



Characterization of the immune response to a TLR4-based adjuvant in murine models

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ÉCOLE DOCTORALE N°569
Innovation thérapeutique : du fondamental à l'appliqué
Discipline: Microbiologie et Thérapeutiques anti-infectieuses

Par
Natasha Dubois Cauwelaert

Caractérisation de la réponse immune induite par un adjuvant comprenant
un agoniste du TLR4 dans des modèles murins

*Characterization of the immune response to a TLR4-based adjuvant in murine
models*

Thèse soutenue à Châtenay-Malabry le 6 Juin 2016

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ABBREVIATIONS

APC: Antigen Presenting Cell

BCG: Bacillus Calmette-Guérin

CpG: Cytosine Phosphate Guanine

DAMP: Damage-Associated Molecular Pattern

DC: Dendritic Cell

GLA: Glucopyranosyl Lipid A

IDRI: Infectious Disease Research Institute

INH: Isoniazid

KLRG1: Receptor Killer-cell lectin like Receptor G1

LN: Lymph node

LPS: Lipopolysaccharide

MDR-TB: multi-drug resistant TB

MPEC: Memory Precursor Effector Cells

MPL: Monophosphorylated Lipid A

Mtb: *Mycobacterium tuberculosis*

MVA85A: Modified Vaccinia Ankara 85A

MyD88: Myeloid Differentiation factor 88

NF: Nuclear Factor

NHP: Non-Human Primates

NK: Natural Killer

PAMP: Pathogen Associated Molecular Patterns

PD-1: Programmed cell Death protein 1

PRR: Pattern Recognition Receptors

QS21: *Quillaja saponaria*

RIF: Rifampicin

SCM ϕ : Subcapsular Macrophages

SE: Stable Emulsion

TB: Tuberculosis

TRIF: TIR domain-containing adaptor protein inducing interferon- β

μ MT-/-: B cell deficient mice

WHO: World Health Organization

WT: Wild-Type

RÉSUMÉ EN FRANÇAIS

Introduction

La Tuberculose (TB) est l'une des maladies les plus anciennes de l'humanité. Des signes de TB ont été observés dans des ossements appartenant à des hommes du Néolithique et dans la colonne vertébrale de momies égyptiennes, attestant des ravages causés par ce mal dès 5000 avant JC. Ce n'est qu'en 1882 que Robert Koch identifia *Mycobacterium tuberculosis* (*Mtb*) comme agent causal de la TB. La mortalité associée à la TB a commencé à diminuer avec l'amélioration du niveau de vie (logement, nutrition et revenus) au début du 20^{ème} siècle, bien avant l'avènement des antibiotiques antituberculeux. En 1921, avec le développement du premier vaccin contre la TB, le BCG, et la découverte d'un certain nombre d'antibiotiques efficaces tels que la streptomycine en 1944, l'isoniazide en 1952 et la rifampicine en 1963, l'espoir d'une potentielle éradication de la TB avant la fin du XX^{ème} siècle a émergé. Malheureusement, avec 9,6 millions de nouveaux cas par an et 1,5 millions de décès, représentant la principale cause de décès par maladie infectieuse dans le monde devant le VIH en 2014, il faut reconnaître que l'éradication de la TB est loin d'être atteinte.

D'importants efforts sont investis dans le développement d'un nouveau vaccin contre la TB. La modélisation mathématique prédit qu'un vaccin efficace à 60% résulterait en une baisse de 80% de l'incidence de la maladie d'ici 2050. Aujourd'hui, 16 candidats vaccins sont dans diverses phases d'essais cliniques avec comme objectif principal soit, de booster la vaccination BCG, soit de pouvoir être utilisé de façon autonome chez les individus immunodéprimés pour lesquels une vaccination BCG est déconseillée. En outre, certains vaccins ont également pour but de raccourcir le traitement et/ou le taux de rechute post-thérapeutique lorsqu'ils sont utilisés comme compléments immunothérapeutiques au traitement antibiotique antituberculeux actuel.

Dans le cadre de cette thèse nous nous intéresserons à l'un de ces vaccins candidats en particulier : ID93/GLA-SE. Ce candidat a été développé par l'Infectious Disease Research Institute (IDRI, Seattle, WA, USA) et est aujourd'hui en essai clinique de phase IIa dans le but d'évaluer son innocuité et son immunogénicité chez des patients adultes atteints de tuberculose pulmonaire et ayant suivi un traitement antibiotique efficace. ID93/GLA-SE est un vaccin sous-unitaire associant la protéine ID93, qui résulte de la fusion de quatre protéines de *Mtb*, avec un agoniste du TLR4, le Glucopyranosyl lipide A (GLA) formulé dans une émulsion stable squalène huile dans l'eau (SE).

L'adjuvant GLA-SE, favorise une réponse CD4 T_H1 importante, considérée comme centrale dans la protection contre la TB, et la production d'IgG2 par les lymphocytes B contre l'antigène utilisé. Cet adjuvant est aujourd'hui évalué dans plusieurs essais cliniques en association avec d'autres antigènes qu'ID93, y compris LEISHF3 contre la Leishmaniose, sm14 contre la Schistosomiase, P27A contre le Paludisme et les particules pseudo-virales H5 contre le Virus *Influenza*. Néanmoins, les mécanismes d'action de cet adjuvant sont encore peu connus.

Le principal objectif de cette thèse est de mieux comprendre l'effet adjuvant médié par GLA-SE. On sait aujourd'hui que le système immunitaire inné joue un rôle primordial dans le déclenchement et l'élaboration de la réponse adaptative aux vaccins. Nous essayons donc de caractériser les principaux acteurs (cellules immunitaires, cytokines, facteurs de transcription, voies de signalisation ...) de la réponse innée à GLA-SE qui coordonnent la réponse adaptative subséquente, à savoir la réponse CD4 T_H1 et la production d'IgG2c par les lymphocytes B. Trois hypothèses de recherche principales sont investiguées :

1. Des facteurs spécifiques à la réponse T_H1, à savoir le facteur de transcription T-bet et la cytokine IL-12, sont essentiels à la réponse adaptative à GLA-SE. De plus, les IFN de type I, importants dans l'initiation de la réponse à GLA-SE, sont également nécessaires pour l'induction des réponses T_H1.
2. Les macrophages sous-capsulaires du ganglion lymphatique drainant interagissent directement avec GLA-SE et sont importants dans la réponse adaptative ultérieure.
3. La fonction de présentation d'antigène des lymphocytes B a un rôle non redondant dans l'initiation de la réponse à GLA-SE.

Un objectif secondaire est d'établir un modèle murin de rechute de TB après traitement et d'évaluer l'utilisation d'ID93/GLA-SE en tant que vaccin immuno-thérapeutique dans ce modèle. Nous postulons qu'ID93/GLA-SE réduira le taux de rechute dans un modèle murin de traitement antibiotique sous-optimal.

Les résultats de cette thèse sont discutés dans trois publications, plus une partie complémentaire non soumise à publication; tous les quatre sont résumés ci-dessous :

Première publication : L'adjuvant vaccin GLA-SE agoniste du TLR4 requiert des mécanismes d'action canoniques et atypiques pour l'induction de la réponse T_H1.

Dubois-Cauwelaert N *et al.* PLoS One. 2016; 11(1): e0146372.

Le glucopyranosyl lipide A en émulsion squalène-eau (GLA-SE) est un adjuvant qui favorise une réponse immunitaire lymphocytaire CD4 T_H1 importante et la production d'IgG2 par les lymphocytes B. Cette immunité renforcée est suffisante pour assurer une protection contre de nombreuses maladies, y compris la Tuberculose et la Leishmaniose. Néanmoins le mode d'action de GLA-SE est encore mal compris. Afin de mieux caractériser l'action de cet adjuvant, il est important de comprendre comment les différentes cytokines et facteurs de transcription contribuent à l'initiation de la réponse immunitaire. Dans cette étude, nous avons évalué la contribution de T-bet, de l'IL-12 et de la signalisation par le récepteur aux interférons de type I (IFN α R1) dans ces réponses en utilisant des souris T-bet^{-/-} et IL12^{-/-} et un anticorps monoclonal bloquant anti-IFN α R1.

Conformément aux résultats de précédentes études sur différents adjuvants, nous avons démontré que l'induction par GLA-SE d'une réponse T_H1 et la production d'IgG2 est dépendante de T-bet, un facteur de transcription clé pour la production d'IFN γ et la différenciation des lymphocytes T en T_H1. De plus, un déficit en IL-12, une cytokine canonique dans l'induction de la réponse T_H1 a également inhibé le développement de lymphocytes CD4 T_H1 ; la production d'IgG2 n'a en revanche pas été altérée. Finalement, nous démontrons que la réponse immunitaire innée vis-à-vis de GLA-SE, y compris la rapide production d'IFN γ par les lymphocytes CD8 mémoires et les cellules NK et l'expression de CD69 par les cellules des ganglions lymphatiques drainants, est conditionnée par les interférons de type I produits rapidement après l'immunisation. Ces cytokines contribuent par conséquent également à la réponse adaptative vis-à-vis de GLA-SE.

D'après ces résultats, nous proposons un modèle dans lequel GLA-SE induit la production d'IFN α à travers l'expression de CD69, pour piéger dans les ganglions lymphatiques les cellules nécessaires à la réponse à GLA-SE. La production d'IFN α permettrait aussi la production, , d'IFN γ par les lymphocytes CD8 mémoires et les cellules NK pendant la phase innée de la réponse immunitaire. En parallèle, GLA induit la production d'IL-12 qui, en synergie avec IFN α , promeut l'expression de T-bet et l'engagement des lymphocytes CD4 dans la voie T_H1.

La compréhension du mécanisme par lequel les adjuvants incitent la réponse immunitaire revêt une importance cruciale dans le développement de vaccins. Les résultats obtenus lors de cette étude suggèrent que l'induction précoce des cytokines IFN de type I et II pourraient être les signatures de la réponse innée optimale à GLA-SE et prédire la réponse T_H1 résultante. Des investigations supplémentaires seront nécessaires pour déterminer si cette production précoce d'IFN de type I peut être utilisée dans une stratégie de sélection dans le développement de nouveaux vaccins ou comme signature d'adjuvant dans les essais cliniques chez l'homme.

Seconde publication : L'IL-18 et les macrophages sous-capsulaires du ganglion lymphatique sont essentiels pour l'induction des réponses lymphocytes B avec l'adjuvant GLA-SE agoniste du TLR4.

Desbien AL, Dubois Cauwelaert N *et al.* (*Soumise à publication*)

La compréhension des événements cellulaires et moléculaires qui relient les réponses immunitaires innées et adaptatives est cruciale dans la conception de nouveaux adjuvants. GLA-SE augmente les réponses cellulaires et humorales aux antigènes vaccinaux. L'induction de lymphocytes B et la production subséquente d'anticorps sont des facteurs clés dans le développement de vaccins efficaces. Dans le contexte actuel de nouvelles pandémies, de libération intentionnelle d'agents biologiques dans un but terroriste et de vaccins « à la demande » pour les voyageurs, l'initiation d'une réponse humorale rapide avec des approches vaccinales pragmatiques est hautement souhaitable.

Dans cette étude nous démontrons que peu de temps après l'injection, de l'adjuvant GLA-SE, et comparativement à l'alum, l'émulsion à base de squalène (SE) sans GLA ou de GLA sans SE, induit significativement plus de lymphocytes B spécifiques à l'antigène, des titres d'anticorps supérieurs, un plus grand nombre de lymphocytes T CD4 folliculaires (T_{FH}) et une réponse T_H1 plus importante. GLA-SE augmente la différenciation des lymphocytes B spécifiques à l'antigène cible en lymphocytes B du centre germinatif, en lymphocytes B précurseurs de lymphocytes mémoires et en pré-plasmablastes qui sécréteront rapidement des anticorps. De plus, nous montrons qu'après immunisation, les macrophages médullaires sous-capsulaires CD169+ SIGNR1+ sont les premières cellules immunitaires du ganglion lymphatique à capturer GLA-SE et sont critiques pour la réponse innée, y compris la production rapide d'IL-18 induite par GLA-SE.

La déplétion des macrophages sous-capsulaires (dont les macrophages médullaires font partie) ou l'abrogation de la signalisation à travers le récepteur de IL-18, altèrent de façon importante la production de lymphocytes B spécifiques à l'antigène et d'anticorps induite par GLA-SE. La déplétion des macrophages sous-capsulaires réduit aussi considérablement la réponse T_H1 mais pas la réponse T_{FH} . Ainsi l'adjuvant GLA-SE agit en interagissant avec des macrophages sous-capsulaires qui produisent IL-18, permettant alors l'induction de l'expansion et de la différenciation des lymphocytes B, de la sécrétion d'anticorps et l'induction d'une réponse T_H1 . Cependant, la réponse T_{FH} semble être, elle, indépendante de l'action des macrophages sous-capsulaires.

Troisième publication : La présentation d'antigène par les lymphocytes B guide la programmation médiée par TLR4 des lymphocytes CD4 mémoires.

Dubois Cauwelaert N *et al.* (Soumise à publication)

Dans cette étude, nous avons cherché à déterminer le rôle potentiel des lymphocytes B, au-delà de leur capacité de production d'anticorps, dans la réponse immunitaire vis-à-vis de GLA-SE et nous nous sommes particulièrement intéressés à l'induction de la réponse CD4 T_H1 . Pour cela nous avons utilisé des souris déficientes en lymphocytes B ($\mu MT^{-/-}$).

En utilisant l'antigène ID93 en association avec GLA-SE, nous avons montré que la réponse T_H1 mémoire (6 semaines après la dernière immunisation) induite par GLA-SE et mesurée par la production de cytokines T_H1 par les lymphocytes CD4, est fortement altérée dans les souris $\mu MT^{-/-}$. Cependant, une semaine après immunisation, cette même réponse T_H1 semblent peu ou pas affectée chez ces mêmes souris. Ce phénomène avait été préalablement observé suite à l'infection de souris $\mu MT^{-/-}$ par le virus de la chorioméningite lymphocytaire (VCML).

Toutefois, même si les lymphocytes CD4 induits peu de temps après immunisation en absence de lymphocytes B semblent, en effet, être fonctionnellement similaires à ceux induits dans des souris non-déficientes, en particulier dans leur capacité à produire des cytokines T_H1 , ils sont néanmoins phénotypiquement différents. En effet, les souris $\mu MT^{-/-}$ sont déficientes dans leur capacité à produire des lymphocytes CD4 effecteurs précurseurs de cellules mémoires (CEPM), préalablement définis dans d'autres études comme étant soit $PD1^{+}/KLRG1^{-}$, soit $Ly6C^{lo}/T-bet^{lo}$.

Ces résultats démontrent que les lymphocytes B sont nécessaires pour l'initiation d'une réponse T_H1 qui pourra être maintenue dans le temps.

Par la suite, en transférant les lymphocytes CD4 générés une semaine après immunisation dans les souris $\mu MT^{-/-}$ dans des souris non-déficientes, nous avons pu démontrer que ces cellules perdaient leur fonctionnalité (capacité à produire des cytokines T_H1) avec le temps. Ces données confirment nos résultats précédents, démontrant que les lymphocytes B sont nécessaires à la génération de CD4 CEPM après immunisation. D'autres expériences seront nécessaires pour déterminer si les lymphocytes B sont également nécessaires pour le maintien de la réponse T_H1 .

Finalement, en transférant aux souris $\mu MT^{-/-}$ des lymphocytes B sauvages ou n'exprimant pas le CMHII et donc incapables de présenter des antigènes, nous avons montré que cette présentation d'antigènes par les lymphocytes B était nécessaire pour leur rôle dans la génération de CD4 CEPM. Il faut noter que ce point est encore aujourd'hui fortement débattu dans la littérature.

En conclusion, ces résultats soulignent l'importance d'un système immunitaire à multiples facettes travaillant de concert pour la génération d'une immunité à médiation cellulaire. Collectivement, les progrès récents dans la compréhension du rôle des lymphocytes B, dans la réponse humorale mais aussi dans la réponse cellulaire, auront un impact important sur le développement de vaccins contre plusieurs pathogènes et en particulier ceux nécessitant une réponse T_H1 . De plus, ils pourront bénéficier aux médecins qui utilisent des thérapies qui éliminent les lymphocytes B chez les patients souffrant de troubles liés aux lymphocytes B.

ID93/GLA-SE, un vaccin thérapeutique ?

Le traitement contre la Tuberculose est long et complexe, la thérapie de première ligne implique 6 à 9 mois de traitement avec une association d'antibiotiques (isoniazide, rifampicine, pyrazinamide et éthambutol). De plus, l'émergence de résistances à ces antibiotiques, principalement causée par la difficulté qu'ont les patients à suivre une si longue thérapie, a d'autant plus complexifié ce traitement. En effet l'apparition de souches *de Mtb* résistantes aux antibiotiques de première ligne nécessite des traitements de durée plus longue (pouvant aller jusqu'à deux ans) avec des associations d'antibiotiques de seconde et troisième lignes. Ainsi le non-respect du traitement antibiotique dans son intégralité entraîne l'augmentation du risque d'échec du traitement, résultant en une récurrence de la maladie. Dans ce contexte, le

développement de nouveaux schémas thérapeutiques permettant de réduire la durée du traitement antituberculeux (et donc d'améliorer l'observance) et/ou permettant de diminuer le risque de rechute après traitement est nécessaire. Il a été démontré qu'ID93/GLA-SE pourrait potentiellement être utilisé en association avec le traitement antibiotique classique pour améliorer son efficacité et diminuer la durée du traitement. Outre la réduction de la durée du traitement, il serait intéressant de déterminer si l'utilisation de ID93/GLA-SE pourrait également permettre de diminuer les taux de rechute post-traitement.

L'une des limites de l'étude de rechute post-traitement dans le modèle murin est la durée de ce genre d'expérience qui inclue 3 à 6 mois de thérapie, ce qui est extrêmement coûteux en temps et en argent.

Nous avons donc cherché à utiliser un modèle murin de thérapie et rechute qui soit moins complexe. Pour cela nous avons adapté un modèle développé dans un autre laboratoire qui nécessite une thérapie beaucoup moins longue. Grâce à ce modèle, nous avons pu démontrer qu'ID93/GLA-SE réduisait les taux de rechutes dans trois différentes lignées de souris et augmentait le taux de survie des souris SWR, une souche de souris extrêmement susceptible à la Tuberculose. Ces résultats montrent la possibilité d'utiliser ID93/GLA-SE en tant que vaccin thérapeutique et en association avec le traitement antibiotique actuel pour améliorer son efficacité. Une meilleure compréhension des capacités thérapeutiques d'ID93/GLA-SE est cruciale puisque ce vaccin fait actuellement l'objet d'un essai clinique de phase IIa ayant pour but d'évaluer son innocuité et immunogénicité lorsqu'il est administré aux patients atteint de tuberculose pulmonaire, après traitement antibiotique, en vue d'une future phase IIb qui permettrait d'évaluer une potentielle amélioration du taux de rechute chez ces patients.

Conclusion

En conclusion, l'ensemble de ce travail nous a permis de mieux définir les caractéristiques du mécanisme d'action de GLA-SE et d'améliorer la compréhension des différents acteurs et des étapes nécessaires à la réponse adaptative induite par GLA-SE. Cependant, des travaux complémentaires seront nécessaires pour pleinement comprendre le mécanisme d'action et pour caractériser tous les intervenants de ces interactions complexes induites par l'association de GLA, agoniste de TLR4 et de l'émulsion squalène-eau.

INTRODUCTION

Tuberculosis (TB) is one of humanity's oldest diseases. Evidence of TB has been recovered from Neolithic man bones and the spine of Egyptian mummies attesting of the devastation caused by this disease as early as 5000 BC (1). It was not, although, until 1882 that Robert Koch identified *Mycobacterium tuberculosis* (*Mtb*) as the causative agent (2). The death toll from TB began to decrease as living standards (housing, nutrition, and income) improved early in the 20th century, well before the advent of antituberculosis drugs. With the development of BCG, the first vaccines against TB, in 1921 and the discovery of a number of efficient antibiotics (1944-1965), the hope of TB eradication by the end of the 20th century emerged. Unfortunately with 9.6 million new TB cases and 1.5 million TB deaths every year, even surpassing HIV as the leading cause of death by infectious disease worldwide in 2014 (3), we must acknowledge that TB eradication is far from being reached.

Major efforts are focused in the development of a new vaccine against TB as mathematical modeling of the impact that could have a hypothetical new vaccine against TB with 60% efficacy predicts an 80% drop in incidence by 2050 (4). There are 17 vaccine candidates in various clinical trial phases for which main objective is to either boost the current BCG vaccine or be used as a stand-alone vaccine in immuno-compromised individuals for which BCG immunization is not recommended. In addition some vaccines could also be used as immunotherapeutic adjuncts to the standard TB antibiotic therapy with the aim of shortening the therapy and/or reduced relapse rates after treatment completion.

One of those vaccines, ID93/GLA-SE, was developed by the Infectious Disease Research Institute (IDRI) and is today entering a phase II clinical trial to evaluate its safety and immunogenicity when administered to adult pulmonary TB patients, following successful completion of TB treatment with confirmed bacteriologic cure, in preparation for a future Phase 2b prevention of TB recurrence trial in the same population (5). ID93/GLA-SE is a sub-unit vaccine composed of a fusion of four *Mtb* proteins formulated with GLA-SE a Toll-like receptor 4 (TLR4) agonist Glucopyranosyl Lipid A (GLA) in a squalene oil-in-water emulsion (SE) (6, 7).

GLA-SE, IDRI's leading candidate adjuvant, promotes strong T_{H1} CD4 T cells, which is thought to be central for protection against TB, and IgG2-skewed B cell responses to protein vaccine antigens. Beyond ID93/GLA-SE, GLA-SE can be found in several vaccine candidates today in clinical trial against a variety of diseases, including LEISH-F3 GLA-SE against the *Leishmania* parasite (8), sm14/GLA-SE against Shistosomiasis (9), P27A/GLA-SE against Malaria (10), and

H5 virus-like-particle/GLA-SE against Flu (11). Nevertheless still very little is known about this adjuvant its mechanism of actions.

The main objective of this thesis was to broaden the understanding of GLA-SE mediated adjuvanticity and characterize the main actors i.e. immune cells, cytokines, transcription factors and pathways, of the innate response to GLA-SE that trigger and shape the strong adaptative response linked to this adjuvant. Our three main hypotheses for this work were:

1. Canonical T_H1 shaping factors, *i.e.* the transcription factor T-bet and IL-12, will be essential to the adaptive response to GLA-SE. Type I IFN, previously shown to be important for the innate response to GLA-SE (12) would be also important for the subsequent adaptive response.
2. Lymph node (LN) sub-capsular macrophages will take-up GLA-SE and be critical for its adjuvanticity.
3. B cells antigen-presenting function will have a non-redundant role in the shaping to the T_H1 response to GLA-SE.

An additional work pursued during this thesis was to establish a mouse model of TB relapse and evaluate the use of ID93/GLA-SE as immunotherapeutic vaccine to be used in combination with chemotherapy. We hypothesize that ID93/GLA-SE would reduce relapse rates in a suboptimal drug treatment model.

The first section of this thesis is a literature review on TB, the search of correlates of protection against it and development of candidate vaccines; we will pay particular attention to sub-unit vaccines in general and ID93/GLA-SE in particular. In a second section we will present the results of the different studies conducted during this thesis. Finally we will do a general discussion of that work, outline the various resultant perspectives that are currently being pursued and close with a general conclusion on the work that was performed.

SECTION I: LITERATURE REVIEW

I. TUBERCULOSIS

A. HISTORY

TB has had various names throughout history, “consumption”, “phthisis”, “Pott’s disease” and “white plague”; all refer to the same scourge that has been known to mankind since ancient times. Bones recovered from different part of the world showed evidence of TB among Neolithic men and in the spine of mummies from ancient Egypt. These data indicate that the humans suffered from TB as early as 5000 BC (1).

It reached epidemic proportions in Europe and North America during the 18th and 19th centuries earning the appellation of “Captain Among these Men of Death”. The death toll from TB began to decrease as living standards (housing, nutrition, and income) improved early in the 20th century, well before the advent of antituberculosis drugs.

Different forms of TB were thought to be different diseases - pulmonary consumption for example was used to label pulmonary TB, scrofula was the term used to describe TB of the LNs and Lupus vulgaris described TB of the skin. At the beginning of the 19th century Laennec advanced the idea of a single cause for the different forms of TB. He did not, however, realize that the condition was infectious (13). It was not until 1865 that Villemin convincingly demonstrated the infectious nature of TB (2). Later, in 1882, Robert Koch, in his famous presentation “*Die Aetiologie der Tuberculose*”, identified the tubercle bacillus *Mycobacterium tuberculosis* (*Mtb*) as the causative agent of TB (2).

B. EPIDEMIOLOGY

TB is a major global health problem. It even surpassed HIV as the leading cause of death by a single infectious agent worldwide in 2014 (3). A third of the world’s population is thought to be infected with *Mtb* and in 2014, there were an estimated 9.6 million new TB cases and 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people (3). The number of incident TB cases relative to population size (the incidence rate) varies widely among countries. TB is a disease of poverty that is inextricably associated with overcrowding and under-nutrition and affects mostly young adult in their most productive years. Thus, more than 80% of all TB cases are found in 22 low- and middle-income countries (14). The highest prevalence of active TBper capita is found in Africa with over 300 new cases per 100,000

population, driven primarily by the devastating effects of the HIV epidemic. The absolute number of cases is highest in South-East Asia, with India and China having the greatest burden of disease globally (Figure 1) (3). In the United States of America and most Western European countries, the majority of cases occur in foreign-born residents and recent immigrants from countries in which TB is endemic (15, 16).

Left untreated, each person with active TB disease will infect on average between 10 and 15 people every year and this continues the TB transmission. Overall a small proportion (5-15%) of the estimated 2- 3 billion people infected with *Mtb* will develop TB disease during their lifetime. However, the probability of developing TB is much higher among HIV infected people. The risk for developing TB disease is also higher in persons with diabetes, other chronic debilitating diseases leading to immune-deficiency, poor living conditions or tobacco smokers (14).

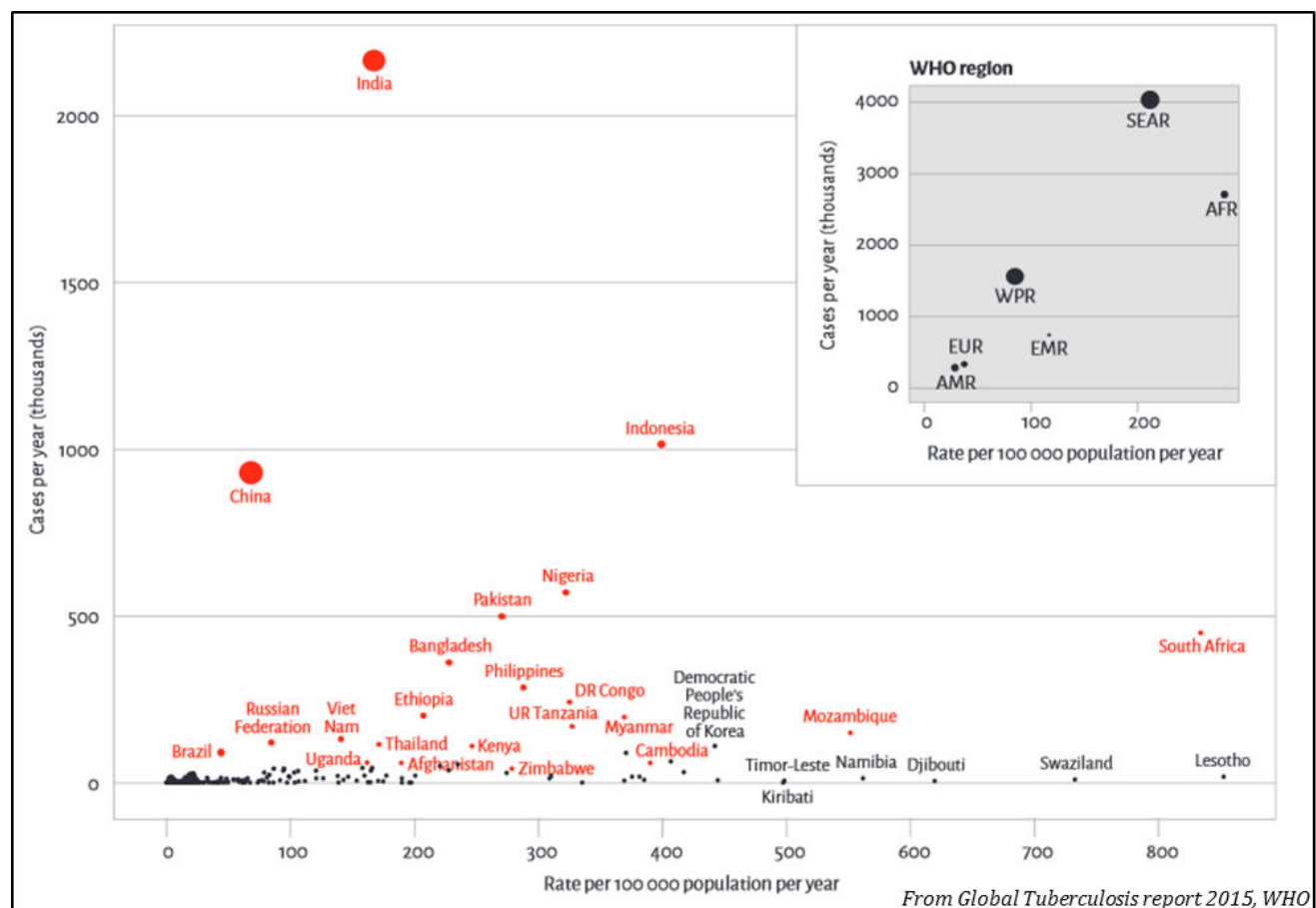


Figure 1. Global distribution of estimated TB incidence by rate and absolute number, 2014. The size of each bubble is proportional to the size of the country's population. High-burden countries are shown in red. WHO regions: African (AFR), Americas (AMR), Eastern Mediterranean (EMR), European (EUR), South-East Asia (SEAR) and Western Pacific (WPR).

C. Pathogenesis

TB is transmitted through the air by aerosol.. The most important source of infection is a patient with pulmonary TB and who is coughing. Coughing produces tiny infectious droplets nuclei that are spread into the air and can remain suspended in it for long periods. The World Health Organization (WHO) divides TB infection into primary and post primary infection (17).

1. PRIMARY INFECTION

Primary infection occurs in people who have not had any previous exposure to tubercle bacilli. Droplets nuclei, which are inhaled into the lungs, are so small that they avoid the defense of the bronchi and penetrate into the terminal alveoli of the lungs, where multiplication and infection begin. Lymphatics drain the bacilli to the hilar LNs. The immune response develops about 4-6 weeks after the primary infection. The size of the infecting dose and the strength of the immune response determine the outcome. In most cases (90% of non-HIV infected people), the immune response stops the multiplication of bacilli but a few latent bacilli may persist. Infected individuals will not have any clinical symptoms nor be contagious, even though the bacteria persist in latent state in the infected organ (18). A positive tuberculin skin test would be the only evidence of latent infection. If the immune response is not strong enough to prevent multiplication of bacilli, disease will occur within a few months (17).

2. POST-PRIMARY TUBERCULOSIS

Post-primary TB occurs after a latent period of months or years following primary infection. It may occur by reactivation where the dormant tubercle bacilli acquired during a primary infection starts to multiply generally in response to a trigger such as weakening of the immune system by HIV infection or ageing. Post primary TB can also be caused by reinfection with a new strain in a person who already had a latent primary infection (17).

3. RECURRENT TUBERCULOSIS

Recurrent TB has been defined as a serial episode of active TB disease occurring after treatment success. Recurrent TB can be categorized as relapse of disease from the original infecting strain or reinfection with a new strain of *Mtb* based on genotyping of the isolates (19). Relapse rates depend on treatment efficacy, program quality, and the patients' compliance with therapy.

The rate of disease due to reinfection, on the other hand, will be determined mainly by the level of TB exposure and transmission in the population (20, 21). The technique of restriction fragment-length polymorphism analysis now allows us to type *Mtb* strains and consequently distinguish between reinfection and relapse as the cause of recurrence. Using this technique it has been shown that in developing countries a high proportion of recurrences are due to reinfections which is consistent with the high incidence of TB in these populations (19, 22, 23).

Recurrence of TB after treatment is a serious problem. On a patient level, recurrent TB requires another round of treatment with a regimen that, in many parts of the world, is more toxic, takes longer to complete, and may amplify drug resistance (24). On a public health level it may account for 10-30% of all cases within some weaker TB control programs, particularly those that do not use at least 6 months of rifampicin (RIF) treatment (20), and contributes to ongoing transmission of infections (25).

D. HOST IMMUNE RESPONSE TO TUBERCULOSIS INFECTION

1. INNATE RESPONSE

Mtb is inhaled through carrier droplets and the innate response develops upon encounter of the microbe and the alveolar macrophages in the lower airways. The establishment of a successful infection depends on this initial encounter; the infection may remain locally limited within the engulfing cells of the innate immune system, or will continue to spread, causing the individual to become a clinical active TB patient. *Mtb* is an intracellular bacterium, and although it can infect different cell types, alveolar macrophages are its favorite niche. The initial stages of infection are characterized by innate immune responses that involve the recruitment of inflammatory cells to the lungs (26). The principal effectors of innate immunity are neutrophils, tissue macrophages (derived from blood-borne monocytes), and natural killer (NK) cells. Neutrophils are generally the first recruited cells to arrive at the infection site and are known to play an important role against TB. Rather than direct bacterial killing, they are believed to play an important role in facilitating the adaptive immune response through cytokine and chemokine signaling (27).

Blood monocytes are less abundant than neutrophils in circulating blood, but are a critical part of the innate immune response to TB. They are recruited through chemokine signals produced by infected alveolar macrophages, and migrate rapidly across the blood vessels to the site of

infection. Within the tissue, they differentiate into macrophages with the ability to ingest and kill the bacteria. The interaction between macrophages and T cells (and in particular, the activation of macrophages by IFN- γ secreted by T cells) is considered central in the elimination of *Mtb* (28, 29). Although alveolar macrophages are thought to be an effective barrier to contain pathogens, *Mtb* has evolved various mechanisms to evade the host immune response and survive in these cells. Several *Mtb* survival mechanisms have been described, including: (i) phagosome-lysosome fusion inhibition by interfering with lipid-mediated signaling, by producing the host-like signaling kinase PknG and by hijacking the calcineurin pathway (reviewed in (30)), (ii) *Mtb* acidification reduction of the *Mtb*-containing phagosome by selectively excluding the proton-ATPase from the phagosome (31), and (iii) protection against nitric oxide through the rearrangement of the actin cytoskeleton which might lead to a reduction in the local concentration of nitric oxide (32) and through the mycobacterial proteasome machinery which provides protection against killing (33).

NK cells are large granular lymphocytes found in circulating blood; their *in vivo* role in controlling *Mtb* is not yet well understood. They have been shown to lyse *Mtb*-infected macrophages *in vitro* and to facilitate an adaptive immune response by producing IFN γ and stimulating macrophages to produce inflammatory cytokines (34).

2. ADAPTIVE RESPONSE

In most cases innate immunity is not sufficient to control *Mtb*. As antigen concentration increases, the adaptive response is activated and it is generally accepted that the long-term outcome of the primary infection is determined by the efficiency of its mobilization.

The induction of an adaptive response occurs after the dissemination of *Mtb* to draining LNs. In the LNs, presentation of bacterial antigens by dendritic cells (DC) leads to T cells priming. This process is quite slow; studies in various animal models suggest that priming of T cells does not occur until 12-21 days post-infection (35). At this time point the infection is already well established consequently, the inflammatory site where the acquired response will be has already been initiated and modulated by the bacterium. This delay is due to two main components. First, the delayed transport of *Mtb* from the lungs to the LN caused by the retention of *Mtb* within the alveolar macrophages which are not a migratory population. It is not until around 8 days post infection that *Mtb*, that has been replicating within the alveolar macrophages, will escape and be

taken up by phagocytes recruited to the infection site (neutrophils, DCs, inflammatory monocytes) (36). Then, even after its transport to the LN *Mtb* further slows the initiation of the adaptive immunity by promoting the induction of regulatory T cells that act to restrict T cell effector priming (37). The resulting delay represents a critical bottleneck to *Mtb*'s control, and possibly to its eradication by adaptive immunity (Figure 2) (38, 39). Once primed Ag-specific T cells will expand and differentiate from naïve T cells into effector T cells which then migrate to the infected lung and, in combination with other leucocytes, stimulate the formation of granulomas (40).

