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NOVEL STRATEGIES TO IMPROVE ANTI-INFLUENZA VACCINES

Positive contribution of adjuvanted immunization strategies during aging and in the resolution of viral-bacterial co-infections

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If you fail, never give up
because F.A.I.L. means "First Attempt In Learning".
End is not the end,
in fact E.N.D. means "Effort Never Dies".
If you get "No" as an answer,
remember N.O. means "Next Opportunity".
So let's be positive!

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SUMMARY

Despite the extensive use of anti-influenza vaccines during the last decades, influenza and its complications are still a major cause of morbidity and mortality worldwide. Older adults (>65 years) are particularly susceptible to influenza illness and it is estimated that approximately 90% of influenza deaths occurs in this population [1]. Notably secondary bacterial infections (SBI), the majority of which are associated with *S. pneumoniae* or *S. aureus*, make a significant contribution to deaths during influenza epidemics and pandemics, through a phenomenon known as "excess mortality" [2]. In order to reduce influenza-driven mortality, broader protective vaccines are needed and different strategies are possible. Among these, universal influenza vaccines or even broad-spectrum "pneumonia" vaccines targeting a range of different viral and bacterial respiratory pathogens are thinkable. To allow the design of such vaccines, a multitude of basic questions - such as the ideal vaccine composition, appropriate vaccine adjuvants and an understanding of the complex pathogen interactions - have to be addressed.

With the current work we wanted to address this specific medical need. In the first part we studied the special immunological pre-requisites for successful influenza vaccination in the elderly, while in the second part we extended our focus on the impact of different influenza vaccines on viral-bacterial co-infection.

Elderly people are particularly susceptible to influenza infection and its complications, but respond poorly to conventional vaccines. MF59-adjuvanted influenza vaccines have been specifically developed and licensed to target this age population and are considered - together with similar formulations - as the best strategy to prevent influenza disease in the context of immunosenescence [3]. Yet, the development process was entirely empirical and it is only poorly understood how MF59 contributes to successfully restoring responsiveness to influenza vaccines in the elderly.

To deeply investigate the mechanism of action of MF59 in elderly subjects, we assessed immune response elicited by this adjuvant in old mice (>18 months). Our results showed that MF59 is able to potentiate responses against influenza antigens not only in young mice (6-8 weeks), but also in older ones: it enhanced immune cell recruitment at the site of injection, antigen-translocation to draining lymph nodes, CD4$^+$ T cell response and germinal center formation. Yet, in line with clinical data, we noticed that hemagglutination inhibition (HI) antibody titers induced by MF59-adjuvanted vaccine in old mice were similar to those obtained in young ones immunized with not adjuvanted antigens arguing for the fact that MF59 can overcome some but not all aspects of immunosenescence. Accordingly, we

wanted to dissect, which of the MF59-induced signaling cascades were impacted by aging. We recently showed in young mice, that transient ATP-release in injected muscle is an important contributor to adjuvanticity of MF59 [4]. Here we verified that also in aged mice ATP plays a central role for adjuvant activity. Yet, while in young mice it is not the only actor of adjuvanticity, in elderly other MF59-targeted immune pathways seem to be reduced due to "immunosenescence" or "inflammaging".

MF59 is a safe, effective and well established vaccine adjuvant for influenza vaccine in humans with millions of doses administered. Whether there is room for further improvement of anti-influenza responses especially in the vulnerable elderly population has to be assessed.

Complications from secondary bacterial infection are a leading cause of influenza-associated morbidity and mortality. Anti-influenza vaccination is considered the best strategy to counteract primary viral disease spread. Moreover data from animal models suggest that it is also an effective method to prevent subsequent secondary bacterial pneumonia [5]. Yet, currently approved influenza vaccines are typically assessed only for their capacity to elicit neutralizing antibodies specific for the homologous (vaccine-type) influenza strain. Protection against heterotypic (antigenic shift by mutations within influenza strain) or against heterologous (HA and/ or NA differing from those in the vaccine strain) influenza infection is studied to a lesser extent. And importantly, studies in humans have typically not been designed or appropriately powered to assess effectiveness against SBI.

It can be assumed that prevention of influenza infection through vaccination would also prevent complications such as SBI, but in case of heterotypic or heterologous virus challenge - as would easily occur during a normal influenza season - does partial protection significantly affect bacterial super-infections? Furthermore different types of influenza vaccines induce differential innate and adaptive responses in infected individuals that might impact positively or negatively on SBI. Does this occur and can it be measured?

We aimed to answer these questions in pre-clinical models of differently anti-influenza immunized mice. To that extent, we vaccinated BALB/c mice systemically with an A/California/7/2009 (H1N1) subunit vaccine either as plain antigens or adjuvanted with i) MF59 to induce a mixed Th1/Th2 response [6], ii) a combination of MF59 and CpG to get a more Th1-prone response [6] or with iii) LTK63 administered via the mucosal route to obtain a Th1/Th17 polarized response [7]. After vaccination mice were challenged with the heterologous mouse adapted strain A/Puerto Rico/8/1934 (H1N1) (PR8) and infection course and various aspects of immune response were dissected. We found that vaccination via different administration routes and adjuvants enhances immune responses to influenza virus infection by creating in the host a differently Th-polarized environment: all tested priming conditions induced

strong vaccine-specific Th1, Th2 or Th17-polarized responses and anti-influenza antibody titers that quickly restored pre-infection immune environment in lung. On the contrary, plain immunization was significantly less effective: mice showed high viral titers similar to those of naïve ones and had overall higher influx of immune cells into the lung, an indication of ongoing inflammation. Notably mucosal vaccination with LTK63, though inducing lower HI titers, was equally good in protecting mice from influenza infection as systemic vaccination with MF59±CpG, strongly arguing for an important contribution of additional immune responses to protection in the setting of heterologous infection.

Secondly we asked if different flavors of immune responses during influenza infection would have a beneficial or detrimental impact on SBI caused by Methicillin-resistant *S. aureus* (MRSA) USA300, which has been recently associated with increasing cases of fulminant post-influenza pneumonia in humans [8]. To this end we set up a new influenza-bacterial co-infection model in previously anti-influenza vaccinated mice. Immunizations were performed as before to skew the immune response towards different Th profiles. Mice were then infected with influenza PR8 virus and six days later co-infected with *S. aureus*. In this co-infection model we followed disease evolution by measuring mouse weight loss and pathogen clearance in lungs.

In this setting the differences between the single vaccination strategies became even more evident. While non-adjuvanted vaccine protected significantly from single influenza infection, it conferred little protection from viral-bacterial co-infection. Plain vaccinated mice were subjected to severe bacterial overgrowth and to high morbidity and mortality during SBI similarly to naïve mice. They just differed from naïve mice by their capability to control virus loads during SBI, while naïve mice showed a second wave of lung viral titer increase after bacterial infection that is a typical consequence of SBI [9]. In contrast, we demonstrated that all adjuvanted vaccines were superior in preventing not only viral infection but also bacterial superinfection as compared to plain antigens vaccination. In particular Th1-prone mice efficiently controlled influenza infection better than those receiving other formulations and were nearly not affected by SBI.

Altogether our results showed that adjuvanted-influenza vaccines are an efficient method to counteract not only heterologous influenza infection, but also eventual SBI. Moreover we demonstrated that the adjuvant MF59 is extremely important to enhance immunity against virus antigens in aged preclinical models. MF59 could eventually be improved by adding immunopotentiators like CpG to further enhance Th1-prone immune responses. These responses seem to be superior for preventing both viral and viral-bacterial infection.

RIASSUNTO

Nonostante che negli scorsi decenni si sia fatto un ampio uso dei vaccini anti-influenzali, l'influenza e le relative complicazioni sono tuttora tra le maggiori cause mondiali di morbilità e mortalità. Le persone più anziane (>65 anni di età) sono particolarmente sensibili all'influenza e si stima che all'interno di tale popolazione si ritrovi circa il 90% delle morti dovute alla malattia [1]. Le infezioni batteriche secondarie (SBI) causate principalmente da *S. pneumoniae* e *S. aureus* rappresentano un'importante causa di morte durante le epidemie e pandemie influenzali attraverso un fenomeno conosciuto come "mortalità eccessiva" [2]. Affinché si riesca a ridurre la mortalità dovuta all'influenza, occorrono vaccini con un più ampio spettro di protezione. Tra le possibili strategie troviamo vaccini influenzali universali o addirittura vaccini "generici" contro la polmonite in grado di difendere l'organismo da un'ampia gamma di virus e batteri patogeni per l'apparato respiratorio. Affinché si arrivi allo sviluppo di tali vaccini innovativi, occorre definire innanzitutto alcuni aspetti basilari, quali ad esempio la loro composizione ideale e la scelta degli adiuvanti appropriati, il tutto insieme ad una maggiore conoscenza delle complesse interazioni tra i patogeni target.

Nel presente lavoro di tesi abbiamo voluto approfondire questo specifico aspetto medico. Nella prima parte dello studio abbiamo definito quali sono i particolari prerequisiti immunologici per la buona riuscita della vaccinazione anti-influenzale negli anziani. Nella seconda parte invece ci siamo focalizzati sull'impatto che differenti tipologie di vaccini anti-influenzali possono avere sulla co-infezione tra il virus e un batterio.

La popolazione anziana, che è particolarmente suscettibile all'influenza e alle sue complicazioni, risponde scarsamente ai vaccini convenzionali. I vaccini adiuvantati con MF59 sono stati sviluppati e approvati specificatamente per questa popolazione target e, insieme a formulazioni simili, sono considerati ad oggi la migliore strategia per prevenire l'influenza nell'ambito dell'immunosenescenza [3]. Tuttavia lo sviluppo di tali vaccini è stato puramente empirico e ben poco si sa di come MF59 contribuisca a ristabilire nelle persone anziane un'efficiente risposta al vaccino.

In questo studio abbiamo analizzato la risposta immunitaria indotta da MF59 in topi anziani (>18 mesi) in modo da definire meglio il meccanismo di azione dell'adiuvante nei soggetti in età avanzata. Dai nostri risultati si evince che MF59 è in grado di potenziare la risposta immunitaria nei confronti dell'influenza non solo nei topi giovani (6-8 settimane), ma anche in quelli più vecchi. Abbiamo dimostrato infatti che l'adiuvante induce robusto reclutamento di cellule immunitarie al sito d'iniezione

del vaccino, potenzia la traslocazione dell'antigene ai linfonodi drenanti e incrementa la risposta delle cellule T CD4$^+$ e la formazione dei centri germinativi. Tuttavia, in linea con i risultati clinici, i titoli anticorpali d'inibizione dell'emoagglutinazione (HI) indotti dalla vaccinazione con MF59 nei topi anziani raggiungono livelli simili a quelli ottenuti nei topi più giovani vaccinati senza l'adiuvante. Da questo risultato possiamo dedurre che MF59 è in grado di porre rimedio ad alcuni degli aspetti caratterizzanti l'immunosenescenza, ma non a tutti. In accordo con ciò, abbiamo voluto definire meglio quali tra le cascate di segnalazione indotte da MF59 è impattata dall'invecchiamento. In nostro gruppo ha recentemente dimostrato in topi giovani che l'iniezione di MF59 nel muscolo induce un rilascio transiente di ATP che si rivela poi importante per l'effetto adiuvante del prodotto [4]. In questo lavoro di tesi abbiamo verificato che anche nei topi anziani il rilascio di ATP gioca un ruolo centrale per l'attività dell'adiuvante. Tuttavia, mentre nei topi più giovani tale rilascio non è l'unico "attore" del potenziamento immunologico indotto dall'adiuvante, in quelli più vecchi gli altri pathway avviati da MF59 sembrano essere impattati negativamente dall'immunosenescenza e dallo stato di continua infiammazione tipico degli anziani.

MF59 è un adiuvante sicuro ed efficace e il suo utilizzo nella vaccinazione anti-influenzale umana è ormai consolidato con milioni di dosi somministrate. Quello che resta da definire è se c'è la possibilità di un ulteriore miglioramento della risposta anti-influenzale soprattutto in una popolazione così vulnerabile come quella degli anziani.

Le cause principali di morbilità e mortalità associate con l'influenza sono da imputarsi alle SBI. La vaccinazione anti-influenzale è considerata ad oggi la migliore strategia per combattere la diffusione della malattia. Inoltre, dati risultanti da studi su modelli animali, rivelano che la vaccinazione anti-influenzale è anche un metodo efficace nella prevenzione di polmoniti batteriche conseguenti all'influenza [5]. Purtroppo i vaccini attualmente in commercio sono testati soltanto per la loro capacità di indurre anticorpi neutralizzanti specifici per il virus influenzale omologo al ceppo contenuto nel vaccino stesso. Non sono molto diffusi studi riguardanti la protezione indotta dai vaccini nei casi d'infezioni di virus influenzali eterosubtipici (cioè varianti antigeniche dovute a mutazioni all'interno di un ceppo influenzale) o eterologhi (le cui proteine HA e/ o NA differiscono da quelle presenti nel vaccino). Inoltre occorre notare che non sono stati ancora stabiliti studi clinici appropriati per definire l'effettiva efficienza dei vaccini influenzali nei confronti delle SBI.

Si può facilmente assumere che la prevenzione dell'infezione influenzale indotta dalla vaccinazione possa anche prevenire le relative complicazioni come le SBI, ma in caso d'infezione di virus eterosubtipici o eterologhi - situazione che può normalmente verificarsi durante la stagione influenzale – quale impatto

può avere una protezione parziale dall'influenza sulle superinfezioni batteriche? Inoltre formulazioni diverse dei vaccini anti-influenzali inducono negli individui infettati risposte immunitarie innate e adattative diverse che possono avere un impatto positivo o negativo sulle SBI. Questa situazione si verifica realmente e come può essere quantificata?

In questo lavoro ci siamo fissati l'obiettivo di rispondere a queste domande utilizzando come modello di studio pre-clinico topi immunizzati contro l'influenza mediante svariate formulazioni di vaccini. Brevemente i topi BALB/c sono stati vaccinati per via sistemica con il vaccino a subunità specifico per il virus A/California/7/2009 (H1N1) sia utilizzando gli antigeni influenzali da soli, sia in formulazioni adiuvantate con i) MF59 in modo da indurre una risposta mista Th1/Th2 [6], ii) MF59+CpG per ottenere una risposta polarizzata verso il profilo Th1 [6] o con iii) LTK63 somministrato per via mucosale affinché la risposta immunitaria fosse indirizzata verso un profilo Th1/Th17 [7]. Dopo la vaccinazione, i topi sono stati infettati col virus A/Puerto Rico/8/1934 (H1N1) (PR8): tale virus è eterologo rispetto agli antigeni contenuti nel vaccino utilizzato ed è un ceppo virale adattato al topo. Nel corso dello studio abbiamo seguito l'evoluzione dell'infezione e vari aspetti della risposta immunitaria. I nostri risultati dimostrano che la somministrazione del vaccino mediante vie diverse e l'utilizzo di svariati adiuvanti potenziano la risposta immunitaria nei confronti dell'infezione influenzale creando nell'ospite un ambiente polarizzato verso i diversi profili Th: tutte le condizioni d'immunizzazione testate inducono elevate risposte immunitarie polarizzate verso i profili Th1, Th2 o Th17 e titoli anticorpali in grado di ristabilire velocemente la situazione immunitaria del polmone ad un livello pari a quello presente prima dell'infezione. Al contrario, il vaccino non adiuvantato si è dimostrato significativamente meno efficiente: i topi mostrano elevati titoli virali simili a quelli dei topi naïve ed hanno un robusto influsso di cellule immunitarie all'interno dei polmoni che identifica l'instaurazione di un processo infiammatorio. Occorre notare che la vaccinazione mucosale adiuvantata con LTK63, pur inducendo titoli HI più bassi, stabilisce un livello di protezione dall'infezione pari a quello della vaccinazione sistemica con MF59±CpG. Questo ci fa supporre che nel contesto di un'infezione eterologa, ai fini della protezione, sia molto importante il contributo di risposte immunitarie addizionali alla risposta anticorpale sistemica.

Partendo dai risultati ottenuti, ci siamo chiesti se le varie tipologie di risposta immunitaria indotte durante l'infezione d'influenza avessero un impatto positivo o negativo su SBI causate da *S. aureus* USA300 resistente alla meticillina (MRSA). Questo ceppo batterico è stato infatti recentemente associato con un numero crescente di casi di polmonite fulminante post-influenzale [8]. A questo scopo abbiamo stabilito un nuovo modello d'infezione influenzale-batterica nei topi vaccinati per l'influenza. Le immunizzazioni sono state eseguite come in precedenza in modo da polarizzare le risposte immunitarie

verso i vari profili Th. In seguito i topi sono stati infettati col virus influenzale PR8 e sei giorni dopo co-infettati con *S. aureus*. In questo modello di co-infezione abbiamo seguito l'evolversi della malattia misurando il peso corporeo dei topi e quantificando la replicazione dei patogeni nei polmoni.

Nel nostro modello di co-infezione le differenze tra le singole strategie di vaccinazione si sono marcate ancora di più. Sebbene il vaccino non adiuvantato proteggesse abbastanza bene dalla semplice infezione influenzale, è in grado di conferire soltanto una protezione parziale durante la co-infezione. Infatti, i topi vaccinati con tale formulazione sono soggetti a un'incontrollata crescita batterica e mostrano elevati livelli di morbilità e mortalità comparabili a quelli dei topi naïve. Si discostano dai topi naïve soltanto per la loro capacità di controllare la replicazione virale durante la SBI: mentre i topi naïve mostrano una seconda ondata d'incremento del titolo virale nei polmoni dopo l'infezione batterica - tipica conseguenza della SBI [9] -, i topi che avevano ricevuto il vaccino non adiuvantato continuano il controllo del virus indipendentemente dalla SBI. Comparando i risultati del vaccino non adiuvantato con quelli ottenuti dalle tre formulazioni contenenti adiuvanti, abbiamo dimostrato che tutti i vaccini adiuvantati sono superiori non solo nella prevenzione dell'influenza, ma anche nel caso della superinfezione batterica. In particolare i topi il cui sistema immunitario aveva una polarizzazione verso il profilo Th1 sono in grado di controllare più efficientemente l'infezione influenzale rispetto ai topi che avevano ricevuto una delle altre due formulazioni adiuvantate e inoltre la SBI non ha quasi impatto negativo su di loro.

Nel complesso i nostri risultati dimostrano che i vaccini influenzali adiuvantati sono un metodo efficiente per combattere non solo un'infezione influenzale eterologa, ma anche un'eventuale SBI. Abbiamo inoltre dimostrato che l'adiuvante MF59 è di estrema importanza per potenziare la risposta immunitaria nei confronti degli antigeni virali nel modello pre-clinico di topi anziani. MF59 può essere eventualmente implementato mediante l'aggiunta di "potenziatori" del sistema immunitario come ad esempio il CpG, in modo da rafforzare le risposte polarizzate verso il profilo Th1. Queste risposte, infatti, risultano essere superiori per la prevenzione sia della semplice infezione virale sia della co-infezione.

INTRODUCTION

INFLUENZA VIRUS

Influenza, commonly known as flu, is a contagious respiratory viral disease of global importance. Yearly, influenza viruses cause an estimated 3-5 million cases of severe illness, and 250,000-500,000 fatal cases [1, 10]. The global importance of influenza is evidenced by the World Health Organization's (WHO) establishment of the Global Influenza Surveillance Network (GISN) in 1952 and an international vaccine development program on a scale not comparable to any other infectious disease [11].

The term *Influenza* derived from Italian language and was initially used to ascribe the cause of disease to some unfavorable astrological influences. Then, with the progress in medical science, the word came to designate, in all languages, human and animal respiratory illness caused by influenza virus infections.

