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Uso de Nanopartículas Metálicas na Vacinologia: Implicações para o Desenvolvimento de Vacinas contra Doenças Infecciosas.

LÁZARO MOREIRA MARQUES NETO

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ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE TESE DE LÁZARO MOREIRA MARQUES NETO - Aos nove dias do mês de outubro do ano de 2018 (09/10/2018), às 14:00 horas, reuniram-se os componentes da Banca Examinadora: Profs. Drs. Ana Paula Junqueira Kipnis, Lidia Andreu Guilo, Simone Gonçalves da Fonseca, Roosevelt Alves da Silva, Helioswilton Sales de Campos para, sob a presidência da primeira, e em sessão pública realizada no INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA DA UFG, procederem à avaliação da defesa de tese intitulada: **"USO DE NANOPARTÍCULAS METÁLICAS NA VACINOLOGIA: IMPLICAÇÕES PARA O DESENVOLVIMENTO DE VACINAS CONTRA DOENÇAS INFECCIOSAS."**, em nível de **DOUTORADO**, área de concentração em **BIOTECNOLOGIA**, de autoria de **LÁZARO MOREIRA MARQUES NETO,** discente do PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA E BIODIVERSIDADE, da Universidade Federal de Goiás. A sessão foi aberta pela orientadora do discente, Profa. Dra. ANA PAULA JUNQUEIRA KIPNIS, que fez a apresentação formal dos membros da Banca e orientou o Candidato sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida ao autor da tese que, em 30 minutos procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca arguiu o Candidato, tendo-se adotado o sistema de diálogo seqüencial. Terminada a fase de argüição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1181/2013 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Biotecnologia e Biodiversidade a Banca, em sessão secreta, expressou seu Julgamento, considerando o candidato (**Aprovado** ou **Reprovado**:

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DEDICATÓRIA

À minha família: minha mãe Marlene, minhas irmãs Calu e Lídia, meus sobrinhos Juju, Arthur e Catarina e meu companheiro Fábio. Por mostrarem para mim que felicidade e amor são dádivas que recebemos todos os dias.

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Na verdade, nem deveríamos estar aqui, mas estamos. São como nas grandes histórias. As que tinham mesmo importância. Eram repletas de escuridão e perigo. E, às vezes, você não queria saber o fim... Porque como podiam ter um final feliz? Como podia o mundo voltar a ser o que era depois de tudo isso? Mas, no fim, é só uma coisa passageira, até tudo passar. Um novo dia virá. E, quando o sol brilhar, brilhará ainda mais forte. Eram essas as histórias que ficavam nas lembranças, que significavam algo. Mesmo que você fosse pequeno demais para entender o porquê. Mas acho, que entendo, sim. Agora eu sei. As pessoas dessas histórias tinham várias oportunidades de voltar atrás, mas não voltavam. Elas seguiam em frente, porque tinham no que se agarrar."

J.R.R Tolkien, O Senhor dos Anéis.

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SUMÁRIO

QUADROS, TABELAS, FIGURAS E ANEXOS

SÍMBOLOS, SÍGLAS E ABREVIATURAS

IFN-γ	Interferon Gama
Ag	Antígeno
Ag85	Antígeno 85
AgNP	Nanopartícula de prata
AIDS	Acquired Immunodeficiency Syndrome
APCs	antigen presenting cells
AuNP	Nanopartícula de ouro
BCG	Bacilo de Calmette e Guerrin
BCR	B Cell Receptor
CD	Cluster of differentiation
CFP-10	Culture Filtrate Antigen 10
CTAB/PSS-MA	Cetyl trimethylammonium bromide/4-styrenesulfonic acid-comaleic acid
CuNP	Nanopartícula de Cobre
CuO_2NP	Nanopartícula de Óxido de Cobre
DAMPs	damage-associated molecular patterns
DCs	Dendritic Cells
DOTS	tratamento diretamente supervisionado
ESAT-6	Early Secreted Antigen 6
FDA	Food and Drug Agency
FE-SEM	Field Emission Scanning Electron Microscopy
GM-CSF	granulocyte macrophage colony stimulating factor
gp38	Glicoproteina 38
HIV	Human Immunodeficiency Virus
HspX	Heat Shock Protein X
IgA1	Imunoglobulina A1
IgA2	Imunoglobulina A2
IgD	Imunoglobulina D
IgG1	Imunoglobulina G1
IgG2a	Imunoglobulina G2
IgG2b	Imunoglobulina G2b

IgG3	Imunoglobulina G3
IL-1	Interleucina 1
IL-17	Interleucina 17
IL-21	Interleucina 21
IL-23	Interleucina 23
IL-6	Interleucina 6
IL-8	Interleucina 8
IONP	Iron Oxide Nanoparticle
ISCOMs	Immune stimulating complexes
LECs	Lymphatic Endothelial Cells
LPS	Lipopolissacaride
MAPK	mitogen-activated protein kinase
MeNP	Nanopartícula Metálica
MHCI	complexo principal de histocompatibilidade de classe I
MHCII	complexo principal de histocompatibilidade de classe II
$MnFe_2O_4NP$	Nanopartícula de Ferrita de Manganês
MPI	magnetic particle imaging
Mtb	*Mycobacterium tuberculosis*
MyD-88	Myeloid Differentiation primary response
NCMX	citrate-coated MnFe2O4 NPs coated with CMX
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor-κB
NIH	National Institute of Health
NiNLPs	nickel-functionalized nanolipoprotein particles
NK	Natural Killer Cells
NLR	NOD-Like Receptor
NP	Nanopartículas
OMS	Organização Mundial da Saúde
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PfCSP	P. falciparum circumsporozoite protein
PLGA	poly lactic-*co*-glycolic acid
PlNP	Nanopartícula de platina

PRR	Pattern Recognition Receptor
RM	Ressonância Magnética
ROS	Reactive Oxygen Species
SBCAL	Sociedade Brasileira de Ciência em Animais de Laboratório
SIDA	Síndrome da Imunodeficiência Adquirida
T CD8	T Citotoxic Cells
TB	Tuberculose
TCR	T Cell Receptor
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
Th1	T Helper Cells Type 1
Th17	T Helper Cells Type 17
Th2	T Helper Cells Type 2
TiO2NP	Nanopartícula de Óxido de Titânio
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor α
VLPs	Virus-Like Particle
WNV	West Nile virus
ZnO_2NP	Nanopartícula de Óxido de Zinco

RESUMO

MARQUES-NETO, Lázaro Moreria, Me, Universidade Federal de Goiás, setembro de 2018. **Uso de nanopartículas metálicas na vacinologia: Implicações para o Desenvolvimento de Vacinas em Doenças Infecciosas**. Orientador: Dra. Ana Paula Junqueira-Kipnis. Co-orientadora: Dr. André Kipnis

A busca por novos adjuvantes é um dos objetivos principais dentro da vacinologia. Juntamente com isso, entender o impacto do uso de nanopartículas como sistema de entrega e imunomodulador em sistemas vacinais impacta diretamente no desenvolvimento de novas vacinas. Nesse trabalho, buscamos estudar e elucidar a adjuvanticidade de nanopartículas magnéticas, bem como a imunogenicidade e proteção de sistemas vacinais utilizando essas nanopartículas. Inicialmente foi feito uma revisão da literatura buscando bases científicas que demonstrassem a possiblidade do uso de nanopartículas metálicas (MeNPs) como estimuladores do sistema imune inato. Buscou-se também encontrar elementos em que as nanopartículas metálicas pudessem auxiliar na geração de uma resposta celular do tipo Th1, Th17 e T CD8. A partir dessa revisão, verificou-se que as nanopartículas magnéticas, ou com íons metálicos, eram capazes de estimular a ativação de moléculas coestimuladoras (CD80, CD40 e CD86), induzir secreção de citocinas (IL-1, IL-6, IFN-γ e TNF-α) bem como a resposta imune humoral, mas nenhum trabalho demonstrou se essas nanopartículas eram capazes de induzir resposta celular. Consequentemente, na segunda parte do trabalho utilizou-se a tuberculose como modelo de estudo para verificar se uma formulação vacinal com uma nanopartícula magnética de ferrita de manganês combinada com proteína de fusão recombinante, teria capacidade indutora de resposta imune celular protetora, sem adição de outros adjuvantes. A nanopartícula foi recoberta com a proteína de fusão recombinante CMX e os camundongos BALB/c foram vacinados com essa formulação, em protocolo com três vacinações com intervalos de 21 dias. Posteriormente, os animais vacinados foram infectados com *Mycobacterium tuberculosis* (H37Rv) para se avaliar a proteção conferida pela vacina. Os resultados mostraram que a nanopartícula teve capacidade de gerar resposta imune celular dos tipos Th1, Th17 e T CD8, dependendo da via de inoculação (subcutânea, intranasal ou mista). Essa resposta foi principalmente do tipo Tc1 e foi capaz de proteger contra o desafio com Mtb. Adicionalmente, não houve qualquer aparecimento de efeito colateral ou danos em órgãos dos animais infectados, demonstrando que a formulação é segura. Por fim, as formulações vacinais com MeNPs, mais especificamente com ferrita de manganês, então demonstram potencial aplicação em vacinologia, podendo ser aplicada em formulações vacinais para gerar resposta imune celular, mas deve-se levar em conta a rota e, caso for utilizar outros adjuvantes complementares, deve-se pensar na possível interação da NP com o adjuvantes e seus ligantes.

Palavras-chaves: Vacinologia, nanopartículas, tuberculose, nanovacina, doença infecciosa.

ABSTRACT

MARQUES-NETO, Lázaro Moreira, Me, Universidade Federal de Goiás, September, 2018. **Role of Metallic Nanoparticles in Vaccinology: Implications for Infectious Disease Vaccine Development**. Advisor: Dra. Ana Paula Junqueira-Kipnis. Co-advisor: Dr. André Kipnis.

The search for new adjuvants is the main goal in vaccinology. Along with this, understanding the impact of using nanoparticles as a delivery system and immunomodulator in vaccine systems directly impacts the development of new vaccines. In this work, we seek to study and elucidate the adjuvanticity of magnetic nanoparticles, as well as its immunogenicity and protection of the vaccine systems. Initially, a literature review was made seeking scientific bases that demonstrated the possibility of using metallic nanoparticles (MeNPs) as innate immune system stimulators. It was also sought to find elements in which metallic nanoparticles could aid in the generation Th1, Th17 and T CD8 type cellular response. From this review, it was verified that the magnetic nanoparticles, or with metallic ions, were able to stimulate the activation of costimulatory molecules (CD80, CD40 and CD86), to induce secretion of cytokines (IL-1, IL-6, IFN-γ and TNF-α) as well as the humoral immune response, but no work demonstrated whether these nanoparticles were able to induce cellular response. Consequently, in the second part of the study, tuberculosis was used as model to verify if a vaccine formulation with a magnetic nanoparticle of manganese ferrite combined with recombinant fusion protein would have the ability to induce a protective cellular immune response, without adding other adjuvants. The nanoparticle was coated with recombinant CMX fusion protein and BALB/c mice were vaccinated with this formulation, in protocol with three vaccinations with 21-day intervals. Subsequently, the vaccinated animals were infected with Mycobacterium tuberculosis (H37Rv) to evaluate the protection conferred by the vaccine. The results showed that the nanoparticle was able to generate cellular immune responses of Th1, Th17 and T CD8 types, depending on the route of inoculation (subcutaneous, intranasal and mixed). The most preeminent response was Tc1 which was recalled after infection was able to protect against the challenge with Mtb. In addition, there was no appearance of side effects or damage to organs of infected animals, demonstrating that the formulation is safe. Finally, the vaccine formulations with MeNPs, more specifically with manganese ferrite, demonstrate potential application in vaccinology, and may be applied in vaccine formulations to generate cellular immune response, but the route must be considered and in case of use other adjuvants it should consider the possible interaction of NP with the molecule and their ligand.

Keywords: Vaccinology, nanoparticles, tuberculosis, nanovaccine, infectious disease.

1. INTRODUÇÃO

O desenvolvimento de nanopartículas para aplicação sobre o sistema imunológico é um campo emergente da nanociência e o seu uso um campo promissor da nanomedicina. Até pouco tempo atrás as principais características dos nanomateriais sobre o sistema imune, eram estudadas sob o ponto de vista da imunotoxicologia, ou seja, avaliando efeitos adversos e/ou negativos para o uso desses materiais na nanomedicina (Elsabahy and Wooley, 2013). No entanto, as características imunomoduladoras dessas partículas podem ser exploradas em várias das estratégias em que as nanopartículas já são utilizadas, além de abrir portar para que sejam explorados em outros usos para esses nanomateriais (Du et al., 2017; Granucci and Prosperi, 2017). Por exemplo, não gerar resposta alguma, anti-inflamatória ou pró-inflamatória, é o objetivo de uma nanopartícula utilizada como contraste em um exame de imagem. No entanto, uma nanopartícula com atividade anti-inflamatória própria seria ótima para ser utilizada em tratamentos de feridas para cicatrização ou em alguns casos de tratamentos de infecção. Por fim, nanopartículas com atividade pró-inflamatória, de maneira controlada pode ser utilizada para tratamento de tumores, podendo modificar o microambiente em que o tumor está melhorando a resposta imune do próprio hospedeiro contra as células cancerígenas, em adição aos quimioterápicos (Du et al., 2017; Granucci and Prosperi, 2017).

Há uma busca por novas moléculas com capacidade adjuvante, para serem usadas em conjunto com antígenos em formulações de vacinas de subunidade proteica. Adjuvantes podem ter vários mecanismos de ação e devem ser selecionados de acordo com o tipo de resposta final almejado. Sendo que um adjuvante para combater microrganismos intracelulares que infectam mucosas, como a tuberculose, deve ser capaz de gerar resposta imune do tipo Th1, Th17 e CD8 (Di Pasquale et al., 2015). Apesar de nanopartículas metálicas terem seu potencial imunoestimulatório estudado é importante entender realmente se elas têm capacidade adjuvante e para quais tipos de resposta almejada podem ser usadas.

Esse trabalho, portanto, busca avaliar a capacidade adjuvante de nanopartículas, utilizando como modelo uma nanopartícula de ferrita de manganês. O foco principal é verificar a possibilidade de essas nanopartículas induzirem resposta Th1, Th17 e CD8, sem a adição dos adjuvantes classicamente utilizados com esse objetivo (os agonistas de

TLR). Para tal, uma revisão da literatura foi feita com objetivo de enriquecer as evidências para a utilização dessas nanopartículas nessa estratégia. Em seguida, utilizamos uma nanopartícula composta de ferrita de manganês ($MnFe_2O_4$) coberta com citrato, em formulação com a proteína de fusão CMX em modelo de vacinação e proteção contra tuberculose objetivando avaliar a geração da resposta celular, bem como verificar se essa resposta é capaz proteger contra a infecção com a bactéria, reduzindo a carga bacilar e a patologia gerada pela infecção no pulmão dos camundongos infectados.

2. REVISÃO DA LITERATURA

2.1 Um panorama do uso de nanopartículas metálicas em nanomedicina

No final da década de 50, o físico Richard P. Feynman fez uma palestra na "Annual Meeting of the American Physical Society" intitulada “There’s Plenty of Room at the Bottom” e inaugurou a ideia de no futuro se poder construir objetos átomos por átomos. Por sua vez, a palavra nanotecnologia foi introduzida por N. Taniguchi em Tokio, no ano de 1974, ao descrever um processo de criação de materiais superfinos com precisão nanométrica. Desde então, esse conceito vem evoluindo até os dias atuais, sendo que em 2013 o NNI (National Nanotechnology Initiaves) departamento do NIH (National Institute of Health) dos Estados Unidos, define como nanotecnologia “a ciência, engenharia e tecnologia feita em escala nanométrica (entre 1 e 100 nanômetros)” (Figura 1) (Toumey, 2012; Hulla et al., 2015).

Nanopartículas (NP) ou nanomateriais são estruturas que apresentam ao menos uma de suas dimensões em escala nanométrica. Podem ser amplamente divididos em várias categorias, dependendo da sua morfologia, tamanho e propriedades químicas. Contudo, uma das classificações mais utilizadas se dá com base nas características físicas e químicas: (I) nanopartículas à base de carbono que compreendem as nanopartículas de fulereno e nanotubos de carbono; (II) nanopartículas à base de íons metálicos que podem ser formadas a partir íons de ferro (Fe), ouro (Au), níquel (Ni), zinco (Zn), dentre outros; (III) nanopartículas inorgânicas compreendem as nanopartículas sólidas não compostas de íons metálicos, como cerâmica e sílica; (IV) nanopartículas semicondutoras, compostas de matérias com características de materiais metálicos e não metálicos; (V) nanopartícula poliméricas compostas de matérias poliméricos como PEG/PLGA; (VI) nanopartículas lipídicas, compostas de frações lipídicas como os lipossomas; (VII) nanocompósitos, nanopartículas formadas de duas partes, por exemplo uma nanopartícula metálica coberta com PLGA(Dobrovolskaia and McNeil, 2007; Gatoo et al., 2014).

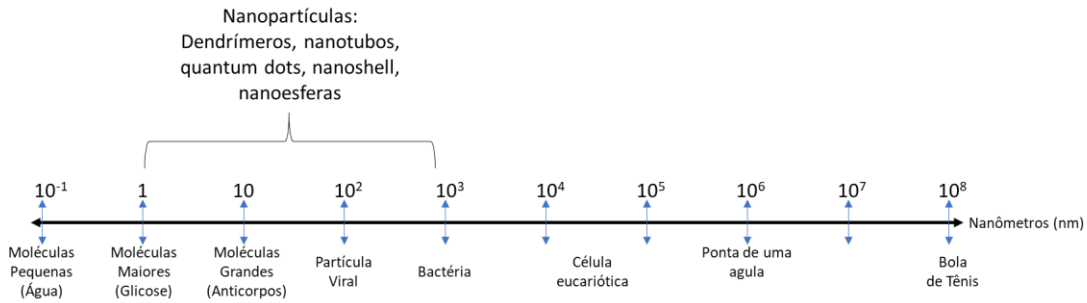

Figura 1. **Comparação do tamanho de estruturas e objetos**.

A nanomedicina é uma área relativamente nova da nanociência que foi definida pela Fundação Europeia para a Ciência como "a ciência e a tecnologia para diagnosticar, tratar e prevenir doenças e lesões traumáticas, aliviar a dor além de preservar e melhorar a saúde humana, utilizando ferramentas moleculares e o conhecimento molecular do corpo humano". Essa ciência busca a aplicação da nanotecnologia e nanomateriais para o desenvolvimento de novos dispositivos médicos, propiciando melhores ferramentas de diagnóstico, agentes de contraste para métodos imagem, sistemas de distribuição de fármacos e produtos farmacêuticos, terapias, implantes, construções de engenharia de tecidos, vacinas dentre outros (Zhang et al., 2008; Chiesa et al., 2009).

As nanopartículas de íons metálicos (MeNP) são uma classe de nanopartículas com grande potencial gerado por suas características únicas como: comportamento óptico, elétrico, catalítico, magnético, químico, estabilidade mecânica, fácil modificação de superfície e etc. No entanto, essas propriedades variam de acordo com o material com que a nanopartícula é sintetizado, de maneira que elas são subdividas em: nanopartículas de íons metálicos puros como nanopartículas de ouro (AuNP) prata (AgNP), cobre (CuNP) ou platina (PlNP) e etc; nanopartículas de óxidos de metais como as nanopartículas de óxido de titânio (TiO_2NP), óxido de zinco (ZnO_2NP) e óxido de cobre (CuO_2NP); nanopartículas magnéticas formadas de ferro (IONP – iron oxide nanoparticles), níquel e cobalto; quantum dots que são nanopartículas formadas de materiais semicondutores (Mody et al., 2010).

As MeNPs em particular, apresentam propriedades, por exemplo, tamanho e forma controláveis, composição, fácil preparação, absorbância de luz, potencial de dispersão e propriedades ópticas dependentes do tamanho fazem com que elas possam

ser utilizadas para direcionamento de tratamento (carreadores e sistemas de entrega) e diagnóstico de câncer, tratamento de doenças inclusive com atividade antimicrobiana, engenharia de tecidos e muitos mais (Zhang et al., 2008; Mody et al., 2010; Hulla et al., 2015).

Dentre os usos de MeNP aprovados pelo FDA (Food and Drug Administration) ou EMA (European Medicine Agency) estão as terapias de reposição de ferro para o tratamento da anemia. Nessas aplicações, a nanopartícula (coloides de óxido de ferro) é a utilizada com o objetivo de aumentar a concentração de ferro no corpo. Essas abordagens de nanopartículas originaram-se da necessidade de abordar as questões de toxicidade associadas à injeção de ferro. Usando ferro coloidal revestido com açúcares, muitos dessas questões foram resolvidas, pois isso altera a capacidade do corpo metabolizar e utilizar esses íons (Tabela 1) (Eifler and Thaxton, 2011; Bobo et al., 2016).

Juntamente com terapias de substituição de ferro baseadas em coloides de óxido de ferro, MeNPs foram aprovadas clinicamente como agentes de contraste para ressonância magnética. Para aplicações em imagem, a capacidade de resposta magnética inata de nanopartículas de óxido de ferro é usado para ressonância magnética para gerar contraste para imagens de uma variedade de cânceres e patologias. A combinação da capacidade de resposta da RM de nanopartículas de óxido de ferro e seu tamanho controlável, facilita o controle da biodistribuição, para tumores ou outros órgãos alvos, principalmente tumores hepáticos, auxiliando na geração de informações precisas (Tabela 1)(Eifler and Thaxton, 2011; Estelrich et al., 2015; Bobo et al., 2016).

Há ainda apenas uma única nanopartícula utilizada no tratamento para o câncer por hipertermia que utiliza nanopartículas sob campos magnéticos, produzindo calor e provocando morte térmica dessas células, o Nanotherm® utilizado no tratamento de glioblastoma (Tabela 1) (Hadjipanayis et al., 2008; Maier-Hauff et al., 2011). Essa estratégia, contudo, já foi utilizada em pacientes terminais, com recidiva de câncer de próstata e essa terapia foi capaz de causar morte das células cancerígenas e diminuir a dimensão do tumor (Johannsen et al., 2010; Attaluri et al., 2015). Estratégias mais arrojadas também têm sido desenvolvida, procurando guiar NPs para direcioná-las para células tumorais alvo, aumentando a especificidade pela ligação a um ligante que induza endocitose, ou a qualquer outro composto ativo, utilizando magnetismo para forçar a entrada das nanopartículas magnéticas nessas células. Dessa forma, AgNP e CuO_2NPs

foram capazes de induzir apoptose em células tumorais como A549 ao inibir Bcl-2, um gene de sobrevivência celular (Sanpui et al., 2011; Sun et al., 2012).

Tabela 1. Nanopartículas metálicas e magnéticas aprovadas por agências reguladoras europeias e/ou norte americanas.

Produto	*Nanopartícula*	*Uso*	*Ano de Aprovação*
Nanotherm® (MagForce)	SPION coberta com ácido aminosilano	Termoterapia de glioblastoma	FDA (2009)
Feraheme™/ferumoxytol (AMAG pharmaceuticals)	SPION coberta com poliglicose sorbitol carboxymetileter	Tratamento de deficiência de ferro em pacientes com doenças renais	FDA (2009)
Venofer® (Luitpold harmaceuticals)	IONP coberto com sacarose	Tratamento de deficiência de ferro em pacientes com doenças renais	FDA (2000)
Ferrlecit® (Sanofi Avertis)	Gluconato férrico e sódio	Tratamento de deficiência de ferro em pacientes com doenças renais	FDA (1999)
INFeD® (Sanofi Avertis)	IONP coberto com dextrana (baixo peso molecular)	Tratamento de deficiência de ferro em pacientes com doenças renais	FDA (1957)
DexIron®/Dexferrum® (Sanofi Avertis)	IONP coberto com dextrana (baixo peso molecular)	Tratamento de deficiência de ferro em pacientes com doenças renais	FDA (1957)
Feridex®/Endorem® (AMAG pharmaceuticals)	SPION coberta com dextrana	Agente de contraste para exame de imagem	FDA (1996)
GastroMARK™; umirem® (AMAG pharmaceuticals)	SPION supermagnético coberta com silicone	Agente de contraste para exame de imagem	FDA (2001)
Resovist (Bayer Schering Pharma)/Cliavist	IONP coberta com carboxidextrana	Agente de contraste para exame de imagem de lesão de fígado	Europa (2009)
Ferumoxtran-10/Combidex/Sinerem (AMAG)	IONP coberto com dextrana	Agente de contraste para exame de imagem de metástase de linfonodo	Aprovado apenas na Holanda

Pesquisas utilizando nanopartículas metálicas em nanomedicina vão muito além dos já aprovados. Tem sido utilizadas para diagnósticos sorológicos de tumores, através da detecção de marcadores tumorais ou células tumorais na circulação, por exemplo, Lin et al. (2011) desenvolveram um sistema misturando proteínas séricas totais com AgNPs, as proteínas aderidas a nanopartícula foram então analisadas por espectroscopia e com base nas diferenças de espectro, foi possível detectar pacientes doentes (com câncer de

estômago) com uma sensibilidade e especificidade de 100% (Lin et al., 2011). Outro estudo utilizou AgNP para rastreamento e detecção ultrassensível de marcadores tumorais utilizando a deposição de AgNPs funcionalizada com estreptavidina, de maneira que a nanopartícula se ligou ao anticorpo marcado com avidina e foi detectada por análise voltamétrica. Essa técnica detectou antígenos tumorais como o antígeno carcinoembriogenico e a α-fetoproteína, em concentrações extremamente baixas (Lai et al., 2011).