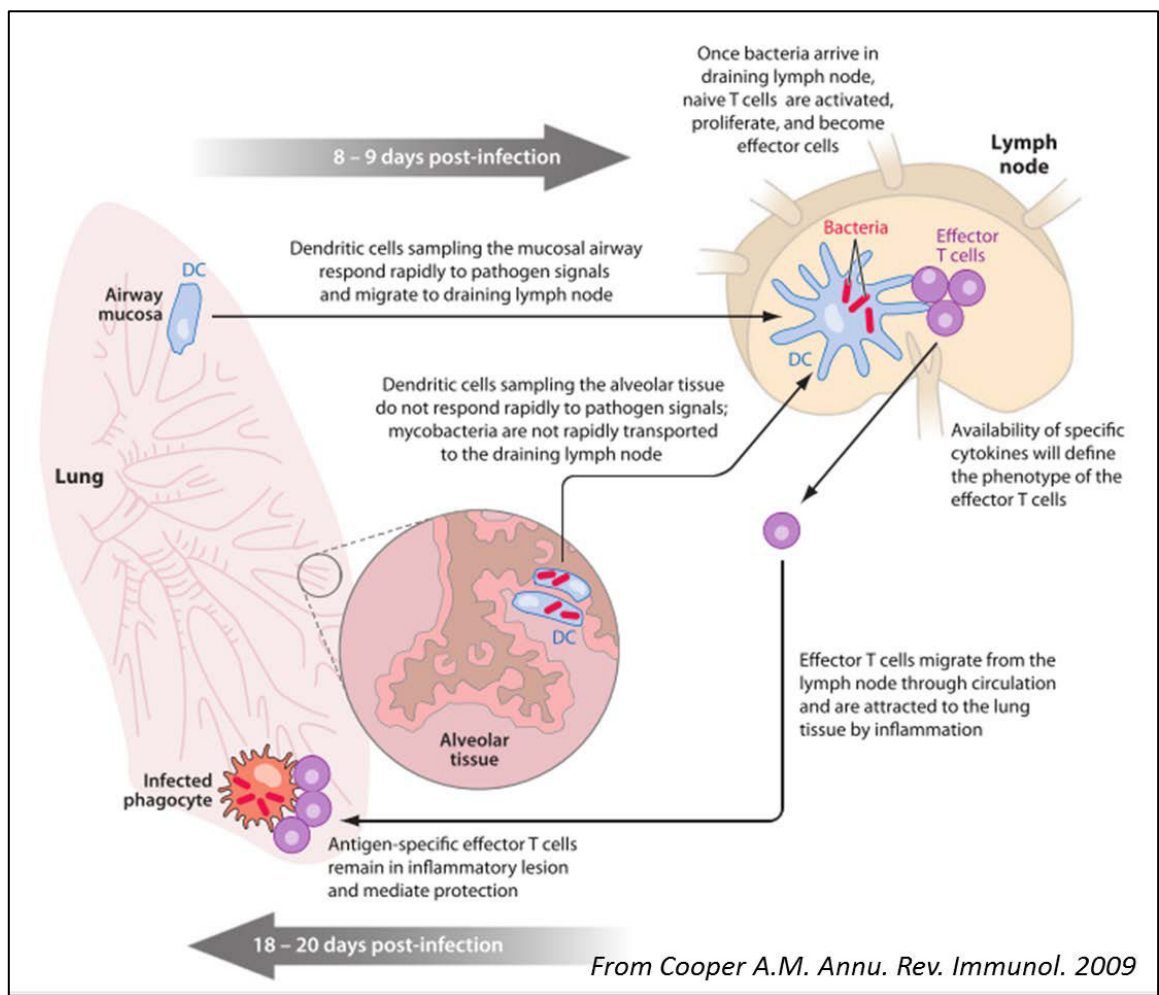


Figure 2. Delayed adaptive response to TB. Low-dose aerosol infection, which approximates the natural delivery route for induction of TB, results in low numbers of *Mtb* (red) being deposited in the lower airways and the alveolar tissue. Bacteria do not disseminate from the lung until 9 days post infection, when they can be detected in the draining lymph nodes. This dissemination coincides with the first activation of naïve T cells (purple). Activation of naïve T cells occurs in the presence of live bacteria, and effector cells develop with expected kinetics. The effector cell phenotype will depend on the availability of specific cytokines. These effector cells migrate to the lung in response to inflammation and mediate protection by activating infected phagocytes (pale red). The response takes 18–20 days to reach an effective level and thereby to stop bacterial growth (38).

3. THE TUBERCULOUS GRANULOMA

Granulomas are organized structures that contain macrophages, lymphocytes and fibroblasts. Within the granuloma, macrophages are activated, for example by CD4⁺ T cells secreted IFN γ , which is thought to restrict the dispersal and replication of *Mtb* (40). The granuloma is the hallmark of TB, and functions as a niche in which *Mtb* can grow and persist and as an immunologic microenvironment in which immune cells interact to control and prevent dissemination of infection.

Granulomas are observed in active, latent and recurrent TB, thus its mere formation is insufficient for control of infection, and rather the granuloma must be functioning properly. Indeed, the granuloma must be carefully balanced in terms of immune responses to provide sufficient immune cell activation to inhibit the growth of *Mtb*, yet modulate the inflammation to prevent pathology (40). Many different chemokines are involved in the granuloma formation. Some are produced by the epithelial cells of the respiratory tract, and others are produced by the immune cells themselves. In particular, the chemokines binding to the CCR2 receptor (CCL2/MCP-1, CCL12, and CCL13) are important for the early recruitment of macrophages to chronic inflammatory sites to initiate granuloma formation (41). CCL19 and, possibly, CCL21 are involved in the recruitment and priming of IFN γ -producing T cells. CXCL13 is involved in B-cell recruitment and the formation of follicular structures (42, 43). Although in the first instance the granuloma acts to constrain the infection, some bacilli can actually survive inside these structures for a long time in a nonreplicating hypometabolic state, as an adaptation to the unfavorable milieu. This altered physiologic state, termed latent TB infection, can endure for the lifetime of the infected individual, but for some reasons, which are still unclear, the bacilli will reactivate in 10% of the latently infected individuals, escape the granuloma and spread throughout the body, thus giving rise to clinical disease, and are finally disseminated throughout the environment (43).

E. TREATMENT AND MULTI-DRUG-RESISTANCE

Before the advent of chemotherapy, TB was one of the major causes of death in both Western and also several non-Western countries (44). Studies from the pre-chemotherapy era found that

about 70% of people with sputum smear-positive pulmonary TB died within 10 years, and that this figure was 20% among culture-positive but smear-negative cases of pulmonary TB (patients with a lower bacterial load) (44).

Nowadays, standard drug regimen for effective treatment of active TB involves a combination therapy with first- and/or second-line drugs for 6-8 months. The requirement for combination therapy is a central tenet of TB treatment mainly to avoid the high probability of developing drug resistance. Recommended first line anti-TB drugs includes RIF, isoniazid (INH), pyrazinamide (PZA) and ethambutol for 2 months followed by RIF plus INH for 4 months. Treatment success rates of 85% or more for new cases are regularly reported to WHO by its Member States (3).

Antibiotic resistance is an increasing problem in TB and the WHO estimates that each year there are half a million cases of multi-drug resistant TB (MDR-TB) (2), defined as a strain of *Mtb* that is resistant to at least INH and RIF (the two most powerful anti-TB drugs) (45). Of even greater concern are “totally drug-resistant TB” strains, which are resistant to all first- and second-line drugs. Treatment for MDR-TB is longer, and requires more expensive and more toxic drugs (combinations of second- and third-line antibiotics added to the regimen including capreomycin, ethionamide and streptomycin). For most patients with MDR-TB, the current regimens recommended by WHO last 20 months, giving the *Mtb* pathogen time to adapt to the drug regimen (46). The development of new drugs is currently the leading solution to the increasing incidence of resistance to the drugs currently employed for TB therapy. Several new drugs are in development but it is not clear when they will receive regulatory approval for their use in new drug regimens. Moreover, since even drug sensitive TB is treated with combination therapy, it seems naïve to assume drug resistant strains will be effectively treated with a single new drug, and there is no reason to believe that resistance will not eventually develop to new antibiotics. In addition to the need for new antibiotics to treat drug resistant isolates, there is an urgent need for treatment regimens which can shorten therapy for *Mtb* (45). Options for improved treatment require new strategies that take advantage of developments in vaccine design and drug delivery that have occurred in the last 50 years, including novel combination regimens in which oral or inhaled agents can be taken for shorter periods of time. To this end, there are today a small number of immunotherapeutic vaccines that are under development for use against *Mtb* (47).

II. VACCINES AGAINST TUBERCULOSIS

A. BCG VACCINE

The high mortality associated with *Mtb* infection occurs despite the widespread use of a live, attenuated TB vaccine *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG). BCG is effective at preventing disease in newborns and toddlers, but not pulmonary TB in adults (48). More specifically, numerous efficacy trials and epidemiological studies conducted over several decades indicate that BCG has 60-80% efficacy against severe form of TB in children, particularly meningitis (49, 50), and that its efficacy against pulmonary disease varies geographically (51, 52). Furthermore, revaccinating with BCG during adolescence in a population vaccinated with BCG at birth does not improve protective efficacy as shown in a large, randomized controlled trial in Brazil (53). Importantly, when given to people already infected or sensitized to environmental mycobacteria BCG has not shown any protection against disease, which could explain the geographical efficacy variations (54, 55). Additionally, the live attenuated BCG vaccine is unsafe for administration to HIV-positive or other immunocompromised individuals due to the possibility of developing regional BCG infection (BCG-itis) or disseminated BCG (BCG-osis) (56-59). Mathematical modeling of the impact that a hypothetical new vaccine against TB with 60% efficacy could have predicts an 80% decrease in incidence by 2050 (4). Thus there is an urgent need for a new TB vaccine to either boost immunity primed by BCG or to replace BCG.

B. CORRELATES OF PROTECTION

TB vaccine research is confounded by a conundrum: a candidate biomarker for protective immunity can only be validated in the clinical trial of an effective vaccine. However, clinical trials of an effective vaccine may not be feasible without a validated correlate of protection for the selection of the most promising candidates as for determining dose and schedule of vaccination.

1. THE CENTRAL DOGMA OF PROTECTIVE IMMUNITY

During the past 40 years a combination of studies have led to the TB central dogma that production of IFN γ by CD4 T cells is the major driver of immunity against TB.

In 1970 it was found that T cells, and not antibodies, are required for host resistance to TB (60). Later, in the 1980's, CD4+ T cells that produce IFN γ were described as the dominant T cell subset

that participates in the immune response to *Mtb* (61). In 2001, it was shown that mice devoid of CD4-mediated immunity (MHCII-/-) or CD4+ depleted were not able to control *Mtb* infection and would subsequently succumb early-on (62). This experiment established a crucial role for CD4+ T cells mediated immunity in TB control. Finally, the discovery that HIV infection, which is strongly associated with TB, infects and kills CD4+ T cells supported a central role for CD4+ T cells in immunity against TB (63).

IFN γ 's, a cytokine involved in the response against viruses and intracellular bacteria, key role in immunity against TB is based on the extreme susceptibility of IFN γ -deficient mice (64). More arguments supporting this hypothesis include nontuberculous mycobacterial infection or BCG-osis in partial or complete IFN γ -deficient patients (65, 66) and IFN γ depression in whole-blood cultures from advanced TB patients (67). IFN γ activates macrophages to kill intracellular bacteria through antimicrobial effector pathways such as inducible nitric oxide synthase, IFN γ inducible GTPases, phagosomal maturation and acidification, autophagy and vitamin D receptor signaling (40). Furthermore, in humans suffering from recurrent nontuberculous mycobacterial infections, deleterious genetic mutations in the genes encoding IL-12p40 and IL-12R have been identified (68-70). IL-12 has a crucial role in the induction of IFN γ production (71), accordingly these patients display a reduced capacity to produce IFN γ (72), highlighting the potential central role of IFN γ in TB protection.

These discoveries helped to define the central dogma of protective immunity against TB, namely, that the production of IFN γ by CD4+ T cells activates macrophages to kill *Mtb* (Figure 3). Today, detection of IFN γ produced by T cells is still the most widely used for monitoring immune response following TB infection (QuantiFERON®-TB test) or vaccination.

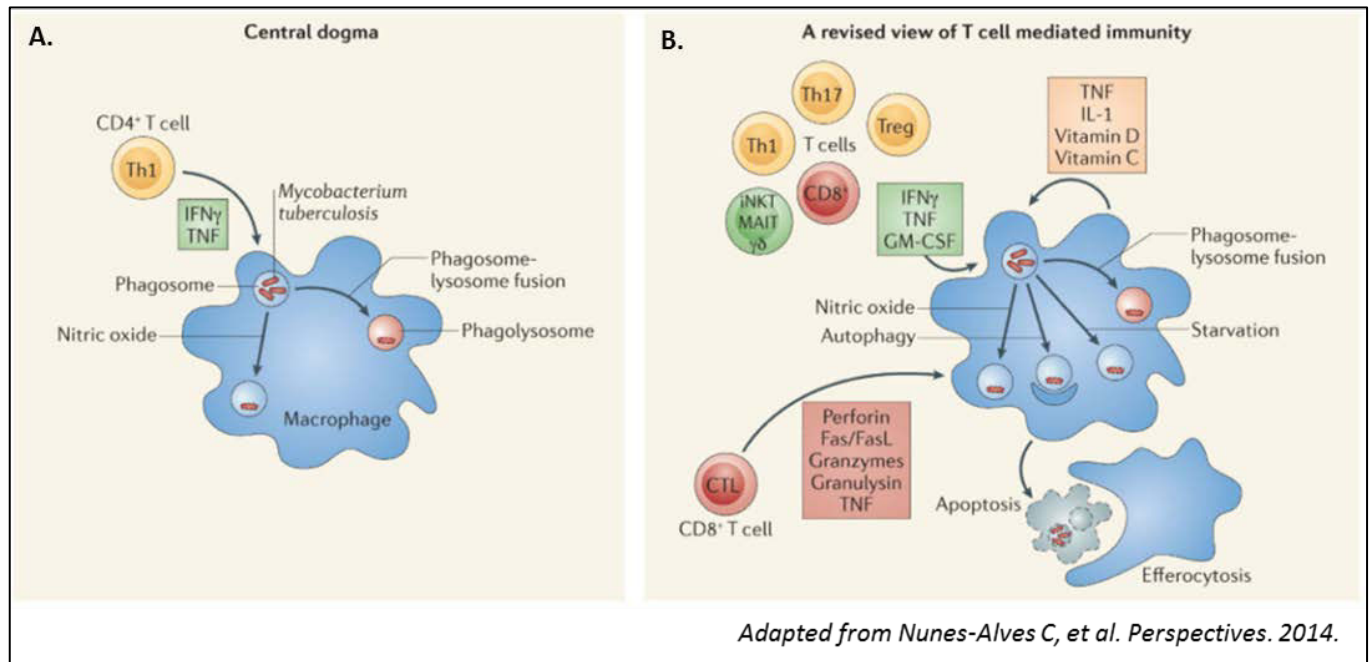


Figure 3. Protective immunity to TB: the central dogma and a revised view of T cell mediated immunity. A. The central dogma of protective immunity to TB is that CD4⁺ T cells produce IFN γ which synergizes with TNF (produced by the T cell or the macrophage), and together these activate macrophage antimicrobial activity capable of restricting *M. TB* growth. Two pathways activated by IFN γ that are capable of killing *Mtb* are nitric oxide (NO) production and phagolysosome fusion, which acidifies the bacterial phagosome. B. “A revised view of protective T cell immunity” incorporates additional T cell subsets (CD4⁺, CD8⁺, and unconventional T cells – $\gamma\delta$ T cells, Mucosal-Associated Invariant T (MAIT) cells and CD1-restricted T cells), and includes additional mechanisms by which T cells mediate killing of *Mtb*.

2. MOVING BEYOND THE CENTRAL DOGMA

Although essential for host resistance to *Mtb*, IFN- γ responses notoriously do not correlate with resistance to TB. More T cells that secrete IFN γ , or greater IFN γ levels, have not been found to correlate with protection (73). Further, a study in 2011 found that patients whose T cells produce greater amounts of IFN γ are more likely to progress to active disease than patients with weaker responses (74), supporting the idea that IFN γ levels actually correlates better with bacterial burden than with disease control (26). Similar conclusions can be taken from vaccination studies. BCG vaccination elicits protective T cells in animals, but IFN γ production by these T cells is not predictive of vaccine induced protection (75, 76). Further, one study in South-African infants vaccinated with BCG found no correlation between the number of BCG-elicited T cells that produce IFN γ and protection against TB (77). Finally some recent studies in murine models suggest that even though T_H1 CD4 T cells might be essential for protection against TB, this mechanism might be IFN γ -independent (78, 79). It seems likely then that even though IFN γ production by CD4⁺ T cells might be a critical component of immunity against *Mtb* it may not

necessary translate into an immune correlate of protection against TB. There is now a wide appreciation for the importance of extending our understanding of anti-TB CD4 T cell effector molecules beyond IFN- γ .

Other immune cells could potentially correlate with protection, such as CD8⁺ T cells, T_{H17} T cells, NK cells, $\gamma\delta$ T cells and CD4⁺ CD8⁺ T cells (Figure 3 B.) (26, 80).

a) CD4⁺ T CELLS POLYFUNCTIONALITY

In addition to T cells that predominantly produce IFN- γ , recent studies have suggested that multifunctional CD4⁺ or CD8⁺ T cells that produce multiple cytokines, especially IFN- γ , tumor necrosis factor alpha (TNF- α), and interleukin-2 (IL-2), could be important in host resistance. For example HIV non progressors expressed a high frequency of such polyfunctional CD4⁺ T cells (81) and similarly Ag-specific polyfunctional CD4⁺ T cells were found to be protective against *Leishmania* infection (82). Consistent with these results, protection in mice against *Mtb* infection correlates with a high frequency of polyfunctional CD4⁺ T cells (83). In humans active TB disease is associated with TNF producing monofunctional CD4 T cells compared to the polyfunctional T cells found in latent *Mtb* infection (84). The Modified Vaccinia Ankara (MVA85A), a modified vaccinia virus Ankara expressing the major *Mtb* antigen Ag85A, was the first TB candidate vaccine to complete an efficacy trial (after BCG). A higher frequency of polyfunctional CD4⁺ T cells were elicited in BCG vaccinated-MVA85A boosted individuals than that for BCG alone (85). However, disappointingly, the results obtained during the phase 2b in infants indicated no enhancement in protection, suggesting that Ag85A-specific polyfunctional CD4⁺ T cells may play a necessary but not sufficient role in protection against TB (86) in that population under the conditions evaluated.

b) CD4⁺ T CELLS PHENOTYPES

The discrepancy between the fact that T_{H1} CD4 T cells are critical in TB immunity, yet higher numbers of these cells do not necessary confer protection, could potentially be explained if different subsets of T_{H1} CD4⁺ T cells differ in their ability to control *Mtb*. Reiley *et al* identified two functionally distinct subsets of effector CD4⁺ cells produced during TB infection that could be distinguished by their expression of the programmed cell death protein 1 (PD-1) and the co-inhibitory receptor killer-cell lectin like receptor G1 (KLRG1). PD-1 expressing activated effector

cells with a higher proliferative and self-renewing capacity than the KLRG1-expressing cells, but a lower capacity of cytokine production, and the capacity of differentiating into KLRG1⁺ cells. KLRG1⁺ cells are terminally differentiated, proliferate poorly, maintain their KLRG1⁺ phenotype and are short-lived (87). There is growing evidence that PD-1⁺ CD4⁺ T cells mediate superior protection against TB than terminally differentiated KLRG1⁺. Indeed, protection against *Mtb* induced by BCG immunization in mice is lost as the infection enters the chronic phase, and this waning of immunity is coincident with an increase of KLRG1⁺ terminally differentiated T cells (88). Furthermore, immunizations that confer superior longer lasting immunity preferentially expand *Mtb*-specific CD4⁺ T cells that are KLRG1⁻, produce IL-2 and are associated to central memory (88, 89). In addition, it was shown that CD4⁺ T cells must make direct contact with infected antigen presenting cells (APC) to induce control of *Mtb* infection (90). Thus, to be protective, *Mtb*-specific CD4⁺ T cells need to exit the circulation and enter the lung parenchyma where the *Mtb* infection occurs. Meanwhile, it was shown that, upon infection with *Mtb*, *Mtb*-specific CD4⁺ T cells residing in the lung vasculature where mainly KLRG1⁺ PD1⁻ and the ones in the infected lung parenchyma where almost exclusively PD1⁺ KLRG1⁻ (91). Furthermore, adoptive transfer of those PD1⁺ KLRG1⁻ cells conferred greater protection against *Mtb* challenge (91, 92). Thus, CD4 T cells with a more central memory polyfunctional phenotype and ability to enter the lung parenchyma might be essential for protection against TB and those functions correlates, at least in mice, with a KLRG1⁻ PD1⁺ phenotype (Figure 4).

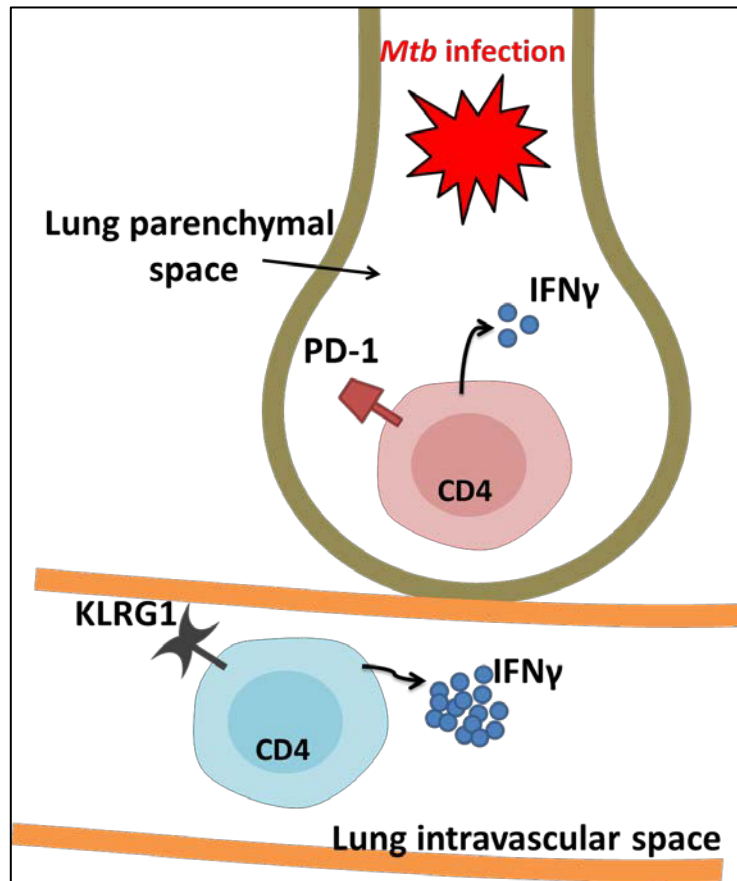


Figure 4. Properties of protective *Mtb*-specific CD4⁺ T cells. Pulmonary *Mtb*-specific Th1 cells display two major phenotypes based on their localization in the lung parenchyma or lung-associated blood vasculature of infected mice. CD4⁺ T cells that can efficiently enter the lung parenchyma (in red) are almost exclusively PD1⁺ KLRG1⁻, secrete low amounts of IFN γ and are highly protective against *Mtb*. CD4⁺ T cells that reside in the blood vessels (in blue), are terminally differentiated, secrete high amounts of IFN γ and are weakly protective against *Mtb*.

C. THE TUBERCULOSIS VACCINES PIPELINE

Due to the complex nature of *Mtb* and resulting pathology within the human host (discussed above), multiple vaccine development strategies are being pursued: (i). prevention of infection (vaccines that are given prior to exposure in order to prevent initial infection), (ii) prevention of disease (vaccines that can be administered after exposure, to infected people that might be asymptomatic and at risk of developing disease in the future), (iii) prevention of recurrence (vaccines that can be administered after infection and during or after drug treatment, to prevent reactivation) (3). Although there is some pessimism about whether a vaccine that averts infection can be developed, the general consensus is that a vaccine that prevents progression to

active disease could reduce the prevalence of pulmonary TB and ultimately break the cycle of transmission.

Efforts are currently underway to develop vaccines against TB that can boost the current BCG vaccine or can be used as a stand-alone prophylactic vaccine in immune-compromised individuals where BCG immunization is not recommended. In addition, immunotherapeutic vaccine are also being developed for individuals with active TB in conjunction with TB drug therapy, with the aim of shortening the duration of the therapy and/or reducing recurrence rates after completion of treatment. There are 17 vaccine candidates in various phases of clinical trials, including five protein or adjuvant vaccines, four viral-vectored vaccines, three mycobacterial whole cell or extract vaccines, one recombinant live and one attenuated *Mtb* vaccine (

Figure 5) (reviewed in (3) and (93)). The two most advanced candidates are *M. vaccae* and M72/AS01. *M. vaccae* is a whole heat-killed agent currently licensed by the China Food and Drug administration as an immuno-therapeutic agent to help shorten TB treatment for patients with drug susceptible TB after it shown improved sputum reversion and X-ray appearances when used in combination with chemotherapy in patients with untreated TB (94). This candidate vaccine is currently in Phase III trial to assess its efficacy and safety in preventing TB disease in people with latent TB (3). M72/AS01 is a recombinant fusion protein of the *Mtb* antigens (Ags) 32A and 39A in combination with the AS01 adjuvant, a liposomal formulation containing the monophosphorylated lipid A (MPL) (TLR4 agonist Cf. IV.A.2.b)) and *Quillaja saponaria* (QS21) a purified plant extract. This candidate vaccine was shown to have a clinically acceptable safety profile and to be highly immunogenic in *Mtb*-infected and uninfected adults (95) and is currently in a large randomized placebo-controlled Phase 2b trial to assess efficacy of two doses of M72/AS01 against pulmonary TB disease (3).

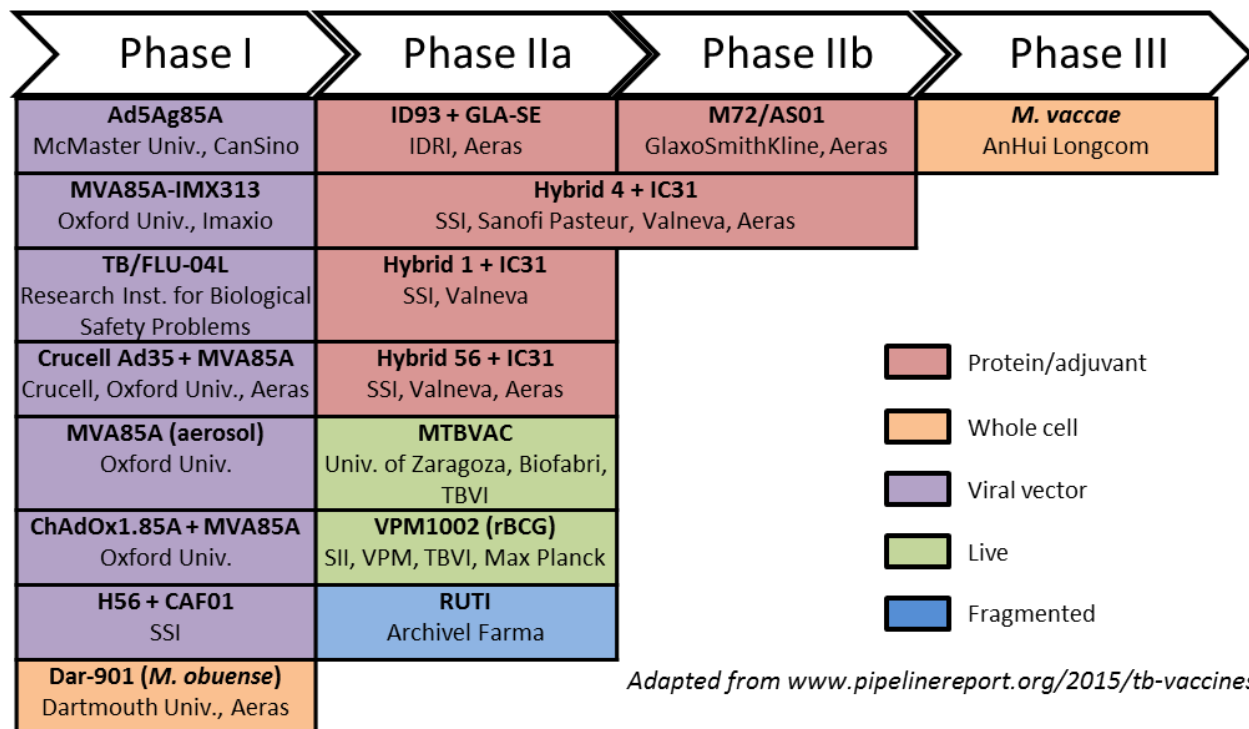


Figure 5. 2015 TB vaccine candidates' pipeline.

III. SUB-UNIT VACCINES

A. WHY WE NEED ADJUVANTS

Vaccines made from live-attenuated or inactivated pathogens can elicit robust protective immune responses because those vaccines contain naturally occurring adjuvants. Vaccination with an antigen alone has advantages in terms of safety and cost-effectiveness, however when separated from their microbial constituents many of these vaccines, such as the acellular pertussis vaccine, are insufficiently immunogenic on their own. Adjuvants become critical to help these proteins become effective vaccines by inducing strong and long lasting immune responses. Adjuvants from the Latin word *adjuvare*, meaning “to help or aid”, were first described by Ramon in 1924 as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone” (96). Most specifically, several important benefits can be found in adjuvants such as enhancement of vaccine efficacy in infants, elders and immunocompromised people, increase of functional antibody titers, induction of more and long-lasting responses, induction of robust cell-mediated immunity, broader protection through cross-reactivity to other strains or pathogens, facilitation of mucosal immunity, overcoming of antigen

competition in combination vaccines, reduction of the dose of antigen needed (dose sparing) and/or reduction of the number of vaccines doses needed (97, 98).

B. PATHOGENS RECOGNITION BY THE HOST

1. DANGER ASSOCIATED MOLECULAR PATTERNS

In some circumstances host inflammatory responses can cause host cell death leading to tissue injury, and the release of host cellular components to the extracellular environment. These self-molecules considered as “messengers” for danger or Damage-Associated Molecular Patterns (DAMP) shape the immune response. Host lipids, sugars, metabolites (e.g. uric acid or ATP), and nucleic acids such as RNA and DNA all contain DAMPs which are recognized when the molecules accumulate in non-steady state locations such as cytosolic DNA or extracellular ATP (99). This leads to activation of inflammasomes. Inflammasome activation leads to caspase-1 activation, which causes the maturation cleavage of pro-IL-1 β and pro-IL-18 into their mature forms IL-1 β and IL-18 which are secreted and shape the innate and adaptive responses to pathogens and adjuvants (100, 101).

2. PATHOGEN-ASSOCIATED MOLECULAR PATTERNS AND TOLL-LIKE RECEPTORS

Host cells are equipped with numerous types of receptors to discriminate self from non-self constituents. Pathogens are first recognized by pattern recognition receptors (PRR) expressed by many cell types including professional APCs such as macrophages and DCs, which prime T cell responses. PRR including TLR, RIG-I like receptors, C-type lectin receptors and Nucleotide oligomerization domain (NOD)-like receptors recognize a diverse array of pathogen associated molecular patterns (PAMP).

TLR are among the best characterized PRR and were first identified in the early 1980s in *Drosophila* where their function are required for responses to fungal and Gram-positive bacterial infection (102). TLRs are the canonical PRR that link innate and adaptive immunity: they sense infection through the recognition of PAMPs and induce appropriately tailored innate and adaptive immune response (103). A summary of known mammalian TLRs and their ligands is shown in Figure 6.

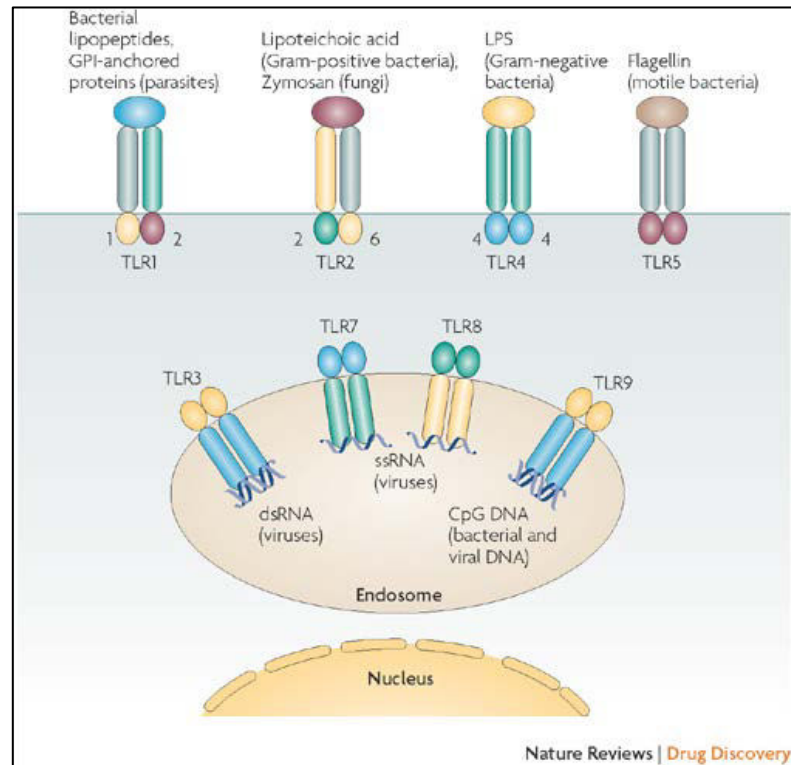


Figure 6. Mammalian TLRs and their ligands.

Binding and TLR homo- or heterodimerization brings the Toll-Interleukin Receptor domains of adjacent TLRs together, providing a conformational change necessary to trigger signaling. Binding of additional adaptor proteins, including the myeloid differentiation factor 88 (MyD88) the MyD88 adaptor-like protein, the TIR domain-containing adaptor protein inducing interferon- β (TRIF) and the TRIF-related adaptor molecule TRAM, is essential for intracellular cascades (104-107) (Figure 7).

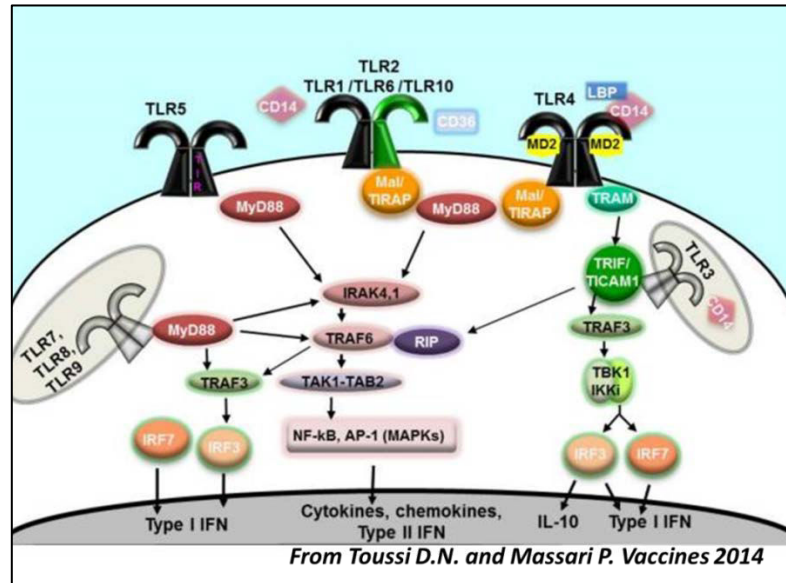


Figure 7. TLR signaling. Extracellular TLR homodimers (TLR4 and TLR5) are represented in black; heterodimers of TLR2 and TLR1, TLR6 or TLR10 are indicated in black/green. Intracellular homodimers (TLR3, TLR7, TLR8 and TLR9) are indicated in gray.

TLRs have been shown to be sufficient to tailor an adaptive immune response characterized by T_H1 induction, IgG2c production, CD8 T cells induction and protection from reinfection to an array of pathogens (Gram-positive and negative bacteria, DNA and RNA viruses, fungi and protozoa) (108, 109). Importantly they have also been shown to be critical for the induction of a strong adaptive immune response to various immunizations (98, 110). They control this responses at multiple levels including: (i) control of antigen uptake by transiently stimulating antigen macropinocytosis and coordinating the redeployment of actin to sustained endocytosis (111) (ii) control of antigen selection for presentation in DCs as efficiency of antigen presentation from phagocytosed cargo is dependent on the presence of TLR ligands within the cargo and the generation of peptide-MHCII complexes is also controlled by TLRs (112), (iii) control of DC migration from the infection site to the draining LN through downregulation of CCR6, an inflammatory chemokine receptor, and upregulation of CCR7, a receptor for lymphoid chemokines (113, 114), (iv) control of DC maturation characterized by the upregulation of the co-stimulatory molecules CD40, CD80 and CD86 and production of the pro-inflammatory cytokines IL-12 and TNF (115), (v) direct co-stimulation of T cell subsets to enhance effector function such as IL-2 and IFN γ production and T cell proliferation (116), and (vi) control of B cells responses to T-dependent and T-independent antigens through B cell intrinsic TLR signaling

that regulates antibody responses (117). Due to their important immunomodulatory capacities TLR are prime- candidates for adjuvant development. In reality, most first generation vaccines, including those consisting of inactivated or attenuated virus or bacteria contain inherent adjuvant activity by possessing molecules that can bind these receptors (Figure 8). The discovery of TLRs and their role in modulation of innate and adaptive immunity has led to exploitation of their ligands as immune modulators. The addition of TLR agonists is not a new concept, since combined vaccine adjuvants have been evaluated since the 1930s, when whole bacterial cells were added to water-in-oil emulsions to create Freund's complete adjuvant. Today, several well-defined and characterized TLR agonists are being selectively developed with the primary purpose to serve as adjuvant components within vaccines. A summary of those TLR based adjuvants and the disease models tested in experimental and clinical trials and human vaccines can be found in Toussi and Massari 2014 paper (107). In particular, TLR3, TLR4 and TLR9 agonists have been shown to improve a number of vaccines, for example against Hepatitis B Virus (118, 119), influenza (120-122), malaria (123-126) and *Leishmania* (8, 127), as well as some types of cancer (128-132).