Structure and classification

Influenza viruses are enveloped RNA viruses, belonging to the *Orthomyxoviridae* family. They are classified into three influenza virus genera, or virus types, influenza A, B and C, according to antigenic differences between their matrix and nucleoproteins. Influenza A, B and C viruses also differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology. Humans are the only host for influenza B viruses, while influenza A viruses can infect a various range of animal species including birds, pigs, horses, dogs, and humans. Particularly influenza A viruses are responsible for pandemic outbreaks of disease and for most of the well-known annual flu epidemics. Influenza C virus, which is substantially different from the A and B viruses, is of minor importance for human influenza infections, causing only a mild common-cold-like disease. [*The entire paragraph refers to* [12]].

The A and B viruses have a segmented genome composed of eight gene fragments of single-stranded negative-sense RNA encoding 12 proteins: surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), two matrix proteins (M1 and M2), the nucleoprotein (NP), three polymerase complex proteins PB1, PB2 and PA, and four non-structural proteins NS1, NS2, PA-X and PB1-F2. The RNA segments are packaged in the viral core which is surrounded by a lipid membrane, or "envelope", derived from the plasma membrane of the infected host cell during the process of budding of progeny virus from the cell

surface. Influenza virion shape is roughly spherical with a diameter ranging from 80 to 120 nm, although pleomorphic particles may occur. The external lipid layer contains numerous copies of the two major spike glycoproteins HA and NA together with lesser amount of M2 proton-selective ion channel protein. HA and NA surface glycoproteins have antigenic properties, indeed they are able to induce antibody response in humans. [*The entire paragraph refers to* [12] *and* Fig. 1A].

In humans, the primary targets for influenza viruses are epithelial cells in the upper and lower respiratory tract. Hemagglutinin binds to sialic acid-containing receptors on the host cell surface and is responsible for the penetration of the virus into the cell cytoplasm [13]. HA-specific antibodies are therefore able to block infection of cells and are believed to be the primary method by which prevention of infection occurs [14]. The second envelope glycoprotein neuraminidase has enzymatic activity catalysing the cleavage of sialic acid residues from glycoproteins or glycolipids on the surface of infected cells. Consequently NA action facilitates the release of viral progeny after inside-cell replication [15]. The third surface protein M2 forms a tetramer with ion channel activity. The inner side of the envelope that surrounds the influenza virion is coated by the antigenic matrix protein M1. By acidifying the environment inside virions, the M2 channel mediates the dissociation of ribonucleoproteins (RNPs) complex from M1-lining leading to virus uncoating process [16]. The viral RNPs complex forms the core of the virus that is constituted by each of the eight RNA segments wrapped in the nucleoprotein and attached to polymerase complex proteins (PB2, PB1, PA) [17]. [*The entire paragraph refers to* Fig. 1A]

Influenza A viruses are classified into subtypes depending on their surface antigens HA and NA: currently 17 different HAs and 9 NAs are known [12, 18]. Virus subtypes are serologically distinguishable, i.e. antibodies to one virus subtype do not react with another. The current nomenclature system for influenza A viruses takes into consideration the virus type, geographic location of first isolation, strain number, year of isolation and virus subtype (Fig. 1B) [19].
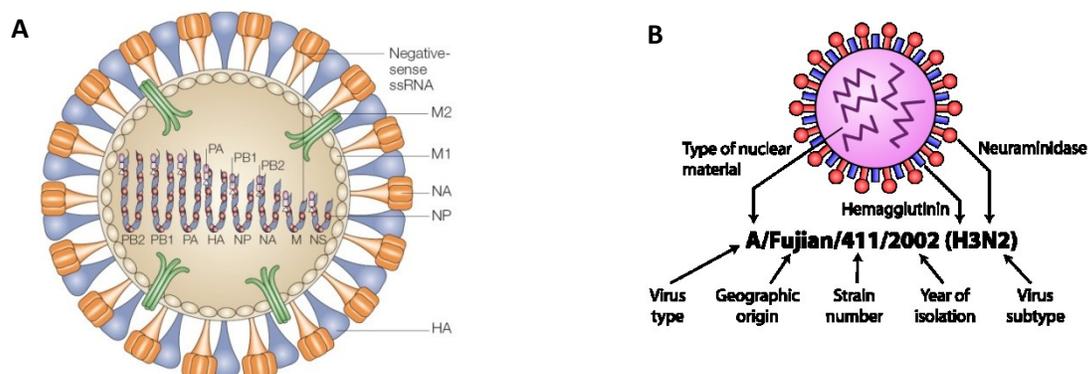

Figure 1 - Influenza virus structure and nomenclature. (A) Model of influenza A virus [20]. (B) Diagram of influenza nomenclature [21].

Antigenic drift and shift

Influenza viruses continuously undergo antigenic evolution to escape the pre-existing immunity [22]. This variation in viral proteins implies that immune responses mounted against earlier forms of the virus are less effective or completely ineffective against newer variants. Since the viral surface glycoprotein HA is the antigen against which virus-neutralizing antibodies are directed, variations in this protein are primarily responsible for the immune escape of influenza viruses. Other viral antigens undergo significantly less variation, but - under immunological pressure - may also contribute to the evasion of the immune defence of the host [23]. The antigenic evolution of influenza viruses is the leading cause of the occurrence of annual influenza epidemics and occasional pandemics. Influenza A viruses change their antigenic properties by two distinct mechanisms: "antigenic drift" and "antigenic shift".

Antigenic drift. Antigenic drift occurs via errors during replication, which are irreparable. The change produced by antigenic drift is due to error-prone polymerase resulting in accumulation of genetic mutations that are selected by immunological pressure for HA and to a lesser extent NA [24, 25]. Most of these mutations do not affect the conformation of the proteins; however, some of them cause alterations to the viral proteins such that the binding of host antibodies is impaired. Consequently, host antibodies raised to previously circulating strains can no longer efficiently inhibit infecting viruses, allowing the virus spread among the population [26]. During the seasonal influenza outbreaks, those virus particles that have successfully accumulated mutations to evade pre-existing immunity will prevail enhancing disease spread [22]. Accordingly, the influenza vaccines have to be reformulated almost every year to take account of the changing virus.

Antigenic shift. Antigenic shift is a more dramatic change in virus antigenicity. Indeed the process leads to the introduction of new influenza A virus with antigenically distinct HA molecules in the human population. When this novel subtype is transmitted efficiently from human to human, it may cause a pandemic influenza outbreak, since neutralizing antibodies to this virus are absent in the population at large [22]. Past pandemic outbreaks are known to have been caused by re-assortment of gene segments between two or more influenza strains (e.g. avian and human) infecting at the same time a "mixing vessel" (e.g. humans or pigs) leading to the creation of a completely new influenza virus with gene segments from both parent viruses [26, 27]. However, recent studies suggest that pandemics could also occur without re-assortment. For example avian influenza viruses, like H5N1, could be directly transmitted from animal reservoirs into the human population, requiring only a small number of adaptive mutations [28].

INFLUENZA DISEASE AND HISTORY

Influenza disease and complications

Influenza viruses pose a grave and unique threat to human health. Most influenza infections are spread by virus-laden respiratory droplets that are expelled during coughing and sneezing. Occasionally, the virus is transmitted to people by pigs or birds. About 20% of children and 5% of adults worldwide develop symptomatic influenza A or B each year [29]. During an influenza illness, the primary causes of disease are related to infection and replication of the virus in the respiratory epithelium. Indeed virus production leads to lysis of the epithelial cells and desquamation of the respiratory lining. Influenza infection causes a broad range of illness, from symptomless condition through various respiratory syndromes, fever, disorders affecting the lung, heart, brain, liver, kidneys, and muscles, to fulminant primary viral and secondary bacterial pneumonia. The common circulating strains of influenza virus normally remain restricted to the respiratory tract and escape only under exceptional circumstances. The severity and course of disease are affected by the patient's age - the very old and the very young are most susceptible to serious illness-, the degree of pre-existing immunity, properties of the virus, smoking, comorbidities (chronic heart, lung and kidney diseases, diabetes or immunosuppressive conditions), and pregnancy. Importantly secondary bacterial infections (SBIs), usually caused by *S. pneumoniae* or *S. aureus*, are known to be the "guilty parties" of a phenomenon known as "excess mortality" during influenza epidemics and pandemics, i.e. extraordinary high morbidity and mortality rate following influenza disease [2]. Although most influenza infections are self-limited, few other diseases exert such a huge toll of absenteeism, suffering, medical consultations, hospital admission, and economic loss. [*The entire paragraph refers to* [30]]

Influenza pandemics

If influenza epidemics are an important medical issue, pandemic outbreaks represent the real danger for human population associated with viral infection. Humanity experienced three influenza pandemics during 20th century and one in 2009. The most terrible outbreak was the Spanish flu (H1N1) in 1918, with an estimated 50 million deaths, justifying its description as "the last great plague of mankind" [31]. Notably studies have shown that bacterial pneumonia had a predominant role as a cause of excess mortality during this pandemic influenza: 95% of all severe illnesses and deaths were complicated by bacterial pathogens, most commonly by *S. pneumoniae* [32]. The successive pandemics in 1957 (Asian flu, H2N2) and 1968 (Hong Kong flu, H3N2) were milder, but nonetheless also caused a total of

approximately 2 million deaths [33]. In 2009, a novel H1N1 virus emerged from swine and caused the first pandemic in more than 40 years [34]. During this influenza outbreak mortality rates were similar to recent seasonal epidemics and most deaths occurred in young adults, often with no underlying chronic conditions [35].

IMMUNE RESPONSE TO INFLUENZA VIRUS INFECTION

When the influenza virus infects cells of the respiratory tract, both innate and adaptive immune responses are stimulated. The innate immune response develops very quickly and controls virus replication during the early stages of infection. While the innate immune system recognizes virus-infected cells through mechanisms that are not antigen-specific, the cytokines produced during this early phase of the host's defense facilitate activation of subsequent antigen-specific adaptive immune mechanisms [36].

Innate immunity

After initial exposure to a novel viral subtype, it takes between 5 and 7 days before specific antibodies and T cells arrive in the lung to definitively clear the virus. Consequently during first days of infection the activity of innate immunity is critical.

First line of defense against influenza virus is represented by physical barriers (e.g. mucus and collectins) that aim to prevent infection of respiratory epithelial cells. Influenza virus that enters the host through the oral or nasal cavities is first countered by the mucus that covers the respiratory epithelium. If the virus is successful in getting through the mucous layer, it must next attach to and invade the respiratory epithelial cells. From there, the virus can spread to both non-immune and immune cells - such as macrophages and dendritic cells (DCs) - in the respiratory tract [37, 38]. Rapid innate cellular immune responses are mounted at this point with the aim to control virus replication.

Respiratory epithelial cells and those of innate immunity detects influenza infection through the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs); these PAMPs are specifically present in the pathogen or are generated during infection [39]. Three distinct classes of PRRs are involved in the recognition of influenza-associated PAMPs: the Toll-like receptors (TLRs) which recognize different forms of viral RNA; retinoic acid-inducible gene I (RIG-I) receptor specific for 5'-triphosphate RNA; and the nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) recognizing various stimuli [27]. RIG-I and NLRP3

detect virus that is present within the cytosol of infected cells (cell-intrinsic recognition), whereas TLR3 detects virus infected cells, and TLR7 (and TLR8 in humans) detect viral RNA that has been taken up into the endosomes of sentinel cells (cell-extrinsic recognition) [40]. The interaction PAMPs-PRRs initiates antiviral signaling cascades, resulting in the production of type I interferons (IFN-α/β), pro-inflammatory cytokines as various interleukins (IL-1β, IL-18, IL-6, IL-12), eicosanoids and chemokines that together lead to recruitment of neutrophils, activation of macrophages and maturation of DCs [41]. Type I IFNs, produced by macrophages, pneumocytes and DCs, stimulate the expression of hundreds of genes that are collectively known as IFN-stimulated genes (ISGs) in neighboring cells, which induce an antiviral state [42-44]. Pro-inflammatory cytokines and eicosanoids cause local and systemic inflammation, induce fever and anorexia, and instruct the adaptive immune response to influenza virus. Chemokines recruit additional immune cells, including neutrophils, monocytes and natural killer (NK) cells, to the airways.

Macrophages. Upon infection of the alveoli, tissue-resident alveolar macrophages become activated and phagocytose apoptotic influenza virus-infected cells and thus limit viral spread [45, 46]. During influenza disease the lung macrophage pool is continuously replenished by circulating monocytes which differentiate into monocyte-derived macrophage after arriving to the site of infection [47]. If macrophages are essential for pathogen clearance, on the other hand once they become activated during influenza infection they produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF-α) and this way contribute to virus induced pathology [48-50].

Natural killer cells. Virally infected epithelial cells are targeted also by NK cells, which mediate viral clearance. NK cells are cytotoxic lymphocytes of the innate immune system that are able to lyse infected cells in a MHC class I independent manner via a direct or indirect mechanism of recognition. The sialylated NKp44 and NKp46 receptors are bound by the HA proteins expressed on the surface of influenza virus-infected cells and this results in direct lysis of the infected cell [51, 52]. In addition, NK cells possess CD16 receptor (FcγRIII) which binds to the Fc portion of antibodies bound to influenza virus-infected cells and mediate lysis of these cells. This process is known as antibody-dependent cell cytotoxicity (ADCC) [53, 54].

Monocytes and neutrophils. Monocytes and neutrophils are rapidly recruited to the site of infection and help to clear infected dead cells. Together with resident alveolar macrophages, phagocytic clearance of virus-infected cells by these recruited cells provides an important mechanism of viral clearance [55]. In addition to maturation to macrophages, monocytes that migrate to influenza virus-infected tissues can also differentiate into monocyte-derived (myeloid) dendritic cells (mDCs) which are the key antigen-

presenting cells for efficient initiation of adaptive immunity. But they contribute also to innate antiviral immune responses secreting type I IFN [56, 57]. The role of neutrophils in viral infection resolution or pathogenesis is not yet fully clear. It is well established that neutrophils - together with monocytes - are major effectors of acute inflammation characterizing anti-viral host response. But as for monocyte/ macrophages their hyperactivation can lead to lung immunopathology [58]. On the other hand recent studies showed that neutrophils can also ameliorate lung injury and the development of severe disease during influenza infection [59].

Dendritic cells. Lung DCs are a heterogeneous population and consist primarily of mDCs - also named conventional DC (cDC) - and plasmacytoid DCs (pDCs). DCs are professional antigen presenting cells (APCs) which form an important bridge between the innate and the adaptive immune system. The DCs are situated underneath the airway epithelium barrier and monitor the airway lumen via their dendrites that are extended through the tight junctions between the airway epithelial cells. DCs can detect opsonized (neutralized) virions and apoptotic bodies from infected cells but can also be infected themselves. In both cases DCs migrate, dependent on CCR7 chemokine receptor, to the draining lymph nodes (dLNs), where they present influenza virus-derived antigens to T cells and activate them [60, 61]. The DCs are able to degrade the viral proteins and present subsequently the immuno-peptides (epitopes) by MHC class I or class II molecules. In case of direct virus infection of DC, proteasomes in the cytosol degrade viral proteins into small peptides which are transported to the endoplasmic reticulum (ER) where they are loaded to MHC class I molecules. These MHC class I peptide complexes are then transported via the Golgi complex onto the cell membrane where they can be recognized by virus-specific CD8$^+$ cytotoxic T cells (CTLs) [62-64]. Instead, for MHC class II presentation DCs need to acquire viral antigens through phagocytosis of virus particles or apoptotic epithelial cells [65, 66]. Viral proteins are degraded into smaller peptides in endosomes/ lysosomes and presented on the cell surface in MHC class II peptide complexes which can be recognized by CD4$^+$ T helper (Th) cells. Th cells assist B cells to proliferate and mature into antibody-producing plasma cells. Via this route of antigen acquisition, DCs can also present epitopes in a MHC class I-mediated fashion to CD8$^+$ T cells. This is also known as cross-presentation. In addition to their critical role in initiating adaptive immune responses, DCs contribute to the antiviral innate immune response by secreting the powerful antiviral cytokine IFN-α/β in response to viral infection [40]. [*Details about innate control of adaptive immunity are resumed in Fig. 2*]
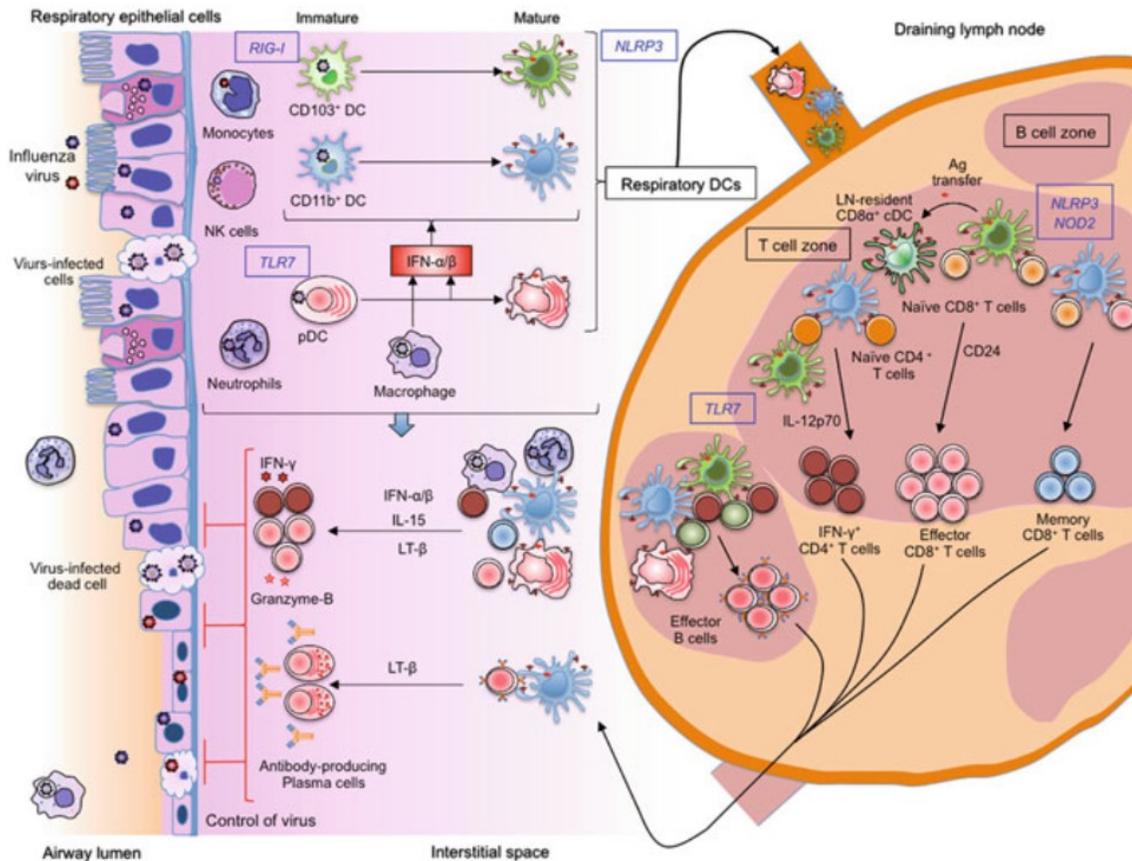
Figure 2 - **Innate control of adaptive immunity to influenza.** Innate immune cells, particularly DCs, in the respiratory tissues acquire antigens either through direct infection or by uptake of influenza-infected dead cells and undergo maturation process triggered by TLR7 or RIG- I-signaling, under the influence of type I IFNs produced by macrophages and pDCs. Respiratory DC subsets (CD103$^+$ cDCs, CD11b$^+$ cDCs and pDCs) migrate to dLNs, where they can transfer influenza antigens (Ag) to LN-resident CD8α$^+$ cDC. In the dLNs respiratory CD103$^+$ cDCs together with CD8α$^+$ cDCs stimulate the naïve CD8$^+$ T cells to proliferate and differentiate into cytotoxic effector CD8$^+$ T cells, in a CD24-dependent manner. On the other hand, CD11b$^+$ cDCs drive the activation of CD8$^+$ T cells, mainly effector T cells at later stage of infection, to induce memory CD8$^+$ T cells. Interaction of naïve CD4$^+$ T cells with cDCs generates IFN-γ-producing Th1 cells, which in turn facilitates the differentiation of effector B cells in a TLR7-dependent manner. These effector cells migrate from dLNs to respiratory tissues, where they have second interaction with Ag-bearing innate immune cells to undergo further activation and differentiation to terminal effector cells that secrete effector molecules to control virus spread [67].