MeNPs de diferentes formas e tamanhos foram descritas como possuindo atividades antimicrobianas (inibindo o crescimento ou matando microrganismos) contra várias espécies de bactérias, fungos e vírus (Dizaj et al., 2014). Essas nanopartículas poderiam ser utilizadas para esterilização de materiais cirúrgicos, assepsia de curativos prevenindo a colonização de materiais e superfícies (Dizaj et al., 2014). Essa atividade é influenciada por fatores tais como o tamanho dos NPs, a concentração utilizada e a sua estabilidade, sendo que quanto menor a NPs maior atividade de inibição bacteriana, sendo hipotetizado que as menores MeNP podem atravessar os poros da membrana e parede celular bacteriana, entrar na célula e interferir com sinalizações e proteínas intracelulares inibindo o crescimento bacteriano (Siritongsuk et al., 2016; Qing et al., 2018; Yan et al., 2018).

O mecanismo de ação antimicrobiana, no entanto, não é bem elucidado e tem dependência direta do material da NP. Por exemplo, IONP (Iron Oxide Nanoparticle) com carga positiva (coberta com quitosana) apresentou atividade antimicrobiana contra bactérias Gram positivas (*Bacillus subtilis*) e bactérias Gram negativas (*Escherichia coli*). Sendo que essa atividade foi diretamente relacionada com a formação de reativos de oxigênio na superfície da parede celular bacteriana através da reação de Fenton (Arakha et al., 2015). Essa reação ocorre entre peróxido de hidrogênio e sais ferrosos e produz espécies reativas de oxigênio capazes de oxidar uma ampla variedade de substratos orgânicos.

As MeNPs também foram demonstradas como tendo potencial para serem utilizadas no tratamento de outras doenças. Por exemplo, AuNP, AgNP e ZnO_2NP foram descritas como antidiabéticas por causarem a diminuição dos níveis de glicose sanguínea e aumentado os níveis de insulina no sangue de camundongos, ao serem administradas por via oral (Alkaladi et al., 2014; Karthick et al., 2014). Nanopartículas de ouro foram

utilizadas como “chaperonas-like” e foram capazes de impedir a formação de proteína fibrilar amiloide, diretamente relacionada com a doença de Alzheimer (Liao et al., 2012). A AuNP também foi utilizada no tratamento contra artrite reumatoide juntamente com inibidor de receptor de IL-6 e hialuronidase (Lee et al., 2014). No entanto, para nenhuma dessas estratégias de uso de nanopartículas seu mecanismo de ação foi elucidado.

Vários trabalhos utilizam essas estruturas de maneira certeira, procurando utilizar-se de suas características físicas. Outros, procuram utilizações menos convencionais e possíveis aplicações dessas nanopartículas sem que se tenha certeza de como essas nanopartículas agem. Independente de qual estratégia, o uso dessas nanopartículas já está consolidado na medicina e o estudo da interação delas com o corpo humano é extremamente importante para que se possa avançar no desenvolvimento de novas tecnologias utilizando esses materiais.

2.2 Nanopartículas Magnéticas: Ferrita de Manganês ($MnFe_2O_4$)

O óxido de ferro é o material mais utilizado em técnicas biomédicas, devido à sua biocompatibilidade em relação a outros materiais magnéticos, sejam baseados em óxidos ou em metais puros. Vários tipos de óxidos de ferro existem na natureza e podem ser preparados em laboratório, mas apenas a maghemita (γ-Fe_2O_3) e a magnetita (Fe_3O_4) são capazes de preencher os requisitos necessários para aplicações biomédicas. Por sua vez, as nanopartículas de ferrita de manganês (MnFeNPs) possuem uma substituição de Fe (II) por Mn (II) na magnetita, o que possibilita uma grande variedade de estados de oxidação disponíveis, por exemplo, Mn (III), Mn (IV), Mn (V), Mn (VII) (Cole et al., 2011a; Karimi et al., 2013). As MnFeNPs têm sido usadas em nanomedicina, por serem pouco tóxicas (Yang et al., 2010; Nunes et al., 2014), em estratégias que estende-se desde o rastreamento e monitoramento de células tronco e de células de tecidos transplantados, sistemas de carreamento de drogas, diagnóstico até o tratamento de tumores (Cole et al., 2011b).

As MnFeNPs foram descritas como um potencial agente de contraste em imagens de RM, pois possuem uma alta magnetização e grande relaxação, devido à sua grande magnitude de spin magnético (Yang et al., 2010). Ou como agente de contrate multimodal, como descritas por Kim et al (2011), que ao conjuga-las com uma molécula

fluorescente, desenvolveu um agente para ser utilizado tanto em ressonância magnética quanto em *near-infrared fluorescence* (Kim et al., 2011).

As MnFeNP também foram avaliadas em tratamento de células cancerígenas por hipertermia. No estudo, a aplicação de campo magnético não teve influência na viabilidade celular, mas em combinação com MnFeNP foi bastante prejudicial para as células testadas em esquema de aquecimento por hipertermia, enquanto linhas celulares saudáveis toleram melhor o tratamento (Makridis et al., 2014).

Essa nanopartícula também foi utilizada como antimicrobiano, em conjunto com o peptídeo antimicrobiano (AMP), Cm-p5, contra o fungo *Candida albicans*. A NP demonstrou atividade intrínseca antifúngica, além disso, houve sinergia na atividade antifúngica quando essas nanopartículas foram formuladas com o peptídeo antimicrobiano. Atualmente são necessárias estratégias para reduzir a toxicidade e aumentar a eficácia e a biodistribuição dos AMPs, essas NP podem ser uma excelente alternativa devido e sua baixa toxicidade (Lopez-Abarrategui et al., 2016).

Por fim, as MnFeNP apresentam boas características para uso em nanomédica, como baixa toxicidade inerente, facilidade de síntese, estabilidade física e química e propriedades magnéticas. Contudo seus efeitos sobre o sistema imune e várias de suas interações com sistemas biológicos ainda não é bem estudada, podendo-se apenas inferir essas características por semelhança com as IONP.

2.3 Tuberculose

A tuberculose é uma doença antiga, que infecta a raça humana e provavelmente se desenvolveu e saiu da África juntamente com o *Homo sapiens*. A doença vem se espalhando ao longo dos séculos e atingiu seu ápice entre os séculos 18 e 19, sendo chamada de “mal do século” nesse período (Daniel, 2006; Barberis et al., 2017). Após a revolução industrial (1840), a doença passou a ser associada com a concentração de trabalhadores nas grandes cidades e aos aspectos socioeconômicos que favoreceram a propagação deste patógeno. Posteriormente a esse período de alta taxa de infecção, houve um declínio progressivo das infecções e mortes pela doença, decorrente da era dos antibióticos (1930) e do desenvolvimento vacina BCG a partir da atenuação de *Mycobacterium bovis* por Albert Calmette e Camile Guerin (1924) (Lawn and Zumla, 2011).

A introdução da vacina e a descoberta de antibióticos fez com que as taxas de mortalidade diminuíssem significativamente a partir do meio do século XX. Contudo, com o aparecimento da pandemia da SIDA (Síndrome da Imunodeficiência Adquirida) e das cepas resistentes, as taxas de mortalidade mais uma vez voltaram a aumentar, e com isso, a TB voltou a ser um problema e preocupação para a saúde pública (Getahun et al., 2010). Em 1993 a OMS (Organização Mundial da Saúde) começou o programa de tratamento diretamente supervisionado (DOTS). Posteriormente, em 1998, houve a complementação do programa para DOTS-plus, com objetivo de controlar a disseminação da doença, bem como a quantidade de mortes causadas principalmente pelos casos de TB multidroga resistente (MDR). Ambos os programas foram centrados no diagnóstico rápido e tratamento das formas mais graves e mais infecciosas (com baciloscopia positiva) de TB, mas também no tratamento de casos de baciloscopia negativa e TB extrapulmonares. Mais recentemente, em 2015, a OMS lançou o programa STOP-TB buscando ampliar e aperfeiçoar o programa DOTS, com objetivo de erradicar a doença até 2050 (Stop, 2006; Korenromp et al., 2012).

Atualmente a tuberculose continua um sério problema de saúde pública, estimando-se que 1/4 da população mundial esteja infectada com *Mycobacterium tuberculosis*. Em 2017, estima-se que houve de 10,4 milhões de casos novos (incidência) de TB em todo o mundo, dos quais 6,2 milhões (56%) entre homens, 3,2 milhões (34%) entre mulheres e 1,0 milhão (10%) entre as crianças. Pessoas vivendo com HIV e tuberculose representaram 1,2 milhões casos no mundo. Apesar de o número de mortes por tuberculose ter caído 22% entre 2000 e 2015, a tuberculose continuou sendo uma das 10 principais causas de morte em todo o mundo e a doença infectocontagiosa que mais mata, sendo que em 2015 houve 1,4 milhão de mortes, dos quais 0,4 milhões de mortes resultantes de coinfecção entre TB e HIV. Isso também está correlacionado com o aumento de casos de infecções multirresistentes (MDR-TB) que foram estimadas em 480 mil casos no ano de 2017 (WHO, 2018).

O grupo de países BRICS (Brasil, Russa, Índia, China e África do Sul), representam cerca de 50% dos casos de tuberculose no mundo. O Brasil também faz parte dos 20 países que contribuem com mais de 80% dos casos de tuberculose no mundo (WHO, 2018). No Brasil, estimou-se um total de 69.569 casos novos de TB em 2016, com aproximadamente 8 mil mortes, sendo que 4426 mortes apenas causados pela

tuberculose sendo que 9,2% apresentaram comorbidade com HIV. A distribuição da doença no país é heterogênea, com estados como o de Goiás com 946 casos novos da doença (em todas as formas), correspondendo a uma taxa de incidência de 14/100.000 habitantes em 2017. Isso faz com que seja o 3º estado com a menor incidência do país. Os casos de coinfecção entre TB e HIV correspondem a 11,5% dos casos. Houve confirmação laboratorial de 74,4% dos casos novos de TB pulmonar. Os dados divulgados, também mostram que apenas 88,2% dos doentes concluíram o tratamento com progressão para cura, o que está abaixo da meta nacional de 85% (SUVUSA/SES-GO, 2018).

Existem vários desafios relacionados com a incapacidade de controlar a TB: (1) detecção precoce de casos e tratamento efetivo de casos de TB resistente; (2) prevenção da TB resistente aos medicamentos; (3) controle e manejo de fatores de risco, como infecção pelo vírus da imunodeficiência humana (HIV), diabetes e tabagismo; (4) controle da infecção para prevenir a transmissão da TB; e (5) uma abordagem eficaz baseada em vacinas (Castro and LoBue, 2011).

Uma vez que a TB é uma doença principalmente de países pobres, o diagnóstico de TB em contextos de recursos limitados depende fortemente da baciloscopia, que é uma técnica barata e rápida. No entanto, apenas a baciloscopia não consegue detectar todos os casos de TB, sendo necessário outros exames complementares que incluem: raio-x e a cultura do escarro. Toda essa cadeia de exames pode levar de 20 a 30 dias para ser finalizada (em alguns casos até 60 dias) o que possibilita a disseminação da bactéria (Small and Pai, 2010). Por conseguinte, novos testes diagnósticos como o ensaio Xpert MTB/RIF, que tem uma sensibilidade maior do que a baciloscopia do esfregaço e é rápido, tendem a favorecer o diagnóstico precoce e favorecer o prognóstico da doença. Esse ensaio também fornece dados sobre possível resistência ao medicamento rifampicina. No entanto, o teste de todos os suspeitos de tuberculose custaria mais do que os diagnósticos convencionais e, logo, impediria o uso geral em situações de recursos limitados. Portanto, investimentos em diagnósticos rápidos, sensíveis, específicos e baratos ainda é um ponto muito importante no combate à tuberculose (WHO, 2018).

Os desafios no tratamento da TB estão intimamente ligados ao fato de a duração do tratamento ser longa (6 meses), resultando numa adesão fraca ao tratamento e alto índice de abandono. Isso potencializa a seleção de cepas resistentes aos fármacos o que

culmina no fracasso do tratamento (Zumla et al., 2013). O número de fármacos disponíveis para tratar a tuberculose é limitado (quatro medicamentos de primeira linha), logo, medidas para evitar a emergência de TB resistente aos medicamentos são cruciais para proteger o já reduzido arsenal que temos para combater a tuberculose. A partir disso o programa DOTS propõe que o regime de tratamento deve ser padronizado, suporte ao paciente deve ser fornecido durante todo o curso do tratamento e o uso irracional de medicamentos contra a TB deve ser proibido em todo o país. Dessa forma, o tratamento padrão contra a TB no Brasil foi proposto em 1999, a partir do programa da OMS, mas isso não impediu que a resistência primária à isoniazida e à rifampicina se desenvolvesse, bem como o número de casos de MDR-TB também tivesse um aumento gradativo (Stop, 2006; Lawn and Zumla, 2011; Zumla et al., 2013).

A BCG é uma cepa atenuada de *Mycobacterium bovis* que perdeu várias regiões genéticas durante sua atenuação, inclusive vários fatores de virulência (por exemplo, CFP-10 e ESAT-6). O que possibilita que as bactérias sejam destruídas pelas células do hospedeiro mais eficientemente (da Costa et al., 2014b). A destruição do patógeno torna disponíveis antígenos de Mtb, permitindo a subsequente ativação de células CD4+ através de células apresentadoras de antígeno e a produção de IFN-γ, uma citocina chave na resposta imunitária contra Mtb (Moliva et al., 2017).

Embora a BCG proteja contra a doença disseminada em crianças pequenas, tem eficácia variável contra a TB pulmonar, particularmente em adultos, com eficácia variando entre 0-80% (Rodrigues et al., 2011). Por conseguinte, é necessária uma vacina mais consistentemente eficaz do que a BCG, tanto em adolescentes como em adultos, para se alcançar a "Estratégia do Fim da TB" estabelecida pela Organização Mundial de Saúde. As limitações e as causas da variabilidade do BCG ainda não estão totalmente compreendidas e os esforços para melhorar a BCG é em parte devido à nossa falta de compreensão do que determina o resultado da infecção por Mtb (Rodrigues et al., 2011; Netea and van Crevel, 2014). É importante manter a eficácia protetora conferida pela BCG contra a doença disseminada e as estratégias para desenvolver uma vacina melhor incluem o desenvolvimento de estirpes melhoradas de BCG, vacinas com vetores micobacterianos alternativos, desenvolvimento de vacinas de subunidade para ser utilizada como reforço (boost) após uma imunização com BCG (da Costa et al., 2014b; Netea and van Crevel, 2014).

2.3.1 Imunopatologia da Tuberculose

Tuberculose é uma doença que classicamente acomete os pulmões, sendo a bactéria um parasita intracelular não obrigatório, que ao ser inalado como gotículas, chega nos espaços alveolares onde são reconhecidas por receptores de reconhecimento de padrões (PRR), presentes nos macrófagos alveolares, sendo que os mais comuns são *Toll-Like Receptors* (TLRs) e NOD-Like Receptor (NLR), e são fagocitados por essas células, justamente as principais células parasitadas pela bactéria. O sinal patognomônico da doença é a necrose caseosa que acontece no órgão. Contudo, existem mecanismos que devem ser levados em conta e que vão culminar no aparecimento da necrose caseosa (Dorhoi and Kaufmann, 2016).

Até a iniciação da imunidade adquirida, os macrófagos permanecem relativamente permissivos ao Mtb intracelular, tendo baixa capacidade de destrui-lo, por conseguinte, inicia-se uma fase replicação exponencial (Kleinnijenhuis et al., 2011). Durante esse período, no entanto, algumas das bactérias são mortas, processadas e apresentadas em associação com moléculas complexo principal de histocompatibilidade de classe II (MHCII) a linfócitos T-helper (Th) CD4 que se diferenciam em diversas subpopulações, e, em menor grau, por via de MHC-I a células T CD8 (Cooper, 2009). As células T ativadas então, através da secreção de interferon (IFN)-γ e do fator de necrose tumoral (TNF)-α ativam os macrófagos infectados ou que fagocitaram as micobactérias a aprimorarem seus mecanismos antimicrobianos, fazendo com que a taxa de replicação bacteriana comece a ser controlada, mas não há a erradicação da bactéria (Kleinnijenhuis et al., 2011).

A não eliminação do bacilo, juntamente com estímulo pró-inflamatório constante, culmina na remodelação do local da infecção e na formação estrutura comum da doença, o granuloma. A estrutura base do granuloma é caracterizada por macrófagos infectados no centro, que podem adquirir diferentes formas e funções na patologia, podendo se tornar macrófagos espumosos, epitelióides ou gigantes além de poderem estar em estado necrótico ou apoptótico. Esses macrófagos serão cercados por linfócitos T, linfócitos B, células dendríticas, células NK além de células tronco mesenquimais e fibroblastos e células epiteliais (Figura 2) (Ramakrishnan, 2012; Orme and Basaraba, 2014).

O granuloma sempre foi considerado como uma estrutura protetora do hospedeiro, capaz de cercear a disseminação e a proliferação da bactéria. No entanto, evidências tem demonstrado que isso pode não ser a verdade, inclusive com a estrutura podendo ser uma forma de patologia e um nicho para sobrevivência e permanência da bactéria (Orme, 2014). O resultado dessa estrutura pode ser uma infecção latente (onde não há sintomas e a doença não evolui) ou pode ocorrer uma infecção e patologia crônica progressiva associada a replicação lenta das bactérias (Orme and Basaraba, 2014). Sendo que concomitante à patologia progressiva o aparecimento de populações celulares relacionadas à proteção também está correlacionado com a o agravamento das lesões pulmonares (Orme, 2014). Por exemplo, as células Th17 podem mesmo coexpressar IL-17 e TNF-α o que pode estar correlacionado com sustentação da inflamação pré-existente e consequentemente podendo ser protetora durante a infecção aguda (início da infecção e ideal para uma vacina) e deletéria durante uma infecção crônica (Gopal et al., 2012).

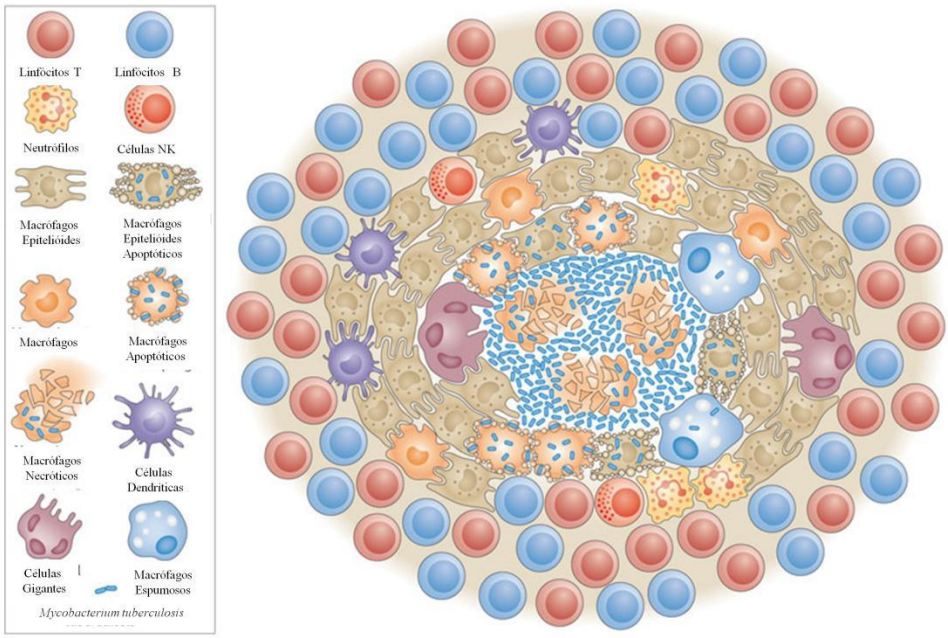

Figura 2. Estrutura base do granuloma na tuberculose. Adaptado de Ramakrishnan L (2012). Revisiting the role of the granuloma in tuberculosis.

Consequentemente, a resposta imune contra tuberculose é muito mais complexa e envolve várias subpopulações de células T, citocinas e mecanismos efetores. O controle da infecção pelo Mtb há muito tempo estava correlacionado com a indução de resposta Th1 e em particular as células T CD4+ tem papel crucial, pois são as principais células

produtoras de IFN-γ. Por exemplo, estudos clássicos com camundongos geneticamente deficientes para essa citocina demonstram maior susceptibilidade à doença, pois os macrófagos infectados perdem muito sua capacidade microbicida, como produção de reativos de oxigênio, e, portanto, são incapazes de restringir e/ou matar o bacilo (Flynn et al., 1993). Essa incapacidade de conter o bacilo se correlaciona também com a disseminação do mesmo para outros órgãos com aumento de lesões e necrose nos mesmos, no entanto, com granuloma malformado ou ausente (Shen et al., 1988; Cooper et al., 1993; de Noronha et al., 2008).

Contudo outras populações celulares foram demonstradas como sendo importantes para combater a evolução da doença ou impedir seu aparecimento. As células T CD8, por exemplo, participam na imunidade da TB por uma atividade citotóxica através da secreção de perforina e granzima além de poderem secretar IFN-γ (Behar, 2013). Consequentemente, a transferência adotiva de células T CD8 pôde gerar proteção em modelos murinos (Woodworth et al., 2008). Além disso, uma vacina capaz de gerar resposta por essa população celular e que seja capaz de proteger contra a tuberculose pode ser uma ótima estratégia para vacinar indivíduos com AIDS, que possuem suas células T CD4 reduzidas (Castro and LoBue, 2011).

Já as células T CD4 produtoras de interleucina (IL) -17 (Th17) são importantes para o recrutamento e início da resposta principalmente pelo recrutamento de neutrófilos e estímulo à granulopoiése e diferenciação de neutrófilos, além de serem importante no recrutamento de outras células produtoras de IFN-γ para o sítio da infecção, uma vez que a produção dessa IL-17 aumenta a expressão das quimiocinas CXCL9, CXCL10 e CXCL11 que recrutarão diversas células do sistema imune para o sítio da infecção (Lyadova and Panteleev, 2015). Mais recentemente, a IL-17 também foi demonstrada como tendo função protetora contra Mtb, de maneira que camundongos não vacinados que receberam por transferência adotivas, células Th17 específicas geradas por vacinação, quando desafiados, tiveram proteção eficiente similar aos camundongos que foram vacinados (Monin et al., 2015). Por transferência adotiva de células T produtoras de IL-17 específica, também induziram uma proteção significativa contra desafio com Mtb mesmo na ausência de células produtoras de IFN-γ (Wozniak et al., 2010).

2.3.2 Tuberculose: Adjuvantes e antígenos vacinais

As vacinas de subunidades de proteína contra a tuberculose que estão atualmente em ensaios clínicos usam moléculas adjuvantes que são agonistas de TLR. A fusão Mtb72 (antígenos Mtb32 (Rv1196) e Mtb39 (Rv0125)) utiliza os adjuvantes AS01B ™ (uma formulação lipossomal) e o AS02A™ (uma emulsão óleo-em-água) foi desenvolvido pela GlaxoSmithKline (GSK). Os adjuvantes acima mencionados são compostos por MPL (monofosforil-lipide A 3-desacilado) e o detergente QS-21. MPL é um derivado desintoxicado do lipídio A das bactérias Gram-negativas Salmonella minnesota R595 LPS, enquanto QS-21 (fração 1) é uma substância purificada e fracionada da casca da árvore sul-americana Quillaja saponaria. A ação conhecida do MPL é através do TLR4, enquanto o QS-21 não tem nenhuma ação agonística relacionada ao TLR (Garcon and Van Mechelen, 2011).

Duas outras proteínas de fusão (Hybrid 4 e Hybrid 56), que estão atualmente em ensaios clínicos, são combinadas com o adjuvante IC31™. Este adjuvante é constituído por dois componentes, um agonista de TLR9 (o oligodesoxinucleótido ODN1a) e um peptídeo catiônico antimicrobiano artificial (KLKL5KLK), que serve como veículo. Seu mecanismo de ação está relacionado à ativação de TLR nos endossomos e, como tal, o IC31 é um bom adjuvante para uso em vacinas contra microrganismos intracelulares (Agger et al., 2006; Kamath et al., 2008).

Já a vacina ID93, criada pela fusão de epítopos de Rv3619, Rv1813, Rv3620 e Rv2608 e proposta para melhorar a profilaxia da TB, inclui GLA-SE [um glucopiranosil lipídio (agonista de TLR4) em emulsão] como um adjuvante. Baldwin et al. relataram que a proteção estava associada à forte estimulação das respostas imunes do tipo Th1, com um aumento de células polifuncionais (produtoras de IL-2, TNF-α e IFN-γ). Para selecionar o melhor adjuvante para combinar com ID-93-GLA, foram testadas diferentes formulações incluindo este agonista de TLR4, incluindo água com sais de alumínio, emulsões e lipossomas (Baldwin et al., 2012).