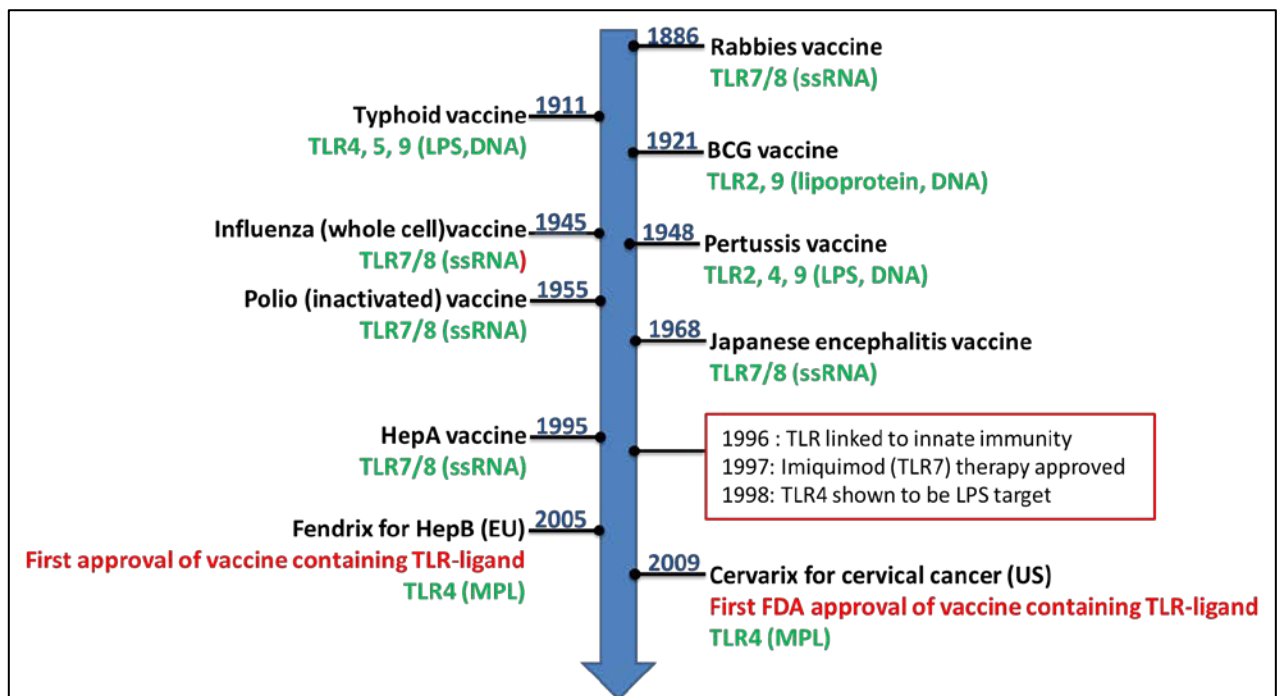


Figure 8. Vaccine development and implications for TLR-agonist use.

C. ADJUVANTS CURRENTLY APPROVED FOR USE IN HUMANS

Approved adjuvants for use in humans include Alum (aluminium salts), oil-in-water emulsions (MF59®, AS03 and AF03), virosomes and AS04 (133).

Alum is the most widely used adjuvant in human and animal vaccines, it elicits a strong humoral immune response that is effective against diphtheria, tetanus and hepatitis B where neutralizing antibodies to the target antigen are required for protection (134-136). It is, however, a poor inducer of cell-mediated immune response and thus unsuitable for vaccines requiring such immune response (137).

Virosomes are spherical lipid vesicles carrying bound vaccine-target antigen on the surface or encapsulated within the lumen (138, 139). Using this technology, the Hepatitis A vaccine is the first vaccine to use an adjuvant other than Alum (140).

Squalene emulsions approved for clinical use, including MF59® and AS03, are liquid droplets dispersed throughout an immiscible liquid. The inclusion of emulsions enables antigen dose sparing and increase Ag-specific Ab titers. Moreover, the Ab response to these emulsions appears to have a more balanced IgG1:IgG2 profile compared to Alum (141).

AS04 contains monophosphoryl lipid A (MPL) a TLR4 agonist (Cf. IV.A.2.b)) adsorbed on aluminum salt. The HPV-16/18-AS04-adjuvanted vaccine was shown to induce significantly higher titers of HPV-specific antibodies and neutralizing antibodies than an aluminum-adjuvanted formulation and was licensed for use after conclusive demonstration of efficacy (142-144). AS04 is also the adjuvant in the licensed vaccines FENDrix™ against Hepatitis B and it is intended to use for active immunization for patients with renal insufficiency which have an increased risk of acute hepatitis B infection and progression to chronic hepatitis but tend to respond less well to Alum-adjuvanted Hepatitis B vaccine than healthy individuals (119, 145).

D. ADJUVANTS IN TUBERCULOSIS CANDIDATE VACCINES

Until fairly recently, there were only two adjuvants approved for use in humans and very few available for human testing: Alum that was used successfully in vaccines for many decades with the key feature being induction of antibodies, and MF59®, a squalene emulsion adjuvant, which also mainly induces antibody responses and is in particular a very effective adjuvant in influenza

vaccines (146). But these adjuvants did not show any evidence of induction of the cell-mediated immunity which is critical for protection against TB, and indeed preclinical studies showed no protective potential of using Alum in TB vaccines (147). Thus, with the first TB fusion proteins getting ready for clinical testing just after the millennium, an important task of developing suitable adjuvants was undertaken.

As shown above, there is no strong correlate of protection in immunity against TB. Thus, adjuvant research is extremely challenging in that the properties associated with efficacious adjuvants is incompletely understood. Today, there are 4 different adjuvants in the TB vaccines pipeline (

Figure 5); all have shown solid evidence of protection in various preclinical models including non-human primates (NHP). AS01 is an adjuvant system containing the immunostimulants MPL (TLR4 agonist) and QS21 combined with liposomes (95); IC31 combines the immunostimulatory effects of an 11-mer antibacterial peptide and a synthetic oligodeoxynucleotide which is a TLR9 agonist without containing cytosine phosphate guanine (CpG) motifs (47, 148); CAF01 is a two-component liposomal adjuvant system composed of a cationic liposome vehicle stabilized with a glycolipid immunomodulator which is a synthetic variant of cord factor located in the mycobacterial cell wall (149), finally GLA-SE is a glucopyranosyl lipid adjuvant-stable squalene-based oil-in-water emulsion, which acts as a TLR4 agonist.

IV. ID93/GLA-SE

A. THE VACCINE

1. ID93 FUSION PROTEIN

ID93 was developed following rigorous screening of a large panel of *Mtb* recombinant proteins constituting the most comprehensive analysis to date of human T cell responses to antigens of *Mtb*. From selected genome mining around 100 candidates were selected and expressed as recombinant proteins. These proteins were then pre-selected based on their ability to induce IFN- γ from the peripheral blood mononuclear cells of healthy human that are purified protein derivative positive [PPD(+)] . A subset of 48 proteins was tested further, each individually combined with CpG in the *Mtb* aerosol mouse model, in order to determine whether they could

reduce lung bacterial load in infected mice (150). ID93 is a fusion of four *Mtb* proteins selected from this analysis it includes Rv2608, Rv3620, Rv1813 and Rv3619 (Figure 9) (7). Each protein is segregated into different *Mtb* protein categories: Rv2608 falls within the PE and PPE (PE/PPE) multigene families of proteins, Rv3619 and Rv3620 are in the ESAT-6 secretion system (EsX) family of virulence factors, and Rv1813 is associated with latent growth of *Mtb* and is expressed during hypoxia which might be useful for optimal immunity and concomitant protection during the later stages of infection (150).

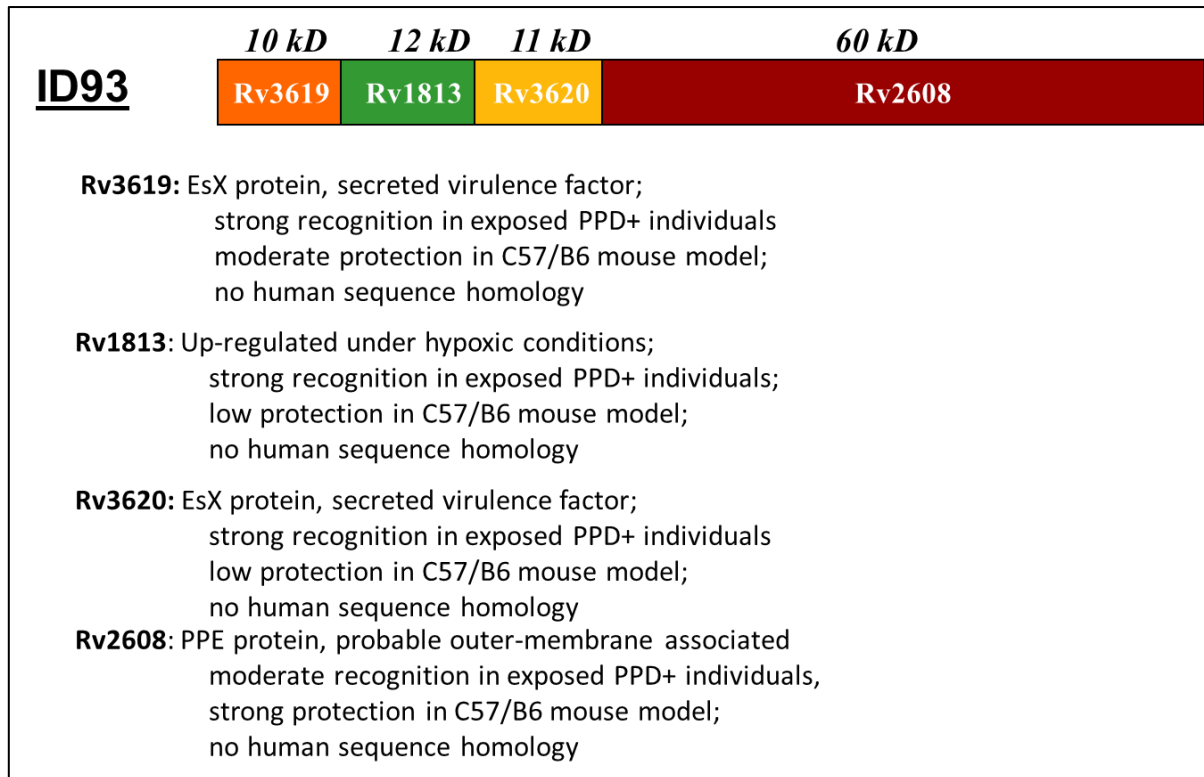


Figure 9. IDRI's lead TB vaccine antigen-ID93

a) PE/PPE FAMILY

PE/PPE gene family, comprising of 176 open reading frames and present exclusively in genus *Mycobacterium*, accounts for approximately 10% of the *M. tuberculosis* genome. It has attracted a lot of interest in the vaccine design world since their discovery more than a decade ago. Indeed, these proteins are found to be localized at cell surface and/or secreted and have been reported to be antigenic in nature. Moreover several members of the family trigger a range of innate immune responses, and many are targets of the adaptive immune system (151). They were shown to be differentially expressed in different species of pathogenic mycobacteria suggesting a key role for the PE/PPE family in virulence and pathogenesis of *Mtb* (152). Interestingly, PE/PPE proteins

having a largely redundant nature in *Mtb*, they also induce a strong cross-reactivity with their numerous homologs (153).

b) ESX FAMILY

The EsX family is composed of small, secreted antigenic proteins that have been demonstrated to likely be required for the growth and pathogenicity of *Mtb* (154). This family is an attractive vaccine target since they are potent T cell antigens that encode several immunodominant molecules, which are strongly recognized by the immune systems in both animals and humans (154-156). Indeed, the most prominent antigens described for induction of cell-mediated immunity have been members of the *esx* family. ESAT-6 and CFP-10 are the most extensively investigated (155, 157).

2. GLA-SE

a) TLR-4 AND LIPOPOLYSACCHARIDE

TLR4 can be found on the plasma membrane of various cell types, including neutrophils, monocytes, eosinophils, basophils, T cells, B cells, NK-cells, immature DCs, and endothelial cells (98). It is a unique receptor among the TLRs as it induces two distinct signaling pathways controlled by (i) MyD88/MAL which results in activation of nuclear factor (NF)- κ B for induction of a number of NF- κ B-dependent genes and inflammatory cytokines, and (ii) TRIF/TRAM which induces production of type I IFN (Figure 7) leading to a strong Th1-type cellular and humoral immune response (158). Lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria was the first bacterial product shown to interact with this receptor (159). LPS has a hydrophilic polysaccharide component and a hydrophobic lipid A, composed of polyacylated diglucosamine lipids. LPS is a potent stimulator of the immune system and it has been shown that its administration in animal can protect against challenges with a variety of bacterial, viral and parasitic pathogens, however, an intrinsic toxicity limits its use in human vaccines (107).

b) MONOPHOSPHORYL LIPID A

MPL[®] is a detoxified derivative of LPS, which retains only ~0.1% of the inflammatory toxicity of its parent LPS molecule (160).

It is also the first TLR agonist ever included in a licensed human vaccine (Fendrix®) (161). It is produced from *Salmonella minnesota* LPS by removal of the core carbohydrate group, the phosphate from the reducing-end glucosamine, and the acyl chain from the 39-position of the disaccharide backbone. In the murine model it was shown to promote T_{H1} response, characterized by increased IFN γ production and IgG2 isotype switch, in combination with a wide variety of Ags such as inactivated rotavirus (162), hepatitis C virus like-particle (163), Leish-111f a leishmanial polyprotein (164), ESAT-6 antigen from *Mtb* (165) and RTS,S malarial antigen (166) among others. In human studies, MPL® alone or in combination with other adjuvants was shown to be well tolerated and to enhance both humoral and cellular immune responses (167-171). The M72/AS01 vaccine candidate contains MPL® and QS21 combined with liposomes and is currently in Phase IIb trial in *Mtb* infected individuals to assess whether this vaccine protects against TB disease progression (II.C, Figure 5) (172). As its parent molecule LPS, MPL signals through both MyD88 and TRIF even though the signal through MyD88 is less efficient than for LPS (173).

c) GLUCOPYRANOSYL LIPID A

Naturally occurring species of LPS and its derivatives can have a variety of chain's number an attachment sites, and the number of carbon within the chain may also vary. For example, MPL® has between four and seven chains depending on the extend of hydrolysis during detoxification and those different species can have different relative potency (174, 175). GLA is a pure synthetic hexaacylated lipid A derivative which holds the advantages of being a more chemically defined molecular adjuvant, in contrast to the heterogeneous mixture of MPL extracted from bacteria (6). Several differences exist between synthetic GLA and naturally occurring LPS endotoxins including (i) the absence of attached residues on the hydroxyl, (ii) the absence of a second phosphate on GLA, and (iii) defined position, number and lengths of chain attachments and carbon number (Figure 10) (6). Because GLA lacks one phosphate group, it still remains several orders of magnitude less endotoxic than its parent molecule LPS, and is safer to use in humans (6). Similarly to MPL, GLA signaled less efficiently than LPS through the MyD88/MAL-dependent pathway, but fully retained the ability to signal through TRIF/TRAM (6).

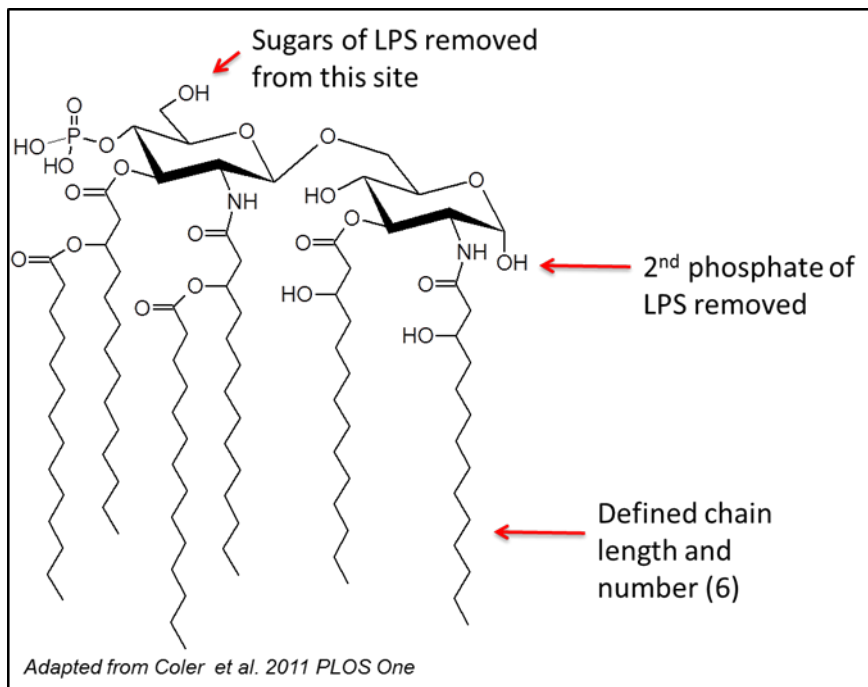


Figure 10. GLA structure.

3. STABLE EMULSION

Typical oil in water emulsions are composed of oil droplets, commonly squalene, stabilized by surfactants in a buffered aqueous phase. The use of stable emulsion as adjuvants has shown promising and has good potential to achieve FDA approval. Indeed, MF59® is today a well-established, safe and potent emulsion-based vaccine adjuvant has been licensed for use in human in Europe in the Flud® influenza vaccine (176). IDRI's stable emulsion (SE) is similar to MF59®, although the squalene dose in SE is 2% (v/v) compared to the 2.3% (v/v) in MF59®. In addition, the primary emulsifier in SE is phospholipid, whereas AS03 and MF59® contain non-ionic surfactant (177). Interestingly, phospholipids which are naturally found in cells, might be more bio-compatible than non-ionic surfactant thus reducing any potential hypersensitivity to the components (178).

B. ID93/GLA-SE AND TUBERCULOSIS

ID93/GLA-SE intra-muscular, recently completed phase I trial and it is currently undergoing Phase 2 immunogenicity and efficacy testing against recurrence of TB in adults.

Mice immunized prophylactically or post-infection with the ID93/GLA-SE candidate vaccine generate a T_H1 -biased response with Ag-specific polyfunctional CD4 T cells producing CD154,

IFN γ , TNF, GM-CSF, and IL-2, as well as cytolytic CD4 T cells (CTL) and a humoral response skewed towards IgG2c class-switching (7, 179, 180). Prophylactic intra-muscular immunization with this candidate vaccine had been shown to be protective in mice against challenge with aerosolized H₃₇Rv, a multidrug-resistant TN5904 strain of *Mtb* and HN878, a recent clinical isolate of the Beijing lineage (7, 180, 181). Additionally, when ID93/GLA-SE is used as a stand-alone or as booster to BCG, post-infection survival of guinea-pigs is increased and it is safe and immunogenic in NHP (7). Finally, therapeutic vaccination with ID93/GLA-SE has been proven to be an effective adjunct to antibiotic therapy in infected SWR mice and NHPs. Indeed it decreased bacterial burden, reduced the duration of conventional chemotherapy required for survival, and decreased *Mtb*-induced lung pathology, compared with chemotherapy alone (182).

It is to noted that, unlike BCG, immunization with ID93/GLA-SE does not cause a reaction to purified protein derivative (PPD) administration (183). Thus, the Tuberculin Skin test, routinely used to identify individuals infected with *Mtb*, particularly in epidemiological studies, won't be compromised upon vaccination with this vaccine.

Interestingly it was recently shown that Induction of T_H1 cells that co-express IFN- γ and TNF is not a requirement for ID93/GLA-SE efficacy against *Mtb* in mice, despite these cytokines being essential for control of *Mtb* in non-vaccinated animals (79).

C. WHAT WE KNOW ABOUT GLA-SE ADJUVANTICITY

When GLA is formulated in the stable emulsion SE (GLA-SE), its adjuvant activity is greatly enhanced compared with that of an aqueous formulation of GLA devoid of a major oil component. Indeed, GLA-SE formulation induces more antigen-specific Th1 CD4 cells (184). It is to be noted that immunization with ID93 formulated in the adjuvant SE alone elicited a predominant type 2 response characterized by IL-5 production and substantial IgG1 antibody responses and that addition of GLA to the formulation eliminated the IL-5 response (12). Furthermore, when formulated with GLA several oil-in-in-water emulsions that are not squalene-based but similar average particle size and size polydispersity than SE, did not elicited detectable T_H1 responses (184). Thus squalene itself seems to contribute to GLA-SE adjuvanticity but exactly how is still unknown.

The innate response to GLA-SE predominantly takes place in the draining LN. Engagement of inflammasome has been implicated in GLA-SE's superior adjuvant activity as the innate response

to GLA-SE and the subsequent adaptive immunity were compromised in the absence of caspase-1/11 or the IL-18 receptor (12). Interestingly, the early response to adjuvant is not dependent on NLR Family, Pyrin Domain Containing 3 (NLRP3), which is consistent with MF59® studies (12, 185). Additionally, IFN γ is a critical mediator of the innate and adaptive responses induced by GLA-SE (12, 79). Early IFN γ production is detected in the draining LNs upon injection with GLA-SE, and the main producer of this early IFN γ had been described to be CD8 $^{+}$ T cells very early on (around 4h after immunization) and neutrophils later in the immune response (detected around 18h). Furthermore, early IFN γ production by neutrophils and CD8 memory cells is dependent upon IL-18 production and caspase 1/11 activity (12) (Table 1 and Figure 11).

GLA-SE induces a strong DC recruitment to the draining LN and quickly renders the DCs functionally mature or immunogenic *in vivo* (186, 187). DCs were shown to be required for its full adjuvant action since no CD4 $^{+}$ T cell response develops when DCs were depleted in CD11c-DTR chimeras (186). Interestingly, unlike CD4 $^{+}$ responses, Ab responses to GLA-SE are not affected by DC depletion (186).

GLA, through recognition by its receptor TLR4, induces activation of both MyD88 and TRIF-associated pathways. T_H1 induction and optimal IgG2c generation by GLA-SE is dependent on both MyD88 and TRIF signaling and their collaboration within the same cell (187, 188). These signaling pathways are also both required for the induction of IL-IL-12, RANTES and TNF by GLA in several different APCs while IFN β production is TRIF dependent/MyD88 independent (188).

The accumulated knowledge about the requirements for a strong immune response to GLA-SE and the suggested mechanism of action are summarized in Table 1 and Figure 11 respectively.

CATEGORIE	COMPONENT	Innate response (strong CD69 induction)	Strong CD4+ TH1 polyfunctional response	Strong B cells IgG2c skewed response	References
Adjuvant	GLA+SE	YES	YES	?	(12, 184)
Pathway components	Caspase 1/11	YES	YES	?	(12)
	NLRP3	NO	?	?	(12)
	MyD88/TRIF	?	YES	YES	(188)
Cytokines	IFN γ	YES	YES	?	(12, 79)
	IL-18	YES	YES	?	(12)
Cells	DC	?	YES	NO	(186)

Table 1. Components, cytokines and pathways required for the different elements of the immune response to GLA-SE solely based on what has been published about GLA-SE. YES = required, NO = not required, ? = no data.

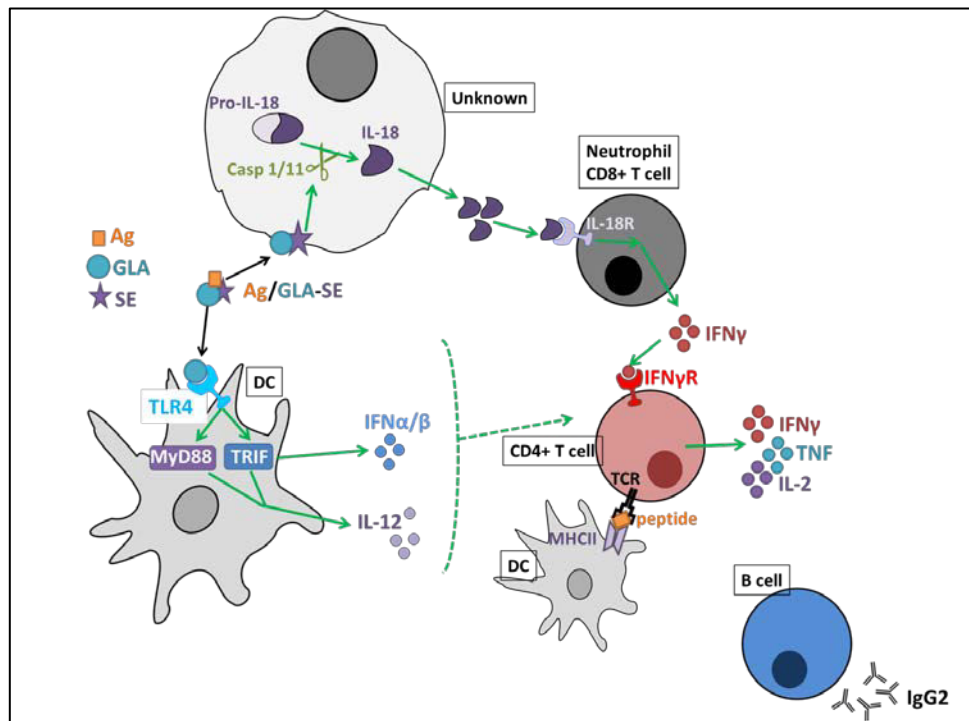


Figure 11. Suggested GLA-SE mechanisms of action in the draining LN solely based on what has been published about GLA-SE. Top: GLA-SE will activate the Inflammasome in an unknown target cell, leading to caspase 1 activation and the subsequent cleavage of the pro-IL-18 in its mature form IL-18. IL-18 will be recognized by neutrophils and possibly CD8+ T cells which will lead to innate IFN γ secretion by these cells. IFN γ will be recognized by its receptor on CD4+ T cells and will help in the establishment of the TH1 response and the subsequent secretion of TH1 cytokines (IFN γ , TNF and IL-2) by the activated CD4+ T cells. Bottom: GLA will be recognized by DC through TLR4 leading to the engagement of MyD88 and TRIF pathways which will result in IL-12 expression (MyD88 and TRIF dependent) and type I IFN production (IFN α/β) (TRIF dependent). DC, MyD88 and TRIF are all required for TH1 CD4+ T cells induction but the exact pathway is not defined yet. GLA-SE also induces an IgG2 skewed response which is DC independent but MyD88 and TRIF dependent. Little is known about the mechanisms of actions induced by GLA-SE that will ultimately result in the observed IgG2 skewed B cell response.

SECTION II: EXPERIMENTAL RESULTS

SCIENTIFIC OBJECTIVES

A. GLA-SE ADJUVANTICITY MECHANISMS OF ACTION

The primary objective of this thesis was to broaden the knowledge that we have on the mechanisms of action of IDRI's leading adjuvant GLA-SE. Recent insight into the functioning of the innate immune system has demonstrated its important role in triggering and shaping the adaptive immune response to vaccines (189). Our main focus was then to better understand which are the main actors in GLA-SE adjuvanticity *i.e.* immune cells, cytokines, transcription factors and pathways, of the innate response to GLA-SE creating the “immune-competent” response in the LN that will eventually trigger and shape the strong adaptive response linked to this adjuvant *i.e.* promotion of a strong CD4 T_{H1} induction and a very quick augmentation of IgG2 skewed B cell response and subsequent antibody production.

Our three main hypotheses for this work were:

1. Canonical T_{H1} shaping factors, *i.e.* the transcription factor T-bet and IL-12, will be essential to the adaptive response to GLA-SE. Type I IFN, previously shown to be important for the innate response to GLA-SE (12) would be also important for the subsequent adaptive response.
2. Lymph node (LN) sub-capsular macrophages will take-up GLA-SE and be critical for its adjuvanticity.
3. B cells antigen-presenting (APC) function will have a non-redundant role in the shaping to the T_{H1} response to GLA-SE.

In the first study we used T-bet^{-/-} and IL-12^{-/-} mice and a blocking IFN α R1 monoclonal antibody to characterize the requirement of the transcription factor T-bet and the cytokines IL-12 and type I IFN for the adaptive response to GLA-SE. The requirement of type I IFN signaling for the initiation of the response was also evaluated.

In the second study we used GLA-SE labelled with the lipophilic tracer DiD (1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) to characterize the main immune cells interacting with GLA-SE in the LN very early after immunization. Subsequently, those cells were depleted and their requirement for the subsequent innate and

adaptive responses investigated. The requirement of the IL-18 cytokine was also investigated using IL-18R^{-/-} mice.

In the third study we focused in the function of B cells in the induction of T_{H1} response and more specifically in the induction of a long lasting T_{H1} response. Using B-cell depleted mice and B and T cell transfers the role of antigen presentation by B cells in the generation of memory precursor T_{H1} effector cells was elucidated.

B. ID93/GLA-SE AS AN IMMUNOTHERAPEUTIC VACCINE

A secondary objective of this thesis was to investigate the possibility of using ID93/GLA-SE as adjunct therapy to existing antibiotic treatments to reduce relapse rates after chemotherapy treatment. We hypothesize that ID93/GLA-SE would reduce relapse rates in a suboptimal drug treatment model. A mouse model of relapse using a sub-optimal, short duration antibiotic treatment was defined and the capacities of ID93/GLA-SE post-exposure to better the post-chemotherapy outcome was assessed in three different mouse strains.

In this second section we will present the different studies, main obtained results and the resulting scientific papers. Those results will be discussed and the different perspective outlined in the third and final section of this manuscript.

- ❖ **Natasha Dubois Cauwelaert**, Anthony L. Desbien, Thomas E. Hudson, Samuel O. Pine, Steven G. Reed, Rhea N. Coler, and Mark T. Orr (2015)
“The TLR4 Agonist Vaccine Adjuvant, GLA-SE, Requires Canonical and Atypical Mechanisms of Action for T_H1 Induction”
PLoS One. 2016; 11(1): e0146372.

- ❖ Anthony L. Desbien, **Natasha Dubois Cauwelaert**, Steven J. Reed, Hilton R. Bailor, Hong Liang, Darrick Carter, Malcolm S. Duthie, Christopher B. Fox, Steven G. Reed, Mark T. Orr
“IL-18 and subcapsular lymph node macrophages are essential for enhanced B cell responses with the TLR4 agonist adjuvant GLA-SE”
Submitted

- ❖ **Natasha Dubois Cauwelaert***, Susan L. Baldwin*, Mark T. Orr, Anthony L. Desbien, Emily Gage, Kimberly A. Hofmeyer, Rhea N. Coler
“Antigen presentation by B cells guides TLR-4 mediated programming of memory CD4 T cell responses”
Submitted

ARTICLE 1: THE TLR4 AGONIST VACCINE ADJUVANT, GLA-SE, REQUIRES CANONICAL AND ATYPICAL MECHANISMS OF ACTION FOR T_H1 INDUCTION

Natasha Dubois Cauwelaert, Anthony L. Desbien, Thomas E. Hudson, Samuel O. Pine, Steven G. Reed, Rhea N. Coler, and Mark T. Orr

PLoS One. 2016; 11(1): e0146372.

PRESENTATION

In order to better define the role of different cytokines and transcription factors in initiating the immune response to GLA-SE from the naive polyclonal repertoire we evaluated the requirement of the transcription factor T-bet and the inflammatory cytokines IL-12 and type I IFN for GLA-SE adjuvanticity.

In a first section, T-bet deficient and IL-12 deficient mice were used to determine T-bet and IL-12 roles in T_H1 CD4 induction and antibodies production.

In the second section the kinetics of the IFN α cytokine production in the draining LN after GLA-SE immunization was assessed. Then, a blockade monoclonal antibody against the type I IFN receptor IFN α R1 was used to determine the requirement of type I IFN signaling in the induction of the innate response to GLA-SE including CD69 and innate IFN γ production in the LN and the subsequent adaptive response.

PRINCIPAL RESULTS

The main obtained results were:

- ❖ GLA-SE induced CD4 T cells T_H1 responses and IgG2c skewing were completely dependent upon T-bet expression.
- ❖ IL-12 was required for GLA-SE induced T_H1 responses but IgG2c skewing was IL-12 independent.
- ❖ IFN α is secreted in the LN early on after GLA-SE immunization.
- ❖ The innate response to GLA-SE immunization including, early innate IFN γ production by CD8⁺ and NK cells and CD69 induction in the LN, is dependent on type I IFN receptor I (IFN α R1) signaling.

- ❖ IFN α R1 signaling partly inhibits IL-12 production in the LN
- ❖ IFN α R1 signaling is required for the strong T_{H1} response to GLA-SE

RESEARCH ARTICLE

The TLR4 Agonist Vaccine Adjuvant, GLA-SE, Requires Canonical and Atypical Mechanisms of Action for T_H1 Induction

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Abstract

The Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant formulated in a stable emulsion (GLA-SE) promotes strong T_H1 and balanced IgG1/IgG2 responses to protein vaccine antigens. This enhanced immunity is sufficient to provide protection against many diseases including tuberculosis and leishmaniasis. To better characterize the adjuvant action it is important to understand how the different cytokines and transcription factors contribute to the initiation of immunity. In the present study using T-bet^{-/-} and IL-12^{-/-} mice and a blocking anti-IFN α R1 monoclonal antibody, we define mechanisms of adjuvant activity of GLA-SE. In accordance with previous studies of TLR4 agonist based adjuvants, we found that T_H1 induction via GLA-SE was completely dependent upon T-bet, a key transcription factor for IFN γ production and T_H1 differentiation. Consistent with this, deficiency of IL-12, a cytokine canonical to T_H1 induction, ablated T_H1 induction via GLA-SE. Finally we demonstrate that the innate immune response to GLA-SE, including rapid IFN γ production by memory CD8+ T cells and NK cells, was contingent on type I interferon, a cytokine group whose association with T_H1 induction is contextual, and that they contributed to the adjuvant activity of GLA-SE.

Introduction

Subunit vaccines, in combination with adjuvants to enhance the immune response to the target antigens, represent a significant advance in the development of better defined, easier to produce and safer vaccines. Importantly an optimal adjuvant should induce a targeted innate response to tailor the desired adaptive response needed for vaccine efficacy. To that end we have developed a number of Toll-like receptor (TLR) agonists containing adjuvants that promote T_H1 T cell responses to vaccine antigens. The most notable of these is the GLA-SE adjuvant which contains a synthetic TLR4 agonist, Glucopyranosyl Lipid Adjuvant (GLA), formulated in a stable nano-emulsion of squalene oil-in-water (SE) [1, 2]. GLA-SE drives

strong T_H1 responses to a variety of antigens that are protective against intracellular infections [2–7]. In combination with the tuberculosis vaccine fusion protein antigen ID93, GLA-SE induces a poly-functional T_H1 response characterized by CD4 T cells producing CD154, IFN γ , TNF, GM-CSF, and IL-2, as well as a humoral response skewed towards IgG2c class-switching [8–10]. In order to better understand the mechanism of action of adjuvants it is important to define the role of different cytokines and transcription factors in initiating the immune response from the naive polyclonal repertoire.

The differentiation of CD4 T cells into T_H1 effectors is orchestrated by the transcription factor T-bet [11]. This differentiation occurs in two steps: first, during the initial polarization phase, simultaneous signaling via the T cell antigen receptor TCR and the IFN γ receptor of the naive T cell results in T-bet expression which enables IL-12 receptor expression, and subsequently a second wave of T-bet expression is induced by IL-12 signaling in the absence of TCR stimulation [12–15]. T-bet induction and IL-12 production are therefore likely necessary for the potent T_H1 response induction to vaccination with GLA-SE.

Type I interferons (IFN α and IFN β) induce an antiviral state in most nucleated cells, providing protection against infection [16, 17]. Furthermore, type I IFN can shape the adaptive responses to infection (reviewed in [18]). These cytokines signal via the heterodimeric IFN α R1/2 receptor and act on both antigen presenting cells (APC) and lymphocytes to enhance maturation, proliferation and survival to a variety of stimuli [19]. In the present study, using T-bet^{-/-} and IL-12^{-/-} mice and IFN α R1 antibody blockade we demonstrate that T-bet induction, IL-12 production and IFN α R1 signaling are necessary for the adjuvant activity of GLA-SE and that IFN α R1 signaling is also crucial for the early innate response initiation to this adjuvant.

Results

GLA-SE adjuvant activity is dependent on T-bet expression and IL-12 production

GLA-SE, a synthetic TLR4 agonist formulated in a stable nano-emulsion of squalene oil induces a strong T_H1 response to vaccine antigens that otherwise elicit minimal cellular immune responses with a T_H2 bias [1, 9, 10, 20]. IL-12 is important for T_H1 induction with LPS, another TLR4 agonist, and monophosphorylated lipid A (MPLA), a detoxified derivative of LPS [21, 22]. Mouse and human dendritic cells stimulated with GLA produce IL-12 in a MyD88 and TRIF dependent manner [2, 9]. To determine whether IL-12 production and/or T-bet expression are important for GLA-SE driven antibody and CD4 T cell responses, we immunized wild type (B6) or IL-12 or T-bet deficient mice with GLA-SE and the recombinant antigen ID93. Both T-bet and IL-12 were essential for T_H1 induction as indicated by CD4⁺ T cells production of CD154, IFN- γ , or TNF upon stimulation with ID93 (Fig 1A). Following immunization with ID93+GLA-SE induction of a strong IgG2c skewed response to ID93 was completely dependent on T-bet expression, but, surprisingly, not on IL-12 (Fig 1B). These data suggest discordance between an IL-12 dependent induction of IFN γ -producing CD4 T cells and an IL-12 independent class-switching to IgG2c, with T-bet being essential for both.