Adaptive immunity

If the influenza virus is successful in establishing infection despite defenses mounted by innate immunity, the ultimate clearance of the virus requires the action of adaptive immunity. This consists of humoral and cellular responses mediated by virus-specific antibodies and T cells respectively (Fig. 3).

The lung presents especially delicate anatomical structures which necessitate a fine balance of pro- and anti-inflammatory responses for their preservation. Well-timed, appropriately placed and strictly regulated T cell and B cell responses are essential for protection from infection and limitation of symptoms, whereas poorly regulated inflammation contributes to tissue damage and disease. The specialized needs of the pulmonary environment impose that highly compartmentalized and sequential immune responses are essential for minimizing loss of function during the inflammatory processes of antiviral defense.
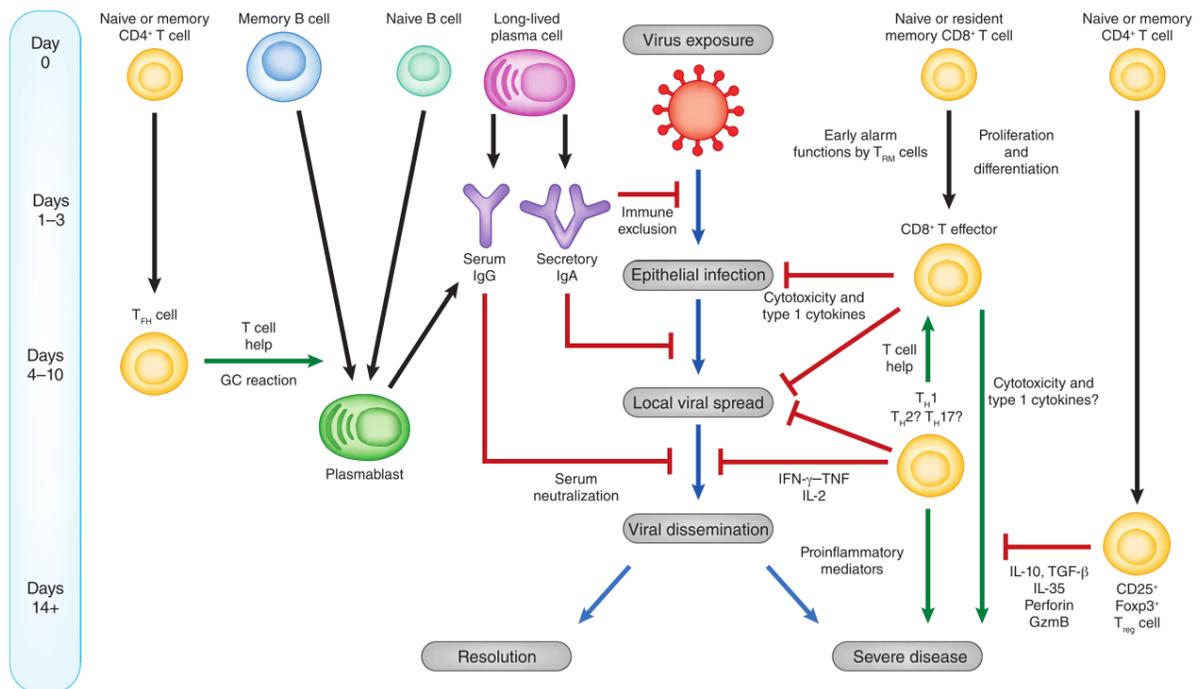
Figure 3 - The roles of adaptive T cells and B cells in respiratory viral infection. During acute respiratory viral infection, humoral and cell-mediated immunity act at different points in time to limit disease. Mucosal immunoglobulins A (IgA) generated during previous encounter with virus can prevent or limit infection. IgG in the lungs can limit more severe disease. T cells are beneficial in terms of eliminating virus-infected cells; they coordinate a regulated immune response and, as T follicular helper (T_{FH}) cells, promote high-affinity durable antibodies. Failure to control viral dissemination can lead to severe disease. Regulatory T (T_{reg}) cells restrain effector responses through various mechanisms, including suppressive cytokines (IL-10, IL-35 and TGF-β) and possibly active killing via perforin and granzyme B (GzmB). An over-exuberant or poorly regulated immune response can also lead to damaging immunopathology [68].

Humoral immune response. Influenza virus infection induces the production of virus-specific antibodies by B cells [26]. In particular, antibodies directed to the viral HA and NA surface glycoproteins correlate with protective immunity [69]. The HA-specific antibodies bind to the trimeric globular head of the HA predominantly and inhibit virus attachment and entry in the host cell leading to the neutralization of the pathogen [70-72]. Additionally these antibodies facilitate phagocytosis of virus particles by Fc receptor expressing cells and their binding to HA expressed on infected cells mediates ADCC [53]. Unfortunately due to the high variability in the HA globular head most antibodies directed against this glycoprotein are strain-specific and fail to neutralize heterologous variants and viruses of other subtypes [19, 22, 73, 74].

Also antibodies specific for NA have anti-influenza protective potential. NA enzymatic activity is essential for the release of newly formed virus particles from infected cell [12]. Therefore by binding NA, antibodies do not directly neutralize the virus - as HA-specific antibodies - but inhibiting its enzymatic activity they limit virus spread and thus shorten severity and duration of illness [75-78]. Furthermore, NA-specific antibodies may also contribute to clearance of virus-infected cells by facilitating ADCC [53].

M2 protein is the third component of influenza virus envelope. Since the protein is highly conserved among influenza viruses of different subtypes, M2-specific antibodies are likely to afford heterosubtypic immunity [79-81]. Unfortunately the protein is present at low concentrations in infected cells and thus M2-specific antibodies are raised after natural infection only to a limited extent.

After infection, antibodies are also induced against other viral proteins, including NP [82]. As M2, also NP is greatly conserved between influenza viruses and consequently NP-specific antibodies could potentially contribute to heterosubtypic immunity. Although NP-specific antibodies are non-neutralizing, it was shown in mice that they contribute to protective immunity [83, 84]. However, their exact mechanism of protection remains to be elucidated, but may include ADCC of infected cells and opsonisation of NP, resulting in improved T cell responses [85, 86].

Antibodies are produced by activated B cells in different isotypes: by a process called "immunoglobulin class switching" B cells are able to progressively produce antibodies of various isotypes without changing their antigen specificity [87]. The main antibody isotypes in the influenza-specific humoral immune response are IgA, IgM and IgG. IgM antibodies initiate complement mediated neutralization of influenza virus and are a hallmark of primary infection [88, 89]. Mucosal or secretory IgA (sIgA) antibodies are produced locally and transported along the mucus of the respiratory tract by transepithelial transport and can protect airway epithelial cells from infection [90, 91]. Serum IgAs are produced rapidly after influenza virus infection and their presence is indicative for recent influenza virus

infection [92, 93]. Serum virus-specific IgG antibodies predominantly transudate into the respiratory tract and correlate with long-lived protection, provided that the antibodies match the strains causing the infection [94-96].

Memory B cells are "stored" to generate a greater and more rapid secondary response on reencounter with antigen. B cell memory is particularly important for responses to respiratory viruses, as influenza virus, to which people are commonly exposed multiple times throughout their lives. Classically, recurrent encounter with viral antigens should lead to boosting of antibody titers that ultimately leads to complete protection. However, influenza virus has evolved various ways to evade host immunity. Host antibody response is extremely antigen-specific and influenza virus, thanks to the high variability rate of its major antigenic proteins (i.e. HA and NA) via antigenic drift, is able to escape pre-existing humoral response [24, 25].

Besides B cell responses, influenza virus infection induces a cellular immune response, including virus-specific CD4$^+$ T cells and CD8$^+$ T cells. These cells together play an important role in regulation of the immune response and viral clearance.

CD4$^+$ T cell immune response. Efficient activation of naïve CD4$^+$ T cells depends on three distinct signals: they are activated after recognizing virus-derived MHC class II-associated peptides on APCs (1st signal) that also express co-stimulatory molecules (2nd signal) [65]. The activated CD4$^+$ T cells undergo extensive cell division and differentiation, giving rise to distinct subsets of effector T cells. The differentiation of polarized effector T cells is controlled by unique sets of transcription factors, the expression of which is determined by multiple signals but particularly by soluble factors as cytokines that act on responding CD4$^+$ T cells during their activation (3rd signal). The best known CD4$^+$ T cells subsets are Th1 and Th2 cells, which are characterized by their production of IFNγ and IL-4 cytokine, respectively [97]. Specialized B cell helpers, known as T$_{FH}$ cells and the pro-inflammatory Th17 cell subset also develop, along with T$_{reg}$ cells, which are essential for avoiding over-exuberant immune responses and consequent immunopathology [98].

The successful clearance of viral pathogens is often depends on complex CD4$^+$ T cell responses that encompass multiple Th cell subsets. A key role of CD4$^+$ T cells is to ensure optimal responses by other lymphocytes. CD4$^+$ T cells are necessary as helpers to promote B cell antibody production [99, 100] and are often required for the generation of cytotoxic and memory CD8$^+$ T cell populations [101, 102]. Moreover recent studies have defined additional roles for CD4$^+$ T cells in enhancing innate immune responses and in mediating non-helper antiviral effector functions. Interestingly CD4$^+$ T cells with

intrinsic cytotoxic activity toward infected cells have been described in several models of viral disease as well as in the clinic [103, 104].

Importantly CD4^{+} T cell responses can target relatively conserved internal influenza proteins (e.g. M1 and NP), implying that these cells may have the potential to provide influenza-specific heterologous immunity. In the absence of neutralizing antibody response, CD4^{+} T cells have a fundamental role in limiting severity of influenza infection by new strains [105, 106].

CD4^{+} Th cell subsets. In contrast with canonical definitions, effector T cells that are found *in vivo* are often characterized by plasticity and heterogeneity in terms of their cytokine-producing potential. Nevertheless, the CD4^{+} T cells that are generated in response to influenza infection mainly have a Th1 phenotype and produce large amounts of IFNγ [102]. It was historically thought that Th1 cells exclusively promoted cytolytic activity of CD8^{+} T cell during viral clearance [97] while Th2 cells were needed to drive optimal humoral immune responses [107, 108]. Thus, the predominance of Th1 cells over Th2 cells during influenza infection was somewhat surprising, given the important role of neutralizing antibodies in pathogen clearance and in providing long-term immunity to re-infection. However deepened studies have shown that both Th1 and Th2 cells provide efficient help for the generation of neutralizing antiviral IgG responses [109]. In particular the Th1-signature cytokine, IFNγ, enhances IgG2a class switching, and this explains why IgG2a is usually the dominant isotype in IgG responses generated against viruses [110]. Interestingly, several studies have found that, far from promoting antiviral responses, Th2 cell-associated cytokines (IL-4 in particular) have a strong negative impact on immune protection and drive immunopathology during infection with many viruses, including influenza virus [111, 112]. However Th2 - together with T$_{FH}$ cells - remain extremely important for the development of a robust antibody response against influenza virus [107]. The roles of Th17-type effector responses during viral infection are not well understood, but virus-specific IL-17-producing CD4^{+} T cells have been detected in mice following infection with influenza virus [113], although at levels lower than those of Th1 cells. Th17 cells are implicated in driving harmful inflammation during autoimmunity, and IL-17 may also contribute to immunopathology during responses against influenza virus [114]. However, in some cases, Th17 cells contribute to host protection against viruses. One protective mechanism mediated by Th17 cells might be the promotion of enhanced neutrophil responses at sites of infection. IL-17 upregulates CXC-chemokines that promote neutrophil recruitment [115], and neutrophils can contribute to protection against certain viruses, including influenza virus [59].

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CD4+ T follicular helper cell. Following influenza virus infection, T$_{FH}$ cells direct the formation of germinal centers (GCs), where they promote the generation of B cell memory and long-lived antibody-producing plasma cells [116, 117]. T$_{FH}$ can address B cells differentiation towards higher affinity antibodies producing cells and moreover they modulate antibody class switching process. Thus, T$_{FH}$ cells are extremely important for generating durable antibody responses and protective immunity towards influenza infection. T$_{FH}$ exert their functions both by direct cell-to-cell signaling and secretion of modulators as IL-21, which promotes isotype switching to IgA and enhances Bcl-6 expression in B cells, thus augmenting memory generation [118]. Unfortunately it is not completely clear how distinct cytokine-polarized CD4+ T cell subsets influence the B cell response during primary viral infection. One possibility is that distinct Th cell subsets, such as Th1, Th2 and Th17 cells, can each develop into T$_{FH}$ cells and provide efficient help for B cells [119].

CD8+ T cell immune response. Naïve CD8+ T cells are activated after recognition of viral epitopes associated with MHC class I molecules on APCs in the dLNs, and subsequently differentiate into CTLs [62]. After activation, these cells are recruited from dLNs to the site of infection where they recognize and eliminate influenza virus-infected cells and thus prevent production of progeny virus [120]. As CD4+ T cells, also influenza virus-specific CTLs are mainly directed against epitopes of the highly conserved internal viral proteins, like M1, NP, PA and PB2. Therefore, CTLs display a high degree of cross-reactivity with influenza viruses of various subtypes [105, 121, 122]. CTLs lytic activity is mediated by the release of perforin and granzymes (e.g. GrA and GrB). Perforin permeabilizes the membrane of the infected cells and subsequently granzymes enter the cell and induce apoptosis [123, 124]. Furthermore, pro-inflammatory cytokines are produced, like TNF-α, which also inhibit virus replication and enhance lytic activity [125, 126]. Finally CTLs also induce apoptosis of infected cells through Fas/FasL interactions [124].

IMMUNOSENESCENCE AND INFLUENZA INFECTION IN ELDERLY

According to the United Nations Population Division, the number of elderly persons is expected to increase from currently 600 million to nearly 2 billion worldwide by 2050, and in developed countries 25% of the population will be older than 65 years because of advances in average life expectancy [127]. Influenza directly or indirectly contributes to the four leading causes of global mortality, at rates that are highest in older adults. Currently, between 250,000 and 500,000 influenza-related deaths occur annually in the over 65 years of age population worldwide [1].

Immunosenescence and Inflammaging

As we age, changes occur in both the innate and adaptive immune compartments leading to increased susceptibility to developing diseases. "Immunosenescence" is the biological aging process associated with progressive decline in systemic immunity and increased prevalence of cancer, auto-immune and chronic diseases, poor responses to vaccination, and increased vulnerability to common infectious diseases such as influenza [128]. "Immunosenescence" does not mean "immunodeficiency". Although a decline of immunological functions is evident, there are elements of the system that are preserved (e.g. $CD8^+$ T cell poly-functionality and number of resident macrophages) [129], while others are even increased (e.g. innate/ inflammatory cytokine production by macrophages) [130]. Therefore, it has been suggested replacing the term "immunosenescence" with "senescent immune remodeling", which better describes the plasticity of the ageing immune system [131].

The changes in the immune response of the elderly are principally due to intrinsic defects within immune cells that show altered phenotype and function [132-134], and possibly to defects in the bone marrow and thymic stroma microenvironment [135]. Other contributing factors include changes occurring in the ageing body, such as increased cellular death [136], increased oxidative stress events [137], nutritional status [138], hormonal dysregulation [139], comorbidities [140], and chronic diseases (e.g. diabetes and cardiovascular diseases) [141]. All these factors contribute to create a basal chronic low grade inflammation called "inflammaging" that maintains innate immune cells in a permanent low activation status [142]. This may cause excessive inflammation and tissue damage upon infectious challenges.

Influenza infection in elderly

Older adults (>65 years of age) are particularly vulnerable to influenza illness [1]. This is due in part to reduced immune responsiveness, but also to the dramatic increase in high-risk chronic conditions that impact on influenza outcomes among the elderly [143-145]. Among community-dwelling older adults, increasing age has been correlated with increased risk of hospitalization due to secondary infections or pneumonia and influenza [146, 147].

Pathogenesis and clinical features. Mortality rates for infectious diseases are often higher among older adults than among younger adults with the same infection [148]. In the case of influenza, older adults account for 90% of the total number of deaths [149] and the risk of death increases among older adults afflicted with chronic disease [145]. However, mortality is not the bigger issue about influenza disease burden in older population, indeed for each death there are many more hospitalizations resulting from complications [146, 150], which are more frequent among older adults [147]. Notably, in comparison to younger population, elderly patients experience higher rate of lower respiratory tract symptoms, with productive cough, wheeze and chest pain [151]. Considering that older people may lose 2-3% of muscle power per day of bed rest and that influenza is also associated with a decline in major physical functions in this subjects, it results obvious that the disease often becomes a trigger for serious disability in older patients [152]. Indeed, it is likely that the majority of influenza-related disability occurs in older adults due to the higher risk of hospitalization and long hospital stays in this population.

Influenza is also the cause of exacerbations of pulmonary and cardiovascular disease [153], and is the primary source of the winter-season increase in mortality among patients suffering from chronic disease such as ischemic heart disease, stroke and diabetes [154-156]. Finally pneumonia has become an increasingly significant cause of morbidity and mortality in the aging population [157], and interestingly, although primary influenza infection alone can lead to adverse outcomes, SBIs during and shortly after recovery from influenza infections are more common reasons for influenza-associated illness in elderly [147, 158].

Immunosenescence impact on influenza infection and vaccination. In humans and mouse models studies are emerging to understand the correlation between immunosenescence and higher rate of influenza infection in elderly, with the final aim to potentiate currently available influenza vaccines.

Distinct studies suggest that age-related dysfunction occurs in pathogen sensing pathways and/ or cytokine production by innate immune cells. The functions of DCs are impaired by aging: cells appear to be constitutively activated in people >65 years of age and are characterized by dysregulated cytokine

production that may limit further activation via TLRs engagement. Interestingly this impairment of DCs response has been shown to be strongly associated with poor antibody response to influenza immunization in aged subjects [159]. Furthermore aged mice infected with influenza have shown impaired activation of the NLRP3 inflammasome in DCs, leading to reduced production of mature IL-1β and IL-18, and impaired caspase-1 activation [160]. Ageing is also associated with an increase in the number of phagocytes, macrophages and neutrophils. In particular numbers of alveolar macrophages, which are the first cells encountered by pathogens or antigens in the respiratory airway, are increased with age. However they have been shown to have decreased phagocytic potential, inefficient chemotaxis and can also contribute to "inflammaging", reducing influenza virus clearance while increasing immunopathology [142, 144, 161]. Notably similar results have been obtained from *in vitro* studies on neutrophils from older adults [162].

