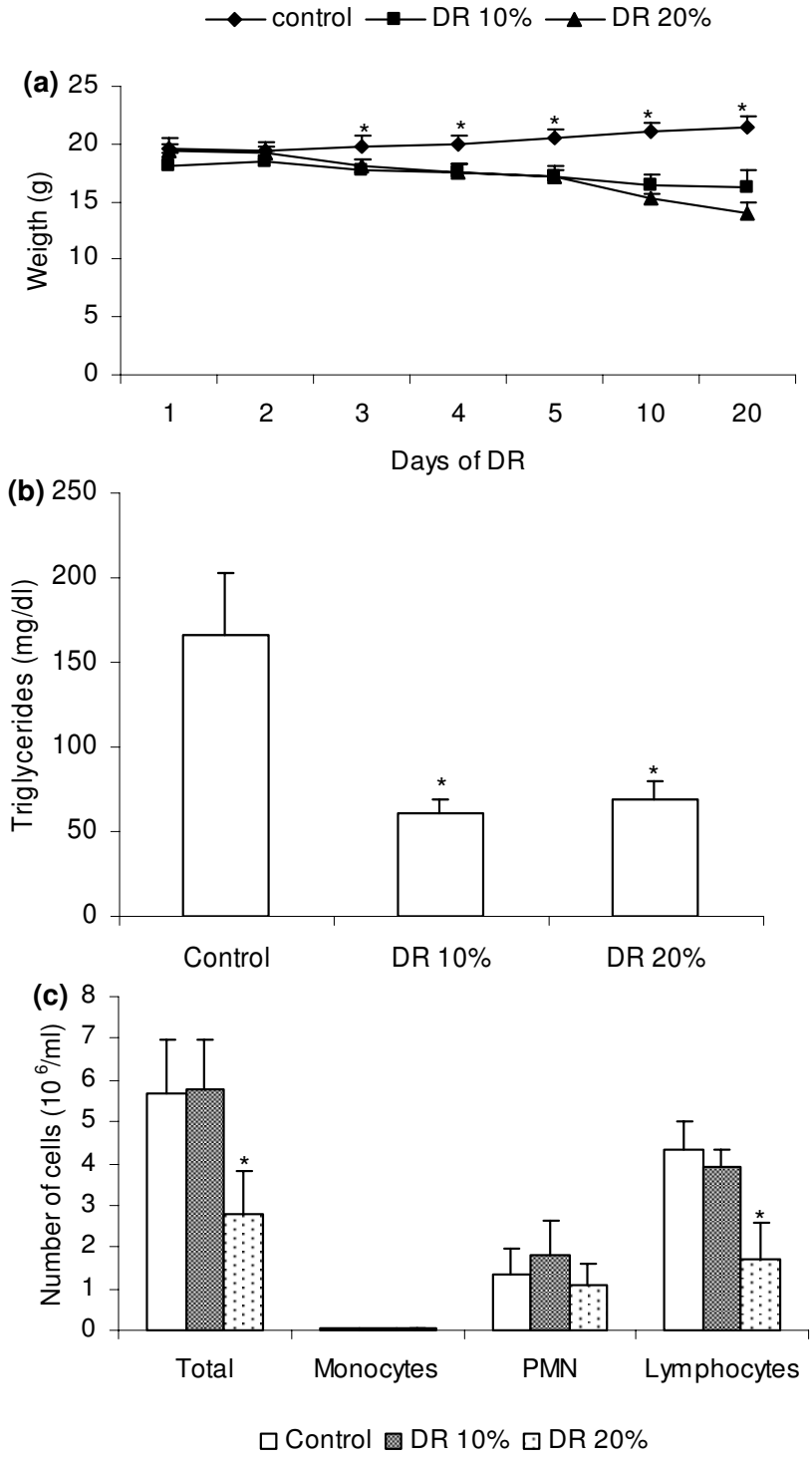

Figure 1

Alterations determined by undernutrition. BALB/c mice were submitted to two percentages of dietary restriction (10 and 20%) and evaluated in relation to weight loss (a), seric triglyceride concentration (b) and differential blood cell count (c). Results are expressed as mean ± SD of 5 animals per group (*$p < 0.05$) in relation to well nourished group.