GLA-SE induces IFN α *in vivo*

GLA induces transcription of type I IFN by human dendritic cells *in vitro*. *In vivo* induction of CD69 on T cells by GLA-SE is partially dependent on cell-intrinsic expression of type I IFN receptor [2, 23]. Type I IFN are TRIF-dependent cytokines produced by TLR4 stimulation with GLA or similar agonists [2, 24, 25]. MyD88 and TRIF are both necessary for GLA-SE

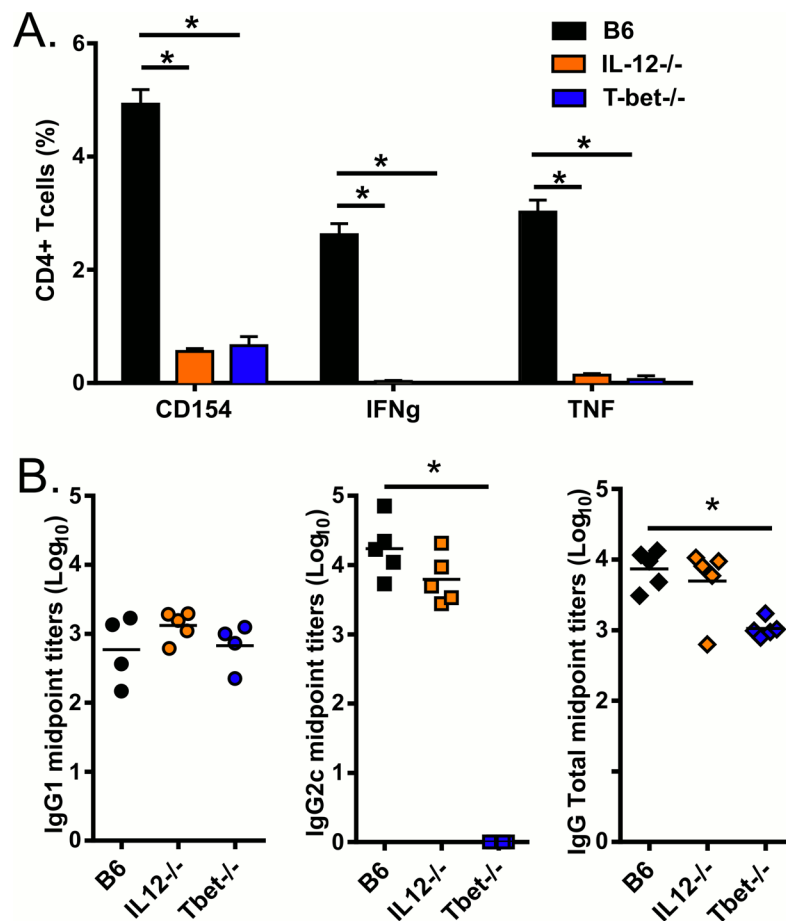


Fig 1. ID93+GLA-SE T_H1 response is dependent on T-bet and IL-12 whereas antibody production is only dependent on T-bet. B6, IL12^{-/-} and T-bet^{-/-} mice were immunized with ID93+GLA-SE. (A) One week after boost, splenocytes were isolated and stimulated with ID93. CD4 T cells were analyzed for the production of CD154, IFN-γ, and TNF. (B) Sera were collected three weeks after the first immunization and serially diluted to assess levels of anti-ID93 IgG1, IgG2c and IgG Total. Data are shown as mean ± SEM of N = 4–5 animal/group and are from one experiment representative of two experiments performed. Statistics by two-way ANOVA with Dunnett's correction for multiple comparison test within IgG and cytokine groups versus B6; *p ≤ 0.05.

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adjuvant activity [9]; suggesting a role for type I IFN in the T_H1 differentiation induction by GLA-SE. To determine whether GLA-SE induced type I IFN *in vivo*, we measured IFNα levels in the draining lymph node (LN) and sera shortly after immunization with GLA-SE. IFNα levels in the draining LN peaked between 6 and 24 hours and returned to almost undetectable levels at 48 hours (Fig 2). No IFNα was detected in the serum indicating that the response was highly localized to draining LNs (Fig 2).

Type I IFN receptor signaling is critical during the innate response to GLA-SE

Type I IFN drive expression of the cell surface activation marker CD69 which in turn facilitates trapping of naïve lymphocytes in the draining LN with antigen-loaded APCs [26]. Immunization with GLA-SE induces CD69 on lymphocytes, independent of antigen-specificity and this induction is partially dependent on cell intrinsic expression of type I IFN receptor as well as

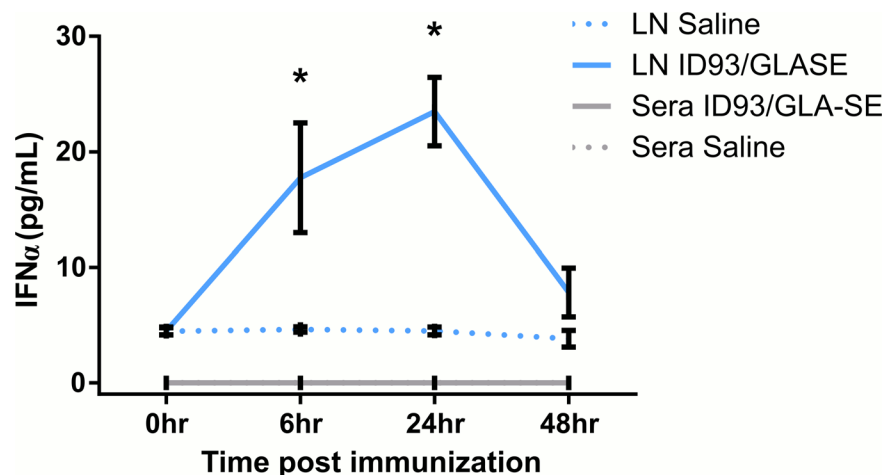


Fig 2. IFN α is produced early after GLA-SE immunization. B6 mice were immunized with saline or ID93 +GLA-SE. Sera and draining inguinal LNs were collected 0, 6, 24, or 48 hours later and analyzed for IFN α protein expression by ELISA. Data are shown as mean \pm SEM and are the combined results of two independent experiments with similar results with 3 or 4 mice/group. Statistics by unpaired t test; * $p \leq 0.05$ compared to Saline group.

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innate phase production of IFN γ . Similar to our previous findings [23] immunization with GLA-SE drove a transient expression of CD69 on the total lymphocyte population in the draining LN, which was dependent on type I IFN signaling (Fig 3A). GLA-SE immunization also produced a strong burst of IFN γ in the draining LN 6hr after immunization which returned to baseline by 24 hours (Fig 3B). This was also dependent on type I IFN as blocking the IFN α R1 ablated most of the IFN γ production (Fig 3B).

The occupancy of IFN- γ receptor, and therefore the amount of IFN γ present on the surface of cells, can be determined by staining with the empty-IFN γ receptor-specific antibody clone GR-20 [14]. Correlating with our IFN γ production results, 12hrs after immunization the empty-IFN γ receptor staining on lymphocytes from draining LNs was substantially reduced in mice treated with GLA-SE compared with contralateral LNs. IFN α R1 blockade preserved empty-IFN γ receptor staining confirming that the early IFN γ burst depends on IFN α R1 signaling (Fig 3C). This early burst of IFN γ at 6 hr was produced by both memory CD8 T cells and NK cells (Fig 3D and 3E). This is likely driven by IL-18 expression which is induced by GLA-SE and necessary for the adjuvanticity of GLA-SE [23]. IFN γ production by both cell types was strongly reduced in mice that were previously treated with anti-IFN α R1 (Fig 3D and 3E). These results indicate that type I IFN, in sequential manner, was necessary for the early innate production of IFN γ by CD8+ T cells and NK cells in response to GLA-SE.

Concurrent with the early IFN γ production there is an increase in IL-12 production in the draining LN following GLA-SE immunization as early as 6 hours post immunization which is sustained for at least 2 days and is critical for the adjuvant activity of GLA-SE (Figs 1 and 3F). Surprisingly IFN α R1 blockade augmented the levels of IL-12 in the draining LN despite IFN γ levels being lower in the treated animals at the same time-points (Fig 3F). These results suggest that type I IFN production inhibits early IL-12 production, which is supported by prior reports [27–30]. Overall the innate activity of GLA-SE is strongly dependent on type I IFN which is necessary for lymphocyte activation, controls IL-12p40 production and promotes early IFN γ production, a feature associated with T-bet expression in T cells, and subsequently T_H1 induction [31].

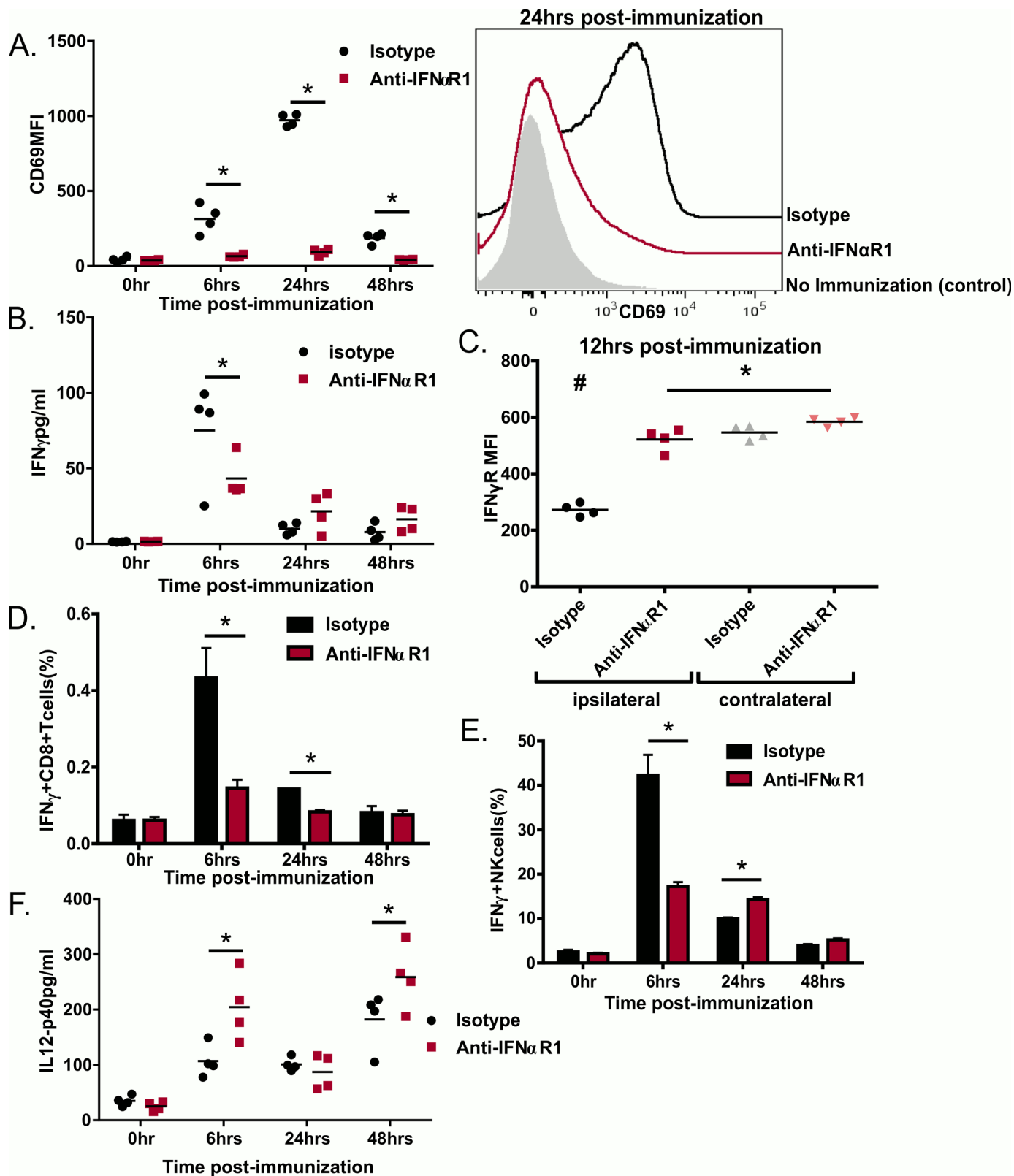


Fig 3. IFN α R1 signaling is essential for lymphocyte activation and IFN γ production upon immunization with ID93+GLA-SE. B6 mice were treated with IFN α R1 antibody or its isotype and immunized with ID93+GLA-SE. Innate responses were assessed in the ipsilateral draining LN or in the contralateral LN by flow cytometry on LN cells or ELISA on LN supernatant. The results are shown for the ipsilateral LN when not stated otherwise. (A) MFI and

representative histograms of CD69 staining (B) IFN γ production assessed by ELISA, (C) IFN γ R occupancy as indicated by decreased MFI of IFN γ R staining with the monoclonal antibody GR-20 at 12 hrs after immunization. (D) IFN γ staining on CD8⁺ T cells. (E) IFN γ staining on NK cells. (F) IL-12 production assessed by ELISA. Data are shown as mean \pm SEM of N = 4 animal/group and are from one experiment. Statistics by one-way ANOVA, * $p \leq 0.05$ compare to anti-IFN α R1 treated animals, # $p \leq 0.05$ compare to all the other groups.

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GLA-SE T_H1 induction and T_H2 counter-regulation is partially dependent on type I IFN production

To further investigate the role of type I IFN in the adaptive response elicited by immunization with GLA-SE and ID93, we blocked IFN α R1 and examined the cellular and humoral responses one week after immunization. IFN α R1 blockade reduced the frequency of antigen-specific CD4 T cells as measured by CD154 expression upon stimulation (Fig 4A). Further, T_H1 responses were reduced and T_H2 responses enhanced, as measured by antigen induced production of IFN γ , TNF and IL-5 (Fig 4A). In parallel, samples were stained for ID93-specific CD4 T cells via peptide-MCHII tetramer (Tet) staining. Consistent with CD154 expression upon stimulation there was a significant reduction in the frequency of Tet⁺ CD4 T cells from the IFN α R1 blockade group (Fig 4B). Congruent with the reduction in IFN γ observed after antigen stimulation, the remaining Tet⁺ T cells from the IFN α R1 blockade group had lower levels of T-bet staining (Fig 4C). The reduction in T_H1 skewing correlated with modestly decreased isotype switching to IgG2c in ID93-specific antibody titers (Fig 4D). Taken together these results suggest that GLA-SE induced type I IFN promote T_H1 responses by enhancing CD69 expression and increasing innate IFN γ production.

Discussion

GLA-SE is a clinical stage vaccine adjuvant that robustly augments T_H1 immunity yet its mode of action is still incompletely understood. In this study we assessed the contribution of T-bet, IL-12 and IFN α R1 signaling to the adjuvant activity of GLA-SE. We show that T-bet is required for IgG2c isotype skewing as well as T_H1 induction by GLA-SE. Thereupon we sought to investigate the role of the pro-inflammatory cytokine IL-12 and, surprisingly, found that IgG2c class-switching was not impaired even though production of IFN- γ secreting CD4 T_H1 cells was ablated. Finally, our results suggest that type I IFN, which are induced early after immunization with GLA-SE, are necessary for an optimal innate response including rapid lymphocyte activation and IFN γ expression by NK cells and memory CD8 T cells and, consequently, the induction of T_H1 immunity.

IL-12 is a key factor that drives T_H1 responses and IFN γ production [23, 32–35]. However, the relevance of IL-12 *in vivo* depends on the nature of the infection or immunization. In IL-12-deficient mice, T_H1-type responses were generated upon immunization with inactivated MHV, demonstrating the existence of an IL-12 independent T_H1 induction that has been since then described in several models of viral infection and immunization [36–41]. Conversely, IL-12 has been shown to be important for T_H1 induction by the TLR4 agonists LPS and MPL, although antibody production was not evaluated in those papers [21, 22]. Using IL-12^{-/-} and T-bet^{-/-} mice, we demonstrated that IL-12 is needed for the CD4 T_H1 induction by GLA-SE but not for IgG2c skewing, whereas T-bet is necessary for both. Since B-cell intrinsic T-bet has been shown to mediate early IgG2c production [42] further experiments will be needed to determine if T-bet expression upon ID93-GLA-SE vaccination is required in the CD4 T cells to promote IgG2c skewing. Furthermore we have previously shown that MyD88 and TRIF are both necessary for GLA-SE T_H1 skewing capacity as well as IL-12 production by BMDC, DC and macrophages [9]. This suggests that IL-12 production might be a necessary point of intersection for the previously demonstrated MyD88- and TRIF-dependent induction of T_H1

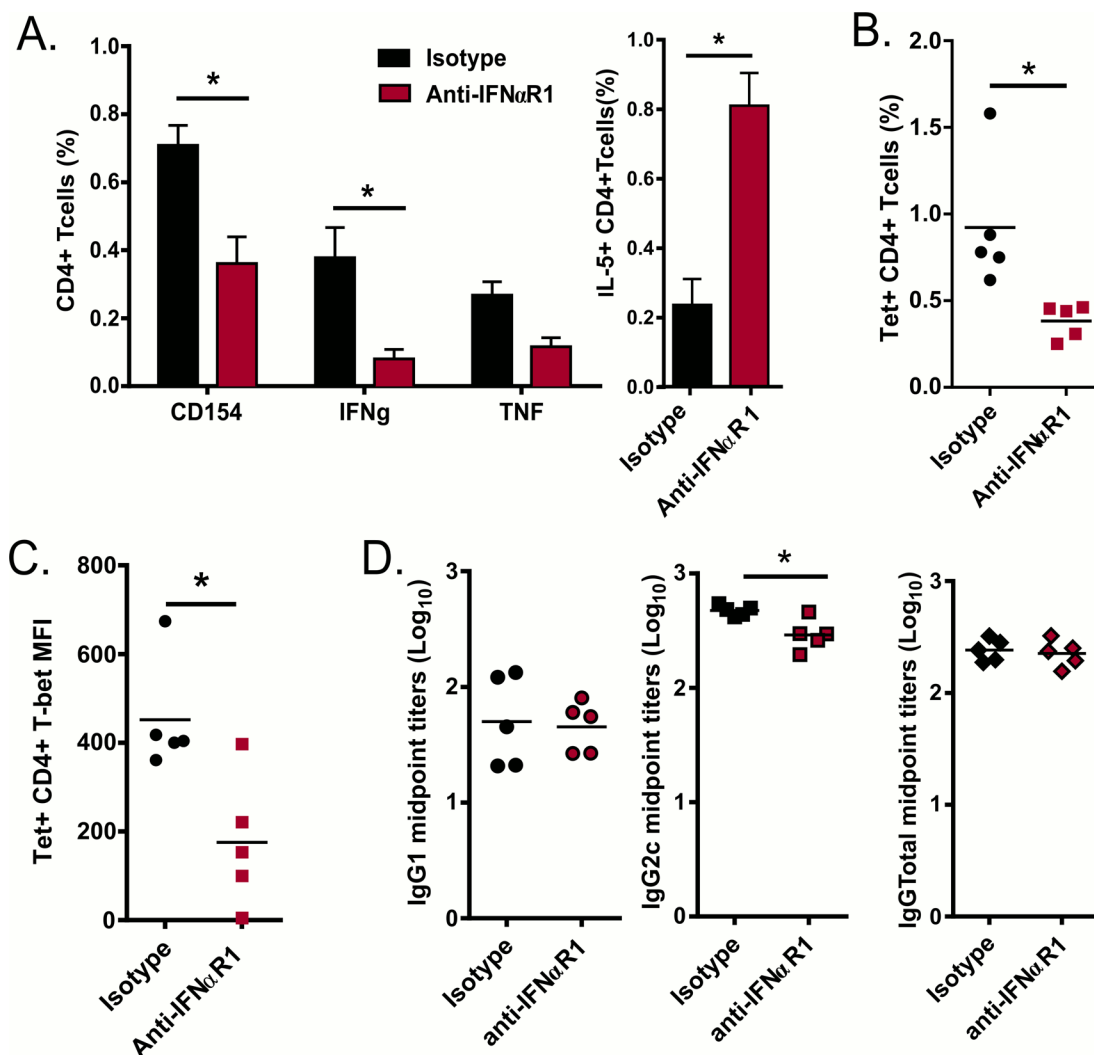


Fig 4. IFN α R1 signaling contributes to T_H1 skewing. B6 mice were treated with IFN α R1 antibody or its isotype and immunized with ID93+GLA-SE and responses were analyzed one week after prime. (A) ID93 stimulated splenocytes were analyzed for the production of CD154, IFN γ , TNF and IL-5 by CD4 $^{+}$ T cells. (B) One week after prime CD4 $^{+}$ T cells were isolated and stained with an I-A b tetramer presenting the dominant epitope for Rv3619 and analyzed for T-bet induction. (C) Sera were collected one week after prime and serially diluted to assess levels of anti-ID93 IgG1 and IgG2c. Data are shown as mean \pm SEM of N = 5 animal/group and are from one experiment representative of two experiments performed except for IL-5 and T-bet levels measurement which were only done once. Statistics by simple or multiple t test corrected for multiple comparisons using the Holm-Sidak method between IgG or cytokine groups; * $p \leq 0.05$

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responses by ID93+GLA-SE immunization. Moreover IL-18 has been shown to synergize with IL-12 to induce IFN γ in T cells [34, 35]. IL-18 is produced very early upon immunization with GLA-SE and IL-18R $^{-/-}$ mice have a reduced antigen-specific T_H1 response [23]. We hypothesize that this IL-18/IL-12 synergy is required for GLA-SE-induced T_H1 responses.

Type I IFN links the innate and adaptive immune responses. They cause APC maturation *in vitro* while inhibiting secretion of T_H2 cytokines leading to IFN γ producing T cells [43–45]. Type I IFN inhibit the death of CD4 activated T cells and their key role in priming adaptive T cells has been demonstrated in a wide variety of immunization schemes [46, 47]. Recombinant type I IFN given *in vivo* has direct adjuvant activity, enhancing both antibody and T cell responses [48, 49]. TLR agonist and non-TLR agonist require type I IFN activity to exert their adjuvant activities [18, 48, 50]. In some cases this may be due to IFN α R signaling altering the

expression of some TLRs [43]. Furthermore Longhi *et al* observed an IL-12 independent impairment of T_H1 responses to poly (I:C) when blocking IFN α R1 and in IFN α R^{-/-} mice [39]. Thus it has been hypothesized that robust type I IFN induction may be necessary for effective adjuvant activity [51]. However other adjuvants including MF59 and Pam3CSK (TLR2) do not elicit type I IFN and do not require IFN α R signaling to enhance antibody responses [50].

Our results show that that type I IFN is critical for the adjuvanticity of GLA-SE. Type I IFN was required for maximal response to this adjuvant illustrated by defects in early innate responses as indicated by reduced CD69 expression and defects in innate IFN γ production by CD8⁺ T cells and NK cells. CD69 expression inhibits lymphocytes egress from the LN which is a mechanism to retain useful clonal specificities in the activated LN [26, 52]. Early IFN γ produced by innate cells upon immunization with GLA-SE and other adjuvants is critical for CD4 T cells T_H1 polarization in the LN [13, 15, 23]. IFN α R1 blockade caused multiple defects in T_H1 adaptive responses including: (1) reduction in the frequency of antigen-specific cells determined by CD154 production or tetramer staining, (2) reduction in T_H1 skewing measured by IFN γ or TNF production and lower amounts of the T_H1 controlling transcription factor Tbet in the remaining tetramer positive cells, and (3) enhanced frequencies of CD4 T cells that produced IL-5, a hallmark of T_H2 differentiation.

In some settings type I IFN is necessary for IL-12 production by murine and human dendritic cells [53, 54] but they also have been shown to decrease IL-12 production in other studies [27–30]. IFN α R1 blockade increases IL-12 production early after immunization with GLA-SE but diminished IFN γ production at the same time indicating a complex regulation of IFN γ upon vaccination. We hypothesize that type I IFN driven IFN γ is necessary to increase expression of the IL-12R on CD4 T cells, thus making them responsive to IL-12. Alternatively production of the IL-12p35 subunit to form active IL-12p70 may be dependent on IFN α R1 signaling. In addition to their role in stimulating APCs to enhance CD4 responses, type I IFN can also have a direct effect on T cells. CD8 T cells require direct type I IFN signals for their expansion in response to some infections [55]. Likewise direct type I IFN action on CD4 T cells is important for clonal expansion *in vivo* following LCMV, but inhibitory during *Listeria* infection [56] showing that the priming milieu determines the extent to which CD4 T cells are dependent on direct signal mediated by type I IFN. Conversely CD4 T cell responses are dependent on type I IFN in a cell extrinsic manner in response to polyIC:CD40 vaccination [57]. Interestingly deletion of IFN α R2 on T cells significantly diminished CD69 expression induced by GLA-SE at 18h after immunization suggesting a dependence on T cell intrinsic expression of the type I IFN receptor for GLA-SE innate response induction [23]. Based on our results we suggest a model where GLA-SE induces IFN α to trap cells in the LN by CD69 expression and to augment the early innate IFN γ production by CD8⁺ T cells and NK cells. In parallel GLA-SE induces production of IL-12 *in vivo* which synergizes with IFN α effects to promote Tbet expression and T_H1 commitment. Understanding the mechanism by which adjuvants engage the immune responses is critically important for development of vaccines. Our results suggest that early type I and II IFNs are signatures of the optimal innate response to the GLA-SE adjuvant, predicting subsequent T_H1 responses. Further research is warranted to determine if this early type I IFN induction can be used as an early gating strategy in developing new adjuvants or as a signature of adjuvanticity in human clinical trials.

Materials and Methods

Ethics statement

The study was conducted under protocols approved by the Infectious Disease Research Institute Institutional Animal Care and Use Committee.

Animals

6–8 week old female C57BL/6 (B6), IL-12^{-/-} and T-bet^{-/-} mice were purchased from Jackson Lab and maintained in Specific Pathogen Free conditions. All animal study protocols were approved by the IDRI Institutional Animal Care and Use Committee (IACUC) and were performed according to IACUC regulations and guidelines.

Immunizations and IFN α R1 blockade

Mice were immunized with 0.5 μ g of ID93 recombinant protein [8] formulated in 5 μ g of GLA (Avanti Polar Lipids) in IDRI's stable emulsion (SE) [2] by intramuscular injection. For IFN α R1 blockade, 1 mg of anti-IFN α R1 antibody (BioXCell, clone MAR1-5A3) or its IgG1 isotype control (BioXCell, clone MOPC-21) in PBS were injected i.p. 6h prior to immunization and on day 3 after immunization.

IFN α ELISA

At indicated times following immunization peripheral blood and inguinal LNs were collected and LNs were homogenized in 500 μ L PBS. Total production of IFN α was assessed using the Mouse IFN α ELISA kits (R&D Systems) according to the manufacturer's instructions. Concentrations below the limit of detection were reported as 0.

Antibody responses

Mouse sera were prepared by collection of retro-orbital blood into microtainer serum collection tubes (VWR International, West Chester, PA), followed by centrifugation at 10,000 rpm for 5 minutes. Each serum sample was then analyzed by antibody capture ELISA. Briefly, ELISA plates (Nunc, Rochester, NY) were coated with 2 μ g/ml recombinant antigen ID93 in 0.1 M bicarbonate buffer and blocked with 1% BSA-PBS. Then, in consecutive order and following washes in PBS/Tween20, serially diluted serum samples, anti-mouse IgG1 or IgG2c-HRP (all Southern Biotech, Birmingham, AL) and ABTS-H₂O₂ (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added to the plates. Plates were analyzed at 405nm (ELx808, Bio-Tek Instruments Inc, Winooski, VT).

Splenocytes recalls and intracellular cytokine staining

Splenocytes were isolated from three to five animals per treatment regimen. Red blood cells were lysed using Red Blood Cell Lysis Buffer (eBioscience) and resuspended in RPMI 1640, 10% FBS. Total viable cells were enumerated using ViaCount assay with a PCA system (Guava Technologies), plated at 2x10⁶ cells/well in 96-well plates and stimulated for 2 hours with media or ID93 (10 μ g/mL) at 37°C. GolgiPlug (BD Biosciences) was added and the cells were incubated for an additional 8 hours at 37°C. Cells were washed and surface stained with fluorochrome labeled antibodies to CD4 (clone GK1.5) and CD8 (clone 53–6.7) (BioLegend and eBioscience) in the presence of 20% normal mouse serum for 20 minutes at 4°C. Cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at room temperature. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome labeled antibodies to CD154 (clone MR1) IFN- γ (clone XMG-1.2), TNF (MP6-XT22) and IL-5 (TRFK5) (BioLegend and eBioscience) for 20 minutes at room temperature. Cells were washed and resuspended in PBS. Up to 10⁶ events were collected on a four laser LSRFortessa flow cytometer (BD Biosciences). Cells were gated as singlets > lymphocytes > CD4⁺ CD8⁻ > cytokine positive. ID93-specific response frequencies were determined by subtracting the frequency of response positives of unstimulated cells from ID93 stimulated cells in matched samples.

For T-bet staining, CD4 T cells were stained with an I-A^b tetramer presenting the dominant epitope for Rv3619 [20] in the presence of 20% normal mouse serum for 1h at 37°C. APC labeled tetramers were provided by the NIH Tetramer Core Facility. Cells were washed and surface stained with antibodies to CD4 (clone GK1.5), CD8 (clone 53–6.7), CD19 (clone 1D3), Ly6G (clone 1A8), Ter119 (clone TER-119), F4/80 (clone BM8), CD11b (clone M1/70) and CD11c (clone N418) (Lin) (BioLegend and eBioscience). Cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at room temperature. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome labeled antibodies to T-bet (clone 4b10) overnight at 4°C. Cells were washed and resuspended in PBS. Up to 10⁷ events were collected on a four laser LSRFortessa flow cytometer (BD Biosciences). Cells were gated as singlets > lymphocytes > CD4+ Lin- > Tetramer+. Data were analyzed with FlowJo v9.8.

LN cell staining and cytokine detection

Inguinal LN were collected in PBS 0.5%BSA with protease inhibitors and 10μg/mL BrefeldinA on ice and mechanically homogenized in PBS. Cells were surface stained with the labeled antibodies to IFNγR (clone GR20), CD69 (clone H1.2F3), CD8 (clone 53–6.7), CD90.2 (clone 30-H12) and NK1.1 (clone PK136) (BioLegend and eBioscience) in the presence of 20% normal mouse serum for 20 minutes at 4°C. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome labeled antibodies to IFNγ (clone XMG1.2) overnight at 4°C. Cells were washed twice with Perm/Wash (BD Biosciences) washed and resuspended in PBS. Up to 10⁶ events were collected on a four laser LSRFortessa flow cytometer (BD Biosciences). Lymphocytes were gated as singlets > lymphocytes (based on SSC x FSC) and CD69 and IFNγR MFI were assessed. CD8+ T cells were gated as singlets > lymphocytes > CD90.2+ CD8+ and NK cells were gated as singlets > lymphocytes CD8- NK1.1+. Data was analyzed with FlowJo v9.8.

Statistical methods and figures

Statistical analyses and figures were performed using Prism software (GraphPad Software, Inc., La Jolla, CA). T-test were used when comparing one group against another and one-way or two ways ANOVA analyses were used when more than two groups were compared over. Non-normal data sets were log-transformed prior to analysis. Statistical significance was considered when the p-values were <0.05 and noted * or # when the group was statistically different to all the other groups.

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The authors declare no financial or commercial conflicts of interest.

Author Contributions

Conceived and designed the experiments: NDC ALD TEH SOP MTO. Performed the experiments: NDC TEH SOP MTO. Analyzed the data: NDC ALD TEH SOP MTO. Wrote the paper: NDC ALD SGR RNC MTO.

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ARTICLE 2: IL-18 AND SUBCAPSULAR LYMPH NODE MACROPHAGES ARE ESSENTIAL FOR ENHANCED B CELL RESPONSES WITH THE TLR4 AGONIST ADJUVANT GLA-SE

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(Submitted)

PRESENTATION

In this second study the main objective was to define the primary cells interacting with GLA-SE early after immunization and characterize the requirements of those cells in the innate and adaptive response to this adjuvant.

A DiD-labelled GLA-SE was used to track the adjuvant in the LN 15 mins after immunization and microscopic and flow cytometry analysis revealed that the LN subcapsular macrophages were the primary immune cell population uptaking GLA-SE early after immunization. Clodronate loaded liposomes were then used to deplete subcapsular macrophages (SCM ϕ) from the draining LN. Using this technique, the requirement of those cells for the induction of the innate response to GLA-SE including inflammatory cytokines and chemokines' induction and CD69 upregulation in the LN was assessed. Further we used clodronate treated mice to assess the role of the subcapsular macrophages in the induction of an adaptive response to GLA-SE namely B cells, antibodies and T_{H1} responses promotion. Finally, using IL-18R^{-/-} mice we define the requirement of the IL-18 inflammatory cytokine to the adaptive response induced by GLA-SE.

PRINCIPAL RESULTS

The main obtained results were:

- ❖ CD169⁺ SIGNR1⁺ subcapsular medullary macrophages are the primary cells that are associated with GLA-SE very early after immunization (15mins).
- ❖ SCM ϕ are critical to early GLA-SE capture and subsequent distribution in the draining LN.
- ❖ SCM ϕ are essential for the innate response to GLA-SE immunization, including production of early IFN γ , IL-18 and a number of chemoattractants and CD69 induction in the LN.
- ❖ SCM ϕ are required for GLA-SE induced CD4 T_{H1} responses and B cell responses.
- ❖ IL-18 also contributes to GLA-SE induced CD4 T_{H1} responses and B cell responses.

IL-18 and subcapsular lymph node macrophages are essential for enhanced B cell responses with the TLR4 agonist adjuvant GLA-SE

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Abbreviations used:

GLA, Glucopyranosyl lipid adjuvant

SE, squalene oil-in-water emulsion

SCM ϕ , subcapsular macrophage

pMHCII, peptide-major histocompatibility complex II tetramer

IL-18R1^{-/-}, IL-18 receptor 1 deficient

Running title: SCM ϕ are required for B cell responses to adjuvant

Abstract

Designing modern vaccine adjuvants depends on understanding the cellular and molecular events that connect innate and adaptive immune responses. The synthetic TLR4 agonist GLA formulated in a stable emulsion (GLA-SE) augments both cellular and humoral immune responses to vaccine antigens. Early after injection, GLA-SE induces substantially more antigen-specific B cells, higher serum antibody titers and greater numbers of T follicular helper (T_{FH}) and T_{H1} cells than alum, the squalene-in-water emulsion (SE) alone, or GLA without SE. GLA-SE augments antigen-specific B cell differentiation into germinal center and memory precursor B cells as well as pre-plasmablasts that rapidly secrete antibodies. $CD169^+$ $SIGIRR1^+$ subcapsular medullary macrophages are the primary cells to take up GLA-SE after immunization and are critical for the innate immune responses, including rapid IL-18 production, induced by GLA-SE. Depletion of subcapsular macrophages (SCM ϕ) or abrogation of IL-18 signaling dramatically impairs the antigen-specific B cell and antibody responses augmented by GLA-SE. Depletion of SCM ϕ also drastically reduces the T_{H1} but not T_{FH} response. Thus the GLA-SE adjuvant operates through interaction with IL-18-producing SCM ϕ for the rapid induction of B cell expansion and differentiation, antibody secretion and T_{H1} responses, whereas augmentation of T_{FH} numbers by GLA-SE is independent of SCM ϕ .

Introduction

Augmentation of antigen-specific B cell responses and subsequent antibody production is central to the development of effective vaccines. In settings such as the emergence of new pandemics, intentional release of bioterror agents, and on-demand traveler's vaccines, the rapid initiation of humoral immunity with practical vaccine approaches is highly desirable. The last decade has seen the licensure of several vaccine adjuvants that augment humoral immunity, including the squalene oil-in-water adjuvants, MF59 and AS03 and the TLR4 agonist-containing adjuvants AS01 and AS04. The study of B cell responses following immunization with clinically relevant adjuvants has been primarily limited to serum antibody analyses and the identification of antibody secreting cells. Compared to the wealth of information regarding T cell and antibody responses to vaccine adjuvants, much less is known regarding the early events following immunization that induce antigen-specific B cell and T_{FH} responses and the mechanisms by which these adjuvants determine the course of these humoral responses.

The initial wave of secreted IgM and class-switched antibodies is produced by CD138⁺ pre-plasmablasts residing in extra-follicular spaces. Concurrently, CD95⁺ GL7⁺ germinal center B cells differentiate within follicles, resulting in affinity-matured antibodies, memory cells and long-lived plasmablasts. Memory B cells expressing IgM and CD38 are generated early in the response - independently of germinal centers - and are distinguished from the bulk population of naïve B cells by their antigen specificity (1). The production of a B cell response is coordinated by multiple innate and adaptive responses. The earliest encounter of foreign material in the draining lymph node (LN) can occur within minutes via cell-free transport from the infection or injection site to the draining LNs (2). This material is captured by CD169⁺ subcapsular macrophages (SCM ϕ) that reside in lymph node sinusoidal spaces, allowing delivery of material to B cell follicles (3). Subsequent T-dependent B cell differentiation is regulated by multiple subsets of CD4 T helper cells. PD1⁺ CXCR5⁺ T follicular helper cells (T_{FH}) expressing the transcription factor Bcl-6 and localized within B cell follicle are particularly important for germinal center responses and the generation of long-lived memory responses (4). T_{FH} cells, or perhaps their progenitors, have also been shown to be necessary for extra-follicular responses (5).

The TLR4 agonist glucopyranosyl lipid adjuvant (GLA) is an effective vaccine adjuvant when formulated in a squalene oil-in-water emulsion. It generates a robust T_{H1} response and

augmented antibody production skewed towards IgG2 class-switching against numerous infectious disease- and cancer-associated antigens (6). The adjuvant activity of GLA is critically dependent on its formulation. When GLA is formulated as a squalene oil-in-water emulsion (referred to as GLA-SE) compared to aqueous formulations of GLA, unique innate and adaptive features emerge. (7–9). We have previously demonstrated that the T_{H1} potentiating activity of GLA-SE is mediated in part by MyD88 and TRIF signaling, inflammatory caspases and IL-18 as well as by type I and II interferons, IL-12, and the transcription factor T-bet (8, 10, 11). In the present paper we evaluate the cellular and molecular events necessary for augmentation of B cell- antibody and T_{FH} responses by the GLA-SE adjuvant.