As innate immunity, also the adaptive response is impaired by immunosenescence [163]. Both naïve T and B cells are still able to undergo renewal, but a preponderance of memory T and B cells has been observed [134, 164, 165]. Age-related defects in naïve T cell activation, expansion, and differentiation may affect their helper function to B cells and lead to reduced humoral immune responses [166, 167]. Influenza-related studies have shown that CD4^{+} T cell functionality declines in the elderly leading to poor antibody and cellular responses to influenza vaccine [168, 169]. Particularly, several studies indicate that human age related susceptibility to influenza virus may be associated with a reduction in Th1 CD4^{+} responses and a relative diminished ability to kill virally infected cells [170, 171]. Likewise, Th17 cells are critical in mediating vaccine-induced immunity against several mucosal infectious diseases, and studies by Lee *et al.* have reported a reduced number in the elderly [172].

Recent studies showed that CD8^{+} T cells responses to influenza are decreased in magnitude and show altered kinetics in aged mice [173, 174]. Similarly to T cell responses, also the humoral response clearly declines with age due to reduced naïve B cell numbers or repertoire diversity [133]. Consequently in aged individuals the antibody response to new antigens is quantitatively decreased, less efficient and with lower avidity [175, 176].

INFLUENZA-BACTERIAL CO-INFECTION

Post-influenza bacterial pneumonia is a major cause of increased morbidity and mortality associated with both seasonal and pandemic influenza virus illness. The influenza-bacteria co-pathogenesis is characterized by complex interactions between co-infecting pathogens and the host, leading to the disruption of physical barriers, dysregulation of immune responses and delays in a return to homeostasis [2]. The net effect of this cascade can be the overgrowth of the pathogens, immune-mediated pathology and increased morbidity.

Epidemiology and microbiology of co-infections

The epidemiology of co-infections remains difficult to assess with sufficient accuracy. The attribution of mortality to influenza or relative complications is complex [177], as most deaths are from complications of influenza, rather than the primary disease. Moreover, a precise viral etiology is infrequently confirmed by diagnostic testing or confounded by co-circulating pathogens and co-infections [178].

Most commonly associated pathogens in post-influenza SBIs are primarily *S. pneumoniae* and *S. aureus* followed by *H. influenza* and *S. pyogenes*. *S. pneumoniae* is the most frequently isolated pathogen associated with influenza [179], although deaths, especially in children, are also associated with *S. aureus* infection, as highlighted by the recent emergence of community-acquired methicillin-resistant *S. aureus* (MRSA) [180].

During non-pandemic years and most pandemic years, the age distribution of severe influenza-related morbidity and mortality (which generally reflects bacterial pneumonia rates) exhibits a U- shaped pattern, with infants and the elderly most frequently affected [147]. Notably the three influenza pandemics of the 20th century (1918 H1N1, 1957 H2N2 and 1968 H3N2) and that of 2009 (pN1N1) are all associated with secondary bacterial pneumonia [32] (Fig. 4).

The 1918 influenza pandemic caused 40-50 million of deaths. Notably the majority of disease cases were complicated by bacterial pathogens, most commonly by *S. pneumoniae* [32]. The patterns of mortality in the next two pandemics in 1957 and 1968 resembled those of seasonal influenza in the respect that bacterial co-infections were a less likely cause of death than they were during the 1918 pandemic. However bacterial pneumonia, caused predominantly by *S. aureus*, still accounted for 44% of deaths in 1957 [181]. Although the incidence and distribution data of pneumonia-associated mortality were similar between 1957 and 1968, *S. pneumoniae* was the primary pathogen of bacterial superinfection in the 1968 pandemic.

Even if *S. pneumoniae* was considered to be the most common cause of SBI in the decades after the 1968 pandemic, *S. aureus* is emerging as a cause of fulminant pneumonia in association with influenza in many parts of the world [180, 182]. In particular the USA300 and USA400 clonotypes of *S. aureus* seem to be likely to cause SBIs with influenza, compared with other circulating strains, probably due to altered expression or regulation of particular bacterial virulence factors, such as cytotoxins or adherence factors [183, 184]. *H. influenzae* has become less frequently associated to SBIs following the introduction of the *H. influenzae* type B conjugate vaccine in 1985 [185].

The first pandemic of the 21st century in 2009 (pH1N1) is still being researched. In contrast to the 1957 and 1968 pandemics, mortality rates were similar to recent seasonal epidemics and high proportion of cases and deaths occurred in young adults, often with no underlying chronic conditions [35]. The precise effect of SBIs remains unclear; some estimates put excess mortality from influenza and pneumonia as low as 10%, which is lower than that of many seasonal influenza epidemics [186]. *S. pneumoniae* and *S. aureus* were the most common causes of SBIs, with regional variations in their frequency [187-190]. The importance of *S. aureus* in 2009-2010 was probably due to strain-specific features of the recently emerged USA300 clonotype, such as Panton-Valentine leukotoxin (PVL) expression [182], coupled with increased effects of pneumococcal vaccines in the past years [190].
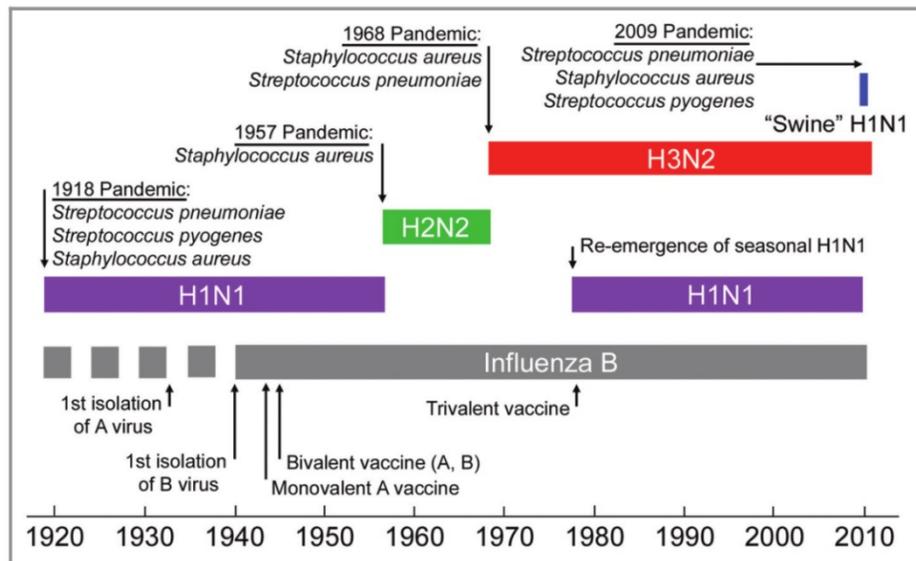
Fig. 4: Timeline of influenza-bacteria co-infections since 1918. The bacterial species most commonly isolated from serious or fatal cases of disease are listed for each pandemic. Notes: (1) The broken gray line indicates that although influenza B virus was not isolated until 1940, it is inferred to have circulated prior to this time. (2) Although the first isolation of influenza A virus was in 1933, the 1918 pandemic strain has been resurrected from frozen material. (3) The seasonal H1N1 strain that re-emerged in 1977 was most similar to viruses that circulated around 1950; the 2009 "swine" H1N1 had an H1 hemagglutinin that differs phylogenetically from the previously circulating human H1N1 strains [191].

Mechanisms of co-pathogenesis

During an influenza virus infection, the respiratory tract environment is remodeled facilitating efficient bacterial invasion. Although mostly based on animal-model data, it is clear that co-pathogenesis between influenza and superinfecting bacteria has a multifactorial basis [2, 192, 193] (Fig. 5). Interestingly, one of the critical factors is the timing of SBI: several studies showed that early after influenza infection (2-3 days) mice have reduced susceptibility to superinfections, while during the recovery stage (6-7 days) they are extremely vulnerable [193, 194].
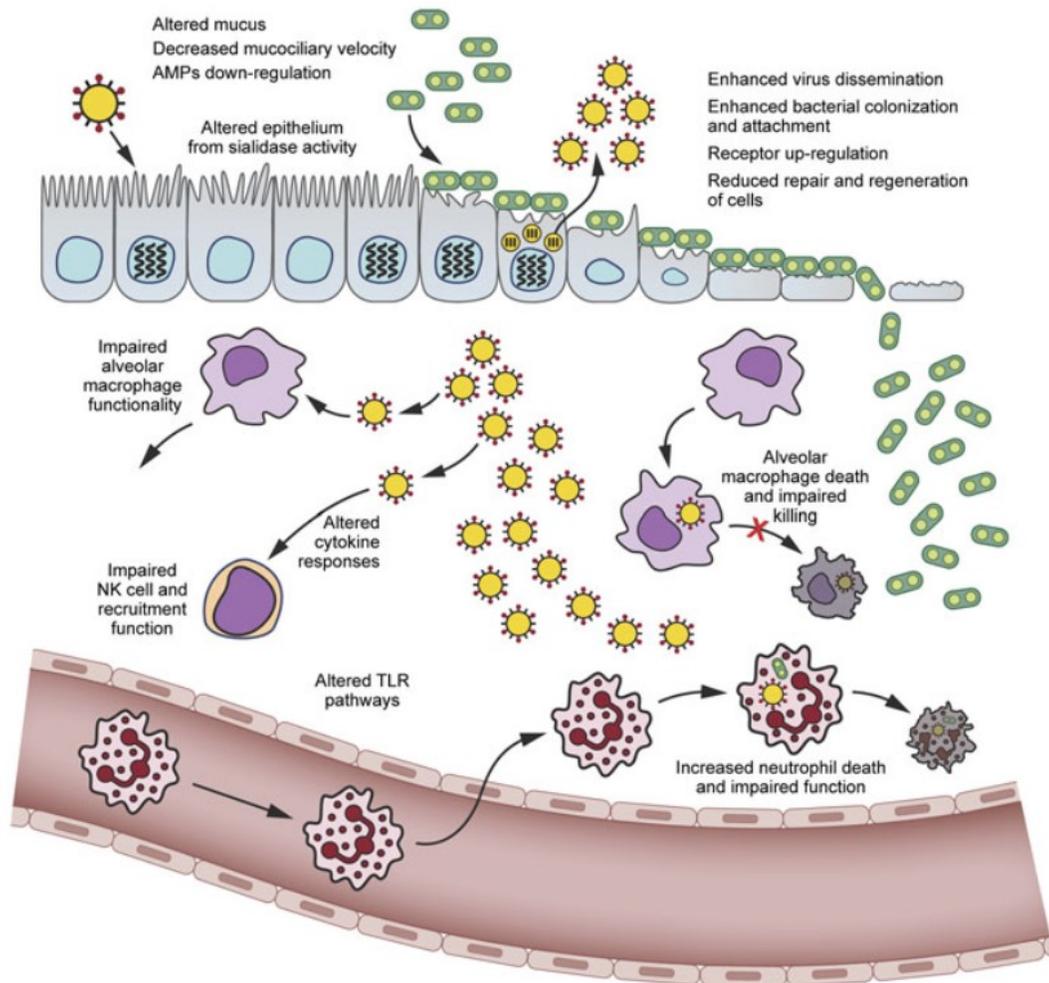
Figure 5 - Influenza-bacterial interaction during co-infections. Numerous alterations of the respiratory epithelium and host immune responses occur during influenza infection that predisposes the host to co-infection with bacterial pathogens. Influenza infection results in epithelial surfaces exposure to bacterial attachment. Physical barriers are damaged, pathogen detection is decreased, anti-microbial peptides are downregulated, receptors are upregulated, virus production is enhanced, bacterial transepithelial migration is permitted, and repair mechanisms are lost. Several host responses are also dampened or altered. Macrophages, neutrophils, DCs, and NK cells have altered cytokine profiles and become impaired and/ or depleted. These changes result in decreased bacterial surveillance and eradication [192].

Dysfunction of lung physiology. Historically, the generally accepted mechanism responsible for microbial synergy is that influenza virus-induced damage to the epithelial barrier provides increased attachment sites for bacteria, resulting in invasive disease [195]. Moreover the host depends on the mucociliary apparatus in the lung and nasal passages to clear invading pathogens, but during influenza infection ciliary beat frequency is decreased and ciliary motion becomes uncoordinated, thus this mode of bacterial removal is inhibited [196]. An important contribution to tissue damage is given by the viral cytotoxin PB1-F2 that can cause cell death and a cytokine storm [197]. Influenza-induced lung tissue damage in both humans and mice is greatest on day 6-7 after infection [198], which generally correlates with the time of greatest susceptibility to bacteria. However, viral strains that cause minimal epithelial cell damage still enhance subsequent bacterial infection in mice [199, 200].

Increased receptor availability. Bacteria express a range of virulence factors that can be used inside infected host for adherence to cell basement membrane or elements of the extracellular matrix such as fibrin, fibrinogen and collagen [201, 202]. Most virulent viruses, such as the mouse-adapted influenza virus strain PR8, cause substantial epithelial cell death *in vivo*, which exposes sites for adherence in the tracheobronchial tree [200, 203]. However most seasonal influenza strains do not cause severe lung injury but can still facilitate bacterial superinfection, although with lesser degree [204]. Three additional mechanisms have been discovered which might increase bacterial receptor availability. First, the influenza virus neuraminidase cleaves sialic acids exposing hidden receptors for bacteria adherence on host cells and disrupts sialylated mucins that can function as decoy receptors for the bacteria [205]. Notably bacteria causing lung infection as *S. pneumoniae* often produce NAs themselves in order to access receptors and avoid host defenses [206]. Second, the host inflammatory response to influenza infections can alter the regulatory state and surface display of multiple proteins, including some, such as the platelet activating factor (PAF) receptor that can be used to facilitate bacterial invasion [207]. Third, tissue wound healing generates changes in the airway that might provide adherence sites for bacteria during recovery from influenza [208].

Modulation of anti-bacterial immunity. Increasing studies about influenza and bacteria synergism have been focused into modulation of host immune responses by the virus. Respiratory bacteria activate, modulate and are eventually controlled by multiple responses of the immune system during invasion of the lungs [209, 210]. Influenza virus also manipulates many of these pathways by the expression of multi-functional accessory proteins, such as NS1, which potentially interfere with lung immune responses to bacterial invaders [211]. It has been demonstrated that early innate responses to

bacteria are compromised by the preceding influenza induction of interferons [212-214]. Type I IFNs are produced early after influenza recognition by innate sensors [42-44] and several possible mechanisms have been identified for their modulation of innate immunity towards secondary infecting bacteria. First of all type I IFNs signaling may suppress recruitment of macrophages and neutrophils and/ or impair their bactericidal capacity during superinfection at days 5 to 7 after influenza virus infection [214-216]. Moreover type I IFNs are able to block IL-17 responses by canonical αβ T cells or γδ T cells present in the lung tissue weakening Th17 local immunity which is essential to counteract respiratory bacteria invaders [213, 217]. Notably the antiviral state promoted by type I IFNs impairs also NK cell responses and this has been shown to be an upstream mechanism of depressed antibacterial activities by macrophages and neutrophils [218]. In addition, influenza viruses specifically deplete the airway-resident alveolar macrophages that are responsible for early bacterial clearance, which leads to a deficit in early bacterial surveillance and killing [219]. These alveolar macrophages die during the initial stages of infection and are replaced over the next two weeks by the proliferation and differentiation of macrophages from other cell classes, which creates a window of primary susceptibility that extends beyond the immediate viral infection. The impact of neutrophils on SBI is strictly related to the timing of viral-bacterial co-infection, indeed although properly functioning neutrophils are important to bacterial clearance early after influenza virus infection [194, 220], it is unclear the role of their dysfunction during the time period of enhanced susceptibility to superinfection at days 6 and 7 after influenza virus infection. It has been supposed that an increased accumulation of dysfunctional neutrophils in the lung late after influenza infection may contribute to increased susceptibility to superinfection via both impaired bacterial clearance and damage to the lung tissue [58, 203, 217, 221, 222]. Rynda-Apple *et al.* have showed that a fine regulation of IFNγ and IL-13 dictates susceptibility to SBI [194, 220]. Particularly at the early onset of influenza infection IL-13 blocks production of IFNγ leading to appropriate phagocytosis and clearance of bacteria [194, 220]. Then during recovery from influenza high amounts of IFNγ are present in lungs and this leads to downregulation of MARCO receptor on phagocytic cells with consequent impairment of co-infecting bacteria phagocytosis and killing [212]. Finally during the clearance of influenza virus and the onset of wound healing, a general anti-inflammatory state is established with the aim to restore lung immune homeostasis [223]. This is characterized by increased IL-10 production, which in turn broadly suppresses multiple mechanisms that are involved in pathogen recognition and clearance [193, 224]. Influenza-induced desensitization of PRRs that are used by phagocytes to detect and respond to bacteria can persist for weeks or months and can contribute to late secondary infections after apparent recovery from the preceding viral illness [225].

Increased inflammation. Pneumonia is an inflammatory state of the lungs. Therefore, viral factors or host responses that increase inflammation in response to the pathogens contribute to the co-pathogenesis of SBI. Particularly virulent viruses express the cytotoxin PB1-F2 that drives over-exuberant inflammatory responses with consequent increased cellular infiltration of the airways and cytokine storm [197]. Notably bacteria also express cytotoxins that contribute to inflammation, such as pneumolysin and *S. aureus* PVL, and these might synergize with the effects of PB1-F2 resulting in necrotizing pneumonia [210, 226]. Furthermore many of the inflammatory pathways activated by PPRs after recognition of viral or bacterial PAMPs overlap, leading to the synergistic activation of immune responses, with resulting excessive morbidity. Finally in the contest of depressed phagocytic activity [212, 214], neutrophil-mediated inflammatory damage may occur in the lungs without effective pathogen control [227].

Facilitation of the viral infection. Although most of the mechanisms that are known involve viral facilitation of subsequent bacterial superinfection, it is now evident that factors that are expressed by co-infecting bacteria affect the virus as well. Bacterial modulation of viral infection may be mediated via direct interactions, such as by interference with antiviral immunity or by synergism or complementation by virulence factors that have similar functions. Studies in animal models have shown an increase in influenza virus titers during bacterial superinfections in which bacterial challenge follows the viral infection; this increased viral lung load is typically accompanied by delayed clearance via unclear mechanisms [183, 228, 229]. However, viral replication may be suppressed and morbidity may be diminished via the induction of innate immune responses if the bacterial infection precedes viral challenge [230]. Even if at this time no data are available, it is reasonable to hypothesize that the bacterial mechanisms that broadly interfere with innate immune mechanisms might also affect the immune response to respiratory viruses [231]. Finally there is evidence that the composition of the microbiome alters immune responses to influenza both by changing the activation set-point for antiviral responses and by influencing the development of adaptive immune responses [232, 233].

Approaches for prevention and treatment of SBI

Considering the strong association of bacterial disease with antecedent influenza infections, there has been reasonable interest in determining whether prevention or treatment of the virus can eliminate or at least ameliorate SBI. Two classes of antiviral drugs are currently approved for use in patients with influenza: the M2 inhibitors, amantadine and rimantadine, and the neuraminidase inhibitors (NIs), zanamivir and oseltamivir [234]. Unlike antibiotics, which can eliminate or greatly reduce pathogen burden, existing influenza antiviral drugs are only able to stop progression of disease by preventing new

host cells from being infected. Nevertheless if treatments are administered early enough in the clinical course, they may delay progression of infection, allowing normal immune clearance mechanisms to gain the upper hand [235]. Unfortunately the major effects of anti-influenza treatment are symptom reduction and a more rapid recovery, not immediate clinical cure. Thus, the continued presence of the virus and the ongoing host response may predispose to SBI despite anti-viral treatment.

NA enzyme has demonstrated to have an important role in post-influenza susceptibility to SBI [205], and preclinical studies in mouse models demonstrated that prophylaxis or early treatment with NIs improved influenza outcome and significantly reduced SBI [236]. Notably antiviral use decreases the incidence of SBIs, prolongs the interval between exposure to bacteria and development of disease, slows progression of pneumonia when it developed, and facilitates antibiotic treatment of the superinfecting bacteria.