Embora não seja um adjuvante atualmente em ensaios clínicos, TDB (trealose-6,6-dibehenate) chamou a atenção porque é um análogo menos tóxico de TDM (trealose-6,6-dimicolato), um componente crítico da parede celular de Mtb. TDB surgiu a partir de alterações no TDM, também conhecido como fator corda, que é um indutor potente de respostas do tipo Th1 com uso restrito em humanos devido à sua toxicidade. O efeito do TDB foi avaliado com a proteína de fusão H1 (Ag85B-ESAT-6) e demonstrou ser um

auxiliar poderoso na estimulação da resposta celular das populações Th1 e Th17, bem como respostas imunes humorais (Holten-Andersen et al., 2004; Davidsen et al., 2005). O receptor de Mincle (uma lectina do tipo C) é responsável pelo reconhecimento de TDB (38) e TDM (39). Tem sido demonstrado que a associação de Mincle-Fcrγ-Syk-CARD9 está envolvida na resposta ao TDM/TDB para gerar respostas imunes do tipo Th1 e Th17, além de ativar o inflamassoma Nlrp3, induzindo a produção de IL-1β (Desel et al., 2013; Schweneker et al., 2013).

Outra atividade chave em pesquisa e desenvolvimento de vacinas é a identificação de antígenos-alvo para uso em formulações. O Mtb possui fases distintas de crescimento, que podem estar associadas à replicação bacteriana ativa, persistência e dormência. Os antígenos associados à replicação bacteriana ativa incluem os antígenos secretados precocemente, como a família Ag85, ESAT-6 e CFP-10 (Fletcher and Schrager, 2016). Esses antígenos têm sido amplamente utilizados no desenvolvimento de vacinas contra a tuberculose, pois são altamente imunogênicos e têm mostrado proteção em modelos animais (Ahsan, 2015). Já os antígenos relacionados ao operon regulador DosR, estão associados com a dormência e seu uso oferece a possibilidade de projetar vacinas para a fase latente da infecção (Pandey et al., 2016). Por conseguinte, a resposta protetora almejada também é diversa, necessitando de resposta de células T CD4+ tipo Th1 e Th17, bem como T CD8+. Sendo assim, estratégias que combinem múltiplos antígenos, de múltiplas fases e correlacionados com vários tipos de respostas, são as mais promissoras (Fletcher and Schrager, 2016; Kaufmann et al., 2017b).

Vacinas de subunidade demonstraram ser eficientes e capazes de proteger sendo que a vacina M72f é uma formulação que contém uma proteína de fusão (antígenos Mtb 32A e Mtb 39A), em combinação com o adjuvante AS01E, e é atualmente a vacina de subunidade no nível mais avançado de ensaios clínicos (Von Eschen et al., 2009; van den Berg et al., 2018). Contudo outras vacinas de subunidade também estão em testes clínicos, como a vacina de proteína de fusão H1 contém os antígenos Ag85B e ESAT-6 fusionados, a vacina H4 contém os antígenos de Mtb 85B e TB10.4, enquanto a H56 contém Mtb 85B, ESAT6 e Rv2660c sendo que as três vacinas são utilizadas em combinação com o adjuvante IC31. Por fim, a vacina ID93 é composta de quatro antígenos de Mtb, RV2608, Rv3619, Rv3620 e Rv1813, em combinação com o adjuvante GLA-SE7 (Fletcher and Schrager, 2016; Kaufmann et al., 2017b).

A escolha de antígenos para ser utilizado nas vacinas pode ser feito de várias maneiras. Classicamente os antígenos de escolha são os imunodominantes, contudo antígenos relacionados as distintas fases da infecção (fase ativa e fase latente) também são alvos promissores, bem como antígenos estruturais e de virulência. A partir disso, nosso grupo de pesquisa tem focado também na busca, avaliação e desenvolvimento de antígenos como alvos vacinais e para desenvolvimento de diagnóstico, como os antígenos HspX, MPT51, Ag85c, GroES, GlcB (Rabahi et al., 2007; Reis et al., 2009; Silva et al., 2014; Trentini et al., 2014).

A família de Ag85 são os antígenos mais utilizados em vacinas para tuberculose, sendo que das 11 vacinas em testes clínicos atualmente 7 usam o antígeno 85 a ou b (Kaufmann et al., 2017a). Essas proteínas são relacionadas à estrutura da parede celular bacteriana e secretadas durante toda a infecção, contudo são principalmente relacionados à fase de replicação do bacilo. Do grupo, o Ag85c é o mais importante para a atividade micoliltransferase, sendo sozinho responsável por 40% dessa atividade, não podendo ser substituído pelas outras duas micoliltransferases, como demonstrado em mutantes deficientes do gene que codifica a proteína (Kremer et al., 2002; Backus et al., 2014). Adicionalmente, o Ag85c demonstrou ser altamente imunodominante, tendo vários epítopos reconhecidos por células T CD4+ e CD8+ em indivíduos doentes (Silver et al., 1995; Valle et al., 2001; Huygen, 2014). Lim et al. também reportaram uma resposta imune positiva de células T de pacientes PPD positivo (Lim et al., 1999), além de que ao avaliar o potencial dos antígenos Ag85a, Ag85b, Ag85c e CFP-10 como marcador de diagnóstico para Tb em crianças, foi demonstrado que o Ag85c é o que apresenta maior sensibilidade (89,77%) e maior especificidade (92%) (Kumar et al., 2008).

A proteína HspX foi um dos alvos mais estudados pelo nosso laboratório por ser um antígeno da fase de latência da infecção (pertence ao operon DosR/DosS), sendo que é correlacionada à regulação da multiplicação bacteriana frente a estímulos como hipóxia e estresse oxidativo (Hu et al., 2015). Kaushik et al, avaliaram a proteína HspX como antígeno para diagnóstico, frente ao soro de pacientes com tuberculose pulmonar, extrapulmonar ela foi capaz de discriminar indivíduos doentes de não doentes (Kaushik et al., 2012). Geluk et al (2007) verificaram uma imunodominância em indivíduos expostos ou infectados pelo Mtb para resposta de células T contra a HspX o que não acontecia em indivíduos vacinados com BCG (Geluk et al., 2007). Rabahi et al.

evidenciaram que a resposta de anticorpos ao HspX também é um bom indicador para novas infecções e um bom marcador de infecção latente (Rabahi et al., 2007). Trentini et al (2014) utilizaram essa proteína como vacina de subunidade proteica encapsulada em um lipossoma em adição ao adjuvante MPL (agonista de TLR4) e CpG/DNA (agonista de TLR9) e verificou-se imunogenicidade humoral e celular do tipo Th1 gerada pela vacinação. Por sua vez a formulação teve capacidade proteger contra a infecção por Mtb, diminuindo a carga bacteriana e as lesões após desafio.

O antígeno MPT51, também chamado de Ag85d devido a sua homologia (40%) com a família de Ag85, contudo não é uma micoliltransferase e tem papel relacionado à virulência e aderência da bactéria à célula alvo (Wilson et al., 2004). Esse antígeno é bom marcador para diagnóstico de tuberculose, principalmente em pacientes coinfectados com HIV (Singh et al., 2005). Nosso grupo, demonstrou que ao vacinar camundongos BALB/C, com a uma vacina composta da proteína recombinante MPT51 e adjuvantes (tanto CPG DNA quanto adjuvante incompleto de Freud) houve uma proteção contra a infecção (Silva et al., 2009).

Os estudos com diferentes antígenos culminaram no desenvolvimento da proteína de fusão CMX, a partir da fusão dos três antígenos, HspX, Ag85c e MPT51. Esses antígenos foram escolhidos também por serem imunodominantes, mas além disso, foram escolhidos devido a sua importância estrutural e para a virulência e desenvolvimento da doença (de Sousa et al., 2012). Essa proteína foi avaliada como vacina de subunidade proteína encapsulada em lipossoma e com adição adjuvante CpG/DNA (um agonista de TLR9) e demonstrou imunogenicidade para células Th1 (produtoras de IFN-γ e TNF-α) e resposta humoral IgG1 e IgG2a (de Sousa et al., 2012).

As outras populações celulares (Th17 e Tc1) importantes para a proteção contra tuberculose não foram avaliadas para a vacina de subunidade e a resposta Th17 e Tc1 só foi evidenciada como sendo crucial na proteção contra infecção por Mtb quando foi utilizada no contexto de vacina de vetor vivo recombinante, na bactéria *M. smegmatis* (MC^2) e BCG (Junqueira-Kipnis et al., 2013; da Costa et al., 2014a). Uma vez que vacinas de subunidade são mais seguras, o desenvolvimento de uma formulação vacinal utilizando vacina de subunidade, que seja capaz de gerar as três respostas necessárias para a proteção contra a tuberculose é um dos objetivos principais das pesquisas desenvolvidas

em nosso laboratório. Para isso a procura de novos adjuvantes, que possam ser utilizados em adição à proteína CMX é o próximo passo.

A utilização de Nanopartículas com íons metálicos e nanopartículas magnéticas em vacinologia foi explorada em um artigo de revisão intitulado: Role of Metallic Nanoparticles in Vaccinology: Implications for Infectious Disease Vaccine Development. O artigo foi publicano na revista Frontiers in Immunology, editora Frontiers, Fator de Impacto 6.49 (qualis A1 em Biotecnologia)

2.4 Artigo 1 – Título : Role of Metallic Nanoparticles in Vaccinology: Implications for Infectious Disease Vaccine Development.

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Role of Metallic Nanoparticles in Vaccinology: Implications for Infectious Disease Vaccine Development.

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Running title: Nanoparticles as vaccine adjuvant

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Key Words: particulate vaccine, adjuvant, immune response, Th1, Th17

Abstract

Subunit vaccines are safer but less immunogenic than live-attenuated vaccines or whole cell inactivated vaccines. Adjuvants are used to enhance and modulate antigen

immunogenicity, aiming to induce a protective and long lasting immune response. Several molecules and formulations have been studied for their adjuvanticity, but only seven have been approved to formulate human vaccines. Metallic nanoparticles (MeNPs), particularly those containing gold and iron oxides, are widely used in medicine for diagnosis and therapy and have been used as carriers for drugs and vaccines. However, little is known about the immune response elicited by MeNPs or about their importance in the development of new vaccines. There is evidence that these particles display adjuvant characteristics, promoting cell recruitment, antigen presenting cell activation, cytokine production, and inducing a humoral immune response. This review focuses on the characteristics of MeNPs that could facilitate the induction of a cellular immune response, particularly T-helper 1 (Th1) and Th17, and their potential functions as adjuvants for subunit vaccines.

1. Introduction

Adjuvant selection for subunit vaccines is key to increasing immunogenicity and therefore guiding stimulation of innate immunity and the development of the appropriate protective response to combat the microorganism of interest. Adjuvants are classified as particulate formulations or immunomodulatory molecules, or may display a combination of both characteristics. In addition to acting on the diversity of the humoral and cellular immune response, they can act in several different ways: by decreasing the vaccine dose, accelerating the immune response, or prolonging the immune response (Reed et al., 2013; Agger, 2016). Among the seven approved vaccine adjuvants for human use, aluminum salts (alum), emulsions (e.g., MF59), and virosomes are particulate formulations. While alum induces efficient antibody (Ab) production and a predominant T-helper 2 (Th2) response, the other two have the capacity to induce Th1 and Th2 as well as Ab. Adjuvant system (AS) 01 and 04 used the combination of an immunomodulatory molecule and a particulate formulation composed of a toll-like receptor 4 (TLR4) agonist, monophosphoryl lipid A (MPL), that also induces Ab. The incorporation of alum in AS04 improved the humoral response, while the association of saponin (QS-21) and liposome in AS01 favored Th1 responses (Morrison et al., 2015;Didierlaurent et al., 2017). Imidazoquinolines (TLR7 and TLR8 agonists) and lipid A analogs (TLR4 agonists) are immunomodulatory molecules, capable of generating a Th1 response (Di Pasquale et al., 2015).

There is a demand for safe adjuvants capable of inducing efficient cellular immunity, especially Th1 and Th17, to be used against tuberculosis, leishmaniasis, malaria, and other diseases caused by intracellular microorganisms (Damsker et al., 2010; Reed et al., 2013).The majority of molecules with this type of adjuvanticity (Th1 driven) are related toward the response of danger receptors to trigger inflammation, thus safety and tolerance could be major barriers that prevent their use in human vaccines (Knudsen et al., 2016). However, comparing Alum and CpG/DNA adjuvants in human trials, only common adverse effects, including local site reaction, flu-like symptoms and headache were observed when CpG/DNA was used (Cooper et al., 2004). Also, Verstraeten et al.

(2009), analyzing more than 30,000 individuals, who received vaccine-containing AS01, observed that only common side effects occurred.

Nanoparticles (NPs) are classically described as structures smaller than 100 nm and can be classified, based on their composition, as polymeric, inorganic, liposomes, immunostimulating complexes (ISCOMs), virus-like particles (VLPs), emulsions, or self-assembled proteins (Zhao et al., 2014). They are made of different materials and differ in size, shape, and surface properties; interactions with biological systems, therefore, are varied, with several applications in modern medicine. In vaccinology, they are classically thought to have delivery and deposit properties. However, many NPs have been shown to stimulate immune responses, including cell recruitment, activation of antigen (Ag) presenting cells (APCs), and induction of cytokine and chemokine release. The development of nanostructures and nanoadjuvants may therefore offer alternatives to currently used adjuvants once studies establish ways for them to elicit innate immune response and support the development of adaptive immune response in the context of vaccine formulations (Zhao et al., 2014).

Metallic nanoparticle (MeNPs) are relatively non-biodegradable, have rigid structures, and possess simple synthesis methodology. Many have been studied for their immunological properties (Hofmann-Amtenbrink et al., 2015). However, there are still gaps in understanding the immune response generated by NPs, especially MeNPs. Few studies have compared NPs of different types and there is no standardization among published methodologies, which hampers comparisons of immunostimulatory characteristics. Several important characteristics, therefore, have not been well studied. For example, how chemical and physical properties (including material composition, size, shape, surface charge, and hydrophobicity) impact vaccine immune response (Di Pasquale et al., 2015). This review focuses on the use of MeNPs in formulations against infectious diseases, aiming to assess progress of their use in vaccinology, and their possible applications as adjuvant.

2. The immune response generated by MeNP-formulated vaccines

Table 1 summarizes the articles that report the use of MeNPs (Metallic nanoparticle) as part of vaccine formulations against infectious diseases and the immune responses they elicited. A range of immune responses is required to fight a diverse group of microorganisms. The type of protective immune response can be simplistically divided based on the type of microorganism: extracellular bacteria and toxin; intracellular bacteria; viruses; fungi; and protozoa. Among the vaccines targeting extracellular bacteria and toxin, two were formulated with lypopolissacaride (LPS) in glycopeptide Ag. The use of glycoantigen and LPS can trigger an intense response through TLR(Toll-Like Receptor) 4 and B cell receptor (BCR) activation; the presence of gold NPs (AuNPs) may have minimal influence on this response. However, in the work of Gregory et al. (2015) and Torres et al. (2015), the use of AuNPs in the formulation generated a different response, improving anti-LPS immunoglobulin G (IgG) response,

decreasing bacterial burden, generating a more efficient humoral response, and improving animal survival, showing that AuNPs may influence immune response and protection.

Using protein Ag, Barhate et al. (2014) formulated a vaccine using AuNPs and toxoid Ag and demonstrated that their formulation could induce a mucosal and systemic IgG and IgA response. When co-administered with Asparagus racemosus extract (ARE), a botanically-derived adjuvant, the response was further enhanced (Barhate et al., 2014). Dakterzada et al. (2016) developed a vaccine against *Pseudomonas aeruginosa* using the flagelin subunit and AuNPs that elicited an IgG response comparable to that induced by Freund Adjuvant. Flagellin is a TLR5 agonist but the recognition and signaling is structure dependent. In this study, however, it was used only the 1–161 aa from flagellin and its ability to activated TLR5 could not be maintained (Dakterzada et al., 2016). Gregory et al. (2012) used an F1 *Yersinia pestis* Ag conjugated to AuNPs that induced an Ab (antibody) response with higher IgG2a associated with higher levels of interferon gamma (IFNγ), suggesting activation of Th1 cells.

Among the studies that used MeNPs in vaccine formulation, only one targeted intracellular bacteria (*Listeria monocytogenes*). The protective immune response against intracellular bacterial infections requires Th1 activation and therefore antigen presenting cells activation and Ag antigen presentation through major histocompatibility complex II (MHCII). To generate a Th1 response, an AuNP and Listeria Ag formulation were used in different strategies. Although the authors tested direct vaccination, when dendritic cells (DC), in vitro loaded with AuNP plus Listeria Ag, were transferred adoptively to a naïve animal, they induced Th1, CD8+, and natural killer (NK) cells that provided better protection against *L. monocytogenes* than the traditional vaccine approach (Rodriguez-Del Rio et al., 2015).

In evaluating vaccines developed with MeNPs against viral infections, Niikura et al. (2013) used West Nile virus (WNV); Tao et al. (2015) used the extracellular portion of Matrix 2 protein (M2) of the influenza virus; Chen et al. (2010) conjugated AuNPs with a 28 amino acid VP1-foot-and-mouth virus protein (pFMDV), and Staroverov et al. (2011) co-administered AuNPs and partially purified enteropathogenic swine-transmissible gastroenteritis (STG) virus. All the above studies evaluated the Ab immune responses and all formulations demonstrated efficient humoral response induction. Tao et al. (2015) also evaluated the addition of cytosine and guanine linked by phosphodiester unmethylated (CpG/DNA) and found that it improved Ab levels and animals' survival rates. Another important feature of studies by Niikura et al. (2013) and Chen et al. (2010) was the use of various NP sizes and the demonstration that all different NP shapes were capable of inducing a humoral response. The levels of Ab were size dependent, but the results were inconsistent: the first study found that a 40 nm sphere was the most efficient Ab inducer and the second found that the 8 nm and 12 nm spheres performed best.

A special case of the use of MeNPs was in the use of nickel-functionalized nanolipoprotein particles (NiNLPs) by Yan et al. (2009) and Wadhwa et al. (2012) in combination with HIV Ag. NiNLPs are nanometer-sized nanolipoprotein particles (NLPs) with nickel incorporation into their surface in order to induce polyhistidine tagged proteins adsorption (Fischer et al., 2009). They demonstrated that specific IgG (IgG1 and IgG2a) levels were greater than those obtained when alum was used in the formulation. Fischer et al. (2010) used truncated WNV envelope protein Ag and found that a single dose vaccination induced a superior anti-WNV IgG response and improved protection against a WNV challenge (Fischer et al., 2010). These responses were associated with nickel functionalization, described as a hapten, and triggered responses through activation of human TLR4 and intracellular transduction signals through myeloid differentiation primary response (MyD-88), nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK), inducing pro-inflammatory responses (tumor necrosis factor [TNF]-α and interleukin [IL]-8) (Schmidt et al., 2010; Schmidt and Goebeler, 2011).

For protozoan infections, Parween et al. (2011), using *Plasmodium falciparum* merozoite surface protein subunit and AuNPs, evaluated the humoral immune response (IgG1, IgG2a, IgG2b, and IgG3) and found an intense IgG1 response compared with the alum formulation (Parween et al., 2011). Kaba et al. (2009), using *P. berghei* circumsporozoite protein (Pb CSP) and AuNPs, generated long-lasting protective immunity with Th that produced IL-2 and mixed high avidity IgG1/IgG2a (Th2/Th1) (Kaba et al., 2009). In other studies, these authors replaced the Ag with *P. falciparum* circumsporozoite protein (Pf CSP); vaccination was shown to induce protective cytotoxic (CD8+) cells, high avidity Ab titers, and specific effector memory, central memory, and long-term central memory CD8+ T cells in draining lymph nodes, spleen, and liver (Kaba et al., 2012). This response was shown to be generated by cross-presentation by DC which had delayed fusion and interaction of endosomes with lysosomes caused by the AuNP formulation (McCoy et al., 2013). Finally, PfMSP was used with dextran-coated iron oxide NPs (IONPs) and was capable of inducing a humoral response in two animal models (mouse and monkey). This response was also shown to inhibit parasite growth by 55–100% (Pusic et al., 2013).

Most studies evaluated immunogenicity through measurement of the humoral immune response. According to their findings; the use of NPs was efficient in inducing an Ab-based response. Based on heavy chain structure, there are five types of Ab, each with a different role: IgG; IgM; IgA; IgD; and IgE. IgG and IgA can be subdivided, based on additional small differences in their heavy chain, in IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. Concerning to vaccination, humoral immunity is especially important in responding to infection by extracellular pathogens, toxins, protozoa, and viruses. Its importance is associated with the biological activities of immunoglobulins, including microorganism opsonization and phagocytosis; complement activation (Schroeder and Cavacini, 2010); toxins and microorganism neutralization (Woof and Kerr, 2006); and

mast cells and basophil activation (Kawakami and Galli, 2002; Schroeder and Cavacini, 2010). In addition, immunoglobulins can help target cytotoxicity against infected cells (Ab-dependent cell cytotoxicity of CD8 T cells and NK). In some cases, however, the pathogens have the ability to evade the humoral system or can even use immunoglobulins as a way to facilitate cell invasion, as in the cases of *Mycobacterium tuberculosis* and Leishmania spp. (Schorey et al., 1997; Dominguez and Torano, 1999).

The studies described above clearly show that MeNPs (gold, iron, and nickel) can be used for vaccine development. Different MeNPs were used in conjunction with several Ag for distinct microorganisms and showed the ability to generate humoral and cytotoxic responses. Although the generation of IgG2a and IFN-γ shown in some studies are indicators of Th1 responses using MeNPs as adjuvant, further research is needed to specifically assess the role of different MeNP vaccines in Th1 induction.

3. Important physicochemical characteristics of MeNPs as activators of immune responses

To understand the possible uses of MeNPs (Metallic Nanoparticle) as platforms for vaccines against infectious diseases, analysis is needed of the impact of different physicochemical characteristics of NPs (nanoparticle) on the innate immune response (Figure 1). Several strategies have included MeNPs as vaccine platforms, involving MeNPs of different materials (including gold, iron oxide, and nickel); shapes (including spheres, cubes, rods, and discs), sizes (from 2 nm to over 200 nm); and types of coating (e.g., citrate, chitosan, dextran, or Cetyl trimethylammonium bromide/4-styrenesulfonic acid-comaleic acid (CTAB/PSS-MA).

The material from which an NP is made has a direct influence on the functions of APCs (Antigen Presentig Cells); Gold Nanoparticle (AuNPs) have been most commonly used in vaccinology (Table 2). The most recent studies involving AuNPs demonstrate the effects of gold sodium thiomalate (AuTM) on macrophage function, showing lysosomal enzyme inhibition and reducing phagocytosis (Turkall et al., 1982). Similar effects were seen in macrophages of several origins, which, when stimulated with AuNPs, showed diminished bactericidal activity against *Staphylococcus aureus* (Davis and Johnston, 1986) and low or absent cytokine production (IL[interleukin]-6, IL-10 and TNF [Tumor Necrosis Factor]-α) (Bancos et al., 2015; Kingston et al., 2016). Moreover, when splenocytes were stimulated with LPS (Lipopolysaccharide), the addition of AuNP reduced IL-17 and TNF-α release (Kingston et al., 2016). Some of these results raise the concern on the use of AuNPs as adjuvants, since these immunomodulatory properties can act inhibiting the generation of Th1. However, the response to AuNPs is also correlated with other physicochemical characteristics, that will be discussed below, which may be tailored to improve immunostimulant or immunomodulatory capacity.

Iron oxide nanoparticle (IONP) have also been used as adjuvants. Iron is an important ion in the homeostasis of all cells and in generating immune responses to several microorganisms. The impacts of iron phagocytosis have been explored in several studies, for example, M2 macrophages after exposure to IONPs induced reactive oxygen species (ROS), but after 24 hours induced IL-10 production (Rojas et al., 2016). The use of IONPs in BALB/c mice demonstrated the immunomodulatory capacity of this NP by diminishing splenocyte cytokine production (IL-4 and IFN[interferon]-γ) (Shen et al., 2011) as well as suppressing the response to pancreatic antigen (Ag) in diabetic mice (Tsai et al., 2010). Sindrilaru (2011), however, showed that macrophages, under iron overloaded conditions, became unrestrained M1 (with an incomplete switch to M2 macrophages) and produced more TNF-α, which impaired wound healing and had an important role in the immunopathology of chronic venous leg ulcers. Consequently, IONP response seems to have direct correlation with time and dose, once iron overload seems to be a requisite to developed pro-inflammatory response and this aspect must be evaluated to avoid the inhibition of the desired immune response.

Other critical characteristics are the shape and size of NPs, which have a direct impact on vaccine efficiency, Ag load capacity, and interaction with cells (phagocytes and APCs). These characteristics have been studied in different NPs: Shah et al. (2014) published a review focusing on the impact of size for alum, oil-in-water, emulsion, polymeric particles, and liposome adjuvanticity, but did not evaluated MeNPs. In the studies reviewed here, NP sizes range from 2 nm nanospheres to 270 nm nanocapsules. Two authors have evaluated the impact of size and shape for MeNPs (Table 2): Chen et al. (2008) evaluated differences in immune response based on AuNP sizes (ranging from 2–50 nm nanospheres) and found that 8 nm and 12 nm were the most drained NP (Chen et al., 2010); Niikura et al. (2013) went further and, using four different shapes of NP (20 nm sphere, 40 nm sphere, cube, and rod), showed that antibody responses and TNF-α were directly correlated with the specific NP surface area (the ratio of the total surface area per single NP volume). Furthermore, 40 nm spheres appear to be the most efficient in generating immune responses (IL-6, IL-12,) and granulocyte macrophage colony stimulating factor [GM-CSF] production.