Results

GLA-SE induces rapid B cell responses

To better understand the B cells responses induced by GLA-SE, we analyzed antigen-specific B cells via immunization with phycoerythrin (PE) either alone or adjuvanted with GLA-SE or its constituent components, SE or GLA, a strategy previously described to identify antigen-specific B cells by flow cytometry (12, 13). We also included alum as a well-established adjuvant with which to benchmark the adjuvanticity of GLA-SE. Seven days after immunization with PE alone, very few antigen-specific B cells were detected in the draining LN (Figure 1A) or spleens (data not shown) (See Supplemental Figure 1 for PE-specific B cell gating strategy). These rare PE⁺ B cells elicited by immunization with unadjuvanted antigen were predominately IgD⁺, CD38⁺, IgM⁺ putative memory B cells (Figure 1B). Adjuvanting PE with alum, GLA or SE elicited a minor but not statistically significant increase in PE⁺ B cells with an increase in the proportion of CD95⁺, GL7⁺, IgD⁻ germinal center B cells (Figure 1A and B). In contrast GLA and SE synergized to elicit significantly more PE-specific B cells (Figure 1A). In addition to inducing germinal center and putative memory B cells, GLA-SE uniquely produced isotype switched pre-plasma blasts (B220^{lo}, CD138⁺, IgD⁻, IgM⁻) which are an early source for circulating antibody (Figure 1B). Accordingly PE-specific serum antibody titers were dramatically increased with GLA-SE compared to the other immunizations including alum (Figure 1A). Furthermore, compared to alum, GLA or SE, GLA-SE uniquely augmented the frequency of antigen-specific B cells expressing intracellular IgG2c and the IgG2c serum antibody titers (Figure 1C). Of note, at this time point (seven days after immunization) few PE⁺ B cells were detected in spleens of immunized animals regardless of

the adjuvant used, suggesting that the site of early-antibody production was predominately the LN (data not shown).

Given the importance of T_{FH} cells to humoral immunity (4) we also enumerated T_{FH} cells based on surface expression of CXCR5 and PD1 and expression of the transcription factor Bcl-6 (Supplemental Figure 2). GLA-SE induced significantly more T_{FH} cells in the draining LNs when compared to the antigen alone or adjuvanted with alum, GLA, or SE (Figure 1D). Taken together, these results suggest that the rapid and dramatic expansion of early B cell responses, especially pre-plasmablasts, antibody production and T_{FH} cells is unique to the combination GLA-SE adjuvant formulation.

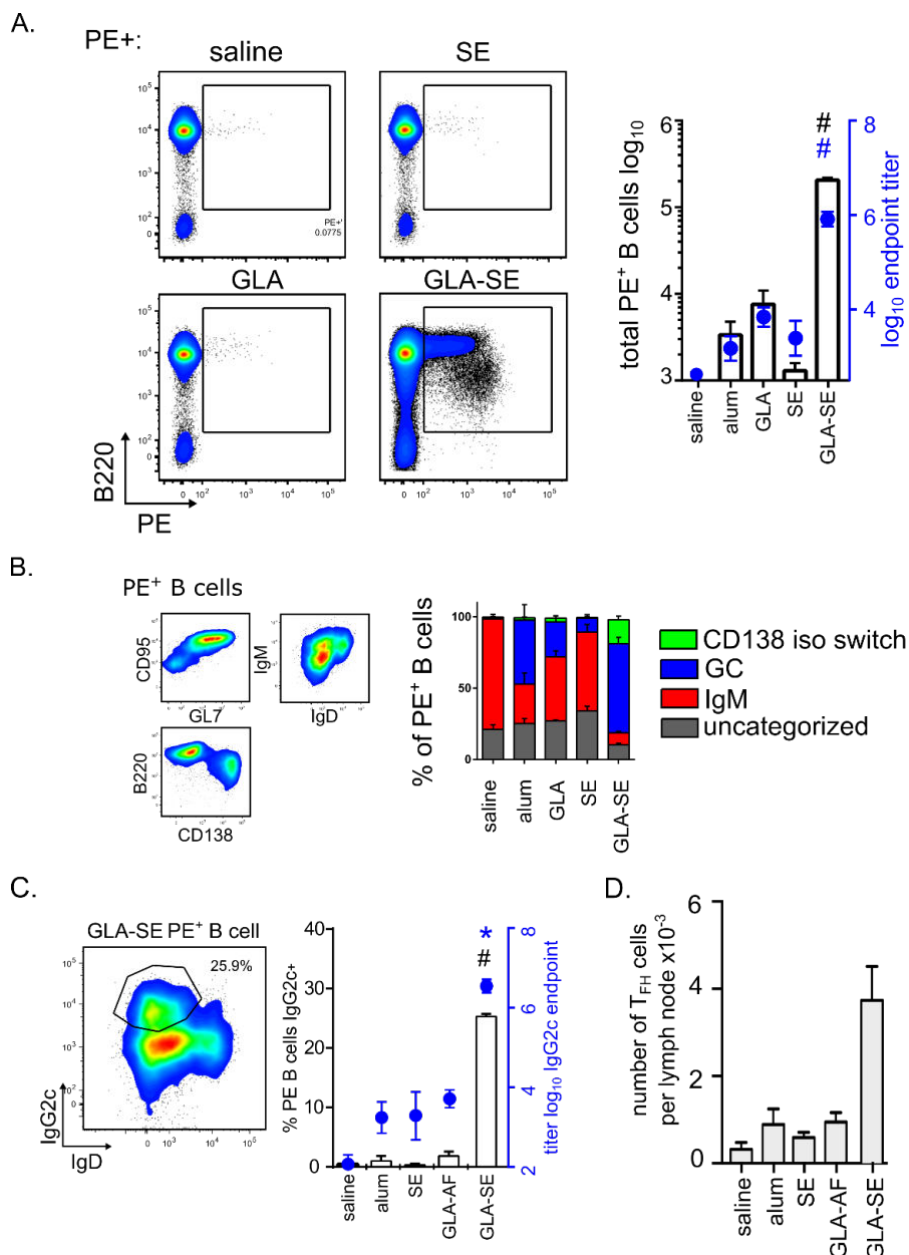


Figure 1. Rapid B cell responses elicited by different formulations of the TLR4 adjuvant GLA

B cell responses in the injection site draining inguinal and axillary LN of mice immunized with the indicated adjuvant and PE were analyzed 7 days after immunization. (A) Antigen specific B cells were enumerated by PE staining and cell populations were determined by cell surface marker staining. Total numbers of PE staining B cells in draining LNs were determined (bars). Overlaid are the total Ig (H+L) serum antibody endpoint titers (circles). (B) FACS plots of GLA-SE induced PE⁺ B cells are shown. Subsets of B cells were identified by surface marker expression as CD38⁺ IgM⁺ memory B cells (IgM), IgD⁻ CD95⁺ GL7⁺ germinal center cells (GC), CD138⁺ B220^{lo} CD38⁻ IgM⁻ IgD⁻ pre-plasmablasts (CD138 iso switch), or uncategorized. The stacked histograms represent the draining LN PE-specific B cell composition for each immunization. (C) Representative FACS plot for the identification of PE⁺ IgG2c B cells in draining LNs are shown. Frequency of IgG2c⁺ PE-specific B cells (bars) are overlaid with serum IgG2c endpoint titers (circles). (D) The numbers of T_{FH} cells per LN were determined. Bars and circles are drawn to the mean values + s.e.m p values were determined by one-way ANOVA/Dunnett's test #p < 0.001 compared with all other groups, *p<0.05 compared to saline. Data are representative of two independent experiments with three or four animals per group.

Subcapsular Mφ are essential for GLA-SE uptake

To better understand how GLA-SE augments rapid B cell responses we identified the cells interacting with GLA-SE by labeling the adjuvant with the lipophilic fluorescent dye DiD. 15 mins after injection, GLA-SE co-localized with the CD169⁺ SIGNR1⁺ medullary macrophage population (14) (Figures 2A and C and Supplemental Figures 3 and 4). This is consistent with microscopic analysis of DiD-labeled MF59 (15). Other populations including monocytes (CD11b⁺ Ly6C⁺) also took up the adjuvant, but in lesser numbers and with less intense DiD signal intensity (Figures 2C and D and Supplemental Figure 4). Of note, DiD^{dim} B cells were the most abundant DiD⁺ population by absolute numbers, although these cells represented only a small fraction of the total LN B cell population (~3%) (Figure 2D).

To determine the significance of GLA-SE capture by SCMφ including the medullary and sinusoidal macrophage subsets we depleted these cells by injection of clodronate-loaded liposomes (CLL) and waited for 6 days to allow the re-population of resident phagocytes (monocytes, DCs and neutrophils) but not the SCMφ (3) as confirmed by analysis of the cellular content of the lymph nodes of CLL-treated animals (Data not shown). SCMφ depletion dramatically impacted GLA-SE uptake by lymph-node cells with less than 0.2% of the LN cells being DiD⁺ 15 mins post-injection compared to an average of 1.5% in the mock-treated mice (Figure 2B). Surprisingly, adjuvant uptake was abrogated in cell populations that were not depleted by CLL treatment including monocytes, macrophages, DCs and T and B cells (Figures

2D). These results indicate that SCM ϕ , and most probably the medullary macrophages, are critical to early GLA-SE capture and subsequent distribution in the LN.

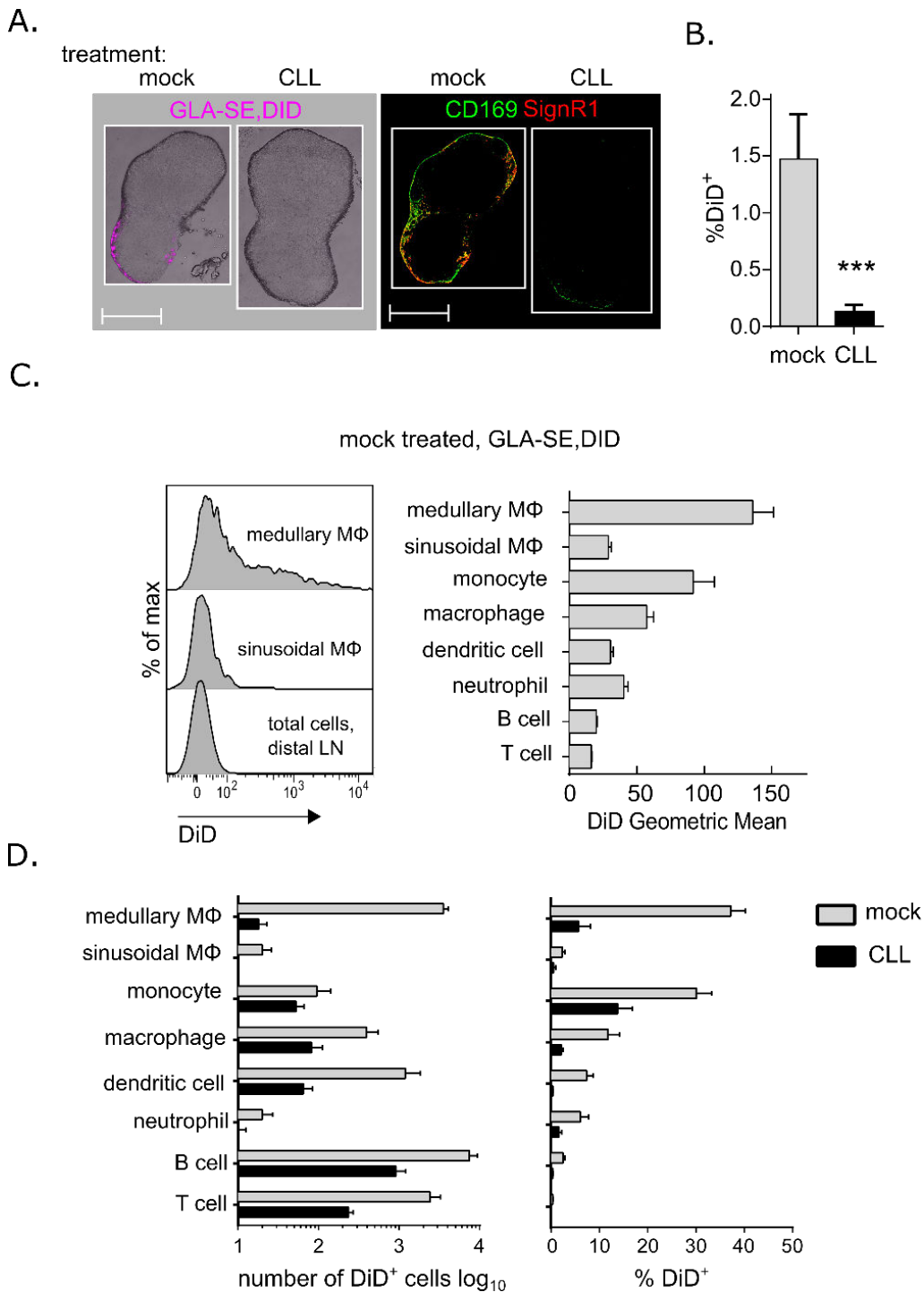


Figure 2: GLA-SE-DiD cellular uptake after SCM ϕ depletion

Mice were treated with clodronate-loaded liposomes (CLL) or mock treated with saline six days prior to injection with DiD-labelled GLA-SE or saline. Draining inguinal LN were harvested 15 minutes after immunization. (A) Immunofluorescence microscopic images of representative LN sections were taken before and after staining with CD169 and SIGNR1 to assess GLA-SE

distribution. (B) FACS analysis of total DiD staining cells per LN were determined. (C) A representative histogram of DiD-GLA-SE staining per cellular population in a mock treated DiD-GLA-SE immunized mouse is shown and DiD-GLA-SE geometric means for each cellular population were plotted. (D) The number of DiD+ cells per population and the percentage of DiD+ cells within each population was determined for mock and CLL treated groups. p values were determined by Student t test ***p<0.005. Bars are drawn to the mean + s.e.m.. Data are representative of two independent experiments with four or five animals per group.

SCM ϕ are critical for the innate response to GLA-SE

Immunization with GLA-SE initiates a complex innate immune response including production of IFN- γ which is essential for the adjuvanticity of GLA-SE (8) and a number of chemoattractants including RANTES, MIP1 α and β , Eotaxin, and CXCL10 (Figure 3A). Depletion of medullary SCM ϕ by CLL treatment severely compromised the innate response to GLA-SE (Figure 3A). CD69 expression increases upon GLA-SE immunization in a type I and II IFN-dependent manner and is thus a sensitive readout for the response to GLA-SE (8, 11). Treatment with CLL resulted in a substantial decrease in the induction of CD69 on total lymphocytes 18h after injection with GLA-SE (Figure 3B). The decrease in these early immune responses to GLA-SE after SCM ϕ depletion indicated that these cells were critical for the innate immune response to GLA-SE.

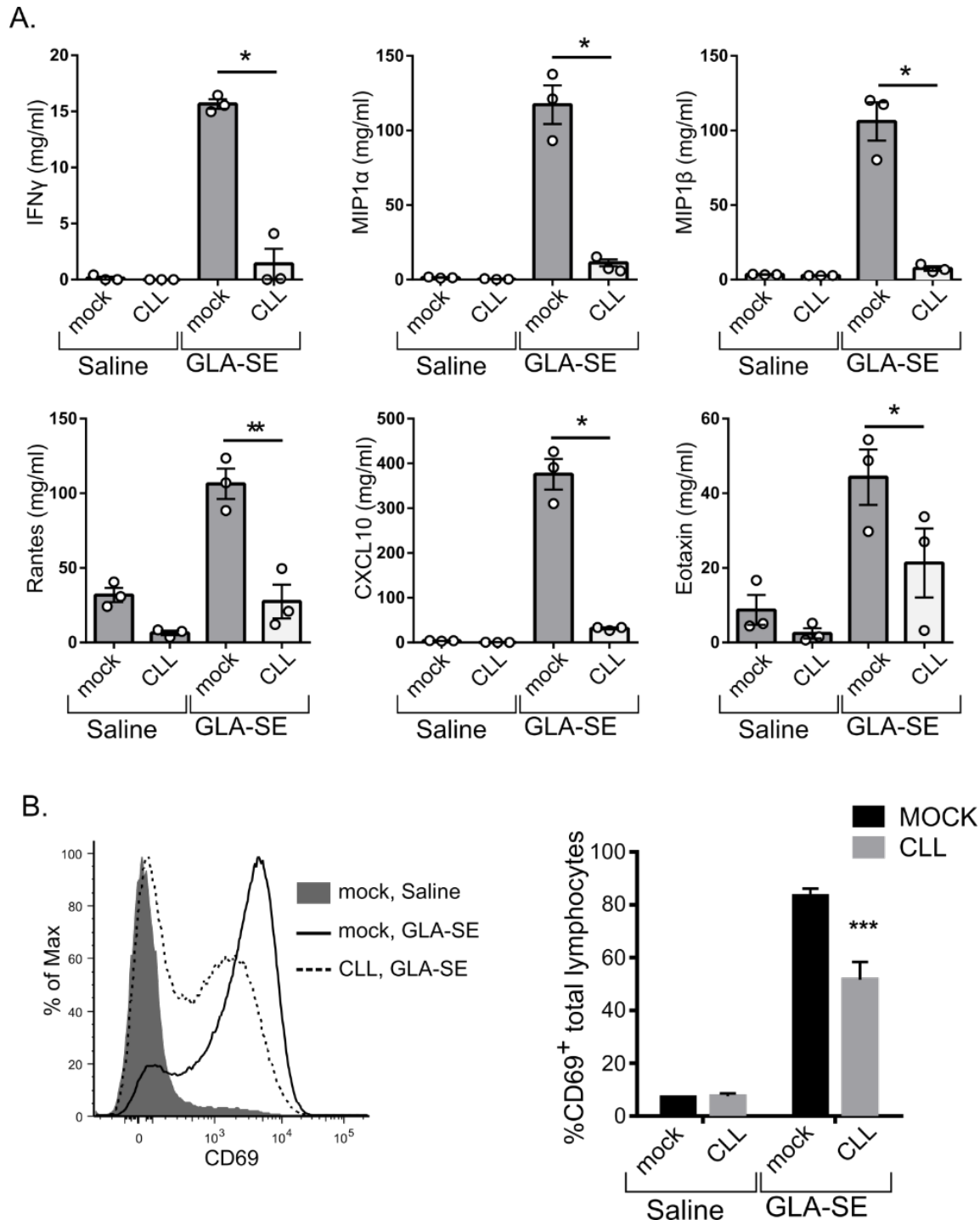


Figure 3 Innate responses to GLA-SE after SCM ϕ depletion

Mice were treated with clodronate-loaded liposomes (CLL) or saline (mock) 6 days prior to injection with GLA-SE or saline and draining LNs were harvested either 4h or 18h after immunization. (A) The concentration of cytokines and chemokines 4h after injection are plotted. (B) A representative histogram of CD69 staining on total lymphocytes 18h after injection is shown and the percentage of CD69⁺ lymphocytes is plotted. Bars are drawn to the mean + s.e.m.. p values were determined by one-way ANOVA/Dunnett's test *** p<0.001, **p<0.01, *p<0.05. Data are representative of two independent experiments with three animals per group.

SCM ϕ are necessary for B cell and T_{H1} but not T_{FH} induction by GLA-SE

To assess the contribution of the SCM ϕ to the adjuvanticity of GLA-SE, we examined the adaptive immune response to PE+GLA-SE immunization in CLL-treated mice. Seven days after immunization, the number of PE-specific B cells were severely diminished by CLL treatment (Figure 4A). PE-specific Ig (H+L) and IgG2c serum titers were reduced as well (Figure 4B). This demonstrated that SCM ϕ play a central role in the B cell expansion and the antibody responses elicited by immunization with GLA-SE. Surprisingly, we observed no difference in the total numbers of T_{FH} cells in the draining LNs between mock treated and the CLL-treated mice in response to PE+GLA-SE immunization (Figure 4C).

To determine whether antigen-specific T_{FH} numbers are specifically impacted by CLL depletion we immunized mock or CLL-treated mice with the recombinant TB antigen ID87 which contains the Ag85B P25 epitope (16) and analyzed peptide-MHCII (pMHCII) tetramer staining (Supplemental Figure 6). The number of antigen specific T_{FH} cells (pMHCII⁺,CD44⁺,CXCR5⁺,PD1⁺,Bcl-6⁺) was virtually unaffected by CLL treatment. In contrast, significantly fewer T_{H1} cells (pMHCII⁺,CD44⁺,CXCR5⁺,PD1⁺,Tbet⁺) were present in LNs from CLL treated mice compared to mock treated mice (Figure 5). These data suggest that antigen-specific T_{H1} cell numbers, not T_{FH} cell numbers, may be important for the early B cell response in GLA-SE immunized animals.

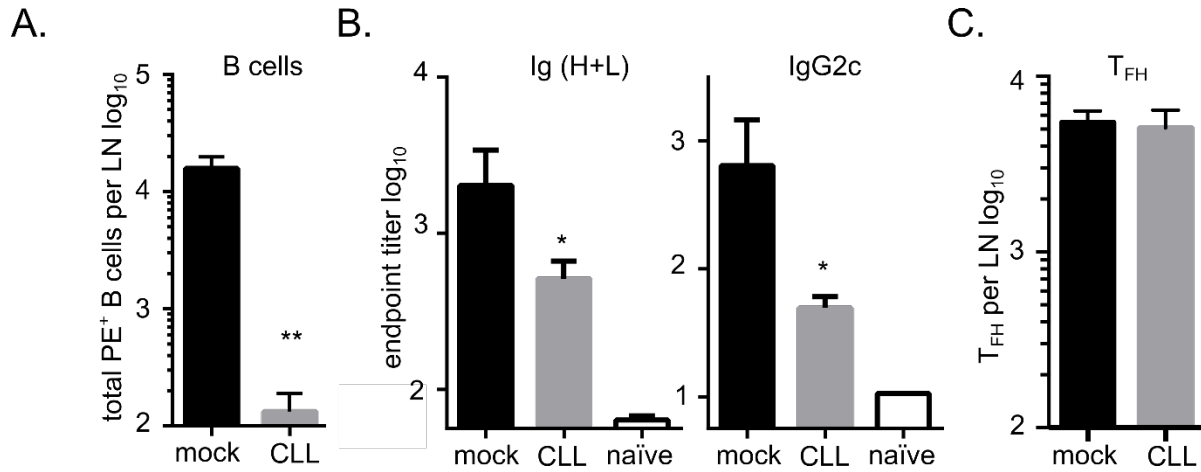


Figure 4. B cell and T_{FH} induction via GLA-SE after SCMφ depletion

Mice were treated with clodronate-loaded liposomes (CLL) or saline (mock) 6 days prior to immunization with PE+GLA-SE and responses were analyzed 7 days after immunization. (A) Shown are the number of PE⁺ B cells in draining inguinal lymph nodes, (B) Shown are the total Ig (H+L) and IgG2c endpoint titers. (C) The total numbers of T_{FH} cells (CD4⁺, CD44⁺, CXCR5⁺, PD1⁺, Bcl-6⁺) in draining LNs were enumerated. Bars are drawn to the mean + s.e.m.. p values were determined by one-way ANOVA/Dunnett's test ** p<0.01, * p<0.05. Data are representative of two independent experiments with five animals per group.

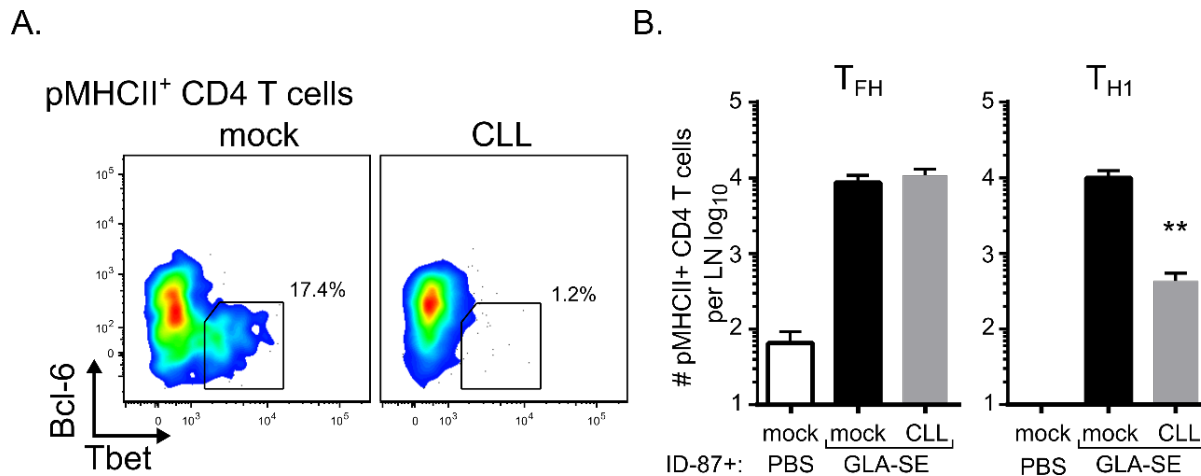


Figure 5. T_{FH} and T_{H1} dependency upon SCMφ

Peptide-MHCII tetramer staining was used to identify T cell subsets in the context of CLL treatment. Mice were treated with clodronate-loaded liposomes (CLL) or saline (mock) 6 days prior to immunization with GLA-SE and the recombinant protein ID87 and responses were analyzed 7 days after immunization. (A) FACS plots of Tbet and Bcl-6 staining of CD4⁺pMHCII⁺ cells from draining lymph nodes. (B) The total numbers of T_{FH} (pMHCII⁺, CD4⁺, CD44⁺, CXCR5⁺, PD1⁺, Bcl-6⁺) and T_{H1} (pMHCII⁺, CD4⁺, CD44⁺, CXCR5⁺, PD1⁺, Tbet⁺) per LN were enumerated. Bars are drawn to the mean + s.e.m.. p values were determined by one-way ANOVA/Dunnett's test ** p<0.01. Data are representative of two independent experiments with three or four animals per group.

IL-18R1 contributes to the induction of early B cell responses elicited by GLA-SE

T_{H1} induction with GLA-SE is dependent on the caspase-1 mediated inflammasome and rapid IL-18 production which is essential for IFN γ production by memory CD8 T cells and neutrophils upon GLA-SE immunization (8). SCM ϕ are an important source of IL-18 in draining lymph nodes (17). Thus, we hypothesized that SCM ϕ played a role in the innate and adaptive responses elicited by immunization with GLA-SE in part by producing IL-18. CLL depletion prior to GLA-SE immunization significantly diminished the amount of secreted IL-18 in the draining LN one hour after immunization indicating that the SCM ϕ are the main source of early IL-18 after immunization with GLA-SE (Figure 6A). To determine whether IL-18 was important for the induction of antigen-specific B cells and antibody production in response to GLA-SE, we compared responses to PE+GLA-SE immunization in wildtype or IL18R1 deficient (IL-18R1^{-/-}) mice. Total Ig (H+L) and IgG2c PE-specific serum titers were diminished in IL-18R1^{-/-} mice (Figure 6B). Concordantly, significantly fewer PE-specific B cells were present in the immunized IL-18R1^{-/-} mice (Figure 6C). IgG2c⁺ class-switching was also impaired in IL-18R1^{-/-} mice (Figure 6D). . These data indicate that, similar to CLL depletion of SCM ϕ , ablation of IL-18 signaling impaired the humoral responses elicited by immunization with GLA-SE. Of note CLL-depletion had a more dramatic impact on adaptive immune responses than IL-18R1 deficiency indicating CLL-dependent cells also control adjuvant driven responses in an IL-18R1-independent manner.

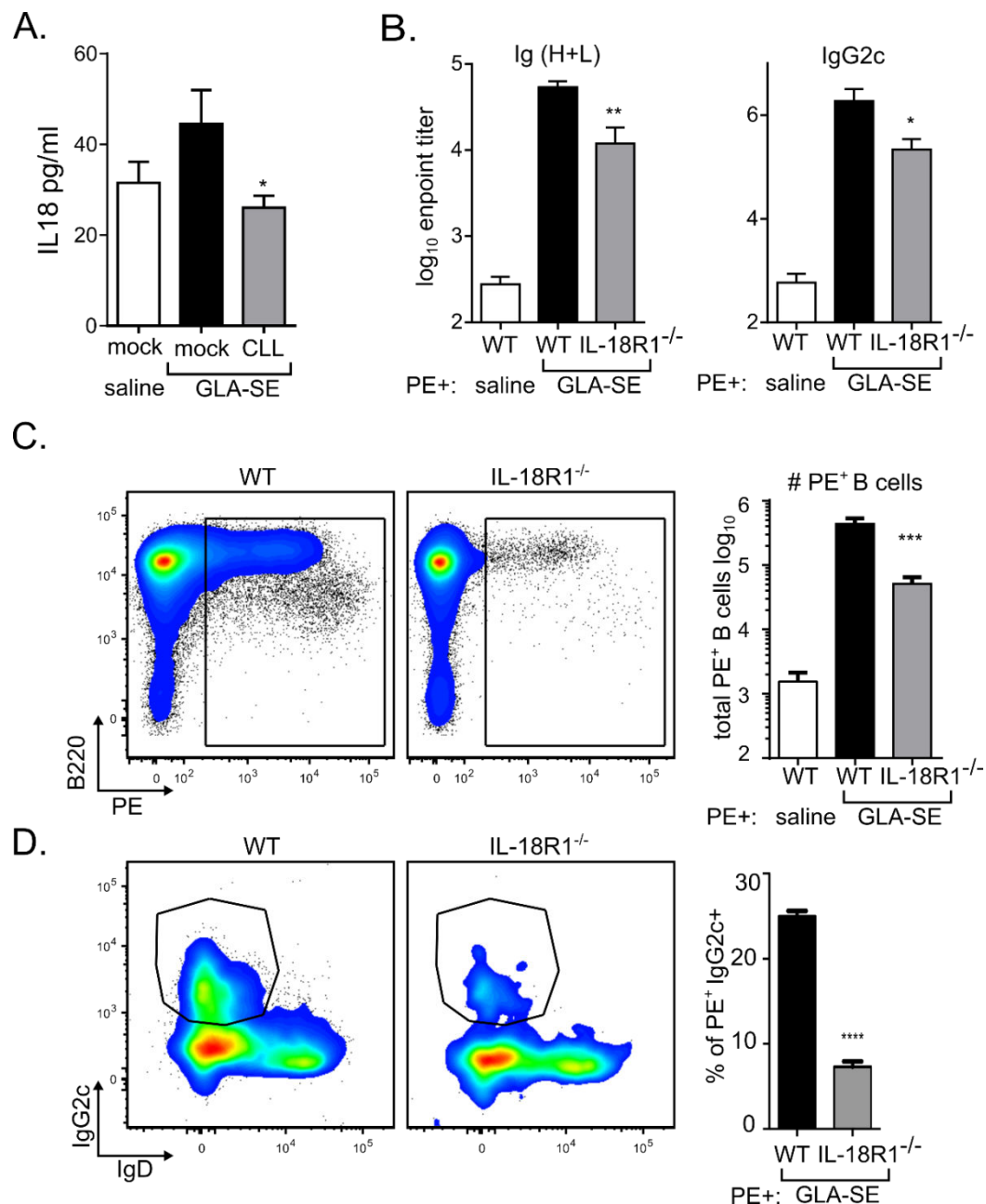


Figure 6. IL-18 is important for GLA-SE augmentation of B cell responses

Mice were treated with clodronate-loaded liposomes (CLL) or saline (mock) prior to immunization. (A) Mice were immunized with GLA-SE and IL-18 production in the draining lymph node was assessed 1h post-injection. (B-D) WT or IL18R^{-/-} mice were immunized with PE+/-GLA-SE and responses were analyzed 7 days after immunization. (B) Total Ig (H+L) and IgG2c PE reactive endpoint titers. (C) FACS plots of PE⁺ staining are shown and the total numbers of PE-specific B cells in the draining LNs are plotted. (D) FACS plots of IgG2c and IgD staining of PE⁺ B cells are shown and frequencies of IgG2c⁺ PE⁺ B cells are plotted. Bars are drawn to the mean + s.e.m.. p values were determined by one-way ANOVA/Dunnett's test **** p<0.0001, *** p<0.001, **p<0.01, *p<0.05. Data are representative of two independent experiments with four or five animals per group.

Discussion

Rapid antibody production after immunization could mean the difference between protection and infection against a new strain of disease such as a pandemic avian influenza virus or biological weapons. As evident in the data presented here, different adjuvants can alter the kinetics and quality of the humoral immune response. Few studies have examined changes in antigen-specific B cell populations elicited by adjuvants, particularly those destined for clinical use. Of note, antigen-staining was recently used to identify germinal center B-cell responses during a prime-boost regimen with the emulsion adjuvant MF59 (18). Overall, those results were consistent with our studies of the similar adjuvant SE. Alum, GLA alone or SE alone only weakly elicited early B cell responses - primarily GC and memory B cells - whereas the combination GLA-SE drove a rapid and robust B cell and antibody response that also included pre-plasmablasts important early antibody secretion. We previously found that a rapid humoral response induced with GLA-SE correlated with early onset protection against the highly pathogenic avian influenza virus H5N1 as early as 4-6 days after immunization, underlining the importance that such an adjuvant could have in a rapidly emerging epidemic (19).

After injection, GLA-SE was predominantly associated with the SIGNR1⁺ medullary subset of SCM ϕ and this correlated with the ability to induce strong B-cell responses. Strikingly, when the SCM ϕ population was disrupted by CLL injection, the number of B cells that were associated with labelled GLA-SE fell by nearly ten-fold. Consistent with this result, it has been previously demonstrated that subcapsular sinusoidal macrophages facilitate transport of virus particles to B cell follicles (3). Presumably this process facilitates the induction of germinal center responses as the disruption of sinusoidal macrophage lattice architecture inhibits the induction of germinal center responses (20). GLA-SE also uniquely induced pre-plasmablasts which have been shown to localize to medullary regions of the lymph node (21). This result suggested that initial priming could occur at sites distal to the medullary sinus, but it is conceivable that medullary macrophages may promote the activation of B cells and their differentiation into pre-plasmablasts in situ. Furthermore, antibody responses in transiently DC-depleted mice (CD11c-DTR) were mostly unaffected, suggesting that DC are not required for B cells priming (22). Based on this results we suggest that SCM ϕ , and not DC, might be critical for B cell priming upon GLA-SE immunization.

SCM ϕ were also important for the production of IL-18 upon immunization. We previously demonstrated that IL-18 production mediated by caspase 1 activation was important for the production of IFN- γ by memory CD8 T cells and neutrophils (8). This pathway in turn was important for the generation of antigen-specific CD4 T cells with GLA-SE. This IL-18 axis was also at least partially responsible for the SCM ϕ -dependent expansion of antigen-specific B cells. It remains to be determined whether IL-18 is acting directly on B cells to drive their expansion, through the IFN γ pathway, or via a distinct pathway.

Substantial evidence has accumulated describing the relationship between T_{FH} cells and the induction of B cell responses (4). Importantly, T_{FH} and GC B cell numbers correlate during the induction of an immune response by vaccination (23). In agreement with these observations, GLA-SE drives an expansion of antigen-specific T_{FH} cells concordant with the increase in antigen-specific B cells and this is superior in magnitude to the other adjuvants tested. However, the contribution of antigen-specific T_{FH} cells to B cell expansion and early antibody titers during immunization with GLA-SE is unclear. Surprisingly antigen-specific T_{FH} cells were unaffected by SCM ϕ depletion, whereas B cell and T_{H1} responses were dramatically impaired. Canonically T_{H1} cells are associated with CD8 T cell responses, not B cell responses, although T_{H1} cells can facilitate B cell priming in some circumstances (24). This underlines the current models suggesting that separate subsets of antigen presenting cells mediate the induction of T_{H1} cells and T_{FH} cells (25, 26). In support of this we have recently shown that T-bet deficient mice which have no T_{H1} responses to GLA-SE, do not induce an IgG2c skewed response further supporting a role for T_{H1} cells in B cell responses (11). Although this does not demonstrate that T_{FH} cells are irrelevant to the early B cell response, it does indicate that T_{FH} cells are insufficient to support all B cell responses.

The unique adjuvant properties of GLA-SE, relative to GLA and SE alone, are likely due both to its biophysical characteristics, as well as known and unknown molecular signaling capacities. For example, the innate response to GLA-SE depends not only upon MyD88 and TRIF, which are critical components of TLR4 signaling, but also upon the inflammasome (8, 10), which is only engaged by TLR4 agonists upon cytoplasmic recognition (27, 28). On their own, squalene oil-in-water emulsions such as MF59 or SE promote humoral immunity via activation of canonical and non-canonical inflammasome pathways (29–32). Inflammasome activation could be due to direct recognition of the squalene via distinct, but unknown, receptors. Additionally,

the nanoparticle nature of GLA-SE may result in more efficient delivery to the lymph node. Nevertheless the particulate nature cannot fully explain the enhancement mediated by GLA-SE as other oil-in-water/TLR4 agonist compositions, with similar biophysical properties, do recapitulate the adjuvanticity of GLA-SE (9, 33). Defining the key pathways that determine the activity of clinical stage adjuvants including GLA-SE, MF59, and the AS01-04 series is crucial to rational development of next generation vaccine adjuvants tailored to elicit specific immune responses.

In summary, when formulated as a squalene oil-in-water emulsion (GLA-SE) the TLR4 agonist GLA induces rapid B cell expansion and differentiation, augmented antibody production and T_{FH} expansion. These responses were superior in magnitude to the widely used adjuvant alum, as well as GLA in an aqueous base or the oil-in-water emulsion alone adjuvant. $SCM\phi$ and IL-18 production were central to successful immunization with GLA-SE as the innate immune response, B cell expansion and T_{H1} induction were impaired upon $SCM\phi$ depletion or abrogation of IL-18R signaling. Conversely, T_{FH} induction after GLA-SE immunization was not affected in the absence of $SCM\phi$. Thus, GLA-SE may be ideally suited for use in vaccines that require a rapid-onset response with a single immunization.