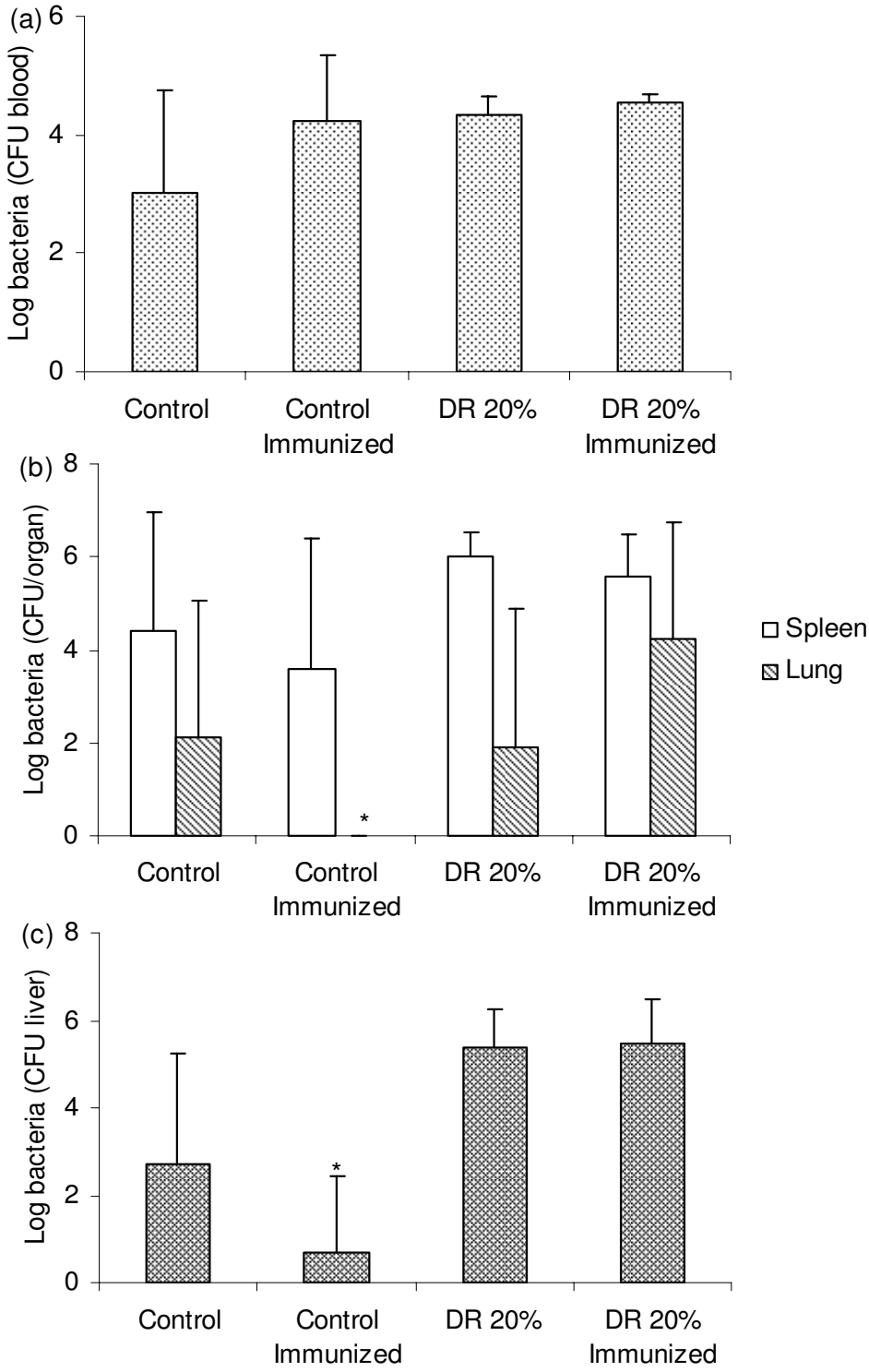

Figure 2

Effect of dietary restriction and immunization on bacterial load. BALB/c mice were submitted to dietary restriction (20%), immunized with the formolized bacteria and infected with 5×10^8 CFU/0.5 ml of *S. aureus*. The bacterial load was determined 24 hours later in the blood (a), spleen and lung (b) and liver (c). Results are expressed as mean ± SD of 5 animals per group (*$p < 0.05$) in relation to well nourished group.