Other anti-viral therapies have been researched in the field of small molecules, monoclonal antibodies or small interfering RNAs. Specific blockade of the pro-inflammatory effects of PB1-F2 may diminish the severity of highly pathogenic influenza viruses and reduce SBI [197]. Moreover explorations have been started to find complementary strategies targeting bacterial toxins that are shown to synergistically interact with influenza viruses to enhance disease. Inhibition of the interferon antagonist NS1 should allow enhanced clearance of the virus, which could prevent several downstream effects on host immunity that likely increase susceptibility to bacteria [211]. Moreover use of collectin-like molecules that recognize the HA might diminish the diffuse alveolar damage caused by viruses that facilitate access to the lower respiratory tract to secondary invaders [236, 237]. Finally strategies of immunomodulation to reduce the inflammatory response during SBI have been proposed and data from animal models suggest that targeting specific pathways involved in inflammation might have success [238].

Interestingly antibiotic therapy is often unsuccessful in SBI cases: the use of bacteriostatic protein synthesis inhibitors has been shown to improve outcomes of secondary bacterial pneumonia, while bacteriolytic drug (e.g. ampicillin) can worse the pathology through enhanced inflammation during bacterial lysis [239].

In conclusion, the most obvious way to prevent SBI is to prevent the antecedent viral infection entirely. Even if animal model data suggest that vaccination against influenza is an effective method to prevent subsequent secondary pneumonia [5, 240-242], influenza vaccine studies in humans have typically not been designed or appropriately powered to assess effectiveness against SBI.

ANTI-INFLUENZA VACCINES

Vaccination against influenza is the most cost-effective method to prevent influenza infections [243, 244]. Active immunization against any infectious disease, including influenza, aims at induction of antimicrobial immunity by inoculating the person with an attenuated or inactivated form of the pathogen involved. Generally immunization attempts to closely mimic the immune response to a natural infection, which is often considered the best strategy for protection. Consequently vaccination targets the adaptive immune response to stimulate B cells to differentiate to plasma cells that produce circulating antibodies and to develop sufficient number of pathogen-specific T cells that are activated upon re-exposure to the microbe during a natural infection.

Background. Flu vaccine development began just few years after the first isolation of the influenza virus in 1933 [245, 246]. Pioneering observations demonstrated that influenza A/PR/8/34 (H1N1) virus would infect humans upon subcutaneous administration, inducing virus-neutralizing antibodies. Soon studies using formalin-inactivated whole-virus preparations were conducted and the first inactivated influenza vaccines were introduced in the 1940s [245, 246]. Even if early anti-influenza vaccine preparations were not consistently successful in reducing the incidence of febrile illnesses, they highlighted the importance of antigen potency and matching vaccine strains [246]. Subsequent multicenter studies commissioned by the U.S. Armed Forces Epidemiological Board used inactivated concentrated virus stocks incorporating multiple virus strains and found prophylactic protection and a much lower incidence of febrile illnesses in vaccinated groups than in controls, setting the foundations for influenza vaccination programs [247]. Finally in 1970s split-virus and subunit formulations were developed and proven to be less reactogenic compared to the whole-virus vaccine preparations despite comparable immunogenicity in primed populations [248, 249].

From surveillance data to vaccine delivery. Global influenza epidemics emerge seasonally and typically occur during the winter seasons of the northern and southern hemispheres. The WHO meets twice annually to review surveillance data and make recommendations as to which strains should be contained in the following season influenza vaccine [11]. Usually two influenza A strains (one H3N2 and one H1N1) and an influenza B strain are recommended for vaccine preparation [11]. Several factors are taken into consideration when making recommendations regarding influenza vaccine composition: (1) predictions of what viral strains are likely to cause in the following season epidemics on the basis of worldwide surveillance data, (2) the antigenic similarity of a chosen vaccine strain to the predicted

circulating strain, (3) the immunogenicity of a selected strain to develop adequate humoral immunity, and (4) the suitability of a viral strain for use in vaccine production [11]. After the 3 strains are selected for influenza vaccine inclusion, the Center for Disease Control (CDC) provides reference viral seed strains to the national drugs regulatory agencies (as FDA and EMA), which subsequently distributes them to the vaccine manufacturers for production. Finally each vaccine lot is evaluated by national drugs regulatory agencies before delivery [11].

Current influenza vaccines

Current influenza vaccines are mainly produced by egg-based production methods: influenza viruses are propagated on embryonated chicken eggs and then viral particles are harvested from allantoic fluid and processed depending on the type of vaccine formulation [244]. Because vaccine manufacturers are dependent on the supply of vaccine-quality eggs, they cannot be flexible in the amount of doses produced. This can lead to vaccine shortages, especially during pandemic situations. To overcome this issue alternative production platforms, such as cell culture-based [250-252] or plant-based [253] vaccine production and synthetic DNA [254, 255] or RNA [256, 257] vaccines, are now under careful examination in preclinical and clinical studies.

Seasonal trivalent influenza vaccine (TIV) formulations are made either with antigens from inactivated influenza or with live attenuated influenza viruses (LAIV), derived from two influenza A strains and one influenza B strain. In addition, even if TIVs are currently the most diffuse anti-influenza vaccination strategy, also quadrivalent influenza vaccine (QIV) formulations have entered the market recently, which adds an additional influenza B strain [258].

The induction of HA-specific serum antibodies, as typically measured by a hemagglutination inhibition (HI) or virus neutralization (VN) assay, is used as an indicator vaccine efficacy. On the basis of clinical studies, it has been established that an antibody titer ≥40 may be considered as immune correlate for protection for inactivated influenza vaccines in adults [70, 259, 260]. Immune correlates of protection for other vaccine platforms are still poorly characterized.

Inactivated influenza vaccines. Inactivated influenza vaccines include 4 types of vaccine preparation with characteristic structural organization or viral components: whole inactivated virus (WIV), split, virosomal or subunit antigen. In WIV formulation viruses are chemically inactivated with formalin or β-propiolactone and subsequently concentrated and purified to remove non-viral protein contaminants [245]. WIV vaccines were the first to be used in widespread annual influenza vaccination campaigns. However, these preparations caused local and systemic adverse effects upon administration probably

due to the presence of impurities, such as egg proteins, in the vaccine [261]. Split and subunit vaccines have been shown to be less reactogenic and consequently WIV vaccines were mostly abandoned when the other two preparations entered the market.

Currently the majority of influenza vaccines available consist of either split viruses or subunit influenza antigens. Split vaccines are influenza virus particles treated with detergent or diethyl ether to dissociate the viral lipid envelope and expose all viral components [262]. Split viruses are now widely used in TIV formulations due to their adequate immunogenicity and relative ease of production [244]. In subunit vaccines the HA and NA proteins are separated from the viral nucleocapsid and lipids and are further enriched through additional purification steps [263, 264]. Due to their "high purity", subunit vaccines are the least reactogenic vaccine preparation currently available, but on the other hand the addition of adjuvants to the antigens is sometimes required to reach adequate immunogenicity in the elderly [265, 266]. Moreover in unprimed populations, such as young children, split virus and subunit vaccines are less immunogenic than in adults and two doses are required to achieve a sufficient antibody titer [245, 267].

A standard dose of TIV contains 15 μg of HA per strain (total HA concentration of 45 μg) and is administered as a single dose in people >9 years [268]. Younger children (between 6 months and 8 years of age) require two doses administered 4 weeks apart, if they have not been vaccinated in previous influenza seasons [268]. Usually TIV is delivered as an intramuscular (i.m.) injection, although intradermal (i.d.) formulations are also available [268, 269].

In addition to split and subunit vaccines, two other vaccine preparations are currently used though with less frequency. A recombinant HA subunit vaccine has been recently approved: it contains a high dose of antigen (45 μg per strain) to reach the required immunogenicity. It has shown high efficacy in healthy adults and the elderly while lower applicability in children requiring additional formulation with adjuvants [270, 271]. Finally virosomal TIV formulations have been used mainly in Europe since 1997 [272]. Virosomes are reconstituted influenza virus envelopes consisting of HA, NA and viral phospholipids. Their particulate structure enables virosomes to retain viral membrane fusion and cell-binding capabilities, which could increase their immunogenicity, compared to subunit and split vaccines [244].

Live-attenuated influenza vaccines. LAIVs are administered intranasally (i.n.) and aim to mimic the natural infection inducing localized mucosal cellular and humoral immune response [273]. They are produced by serial passage of the virus in eggs under suboptimal conditions resulting in viruses with a temperature-sensitive phenotype: attenuated viruses are able to grow at 25°C - i.e. the temperature of

nasal passage - but not at temperature higher than 35°C which is characteristics of the respiratory tract [245]. Although LAIVs are proven effective, their use has raised concerns about their safety, considering that the virus can theoretically undergo genetic reversion into a pathogenic, transmissible influenza strain [274].

Adjuvants: tools to improve inactivated influenza vaccines

Despite the fact that inactivated influenza vaccines are on the market since the 1940s, several limitations still exist regarding both their availability and their effectiveness.

There are two major issues concerning currently licensed inactivated influenza vaccines: firstly they have low efficacy in elderly and unprimed people (as very young children); secondly they have very poor cross-reactivity [244, 245]. Inactivated influenza vaccines aim at the induction of virus neutralizing antibodies specific for the HA protein, but in aged subjects the immune system is compromised by immunosenescence which reduces immunogenicity of vaccines through impaired humoral response [168, 169, 176, 266]. Furthermore approved influenza vaccination strategies, due to their viral strain specificity, are not cross-reactive towards antigenic drifted viruses that may spread during annual influenza season and this may reduce a lot the vaccination success [275].

Optimizing influenza vaccination for infants and the elderly as well as expanding its viral subtypes coverage are currently two crucial objectives in immunological research and three major strategies have been proposed to solve this unmet need: -i) increase vaccine antigens dosage, ii) try different routes of administration or iii) add adjuvants to the formulation.

Adjuvants are compounds that can be added to influenza vaccine formulations in order to enhance immunogenicity of vaccine antigens and consequently potentiate immunization effectiveness [276, 277]. Generally, adjuvants exert their effect on innate immunity by improving antigen delivery or by targeting specific immune pathways to enhance immune responses towards the vaccine [278]. The preparation of influenza vaccines together with adjuvants has been shown not only to improve vaccine efficacy in elderly, unprimed and immunocompromised individuals, but also to broaden influenza-specific immune response [3, 278-280]. Moreover, considering their immunopotentiator activity, the addition of adjuvants to influenza vaccine facilitates the use of lower doses of antigen. This is an advantage that gets important during large-scale vaccination emergency as in case of a pandemic influenza strain outbreak [281, 282]. Finally, adjuvants may also be incorporated in vaccine formulations to achieve qualitative shifts of the immune response. Therefore the ability of adjuvants to promote functionally appropriate types of immunity (e.g. Th1 versus Th2 cell, CD8^{+} versus CD4^{+}, specific antibody isotypes) not effectively generated by the non adjuvanted antigens has been evaluated in preclinical and clinical studies [278].

Adjuvants in influenza vaccines. Currently licensed adjuvants for vaccine usage in humans include aluminum salt (alum) and the squalene oil-in-water emulsion systems MF59 (Novartis) and AS03 (GlaxoSmithKline). Although alum has been successfully used as an adjuvant in many other vaccines, no beneficial effect of alum was observed in influenza H5N1 or H1N1 pandemic vaccines in comparison to non-adjuvanted formulations [283-285]. On the other hand, oil-in-water emulsions have proven to be suitable adjuvants for influenza vaccines. MF59 was the first of these adjuvants approved for use in human influenza vaccines in 1997. It consists of squalene oil droplets stabilized by non-ionic surfactants and has been proven to be fundamental to overcome immunosenescence-induced weak response to unadjuvanted vaccines in aged people (>65 years) [286, 287]. Moreover numerous reports observed increased immunogenicity and efficacy of MF59-adjuvanted subunit vaccine in young children and healthy adults [288-290]. Overall, MF59 has been demonstrated to be safe and a very effective adjuvant for the stimulation of humoral and cellular responses against seasonal, pre-pandemic and pandemic influenza vaccines [3, 291]. AS03 is also an oil-in-water emulsion based on squalene droplets, but, unlike MF59, it contains α-tocopherol as additional immunostimulant and is currently used only in pre-pandemic and pandemic influenza vaccines [292, 293]. Even if AS03 adjuvanted influenza vaccines were significantly more immunogenic than their unadjuvanted counterparts both in primed and unprimed individuals [282, 294], their efficacy in immunocompromised patients remains controversial [295, 296]. *[A complete list of adjuvants for influenza vaccines is reported in Table 1]*

Adjuvant category	Adjuvant	Antigen(s)	Stage of development
Salts	Alum	Split, WIV (pandemic)	Licensed
Oil-in-water emulsions	**MF59** (squalene, Span 85, Tween 80)	Subunit (seasonal and pandemic)	Licensed
	AS03 (squalene, DL-a-tocopherol, Tween 80)	Split, WIV (pandemic)	Licensed
	AF03 (squalene, Brij 76)	Split (pandemic)	Licensed
	CoVaccine HT (squalene, Tween 80, sucrose fatty acid sulfate esters)	WIV (pandemic)	Animal model
Saponins	Iscomatrix	WIV (seasonal)	Clinical development
	Matrix-M	Virosomes (pandemic)	Clinical development
Glycolipids	Alpha-GalCer (alpha-galactosylceramide)	LAIV (seasonal)	Animal model
		DNA (HA-encoding)	
Liposomes	CCS/c (cationic liposomes of ceramide carbamoyl-spermine/cholesterol)	Subunit (seasonal)	Animal model
	CAF01 (cationic liposomes of DDA/TDB)	Split (seasonal)	Animal model
	Vaxfectin (cationic liposomes of GAP-DMORIE/DPyPE)	Split (seasonal), WIV (pandemic)	Animal model
		DNA (HA-encoding)	Clinical development
Bacterial components	CTA1-DD (Cholera toxin subunit A)	Peptide (M2e-based)	Animal model
	LT patch (*E. coli* enterotoxin)	Split (pandemic)	Clinical development
	Salmonella and *E. coli* flagellin	rHA (pandemic) rM2e	Clinical development
Cytokines	IL-12, IL-23	WIV (laboratory strain)	Animal model
	GM-CSF	DNA (HA-encoding)	Animal model
	Type I IFN (IFNα)	Subunit (seasonal)	Animal model
TLR agonists/ immunomodulators	GLA (glucopyranosyl lipid A) (TLR4)	rHA (pandemic)	Clinical development
	Bacterial flagellins (TLR5)	rHA (pandemic) rM2e	Clinical development
	CpG oligodeoxynucleotide (TLR9)	Split (seasonal)	Clinical development
	PolyI:C (TLR3)	Split (seasonal) LAIV (laboratory strain)	Animal model
	IC31 oligodeoxynucleotide (TLR9)	Subunit (seasonal)	Animal model
	sLAG-3 (IMP321) (MHC class II ligand)	Split (seasonal)	Clinical development
Polymers	Chitosan	Subunit (laboratory strain) DNA (M2 and NP)	Animal model
	PCPP (poly[di(carboxylatophenoxy)phosphazene])	Subunit (pandemic)	Animal model
	Advax (delta inulin)	Split (pandemic) rHA (pandemic)	Clinical development

Table 1 - Adjuvants for influenza vaccines. [Modified from [244]]

MF59-adjuvanted influenza vaccines. MF59 is an oil-in-water (o/w) emulsion, prepared with a low content of the biodegradable oil squalene (4.3%), which is a natural component of the human body and a precursor in the synthesis of cholesterol and steroid hormones. The oil droplets, which have a mean size of about 160 nm, are stabilized by two non-ionic surfactants (Tween 80 and Span 85) [297], which are commonly used as emulsifiers in foods, cosmetics and pharmaceutical formulations. MF59 is a potent and safe vaccine adjuvant developed in the early 1990s by Chiron Vaccines (Emeryville, CA) [298] and has been included in licensed vaccines in more than 30 countries since 1997 [291]. The adjuvant has been initially developed for the use in seasonal TIVs focused on elderly subjects (Fluad®), indeed extensive clinical studies have proved that MF59-adjuvanted vaccine is more immunogenic than non-adjuvanted ones in aged people (≥65 years) and is also well tolerated [287, 299, 300]. Notably influenza vaccination using MF59-adjuvanted TIVs has significantly reduced the probability of being hospitalized for pneumonia and cardiovascular or cerebrovascular disease in aged people [301].

Recent studies have shown that the addition of MF59 to TIV significantly potentiates efficacy of vaccination also in young children that are unprimed and thus poorly responsive to standard influenza vaccines, and highlighted the necessity of its usage also in these subjects [288, 289, 302].

Similarly, MF59 has been recognized as key component for the success of pre- and pandemic vaccines and proven to be able to broaden the immune response beyond the influenza strains included in the vaccine. This has been demonstrated by several studies showing that MF59 can induce fast priming of influenza antigen-specific CD4$^+$ T cell responses and strong and long-lasting memory cross-reactive T- and B-cell responses [280, 281, 303-307].