Materials and methods

Animals and immunizations

Female C57BL/6 mice and IL-18R1 $-/-$ mice aged 6-10 weeks were purchased from The Jackson Laboratory. All animal experiments and protocols used in this study were approved by IDRI's IACUC. For the adaptive response assessments mice were immunized subcutaneously via foot pad or base of tail injection with 1.25 or 5 μ g of GLA respectively in combination with 1 μ g of R-phycoerythrin (Prozyme, Hayward, CA) or 1 μ g of the *M. tuberculosis* antigen ID87 (16). Squalene oil formulations were emulsified with egg phosphatidylcholine or synthetic dimyristoyl phosphatidylcholine as described (34) and used after dilution in saline for injection to 2% oil. For the innate response assessment mice were immunized via intramuscular in the quadriceps injection with 5 μ g of DiD-labelled GLA-SE. To label GLA-SE, DiD Oil; DilC18(5) (Molecular Probes) was solubilized in DMSO at 25 mg/mL. DiD was added to GLA-SE at 25 μ g/mL, mix well

and incubate at room temperature for 10 minutes. Excess DiD was removed using a PD-10 desalting column (GE).

SCM ϕ depletion

Mice were treated with 30 μ L of clodronate-loaded liposomes (5mg/mL clodronate disodium salt) (Encapsula Nano Sciences, Brentwood, TN) via intradermal hock injection 6 days prior to subsequent immunizations. Mice were immunized six days later via footpad, hock or intramuscular injection in the quadriceps.

Serum endpoint titer determination

Serum endpoint titer ELISA's were performed on Corning high bind 384 well plates. Plates were coated overnight at 4 degrees with 1 μ g/ μ L PE. Detection antibodies were Ig (H+L) HRP and IgG2c HRP (Southern Biotech). Endpoints were set as the minimum dilution at which values were \leq the mean + 3 s.d. of naïve controls.

Flow cytometry

Samples were prepared by Fc receptor blocking (clone 2.4G2). B cells were stained in 1% FBS, 2mM EDTA in PBS with the following: CD95 (clone JO2), CD138 (clone 16A8), B220 (clone RA3-6B2) or CD19 (clone 1D3), IgM (clone IL/41), IgD (clone 1126C.2A), CD38 (clone 90), lineage cocktail: Ly6G (clone 1A8), CD11b (clone M1/70), CD11c (clone N418), F4/80 (clone BM8), Ter119 (clone TER119), CD90.2 (clone OX-7). Cells were fixed and permeabilized with Fix/Perm buffer (BD Biosciences) and stained for intracellular IgG2 (clone 5.7). PE-specific B cells were identified using 1 μ g/ml PE for surface staining, and 0.1-0.3 μ g/ml PE for intracellular staining.

For peptide-MHCII tetramer staining, the Ag85B p25 tetramer (NIH Tetramer Core Facility) was incubated in a 100 μ L with a maximum of 4×10^6 cells at 37° C for 1.5 hours at 13 μ g/ml with anti-CD16/32 antibody. Cells were washed and stained for CD4 (clone RM4-5), CD44 (clone IM7), PD-1 (clone 29F1A12), CD95, CXCR5 (clone SPRCL5) and lineage cocktail: CD8a (clone 53-6.7), CD11b, CD11c, Ly6G, Ter-119, F4/80, CD19 at room temperature for 30 minutes. Cells were then fixed and permeabilized as above and stained for Bcl6 (clone BCL-DWN) and Tbet (clone 4B10) overnight at 4°C.

Identification of DiD⁺ cells was performed by surface staining draining lymph node cells by blocking CD16/32 and staining for CD11b, CD90.2 (clone 53-2.1), CD19 (clone 6D5), SIGNR1 (clone eBio22D1), Ly6C (clone HK1.4), CD169 (clone 3D6.112), Ly6G, and CD11c.

Quantitation of cells was carried out by applying the frequencies of cell populations to cells counts obtained with a GUAVA EasyCyteHT (Millipore, Billerica, MA). Data were collected on LSRII or Fortessa flow cytometers (BD). Data were analyzed using FlowJo (Tree Star Inc., Ashland, OR), Pestle and SPICE (NIAID, NIH (BCBB)) (35). Statistical analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

Immunofluorescence microscopy

LN's were collected and embedded in OCT Compound (Tissue-Tek). Cryostat sections (6 μ m thick) were mounted on Poly-lysine microscope slides (Thermo Scientific). Brightfield and DiD images of cryostat sections were acquired using Nikon Eclipse Ti-5 before acetone fixation. Sections were subsequently dried and fixed in cold acetone for 5 min at -20° C, blocked with 3% BSA in PBS blocking buffer at room temperature for 30 min, and incubated with anti-CD169 (ThermoScientific) and SIGNR1 (eBioSciences) antibodies diluted in the blocking buffer for 1 hour. Primary antibodies were detected with Goat-anti-Rat-AF488 (against CD169, BioLegend) and Goat-Anti-Hamster IgG-568 (against SIGNR1, Abcam). Images were acquired with the Nikon Eclipse Ti-5 microscope, using 4 \times objective lens and NIS-Elements D3.2 Software and processed with Fiji software.

Cytokine and chemokine protein levels quantitation

The concentration of cytokines and chemokines were measured using a microbead-based ELISA system (ProcartaPlex Mouse, Affymetrix eBioscience), according to the manufacturer's directions.

Acknowledgments

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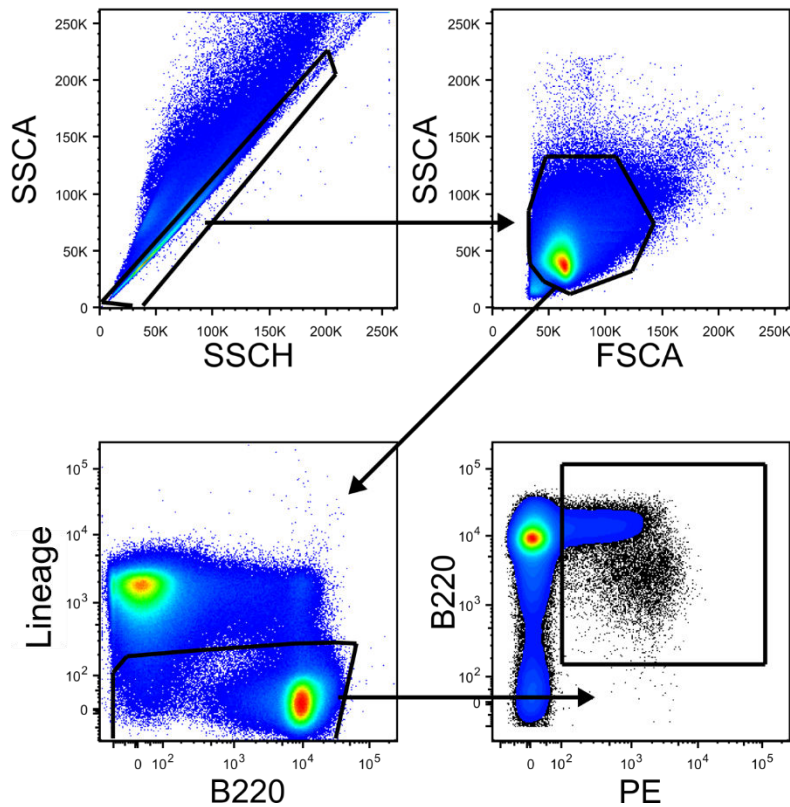
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Supplemental Figures

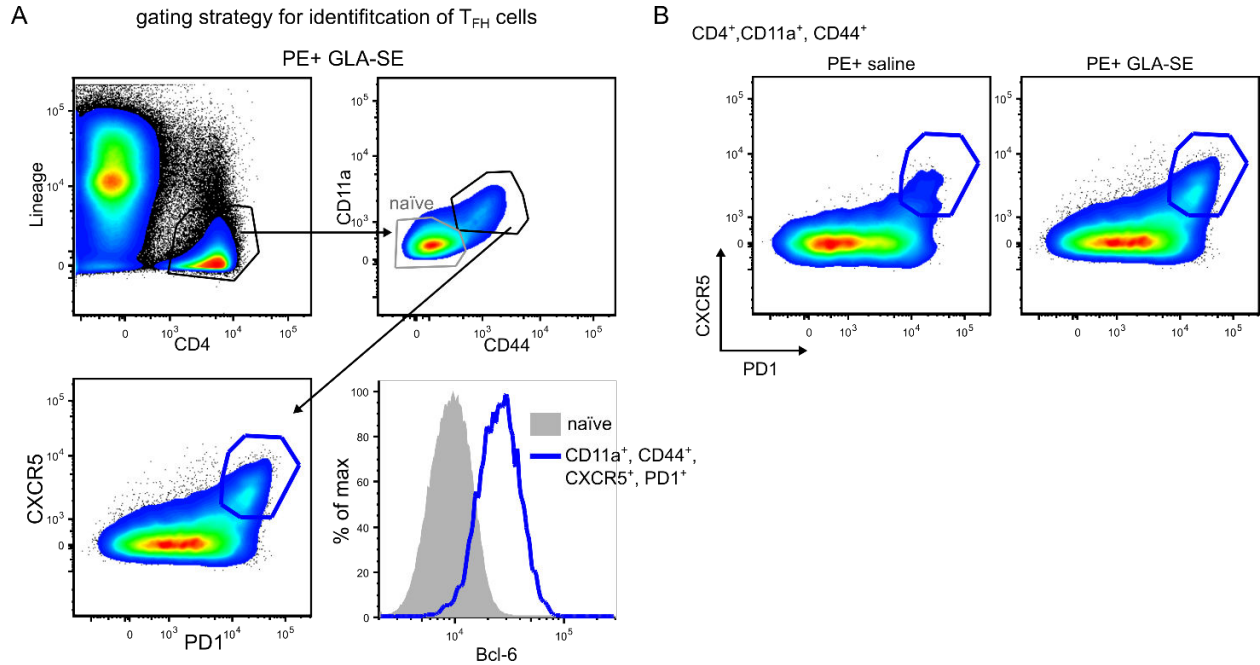
PE⁺ B cell gating

1 week post: PE+GLA-SE



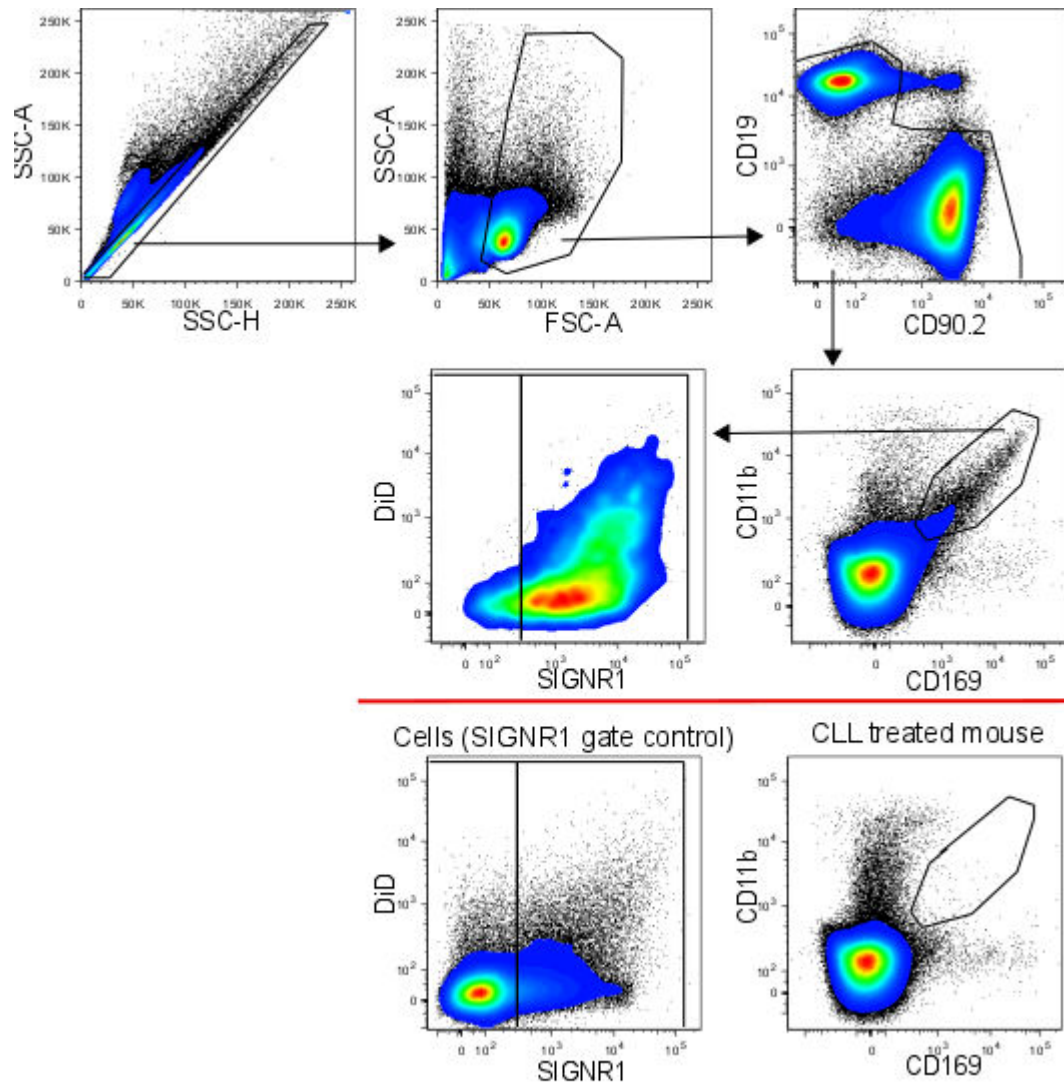
Supplemental Figure 1. PE⁺ B cell gating strategy

Representative FACS plots of the gating strategy used to determine the numbers of antigen specific B cells in Figure 1. B cells were gated as B220 or CD19⁺ and FITC lineage cocktail- (Ly6G, CD11b, CD11c, F4/80, Ter119, Thy1.2)- B220⁺.



Supplemental Figure 2. T-FH CD4 T cells gating strategy and induction with GLA-SE.

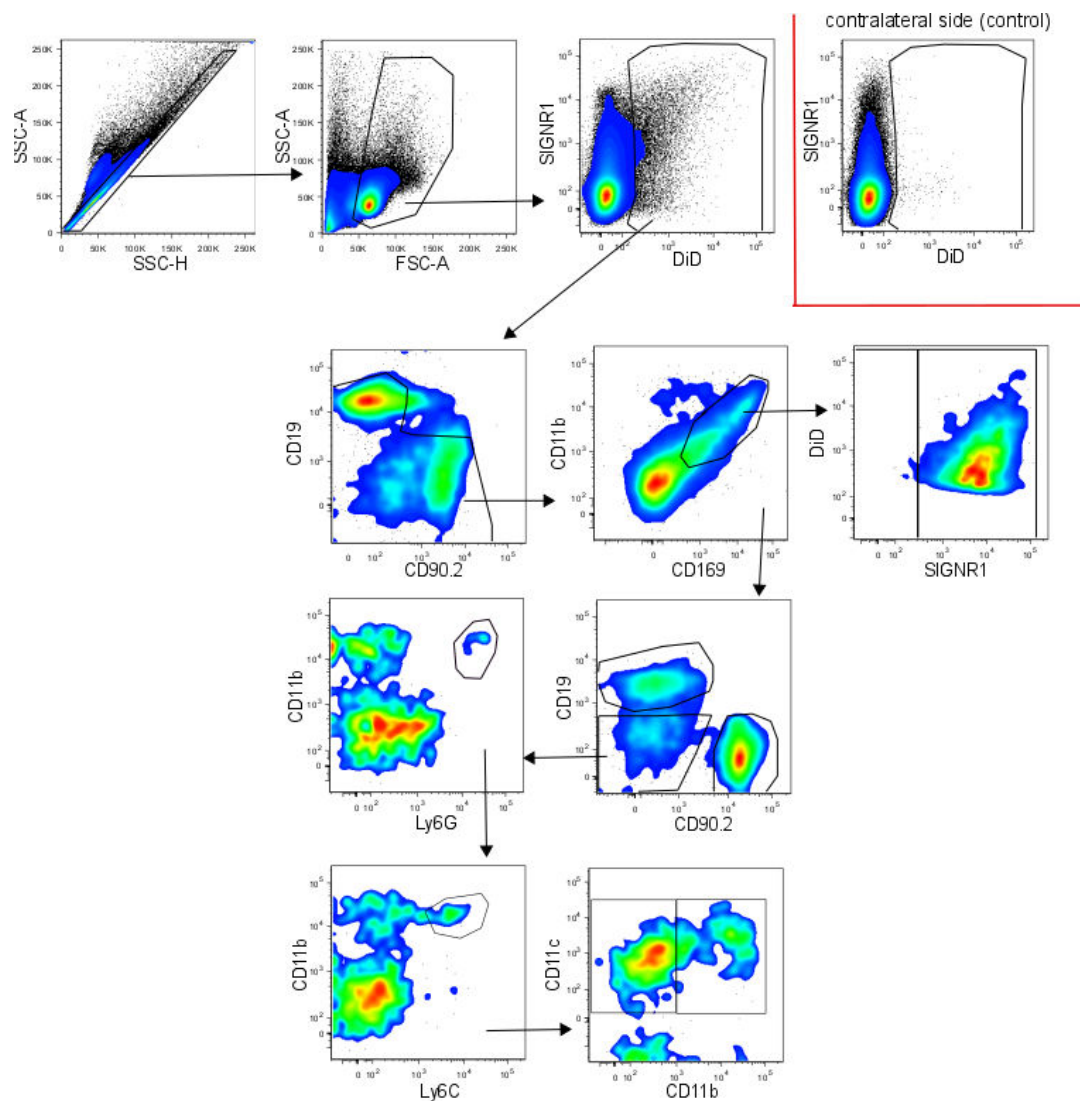
Parallel samples from Figure 1 were used. (A) Representative FACS plots of the TFH gating strategy. TFH cells were gated as CD4⁺, CD44⁺, CD11a⁺, CXCR5⁺, PD1⁺. The Bcl-6 staining of such cells was compared to naïve CD4⁺ T cells (CD11a⁻, CD44⁻). (B) TFH cells in draining LN one week after immunization with PE and saline or PE+GLA-SE.



Supplemental Figure 3. Gating strategy for the identification of LN subcapsular sinus macrophages and medullary sinus macrophages.

Representative FACS plots of the gating strategy used to identify the subcapsular sinus macrophages and medullary sinus macrophages. SCM ϕ were gated as SSC x FSC lymphocytes > not CD19+ or CD90.2+ > CD11b+CD169+. Sinusoidal SCM ϕ are SIGNR1- and medullary SCM ϕ are SIGNR1+.

Shown are also representatives FACS plot of SIGNR1 staining in total cells (SIGNR1+ gating control) and CD11b x CD169 staining in CLL treated mice (CD11b+ CD169+ gating control).

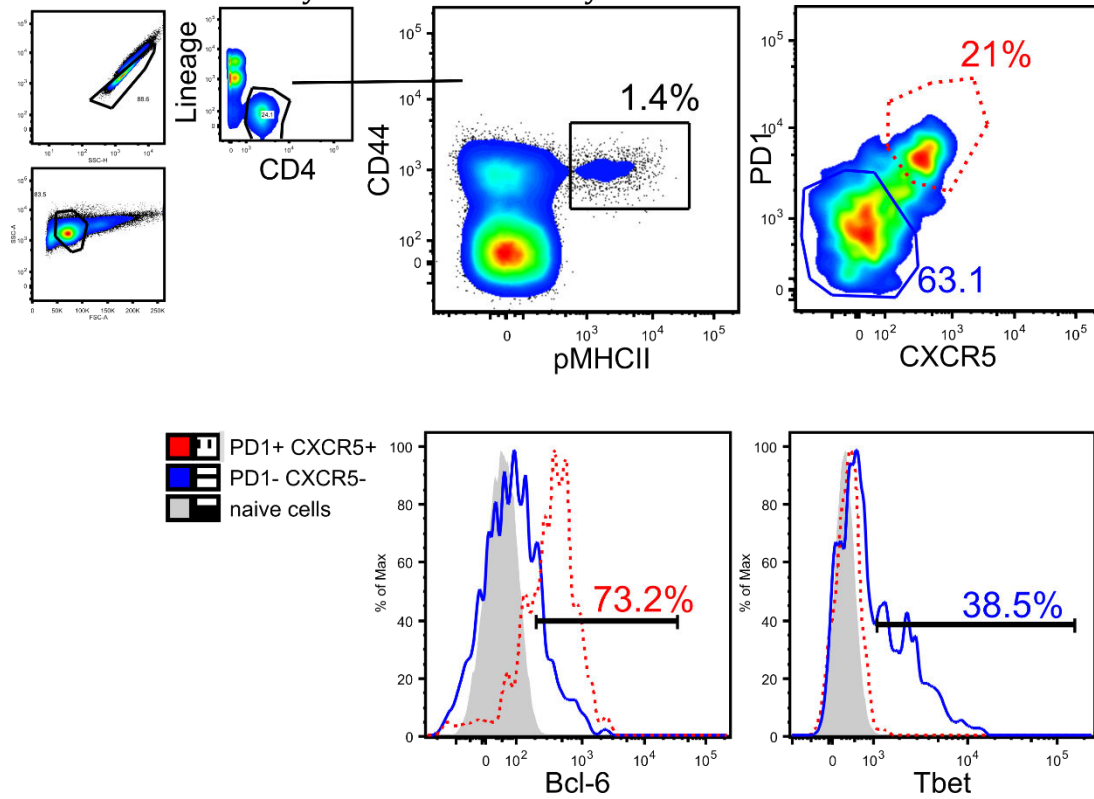


Supplemental Figure 4. Gating strategy for the identification of DiD+ LN cells.

Representative FACS plots of the gating strategy used to identify the main DiD+ cell populations. DiD+ cells were gated by comparing with a contralateral LN population. The different DiD+ cell populations were gated as follows:

- Medullary SCM ϕ : SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > CD11b+CD169+ > SIGNR1+.
- Sinusoidal SCM ϕ : SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > CD11b+CD169+ > SIGNR1-.
- B cells: SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > not CD11b+CD169+ > CD19+ CD90.2-.
- T cells: SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > not CD11b+CD169+ > CD90.2+ CD19-.
- Neutrophils: SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > not CD11b+CD169+ > CD90.2- CD19- > CD11b+ Ly6G+.
- Monocytes: SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > not CD11b+CD169+ > CD90.2- CD19- > not CD11b+ Ly6G+ > CD11b+ Ly6C+.
- Macrophages: SSC x FSC lymphocytes > DiD+ > not CD19+ CD90.2+ > not CD11b+CD169+ > CD90.2- CD19- > not CD11b+ Ly6G+ > not CD11b+ Ly6C+ > CD11b+ CD11c+.

- DC: SSC x FSC lymphocytes > DiD+> not CD19+ T cell+ > not CD11b+CD169+> CD90.2-CD19-> not CD11b+ Ly6G+> not CD11b+ Ly6C+> CD11b- CD11c+.



Supplemental Figure 5. Identification of T_{FH} and T_{H1} cells via transcription factor and peptide-MHCII tetramer staining.

(A) Representative FACs plots used to determine numbers antigen specific T_{FH} ($CD4^+$, $pMHCII^+$, $CXCR5^+$, $PD1^+$, $Bcl-6^+$) and T_{H1} T_{FH} ($CD4^+$, $pMHCII^+$, $CXCR5^-$, $PD1^-$, $T-bet^+$) $CD4$ T cells in Figure 6. (B) Overlays of T-bet and Bcl-6 staining of naïve, T_{FH} and T_{H1} $CD4$ T cell populations described in (A).

ARTICLE 3: ANTIGEN PRESENTATION BY B CELLS GUIDES TLR-4 MEDIATED PROGRAMMING OF MEMORY CD4 T CELL RESPONSES

Natasha Dubois Cauwelaert*, Susan L. Baldwin*, Mark T. Orr, Anthony L. Desbien, Emily Gage, Kimberly A. Hofmeyer, Rhea N. Coler.

(Submitted)

PRESENTATION

The main objective of this study was to assess the role of B cells in the induction of T_{H1} responses by the adjuvant GLA-SE.

We used the B cell deficient mice μ MT^{-/-} to characterize early and memory CD4 T_{H1} responses to GLA-SE in the absence of B cells. Early T_{H1} response in Wild Type (WT) and μ MT^{-/-} was further characterized using the Memory Precursor Effector Cells markers T-bet/Ly6C and PD1/KLRG1.

In order to assess the survival capacities of the antigen specific CD4 T cells generated in B-cell deficient mice we harvested CD4 T cells generated early on after ID93/GLA-SE immunization either in WT or μ MT^{-/-} mice and analyzed their fate when transferred in B-cell sufficient mice.

Finally we used B cell transfers from WT or MHCII^{-/-} mice into μ MT^{-/-} mice to assess the capacity of those B cells to rescue the phenotype observed in μ MT^{-/-} and define the requirement of B cells and their antigen presentation capacities in the generation of CD4 Memory Precursor Effector Cells T cells.

PRINCIPAL RESULTS

The main obtained results were:

- ❖ Early T_{H1} responses to GLA-SE (one week after immunization) are only modestly affected in the absence of B cells.
- ❖ CD4 Memory Precursor Effector Cells (MPEC) induction is impaired in the absence of B cells
- ❖ Memory CD4 T_{H1} response to GLA-SE is severely impaired in the absence of B cells
- ❖ CD4 T_{H1} T cells generated in the absence of B cells lose their T_{H1} cytokine production and proliferative capacities over time even when transferred into B-cell sufficient mice.

- ❖ CD4 MPEC impairment is partially restored in B-cell deficient mice if WT, but not MHCII-/, B cells are transferred before immunization with GLA-SE.

Antigen presentation by B cells guides TLR-4 mediated programming of memory CD4 T cell responses

Running title: B cell antigen presentation guides T cell memory.

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ABSTRACT

The contribution of B cells to immunity against many infectious diseases is unquestionably important and well characterized. Here we sought to determine the role of B cells in the induction of T helper 1 (T_H1) CD4⁺ T cells upon vaccination with a TB antigen combined with a TLR4 agonist. We used B cell deficient mice ($\mu MT^{-/-}$), tetramer-positive CD4⁺ T cells, markers of memory 'precursor' effector cells (MPEC), and T cell adoptive transfers and demonstrated that the early antigen-specific cytokine-producing T_H1 responses are unaffected in the absence of B cells, however MPEC induction is strongly impaired resulting in a deficiency of the memory T_H1 response in $\mu MT^{-/-}$ mice. We further show that antigen-presentation by B cells was necessary for their role in MPEC generation using B cell adoptive transfers from wildtype or MHC class II knock-out mice into $\mu MT^{-/-}$ mice. Our study challenges the view that B cell deficiency exclusively alters the T_H1 response at memory time-points. Collectively, our results and other recent studies provide new insights on the multifaceted roles of B cells which will have a high impact on vaccine development against several pathogens including those requiring T_H1 cell-mediated immunity.

INTRODUCTION

Since the primary objective and hallmark of vaccine design is to generate memory immune responses, a better understanding of the machinery that results in a robust memory response is crucial. Several sequential processes are required for the development of antigen-specific central memory T cell (T_{cm}) production upon protein-antigen immunization. Primary responses depend on additional components in the vaccine formulation, usually in the form of adjuvants. Adjuvants containing Toll like receptor (TLR) agonists, such as the clinically tested synthetic TLR-4 agonist Glucopyranosyl Lipid Adjuvant (GLA), help promote and influence the fate of a desirable T response through enhanced antigen presentation on dendritic cells (DCs), DC maturation, and production of innate cytokines (1). Secondary responses require expansion and subsequent contraction of T cells, leaving behind a small percentage of memory T cells that retain proliferative capabilities and are available for future encounters with the specific pathogen. Short lived effector T cells are instead terminally differentiated but provide effector helper functions such as cytokine production or cytotoxic functions that contribute to enhanced magnitude and quality of immunity against subsequent infection (for a review see (2)). It is thought that the long-term fate of antigen experienced T cells can be predicted based on expression of different surface markers and transcription factors. Using expression of two inhibitory surface molecules Woodland and colleagues proposed that memory precursor effector cell (MPEC) could be distinguished as being PD-1⁺ and KLRG1⁻ (3). Later, Kaech and colleagues showed that MPEC expressed lower levels of the T_H1 committing transcription factor T-bet and the surface marker Ly6C and persist to transition into memory T cells (4). MPEC cells display enhanced survival during the contraction phase and elicit greater proliferative responses to secondary infection.

Despite the importance of CD4 memory establishment for long-term immunity against pathogens, little is known of the factors influencing the survival of effector T cells and their transition to memory CD4 T cells. Of particular interest is the role of B cells in maintaining long-term T cell memory. The mouse model has provided insight into how B cells affect T cell responses. Diminished T cell memory responses and/or protective immunity generated to several intracellular pathogens including lymphocytic choriomeningitis virus (LCMV) (5, 6), *Listeria monocytogenes* (7), *Francisella tularensis* (8), and *Mycobacterium tuberculosis* (9) have been shown in animals with B cell deficits. Furthermore B cells were shown to be essential for T

cell immunity against tumors, where enhanced B16 melanoma growth was observed following anti-CD20 Mab mediated B cell depletion(10).

There are many ways B cells could influence antigen-specific T cell generation and subsequent generation of T cell memory. First, B cells effectively present antigen to T cells through MHC class II (MHC-II) molecules and drive antigen-specific proliferation (11, 12). Second, B cells produce antibodies that bind antigen and enable the formation of complexes that follicular dendritic cells engulf and use for antigen presentation to circulating T cells, and could additionally be involved in the maintenance of memory T cells (13). Lastly, cytokine production by B cells and cellular localization are also important factors for shaping CD4 T cell responses (14-16). B cell toll like receptor (TLR) activation and cytokine production leading to T helper cell differentiation and function, including T_H2 (17) and T_H1 (18) polarization have also been reported. All of these B cell functions underlie the importance of these cells in protection against a large number of pathogens.

In this study, the mechanistic contribution of B cells to MPEC induction following vaccination was examined. We used the ID93/GLA-SE clinical Phase tuberculosis vaccine, which drives a strong T_H1 response, in addition to a vaccine-specific MHC-II tetramer bound to a dominant epitope of Rv3619 (one of the component antigen of the ID93 polyprotein fusion) to follow specific memory T cell responses. In addition to the use of μ MT^{-/-} B cell deficient mice, adoptive transfer studies were done with MHC-II knockout mice to determine the role that antigen presentation by B cells play in vaccine-derived T cell immunity.

RESULTS

Memory T_H1 responses are severely impaired in B-cell deficient mice.

GLA-SE, a TLR4 agonist formulated in a stable nano-emulsion of squalene oil, induces a strong T helper 1 (T_H1) response in mice when formulated with the clinically tested tuberculosis (TB) vaccine polyprotein fusion antigen designated as ID93 (1, 19-21). Since diminished T cell memory responses have been shown with B cell deficits in several models of infection (5-9) we asked whether B cells were necessary for the induction of T_H1 responses to this vaccine. Wild type (wt) and μ MT^{-/-} mice were immunized three times at three-week intervals with ID93/GLA-SE. Six weeks after the last immunization, T_H1 responses in the spleen and draining lymph node (dLN) were significantly reduced in μ MT^{-/-} compared to wt mice as indicated by CD4 T cells up-regulation of CD154 and production of the T_H1 cytokines IFN- γ , TNF and IL-2 upon stimulation with ID93 (Fig. 1a). The induction of poly-functional antigen-specific T_H1 CD4 T cells, expressing IFN γ , TNF and IL-2, was also severely impaired in the μ MT^{-/-} mice (Fig. 1b).

Figure 1.

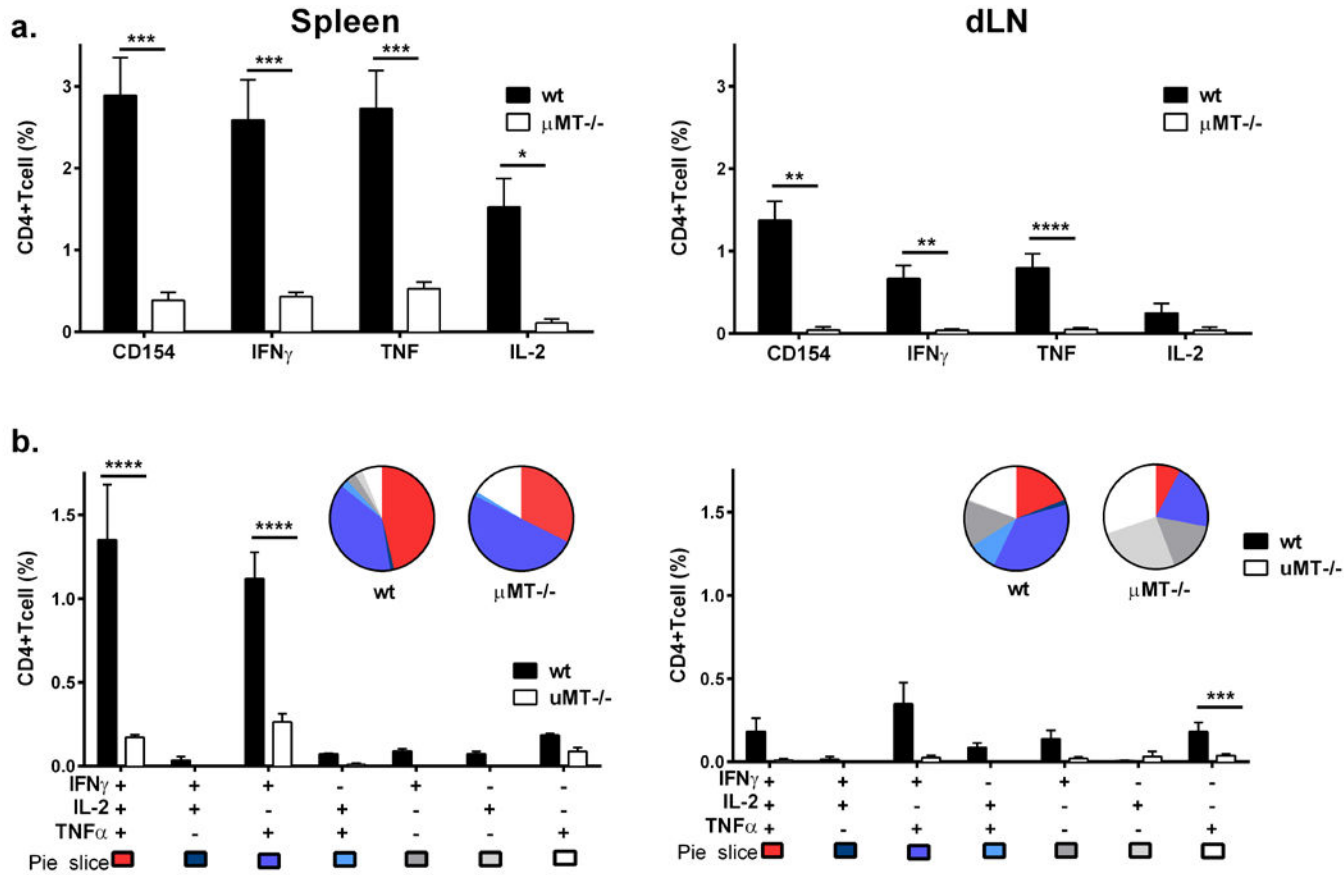


Figure 1. Memory TH1 responses are significantly reduced in μ MT^{-/-} mice. We immunized wt and μ MT^{-/-} mice with ID93/GLA-SE three times, at 3-week intervals, for the analysis of ID93-specific immune responses. We assessed ID93-specific recall immune responses following ID93 stimulation of splenocytes (a) or dLN cells (b) with 10 μ g ml⁻¹ ID93 6 weeks after the final immunization. Graphs show mean values \pm SEM for each group. The p values were determined by two-way ANOVA with Bonferroni correction for multiple comparisons (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001). Data are representative of three independent experiments with three mice per group.

B cells are necessary for CD4 MPEC induction.

To further define the role of B cells in CD4 TH1 response generation we sought to investigate whether early TH1 responses after the first immunization with ID93/GLA-SE were also impaired. Wt and μ MT^{-/-} mice were immunized with ID93/GLA-SE and TH1 responses were assessed one week later. At this time-point TH1 responses were only modestly affected by B-cell deficiency with no discernable difference in poly-functionality (Fig. 2a,b). In the spleen, TH1 responses were

consistently lower in the μ MT mice but this response, setting aside CD154 expression, was rarely significant (Fig. 2a,b). T_H1 responses in the dLN were comparable in the two strains (Fig. 2a,b).

Figure 2.