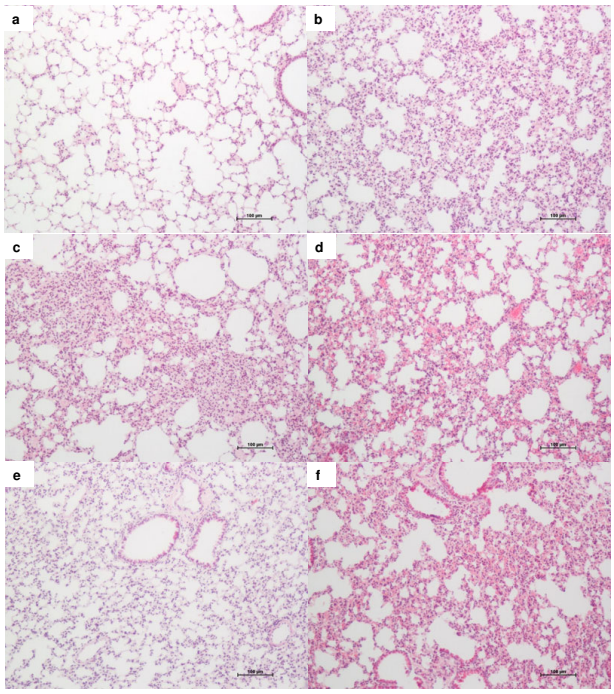

Figure 3
Effect of dietary restriction and immunization on lung histology. BALB/c mice were submitted to dietary restriction (20%), immunized with the formolized bacteria and infected with *S. aureus* (5×10^8 CFU/0.5 ml). Lung sections were obtained 24 hours later, stained with H&E and analysed with a Leica microscope. Lung samples from normal (a), undernourished (b), well nourished and infected (c), undernourished and infected (d), well nourished immunized and infected (e), undernourished immunized and infected (f).

ficiency and consequently increased susceptibility to infectious agents, including to *S. aureus* [13-15]. In this context, this work was done to establish a murine experimental model of PEM and to evaluate the effect of malnutrition on both, susceptibility and ability to mount a protective immunity against a methicillin-resistant *S. aureus* (MRSA). To define a model of dietary restriction, BALB/c female mice were submitted to a reduction of 10 or 20% in their food intake during 20 days and compared to a well nourished experimental group. Both levels of restriction determined a significant decrease in weight and serum triglycerides concentration. However, immunological evaluation indicated that only the group submitted to 20% dietary restriction developed secondary immunodeficiency. Initial comparison of colony forming units (CFU) obtained from spleen, liver and lung homogenates suggested that well nourished and undernourished mice were similarly susceptible to *S. aureus* infection. This methodology also suggested that a previous immunization with formolized *S. aureus* was able to partially protect healthy animals but not undernourished ones. In addition, this vaccine protective effect varied according to the evaluated organ; it was observed in the liver and lungs but not at the spleen. Even though determination of CFU in organs not previously perfused have been used as a parameter to quantify bacterial colonization [16] it is possible that bacteremia could interfere with the results. As lungs are critical targets during MRSA infections, a more detailed investigation was performed at the lungs by doing an histopathological analysis with H&E and Gram stains. This approach would allow a direct evaluation of lung parenchyma, avoiding a possible interference by bacteria present in the blood. As expected, lung structure was totally preserved among the animals from the normal control group that presented very well defined alveolar spaces and no signs of inflammation. Well nourished mice infected with *S. aureus* developed a clear and widespread inflammatory reaction in this organ. Interestingly, there was an evident downmodulation of this inflammatory reaction in well nourished mice previously vaccinated with *S. aureus*. On the other hand, undernourished animals already presented a lung disseminated inflammatory process before infection. This inflammatory reaction did not change in amount or quality after infection with *S. aureus* preceded or not by immunization. The cause of this inflammatory process was not investigated. However, it could be due to the presence of environmental agents or, alternatively, to the overgrow of resident bacteria that could trigger a respiratory infection in these animals but not in the well nourished ones.