Surface charge and hydrophobicity are additional important NP characteristics for immune response induction and are directly influenced by NP functionalization (chemical modification of nanoparticles surface by adding or replacing functional groups) and coating (antigen) (Mout et al., 2012). Most studies used citrate-coated NPs, but dextran and CTAB/PSS-MA have also been used; all three result in negatively charged (anionic) particles. Only one NP, revised here, used positive charged (cationic) functionalization (Barhate et. al., 2014; Table 2). The higher hydrophobicity of AuNP was shown to activate the innate immune system (TNF-α secretion) (Mayano et. al., 2012). Although the surface charge of other non-metallic NPs has been studied (Fromen et al., 2016), to our knowledge the studies using MeNPs did not address the other characteristics associated with immune response induction. For non-metallic NPs, it appears that a positive charge signified a greater ability to induce immune responses

than a negative charge. Interestingly, negatively charged non-metallic NPs were associated with Ag-specific tolerance (Fromen et. al., 2016). Further studies are needed to investigate whether or not the charge imputed by NP coating influences the immune response. Though the size and shape of MeNPs had little to no impact on the innate response elicited, coating modifications may improve the capacity of these molecules to influence immune responses. Finally, it is important to note that the majority of adjuvant characteristics were evaluated using non-metallic NPs.

4. Nanoparticles as adjuvants to generate Th1 and Th17 responses

In this review, only one study investigated the development of the direct Th1 (type 1 T helper cell) and Th17 response. Using a Listeria Ag, Rodriguez-Del Rio et al. (2015) showed that in contrast to Advax™ adjuvant alone, a combination of 25 nm AuNPs and Advax™ was capable of inducing the highest Th1 response. Pusic et al. (2013) immunized mice with IONPs covered with rMSP1, a *P. falciparum* merozoite antigen, and showed that after immunization (intramuscular, subcutaneous, or intraperitoneal), production of IL-4 was greater than that of IFN-γ, suggesting a predominant Th2 response (although the cellular immune response was not directly evaluated).

Th1 cells are pro-inflammatory effectors of immunity: they are associated with immunity against intracellular pathogens and the secretion of IFN-γ, which, in turn, is essential for the activation of mononuclear phagocytes, including macrophages, resulting in enhanced phagocytic activity (Golubovskaya and Wu, 2016). Th17 cells (IL-17A and IL-17F producer cells) are associated mainly with stimulation and chemotaxis of neutrophils to the site of inflammation. However, their function goes beyond this and includes the targeting of various cells types, including non-lymphoid cells, and the stimulation of cytokine, chemokine, and prostaglandin production. Another characteristic of these cells is their memory effector subset, which is maintained in mucosal tissues for extended periods. This subset has high plasticity and is able to transform into Th1 or Th2 phenotypes depending on the cytokine milieu at mucosal sites. This diversity of function and actuation make Th17 cells very important in defense against several microorganisms, mainly those acquired through mucosal routes (Zambrano-Zaragoza et al., 2014; Golubovskaya and Wu, 2016).

Th1 and Th17 have their own distinct sets of functions and differentiation factors; the ways by which nanoparticle may influence their differentiation are shown in Figure 2. While Th1 differentiation requires stimulation by IL-12, Th17 generation requires TGF[Transforming growth factor]-β and IL-6. IL-2 is needed to expand and maintain the Th1 subsets, while IL-21 and IL-23, respectively, are needed to expand and maintain Th17. Both cell types also require the downstream activation of a TCR (T cell receptor) through MHC II (major histocompatibility complex II) antigen presentation (Damsker et al., 2010). The first major determinant in generating Th1 and Th17 populations is the route of vaccine administration, which dictates the cell dynamic and initial response to the vaccine. For example, Mohanan et al. (2010), in a cross-sectional

study using a liposome plus ovalbumin Ag (OVA) vaccine formulation, compared intradermal (high IgG1; intermediate IgG2 and IFN-γ), intralymphatic (high IgG1, IgG2, and IFN-γ), intramuscular (high IgG1; intermediate IgG2 and IFN-γ), and subcutaneous (high IgG1; low IgG2 and IFN-γ) routes of administration (Mohanan et al., 2010). The predominant Th1 response to administration through the intradermal route was most likely due to the cooperation between Langerhans cells, the primary innate immune response cells, and keratinocytes that may also be stimulated by the formulation. These elicited the production of cytokines and chemokines that helped in the activation of other antigen presenting cells(APCs) (Kawase et al., 2006).

The early phase of vaccination is characterized by recruitment of neutrophils and monocytes to the site of inoculation. Both cell types can also act as APCs, delivering Ag-specific and costimulatory signals to T cells. Their collaborative endeavors have been found to modulate (positively or negatively) the activity of different effector T cell subsets (Didierlaurent et al., 2014; Iwasaki and Medzhitov, 2015). Neutrophils are the first cell lineage to migrate to inflammation sites and, when stimulated, they produce cytokines and chemokines that will attract and activate another cell types. For example neutrophils were shown to be an important inducer of Th1 and Th17 cells (Abi Abdallah et al., 2011), but their role in cytokine secretion is much broader (Tecchio et al., 2014). Moreover, signals may elicit different function in neutrophils and therefore influence the quality of T cell responses. For example, AuNPs have been described as capable of inducing neutrophil extracellular traps (NETs) which act as damage-associated molecular patterns (DAMPs) and stimulate immune system through DNA receptors such as TLR9 (Schaefer, 2014). Upon stimulation by NPs (TiO2 –titanium dioxide - and alum), Duffin, et al. (2007) demonstrated neutrophil influx to the lungs and also induced production of IL-18. Silver NPs were also shown to be capable of interacting with neutrophils, inducing apoptosis of these cells, and inducing caspase-1\caspase-4 partially dependent IL-1β secretion (Liz et al., 2015). In another study, cobalt and nickel nanoparticle were shown to induce higher nitric oxide, TNF-α, and CXCL2 chemokine production, by human peripheral blood neutrophils, than titanium nanoparticle (TiO2NP) (Mo et al., 2008). Nonetheless, TiO2NPs also induced polymorphonuclear cell activation through phosphorylation of several proteins, including p38 MAPK (mitogen-activated protein kinase) and extracellular signal-regulated kinases-1/2 (Erk-1/2), which were associated with increased neutrophil life-span and production of several cytokines and chemokines (Goncalves et al., 2010).

Classically, APCs, macrophages, and dendritic cells act at the site of vaccine inoculation by sensing foreign agents, through TLRs and other receptors, and triggering inflammation. APCs play a key role in the initiation, maintenance, and selectivity of inflammation, through their three major functions: endocytosis, Ag presentation, and production of various cytokines and growth factors (Reed et al., 2013). The main family of pattern recognition receptors (PRRs) in microbial recognition are the TLRs, part of the family of transmembrane proteins, which affect the transcription of genes involved in inflammatory and immune response-enhancing cellular activities such as

phagocytosis, endocytosis, cytotoxic functions, and cytokine production (Platt and Wetzler, 2013; Junqueira-Kipnis et al., 2014). The adjuvants used most frequently for the induction of Th1 and Th17 responses are TLR agonists, such as AS04, CPG/DNA, and others. However, NPs, such as TiO2NPs and zirconium oxide NPs, have also been described to interact with those receptors through interaction with TLR7 of the human macrophage U-937 cell line and enhancement of TLR10 expression (Lucarelli et al., 2004), and interaction with TLR2 and TLR4 in mouse liver cells (Cui et al., 2011). Consequently, TiO2NPs were shown to induce DC upregulation of MHC-II, CD80, and CD86 molecules (Schanen et al., 2009; Winter et al., 2011); zinc oxide nanoparticle (plus OVA) generated an inflammatory response in BALB/c mice through TLR-2, -4 and -6 activation, followed by activation of Src family kinases (Roy et al., 2014); silver NPs induced IL-6 production via TLR3 and TLR9 ligands in Raw264.7 macrophages (Castillo et al., 2008); peptide-conjugated AuNPs were shown to generate macrophage proliferation and TNF-α release through TLR-4 (Bastus et al., 2009); and IONPs, internalized by DCs and macrophages, enhanced the expression of CD80 and CD86, but not MHC-II. An association between IONPs and TLR signaling has not yet been elucidated (Pusic et al., 2013).

The next step in the generation of adaptive responses is the tailoring of cytokine secretion by APCs at immunological synapses, which will guide the development of the response. Several NPs have been reported to trigger cytokine and chemokine production, which may be used as biomarkers for immunotoxicity (Elsabahy and Wooley, 2013). Among those described, TiO2NPs were used in mimetic systems composed of blood vein endothelial component (including PBMC) and was reported to trigger pro-inflammatory cytokines (IL-6, IFN-γ, and TNFα) (Schanen et al., 2009); Zinc oxide NPs were shown to be preferentially associated with monocytes and, when used in PBMC, induced IFN-γ, TNF-α, and IL-12 cytokine production (Hanley et al., 2009); AuNP-stimulated bone marrow derived dendritic cells produced IL-6, TNF-α, and IFN-γ (Niikura et al., 2013); and IONPs were shown to induce the activation of APCs with an increase of IL-6, TNF-α , IFN-γ, and IL-12, as well as chemokines. The response generated by IONPs, however, was weaker than that generated by the positive control (LPS) which may be beneficial in controlling possible side effects (Pusic et al., 2013).

The generation of a cellular response associated with protection against intracellular pathogens is the ultimate goal of vaccination. However, the direct effects of NPs on cellular responses have been evaluated in only a few studies. TiO2NPs were shown to activate and induce proliferation of naïve CD4+ T cells and to generate a pronounced Th1 response with IFN-γ and TNF-α production, associated with pro-inflammatory cytokine production (IL-6, IL-1a, IL-1b) and DC maturation (CD86+ and CD83+ expressions increase). Schanen et al. (2013) hypothesized that the oxidative capacity of an NP could impact the response and trigger pro-inflammatory (oxidant capacity) or anti-inflammatory (antioxidant capacity) responses. This oxidant effect could control ROS generation and thus control downstream pro-inflammatory effects while

antioxidants prevent the initiation of the innate immunity in LPS-stimulated macrophages (Schanen et al., 2013). This study was, however, conducted with mitogens (nonspecific stimuli) and not with vaccine stimuli, but nevertheless serves as a warning about the direct action of NPs, not only on the innate immune system, but specifically on T cells.

5. Conclusion

MeNPs clearly have immunostimulatory capacity and can induce several reactions in all phases of vaccine development. There is enough evidence to suggest that they are not only particulate formulations but also immunostimulant molecules with several studies demonstrating their capacity to generate humoral and cytotoxic responses. These capabilities are correlated with NP physicochemical characteristics such as size, charge, and hydrophobicity, but there are several gaps in our understanding of their mechanism of actions and how they may lead to adjuvanticity, immunomodulation, or tolerance of Ag formulated with NPs. In conclusion, MeNPs are able to efficiently stimulate innate immune responses to enable the generation of Th1 and Th17, though their activity in generating this type of response has not yet been well investigated.

6. Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

Lázaro Moreira Marques Neto designed the review and wrote the first draft. Ana Paula Junqueira-Kipnis edited the first draft and critically reviewed the manuscript. André Kipnis edited the first draft and critically reviewed the manuscript. All authors read and approved the final version of the manuscript and agreed to submission.

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Review Paper - Table 1. Studies describing immune responses to vaccination with metallic nanoparticles, listed by nanoparticle material and year of publication (n= 18 studies)

NP material	Complementary adjuvant	Animal model (route of vaccination)	Evaluation of immunogenicity	First author, year of publication
Gold		C57BL/6 (H-2b) and BALB/c (H-2d) mice used for protection experiments (intraperitoneal)	CD4+, IL-2+, and duration and avidity of total IgG (IgG1, IgG2a, IgG2b, and IgG2c)	Kaba, 2009
		BALB/c mice (intraperitoneal and subcutaneous)	IgG (total)	Chen, 2010
	Alum; CFA/IFA	BALB/c mice (subcutaneous)	IgG1, IgG2a, IgG2b, and IgG3	Parween, 2011
		Albino mice and rabbits (intraperitoneal)	IgG, circulant IFN-γ, and ROS in vivo generation by peritoneal macrophages	Staroverov, 2011
	Alhydrogel	BALB/c mice (intramuscular)	IgG1 and IgG2a titer, CD4 and CD8 activation, and IFN-γ release	Gregory, 2012
		C57BL/6 (H-2b) and BALB/c (H-2d) mice used for protection experiments (intramuscular/intraperitoneal)	Total IgG; IgM and IgA titer and avidity; and CD8+ memory population (effector, central, and long-term central)	Kaba, 2012
		C57BL/6 mice (intramuscular/intraperitoneal)	IgG1, IgG2c, IgG3, and IgE titers	McCoy, 2013

		C3H/HeNJc1 mice (intraperitoneal)	IgG	Niikura, 2014
	CpG/DNA (TLR9 agonist)	BALB/c mice (intranasal)	IgG1 and IgG2a	Tao, 2014
	Asparagus racemosus extract	Swiss albino mice (oral)	Serum IgG, serum IgA, intestinal IgA, and fecal IgA	Barhate, 2014
	LPS (TLR4 agonist)	BALB/c mice (intranasal)	IgG1 and IgG2a	Gregory, 2015
	LPS (TLR4 agonist)	Rhesus macaques (subcutaneous)	IgG	Torres, 2015
	Advax™ adjuvant	BALB/c mice (intraperitoneal and intravenous)	Th1, CD8+, and NK cells	Rodriguez-Del Rio, 2015
		BALB/c mice (subcutaneous)	IgG (total)	Dakterzada, 2016
Iron		SW mice (intraperitoneal, intramuscular, and subcutaneous); *Aotus lemurinus trivirgatus* monkeys (intramuscular)	Total Ab response, IFN-γ, and IL-4 (mice) and total Ab response (monkeys)	Pusic, 2013
Nickel		BALB/c mice (subcutaneous)	IgG response	Fischer, 2010

	BALB/c mice (subcutaneous)	IgG1 and IgG2a serum titer and IL-12/p40 and RANTES/CCL5 serum concentration	Wadhwa, 2012
	BALB/c mice (subcutaneous)	Specific serum IgG; IgG1 and IgG2a Ab titers and IFN-γ (splenocytes)	Yan, 2009

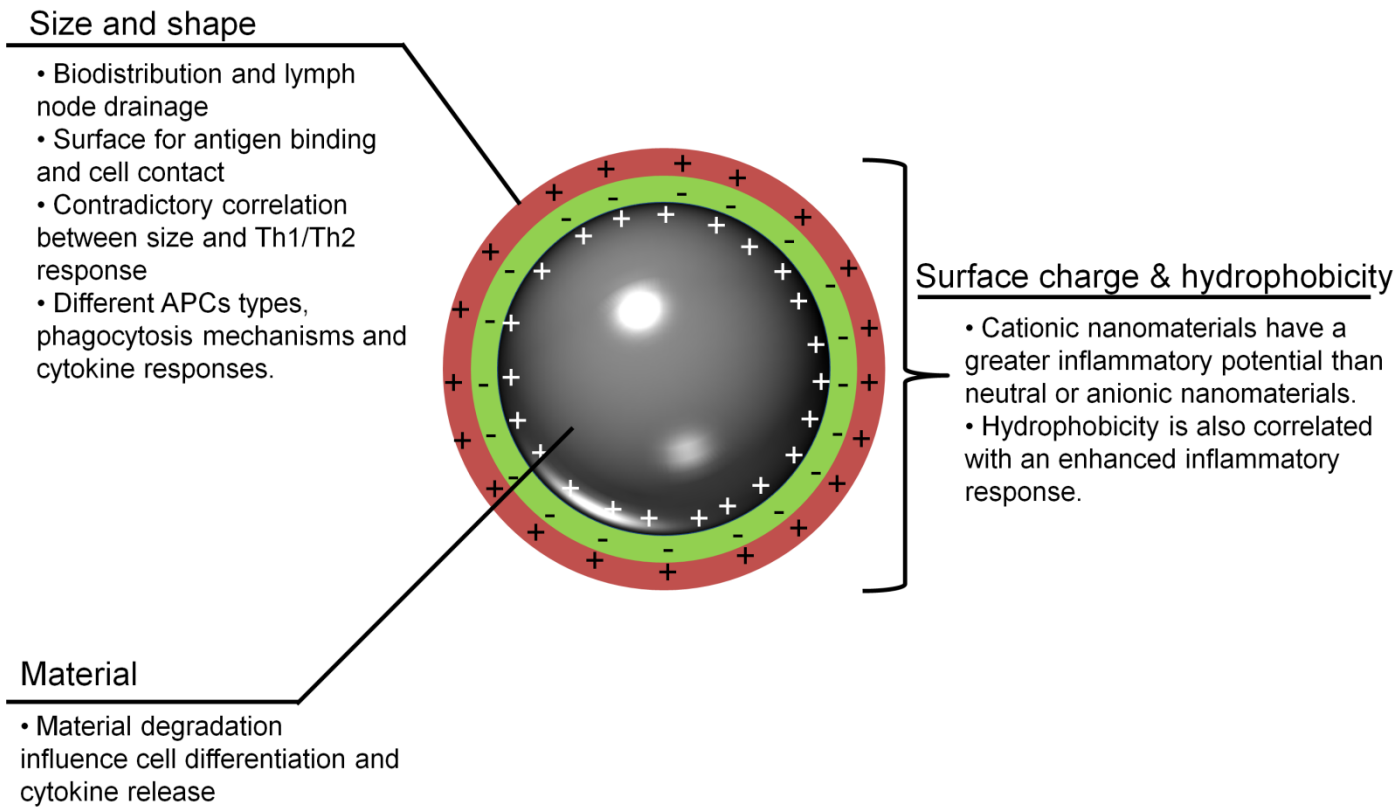
Ab: antibody; Alum: aluminum salts; CFA: complete Freund adjuvant; IFA: incomplete Freund adjuvant; IFN: interferon; Ig: immunoglobulin; IL: interleukin; LPS: lipopolysaccharide; NK: natural killer; NP: nanoparticle; ROS: reactive oxygen species; SW: swiss webster mouse ;Th: T-helper; TLR: Toll-like receptor

Review Paper - Table 2. Studies describing nanoparticles and antigens used as vaccines against infectious diseases, listed by nanoparticle material and year of publication (n= 18 studies)

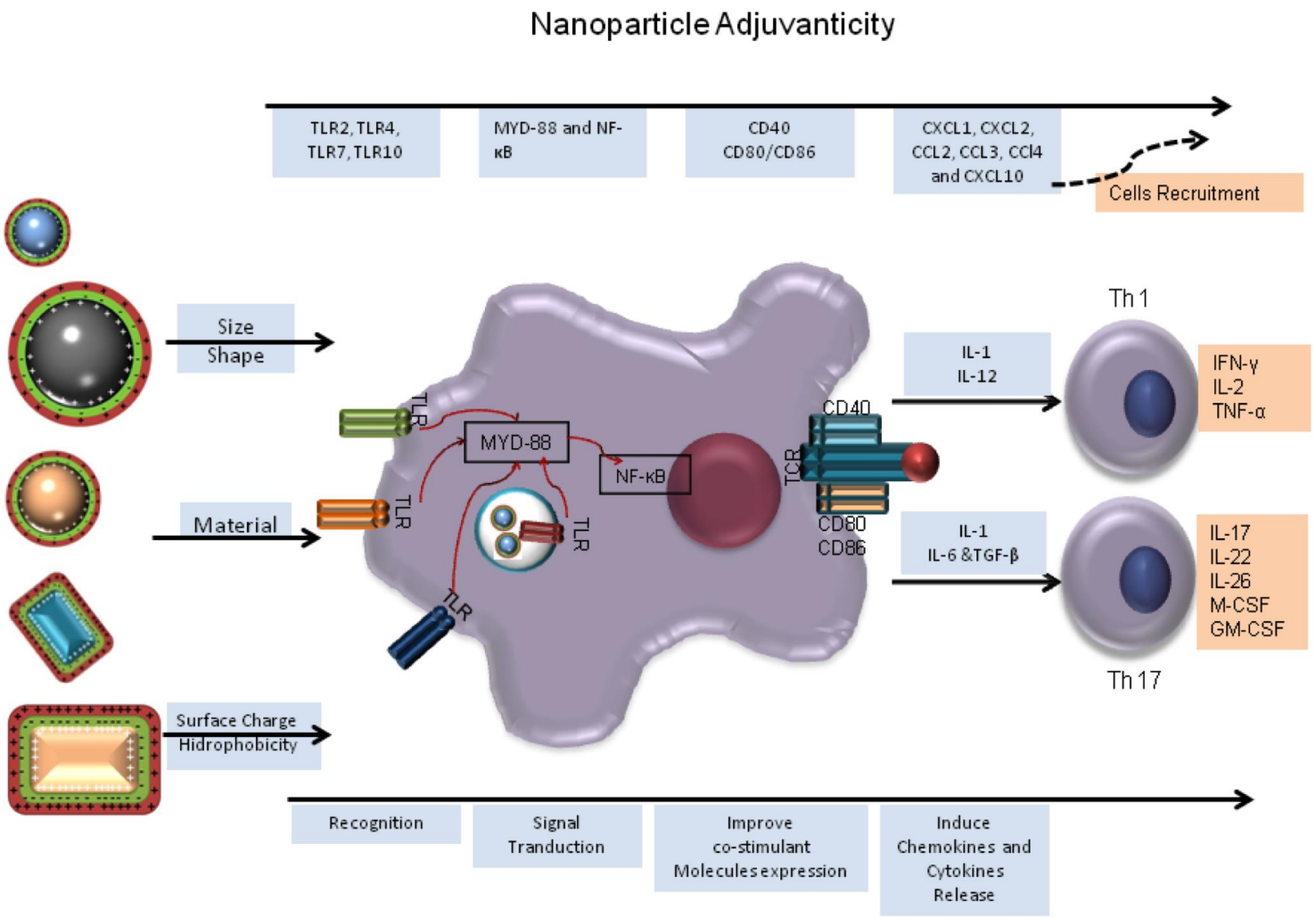
NP material	Size in nm (shape)	Functionalization	Antigen (microorganism)	First author, year of publication
Gold	25 (sphere)		Pb CSP (*Plasmodium berghei*)	Kaba, 2009
	2, 5, 8, 12, 17, 27, 32, and 50 (sphere)	Citrate	pFMDV (foot-and-mouth virus)	Chen, 2010
	17 (sphere)	Citrate	PfMSP-1_{19} (*P. falciparum*)	Parween, 2011
	15 (sphere)	Citrate	Partially purified enteropathogenic STG coronavirus	Staroverov, 2011
	15.6 (sphere)	Citrate	F1-antigen (*Yersinia pestis*)	Gregory, 2012
	40 (sphere)		Pf CSP (*P. falciparum*)	Kaba, 2012
	35–40 (sphere)	Citrate	Pf CSP (*P. falciparum*)	McCoy, 2013
	20 and 40 (sphere); 40×10 (rod); and 40×40×40 (cubic)	CTAB and PSS-MA	WNVE protein (West Nile virus)	Niikura, 2014
	12 (sphere)	Citrate	Extracellular portion of M2 protein (influenza virus)	Tao, 2014

	40 (sphere)	Chitosan	Tetanus toxoid bulk from *Clostridium tetani*	Barhate, 2014
	15 (sphere)	Citrate	TetHC and modified LPS from *Clostridium tetani*	Gregory, 2015
	15 (sphere)	Citrate	LPS conjugated to FliC as glycoantigen (*Burkholderia thailandensis*)	Torres, 2015
	1.5 (sphere)		T cell epitopes, , $LLO_{91–99}$, and $LLO_{189–201}$ (*Listeria monocytogenes*)	Rodriguez-Del Rio, 2015
	15 (sphere)	Citrate	$Flagelin_{1-161}$ (*Pseudomonas aeruginosa*)	Dakterzada, 2016
Iron	20 (sphere)	Dextran	$PfMSP\text{-}1_{1-42}$ (*P. falciparum*)	Pusic, 2013
Nickel	23 (discoidal)		Truncated WNVE protein (WNV)	Fischer, 2010
	199, 214, and 270 (capsule)		Gag p41 (HIV)	Wadhwa, 2012
	100 (capsule)		Gag p41 or p24/his-Nef (HIV)	Yan, 2009

CTAB: cetyltrimethylammonium bromide; HIV: human immunodeficiency virus; LPS: lipopolysaccharide; NP: nanoparticle; Pf CSP: *P. falciparum* circumsporozoite protein ; pFMDV: ; PfMSP: *P. falciparum* merozoite surface protein; PSS-MA: poly(4-styrenesulfonic acid- comaleic acid); nm: nanometer(s); STG: swine-transmissible gastroenteritis; TetHC: ; WNV: West Nile virus; WNVE: WNV envelope;



REVIEW PAPER - FIGURE 1. IMPORTANT NANOPARTICLE characteristics for adjuvanticity. **To be recognized and to stimulate innate immunity, MeNPs must have some physicochemical traits that allow for interactions with host cells and lead to the generation of a response.APCs: antigen presenting cells; MeNPs: metallic nanoparticles; Th: T-helper cell.**



Review Paper - Figure 2. Metallic nanoparticles have been described as capable of inducing stimulation associated with Th1 and Th17 responses. To generate a cellular immune response, the NP must be able to be recognized by the host and stimulate a sequence of events that will lead to the release of a specific milieu of cytokines and better antigen presentation. NF-κB: nuclear factor kappa B; CCL: chemokine ligand; CXCL: chemokine (C-X-C motif) ligand; GM-CSF: granulocyte macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; M-CSF: macrophage colony-stimulating factor; MYD: myeloid differentiation factor; TCR: T cell receptor; Th: T-helper cell; TLR: Toll-like receptor; TNF: tumor necrosis factor.