The adjuvant effect of MF59 on influenza vaccines has been ascribed to its capacity to significantly increase virus neutralizing antibody titers in comparison to those obtained using unadjuvanted formulations [308, 309]. Even if the mechanism of action of MF59 adjuvant has been extensively evaluated, some issues about its activity remain to be solved [310]. Since the first studies, it became evident that antigen binding to the emulsion droplets is not necessary for adjuvant activity and, moreover, MF59 does not establish an antigen "depot" at the site of injection. Instead, both the antigen and the adjuvant are cleared relatively rapidly with independent kinetics [298, 311]. Studies about antigen and adjuvant localization after injection showed that MF59 operates mainly as a "delivery system" that enhances antigen uptake by local cells and subsequent presentation to T cells in dLNs [312]. MF59 activity seems to be localized in the muscle injection site. There, its adjuvanticity is correlated with the induction of a local "immunocompetent environment" initially established by MF59-activated normal tissue-resident cells like macrophages and muscle cells [298, 310]. These cells produce a mixture of

cytokines and chemokines which act as chemoattractants for the recruitment of immune cells, as monocytes and granulocytes, from the blood stream into the injected muscle [313, 314]. A gene expression profile study reported that MF59 is a broad activator of transcriptional changes in the injected muscle and, in particular, it induces the upregulation of genes coding for cytokines and chemokines, cytokine receptors, adhesion molecules involved in leukocyte migration, and antigen-presentation related genes [315]. Moreover a recent study demonstrated that MF59 is able to induce a transient ATP-release at the injection site which is a crucial contributor to innate and adaptive immune responses elicited by influenza adjuvanted vaccine [4]. Newly muscle-recruited cells activate a positive feedback loop by secreting the same panel of factors previously produced by tissue-resident cells and in this way they strongly enhance the influx of phagocytic cells into the injection site [314]. Additionally, MF59 may enhance the differentiation of recruited monocytes towards DCs and alter their phenotype [314]. The presence of an elevated number of immune cells (firstly neutrophils, monocytes and eosinophils and later DCs and macrophages) localized in the muscle increases the probability of interaction between antigen presenting cells (APCs) and the antigen leading to an efficient transport of Ag to the lymph nodes which in turn translates in a strong T cell priming and subsequent wide B cells antibody production [316] (Fig. 6). Finally *in vivo* studies showed that the robust adaptive response induced by adjuvanted influenza vaccine is not biased to a specific profile and MF59 acts as a neutral adjuvant enhancing whichever response is prevalent. In particular MF59-usage in BALB/c mice results in a mixed Th1/Th2 profile with Th2>Th1 [6, 317], while human vaccination with MF59-adjuvated influenza vaccine expands CD4$^+$ T lymphocytes population with a Th1-prone effector/memory phenotype [304] , probably due to some contribution of Th1-cells primed by influenza infection earlier in life.
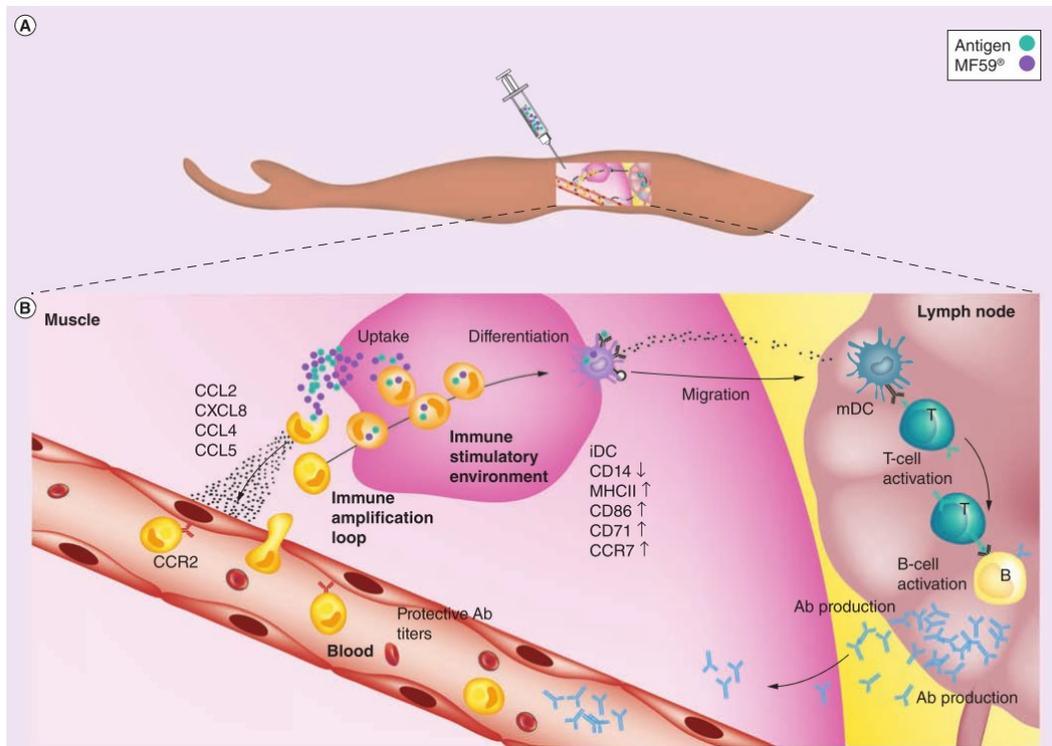
Figure 6 - The mechanism of action of MF59 adjuvant. (A) MF59 adjuvant in combination with a vaccine antigen is injected into the muscle through a normal needle and syringe. (B) In the muscle, tissue-resident monocytes, macrophages and DCs are activated and respond by inducing a mixture of chemokines (CCL2, CXCL8, CCL4, CCL5), which results in a significant influx of phagocytic cells that take up the antigen and differentiate into APCs (iDCs, immature DCs). These cells are responsible for the efficient transport of antigen to the lymph nodes, where the immune response is triggered through the activation of T and B cells and antibody (Ab) production. mDC: monocytic dendritic cell. [291]

MF59+CpG combined adjuvant for influenza vaccine. An optimal adjuvant (or combination of adjuvants) has to play two roles: it must act both as "delivery system", localizing vaccine antigens and targeting them to the appropriate innate immune cell types, and as "immunopotentiator", activating immune cells through specific receptors and/ or pathways [318]. An increasing number of studies are evaluating the use of immunopotentiators targeting PRRs as single or combined adjuvants in vaccines formulation [278, 319]. MF59 is a safe and potent adjuvant for the induction of humoral and cellular response towards influenza antigens [291], but it does not really bias the adaptive Th profile of vaccinated subjects. This, on the contrary, depends on the experimental settings and/ or previous experienced infections [6, 317]. The "neutrality" of MF59 makes it an ideal vehicle to deliver immunopotentiators, which have the ability to strongly skew the immune response towards specific Th profiles. The crucial role of cell- and antibody-mediated Th1-shifted immune response towards influenza infection has been demonstrated by several studies [102, 320, 321] and particularly it has been reported

that aging results in impaired Th1 immunity [170, 171, 322]. Notably MF59 is not a powerful adjuvant for the induction of Th1-polarized immune responses, but it can act as efficient delivery system for TLR-agonists able to bias the adaptive immunity [6, 317]. Different strategies are currently under evaluation and in particular, the use of TLR4- and TLR9-agonists such as MPL, GLA-SE and CpG is giving promising results [317, 322, 323].

Oligonucleotides containing unmethylated cytosine and guanosine dinucleotides, which are called CpG sequences, have been shown to act as potent vaccine adjuvants in a range of species including humans by activating the innate immune response directly through TLR9-stimulation [319, 324-326]. CpG has shown to be a potent vaccine-adjuvant able to induce strong Th1 immune responses characterized by T cells secreting IFNγ and TNFα [325], which are known to synergize against a number of viral infections, including influenza [320]. The combination of MF59 and CpG as vaccine-adjuvant has been explored for influenza antigens with positive results [6, 317]. It has also been demonstrated to be an optimal tool for the improvement of anti-hepatitis C virus vaccines-formulations and tumor vaccines against melanoma [327-329]. Pre-clinical studies on MF59+CpG-adjuvanted influenza vaccines reported that this combination is able to induce potent and long-lasting antibody and T cell responses: the adding of CpG to MF59 does not affect the magnitude of functional antibody titers and cellular response, but on the contrary it polarizes these responses towards a Th1 profile unbalancing the proportion between IgG1 and IgG2a towards the second isotype and expanding IFNγ-producing T cells at the expense of Th2 cells [6, 317].

Mucosal influenza vaccination adjuvanted with LTK63. The respiratory tract mucosa is the site of entry of influenza viruses and, consequently it can be considered also the initial site of possible protection against the pathogen. Several studies showed that mucosal vaccination not only elicits efficient mucosal immune responses which are only poorly induced by parenteral vaccination, but is also able to induce systemic immune responses [330]. Currently LAIVs, as FluMist®, are licensed for i.n. delivery in humans [273]. However other intranasal vaccination strategies have reported safety concerns due to the possible antigens and/ or adjuvants addressing to the central nervous system through the olfactory epithelium [331]. In particular, the use of NasalFlu vaccine adjuvanted with a genetically detoxified mutant of *E. coli* heat labile toxin (LTK63) has reported serious side effects, as facial nerve paralysis, in clinical studies [332]. Considering that the intranasal route for the delivery of adjuvanted influenza vaccine seems to have too many risks for human usage, other mucosal routes are currently under careful examination. Interestingly *in vivo* data demonstrated that sublingual (s.l.) immunization of inactivated influenza virus with a mucosal adjuvant follows the same immunological pathway as i.n.

immunization [333], but without negative effects on the central nervous system [334]. The s.l. administration of LTK63-adjuvanted subunit influenza vaccine was shown to be able to elicit comparable systemic immune responses to i.m. immunization without adjuvant and, moreover, to reach the same level of immune stimulation obtained with the same formulation given i.n. [7]. Finally, differently from parental vaccination, s.l. adjuvanted influenza immunization enhances antigen-specific mucosal IgA and Th17 responses [7], which have been reported to have a crucial role in the neutralization of the virus and the recruitment of neutrophils respectively [90, 91, 113, 115].

Anti-influenza vaccination as prevention from SBI

Vaccination against influenza is becoming an interesting research field, also for its interest as efficient method for the prevention of SBIs. This is particularly significant considering that vaccines for *S. pyogenes* and *S. aureus*, which are frequently associated with influenza, are not currently available and consequently anti-influenza vaccination is the only mean for preventing these bacterial super-infections.

LAIVs have shown potential applications in the prevention of SBIs conferring high level of protection in mouse model of *S. pneumoniae* and *S. aureus* super-infections [242]. Particularly it has been reported that seasonal FluMist® vaccination can induce cross-reactive T cell immunity towards pandemic H1N1 influenza virus and is also able to restore innate immunity against SBIs reducing the susceptibility to the common post-influenza complications [242]. On the other hand, a comparative study between inactivated and live attenuated influenza vaccines demonstrated that, even if the two vaccination strategies can limit expression of pro-inflammatory cytokines that are induced after super-infection and ameliorate morbidity and mortality associated with *S. pyogenes* super-infections, they confer only an incomplete protection from bacterial replication in the lung [240].

Taking into consideration the critical role of NA activity in the induction of SBI [205], Huber and colleagues showed that the immunity towards this component of the virion, even not able to neutralize influenza infection, can limit progression toward secondary bacterial complications in a mouse model of post-influenza *S. pneumoniae* infection [5]. Furthermore a more recent study demonstrated that vaccination with the highly conserved NP also reduces influenza-induced susceptibility to lethal bacterial infections [241]. In particular, focusing on the mechanisms of protection, Haynes *et al.* showed that both T cells and antibodies contribute to defense against influenza-induced pneumococcal diseases, but while influenza cross-reactive T cells reduce viral titers, NP-specific antibodies suppress induction of inflammation in the lung [241]. The findings of these two studies suggest that, in case neutralizing influenza vaccines are not available, non-neutralizing strategies that fail to prevent viral infection may nevertheless protect the public from secondary bacterial diseases.

Despite the evolution in vaccine formulations, and evidence that the HI assay is an imperfect correlate of protective immunity [335, 336], this assay remains the gold standard correlate of vaccine-induced protection against influenza [70, 260]. Several studies are currently ongoing to evaluate alternative correlates of anti-influenza vaccines protection establishing the prevention of SBIs as endpoint in place of neutralization of influenza infection [191]. They highlighted three major parameters that have to be taken into consideration to evaluate vaccine efficacy towards influenza complications: first of all the level of anti-NA antibodies and their functionality evaluated by neuraminidase-inhibition (NI) assay [5]; secondly appropriate correlates of mucosal immunity, as IgA levels, are needed [191, 240, 242]; finally, considering that the cross-reactivity of T cells has been pointed as a key component of the immunity fight against influenza-induced bacterial diseases [240, 241], antigen-specific cytotoxic CD8$^+$ and cytokines secreting CD4$^+$ T cells levels need a careful evaluation [337-339].

OUTLINE OF THE THESIS

Secondary bacterial infections (SBI) contribute to morbidity and mortality from influenza, especially in elderly subjects. This age group, besides being the most vulnerable to influenza, responds only poorly to conventional influenza vaccines. With this work we wanted to address this medical need and investigate on novel strategies for improved vaccination strategies.

We designed this project to deeply investigate immune response induced by different anti-influenza vaccination strategies in mouse model. This thesis is organized in two studies:

- With the first study we wanted to assess how the successful vaccine adjuvant MF59 that was specifically developed for influenza vaccination in the elderly would contribute to overcoming suboptimal immune responses in the elderly. This work resulted in the manuscript "**MF59-induced ATP-signalling pathway is not impaired by immune-senescence thereby contributing to an efficient adjuvant activity in the elderly**".
- With the second study we investigated whether influenza vaccination could be further improved by the use of different adjuvants. Using a novel mouse model of viral-bacterial co-infection we found that there is a "**Positive contribution of adjuvanted influenza vaccines on the resolution of viral-bacterial co-infections**".

In the first study we tested MF59 adjuvanticity in aged mice. This adjuvant is specifically licensed to prevent influenza infection in elderly subjects (>65 years) and its mechanism of action has been deeply characterized in pre-clinical models. Yet all these studies have been performed in young mice (6-8 weeks), which do not reflect immune response in elderly. Which MF59-activated immune events are still active in old mice? Is this adjuvant able to overcome intrinsic defects of immunosenescence? We performed a comparative study in young and aged (>18 months) mice applying most of relevant assays to assess MF59 activity. We vaccinated mice using influenza (Fig. 1) or ovalbumin (OVA) (Fig. 2) MF59-adjuvated vaccines and we measured systemic antibody and cellular response. Moreover we assessed serum cytokine release induced by MF59 and consequent immune cell recruitment to the injection site and antigen translocation to draining lymph nodes (dLNs). Notably ATP release at the site of injection has been shown to be essential for MF59-adjuvaticity. Is this pathway still functioning in MF59-vaccinated aged mice? We aimed to understand the importance of ATP-release for the activity of MF59 also in the context of immunosenescence.
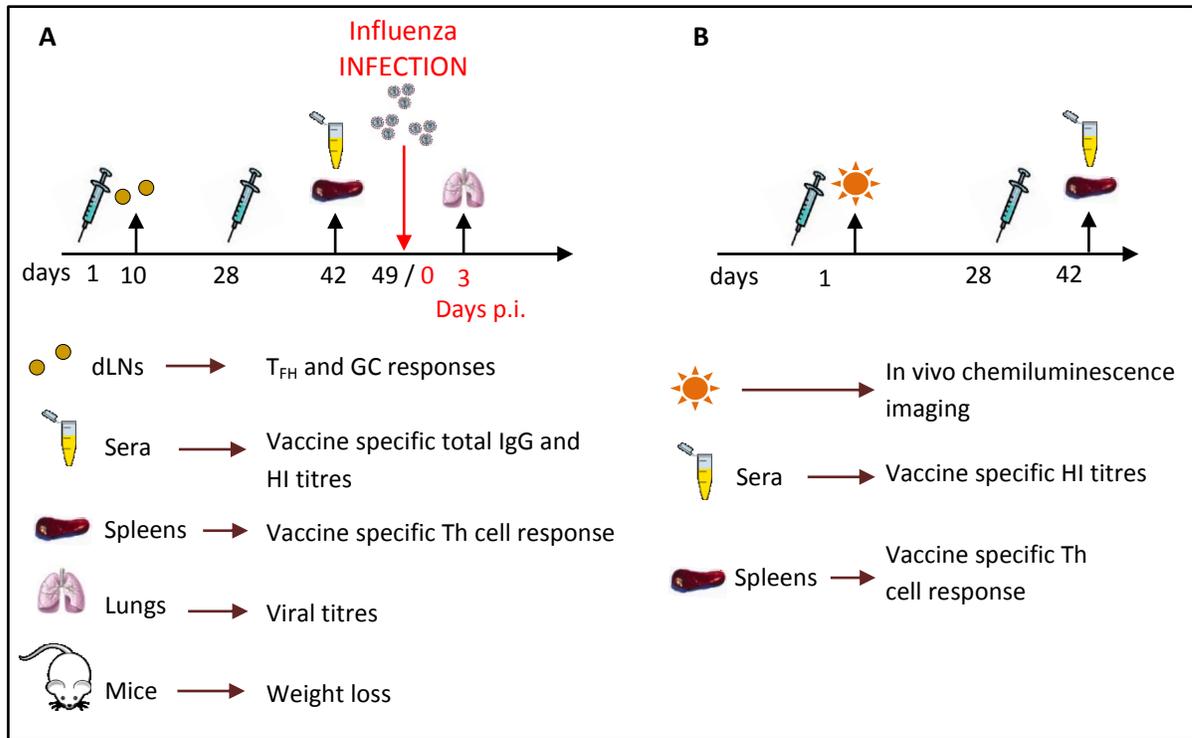
Figure 1 - Characterization of mechanism of action of MF59-adjuvanted anti-influenza vaccine in elderly.

BALB/c mice were used at 6-8 weeks (young) and at 18 months or more (aged) of age. (A) i) Mice have been vaccinated twice intramuscularly (i.m.) four weeks apart with trivalent influenza vaccine (TIV) as plain antigens or adjuvanted with MF59. 10 days after first vaccination we examined T follicular helper (T_{FH}) cell and germinal center (GC) responses in dLNs. We then collected sera and spleens two weeks after second immunization to analyse vaccine specific humoral and cellular response. ii) Mice have been vaccinated twice i.m. four weeks apart with hemagglutinin (HA) protein of H1N1/A/Puerto Rico/8/1934 (PR8) virus as plain antigen or adjuvanted with MF59. Three weeks after second immunization mice have been infected intranasally (i.n.) with 2.1 $TCID_{50}$ of PR8 virus and we followed disease evolution and pathogen clearance in lungs for various days post-infection (p.i.). (B) To verify ATP release in muscles mice have been i.m. injected with MF59 or PBS together with the mixture luciferase-luciferin that reports on ATP changes. We then performed quantitative analysis of chemiluminescence over time. Secondly mice have been vaccinated twice i.m. four weeks apart using TIV, TIV+MF59 or TIV+MF59+apyrase and we collected sera and spleens two weeks after second immunization to test vaccine specific Th cell and antibody responses.
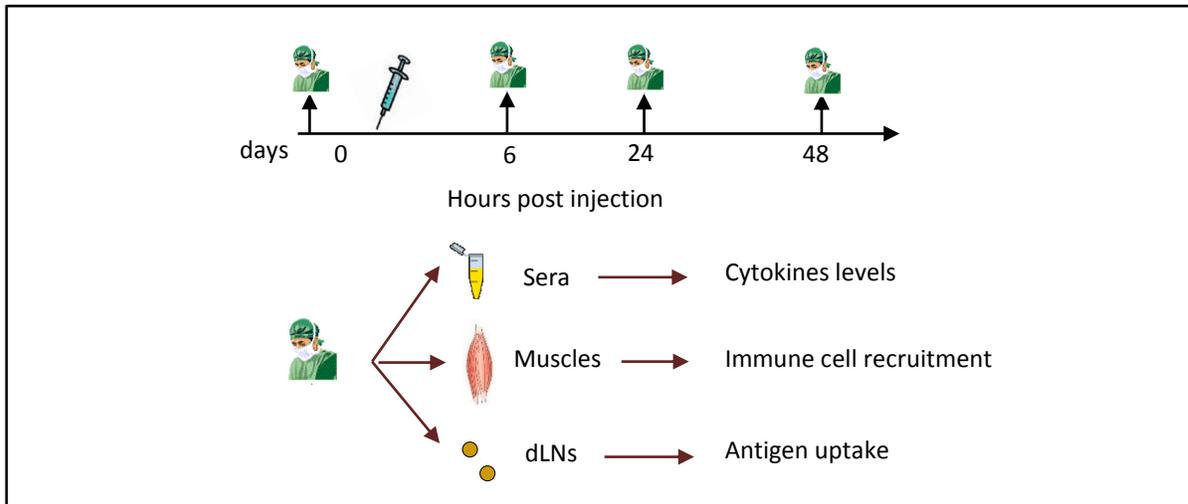
Figure 2 - Analysis of MF59-induced cell recruitment to injected muscle and antigen uptake to dLNs.
BALB/c mice were used at 6-8 weeks (young) and at 18 months or more (aged) of age. Firstly mice have been immunized i.m. with OVA in presence or absence of MF59 and cytokine levels in the serum were assessed at the indicated time points. Secondly we vaccinated mice i.m. with OVA-AlexaFluor467 and we sampled muscles and dLNs at the indicated time points to characterize immune cell recruitment to injection site and antigen translocation to dLNs.

In the second work we wanted to dissect the complex interplay between immune responses towards an influenza virus and a co-infecting bacterium in the mouse model. In particular our final goal was to explore the possibility to modulate the events by anti-influenza vaccination inducing in mice different Th-polarized immune response. Use of different routes of vaccine administration - mucosal versus systemic immunization - as well as different adjuvants, should allow us to assess how diverse conditions can impact on the quantity and quality of vaccine-antigen specific Th cells and antibody response. Moreover we had the opportunity to test the efficacy of our vaccine formulations not only against influenza challenge, but also for SBI.