As expected, staining of lung sections with Gram revealed a great amount of cocci in well nourished mice infected with *S. aureus*. Immunization before infection determined a visible reduction in the amount of bacteria and this coincided with an almost complete resolution of the inflammatory process found at the lung parenchyma. Comparing to these findings, two striking differences were detected in undernourished animals. They presented a much smaller amount of cocci in the lungs. This was initially unexpected because undernutrition has been more commonly associated with increased susceptibility to infectious agents [17]. However, this finding could be explained by competition for nutrients between host and pathogens as described by Prentice & McDermid, 2008 [18]; therefore decreasing the food supply for bacterial growth. Alternatively, endogenous or environmental bacteria could, as we said before, be already present at the pulmonary parenchyma in undernourished mice, competing for nutrients. The fact that *S. aureus* is a poor competitor and does not grow well in the presence of other microorganisms supports this hypothesis [19]. Previous immunization of undernourished mice, differently from the findings in the well nourished group, did not decrease the amount of cocci in the lungs. We believe that this result could be attributed, at least partially, to a decreased antibody production because they are essential to control

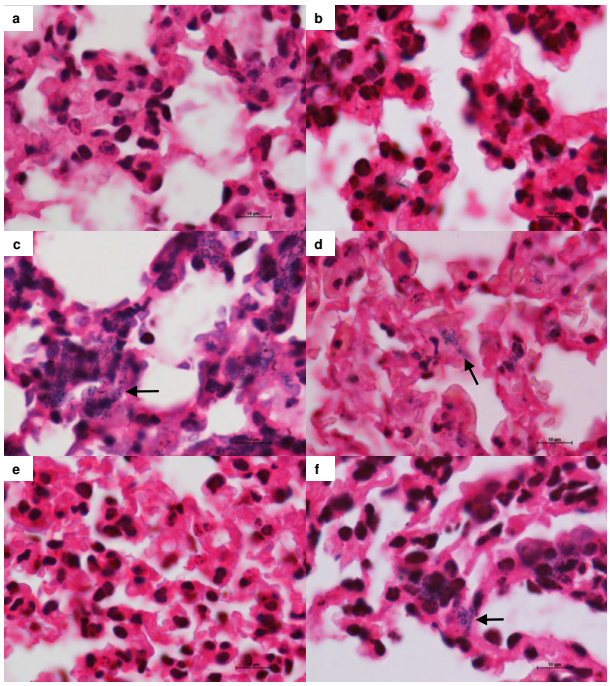

Figure 4
Effect of dietary restriction and immunization on lung bacterial load. BALB/c mice were submitted to dietary restriction (20%), immunized with the formolized bacteria and infected with *S. aureus* (5 × 10^8 CFU/0.5 ml). Lung sections were obtained 24 hours later, stained with Gram and analysed with a Nikon microscope. Lung samples from normal (a), undernourished (b), well nourished and infected (c), undernourished and infected (d), well nourished immunized and infected (e), undernourished immunized and infected (f). Arrows indicate bacteria location.

S. aureus infections, including life-threatening conditions as pneumonia and septicemia [20].

From a practical point of view, these results raise two very relevant aspects. The first one relates to the condition of malnutrition as a high risk factor for nosocomial pulmonary infections caused by MRSA. This possibility has not been directly investigated but it has been suggested by some findings as the ones described by Miyake et al., 2007 [21]. Our results also alert for a possible low efficacy of an MRSA vaccine in undernourished patients, mainly concerning the prevention of pulmonary involvement.

Conclusion

Together these results demonstrated that a 20% dietary restriction in food intake triggered a secondary immunodeficiency in BALB/c mice. This condition determined a very distinctive lung involvement in comparison to well nourished animals. This organ presented an inflammatory process that was not altered by infection with *S. aureus* or by infection preceded by immunization with the formolized bacteria. Absence of required nutrients or a state of resistance by the previous inflammatory process could decrease *S. aureus* growth in lungs of undernourished animals.