3. JUSTIFICATIVA

O estímulo necessário à resposta imune inata para se gerar resposta imune adaptativa celular do tipo Th1, Th17 se dá principalmente através do estímulo de moléculas coestimuladoras (CD40, CD80 e CD86) em células apresentadoras de antígenos, bem como indução da secreção de citocinas específicas (IL-12 para Th1 / IL-6 e TGF-β para Th17) e as nanopartículas foram demonstradas, principalmente *in vitro*, como tendo capacidade de estimular esses fatores.

A revisão da literatura demonstrou que as nanopartículas metálicas tem potencial para serem utilizadas como adjuvantes vacinais. Nessa revisão verificamos que as NP mais estudadas foram as de ouro, contudo NP de níquel e ferro também foram utilizadas em formulações vacinais. Contudo, nenhum trabalho avaliou se essas nanopartículas sozinhas eram capazes de auxiliar na indução da resposta imune adaptativa do tipo Th1, Th17 e CD8.

Dentre as doenças infecciosas mais graves que ainda são problemas de saúde pública mundial, a tuberculose tem um dos patógenos mais evoluídos para causar infecção no homem e ainda hoje é a maior causa de morte por doença infectocontagiosa no mundo. Mesmo a BCG (vacina contra tuberculose) estando em uso desde 1924 ela foi incapaz de erradicar a doença pois é ineficiente em proteger jovens e adultos contra a tuberculose. Por conseguinte, há a necessidade de se desenvolver uma vacina mais eficaz que melhore ou substitua a BCG. Juntamente com isso, outras doenças infecciosas causadoras de grandes problemas para a saúde pública mundial também se beneficiariam do estudo de adjuvantes capazes de auxiliar resposta do tipo T1, Th17 e T CD8, como é o caso da malária e leishmaniose.

Buscamos, portanto, através desse trabalho estudar as características das nanopartículas de íons metálicos, para geração de respostas imune celulares (principalmente Th1, Th17 e T CD8 que são as relacionadas à proteção contra patógenos intracelulares e que infectam mucosas) e utilizamos a tuberculose em modelo murino para estudar as características mais almejadas para uma vacina: a geração da resposta imune, a capacidade protetora da resposta imune gerada e a seguridade da formulação como vacina.

4. OBJETIVOS

Avaliar se nanopartículas metálicas podem ser utilizadas como adjuvantes vacinais para resposta Th1, Th17 e T CD8, em formulações de vacinas de subunidade proteica.

Objetivos Específicos

- Verificar se MeNP de ferrita de manganês podem ser capazes de gerar resposta imune celular Th1, Th17 e Tc1, em associação com proteínas de fusão CMX.

- Verificar se a resposta imune gerada é capaz de proteger contra a infecção com *Mycobacterium tuberculosis*

- Verificar se formulação é segura e possui atividade tóxica, danosa a algum órgão ou efeitos colaterais.

5. RESULTADOS

5.1 Artigo 2 – Título: Specific T cell induction using iron oxide based nanoparticles as subunit vaccine adjuvant

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Artigo 2. Title: Specific T cell induction using iron oxide-based nanoparticles as subunit vaccine adjuvant

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Keywords: Infectious disease; Manganese ferrite; antigenicity; Th1; Th17; TCD8 cells.

Abstract

Metal-based nanoparticles (NPs) stimulate innate immunity; however, they have never been demonstrated to be capable of aiding the generation of specific cellular immune responses. Therefore, our objective was to evaluate whether iron oxide-based NPs have adjuvant properties in generating cellular Th1, Th17 and TCD8 (Tc1) immune responses. For this purpose, a fusion protein (CMX) composed of *Mycobacterium*

tuberculosis antigens was used as a subunit vaccine. Citrate-coated $MnFe_2O_4$ NPs were synthesized by co-precipitation and evaluated by transmission electron microscopy. The vaccine was formulated by homogenizing NPs with the recombinant protein, and protein corona formation was determined by dynamic light scattering and field-emission scanning electron microscopy. The vaccine was evaluated for the best immunization route and strategy using subcutaneous and intranasal routes with 21-day intervals between immunizations. When administered subcutaneously, the vaccine generated specific $CD4^+IFN\text{-}\gamma^+$ (Th1) and $CD8^+IFN\text{-}\gamma^+$ responses. Intranasal vaccination induced specific Th1, Th17 ($CD4^+IL\text{-}17^+$) and Tc1 responses, mainly in the lungs. Finally, a mixed vaccination strategy (2 subcutaneous injections followed by one intranasal vaccination) induced a Th1 (in the spleen and lungs) and splenic Tc1 response but was not capable of inducing a Th17 response in the lungs. This study shows for the first time a subunit vaccine with iron oxide-based NPs as an adjuvant that generated cellular immune responses (Th1, Th17 and TCD8), thereby exhibiting good adjuvant qualities. Additionally, the immune response generated by the subcutaneous administration of the vaccine diminished the bacterial load of Mtb challenged animals, showing the potential for further improvement as a vaccine against tuberculosis.

1. Introduction

Adjuvants are substances that enhance and extend the immune response generated against a co-administered antigen. Only a few adjuvants are licensed for use in human vaccines, and even fewer efficiently induce a cellular immune response. This type of response is required for protection against some diseases that are of concern around the world, such as tuberculosis (TB) and malaria.[1] Therefore, the discovery of new vaccine adjuvants is the main goal of vaccinology. In a vaccine formulation, together with the adjuvant, the antigen guides the adaptive immune response toward the target microorganism and is associated with the long-lasting (memory) response.[2] However, there is evidence indicating that proteins from microorganisms are recognized not only by major histocompatibility complex (MHC) II/T cell receptors (TCRs) but also by the innate immune response via some pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs).[3, 4] Hence, the immunization route and the vaccination dose also impact generation of the immune response, especially the protective mucosal immune response.[5, 6]

Magnetic nanoparticles (MNPs) have several applications in nanomedicine such as contrast agents in magnetic resonance imaging (MRI), magnetic tracers in magnetic particle imaging (MPI) and alternating current biosusceptometry (ACB), and in magnetic hyperthermia therapy.[7-10] For a long time, nanoparticles (NPs) were only considered to be delivery systems in vaccines; however, there is now evidence showing that NPs can act as vaccine adjuvants with immunostimulatory capabilities.[11] Nonetheless, the use of metallic NPs in vaccinology is rare, and many effects and mechanisms of NPs have yet to be elucidated.[12] Some of the most common nanoparticles are iron oxide ones: magnetite – Fe_3O_4 – that consist of a cubic ferrite spinel structure containing one $1Fe^{2+}$; $2Fe^{3+}$; $4O^{2-}$ in the molecular structure. This type

of NP has been utilized as a vaccine platform against malaria only by Pusic et al. (2013), who demonstrated that vaccination with IONPs induces higher titers of parasite-inhibitory antibodies than vaccination with ISA51 (montanide) as an adjuvant. It was also demonstrated that IONPs activated dendritic cells (DCs) *in vitro* and enhanced CD86 expression. In addition, IONPs enhanced IL-6, TNF-α, IL-1β, IFN-γ, and IL-12 production, which correlated with the Th1 (IL-12) and Th17 (IL-6 and IL-1β) responses.[13]

In 2015, TB became the world's leading cause of infectious disease-related death, surpassing acquired immune deficiency syndrome (AIDS). It is estimated that one-third of the global population is infected with this bacillus, and the development of an effective vaccine to prevent disease activation and new infections is very important for TB eradication.[14] Although it is widely accepted that balanced Th1 and Th17 cellular responses correlate with protection against infection, TB is a complex disease whose pathogenesis reflects the interactions between *Mycobacterium tuberculosis* (*Mtb*) and the host immune system, which are not fully understood.[15] Thus, TB represents a model disease for evaluating the adjuvant properties of IONPs in eliciting cellular immune responses.

CMX protein is a fusion protein composed of portions of immunodominant antigens of *Mtb*: one is a peptide from Ag85c, a mycolyl-transferase protein responsible for the synthesis of *Mtb* cell-wall lipids;[16] another is a peptide from MPT51, a homolog of the Ag85 family but with functions associated with cell adhesion and virulence;[17, 18] and the third is the entire HspX protein, a heat shock protein associated with the latent phase of mycobacterial infection.[19, 20] CMX has been used in several vaccination strategies; this protein is capable of generating a Th1 response (together with CpG/DNA) in a subunit vaccine formulation[21] and of producing Th1 and Th17 responses to protect

against TB when expressed in live vectors (*Mycobacterium smegmatis* or *M. bovis* bacille Calmette-Guerin (BCG).[22-24] Recently, it was shown by da Costa and colleagues that CMX protein alone could influence the innate immune response and exhibit immunomodulatory activity, which was partially TLR-4 and TLR-2 dependent, inducing NF-κB, IL-6, TGF-β and IL-1β expression in bone marrow-derived macrophages.[25]

The complete pathway driving T cell responses in the respiratory tract (targeted by TB) is still unclear. However, there is evidence that intranasal vaccination can stimulate DCs responsible for generating T cells and recruiting them back to the lungs through the induction of CCR4 expression.[26] BCG is a globally accepted TB vaccine used in humans and is administered subcutaneously (SC) but does not provide long-term protection. Animals, such as guinea pigs, mice and macaques, are currently used as models for investigating new vaccines against TB. BCG vaccine administered SC is most frequently used as a control; however, the use of BCG or other vaccines (viral vectors or subunit vaccines) via the intranasal route can improve the immune response (Th1), change the response profile in the lungs, increase the mucosal Th17 response, and enhance the protection against aerosol challenges.[27-29]

The objective of this work was to evaluate the adjuvant properties of manganese ferrite ($MnFe_2O_4$)-NPs in the context of a nanoparticulate subunit vaccine composed of CMX fusion protein adsorbed on it toward the generation of specific Th1 (CD4 T lymphocyte, IFN-γ producer), Th17 (CD4 T lymphocyte, IL-17 producer) and Tc1 (CD8 T lymphocyte, IFN-γ producer) responses.

2. Results

NP vaccine characterization

The CMX protein, with a molecular mass of approximately 36 kDa, was used to cover the citrate-coated $MnFe_2O_4$ NPs to form NCMX. The size of the citrate-coated $MnFe_2O_4$ NPs obtained by transmission electron microscopy (TEM) was 15.4 ± 4.6 nm (Figure 1B). However, when dispersed in liquid, attractive intraparticle interactions, mainly van der Waals and magnetic interactions, led to the formation of agglomerates. This result was confirmed by the larger mean hydrodynamic diameter (86 nm) observed by dynamic light scattering (DLS) prior to protein coating (Figure 1A, upper curve). Indeed, DLS also indicated that the CMX protein adsorbed onto the NPs by affinity, as demonstrated by observation of a larger nano-compound with a mean hydrodynamic diameter of approximately 622 nm (Figure 1A, bottom curve). Protein adsorption was also confirmed by field-emission scanning electron microscopy (FE-SEM) analysis. The uncoated $MnFe_2O_4$ NPs, namely Nano, those not coated with CMX, were 151 ± 25 nm in size (Figure 1C). In contrast, the formation of the CMX corona in the NCMX nanostructure yielded particles with an increased size of 881 ± 130 nm (Figure 1D). The larger value measured by FE-SEM than by DLS was expected; in DLS, the particle size was measured in the liquid media, while in FE-SEM, the sample was dried. Therefore, one expects part of the protein corona to disconnect from the nanostructure, resulting in a larger size. The attachment of CMX to the NPs was also confirmed by immunodot blot assays using anti-CMX antibodies (Figure 1E). As shown, the majority of the protein used was coated onto the $MnFe_2O_4$ NPs, and only a residual quantity of the protein remained in the supernatant after magnetic lateral separation (fourth dot of Figure 1E). To verify the amount of CMX linked to the nanostructure, the protein was separated by the addition of sodium dodecyl sulfate (SDS) and centrifuged; then, the supernatant was utilized for western blotting (compared to a known CMX concentration). By doing so, it was possible to determine that the concentration of CMX

protein attached to the NP was very close to the total concentration used to cover the NP, i.e., a final concentration of 200 μg/mL (Figure 1F).

Subcutaneous NCMX vaccination induces Th1 and Tc1 responses and an increase in the number of lymphatic endothelial cells (LECs) (gp38$^+$CD31$^+$ cells)

To determine whether the vaccine formulated with NPs was immunogenic, mice were vaccinated SC three times at 21-day intervals (A). To optimize the dose necessary to induce specific immune responses, two concentrations of NPs were used, 10 μg (SC10) and 50 μg (SC50), which were formulated with 20 μg of CMX. Twenty-one days after the last immunization, the specific immune responses in the spleen and lungs were evaluated (Figure 2B). T CD4$^+$ lymphocytes expressing IFN-γ (Th1), T CD4$^+$ lymphocytes expressing IL-17 (Th17), and T CD8$^+$ lymphocytes expressing IFN-γ (Tc1) were analyzed by flow cytometry. Both formulations demonstrated the capacity to generate Th1 (Figure 2C and 2D) and Tc1 (Figure 2G and 2H) responses in the spleen and lungs of vaccinated mice. None of the groups showed a Th17 response (Figure 2Eand 2F) using this route with these tested doses. The generated CMX-specific humoral response varied among the groups; subcutaneous vaccination using 50 μg of NPs presented the highest IgG1 and IgG2a levels. However, the level of IgG2a to IgG1 was balanced for both vaccine formulations (Figure 2I and 2J).

LECs have been shown to proliferate, store antigens in the lymph nodes, and support the maintenance of T CD8$^+$ memory cells under proinflammatory conditions. Thus, since the subcutaneous route induced a robust Tc1 response in the spleen, the capacity of NCMX to induce LEC (gp38$^+$CD31$^+$) proliferation was evaluated. Evaluation of the axillaries lymph nodes 4 days after subcutaneous vaccination showed that NCMX

induced an increase in the level of LECs, while none the CMX protein or Nano alone was able to induce this proliferation (Figure 3).

Subcutaneous vaccination followed by intranasal boost generates specific Th1 and Tc1 lymphocytes but does not induce Th17 cells

Although both vaccine formulations were immunogenic, an important component of the supposed protective immune response against *Mtb* was missing. Th17 cells, when stimulated together with Th1 cells, are known to aid the generation of a better protective response.[30, 31] The combination of subcutaneous and intranasal mucosal vaccination has been shown to generate Th17 cells.[32, 33] Thus, it was hypothesized that in order to generate specific Th1/Tc1/Th17 responses, a combination immunization route should be used (Figure 4A). The mixed vaccination scheme generated specific Th1 (Figure 4B) cells in the spleen and lungs (Figure 4C) of immunized mice. Additionally, specific Tc1 cells (Figure 4E) were induced in the spleen. However, no Th17-specific response was observed in the spleen (Figure 4D) or lungs (Figure 4E) of vaccinated mice. The nanoparticle alone was also used as control, however as we evaluated the specific response to CMX, no response elicited (Supplementary Figure 5). The humoral response elicited by this vaccination strategy was weaker than that generated by subcutaneous vaccination, and the intranasal booster led to little improvement in the antibody response (Figure 4H and 4I). In summary, the results show that the NCMX formulation efficiently induces Th1 and Tc1 responses but not a Th17 response when the subcutaneous and intranasal routes are used.

Intranasal instillation can stimulate Th17 in addition to Th1 and Tc1 responses in the lungs

Since specific Th17 cells were not generated in the lungs when intranasal vaccination was used as a booster, a vaccination strategy using only intranasal vaccination (a mucosal immune stimulator) was used (Figure 5A). The vaccination stimulated specific Th1 (Figure 5B) and Tc1 (Figure 5D) responses in the lungs as well as the important Th17 (Figure 5C) response; however, no antibody responses were observed (Figure 5E and 5F).

Multiple exposures of the lung parenchyma to inorganic NPs, especially iron and manganese NPs, could result in lung damage. Although the animals did not present any sign of stress or disease (data not shown), the morphology and histology of the lung tissue were evaluated. Mice vaccinated with the $MnFe_2O_4$ NP and NCMX formulations exhibited increased lung cellularity without the formation of arteriolar cuffs, which is indicative of cell migration. However, the $MnFe_2O_4$ NPs resulted in a lower cellularity index, and this difference could be correlated with particle size and aggregation (Figure 6).

The hemolytic effect of the $MnFe_2O_4$ NPs (10 μg, 50 μg, 100 μg and 500 μg) and NCMX (with 20 μg of CMX and 10 μg, 50 μg, 100 μg and 500 μg of $MnFe_2O_4$ NPs) is demonstrated in Supplementary Figure 3. Hemolysis increased slightly with the increasing tested concentrations; however, hemolysis did not exceed 20% for any sample, even with the highest concentration tested being 10-fold greater than the vaccine concentration. Additionally, twenty-one days after two intranasal vaccinations, two subcutaneous and three subcutaneous vaccinations (using 10 μg of $MnFe_2O_4$ NPs and 20 μg of CMX), the heart, kidneys and liver were collected, sectioned, stained with hematoxylin and eosin (H&E) and evaluated for signs of chronic toxicity or persistent organ damage. No disturbances or injuries were found in any of these organs (Supplementary Figure 4).

CMX-specific Th1 and Tc1 recall responses after *Mtb* challenge cause reduction in the bacterial load

Because the subcutaneous and the combined subcutaneous and intranasal vaccination strategies induced cellular immune responses, vaccinated mice were challenged with *Mtb* to determine whether the response could be recalled (Figure 7A). The *Mtb* challenge resulted in increased numbers of specific Th1 cells in the lungs of all infected groups. However, the greatest increase in $CD4^{+}IFN\text{-}\gamma^{+}$ cells was observed in animals SC vaccinated with NCMX (Figure 7B-D). Additionally, the *Mtb* infection induced an increase in the CMX-specific Tc1 population. Vaccination with NCMX recalled the CMX-specific Tc1 population (doubled the amount observed for *Mtb*-challenged or BCG-vaccinated animals) to the lungs of the vaccinated mice (Figure 7C-E).

Preservation of the lung architecture (healthy/saline control; Figure 8A) is an essential feature of vaccines against TB. The lungs of the *Mtb*-infected group exhibited expressive lymphocytic and neutrophilic infiltrates, compromising the entire lung tissue architecture, together with the presence of foamy macrophages (Figure 8B). On the other hand, BCG-vaccinated mice showed fewer lung lesions, preserving the alveolar spaces, and limited lymphocytic tissue infiltration (Figure 8C). However, while the subcutaneous NCMX vaccination preserved the lung architecture but with areas of mononuclear inflammatory cell infiltration (Figure 8D), the mixed (subcutaneous and intranasal) NCMX vaccination showed larger areas of mononuclear inflammatory cell infiltration that compromised the alveolar structure (Figure 8E).

Finally, diminishing the bacterial burden in the lungs of infected hosts is one of the main goals of *Mtb* vaccines. Thus, we assessed whether vaccination with NCMX could generate a response that could protect mice against intravenous *Mtb* challenge. NCMX

vaccination reduced the number of bacteria recovered from the lungs of challenged mice by half log unit compared with the number of bacteria recovered from the lungs of unvaccinated mice (saline control), while BCG vaccination reduced the number of bacteria by approximately 0.8 log unit (Figure 8F).

3. Discussion

The search for new adjuvants is one of the main goals of vaccinology. NPs have been reported in several studies as being able to interact with the immune system and have been demonstrated to serve as adjuvants capable of inducing a humoral immune response. The capacity of NPs to induce T cell responses is still unclear. Consequently, cellular (Th1 and Th17) immune responses, even when antigens are coupled with NPs, are correlated with the utilization of TLR adjuvants or other potent adjuvants.[12, 34] In this work, we presented a new NP adjuvant for a subunit vaccine, which we tested in a murine model of TB. The adjuvant used a fusion protein named CMX, which is a protein composed of three *Mtb* antigens that has previously been shown to induce a protective response against TB in several vaccine formulations.[22-24] The NCMX vaccine was tested using subcutaneous and intranasal vaccination schemes. All strategies showed the ability to induce a specific immune response to CMX, and when the vaccine was administered SC followed by challenge with *Mtb*, CMX-specific Th1 and Tc1 responses were achieved and conferred half log protection against TB.

The NPs used here is very similar to magnetite, but instead of containing Fe^{2+} in this structure it has Mn^{2+}. Note that it still contains Fe^{3+} and O^{2-}, therefore is an iron oxide-based NP. It had a mean individual core size of 15.4 nm, as determined by TEM; however, due to particle agglomeration and the citrate layer, the mean hydrodynamic diameter was larger, on the order of 85.9 nm in phosphate-buffered saline (PBS), which

was the vaccine buffer. The immune responses elicited by metallic NPs have been shown to be related to the size and shape of the NPs, as demonstrated by Niikura (2013);[35] however, in that study, only gold NPs and TNF-α and antibody responses were investigated, and these NPs may have significantly different properties than our NPs. Consequently, the effect of the physical characteristics of metallic NPs (such as IONPs or Mn-doped iron oxide NPs) on the immune response remains to be elucidated.

The formation of a protein corona composed of the fusion protein CMX was also demonstrated, along with NP agglomeration; the particle size increased to more than 600 nm (DLS) and 800 nm (FEG-SEM) after protein adsorption. The larger NPs can serve as a depot at the vaccine inoculation site, guiding the immune response to the site and prolonging antigen release, and consequently, antigen presentation, thereby having a direct impact on lymph node drainage, cellular uptake, and antigen presentation.[36] NPs larger than 500 nm have been shown to need DCs to reach lymph nodes from the injection site,[37] and NPs exhibiting the depot effect (in a cationic liposome model) have been shown to be more capable of inducing IFN-γ release.[38]

We verified the possibility of using different doses of NPs in vaccine formulations (10 μg and 50 μg). Both doses generated Th1 and Tc1 responses; thus, the NP dose seems to have less of an impact on cellular immunity than on the humoral immune response (Figure 2). Sindrilaru et al. (2011) have shown that greater iron uptake by macrophages makes the macrophages less modulatory and capable of secreting more proinflammatory cytokines.[38] Similar to iron, manganese has also been demonstrated to be capable of inducing proinflammatory responses in microglia; however, manganese alone induced moderate only production of IL-6 and TNF-α,[39] as well as superoxide reactivity, H_2O_2 formation and NO production by neutrophils.[40] Therefore, the use of higher concentrations of iron and manganese-based NPs could be directly

correlated with the immune response induced. Yet, from our data, the tendency of the particles to induce a greater CD8 response when 50 µg was used indicates that the NP dose should be investigated to better understand this relationship.

The administration route can affect the immune response elicited by a vaccine. For instance, when mice are immunized through subcutaneous route, CD103+ DC subset present in the dermal compartment can migrate to the site of injection and then migrate to the LN. Dermal CD103+ DC produce IL-12 and can cross present the antigen by MHCII/MHCI generating Th1/Tc1 (CD8) responses.[41] Our results with subcutaneous vaccination also induced Th1/Tc1 responses and probably this was due to production of IL-12 by those DCs. Intranasal vaccination using NCMX generated Th1/Tc1 and Th17 responses, CD103+ DCs also present in the lungs could be involved in the generation of Th1/Tc1 responses.[42] Further, the DCs present in NALT (nasal associated lymphoid tissue) produce more IL-6 than those present in cervical lymph nodes and spleen and consequently might be responsible for preferential Th17 generation when the intranasal route was used.[32]

IONPs and $MnFe_2O_4$ NPs have been used in different nanomedical approaches and are usually tolerated well, sometimes at doses ten-fold higher than those used in this study.[10, 43-45] However, repeated exposure of the airway to manganese (salt) or iron NPs can lead to pulmonary disorders, such as bronchiolitis and pneumonitis.[46-48]It is also through the airway that manganese enters the bloodstream and starts to accumulate in and damage the nervous system, leading to predispositions to neurodegenerative disorders.[47, 49] Despite these disadvantages, passivation of the $MnFe_2O_4$ used here enriched the surface with Fe, and the subsequent coating with CMX might have prevented the release of ions (Fe or Mn) during a short time window, thus reducing the toxicity. Not all adjuvants can be efficiently used for mucosal vaccine administration,

and even established adjuvants, such as aluminum, MF-59 and even muramyl-di-peptide (cellular immune response adjuvant), have failed to induce an immune response via intranasal vaccination.[50] Thus, the diminished immunogenic capability of the intranasal route as a booster, with the need for multiple vaccinations through the same route, can be an obstacle for the use of NPs via this route, once mild inflammation of the lungs observed even after the second intranasal immunization.