Briefly we immunized mice with a H1N1/A/California/7/2009 (H1N1/ Cal) subunit vaccine either as plain antigens or with different adjuvants inducing either Th1-, Th2- or Th17-prone immune responses. Vaccinated mice were challenged with the heterologous influenza PR8 virus and six days later infected with *S. aureus* USA300.

Initially we analyzed different aspects of innate and adaptive immunity in the context of single influenza infection. Is systemic response sufficient to counteract heterologous influenza infection or we need specific mucosal immunity at the site of infection? How differently skewed-immune responses protect mice from influenza disease? To answer these questions we not only evaluated vaccine-specific serum antibody titers and spleen Th cell response but also took into account immune cell recruitment

and humoral and cellular adaptive response in the lung. Moreover we followed mice weight loss and lung viral titers as signs of ongoing disease. Fig. 3 shows details of our study.
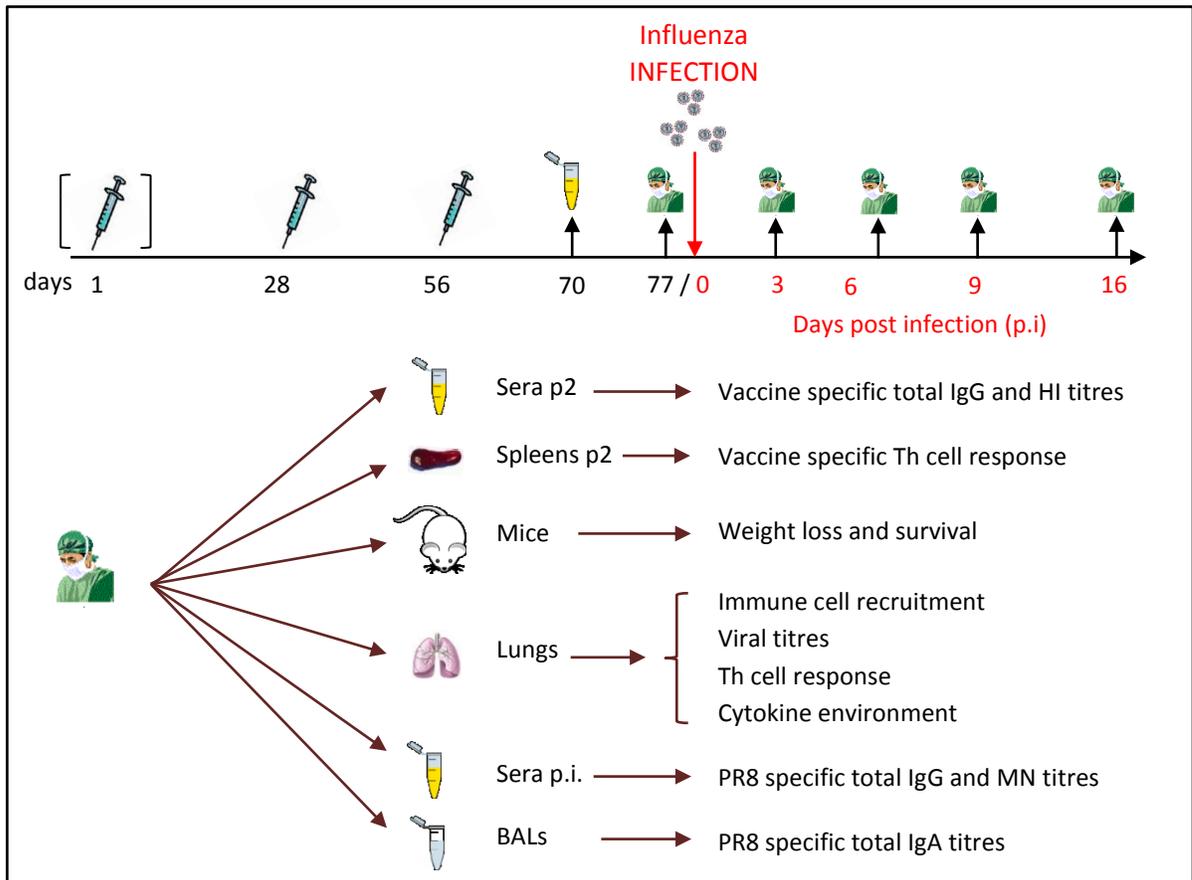

Figure 3 - Characterization of the impact of vaccination route and chosen adjuvant on immune responses and host-pathogen interplay during influenza infection.

BALB/c mice have been vaccinated three times sublingually (s.l.) or two times i.m. four weeks apart. We used H1N1/ Cal antigens either not adjuvanted (i.m.) or formulated with MF59 (i.m.) (Th1/Th2), MF59+CpG (i.m.) (Th1) or LTK63 (s.l.) (Th17). Two weeks after last immunization we collected sera (sera p2) and one week later we collected spleens (spleens p2). At the same time we infected mice i.n. with 2.1 $TCID_{50}$ of PR8 virus and we sacrificed them to sample lungs, sera and bronchoalveolar lavages (BALs) at 3, 6, 9 and 16 days p.i. We used naïve (not vaccinated) mice as negative controls and mice pre-exposed to a sublethal dose of virus as positive controls. Sampling and relative readouts are listed in the figure.

Finally we evaluated the efficacy of previously characterized anti-influenza vaccine formulations in the context of viral-bacterial co-infection. How does the specific cytokine environment experienced during influenza infection affect co-infecting organisms? Which Th profile would impact most positively on bacterial clearance? With these questions in mind we followed disease evolution measuring body weight loss and pathogens clearance from lungs in influenza+*S. aureus* co-infected mice (Fig. 4). With this

study we aimed to induce in the host a proper Th-polarized immune response by anti-influenza vaccination that may help not only to resolve the influenza infection, but also the SBI.
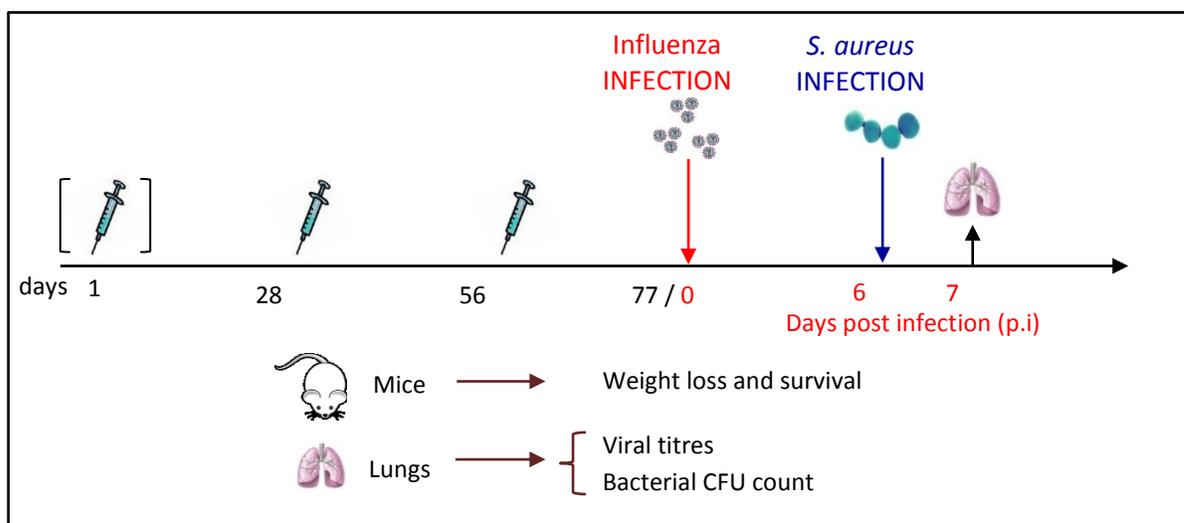

Figure 4 - Analysis of the effects of different anti-influenza vaccination strategies on viral-bacterial co-infection. BALB/c mice have been vaccinated as showed in Fig. 3 and three weeks after last immunization were infected i.n. with 2.1 $TCID_{50}$ of PR8 virus. Six days later we challenged animals i.n. with 10^7 CFU of *S. aureus* USA300. We followed mice weight loss up to sixteen days p.i. and we collected lungs the day after bacterial infection to analyse viral and bacterial load. Mice infected only with influenza or *S. aureus* were used as controls.

Our studies were meant to shed light on new strategies for improved influenza vaccines, which would have to take into consideration the potential combination of protective antigens and adequate adjuvants. Moreover we wanted to stress the need of new correlates of protection for influenza vaccines: it is extremely important to know the power of a vaccine not only in counteracting homologous viral infection, but also heterologous challenge. Notably SBI are frequently diagnosed in previous influenza-infected subjects, especially in elderly population, so it would be extremely important to understand immunological imbalances that are responsible for influenza-bacteria synergy and moreover to design effective broad-spectrum approaches to prevent the susceptibility to bacterial superinfection.

MF59-induced ATP-signaling pathway is not impaired by immune-senescence thereby contributing to an efficient adjuvant activity in the elderly.

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ABSTRACT

MF59-adjuvanted influenza vaccine has been developed specifically for the elderly, since this age group typically responds inadequately to traditional influenza vaccines, due to immunosenescence. Several studies have been performed to identify MF59 mechanism of action in pre-clinical models. However, most of these studies have been conducted in young mice (6-8 weeks, comparable to young adults), which do not reflect the immune response of the elderly. Taking into consideration that several signaling cascades are altered during aging, we wanted to assess which MF59-activated immune events were still active in old mice (> 18 months), and performed a comparison to young mice in most of the relevant assays for MF59 activity.

We found differences in MF59-induced cytokines, with a lower pro-inflammatory response in the elderly. Yet, our results also show that MF59 still acts as adjuvant in elderly mice, enhancing immune cell recruitment, antigen-translocation to draining lymph nodes, $CD4^+$ T helper responses, germinal center reaction and restores HI titers similar to those from young mice immunized with unadjuvanted vaccine. Further, since we recently showed that MF59 induces rapid and transient ATP-release in young mice, we tested functionality of this signaling pathway also in the elderly. Indeed, abrogation of ATP-signaling with apyrase - an ATP-hydrolyzing enzyme - completely blocks MF59-activity also in elderly mice suggesting an important role for extracellular ATP also in this age group. Understanding in elderly mice how the immune system is altered and how MF59 helps to overcome age-related limitations, will give us useful insight for future tailor-made vaccine solutions for this growing patient age group.

INTRODUCTION

Influenza infection can lead to considerable morbidity and even mortality with approximately 90% of influenza deaths occurring in older adults [1]. The best strategy to prevent influenza disease among this vulnerable population is to be immunized with the current influenza vaccine on an annual basis [243]. Yet, traditional seasonal subunit and split-virus influenza vaccines have shown limited effectiveness in people over 65 years of age [145, 266, 340]. There are well-known defects in both the innate and adaptive arms of the immune system in older adults, which contribute to the poor vaccine efficacy in this population [341]. In particular, the decline of the effectiveness of the influenza vaccine in the elderly correlates with a decrease in the excitation of cell-mediated and antibody responses that are essential for providing protection against influenza. To improve vaccine-mediated protection against influenza in older adults, many strategies are being pursued, such as increasing the dose of the vaccine antigens, evaluating alternative routes of delivery to mucosal and dermal compartments compared to intramuscular injection and adding adjuvants to the vaccine.

Adjuvants perform through innate immune mechanisms and are responsible for effective onset of immune responses increasing T cell mediated and humoral responses to vaccine antigens and increase vaccine effectiveness [276, 277, 342, 343]. Activation of the innate immune system is especially critical to the development of protective adaptive immune responses against intracellular pathogens such as influenza. The role of an adjuvant for improving influenza vaccines for the elderly is to increase the level of inflammatory mediators at the site of injection, to activate dendritic cells (DCs) and enhance their antigen-presenting capacity to induce the desired adaptive immune response [344]. Many different adjuvants have been tested for efficacy in the quest to identify a successful adjuvant that would boost the immune response in aged mice [345]. But few have succeeded beyond pre-clinical models.

The current gold standard of vaccines licensed for elderly people are those adjuvanted with oil-in-water-emulsion adjuvants like MF59 and AS03 [3, 286, 310, 346]. Influenza vaccines containing MF59 have been approved with a very well established use in people older than 65 years, since 1997 in Europe. In fact, MF59-adjuvanted influenza vaccine has been developed specifically for the elderly in order to overcome the inadequate response to traditional influenza vaccines due to immunosenescence typically observed in this age group. Besides inducing high hemagglutination inhibition (HI) titers, MF59 is also an efficient inducer of T follicular (T_{FH}) and T helper (Th) responses in mice [4, 6, 347] and men [288, 348]. Both CD4$^+$ T subsets cells have been implied in prediction of vaccine effectiveness. And CD4$^+$ Th cells are believed to contribute to protection against influenza challenge via various pathways [102, 349].

Many studies have been performed to identify the mechanism of action of MF59 adjuvant in pre-clinical models [4, 297, 311-316, 350]. All these studies have been conducted in young mice (6-8 weeks comparable to young adults), which do not reflect the immune response of the elderly. The aim of this study was to perform a comparison between aged mice (> 18 months) and young mice in most of the relevant assays for MF59 activity to assess which MF59-activated immune events were still active in aged mice contributing to MF59 powerful effect on this age group.

MATERIALS AND METHODS

Mice

Female BALB/c mice were used at 6-8 weeks (young) and at 18 months or more (aged) of age in agreement with institutional and European guidelines. All experiments involving animals were approved by the Italian Health Ministry and carried out in accordance with the Italian legislation (Legislative Decree 116/92).

Adjuvant, antigens, immunization

MF59, a Novartis proprietary oil-in-water emulsion, consisting of 4.3% squalene, 0.5% Tween 80, 0.5% Span 85 in citrate buffer (10 mM), was prepared as described before [316]. The mean particle size of the emulsion droplets determined with a Mastersizer X (Malvern Instruments, Southborough, MA) was 194 ± 76 nm. For cell-tracking experiments we used 10 µg/ mouse of ovalbumin (OVA) conjugated with AlexaFluor 647 (Invitrogen) with or without MF59. For adjuvanticity experiments, experimental trivalent influenza vaccine (TIV) composed of equal amounts of hemagglutinin (HA) from influenza strains H1N1/A/California/7/2009, H3N2/A/Perth/16/2009 and B/Brisbane/60/2008 was used. The vaccine contains purified subunit antigens and is standardized for HA content by single-radial-immunodiffusion. Mice were divided into groups and immunized intramuscularly (i.m.) twice on days 0 and 28 in the quadriceps muscles of both hind legs with 50 µl vaccine/ leg (100 µl total per mouse). Doses were 0.3 µg (0.1 µg each antigen) of either influenza soluble trivalent egg-derived antigen alone; antigens were mixed with research grade MF59 (1:1, vol:vol) alone or formulated with apyrase (10 U/ muscle). Serum samples of individual mice were collected 2 weeks after each immunization and evaluated for total IgG antibody titers by ELISA and hemagglutination inhibition (HI) titers by the HI assay. All formulations were optimized for pH and osmolality to physiological conditions. For infection experiments, i.m. immunizations have been performed as reported before but we used 1 µg of HA of H1N1/A/Puerto Rico/8/1934 (PR8) virus (Sino Biological Inc.) as antigen.

ELISA

Titration of HA-specific total IgG was performed on individual serum samples as previously described [316]. Antibody titers are dilutions that give an optical density (OD) higher than the mean plus five times the standard deviation of the average OD obtained in the pre-immune sera. The titers were normalized with respect to the reference serum assayed in parallel.

Determination of antibodies by HI Assay

The HI assay was carried out on individual sera taken 2 weeks after the second immunization as described elsewhere [351].

In vitro re-stimulation of antigen-specific CD4+ T cells

Four mice per group were sacrificed 2 weeks after the second immunization and spleens were collected to assess the frequency and phenotype of antigen (Ag)-specific CD4$^+$/ CD44^{high} T cells induced by vaccination. The assay was performed as described elsewhere [351].

Influenza viral infection

Mice were anesthetized and challenged intranasally (i.n.) with 30 µl (15 µl/ nostril) of 2.1 TCID$_{50}$ of mouse-adapted influenza PR8 virus three weeks after the final vaccination. After viral challenge, mice were monitored daily for 16 days for weight loss and euthanized if humane endpoints (= 25% weight loss) were reached.

Lung viral titers

The infectious PR8 virus titers in homogenized lung samples were determined by TCID$_{50}$ assay performed on MDCK cells by ten-fold serial dilutions of samples as described [352]. Briefly, supernatants from lung homogenates were diluted in Ultra MDCK-Medium (BioWhittaker, Lonza) supplemented with 1% PSG (Life Technologies) and 1 µg/ ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK-treated) trypsin (Sigma-Aldrich). All samples were then transferred on MDCK cells, plated in a 96-well plate (15,000 cells/ well). The final volume was 200 µL/ well. Plates were incubated for 3 days at 37°C in 5% CO$_2$. Cells were microscopically observed and cytopathic effect of virus on cells evaluated. The titer was determined by interpolation using the method of Reed and Muench [353]. TCID$_{50}$ was reported as the dilution in which 50% of the infected wells were positive for virus.

Cytokine analysis

Serum samples were collected at different time point post injection and cytokine concentrations were measured by Luminex Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay (Biorad) according to manufacturer's instruction. The cytokines analyzed were IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-α, MIP-1β, RANTES and TNF-α.

Cell recruitment into muscle and antigen uptake into draining lymph node

Groups of mice were injected with 25 µl/ muscle of MF59 (1:1, vol:vol) or PBS buffer control. Mice were sacrificed 24 h post-injection, quadriceps muscles and draining lymph nodes were processed as previously described [316]. Cells were stained with combinations of the following antibodies: α-Ly6C-FITC, α-CD11b-PE-Cy7, α-Ly6G-PE, α-CD11c-APC, α-CD3-PerCpCy5.5 (all from BD Pharmingen), and α-I-A/I-E-AlexaFluor700, α-F4/80-PacificBlue, α-CD11c-APC-AlexaFluor750 (all from eBioscience). Stained cells were analyzed using a FACS LSR II Special Order System (BD) using BD DIVA software (BD Bioscience).

FACS analysis of T follicular helper and Germinal Center B cells

Draining lymph nodes (dLNs) were harvested and pooled per mouse after i.m. immunization. dLNs single cell suspensions were prepared by homogenization, fixed and stained with the following combination of antibodies: α-IgM-BV421, α-CD19-APC-H7, α-IgD-PE, α-GL7-Alexa647, α-CD73-PE-Cy7 (all from BD Pharmingen), α-CD80-PE-CF594, α-CD4-V500 (both from BD Horizon), α-PD1-FITC, α-CXCR5-PerCP-Cy5.5, α-CD3-BV785 (all from BioLegend), α-CD38-PE-Cy5 (eBioscience). Each staining step was done in PBS + 2% FBS on ice. Samples were acquired on a FACS LSR II Special Order System (BD) and analyzed using FlowJo Software (Tree Star).

In vivo bioluminescence imaging

In vivo bioluminescent imaging was performed as previously described in [4]. An ultra-low-noise, high sensitivity cooled CCD camera mounted on a light-tight imaging chamber (IVIS Lumina System, Caliper, Perkin Elmer) was used. Tracking, monitoring and quantification of signals were controlled by the acquisition and analysis software Living Image.

Statistical analysis

All statistics were performed using GraphPad Prism software (version 6.0). Statistical analysis between results obtained from various groups of mice was performed using the Mann-Whitney test. Differences with p values < 0.05 were considered significant.