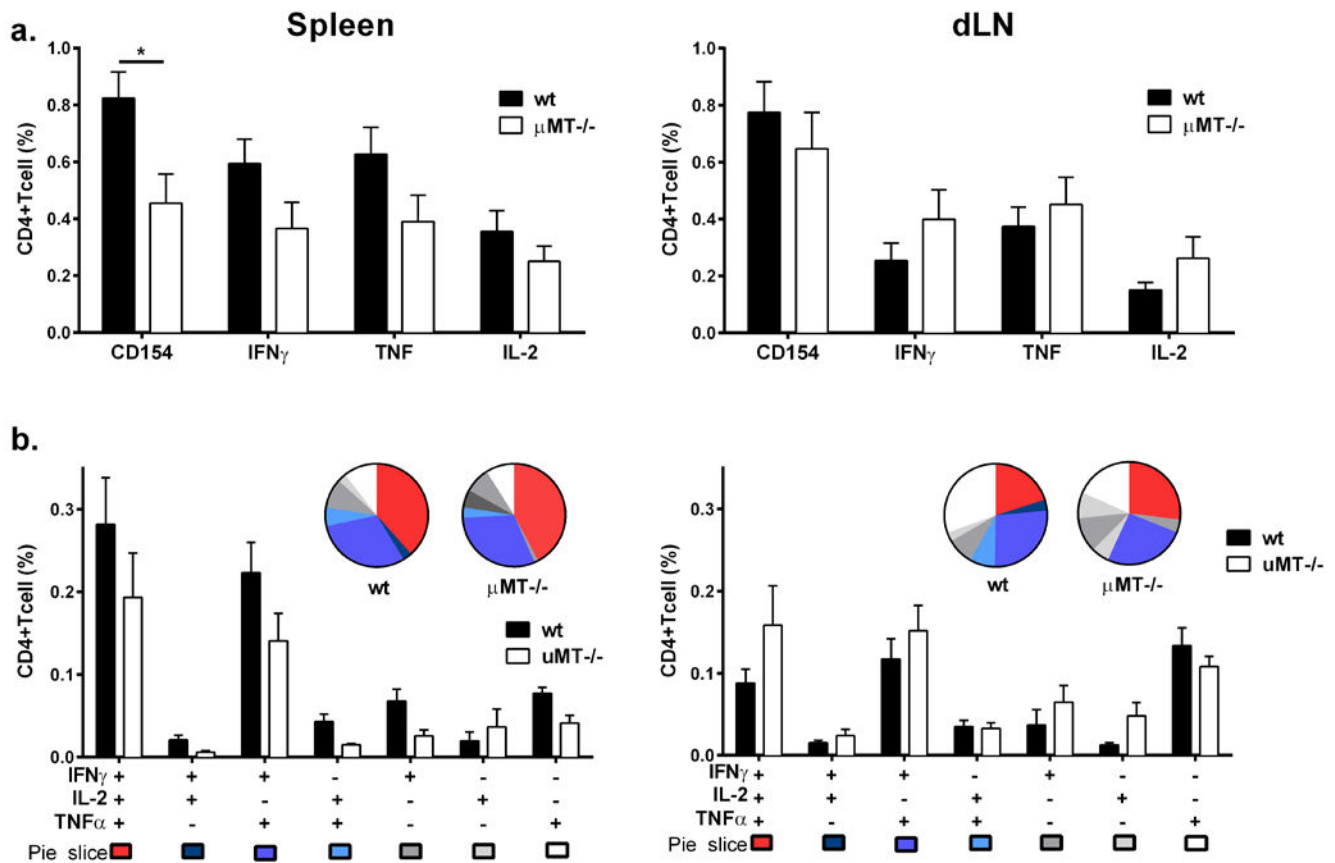


Figure 2. B cell deficiency does not alter the priming of T_H1 responses. We immunized wt and μ MT $^{-/-}$ mice once with ID93/GLA-SE for the analysis of ID93-specific immune responses. We assessed ID93-specific recall immune responses following ID93 stimulation of splenocytes (a) or dLN cells (b) with $10 \mu\text{g ml}^{-1}$ ID93 1 week after prime. Graphs show mean values \pm SEM for each group. p values were determined by two-way ANOVA with Bonferroni correction for multiple comparisons (* $p < 0.05$). Data are representative of four independent experiments with five mice per group.

To further characterize the antigen-specific CD4 T cell response to ID93/GLA-SE in B cell deficient mice, we stained the CD4 T cells generated by immunization with a MHC class II tetramer bound to the dominant epitope from Rv3619, one of the component proteins of ID93 (22). The frequency of tetramer positive CD4 T cells was similar in the dLN in both the wt and μ MT $^{-/-}$ mouse strains and, surprisingly, elevated in the μ MT $^{-/-}$ spleen (Fig. 3a). Marshall and others (3, 4) have reported a division within the primary CD4 T_H1 effector pool similar to the one

observed in CD8⁺ T cells with a population of Memory Precursor Effector Cells (MPEC) that survive contraction and differentiation into competent memory T cells. The MPEC markers PD-1⁺ and KLRG1⁻ or Ly6C^{Lo} and T-bet^{lo} were used to evaluate the formation of these particular memory subsets within the tetramer⁺ CD4 T cells (Fig. 3b,c). B cell deficient μ MT^{-/-} mice had significantly lower frequencies of MPEC by these two memory T cell markers in both LN and spleens; the PD-1⁺ KLRG1⁻ compartment being the most affected (Fig. 3b compared to Fig. 3c). These results show an impairment of CD4 MPEC induction in ID93/GLA-SE immunized mice in the absence of B cells.

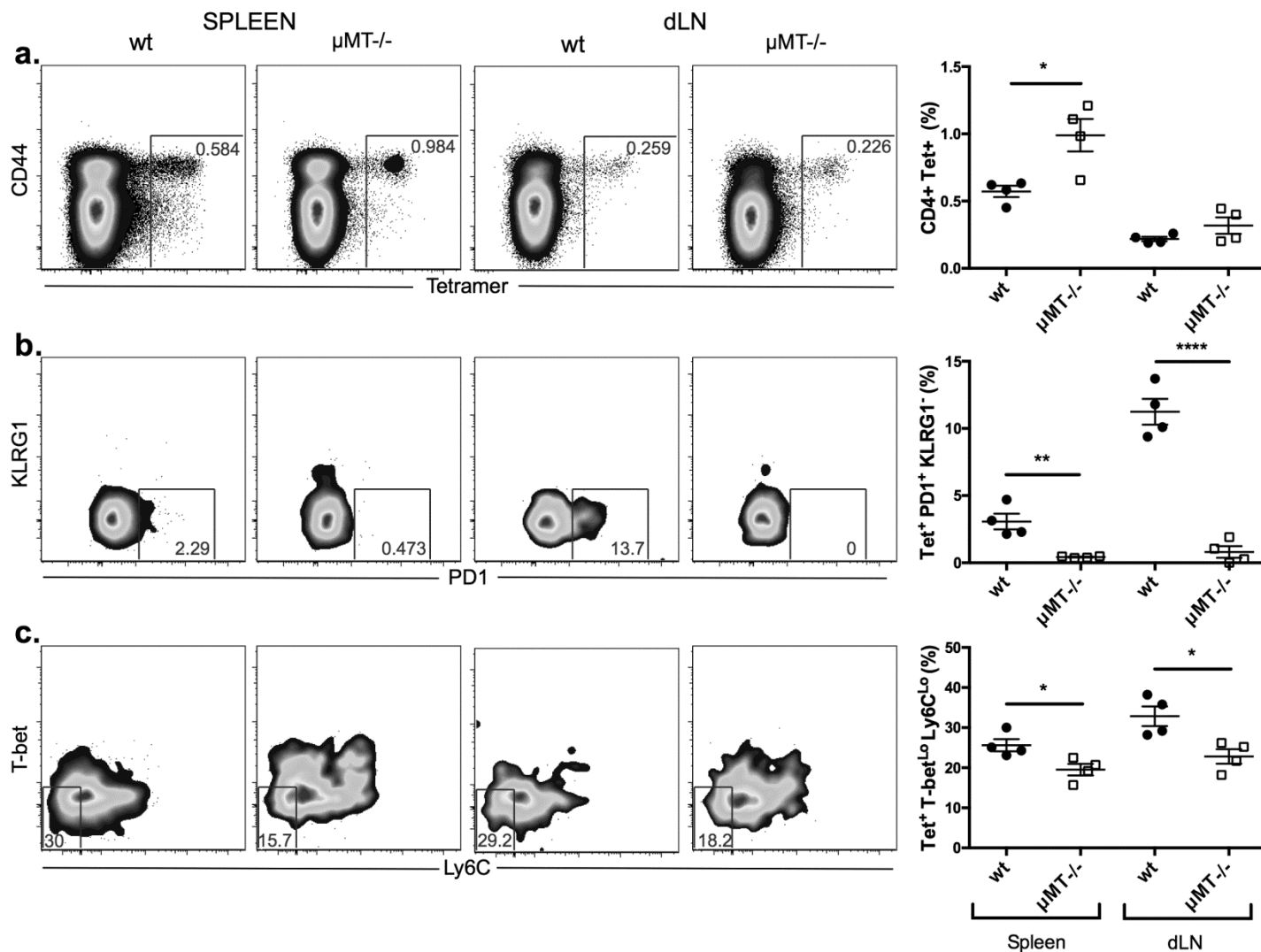


Figure 3. CD4 memory precursor effector T cell (MPEC) induction by ID93/GLA-SE is impaired in μ MT^{-/-} mice. We immunized wt and μ MT^{-/-} mice once with ID93/GLA-SE for the analysis of ID93-specific immune responses. Peptide-MHCII tetramer staining was used to identify different ID93-specific T cell subsets. Representative flow cytometry plots and graphs are shown for the percentage of CD4 T cells that bind the tetramer (a), the percentage of tetramer-binding CD4 T cells that are PD1⁺/KLRG1⁻ (b) and the percentage of tetramer-binding

CD4 T cells that are T-bet^{Lo}/Ly6C^{Lo} (c). Graphs show mean values \pm SEM for each group. The p values were determined by unpaired t-test within each organ (*p<0.05, **p<0.01, ****p<0.001). Data are representative of five independent experiments with 3-5 mice per group.

B cells are necessary for memory CD4 T cell programming.

Next we wanted to further characterize the phenotype of the T_H1 T cells that are induced in the absence of B cells by distinguishing between the role of B cells in the induction and maintenance of antigen-specific MPEC. To determine this, we investigated the survival and stimulation/expansion of T_H1 CD4 T cells generated in μ MT^{-/-} mice following a boost, in B cell-competent wt mice. We immunized wt (CD90.2), μ MT^{-/-} (CD90.2) and wt CD90.1 mice with ID93/GLA-SE and isolated their CD4 T cells one week later. CD45.1 received either a mix of wt CD90.2 and wt CD90.1 cells (as a control) or a mix of μ MT^{-/-} (CD90.2) and wt CD90.1 cells (Fig. 4a).

To analyze the survival capacities of the generated CD4 T cells we compared the ratio of CD90.2/CD45.2 tetramer+ or T_H1 cytokine-producing CD4 T cells 2 weeks after transfer (output) for to the ratio in the donors cells (input) (Fig. 4b). No significant difference was found in the percentage recovery of CD90.2tetramer+ CD4 T cells from μ MT^{-/-} compared to wt mice (Fig. 4b, left graph). Surprisingly, even though total number of tetramer+ CD4 T cells is unaffected the ratios of T_H1 cytokines producing cells are disparate. 2 weeks after transfer into wt mice a consistent reduction of approximately 30% in the recovered percentage of CD90.2 T_H1 cytokine-producing CD4 T from μ MT^{-/-} mice was observed (Fig. 4b, right graph).

We then compared the ratios found in mice one week after ID93/GLA-SE recipient immunization (output) (at week 2 after transfers) to the ratio in the donor cells (input) to assess the memory T cell boosting capacities (Fig. 4c). Again, no significant difference was found in the percentage recovery of CD90.2 tetramer+ CD4 T cells from μ MT^{-/-} compared to wt mice (Fig. 4c, left graph). Further, the reduction observed in the recovery percentage of CD90.2 T_H1 cytokines producing cells from μ MT^{-/-} mice at the survival time-point was maintained, but not further diminished (Fig. 4c compared to Fig. 4b, left graphs).

These results suggest that when T_H1 CD4 T cells are generated in B cell deficient mice they will lose their T_H1 response and proliferative capacities over time even when transferred in B-cell sufficient mice.

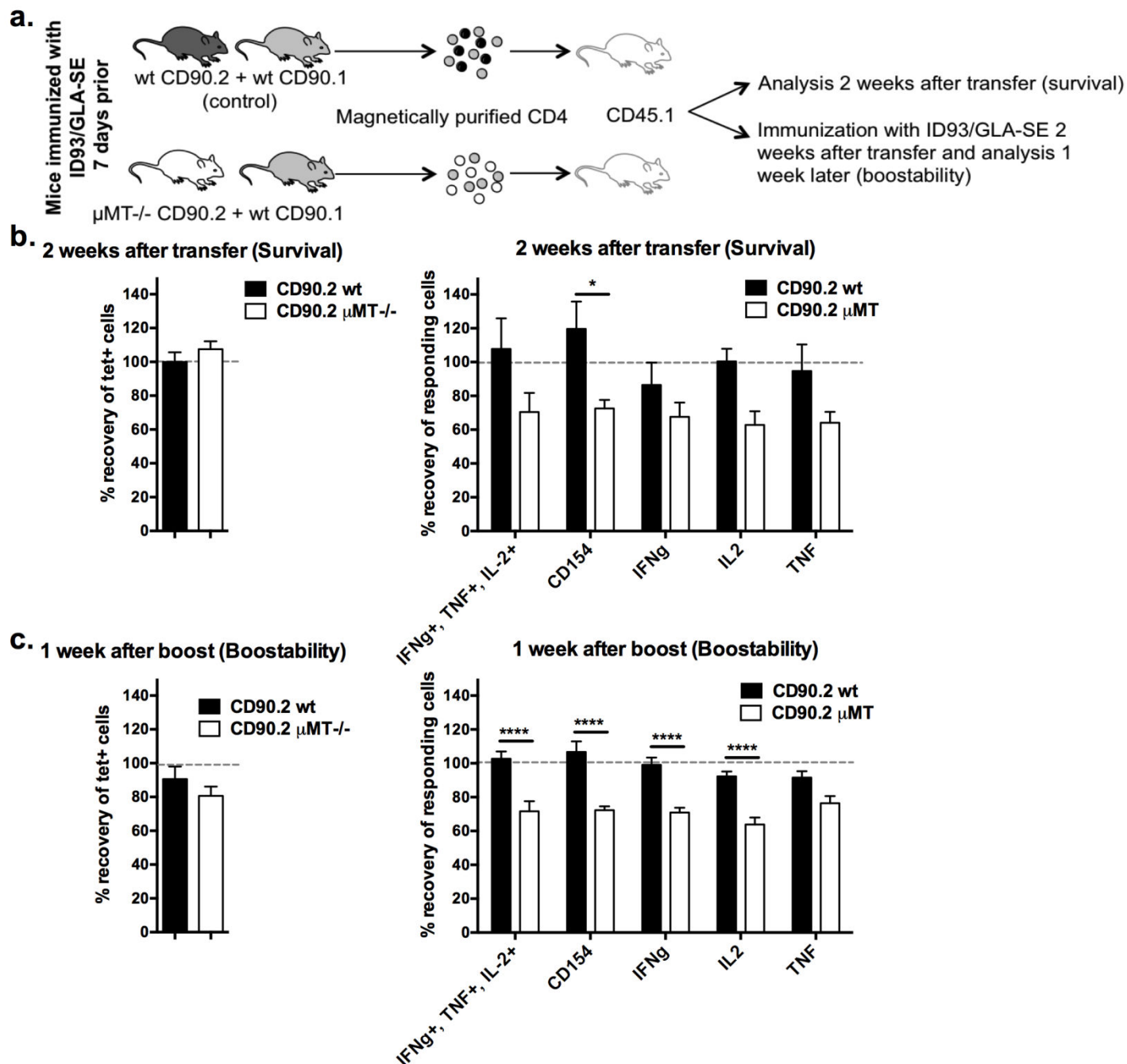


Figure 4. CD4 T_H1 T cells produced in μMT^{-/-} mice are impaired in their survival. We isolated CD4 T cells from spleens and dLN of either wt (CD90.2) mice, μMT^{-/-} (CD90.2) or CD90.1 mice. We performed adoptive transfer into CD45.1 naïve mice using either a mix of wt (CD90.2) and CD90.1 CD4 T cells (control group in black) or a mix of μMT^{-/-} (CD90.2) and CD90.1 CD4 T cells (in red) for the analysis of ID93-specific immune responses. We assessed ID93-specific immune responses by recall of splenocytes with ID93. (A) Schematic representation of the experimental design. (b-c) Percentage recovery of tetramer⁺ or responding CD90.2 cells (see Material and Methods for calculation) (b) two weeks after transfer or (c) one week after boost. Graphs show mean values ± SEM for each group. The p values were determined by two-way

ANOVA with Bonferroni correction for multiple comparisons (* $p < 0.05$, **** $p < 0.0005$). Data represents 9-10 mice pooled from two independent experiments with 4-5 mice per experiment.

MPEC impairment can be partially restored by B cell transfer and is MHC-II dependent.

Even though the capacity of B cells to present antigens to the CD4 T cells is well established and a long-held view, their contribution to T cell priming and maintenance *in vivo* is still debated (11, 18, 23-27). We sought to investigate whether their APC function is implicated in this process. To test this, we transferred B cells from wt or MHC-II^{-/-} mice into μ MT^{-/-} mice and compared the MPEC generation in the dLN, where the MPEC deficiency phenotype observed was strongest. Three days after adoptive transfer, mice were immunized with ID93/GLA-SE and the responses in the dLN assessed 1 week later to determine ID93-specific immune responses. As shown above, there was no significant difference in the frequency of tetramer⁺ CD4 T cells (Fig. 5a) and the PD1⁺ KLRG1⁻ was strongly reduced in μ MT^{-/-} compared to wt (Fig. 5b). This impairment was partially reversed when wt B cells but not MHC-II^{-/-} B cells were transferred to μ MT^{-/-} mice (Fig. 5b). A similar tendency was observed for the Ly6C^{Lo} T-bet^{Lo} subpopulation (Fig. 5c) pointing to a role of B cells as APC for the induction of MPEC by ID93/GLA-SE immunization.

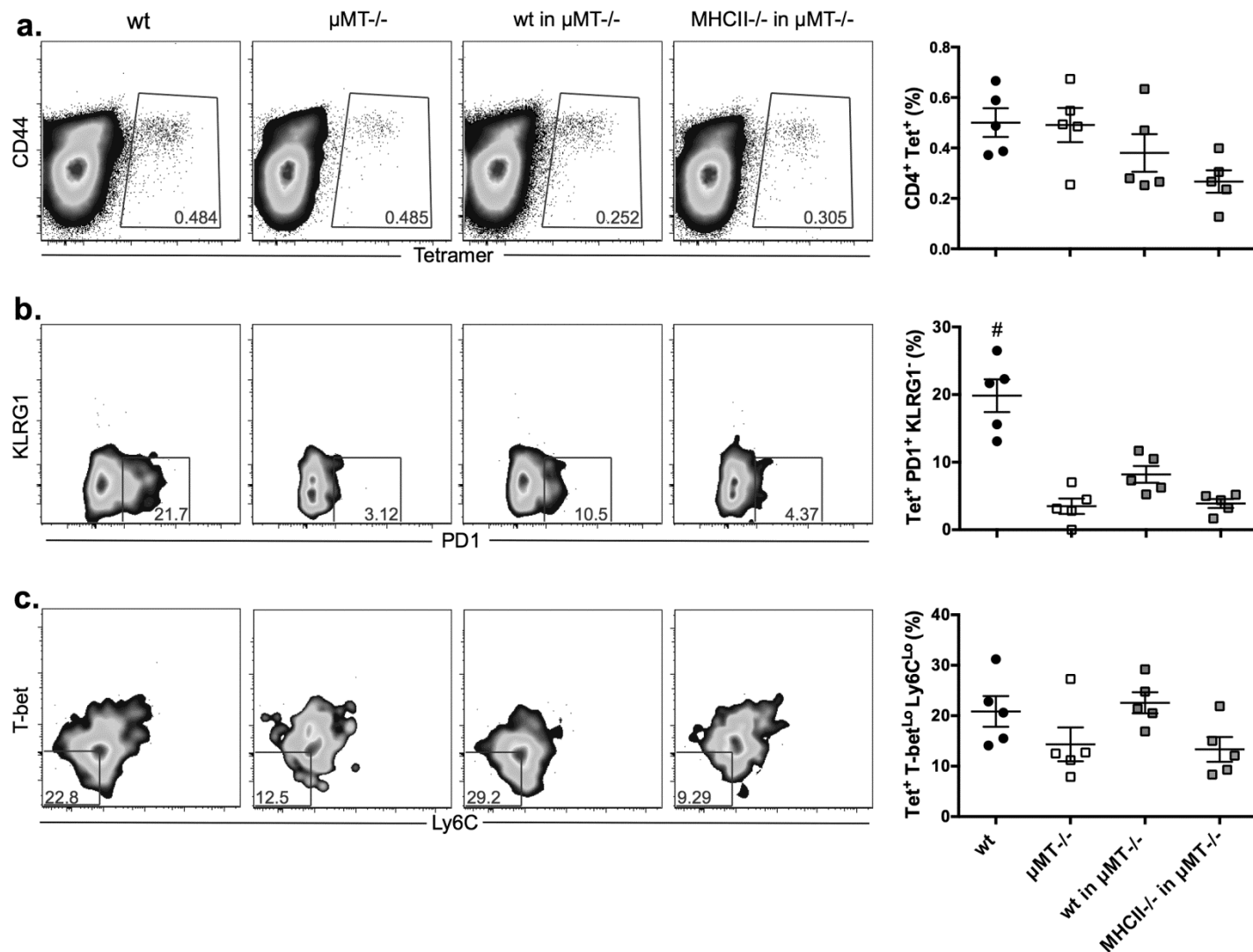


Figure 5. CD4 memory precursor effector T cell (MPEC) impairment is partially restored in μ MT^{-/-} mice with wt B cell transfer but not with MHCII^{-/-} B cells. We adoptively transferred B cells from wt or MHCII^{-/-} mice into μ MT^{-/-} mice. Three days later, we immunized wt, μ MT^{-/-} or μ MT^{-/-} adoptively transferred mice once with ID93/GLA-SE for the analysis of ID93-specific immune responses. Peptide-MHCII tetramer staining was used to identify different ID93-specific T cell subsets. Representative flow cytometry plots and graphs are shown for the percentage of CD4 T cells that bind the tetramer (a), the percentage of tetramer-binding CD4 T cells that are PD1⁺/KLRG1⁻ (b) and the percentage of tetramer-binding CD4 T cells that are T-bet^{Lo}/Ly6C^{Lo} (c). Graphs show mean values \pm SEM for each group. The p values were determined by one-way ANOVA with Bonferroni correction for multiple comparisons (#p<0.005 compared to all other groups). Data are representative of two independent experiments with five mice per group.

DISCUSSION

B cell-T cell cross talk is important for generation of effector adaptive immunity in several systems including in infectious disease settings and in cancer models. Indeed impairment of memory T cell responses to infectious diseases has been observed in the absence of B cells (μ MT^{-/-} mice) (5-9).

Therapeutic use of B cell-depleting monoclonal antibodies in patients diagnosed with B cell lymphomas (28) or autoimmune disorders (29-32) has brought new evidence of B cell involvement in affecting the T cell compartment. For instance, these therapies are well tolerated and adverse problems are relatively rare, but there have been reports of recurrent infections that depend on cellular immunity in these patients (33-37). Furthermore, B cell depleting monoclonal antibody therapies also ameliorate the disease course in patients with CD4⁺ T cell-mediated autoimmunity (38, 39).

Consistent with these previous findings in mice and humans, we show that MPEC CD4 T cell generation upon immunization with a clinical TB vaccine is impaired in B cell deficient mice and results in a dramatically diminished memory T cell recall response.

Our results suggest that while vaccine-specific memory T_H1 responses are affected by the lack of B cells, they are not defective at earlier time points. At 1 week after immunization functional T_H1 responses are mostly unaffected. This phenomenon following acute LCMV infection in mice has been observed and similarly, deficiency was shown only in the memory T cell population and not in the primary response (5). Interestingly, even though these early (1 week after prime) antigen-specific cells seem functionally similar in their capacity to produce T_H1 cytokines when generated in either wt or B cell deficient mice, they are phenotypically different. B-cell deficient mice fail to generate MPEC defined as either PD1⁺ KLRG1⁻ or T-bet^{lo} Ly6C^{Lo} as characterized by others (3, 4). These data demonstrate that B cells might be needed for priming a robust long-lasting T_H1 response characterized by the generation of antigen-specific MPEC.

To further explore whether there was an impairment in the survival of T_H1 memory T cells in μ MT^{-/-} mice, we performed a mixed-cell adoptive transfer experiment using wt CD90.1 T cells combined with either wt CD90.2 or μ MT^{-/-} CD90.2 T cells, where the survival of CD4 T cells derived from either B cell deficient or wt ID93/GLA-SE immunized mice could be followed relative to the internal CD90.1 T cell control. We also examined whether transferred cells could

be boosted with a subsequent immunization. While we observed a significant reduction in the percentage of responding T_H1 CD4 T cells from B cell deficient donors, there was no significant further reduction in the percentage of cells when those surviving cells were boosted at week 3, suggesting that the main impairment in of T_H1 cells generated in B cell deficient mice is their survival, and that this deficiency is not rescued by the presence of B cells after the priming event. When looking strictly at tetramer numbers there was no apparent difference in wt and B cell deficient mice, similar to that observed in prior studies (5). This suggests that the responding T_H1 CD4 T cells generated in μ MT^{-/-} mice might be impaired rather than dying at the time point observed in this study (3 weeks after immunization). These data confirm our results above showing that the MPEC generation is impaired in B cell deficient mice and thus, B cells are required upon priming. Further experiments will be needed to investigate whether B cells are also required for T_H1 CD4⁺ T cells maintenance.

To investigate antigen-specific CD4 T cells further, the MHC-II tetramer-positive T cells that recognize an epitope of the ID93/GLA-SE vaccine were used to determine whether CD4 MPEC could be restored following transfer of wt B cells into μ MT^{-/-} recipient mice. We show that MPECs are partially restored as determined by T-bet^{lo}Ly6C^{lo} or PD1⁺ KLRG1⁻ stained cells (memory phenotype). B cell transfers do not restore B cell numbers to similar levels observed in wt mice, which could explain why only a partial restoration of the phenotype was observed. Interestingly, MHC-II ^{-/-} B cells from wt mice were not capable of restoring the memory phenotype, suggesting that antigen presentation by B cells is required for MPEC generation. The requirement for antigen presentation by B cells is strongly debated (11, 18, 23-27). Gray *et al* showed that antigen presentation by B cells is not necessary for inducing primary T cell responses to *Salmonella enterica* infection and instead is needed later (7-14 days) for effector T cell response sustenance (18), which disagrees with the results presented here where B cells are required earlier. In their study, however, survival of the T cells generated at day 7 in mixed bone marrow chimeras, in which B cells could not present antigen via MHC-II, was not tested. As shown in our study those T_H1 T cells might have normal cytokine producing capacities at that time point, but may already be poised to become impaired later on. In additional studies this function was shown to be needed much earlier (at days 3-4) upon immunization with a model vaccination regimen (11). One caveat of this study is that they used a TCR transgenic model in which the frequency of naïve antigen-specific T cells is artificially increased; they hypothesized that the APC capacity of DCs might be insufficient in this model. Another hypothesis which is in

agreement with the results presented here is that the antigen presenting capacity of B cells may be needed upon immunization, where APC activation is less robust than that observed following infection.

One caveat of our studies is that they were performed in μ MT-/- mice which have altered splenic architecture and which could potentially influence our results (40, 41). However, the B cell deficient phenotype was partially restored when wt and not MHC-II-/- B cells were transferred in the μ MT-/- mice 3 days prior to immunization suggesting that the CD4 MPEC generation impairment observed in the μ MT-/- mice is due to a lack of antigen presentation by B cells rather than a defect in the splenic architecture of those mice. Taken together these data show that B cell antigen presentation is necessary, from the earliest stages of the response, to drive T cells down the memory pathway in the context of vaccination. Previous studies, done with another model antigen, have shown that dendritic cells and their antigen presenting capacity are essential for the generation of a T cell response influenced by GLA-SE (42). Consequently both DC and B cells MHC-II presentation are necessary for the generation of a robust long-lasting T_H1 responses to GLA-SE. Further studies would be necessary to determine why the antigen presentation by DC and B cells is not redundant in this system and particularly what kind of extra and essential antigen presentation capacity is brought about by B cells.

The data presented show that there is failure to develop a strong T_H1 memory response in the absence of B cells or their antigen-presenting capacity. These results highlight the importance of a multi-faceted immune system working in concert for cell-mediated immunity against a number of important pathogens. Collectively, our studies and recent advances in understanding the role B cells play in both humoral and cellular immunity will have a high impact on vaccine development against several pathogens especially those requiring T_H1 cell-dependent immune responses, and will be useful to clinicians that utilize B cell depleting monoclonal antibody therapy in patients with B cell disorders.

MATERIALS AND METHODS

Animals and immunizations

Female wt C57BL/6 mice, μ MT-/- (B cell deficient), CD45.1+, CD90.1+, and MHC-II-/- mice, 6-10 weeks of age, were purchased from The Jackson Laboratory. All strains were on the C57BL/6

background. Mice were immunized by i.m. injection with either saline or ID93 (a recombinant fusion protein comprised of Rv3619, Rv1813, Rv3620, and Rv2608) formulated with the adjuvant GLA-SE to provide a final vaccine dose of 0.5µg ID93 and 5µg GLA-SE (21). All animals were housed in the IDRI animal care facility (Seattle, WA) under specific pathogen-free conditions. All animal experiments and protocols used in this study were approved by IDRI's Animal Care and Use Committee (ACUC).

Spleen and LN processing

Single-cell suspensions of splenocytes or draining LN (dLN) cells were obtained by dissociating the whole organ through a 100-µm nylon cell strainer (BD Falcon). Red blood cells from the spleens were lysed with Red Blood Cell Lysis Buffer (eBioscience) and resuspended in RPMI 1640/10%FBS with pen/strep and glutamine (cRPMI) for further analysis.

Intracellular Cytokine Staining and tetramer staining

Processed splenocytes or dLN (inguinal) cells were stimulated for 2 hours with either ID93 (10 µg/ml) in cRPMI, or cRPMI alone. Stimulated cells were then treated with BrefeldinA (Golgi Plug, BD Biosciences) and incubated for 8 hours at 37°C, then transferred to 4°C. Cells were surface stained with fluorochrome labeled antibodies to CD4 (clone GK1.5), CD8 (clone 53-6. 7), CD90.1 (clone OX-7), CD90.2 (clone 53-2.1), CD45.1(clone A20) and CD45.2 (clone 104) in the presence of 20% normal mouse serum for 20 min at 4°C. Cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min at room temperature. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome labeled antibodies to IFN-γ (clone XMG-1.2), IL-2 (JES6-5H4), TNF (MP6-XT22) and CD154 (clone MR1) for one hour at 4°C. Cells were washed and resuspended in PBS+1% BSA. Up to 10⁷ events were collected on a four laser LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo. Cells were gated as singlets > lymphocytes > CD4⁺ CD8⁻ > cytokine positive; except for the T cell survival analysis where cells were gated as singlets > lymphocytes > CD4⁺ CD8⁻ CD45.1⁻ CD45.2⁺ > cytokine positive or tetramer⁺ > CD90.2⁺. ID93-specific response frequencies were determined by subtracting the frequency of unstimulated cells with positive responses from ID93-stimulated cells in matched samples.

Percentage recovery of CD90.2 cells for each category was calculated as follow:

$$\frac{(\text{number of CD90.2}^+ / \text{number of total CD45}^+)_{\text{output}}}{(\text{number of CD90.2}^+ / \text{number of total CD45}^+)_{\text{input}}} * 100.$$

Alternatively CD4 cells were positively selected with the mouse CD4 (L3T4) microbeads from Miltenyi Biotec and stained, in the presence of 20% normal mouse sera, for 1h at 37°C with an I-A^b tetramer presenting the dominant epitope of Rv3619 (VIYEQANAHGQ), one of the components of ID93 (22). APC-labeled tetramers were provided by the National Institutes of Health Tetramer Core Facility. Cells were washed and stained for surface CD4 (clone RM4-5), CD8 (clone 53-6. 7), Ly6G (clone 1A8), CD11b (clone M1/70), CD19 (clone 1D3), CD11c (clone N418), F4/80 (clone BM8), ter119 (clone TER-119), CD44 (clone IM7), Ly6C (clone HK1.4), PD-1 (clone 29F.1A12) and KLRG1 (clone 2F1). Cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min at room temperature. Cells were washed twice with Perm/Wash (BD Biosciences) and stained intracellularly with fluorochrome labeled antibodies to T-bet (clone 4B100) overnight at 4°C. Cells were washed and resuspended in PBS+1% BSA. Up to 10⁷ events were collected on a four laser LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo. Cells were gated as singlets > lymphocytes > CD4⁺ Dump⁻ (Ly6G, CD11b, CD19, CD11c, CD8, F4/80, ter119) > tetramer⁺ > PD1⁺ KLRG1⁻ or T-bet^{Lo} Ly6C^{Lo}.

B and T cell adoptive transfers

For the B cell adoptive transfer experiments, spleens and LNs (inguinal, popliteal, iliac, axillar and brachial) from 5 naïve donor mice per strain (wt or MHCII^{-/-}) were harvested and processed as described above. Cells were pooled for each strain and B cells were isolated by positive selection with the mouse Pan B Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. Approximately 2.5x10⁷ B cells/mouse in 300µl of PBS were transferred into naïve recipient µMT^{-/-} mice by i.v. injection.

For the CD4 T cell adoptive transfer experiments, 5 wt (CD90.2), 5 µMT^{-/-} (CD90.2) and 10 CD90.1 mice were immunized with ID93/GLA-SE. One week later their spleens and dLN (inguinal, popliteal, iliac and axillar) were harvested and processed as described above. Total cells from the 5 wt (CD90.2) and 5 CD90.1 constituting the first combined donor population (control) and 5 µMT^{-/-} (CD90.2) and 5 CD90.1 mice constituting the second combined donor population (experimental) were pooled and CD4 T cells were isolated with the mouse CD4 T Cell

Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Approximately 3.5×10^7 CD4 T cells/mouse in 300 μ l of PBS were transferred into naïve CD45.1 recipient mice by i.v. injection (see Fig. 5a for a schematic representation of the experimental design).

Statistical Analysis

Statistical analysis was determined by one-way or two-way ANOVA with Bonferroni correction for multiple comparisons, or by student's t-tests as indicated for each experiment. Graphs and statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $p < 0.05$ was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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A. INTRODUCTION

TB (Tuberculosis) treatment is complex and lengthy; the first line therapy involves 6-9 months of combination drug treatment consisting of isoniazid (INH) rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) (190). Treatment for drug-resistant TB (DR-TB) requires even more prolonged (up to 2 years) combinations of second and third line antibiotics added to the regimen, including capreomycin, ethionamide and streptomycin¹. The development of new drugs is currently the leading solution to the increasing incidence of resistance to the drugs currently employed for TB therapy. Several new drugs are in development but it is not clear when they will receive regulatory approval for use in humans. The current (minimal) six-month therapy leads to many problems, among which is the high proportion of patients failing to adhere to the TB treatment. As a result of this non-compliance (non-adherence) of patients, especially in TB- HIV co-infected patients there is an increased risk of therapy failure, resulting in relapse of infection and/or development of resistance(191, 192). Thus, in addition to the need for new antibiotics to treat drug resistant isolates, there is an urgent need for treatment regimens which can shorten therapy for *Mycobacterium tuberculosis* (Mtb) (193). Development of a novel co-treatment approach using classical chemotherapy and a therapeutic vaccine product that addresses alternatives to TB therapy would be a major breakthrough. We would like to develop a novel combination immuno-chemotherapeutic product that addresses alternative strategies to shorten TB therapy and/or reduce relapse rates.

Currently only a handful of therapeutic vaccines are in the TB development pipeline. The most advanced is an inactivated whole-cell *M. vaccae* based vaccine. This vaccine was tested for in human clinical trial in post-exposure settings with encouraging results. When one dose of inactivated *M. vaccae* was administered in combination with antibiotic treatment, significant clinical improvements *i.e.* early sputum culture conversion and greater radiographic improvement were seen in patients in a clinical trial conducted in Uganda (194). Another trial with three doses of *M. vaccae* also showed clinical improvements, *i.e.* improved weigh gains and better clearance of cavities and opacities in chest radiograph, in chemotherapy treated patients (195). This progress however meant that treatment with chemotherapy is still crucial.

IDRI has demonstrated proof of concept that the addition of a vaccination program with ID93/GLA-SE to antibiotic treatment delivers a more effective cure, reducing mortality and relapse rates in a mouse model of TB (182). This was done in a mouse model of fatal TB: the SWR mice. When given either in conjunction with or after drug therapy (INH+RIF), ID93/GLA-SE resulted in a remarkable improvement in survival and a significant reduction in lung bacillary loads. Importantly the survival was increased even when given with a shorter drug treatment, *i.e.* 60 days of drug treatment in combination with ID93/GLA-SE enhanced survival compared to 90 days of chemotherapy alone (182). This is promising since it suggests that therapeutic vaccination might be useful in the treatment of drug-sensitive TB to reduce chemotherapy duration and thus might improve compliance.

Besides therapy shortening, preventing inadequately treated individuals from progressing to active disease could also make a major impact on TB control by not only preventing disease in the infected individual but also future transmission from that individual.

The most widely used model of reactivation of latent TB or relapse is the Cornell model developed by McCune *et al.* in the 1950s (196, 197). In this model, mice receive a chemotherapy treatment until no bacteria can be cultured from any organ. After treatment cessation however, mice can potentially relapse spontaneously and following immunosuppression. Bacteria from the lungs and spleens can be cultured again. This model has been used for evaluating new vaccines and drug candidates for sterilizing activity *in vivo* (198, 199). Nevertheless this model has its limitations, drug treatments last from 3 to 6 months making the model extremely time consuming and laborious and the relapse of infection after treatment is totally unpredictable. Thus a large number of animals are required to gain sufficient statistical power.

In this study, to test whether ID93/GLA-SE can be used as a therapeutic vaccine to reduce relapse rates after drug treatment completion, we sought to use a mouse model that would significantly decrease the length of the treatments, show less variability and immediate relapse of infection.

B. MATERIAL AND METHODS

Mice

Female, 4-6 weeks old, C57BL/6, CB6F1 and SWR mice were purchased from Jackson Laboratory. All mice were maintained in the animal facility of the Infectious Disease Research Institute (IDRI)

in BL3 containment and were treated in accordance with the guidelines of the Animal Care and Use Committee.