To induce a CD8 response, the antigen should be presented by DCs to naïve CD8 cells via cross-presentation, and as demonstrated by Tran and Shen (2009),[51] NPs larger than 500 nm are better at inducing this type of response by reducing the endosomal pH and thus improving the antigen processing. [51] A TCD8 response can directly kill the infected cells or produce cytokines (Tc1) to aid in the activity of other cells;[52] the TCD8 response was the most prominent response induced by our vaccine formulation with NMCX. $CD8^+$ T cell generation is known to be partially CD4 independent; however, the development and maintenance of memory is CD4 dependent, and the priming of the Tc1 population is improved when correlated with the Th1 response.[53] In this study, even at low levels, the Th1 response was observed together with the Tc1 response.

The observed NCMX size of 622-881 nm implies that they will not be uptake by LECs. Therefore, the increase in the number of LECs observed in our experiments may be due to CMX antigen shedding and drainage to the lymph nodes that in turn could be taken by LECs. Previously, CMX alone was shown to induce activation of NF-κB, IL-6 and IL-1α .[25] Pusic and cols showed that iron oxide nanoparticles were capable of inducing IL-6, TNF-α, IL1-b, IFN-γ, and IL-12.[13] As shown by Tamburini and cols, although the adjuvant (polyIC) activates the innate immune response, only when the protein and the adjuvant formulation were used as vaccine LECs proliferated.[54] Thus maybe this is the

case for our results, where the increase in the number of LECs was observed only when Nano was combined with CMX.

CMX is capable of inducing IL-6 and TGF-β expression in macrophages *in vitro*;[25] however, when used alone, it cannot generate an adaptive immune response.[55] This observation is in agreement with the literature showing that protein alone cannot efficiently generate an adaptive immune response.[56] CMX was used by de Sousa et al. (2010) in a formulation with CpG/DNA and liposomes and efficiently induced a Th1 response,[21] which is important for protection against TB. Compared with most potent adjuvants (such as lipopolysaccharide (LPS) or CpG/DNA), NPs have a diminished capacity for stimulating these types of responses,[13] but even without any TLR agonist, our formulation was able to induce a Th1 response.

The induction of the Th17 response is correlated with MHCII presentation and a milieu of IL-6 and TGF-β in the immunological synapse. This response is important for protection against TB by supporting Th1 reactivity and downregulating IL-10 and upregulating IL-12 production by DCs; after *Mtb* challenge, Th17 cells stimulate chemokine production, recruit $CD4^+$ T cells to the lungs, favor granuloma formation, and accelerate pathogen clearance.[30, 57]CMX alone has been shown by da Costa et al. (2015) to induce IL-1α, IL-6 and TGF-β expression in bone marrow macrophages (BMMs), and iron NPs have also been demonstrated to be capable of inducing IL-6 and IL-β expression.[13, 25] However, the stimulatory capacity of CMX is TLR-4 and TLR-2 dependent, and IONPs are capable of interfering with monocyte responses, diminishing the production of IL-1β and TNF-α, when used as co-stimuli with LPS (TLR-4 dependent).[58] This observation supports our finding of a low efficiency in the induction of a Th17 response by our formulations. Finally, we and others have recently demonstrated the role of IL-17 produced by Th17 cells in TB, and the failure to induce

this population in this study was probable the main factor responsible for not achieving better protection against infection.

After the *Mtb* challenge, the subcutaneous and mixed formulations were able to recall the CD8 response, reflecting the capacity of the formulations to maintain and generate that response. Both recalled responses diminished the bacillary load in the lungs of infected mice, reinforcing the importance of this population in the protection against TB. However, the subcutaneous vaccination alone also induced a Th1 response that could be recalled upon challenge and diminished the damage to the lungs, demonstrating that the Th1/Tc1 immune response is responsible not only for killing the bacteria but also for delimiting and controlling the infection.[52, 57]

The scientific community and the WHO have been careful with the use of metallic adjuvants, mainly because of the association of the presence of heavy metals in vaccines and adverse effects. However, there is no evidence of such adverse effects; for example, Taylor et al. (2014) evaluated almost 1.3 million people through a meta-analysis and found no evidence of an association between vaccination and autism.[59] In addition, the presence or absence of thimerosal (mercury-containing preservative) in American vaccines or immunoglobulin preparations does not affect the number of individuals with autism spectrum disorder.[60] Despite these observations, there are still questions about possible side effects. As such, we searched for potential toxic effects of our vaccine formulation; we found no hemolysis, and the vaccinated mice showed no evidence of organ damage or disease in terms of abnormal behavior, weight loss, reflexes, balance, exploration, strength, locomotor activity or any other parameter. The only observed effect was increased cellularity in the lungs when the mice were vaccinated intranasally, which is also correlated with cell migration and development of the immune response.

The biodistribution of nanoparticles can be related to several characteristics such as site of injection, size, shape and charge. Particularly, the size of the nanoparticle also dictates its distribution, once nanoparticle with size closest to the used in our work (600-800 nm) will be found almost equally in the lungs, liver and spleens, while nanoparticles smaller than 10 nm tend to be cleared by the kidneys.[61] Because of that, in toxicity assay we looked for any damage in the lungs, liver and kidneys of vaccinated mice. However, no sign of damage was found in any organ when NPs were utilized through subcutaneous route while only small modification was found in the lungs of animals vaccinated intranasally. Together with this, there are multiples possible cells which are capable of endocytosis, as well as biodegrade these particles, particularly macrophages, dendritic cells and inflammatory monocytes.[62] The amount of NP in our formulations (50µg being the biggest quantity) is well below the amounts used in FDA approved iron oxide based formulations such as Feraheme or Resovist that can be used in mice from 0.6 mg to 12.3 mg, respectively.[63] Furthermore, even with this type of NP taking several weeks to be degraded, the administered amount are not capable to become toxic, while reports in the literature indicate that it can be used by the body as nutrient iron or be excreted in the feces after degradation.[64, 65]

The formulation used here efficiently induced a Tc1 response, which heightens the potential use of this formulation (Tc1 inducer) in cases where the TCD4 response is not efficient, such as in AIDS. In addition, we only used NPs as an adjuvant and immunostimulant, and other approaches, for example, complexing with CpG/DNA or other TLR agonists, can improve the desired immune response. However, the use of such approaches must be carefully evaluated since the presence of NPs can alter the function and response of TLRs, as demonstrated for TLR-2 and TLR-4.[58]

In this study, we found distinct responses when different doses and routes were used, with SC50 generating more IgG1 and IgG2a than SC10. Vaccination strategies that included intranasal vaccination (alone or combined with subcutaneous) did not induce or improve significant antibody titers. In TB, the mechanism of antibody protection is unclear, but it seems to be essential for containing the mycobacterial infection by synergizing with cellular immune response.[66-68] In some studies, comparison of similarly shaped particles revealed an inverse correlation between the particle size and immune response, with smaller particles inducing a more efficient humoral response, while these effects were not observed in other studies.[34, 69, 70]

Although our cell preparations could contain circulating blood cells not representative of the investigated organ, the mucosal immune response was evaluated after perfusing the lungs with ice-cold PBS containing heparin to remove traces of blood.[71] Our group evaluated protection against TB using the intravenous route with relatively high bacillary doses, which is not the usual route of infection for TB but provides a better surrogate of protection due to the higher bacterial load rapidly achieved in several tissues following infection requiring a more efficient protective response that could be missed when low-dose aerosol infection is used.[22, 72] BCG is always used as a control in our studies, and as shown by our results, the protection induced by BCG in our model of infection is similar to that obtained by several groups that work with vaccines and use the aerosol route of TB infection. Moreover, the lesions caused by intravenous infection are similar to those caused by aerosol infection; thus, the host-pathogen interactions are conserved.[23, 24, 55]

Finally, for the first time, this study demonstrates that $MnFe_2O_4$ NPs in a vaccine formulation can generate cellular immune responses. The formulation could generate Th1 and, most prominently, Tc1 (CD8) responses that could be recalled upon TB

infection and could help in protection against the bacteria, as demonstrated by our evaluation of the total immune response present in the lungs and spleen.

4. Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Committee of Sociedade Brasileira de Animais de Laboratório (SBAL). The animals were housed and handled according to the guidelines of the Conselho Nacional de Controle e Experimentação Animal (CONCEA). The protocol for this work was approved by the Committee on the Ethics of Animal Experiments of the University Federal de Goiás (protocol number: 103/14).

Citrate-coated $MnFe_2O_4$ NP synthesis and CMX coating

The citrated-coated $MnFe_2O_4$ NPs were synthesized as described by Nunes et al. (2014).[44] Briefly, 50 mmol of $FeCl_3$ and 25 mmol of $MnCl_2$ (both dissolved in 100 mL of 3% HCl) were introduced into 500 mL of boiling 2.0 mol/L methylamine solution under vigorous stirring for 30 min. The obtained solid was magnetically separated from the supernatant and washed three times with distilled water. The precipitate was acidified with a 0.5 mol/L HNO_3 solution and magnetically separated from the supernatant, which was discarded. Then, the NPs were hydrothermally treated by boiling 0.5 mol/L $Fe(NO_3)_3$ for 30 min, and the excess ferric nitrate was removed from the solution by magnetic decantation. After preparation of the magnetic composite, the coating process was performed by the addition of sodium citrate to the solution under stirring for 30 min using a 1:10 mass ratio of $Na_3C_6H_5O_7$ to $MnFe_2O_4$, in 50 mL of water. The obtained precipitates were magnetically separated, and the supernatants were discarded. Then, the precipitates were washed three times with acetone, the

desired amount of water was added, and the excess acetone was evaporated to yield the magnetic fluid samples.

CMX protein is a recombinant protein developed by de Sousa et al. (2012)[21] and produced in *Escherichia coli* in the Immunopathology and Infectious Diseases Laboratory at the Institute of Tropical Pathology and Public Health (Instituto de Patologia Tropical e Saúde Pública - IPTSP), UFG.

For the CMX coating, we utilized the protocol of Yang and Burkhard (2012)[73] with modifications. Briefly, $MnFe_2O_4$ NPs were diluted in the CMX protein solution (200 μg/mL antigen concentration) to a concentration of 100 μg/ml (SC 10) or 500 μg/mL (SC 50). The solution was then incubated for 2 h under 1000 rpm homogenization and utilized for the other tests and in immunizations.

Characterization of NPs with and without CMX protein corona

The hydrodynamic diameter of citrate-coated $MnFe_2O_4$ NPs and citrate-coated $MnFe_2O_4$ NPs coated with CMX (NCMX) was determined with a Malvern Zetasizer Nano S equipped with a 633-nm laser (Instituto de Química – Laboratório de Química Analítica -UFG). Hellma quartz cuvettes with a 3-mm path length and a 9.65-mm center were used. Measurements were performed at 20°C using 500-μL samples. The hydrodynamic diameters were verified by Malvern DTS software, version 7.01.

In addition, the size of the citrate-coated $MnFe_2O_4$ NPs was characterized by TEM. A drop of the NP solution was deposited onto a glow-discharged carbon-coated grid. The grid was subsequently dried and visualized using a JEOL JSM-6610 microscope (JEOL, Japan) equipped with an energy dispersive spectrometer (EDS; Thermo Fisher Scientific NSS Spectral Imaging) at Laboratório de Microscopia de Alta Resolução LabMic - UFG). The images were analyzed with public domain Java ImageJ software (V1.37).[74] For the NP size measurements by TEM, 230 NPs were evaluated.

The process of coating the NP surface with protein is known in the literature as protein corona formation.[75] To visualize the CMX corona formation, the NCMX suspension was dripped onto ultra-thin carbon membranes and left to dry at room temperature to make the membranes suitable for analysis. The images were acquired using a JEOL JSM-7100F field-emission scanning electron microscope in scanning transmission electron microscope (FE-SEM) mode at Centro Regional de Desenvolvimento Tecnológico (CRTI-UFG). For each sample, a series of images with increasing magnification was recorded. The images were analyzed, and the corona was evaluated using public domain Java ImageJ software (V1.37).[74]

NCMX samples were separated by lateral magnetic separation for 24 h and resuspended in PBS (100 µL). To evaluate unbound protein, the residual supernatant from the lateral magnetic separation was centrifuged at 16,000 ×g for 15 min at 22°C. Then, the supernatant was discarded, and the sediment was resuspended in PBS and applied to a nitrocellulose membrane.

For the immunodot blot assay, a nitrocellulose membrane was spotted with 10 µL of each of the following solutions: a solution containing 5 µg of NPs, a solution containing 10 µg of CMX, 10 µL of the NCMX formulation and 10 µL of the lateral flow NP separation supernatant. After this step, the nitrocellulose membrane was incubated with polyclonal antiserum against CMX (1:10,000 dilution obtained from rCMX-immunized BALB/c mice). The membrane was then incubated with horseradish-peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich). The presence of the CMX protein was detected by incubation with diaminobenzidine (Roche).

Protein corona quantification

Western blotting (Figure 1C) was performed to quantify the recombinant CMX protein linked to the $MnFe_2O_4$ NPs. To release the protein corona from the NPs, NPs in NCMX

(500 µL) were separated by lateral magnetic separation for 24 h. Then, the supernatant was discarded, and the sediment was resuspended in 500 µL of 10% SDS-PBS buffer, incubated for 30 min, and centrifuged at 5000 rpm for 10 min; then, 10 µL of the supernatant was utilized for western blotting. After transferring the proteins to the nitrocellulose membrane, the procedures described above were followed to perform the immunodot blot assay.

Animals

Specific-pathogen-free female BALB/c mice (4–8 weeks old), purchased from CEMIB-UNICAMP, were maintained in ABSL-2 racks fitted with a HEPA-filtered air intake and exhaust system with water and food provided ad libitum at the animal care facility of the Institute of Tropical Pathology and Public Health at Federal University of Goiás. The temperature was maintained from 20–24°C with a relative humidity of 40–70% and a 12-h light/dark cycle. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Committee of SBAL and was approved by the Research Ethical Committee of Federal University of Goiás (approval number: 103/14).

Immunogenicity assay

Three vaccination strategies, with 21-day intervals between immunizations, were performed. The first strategy, to define the vaccine dose, consisted of mice immunized SC three times. Two vaccine formulations were utilized, as follows: (I) the SC10 group utilized 10 µg of NPs mixed with 20 µg of CMX per animal; (II) the SC50 group utilized 50 µg of NPs mixed with 20 µg of CMX per animal. The second strategy consisted of two subcutaneous immunizations with 100 µL of the NCMX formulation (10 µg of NPs mixed with 20 µg of CMX) per animal followed by an additional booster via intranasal instillation at the same dose as the subcutaneous injection. The third vaccination scheme

was performed using two intranasal instillations of 100 µL of the NCMX formulation (10 µg of NPs mixed with 20 µg of CMX) per animal.

Twenty-one days after the last immunization, the mice were euthanized, and the lungs and spleens were collected for flow cytometry analysis and histological immune response evaluation. As a negative control, a group of mice received PBS (100 µL) via the same routes evaluated in each experimental strategy.

Serum collection and ELISA

Serum samples were collected from the immunized mice 21 days after each immunization. The samples were incubated for 1 h at 37°C, centrifuged at 1,200 ×g for 15 min at 4°C to separate the serum, and stored at -20°C.

ELISA was performed as described by Sousa et al. (2012).[21] Briefly, 96-well polystyrene plates (Falcon®) were coated with 10 µg/mL of recombinant CMX fusion protein diluted in 0.05 M sodium carbonate/bicarbonate buffer and incubated at 4°C for 16 h. The wells were blocked with PBS containing 1% skim milk. The serum samples were diluted 1:40, added to the wells, and incubated for 2 h at 37°C. Biotin-conjugated antibodies (anti-IgG1 or anti-mouse IgG2a; Pharmingen®) diluted 1:5000 were added to the plates, which were then incubated for 1 h at 37°C. Streptavidin peroxidase, diluted 1:1000, was added, and the plates were incubated again for 1 h at 37°C. After incubation with the substrate solution, the absorbance at 492 nm was measured on an ELISA reader (LabSystems Multiskan Thermo®).

CMX-specific cellular responses in the lungs and spleen

Cell preparation and the cytometry protocol were performed as described by Junqueira-Kipnis et al. (2013) and da Costa et al. (2014).[22, 23] Briefly, twenty-one days after the last immunization, four BALB/c mice from each group were euthanized, and the spleens and lung lobes were collected. The spleens were prepared as single-cell

suspensions using 70-µm cell strainers (BD Biosciences), and the cells were resuspended in RPMI medium. After erythrocytes were lysed with lysis solution (0.15 M NH_4Cl, 10 mM $KHCO_3$), the cells were washed and resuspended in RPMI medium supplemented with 10% fetal calf serum, 0.15% sodium bicarbonate, 1% L-glutamine (200 mM; Sigma-Aldrich), and 1% nonessential amino acids (Sigma-Aldrich). Cells were counted in a Neubauer chamber, and the concentration was adjusted to 1×10^6 cells/mL. Prior to collection, the lungs were perfused with ice-cold PBS containing 45 U/mL heparin (Sigma-Aldrich). The lungs were digested with DNAse IV (30 µg/mL; Sigma-Aldrich) and collagenase III (0.7 mg/mL; Sigma-Aldrich Brazil) for 30 min at 37°C. The digested lungs were prepared as single-cell suspensions using 70-µm cell strainers and subjected to erythrocyte lysis. The cells were washed and resuspended in RPMI medium, and the concentration was adjusted to 1×10^6 cells/mL.

Cell stimulation was performed in a 96-well cell-culture plate (CellWells™). Spleen or lung cell suspensions were cultivated with media alone, with recombinant CMX (10 µg) or with ConA (positive control) in a 5% CO_2 incubator at 37°C for 4 h. Then, monensin (3 µM; eBioscience) was added to the wells, and the cultures were incubated for 4 h. Cells were treated with 0.1% sodium azide in PBS for 30 min at room temperature and then centrifuged. The cells were stained with FITC-conjugated anti-CD4 (BD Pharmingen; clone RM4-5) or PE-conjugated anti-CD8 for 30 min and then permeabilized with Perm Fix/Perm Wash (BD Pharmingen), washed with 0.1% sodium azide in PBS, and stained with the following antibodies for 30 min to assess the expression of a panel of Th1/Th17 cytokines: PerCP-conjugated anti-IL-17A (eBioscience; clone: eBio17B7) and APC-conjugated anti-IFN-γ (eBioscience; clone: XMG1.2). Cell acquisition at 50,000 events per sample was performed using a BD FACS Verse (Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde

Pública) flow cytometer, and the acquired data were analyzed using FlowJo (V10) software .

LEC evaluation

Specific-pathogen-free female BALB/c mice (4–8 weeks old) were injected SC in the dorsal region with PBS (saline; 100 µL), CMX (20 µg), $MnFe_2O_4$ NPs (10 µg), or NCMX (SC10) prepared as described above. At 1 or 4 days after inoculation, the mice were euthanized, and the axillary lymph nodes were collected and prepared as single-cell suspensions using 70-µm cell strainers (BD Biosciences); the cells were resuspended with RPMI medium supplemented with 20% fetal calf serum, 0.15% sodium bicarbonate, 1% L-glutamine (200 mM; Sigma-Aldrich), and 1% nonessential amino acids (Sigma-Aldrich). The cells were counted in a Neubauer chamber, and the concentration was adjusted to 1×10^6 cells/mL. For staining, the cells were centrifuged and then stained with FITC-conjugated anti-gp38 (clone: 8.1.1; Novus Biologicals) and PE-Cy7-conjugated anti-CD31 (BD bioscience; clone: 390) for 30 min. Cell acquisition at 100,000 events per sample was performed using a BD FACS Verse system, as previously described. LECs were characterized as $gp38^+CD31^+$, as described by Tamburini et al. (2014).[54]

Intravenous infection with *Mtb*

The *Mtb* H37Rv strain was maintained as previously described (Junqueira-Kipnis et al., 2013).[22] A vial from a constant stock was thawed, and the inoculum was adjusted to a concentration of 10^6 CFU/mL by dilution with PBS containing 0.05% Tween 80. The immunized animals were challenged with 10^5 bacilli. Thirty days after immunization, 100 µL of the inoculum was injected into the tail vein. The inoculum/infective bacterial load was determined by plating the lung homogenates on 7H11 agar supplemented with OADC for one mouse in each group on day one after infection. Thirty days after

infection, the mice were euthanized, and the anterior and mediastinal right-lung lobes were collected, homogenized, and plated on 7H11 agar supplemented with OADC. The bacterial load was determined by counting the CFU after 21 days of incubation at 37°**C**.

Histopathology

To assess the possibility of vaccination inducing organ inflammation or causing side effects, mice were euthanized 21 days after the 2 intranasal vaccinations and after 2 and three subcutaneous vaccinations, and the lungs (Figure 4), heart, kidneys and liver (Supplementary Figure 3) were collected for histology. To evaluate the ability of the generated immune response to protect against the pathogenic effect of infection, mice were euthanized 30 days after being challenged with *Mtb*. For histological evaluation of the lungs, the organs were perfused with 0.05% heparin by injection in the right ventricle of the heart, and the caudal right lobes were collected. The lungs, heart, kidneys and liver were conditioned in histological cassettes and fixed with 10% buffered formaldehyde. Samples were sectioned at a thickness of 5 μm and stained with H&E for analysis by optical microscopy (Axio Scope.A1, Zeiss). Two representative images (4x,10x or 40x) for each group were acquired.

Hemolysis assay

Blood from a healthy patient was collected (5 mL) in a heparinized tube, and red blood cells were separated and washed with PBS. The red blood cell volume was adjusted to 2% at a concentration of $1x10^8$ cells/mL. Concentrations of 10, 50, 100 and 500 μg of $MnFe_2O_4$ NPs, i.e., concentrations up to 50-fold greater than the value used in the vaccine (10 or 50 μg), were prepared for volume/volume addition (100 mL) with the red blood cells. Cells were incubated for 1 h at 37°C and centrifuged (1000 g); then, the absorbance at 550 nm was determined. As a positive control, the erythrocytes were treated with Triton-100X, and as a negative control, PBS was added. The percentage of

red blood cell hemolysis was obtained by the following formula: % hemolysis = 100X [(Sample - AbsPBS) / (AbsTriton - AbsPBS)].

Statistical analysis

The results were tabulated using Excel and Prism software (version 7.0, GraphPad). The differences between groups were assessed with a nonparametric Student's *t*-test or one-way ANOVA followed by Dunn's post hoc test. Significant differences were found among the groups. All three repetitions of the experiments showed similar responses. The size distribution of the NPs was evaluated by the Anderson-Darling normality test.

5. Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Author contributions

LMM performed all the experiments and wrote the draft. APJK conceived the experiments. NZ, AAS and AFB developed and characterized the NPs. MMT and ACC developed the toxicity experiments. AFB, AK and APJK critically revised the manuscript. All authors have read the final version of the manuscript.