Methods

Experimental design

Isogenic female BALB/c mice, 4-5 weeks old were manipulated according to the ethical guidelines adopted by the Brazilian College of Animal Experimentation, being the experimental protocol approved by the local Ethics Committee. After weaning the animals received a 10 day acclimation on a standard chow. In the first set of experiments, after being acclimated they were distributed into three experimental groups (with 5-6 animals each) including the control fed *ad libitum* and two others that received 80 or 90% of the amount of food consumed by the control group and that were called DR 20% and DR 10%, respectively. The animals were kept in these conditions during 20 days and then evaluated by clinical (weight), biochemical (triglycerides) and lymphocyte number. In a second set of experiments, after being acclimated, mice were allocated into 4 experimental groups (4-5 animals each). Two groups were kept under normal diet and the other two were submitted to DR 20%. After 10 days, one control and one DR 20% group were immunized with formolized *S. aureus*. Ten days after, i.e, at the 20th day from the beginning of diet, all groups were infected with a fresh *S. aureus* suspension. Twenty four hours later the animals were euthanized to determine the bacterial load by CFU in blood, spleen, liver and lungs. Lung injury was additionally evaluated by hematoxylin & eosin and Gram stains.

Bacterial suspension

A *S. aureus* strain (S-6055/94) initially isolated from a clinical specimen was used for infections. This strain was characterized as being methicillin resistant by mecA gene detection by PCR. The strain was cultivated in blood agar and incubated at 37°C for 24 h. Isolated colonies were inoculated into brain heart broth and incubated at 37°C for 24 h. Bacteria were collected by centrifugation, washed and resuspended at a concentration of 1 × 10^9 CFU/mL. Mice were injected by intraperitoneal route with 5 × 10^8 CFU in 0.5 mL of saline. Control mice received an equal volume of saline. Bacteria were alternatively inactivated by resuspension in formol 3%. Normal and diet restricted groups (10th day of restriction) were immunized by subcutaneous route with 2 × 10^8 CFU/0.2 ml formolized *S. aureus* previously emulsified with Complete Freund's Adjuvant.

Blood evaluations

Blood samples were collected by cardiac puncture and total leukocyte number was counted after blood dilution in Turk's solution. Differential leukocyte count was per-

formed by analysis of blood smears stained with eosin/methylene blue (Leishman's stain). Serum samples were kept al - 20°C and total triglycerides concentration was measured by an enzymatic method (Kits Laborlab, Guarulhos, São Paulo).

Histopathological analysis

Lung sections were obtained 24 hours after infection, were fixed in formalin (10%), embedded in Paraplast plus (McCormick), prepared routinely and then sectioned for light microscopy. Sections (5 μm each) were stained with haematoxylin and eosin (H&E) or with Gram and analyzed by optical microscope and the images acquired with a coupled digital camera.

Determination of blood and tissue bacterial loads

Blood samples, spleens, lungs and livers from infected animals were homogenized in saline and plated. Briefly, 0,1 mL of serially diluted organ homogenates or 50-100 μL of blood were inoculated into baird-parker agar plates and incubated at 37°C. Colonies were counted 24 h later.

Statistical analysis

Statistical analysis was performed using SigmaStat statistical software (Jandel Corp., San Rafael, CA). The Kruskal-Wallis nonparametric test was used to compare CFU determinations in livers. For the parametric variables the results were expressed as mean ± standard deviation (SD) and the comparisons between the groups were made by variance analysis (ANOVA) followed by Tukey's test. A *P* value of less than 0.05% was considered statistically significant.

Abbreviations

PEM: protein energy malnutrition; CFU: colony forming unit; H&E: haematoxylin and eosin; SD: standard deviation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TGDF and LLWI executed most of this work. SFGZP, FCM and CPFG. largely contributed with the immunological experiments and the statistical analysis. MLRSC. participated in the design of the study and contributed with her expertise in *Staphylococcus* and AS conceived the study, coordinated it and revised the manuscript. All authors read and approved the final manuscript.

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