Treatment

Mice were aerogenically infected with a low dose (50–100 bacteria) aerosol (LDA) of *Mtb* H37Rv (ATCC #27294) using a GlasCol aerosol generator. After 4 weeks of infection a subset of mice were started on a drug regimen of INH (250mg/L of drinking water), RIF (100mg/L of drinking water) with or without PZA (150mg/L of drinking water) administered for eight weeks total. Female mice are estimated to drink between 0.15 and 0.37mL/g (200). Assuming a mean intake of 0.26mL/g per day animals would receive approximately 65mg/kg of INH, 26mg/kg of RIF and 39mg/kg of PZA. A subset of groups were immunized with GLA-SE alone or ID93/GLA-SE which were produced as previously reported (7, 201). Mice were immunized 3 times 3 weeks apart, starting 2 or 4 weeks after antibiotic treatment start. ID93 was used at 0.5µg/dose and GLA-SE at 5µg GLA-2% oil/dose.

Bacterial burdens

Lungs and spleens from the infected mice were harvested at the defined time-points and homogenized in 0.1% PBS–Tween 80. 5-fold serial dilutions for lungs and 8-fold serial dilutions for spleens were plated on 7H10 agar plates (Molecular Toxicology) for bacterial growth. Bacterial colonies were counted after incubation at 37°C for 14–21 days.

Statistical analysis

Bacterial burdens were normalized by log₁₀ transformation. Prism 5 (GraphPad Software) was used to determine statistical significance. Differences in bacterial burdens were assessed using one-way analysis of variance when more than two groups were used and the Student T-test for experiments with only two groups. Log Rank test was used to evaluate statistical difference between survival curves. P<0.05 was considered significant.

Addition of ID93/GLA-SE reduces relapse in a C57BL/6 model of disrupted chemotherapy treatment

A ‘short duration’ chemotherapy entails the use of a stronger chemotherapy regimen than the one used in the Cornell model. We used a modified version of the method used by Bucerri *et al.* (202). In this model, 2 weeks after i.n. infection, 250mg of INH and 100mg of RIF per liter of drinking water are given for a period of 4 weeks. According to the authors, this regimen almost completely abrogates recovery from viable *Mtb* from the lungs and spleens of treated mice and is followed by a rapid spontaneous “relapse” (regrowth) of *Mtb* within the 2-4 weeks following the treatment arrest (202). The modifications made to this experiment include (i) an infection using the well-defined low dose aerosol infection model which is thought to more closely recapitulate the human infectious inoculum (203) and (ii) chemotherapy treatment starting 4 weeks after infection when the bacillary load in the lungs is stabilized (data not shown). After 4 weeks of chemotherapy (week 8) the CFU counts in the lung and spleens were still very high. Mice were put in a second round of 4 weeks chemotherapy from week 10 to week 14 to achieve CFU numbers more similar to the one observed in Bucerri *et al.* publication (around 2logs) (202) (Figure 1A). Mice were immunized 3 times 3 weeks apart starting on week 8 (end of first round of chemotherapy) with either GLA-SE (adjuvant alone) or ID93/GLA-SE (Figure 1A). After two rounds of chemotherapy (week 14), CFU counts in treated mice were reduced 4 logs in the lungs and spleens (figure 1B and C). A pronounced spontaneous relapse was observed 4 weeks after chemotherapy arrest (week 18). Neither GLA-SE nor ID93/GLA-SE had a significant effect in viable counts when given in mice not receiving drug treatment. When mice received chemotherapy treatment and immunized with ID93/GLA-SE the mean CFU in the lungs and spleens were significantly reduced at both “relapse” time points, 4 and 10 weeks after chemotherapy arrest (figure 1B and C).

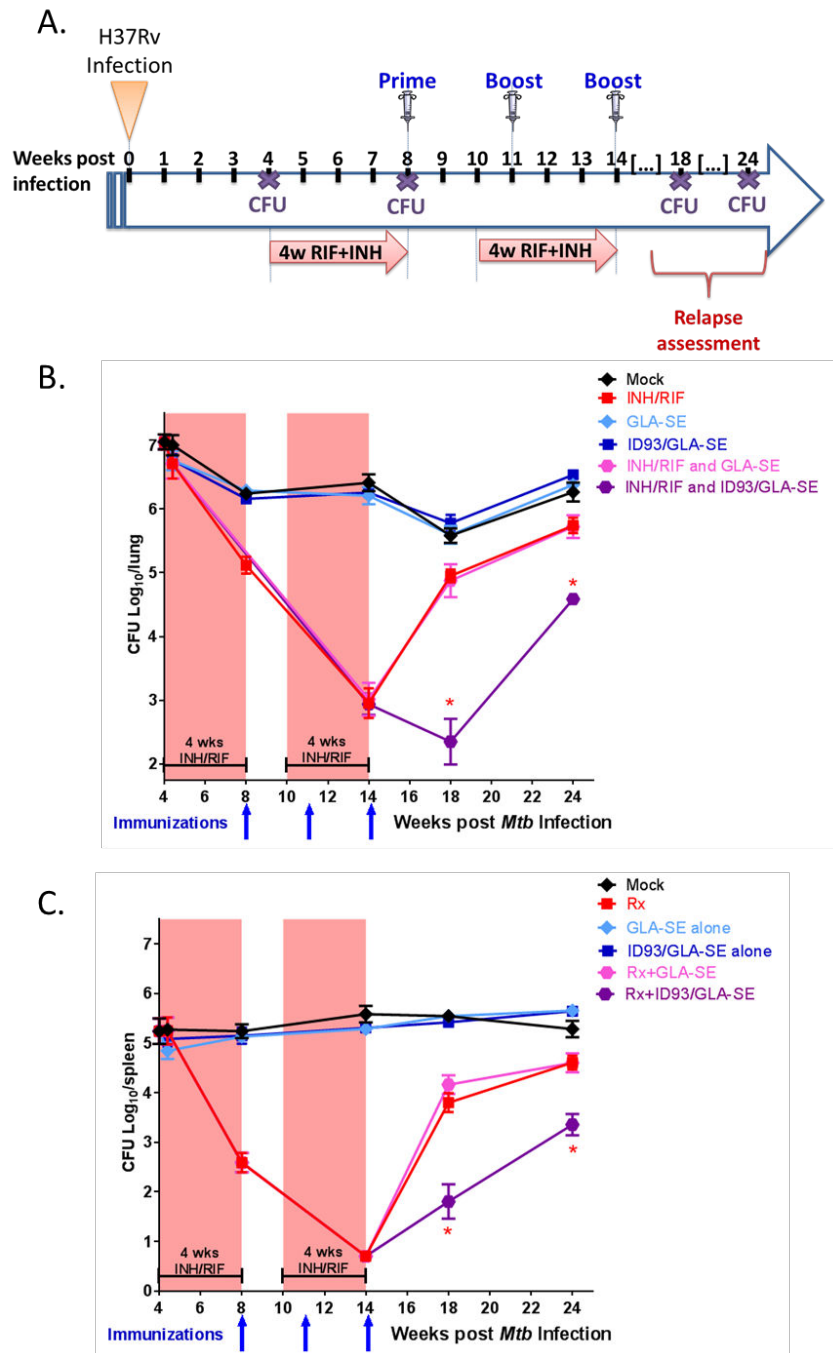


Figure 1. Colony-forming unit counts of C57BL/6 mice treated with mock or antibiotics and/or with GLA-SE or ID93/GLA-SE.

C57BL/6 mice were infected with LDA *Mtb* (day 0). 4 weeks later mice were mock or RIF+INH treated for 4 weeks (weeks 4-8), rested for 2 weeks and then treated again for 4 weeks (weeks 10-14). A subset of mock and antibiotic treated mice were also immunized 3x3 weeks apart with either GLA-SE or ID93/GLA-SE starting at week 8. (A) Scheme of Immunotherapy experiment.

Numbers of viable bacteria in the lung (B) or spleen (C) of animals were determined at the CFU time-points indicated in (A). Mean + s.e.m. values are shown. p values were determined by one-way ANOVA/Dunnett's test * $p < 0.01$. 5 animals per group.

Addition of ID93/GLA-SE reduces relapse rates and increase survival in a suboptimal chemotherapy model

Further, we sought to confirm the above results and analyze whether the shown beneficial adjunct effect of ID93/GLA-SE had also a beneficial effect on survival rates. The chemotherapy treatment used was as above 250mg of INH and 100mg of RIF per liter of drinking water but supplemented with PZA (150mg/L) to more closely mimic standard human chemotherapy treatment and was given for 8 weeks uninterrupted (Figure 2 A). 3 immunizations 3 weeks apart were administered starting at week 6 so the end of chemotherapy coincides with the last immunization. Two mouse strains were used. CB6F1 mice are the offspring of a cross between BALB/CJ females and C57BL/6J males. This strain was chosen since BALB/c mice are the preferred strain for evaluation of *Mtb* drugs, whereas C57BL/6 mice are the preferred strain for most vaccination studies. The second mice strain used are SWR, unlike C57BL/6 and BALB/c mice, which exhibit stabilized pulmonary bacterial growth and survive for more than one year (204-208), SWR/J mice exhibit extreme *Mtb* susceptibility with progressive bacterial growth resulting in fatal disease, thus making them a good post-exposure model to rapidly evaluate immunotherapeutic regimens (206). Relapse rates were evaluated in CB6F1 and SWR and survival rates were determined in SWR. As observed above no difference was observed in the CFU counts during the chemotherapy treatment between INH/RIF/PZA with or without ID93/GLA-SE. ID93/GLA-SE significantly reduce the mean CFU in the lungs and spleens at the "relapse" time point 4 weeks after chemotherapy arrest in both strains and at 10 weeks after chemotherapy arrest in SWR mice (Figure 2 B and C). SWR mice survival (median survival = 36.9 weeks) was concordant with the observed CFU reduction with mice treated with chemotherapy in combination with ID93/GLA-SE surviving close to 2 months more than chemotherapy only treated mice (median survival = 29.4 weeks) (Figure 2D).

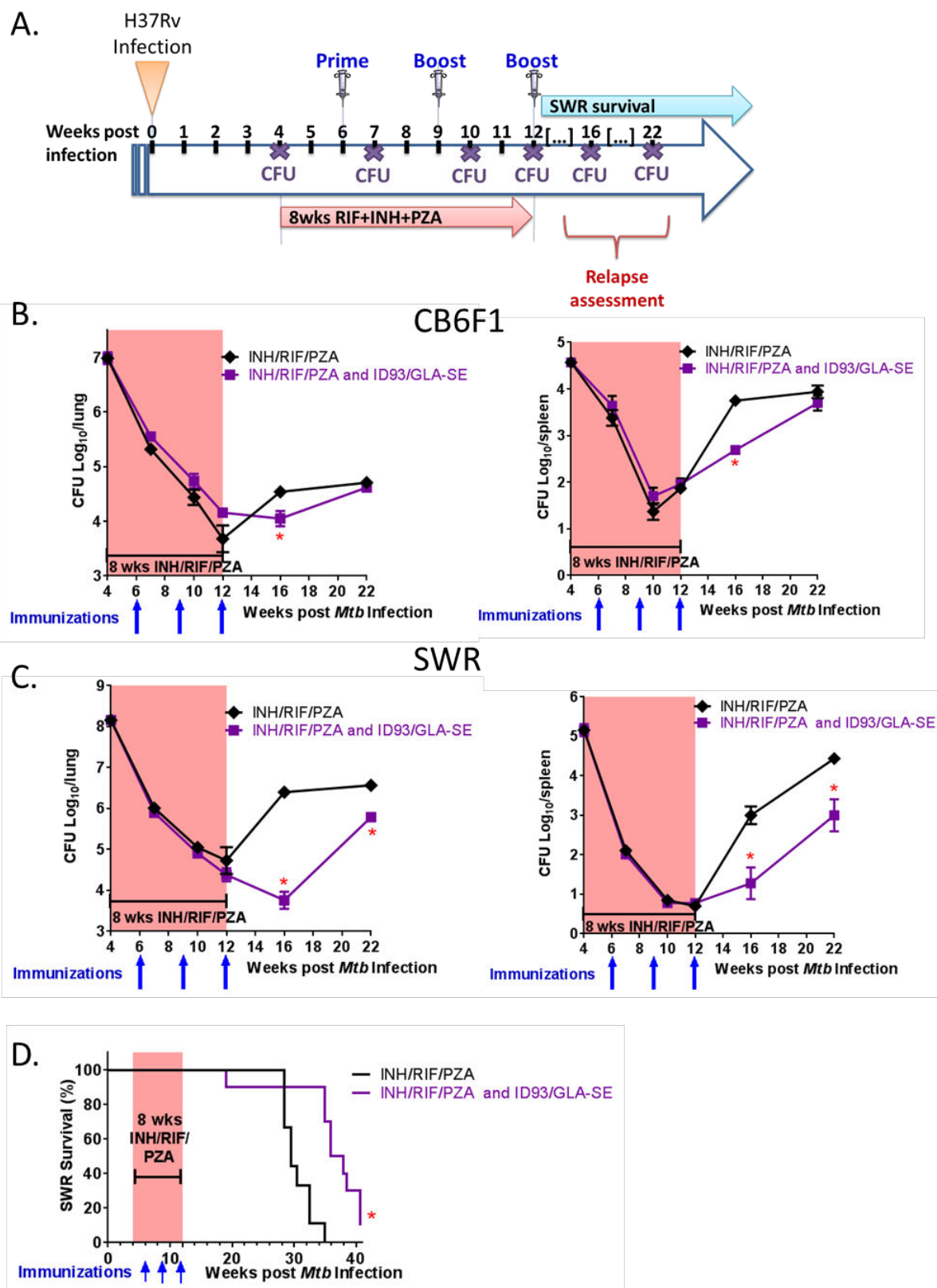


Figure 2. Colony-forming unit counts and survival in two mouse strains treated with antibiotics in combination with ID93/GLA-SE.

CB6F1 or SWR mice were infected with LDA *Mtb* (day 0). 4 weeks later mice were RIF+INH+PZA treated for 8 weeks (weeks 4-12). A subset of mice was also immunized 3x3 weeks apart with either GLA-SE or ID93/GLA-SE starting at week 6. (A) Scheme of Immunotherapy experiment. Number of viable bacteria in the lung and spleen of CB6F1 (B) or SWR (C) were determined at the CFU time-points indicated in (A). (D) Protection was assessed by monitoring animal death. Mean + s.e.m. values are shown. p values were determined by one-way ANOVA/Dunnett's test for CFU counts and Log Rank Test for survival *p<0.01. 5-7 animals per group for CFU and 10 animals per group for survival.

D. DISCUSSION AND CONCLUSION

Mice therapeutic models are difficult to develop due to the number of variables that need to be determines *i.e.* antibiotic concentrations, chemotherapy length, timing, number and distance between immunizations, etc. that are today far less established than the mouse prophylactic model to test vaccine candidates. Another big challenge is that, even with a “short duration chemotherapy treatment”, the length of these studies makes them extremely expensive and time-consuming.

To mimic non-compliant regimen in humans two different chemotherapy regimen were used in this study: RIF+INH for 8 weeks with 2 weeks rest in the middle or RIF+INH+PZA for 8 weeks. Neither of those regimens achieved complete bacterial clearance in the lungs.

No differences were observed during the course of chemotherapy when ID93/GLA-SE was used in combination with chemotherapy, this is not surprising since the chemotherapy regimen used is very strong to achieve a quick CFU reduction in lungs and spleens of mice, this “strong chemotherapy regimen” was expected to hide any benefit of adding a therapeutic vaccine while the treatment was ongoing (202). The endpoint expected was a reduction in “relapse CFUs” when the mice were taken out of the chemotherapy regimen. This latter was achieved in all 3 strains of mice (C57BL/6, CB6F1 and SWR) when the chemotherapy was combined with ID93/GLA-SE immunizations.

GLA-SE alone had no effect on the relapse CFUs, showing that the generation of an immune response against ID93 was required and that the reduction observed in ID93/GLA-SE immunized mice was not due to an overall enhancement of inflammation that could have been triggered by GLA-SE independently of ID93.

Furthermore no effect of GLA-SE or ID93/GLA-SE was observed in the bacterial burdens when they were given in non-chemotherapy treated mice after infection, this was to be expected since at the time-point of immunization (8 weeks after infection) *Mtb* infection is already established and there is a strong immune response against TB generated by infection.

Unfortunately the CFU and survival results showed that in the chosen regimens ID93/GLA-SE is delaying relapse rather than preventing it. Why the elicited protection by ID93/GLA-SE is fading over time remains to be elicited. One hypothesis is that with the chemotherapy regimen used the ongoing infection was still too high titer and established, between 3 and 4 logs in the lungs, for the candidate therapeutic vaccine to be able to control it durably. In the future it would be interesting to test ID93/GLA-SE in combination with a longer or stronger chemotherapy regimen that reduces the bacillary load further but without achieving complete clearance to still allow a quick relapse after chemotherapy arrest. It has to be noted that in this model while having the advantage of a shorter treatment time, the rapid re-growth of *Mtb* might be due to different mechanisms than those involved in the long duration models of relapse.

Nevertheless this study shows that ID93/GLA-SE elicits some protection against *Mtb* when used as therapeutic vaccine in combination with a standard chemotherapy regimen and is a good proof of concept of the possibility of using ID93/GLA-SE in combination with antibiotics as a therapeutic vaccine against TB which would limit further usage of chemical drugs and thus minimize their toxicity and cost.

Further analysis will be needed to understand what component(s) of the response to ID93/GLA-SE administered post-therapeutically are protective and improve the infection outcome and whether the vaccine affects different *Mtb* reservoirs than those targeted by the drugs (*e.g.* latent bacteria). Better understanding of ID93/GLA-SE therapeutic capacities is crucial since this vaccine is currently undergoing a Phase 2a clinical trial to evaluate its safety and immunogenicity when administered to adult pulmonary TB patients, following successful completion of TB treatment with confirmed bacteriologic cure, in preparation for a future Phase 2b prevention of TB recurrence trial in the same population (5).

DISCUSSION AND PERSPECTIVES

A. GLA-SE ADJUVANTICITY MECHANISMS OF ACTION

The development of the appropriate type of immune response is essential for successful immunization. IDRI's leading candidate adjuvant GLA-SE promotes strong T_{H1} CD4 T cells and IgG2-skewed B cell responses to protein vaccine antigens. On one side augmentation of antigen-specific B cell responses and subsequent antibody production has been central to the development of effective vaccines against a variety of pathogens. One of the main characteristic of B cell response to GLA-SE is that it is induced very quickly after immunization correlates with an early onset protection (209). In settings such as the emergence of new pandemics, intentional release of bioterror agents, and on-demand traveler's vaccines, the rapid initiation of humoral immunity with practical vaccine approaches is highly desirable. On the other side robust cell-mediated immunity, which is associated with a $Th1$ type immune response, is thought to be required for the control of intracellular pathogens, including TB, viruses and cancer (60, 210-212).

Recent insights into the functioning of the innate immune system have demonstrated its crucial role in the development of an effective antigen-specific immune response and thus its key role in vaccines adjuvanticity (189).

A better understanding of GLA-SE mechanism of action is crucial for the rational development of next generation vaccine adjuvants tailored to elicit specific immune responses but knowledge on GLA-SE mechanisms of action is still scarce (Section I IV.C). One of the main objectives of this thesis was to broaden that knowledge with a specific focus on determining which are the main actors in the innate immune initiation and subsequent adaptive response. For this purpose we divided our work in three main studies aiming to first elucidate the role of the pro-inflammatory cytokines IL-12, type I IFN and IL18, the transcription factor T-bet, then the LN SCM ϕ and the IL-18 cytokine and finally B cells in the response to GLA-SE.

The newly acquired knowledge brought by the work undertaken during this thesis is summarized in Table 2 and Figure 12.

CATEGORIE	COMPONENT	Innate response (strong CD69 induction)	Strong CD4+ TH1 polyfunctional response	Strong B cells IgG2c skewed response	References
Adjuvant	GLA+SE	YES	YES	YES	(12, 184) Article 2
Pathway components	Caspase 1/11	YES	YES	?	(12)
	NLRP3	NO	?	?	(12)
	MyD88/TRIF	?	YES	YES	(188)
Cytokines	IFN γ	YES	YES	?	(12, 79)
	IL-18	YES	YES	YES	(12) Article 2
	IL-12	?	YES	NO	Article 1
	type I IFN	YES	YES	NO	Article 1
Transcription Factor	T-bet	?	YES	YES	Article 1
Cells	DC	?	YES	NO	(186)
	SCM ϕ	YES	YES	YES	Article 2
	B cells	?	YES (<i>memory</i>)	YES	Article 3

Table 2. Updated components, cytokines, transcription factors and pathways required for the different elements of the immune response to GLA-SE based on the results obtained during this thesis. YES = required, NO = not required, ? = no data, white and italic font = updated knowledge.

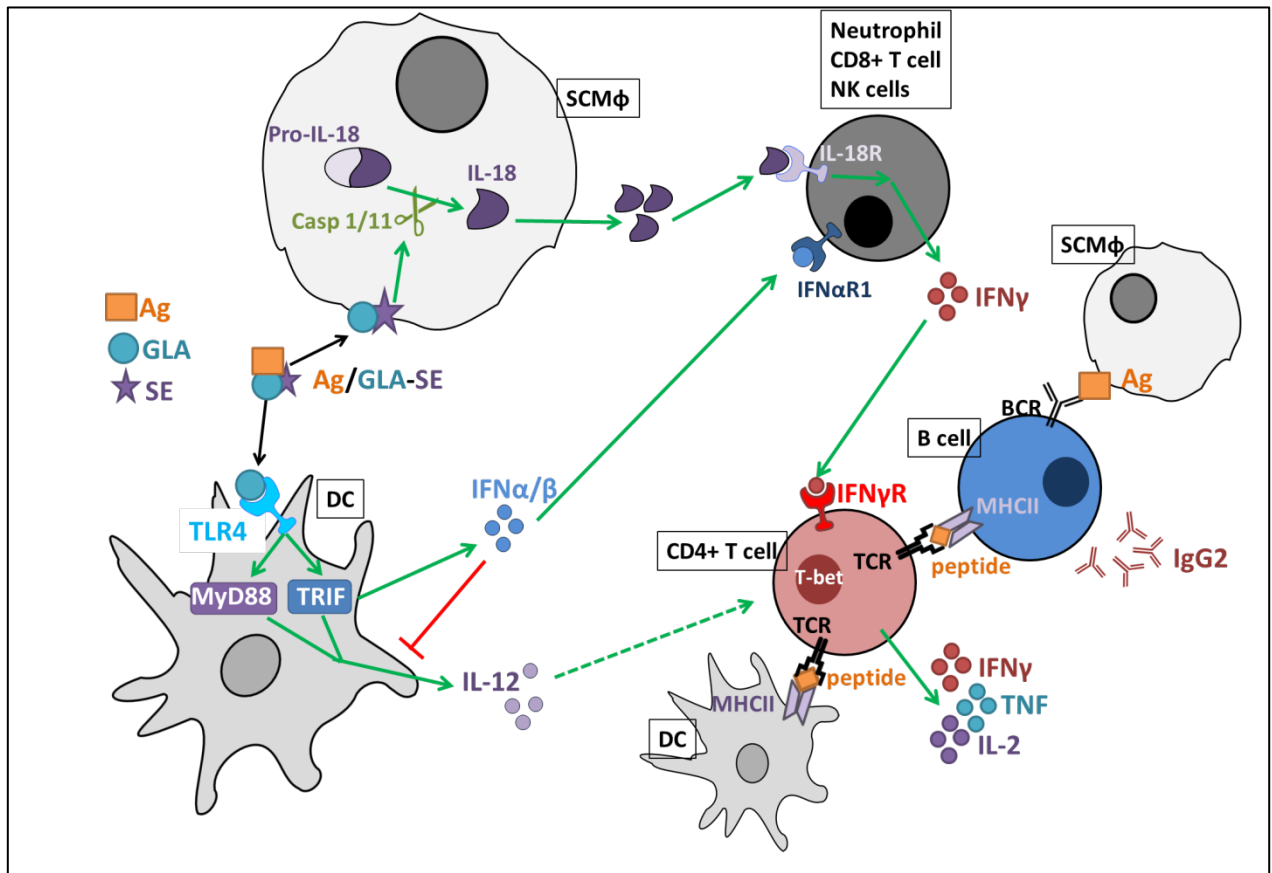


Figure 12. Updated representation of the suggested GLA-SE mechanisms of action in the draining LN based on the results obtained during this thesis. GLA-SE will be quickly recognized by the sub-capsular macrophage (SCMφ) cell population in the draining LN. This will induce the Inflammasome leading to caspase 1 activation and the subsequent cleavage of the pro-IL-18 in its mature form IL-18. GLA will be recognized by DC through TLR4 leading to the engagement of MyD88 and TRIF pathways which will result in IL-12 expression (MyD88 and TRIF dependent) and type I IFN production (IFNα/β) (TRIF dependent). Type I IFN will partially inhibit IL-12 production establishing a regulation loop. IL-18 and type I IFN will be recognized by NK and CD8+ T cells and neutrophils which will lead to innate IFNγ secretion by these cells. IFNγ will be recognized by its receptor on CD4+ T cells and will help in the establishment of the TH1 response and the subsequent secretion of TH1 cytokines (IFNγ, TNF and IL-12) by the activated CD4+ T cells. Another crucial role of the SCMφ is the association of the antigen (Ag) on its surface that will be presented to naïve B cells step crucial for the IgG2 skewed strong B cell proliferation and subsequent antibody production. B cells also play an important role as antigen-presenting cells to CD4 T cells leading to induction of TH1 Memory Precursor Effector CD4 Cells but the main APC for CD4 cells are the DCs. IL-12 production and T-bet induction are crucial for the TH1 induction.

The work conducted during this thesis has helped us in the elucidation of GLA-SE mechanism of action but there still are a lot of unknowns and questions to answer stemming from this work, many of which are being studied in ongoing and future experiments.

We demonstrated that IL-12 is needed for the CD4 TH1 induction by GLA-SE but not for IgG2c skewing, whereas T-bet is necessary for both. Since B-cell intrinsic T-bet has been shown to mediate early IgG2c production (213) further experiments will be needed to determine if CD4 T cell intrinsic T-bet expression upon ID93-GLA-SE vaccination is required to promote IgG2c skewing.

We have shown that IL-12 and IL-18 are required for the induction of T_{H1} responses and IL-18 was shown to be also required for the early innate IFN γ production by neutrophils and memory CD8 cells (12). Moreover IL-18 has been shown to synergize with IL-12 to induce IFN γ production in T cells and NK cells (214). Further studies are ongoing to determine if IL-12 is also required for the induction of the innate IFN γ production by innate immune cells observed upon immunization with GLA-SE.

The clodronate depletion helped us define the SCM ϕ population as a central player in GLA-SE adjuvanticity particularly in the induction of the innate response and subsequent quick and strong B cell response. This population was important for the production of IL-18 upon immunization and IL-18 was shown to be partially responsible for the SCM ϕ -dependent expansion of antigen-specific B cells but it remains to be determined whether IL-18 is acting directly on B cells to drive their expansion, through the IFN γ pathway, or via a distinct pathway. Either way, IL-18 production is most likely not the only role of SCM ϕ in B cell induction since the depletion of this population had much profound effects than the one observed in IL-18R^{-/-} mice. The SCM ϕ population is composed of the sinusoidal SCM ϕ and the medullary SCM ϕ , our hypothesis is that the specific population that is crucial for the rapid induction of the innate response is the medullary macrophages since we have shown that they are the main immune cell population interacting with GLA-SE early after immunization. The main known difference between sinusoidal and medullary macrophages is that the latter express SIGNR1 and the former don't, it would be important to know if SIGNR1's had any role in GLA-SE recognition or adjuvanticity. Studies are ongoing to determine SIGNR1 role in GLA-SE adjuvanticity. Furthermore SIGNR1 is a transmembrane C-type lectin that regulates the immunoglobulin-independent classical complement fixation pathway by interacting with C1q (215). Early results using cobra venom factor, which has been described to induce complement depletion, showed that complement might be implicated in the response to GLA-SE (data not shown). If further studies confirm those results it would be interesting to define if medullary SCM ϕ and the complement pathway roles in the induction of the innate response to GLA-SE are linked. Alternatively SIGNR1 could be implicated in the presentation of the unprocessed antigen to B cells since a crucial role for SCM ϕ in the presentation of antigen to follicular B cells has been recently established (216).

We also showed that B cells and most likely their antigen presentation capacities are required upon priming for CD4 MPEC generation and eventually the establishment of a long lasting CD4 memory response to GLA-SE, supplementary experiments will be needed to investigate how exactly B cells program MPEC generation and whether B cells are also needed at latter time points for CD4 memory maintenance following GLA-SE immunization.

Beyond the different factors studied during this thesis and discussed above, a wide variety of cytokines, pathways and innate immune activators still remain to be studied in the context of GLA-SE immunization, including extracellular ATP release which has been shown to be important for MF59 adjuvanticity (217), IL-6 and IL-1 β roles and others which are currently undergoing further investigation.

Understanding the mechanisms of action of this adjuvant and others provides crucial information on how innate immunity influences the development of adaptive immunity, help in rational design of the next generation of vaccines against a variety of diseases, and can inform on adjuvant safety and thus help inform the potential licensing for human use.

B. ID93/GLA-SE AS AN IMMUNOTHERAPEUTIC VACCINE

The development of new drugs is currently the leading solution to the increasing incidence of resistance to the drugs currently employed for TB therapy. Although new, faster-acting drugs are in development, history predicts that their very usage may lead to selection of resistant *Mtb* strains. Development of a novel co-treatment approach using classical chemotherapy and a post-exposure vaccine product, for which resistance developing is far more unlikely, that addresses alternatives to TB therapy would be a major breakthrough. Many beneficial effects can be expected to result from an efficient therapeutic vaccine including (i) reduction of treatment length and then an improvement in compliance, (ii) prevention of drug-resistant TB development, (iii) reduction of relapse/reactivation caused by insufficient drug efficacy or poor compliance.

The capacity of ID93/GLA-SE to shorten chemotherapy treatment length was previously demonstrated in the SWR mouse model (182) and the work performed during this thesis has shown proof of concept that ID93/GLA-SE can also better the outcome of a sub-optimal antibiotic treatment, but much work still remains to be done.

It will be important to understand the arms of the response to ID93/GLA-SE that play a role in the enhancement of protection against TB in the therapeutic vaccine setting.

It would also be extremely interesting to test ID93/GLA-SE capacities to reduce the development of drug-resistant *Mtb* when given in combination with a standard drug therapy. The hypothesis behind these studies is that the effect of adding a therapeutic vaccine to an antibiotic regimen will not only reduce treatment times, but will be bactericidal and prevent post-treatment disease recurrence and thus drug-resistant development. Additionally, because therapeutic vaccines target a different set of pathogen modalities, it should be effective against both drug-sensitive and drug-resistant *Mtb* thus permitting both the simplification and shortening of treatment duration.

GENERAL CONCLUSION

In conclusion, the research that is the foundation of this thesis has helped us to better define the different characteristics of GLA-SE mechanism of action and to broaden our knowledge and current understanding of the different steps and actors required for the broad adaptive response induced by GLA-SE (Figure 13) (218) (unpublished data). Nevertheless much work remains to be done to fully understand those mechanisms and being able to draw a complete figure highly complex response orchestrated by the combination of the TLR4 agonist GLA and the squalene oil-in water-emulsion SE.

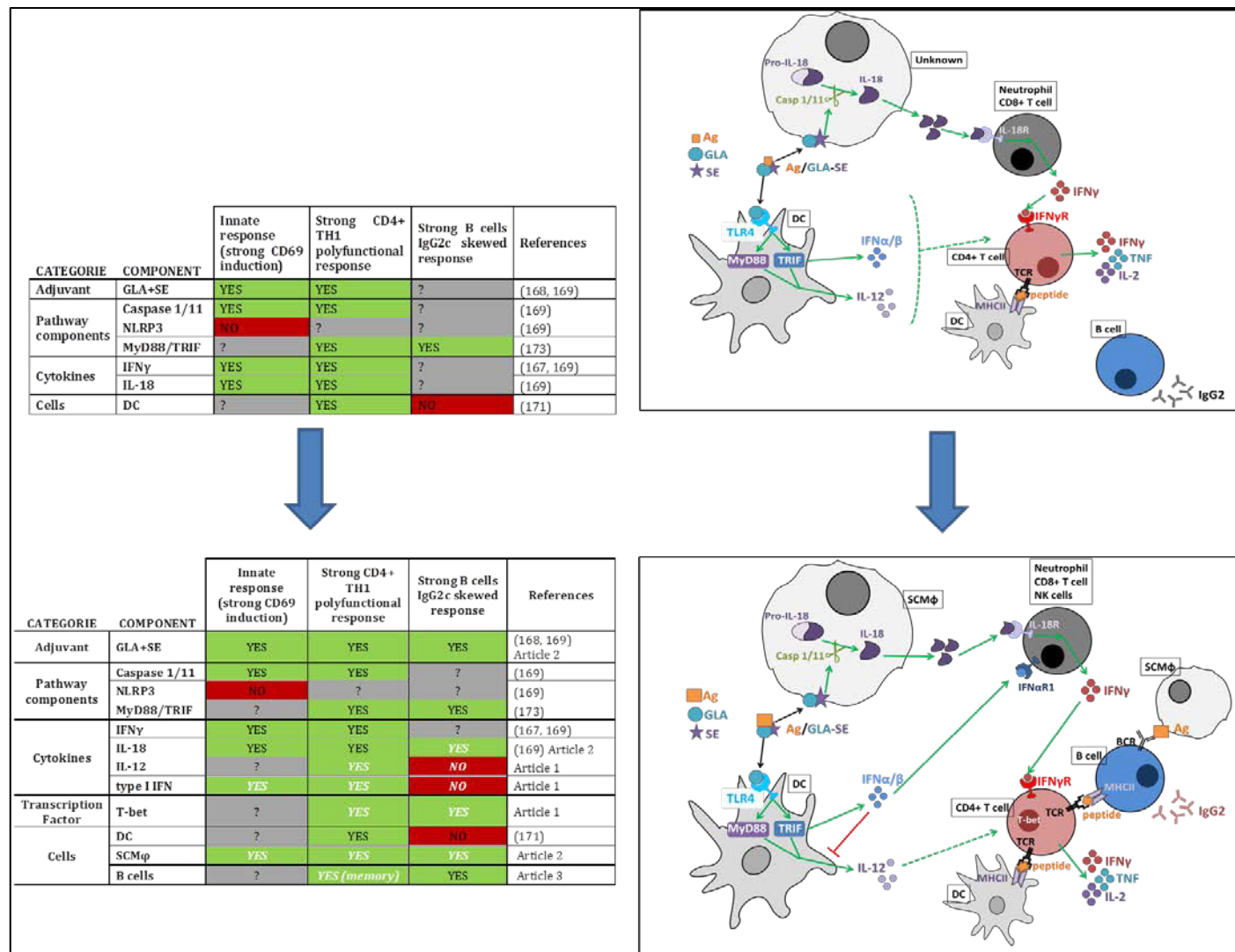


Figure 13. Broadening the knowledge on the mechanism of action associated with GLA-SE.

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Titre : Caractérisation de la réponse immune induite par un adjuvant comprenant un agoniste au TLR4 dans des modèles murins

Mots clés : adjuvant, réponse innée, réponse adaptative, TLR4, tuberculose, immuno-thérapie

Résumé : En 2014 la Tuberculose (TB) à dépassé le VIH comme la principale cause de décès par maladie infectieuse dans le monde soulignant le besoin urgent de développer un vaccin plus efficace contre cette maladie. Le candidat vaccin contre la TB, ID93/GLA-SE, développé à l'Infectious Disease Research Institute (IDRI), est aujourd'hui en essai clinique de phase IIa et a montré des résultats pré-cliniques et cliniques promettants. Dans un modèle murin de TB, ce vaccin induit une forte réponse T_H1 , considérée comme centrale dans la protection contre la TB, et la production d'IgG2 par les lymphocytes B. Néanmoins, les mécanismes d'action de GLA-SE sont encore peu connus.

L'objectif principal de cette thèse est donc d'élucider les mécanismes clés qui relient les réponses innées et adaptatives induites par cet adjuvant dans le modèle murin. Un objectif secondaire est d'établir un modèle murin de rechute de TB après traitement et d'évaluer l'utilisation d'ID93/GLA-SE en tant que vaccin immuno-thérapeutique et sa capacité à réduire les taux de rechute dans ce modèle. L'ensemble de ce travail nous a permis de mieux comprendre les mécanismes impliqués dans la réponse immunitaire adaptative induite par GLA-SE et de montrer la capacité de ID93/GLA-SE à être utilisé comme un vaccin thérapeutique contre la tuberculose dans le but de réduire les taux de rechute post-thérapeutique.

Title : Characterization of the immune response to a TLR4-based adjuvant in murine models

Key words : adjuvant, innate response, adaptive response, TLR4, tuberculosis, immunotherapy

Summary : In 2014 tuberculosis (TB) surpassed HIV as the leading cause of death by an infectious disease worldwide emphasizing the urgent need to develop a more effective vaccine against this airborne disease. The Infectious Disease Research Institute (IDRI) TB candidate vaccine ID93/GLA-SE is currently undergoing a Phase IIa clinical trial and has shown promising preclinical and clinical results. In murine models of TB this vaccine drives a strong CD4 T_H1 response, which is thought to be important for protection against TB, and an IgG2c skewed B cell response. However, little is known about the cellular and molecular events that drive GLA-SE adjuvanticity.

To that end, the main objective of this thesis was to elucidate the key mechanisms that connect innate and adaptive immune responses elicited by this adjuvant in the murine model. A secondary objective was to evaluate the possibility of using ID93/GLA-SE as adjunct therapy to existing antibiotic treatments to reduce relapse rates after TB treatment. Collectively the results obtained during this research project and thesis broaden our knowledge and our current understanding of the mechanisms involved in the adaptive immune response induced by GLA-SE and show the capacity of ID93/GLA-SE to be used as a therapeutic vaccine against TB to reduce post-therapeutic relapse rates.