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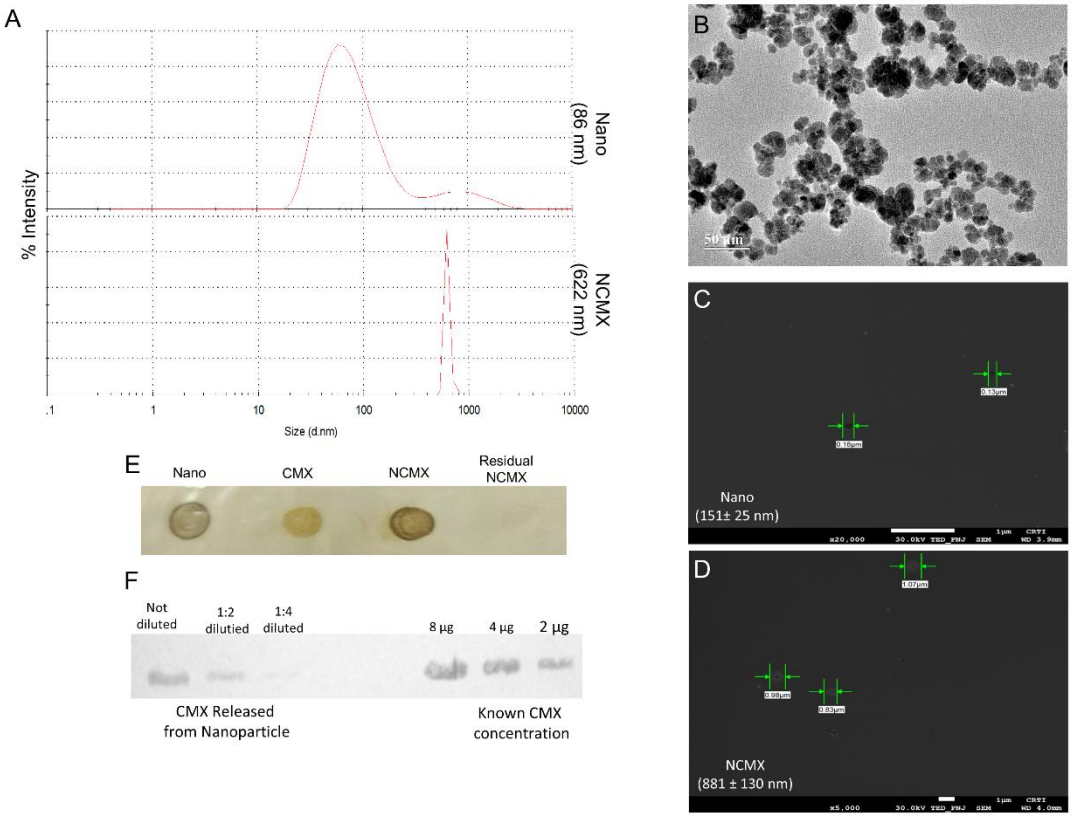
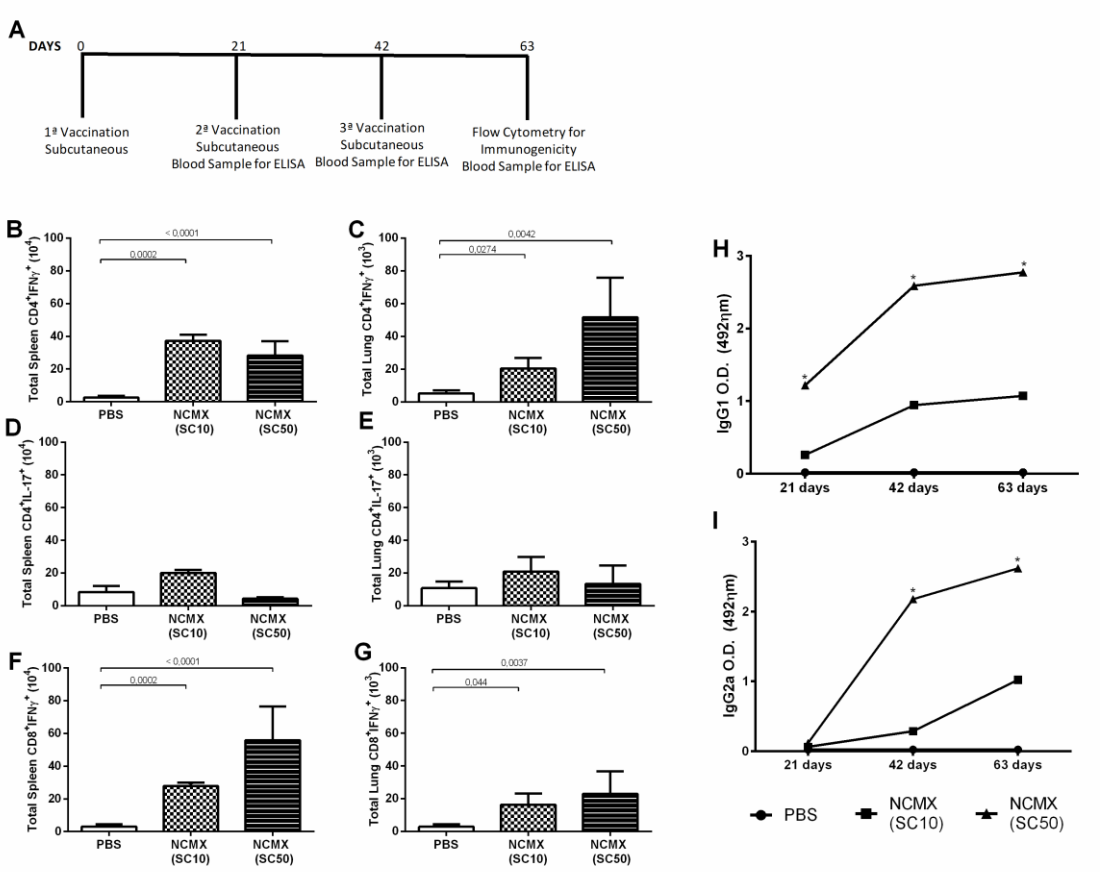
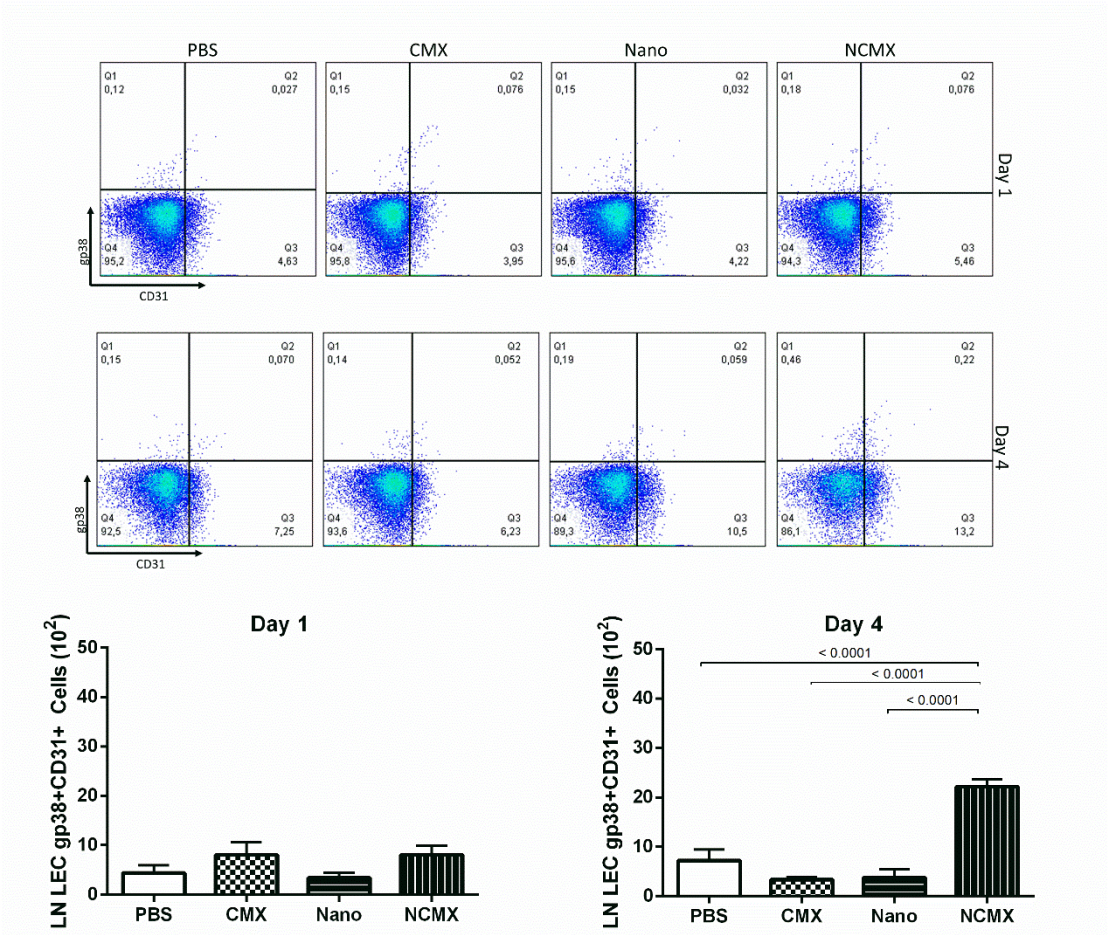


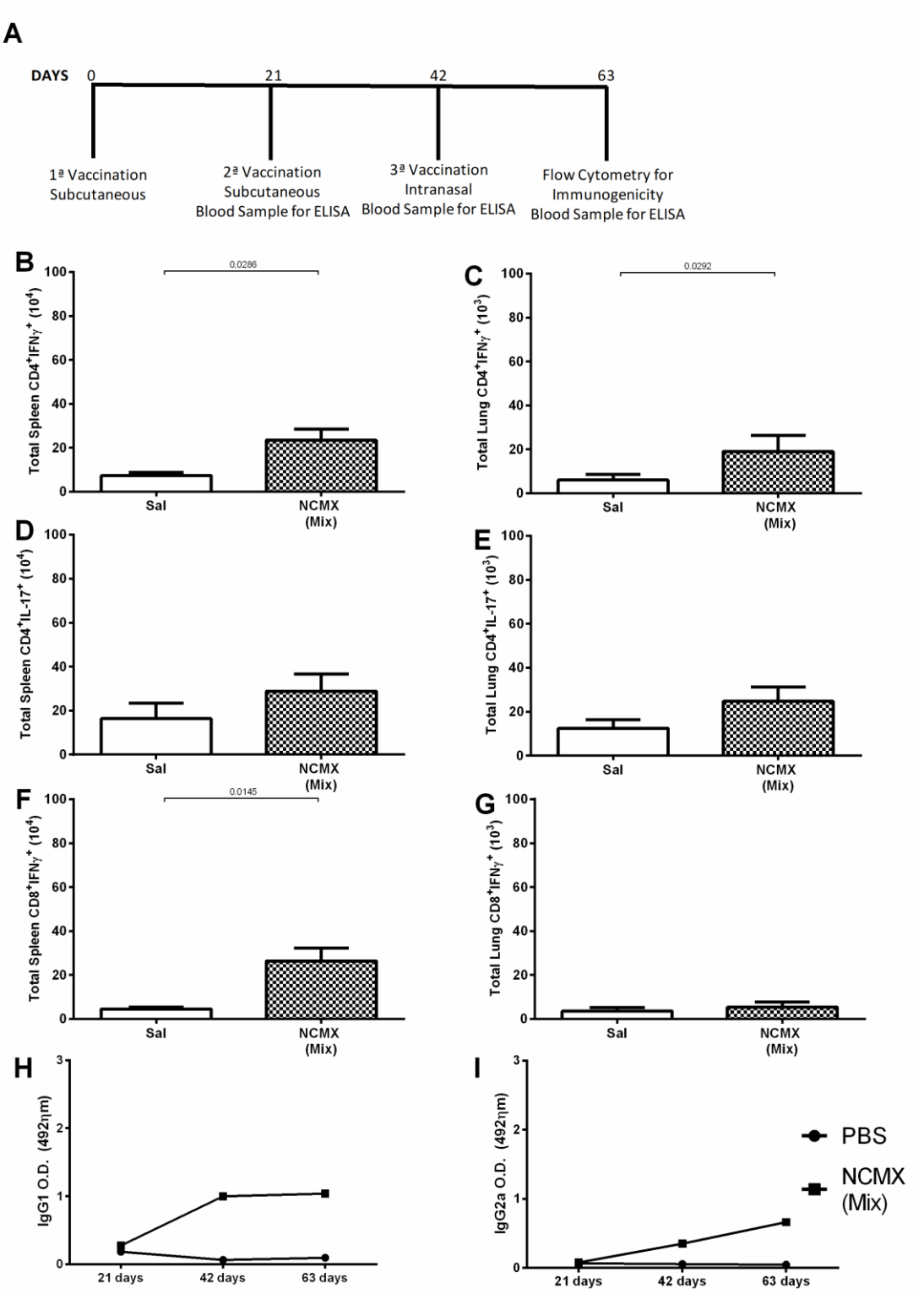
Research Paper - Figure 1. Construction and characterization of $MnFe_2O_4$ NPs coated with CMX. (A) DLS results of citrate-coated $MnFe_2O_4$ NPs (Nano, upper panel) and citrate-coated $MnFe_2O_4$ NPs incubated with CMX (NCMX, lower panel). (B) TEM images of citrate-coated $MnFe_2O_4$ NPs. Characterization of CMX protein corona formation of Nano (C) and NCMX (D). (E) NCMX samples were separated by lateral magnetic separation and utilized for the dot plot. The first dot represents only citrate-coated Nano; the second dot represents only CMX as a positive control; the third dot represents magnetically separated citrate-coated Nano covered with CMX (NCMX); and the last dot is the residual protein in the supernatant after the adsorption of CMX onto the NPs (residual CMX). (F) Quantification of CMX adsorbed onto the NPs. NCMX was incubated with 10% SDS and then centrifuged, and the supernatant left was used for western blotting and comparison with a known concentration of CMX.



Research Paper - Figure 2. Immune response to subcutaneous vaccination is not affected by the NP dose in the formulation. Groups of four mice were immunized SC two times with 100 μL of NCMX, and the third booster was administered via intranasal instillation. Twenty-one days after the last immunization, the spleen and lungs were collected and analyzed by flow cytometry for Th1, Th17, and Tc1 lymphocytes (A).- one days after the last immunization, the spleen and lungs were collected and analyzed by flow cytometry for Th1, Th17, and Tc1 lymphocytes (A). The number of Th1 (CD4+IFN-γ+), Th17 (CD4+IL-17+), and Tc1 (CD8+IFN-γ+) cells in the spleen (B, D and F, respectively) and lungs (C, E and G, respectively) are shown. Differences between the means of the groups were determined by ANOVA and post-hoc test, and p values are shown. Serum samples were collected, and the humoral immune response was evaluated by measuring the levels of IgG1 (H) and IgG2a (I). Differences between the means of the groups were determined by ANOVA and significant differences with $p<0.05$ are shown with an asterisk (*).

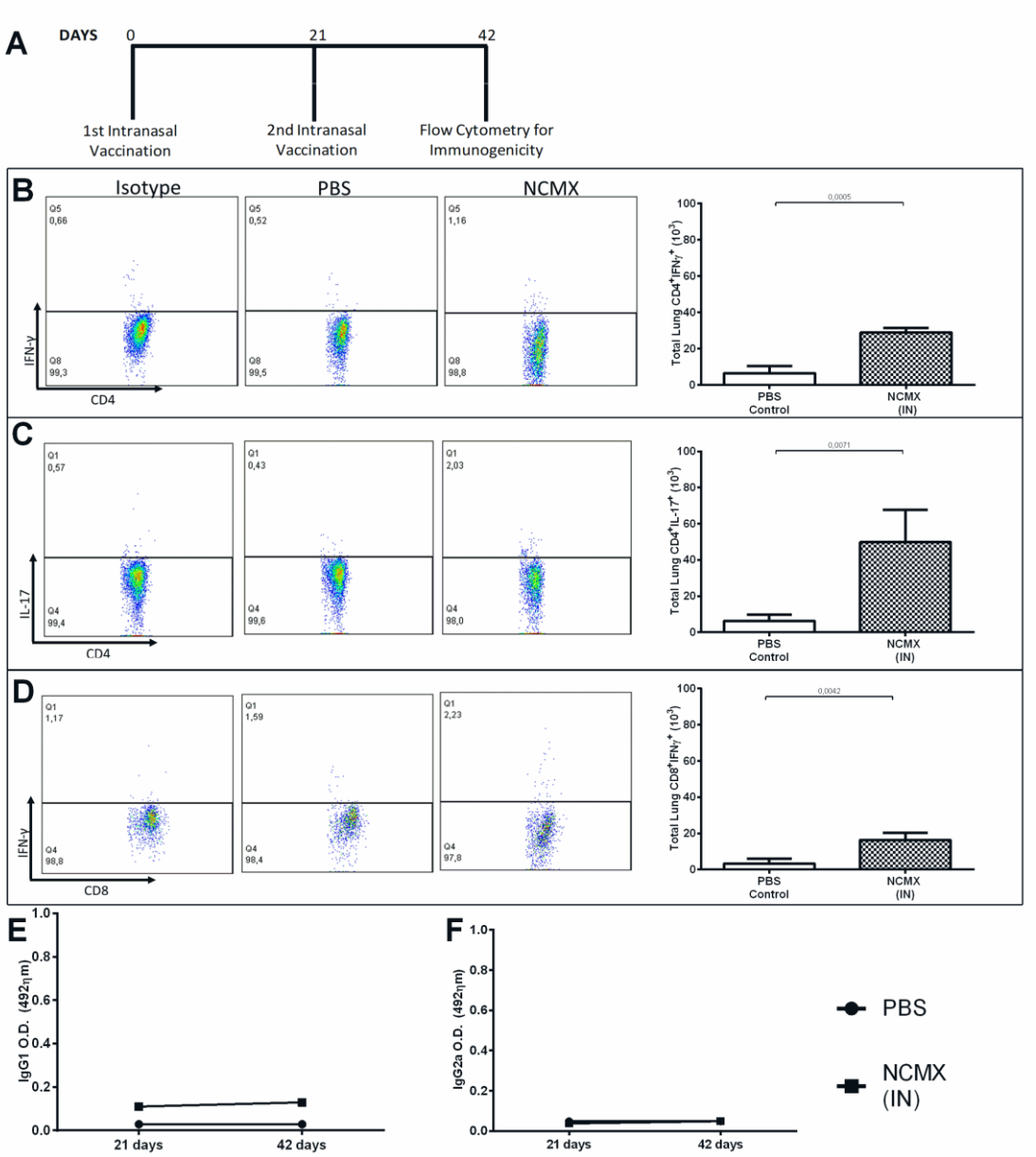


Research Paper - Figure 3. Subcutaneous immunization induces an increase in LECs ($gp38^+CD31^+$) in draining lymph nodes. Representative dot plots for each group are shown above the graphs of lymph node endothelial cell counts evaluated based on CD31, gp38 and CD21/35 staining 1 day (left graph) or 4 days (right graph) after immunization with CMX or Nano only or with the NCMX formulation. Differences among groups were determined by one-way ANOVA, and p values are shown. Significant differences were found among the groups, n=4 mice.

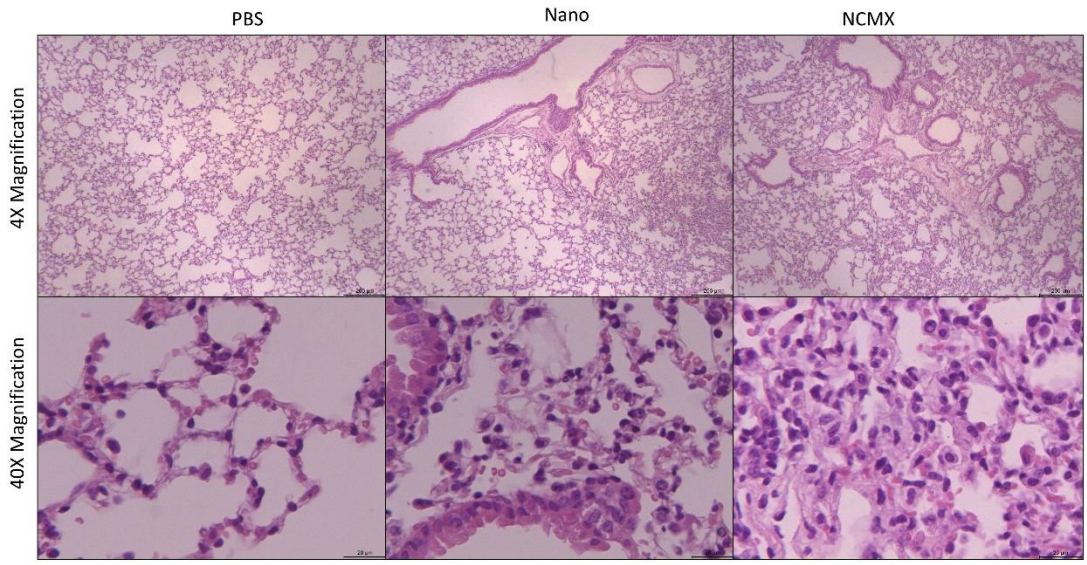


Research Paper - Figure 4. Mixed vaccination induces weak lung Th1 and splenic Th1 and Tc1 responses. Mice were immunized SC two times with 100 µL of NCMX, and the third booster was administered via intranasal instillation. Twenty-one days after the last immunization, the spleen and lungs were collected and analyzed by flow cytometry for Th1, Th17, and Tc1 lymphocytes (A). The number of Th1 ($CD4^+IFN\text{-}\gamma^+$), Th17 ($CD4^+IL\text{-}17^+$), and Tc1 ($CD8^+IFN\text{-}\gamma^+$) cells in the spleen (B, D and F, respectively) and lungs (C, E and G, respectively) are shown. Serum samples were collected, and the humoral immune response was evaluated by measuring the levels of

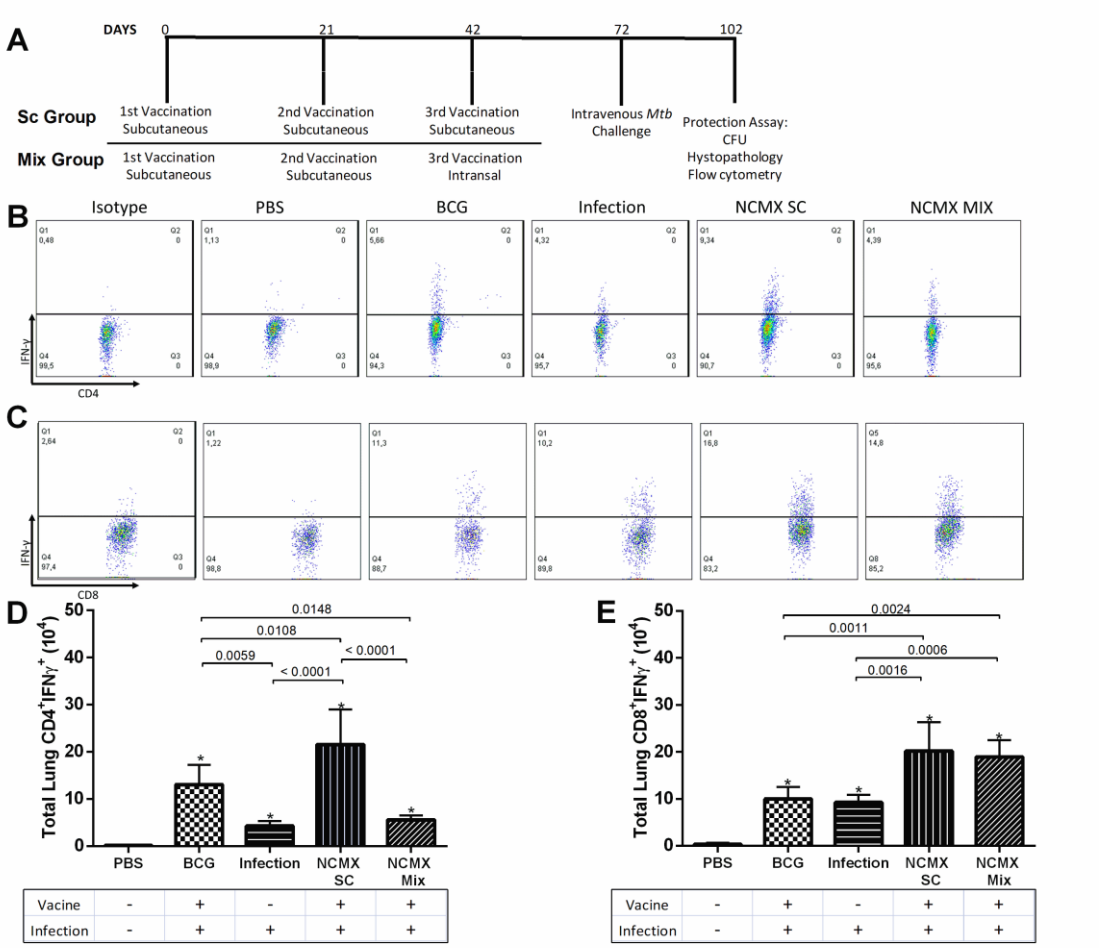
IgG1 (H) and IgG2a (I). Differences between the means of the groups were determined by Student's *t*-test, and p values are shown. Significant differences were found between the groups, n=4.



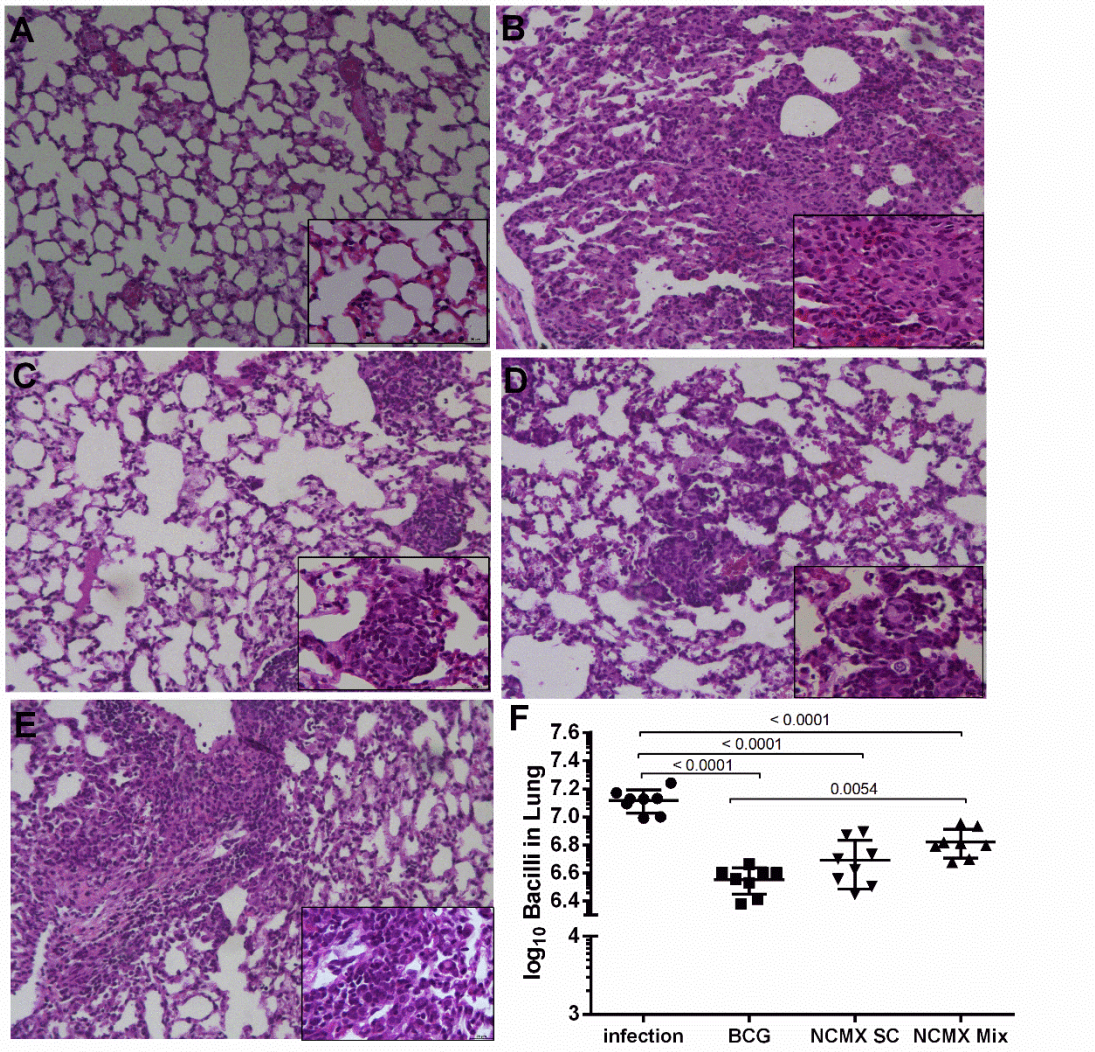
Research Paper - Figure 5. Intranasal vaccination increases the cellular immune response in the mucosa. (A) Mice were vaccinated 2 times at 21-day intervals with NCMX. At 21 days after the last immunization, the lungs were collected and analyzed by flow cytometry for (B) Th1 ($CD4^+IFN\text{-}\gamma^+$), (C) Th17 ($CD4^+IL\text{-}17^+$), and (D) Tc1 ($CD8^+IFN\text{-}\gamma^+$) cells. Mouse serum samples were collected 21 days after each vaccination for evaluation of the elicited humoral immune response by measuring the levels of IgG1 (E) and IgG2a (F). Differences between the means of the groups were determined by Student's *t*-test, and p values are shown. Significant differences were found between the groups, n=4.



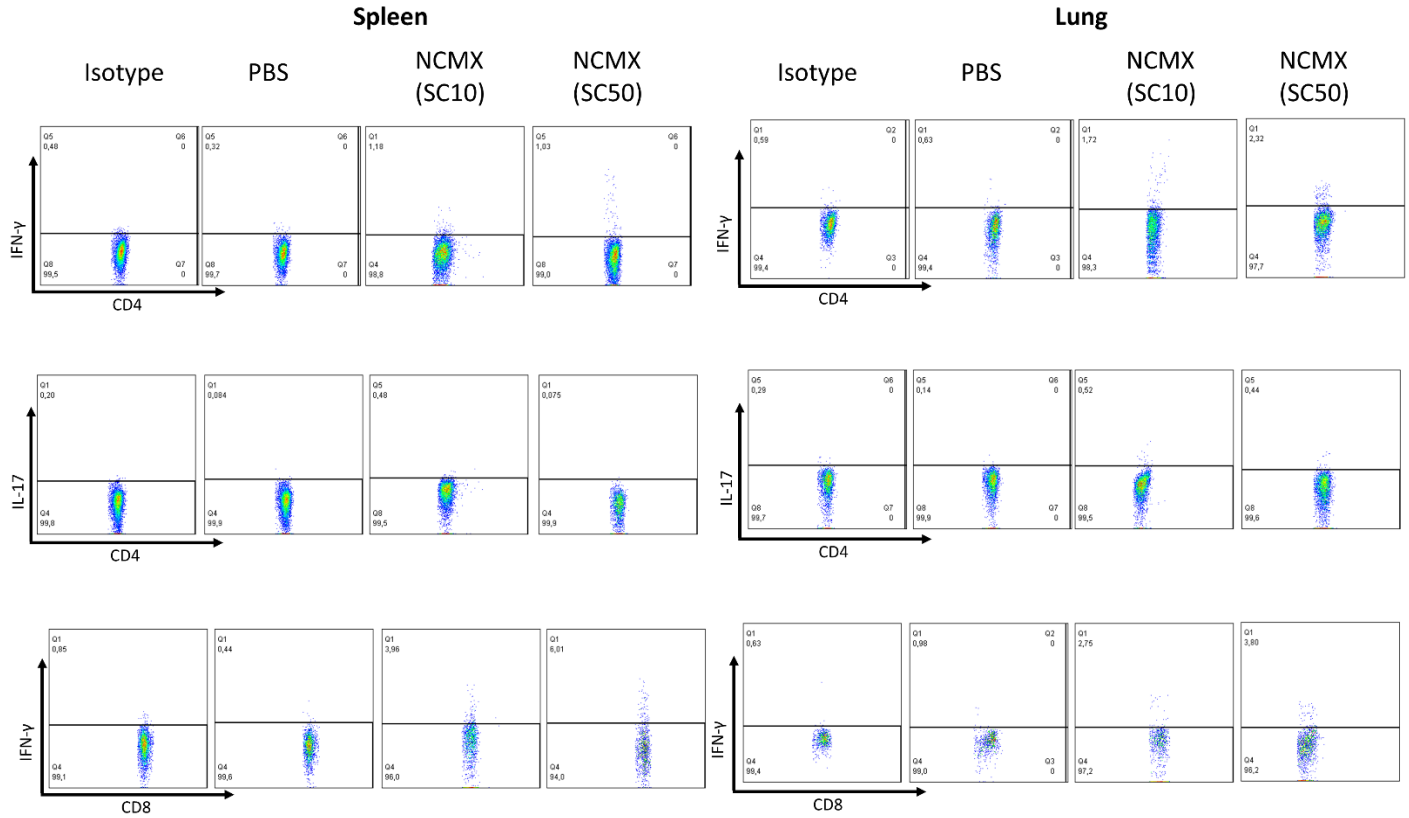
Research Paper - Figure 6. Evaluation of lung injury or tissue changes in intranasally vaccinated mice. Mice were vaccinated 2 times at 21-day intervals with NCMX. At 21 days after the last immunization, the lungs were collected, stained with H&E and analyzed for damage at 4x magnification (upper) and 40x magnification (botton).



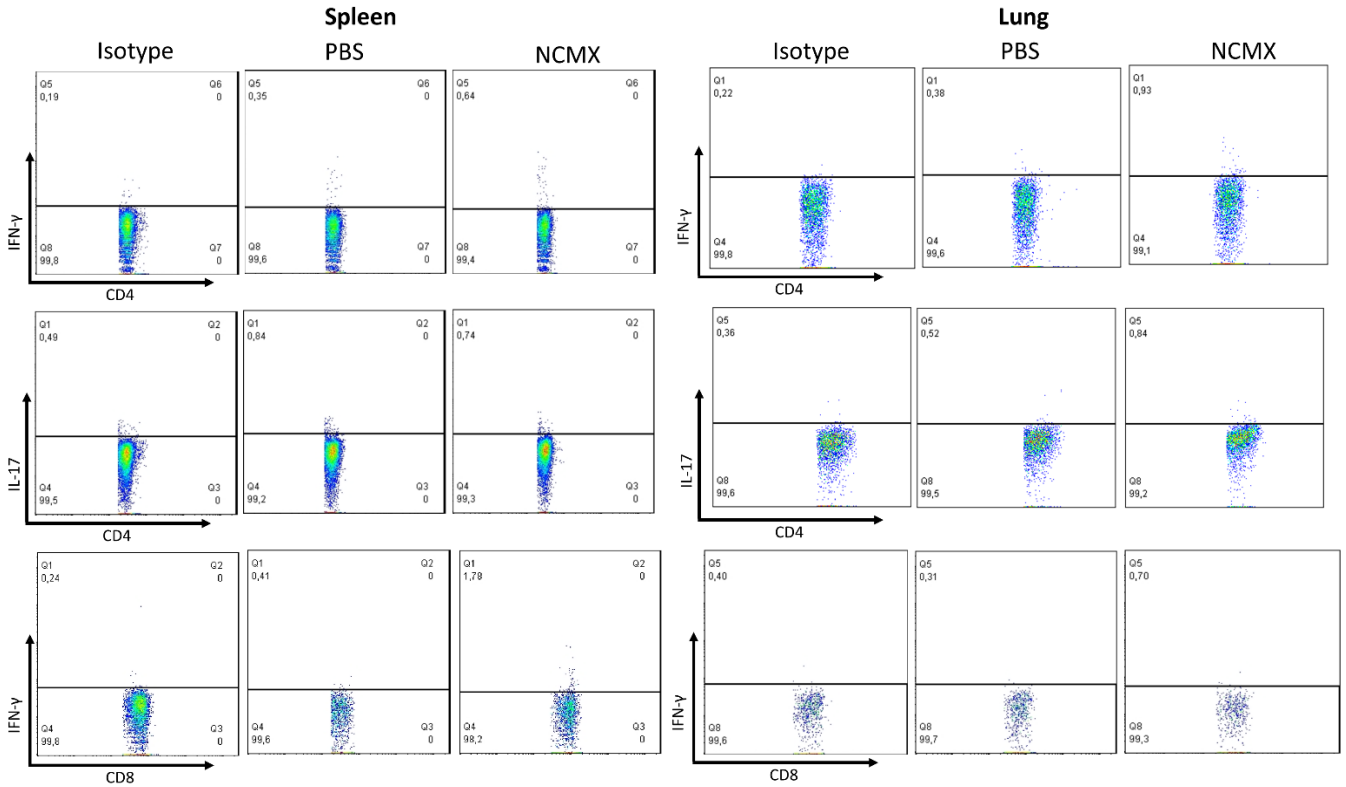
Research Paper - Figure 7. Challenge with *Mtb* after vaccination with NCMX recalls Th1 and Tc1 populations to the lungs. Thirty days after the challenge (A), the lungs were analyzed for the presence of $CD4^+IFN\text{-}\gamma^+$ (B) and $CD8^+IFN\text{-}\gamma^+$ (C) lymphocytes to evaluate the capacity of the immune cells generated by the vaccination to migrate to the lungs and to help protect against infection. The total number of cells was then calculated and is summarized in the graph below the dot plot for $CD4^+IFN\text{-}\gamma^+$ (D) and $CD8^+IFN\text{-}\gamma^+$ (E) cells. Differences among the groups were determined by one-way ANOVA, and p values are shown. Significant differences were found among the groups, n=4.



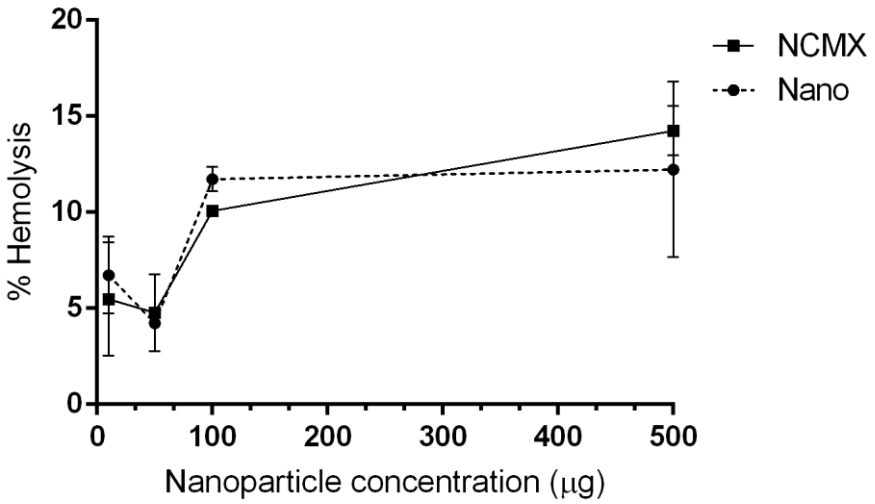
Research Paper - Figure 8. Vaccination with NCMX reduced the histopathological damage and bacterial load in the lungs of *Mtb*-challenged mice. Thirty days after the last vaccination via subcutaneous and mixed administration, mice were challenged with 10^6 CFU per animal. After thirty days, the mice were euthanized, and the lungs were studied to determine the capacity of the vaccination to protect against damage and pathological response (A to E), as well as its capacity to diminish the bacterial load in the lungs (F). Lung samples were also stained with H&E and analyzed for damage at 4x magnification and 40x magnification. Differences among the mean CFU values were determined by one-way ANOVA, and p values are shown. Significant differences were demonstrated among the groups, n=8.



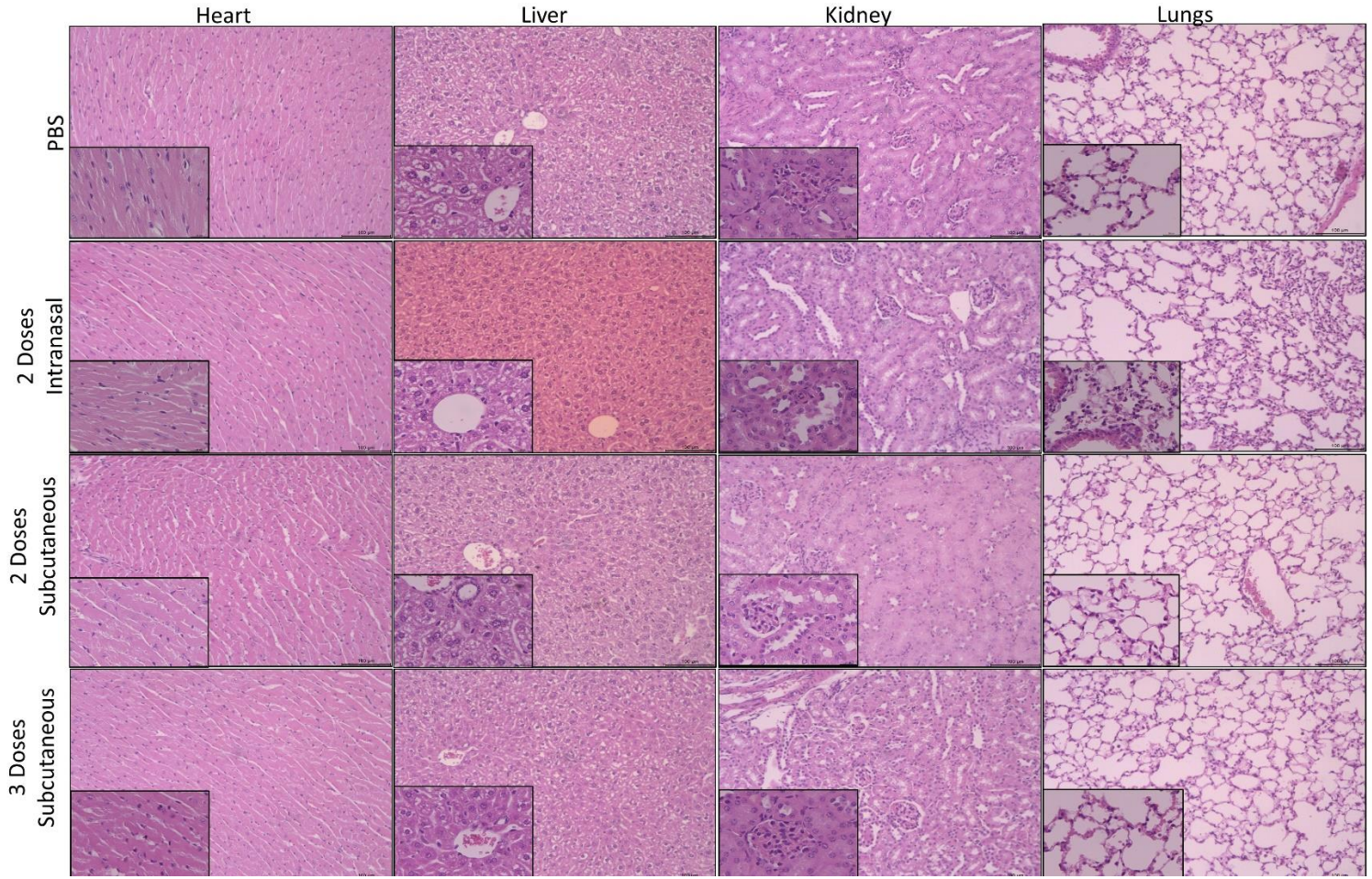
Research Paper - Supplementary Figure 1. Representative flow cytometry quadrant sets to quantify $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}17^+$, and $CD8^+IFN\text{-}\gamma^+$ cells in the lungs and spleen after use of the subcutaneous vaccination strategy. Representative dot plots of spleen $CD4^+$ T cells expressing IFN-γ or IL-17 and $CD8^+$ T cells expressing IFN-γ are shown.



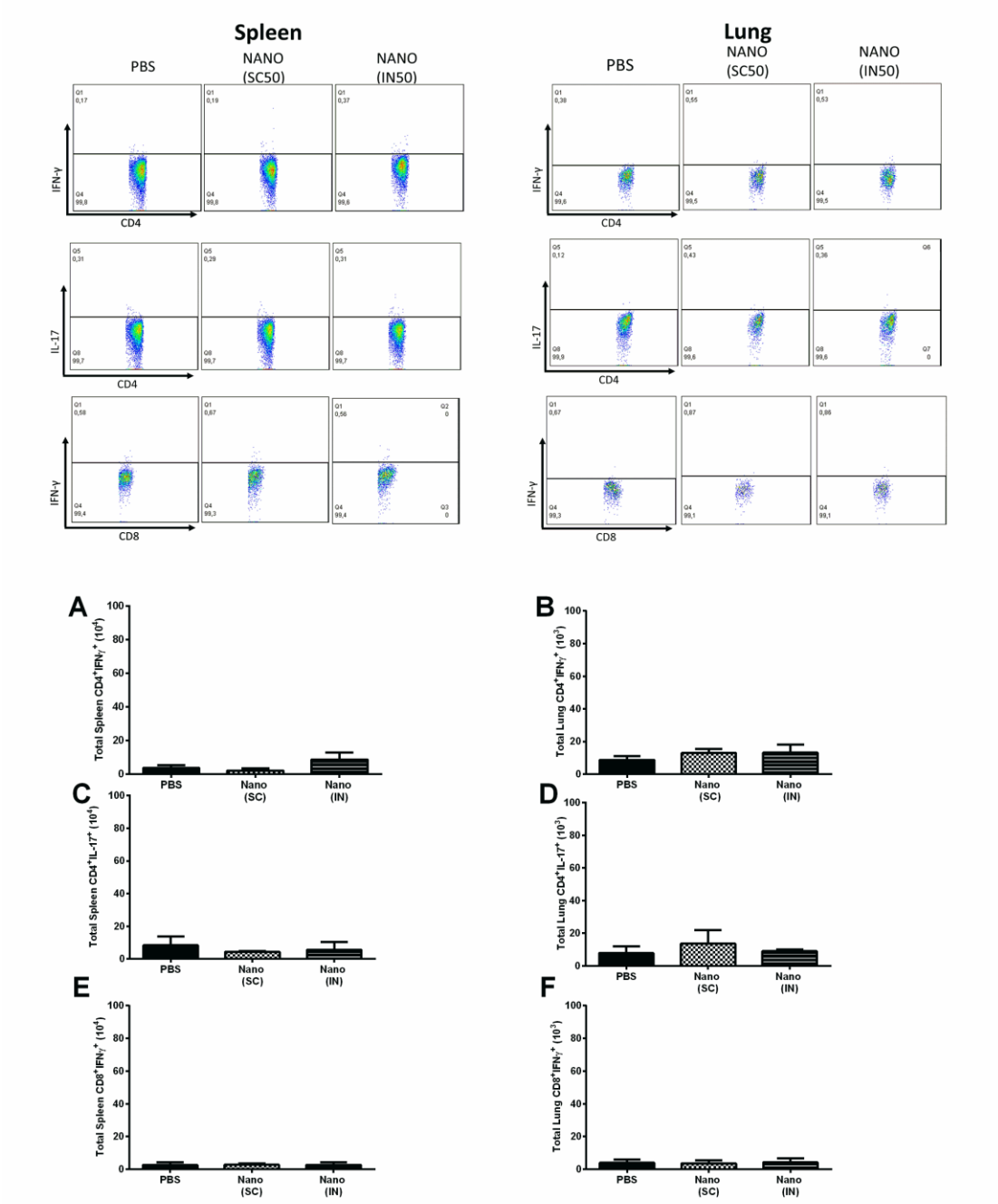
Research Paper - Supplementary Figure 2. Representative flow cytometry quadrant sets to quantify $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}17^+$, $CD8^+IFN\text{-}\gamma^+$ cells in the lungs and spleen after use of the mixed vaccination strategy. Representative dot plots of spleen $CD4^+$ T cells expressing IFN-γ or IL-17 and $CD8^+$ T cells expressing IFN-γ are shown.



Research Paper - Supplementary Figure 3Evaluation of NP-induced hemolysis. Human red blood cells were incubated with the $MnFe_2O_4$ NPs or the NCMX formulation at concentrations ranging from 5, 10, 100 and 500 μg, and the resulting hemolytic activity is summarized in the graph.



Research Paper - Supplementary Figure 4. Evaluation of toxicity and organ damage. Animal kidney (first column), liver (second column), heart (third column) and lung samples were stained with H&E and analyzed for damage and inflammation at 10x magnification and 40x magnification(insert).



Research Paper - Supplementary Figure 5. Nano vaccination does not induce cellular immune response. (A) Mice (N = 4) were vaccinated 3 times at 21-day intervals with 50 µg of Nano (without CMX). Twenty-one days after the last immunization, the spleen and lungs were collected and analyzed by flow cytometry for Th1, Th17, and Tc1 lymphocytes (A). The number of Th1 (CD4+IFN-γ+), Th17 (CD4+IL-17+), and Tc1 (CD8+IFN-γ+) cells in the spleen (A, C and E, respectively) and lungs (B, D and F, respectively) are shown. No differences between the means of the groups were determined by ANOVA.

6. CONCLUSÕES

O desenvolvimento da uma formulação de nanovacina utilizando uma nanopartícula de ferrita de manganês recoberta com a proteína de fusão CMX foi capaz de gerar resposta imune celular do tipo Th1, Th17 e Tc1, contudo essa geração foi dependente da via utilizada. A via também influenciou no aparecimento de processo inflamatório pulmonar quando a vacina foi utilizada por via intranasal em mais de 1 dose, o que foi também um requisito para geração de resposta Th17 no pulmão dos animais vacinados. A vacina foi indutora de resposta do tipo Tc1 em todas as vias de inoculação e estratégias utilizadas e juntamente com essa resposta indutora de resposta Th1. Essas duas respostas foram capazes de juntas proteger contra a infecção, contudo a proteção foi aquém da vacina BCG o que provavelmente se dá pela ineficiência da vacina desenvolvida nesse trabalho em gerar resposta Th17. A formulação vacinal também foi bem tolerada, não apresentando qualquer sinal de efeito colateral no coração, rim e fígado dos animais vacinados. Apenas início de um processo inflamatório pulmonar quando utilizada em duas doses ou mais por via intranasal.

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8. ANEXOS

8.1 Parecer do Comitê de Ética de Uso de Animais (CEUA – UFG)

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

UFG

Goiânia, 23 de março de 2015.

PARECER CONSUBSTANCIADO REFERENTE AO ATENDIMENTO DE PENDÊNCIA DO PROTOCOLO Nº. 103/14

I. IDENTIFICAÇÃO:

1. **Título do projeto**: Desenvolvimento de sistema adjuvante a partir da junção de nanopartícula magnética e peptídeo antimicrobiano derivado de venenos de animais do cerrado.
2. **Pesquisador Responsável:** Lázaro Moreira Marques Neto IPTSP/UFG
3. **Unidade/Órgão do pesquisador:** IPTSP/UFG
4. **Pesquisadores Participantes:** André Kipnis, Ana Paula Junqueira-Kipnis, Monalisa Martins Trentini, Viviane Lopes Rocha, Adeliane Castro da Costa, Bruno de Paula Fonseca, Thaiz K Santos.
5. **Unidade onde será realizado:** IPTSP/UFG
6. **Data de apresentação do protocolo a CEUA: 08/12/2014**
7. **Data de Atendimento das Pendências: 26/02/2015**

II - Parecer da CEUA:

Informamos que a *Comissão de Ética no Uso de Animais/CEUA* da Universidade Federal de Goiás, após análise das adequações solicitadas **Aprovou** o projeto acima referido e o mesmo foi considerado em acordo com os princípios éticos vigentes.
Reiteramos a importância deste Parecer Consubstanciado, e lembramos que o(a) pesquisador(a) responsável deverá encaminhar à CEUA-PRPI-UFG o Relatório Final baseado na conclusão do estudo e na incidência de publicações decorrentes deste, de acordo com o disposto na Lei nº. 11.794 de 08/10/2008, e Resolução Normativa nº. 01, de 09/07/2010 do Conselho Nacional de Controle de Experimentação Animal-CONCEA. O prazo para entrega do Relatório é de até 30 dias após o encerramento da pesquisa, prevista para conclusão em **setembro de 2019**.

III - Data da reunião: 23 de março de 2015

Dra. Renata Mazaro e Costa
Coordenadora da CEUA/PRPI/UFG

Comissão de Ética no Uso de Animais/CEUA
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