RESULTS

MF59 enhances T helper cell and antibody responses as well as vaccine efficacy in young and aged mice

It is well established that MF59-adjuvanted influenza vaccine induces increased antibody titers that correlates with increased protection against influenza virus in both young and older adults compared to the respective age group immunized with un-adjuvanted vaccine. To examine this outcome in an experimental mouse model, we immunized young (6-8 weeks of age) and aged (18 months of age) BALB/c mice with a trivalent influenza vaccine (TIV) given intramuscularly (i.m.) either alone or delivered with the MF59 emulsion. As expected, both young and aged mice immunized with TIV+MF59 had significantly higher serum influenza antigen-specific IgG antibody titers compared to non-adjuvanted TIV vaccine groups (Fig. 1A). The striking adjuvant effect of MF59 not only induced a large increase in total antibody titers but also increased functional Hemagglutination Inhibition (HI) titers, which are considered a correlate of protection for influenza vaccination (Fig. 1B and Suppl. Fig. 1A+B) [308]. While MF59 enhanced functional antibody titers in both young and aged mice, functional antibody titers in young mice were of a higher magnitude than that observed in aged mice. There were significant differences in total IgG and HI titers between young and aged mice vaccinated with non-adjuvanted TIV alone and between the young and aged TIV+MF59-vaccinated mice (Fig. 1A+B). Remarkably, when aged mice were immunized with TIV in presence of MF59, significant total IgG and HI antibody responses were noted, which were essentially equivalent (no significant differences) to the response observed in young mice immunized with TIV alone. These results are in agreement with data from clinical trials comparing elderly to adults [354].

Studies performed in older adults have demonstrated that MF59 induces an increased frequency of circulating CD4$^+$ T cells specific for vaccine antigens compared to un-adjuvanted vaccine [355]. Accordingly, we assessed the effect of MF59 adjuvantation on CD4$^+$ T helper (Th) responses in our experimental murine model. Splenocytes from young and aged mice were collected after the second immunization, stimulated *in vitro* with antigen to reactivate vaccine-specific Th cells and assessed by FACS for intracellular cytokine expression. MF59-adjuvanted vaccine induced higher T cell responses compared to plain vaccine in both young and aged populations, with higher variability observed for aged mice (Fig. 1C). The induced Th profile was confirm with a mixed Th1 (IFNγ)/ Th2 (IL4/13) phenotype as described for this adjuvant (Fig. 1D).

Finally, we tested the effectiveness of the elicited immune responses in conferring protection from challenge with influenza virus. Previous work has illustrated age-associated impairments in immune responses during influenza viral infection with the H1N1/A/Puerto Rico/8/1934 (PR8) strain [356, 357]. To further examine this phenotype, we vaccinated mice using HA protein from PR8 virus as antigen alone or in combination with MF59 and we infected young and aged mice with the homologous strain of influenza. We quantified HI titer in sera from immunized mice and verified that HA protein alone was not sufficient to induce functional antibodies, while the addition of MF59 greatly enhanced HI titers in both young and aged mice (Suppl. Fig. 1C). Notably, in parallel with previous results of TIV immunization, also in case of HA+MF59 vaccine functional antibodies titers were significantly higher in young mice than in aged ones. In our model, naïve aged mice resulted less susceptible to influenza viral infection compared to naïve young mice (Fig. 1E+F). However, young mice vaccinated with non-adjuvanted HA protein showed partial protection, as indicated by significantly reduced weight loss at day 7 post challenge compared to the respective naïve group (Fig. 1G). In contrast, aged mice receiving non-adjuvanted HA immunization were not protected in terms of weight loss at day 7 post challenge compared to the respective naïve group (Fig. 1G). Notably, HA+MF59 vaccine fully protected from weight loss both young and aged mice for the whole observed period post challenge.

At specific time points during influenza viral infection, lung tissue was harvested from young and aged mice, and lung viral loads were assessed by the TCID$_{50}$ assay. A kinetic study of lung viral titers was previously performed in young mice in our laboratory and demonstrated that a peak occurred at day 3 post challenge (Suppl. Fig. 1D). Therefore, we evaluated and compared viral titers from lungs of young and aged mice three days after challenge. As illustrated in Fig. 1H, both young and aged mice vaccinated with HA alone showed significantly lower lung viral titers compared to the respective naïve groups. However, we observed in young mice a considerably higher decrease, reflecting what we observed for body weight loss. Consistent with a full protection from weight loss following MF59-adjuvanted vaccination, a very low detectable lung viral load was found in the HA+MF59 vaccinated young and aged mice.

Comprehensively, our data support the conclusion that while MF59 can boost TIV-specific T cell and antibody responses in aged mice, it does not completely overcome the reduced ability of a senescent immune system to respond to an antigen.
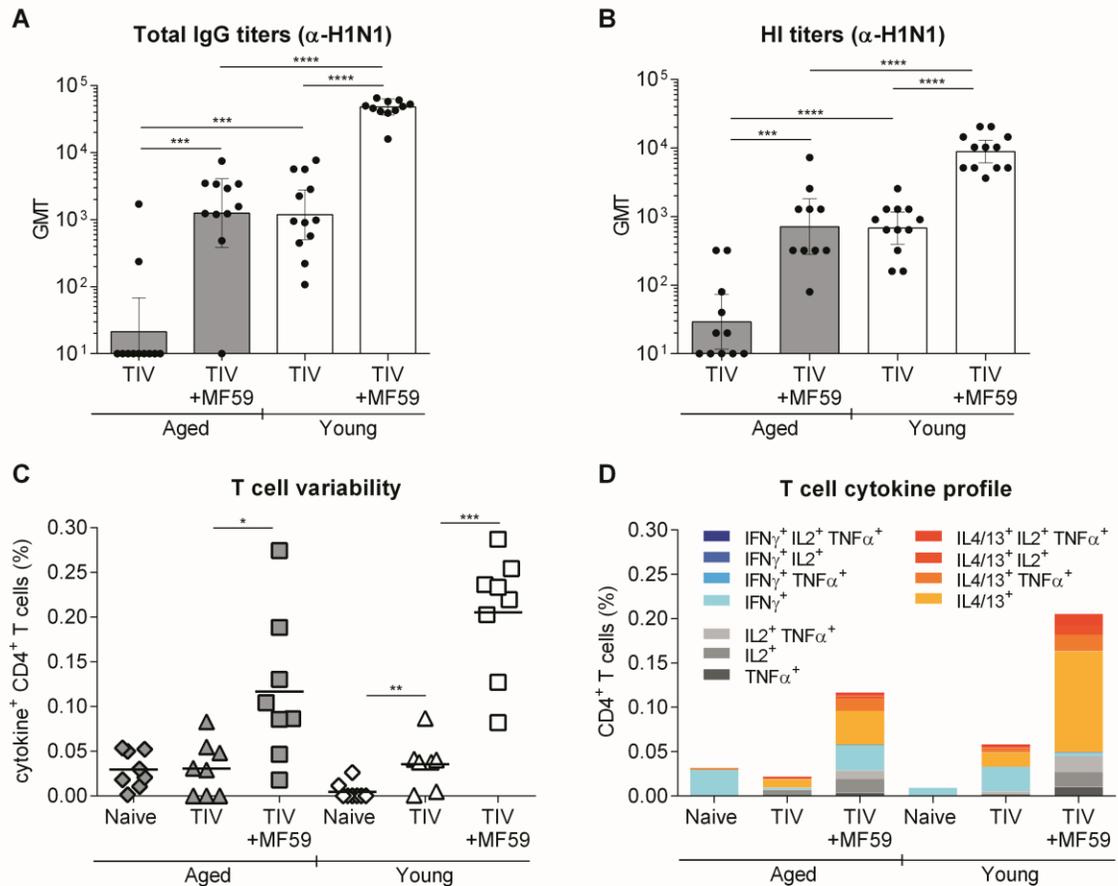
Figure 1 (A-D) - MF59 induces enhanced T helper cell and antibody responses and vaccine efficacy in young and aged mice.

(A) Ag-specific IgG titers in aged and young mice. Groups of mice were immunized twice with TIV or TIV+MF59 with a 4-week interval. Blood samples were drawn 2 weeks after the second immunization and H1N1-specific total IgG antibodies were measured by ELISA. Values represent geometric mean titers (GMT) of 12 mice/ group with 95% CI. (B) Hemagglutination inhibition (HI) titers towards H1N1/California; values represent GMT of 12 mice/ group with 95% CI. (C-D) Spleens from young and aged mice were taken 2 weeks after the second immunization with TIV or TIV+MF59, and TIV-specific CD4$^+$ Th cells were reactivated by *in vitro* stimulation. Their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. (C) Dot plot graph represents the percentage of cytokine$^+$ CD3$^+$CD8$^-$CD4$^+$CD44^{high} T cells for each single mouse. (D) Bars show cumulative numbers of TIV-specific cytokine expressing cells, while the individual color code indicates the type of cytokines expressed by the respective cells, coded as shown in the panel above the graph. Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.
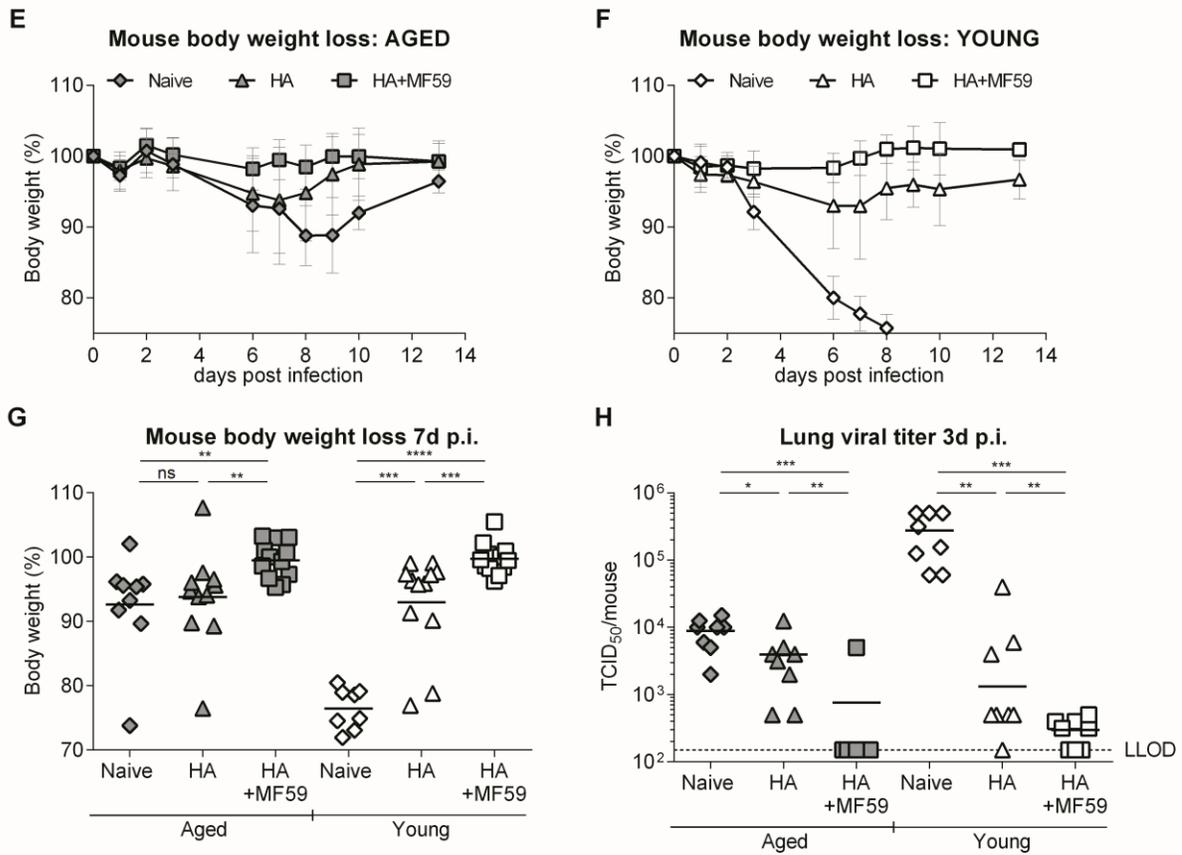
Figure 1 (E-H) - MF59 induces enhanced T helper cell and antibody responses and vaccine efficacy in young and aged mice.

MF59 significantly reduced illness severity and viral load after influenza challenge in aged and young mice. Following infection with PR8 virus, aged mice (E) and young mice (F) were evaluated daily for weight loss. (G) Statistical analysis of weight loss 7 days post infection (7d p.i.). (H) Influenza virus titers were evaluated in lung supernatants of young (white symbols) and aged (grey symbols) mice 3 days post infection and represented as $TCID_{50}$/ mouse. Mann-Whitney test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = not significant.

MF59-induced cytokine release is significantly lower in aged mice

Since several studies published by our group dissected the contribution of individual pathways and immune mechanisms to MF59-adjuvanticity, we set to study further differences in immune responses in aged and young mice.

A set of studies, undertaken both in vivo in young mice and in vitro, has demonstrated that MF59 predominantly act through a mechanism involving chemokine-driven immune cell recruitment to the injection site [297, 314-316, 350]. To compare aged and young mice in cytokines release induced by MF59, the two groups of mice were immunized with ovalbumin (OVA) in presence and in the absence of MF59 and cytokine levels in the serum were assessed at different time points after intramuscular injection. In both aged and young mice, OVA+MF59 treatment induced high cytokine release that reached statistical significance for KC (CXCL1), MCP-1 (CCL2), IL-6, IL-5 and G-CSF at least at one time point compared to OVA alone treatment (Fig. 2A). As shown in Fig. 2B, statistical analysis performed at 6 hours post injection revealed significant lower MF59-induced cytokine release of MCP-1, IL-5 and G-CSF in aged mice compared to young mice.
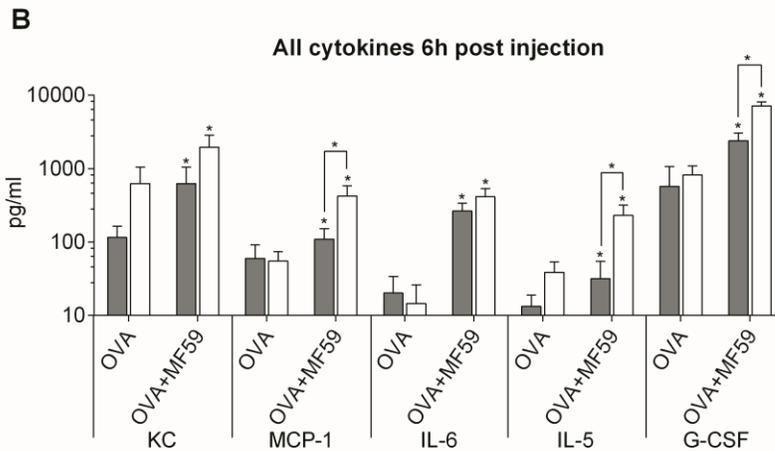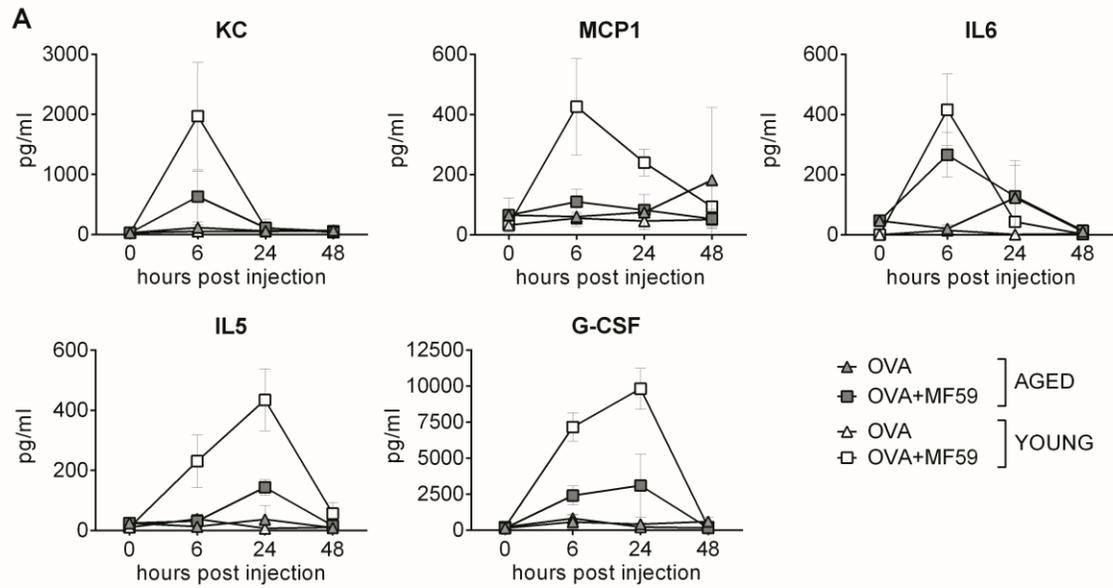
Figure 2 - Cytokines release in the serum of young and aged mice in response to MF59 injection.
(A) Four mice per group were injected in both legs (i.m.) with 50 µl per quadriceps muscle of OVA (10 µg/ mouse) either with or without MF59 (1:1, vol:vol, in PBS). Blood samples were drawn at indicated time points and cytokines in serum were analyzed by multiplex-bead-ELISA. Values represent mean of 4 mice ± SD. Mann-Whitney test was performed at each time point for OVA alone vs. OVA + MF59, and a significant difference was found at least for one time point. (B) Cytokine content in the serum 6 hours post injection. Four mice per group were injected with the indicated compounds as before, and cytokine content was assessed 6 hours p.i. by multiplex-bead-ELISA. Values represent mean of 4 muscles + SD. Aged mice (grey bars) show a lower release of MCP-1, IL-5 and G-CSF compared to young mice (white bars). Mann-Whitney test: *p < 0.05

Analysis of MF59-induced cell recruitment and antigen uptake revealed intrinsic defects in antigen-loaded cDCs in translocation to draining lymph nodes of aged mice.

As the chemoattractive role of these cytokines in mediating innate cell recruitment and activation at the injection site is a key feature of MF59 adjuvanticity [314-316], we asked whether the reduced cytokine release observed in aged mice could affect cell recruitment and/ or antigen uptake. Young and aged mice were treated intramuscularly with the fluorescent model antigen OVA-AF467 in presence and in absence of MF59 and, at different time points, muscle single cell suspensions were prepared and analyzed by multicolor FACS. Injection of antigen alone led to moderate CD11b$^+$ cells infiltration in both mice groups (Fig. 3A). In contrast, in both aged and young mice, MF59 induced strong cellular recruitment that was significantly enhanced over the antigen alone treatment for the total CD11b$^+$ population (Fig. 3A) and for each cell type assessed (Suppl. Fig. S2). No significant differences were observed in the overall number of CD11b$^+$ cells and any of the single cell populations present in the muscle infiltrate at any time points when comparing aged and young mice (Suppl. Fig. S2). MF59-induced cell infiltration occurred with similar magnitude and kinetics in aged and young mice (Fig. 3B).

We further wanted to assess the impact of MF59 on antigen translocation to draining lymph nodes (dLNs). We collected dLNs of the same mice that were used to study cell recruitment into the muscle comparing aged and young mice. MF59 induced a significant increase of total OVA$^+$ cells in dLNs of both mouse populations when compared to antigen alone treatment (Fig. 3C). Again, overall kinetics and magnitude of OVA$^+$ cells were similar in both age groups.

We extended our analysis to the single antigen presenting cell (APC) types usually enhanced by MF59 (Fig. 3D and Suppl Fig. S2B). In aged mice, the influx of all assessed antigen-loaded APC types was observed but conventional dendritic cells (cDCs) remained significantly fewer than in young mice (Fig. 3D+E). This difference was observed 24 hours post treatment both with OVA and OVA+MF59 suggesting an intrinsic defect associated with aging.
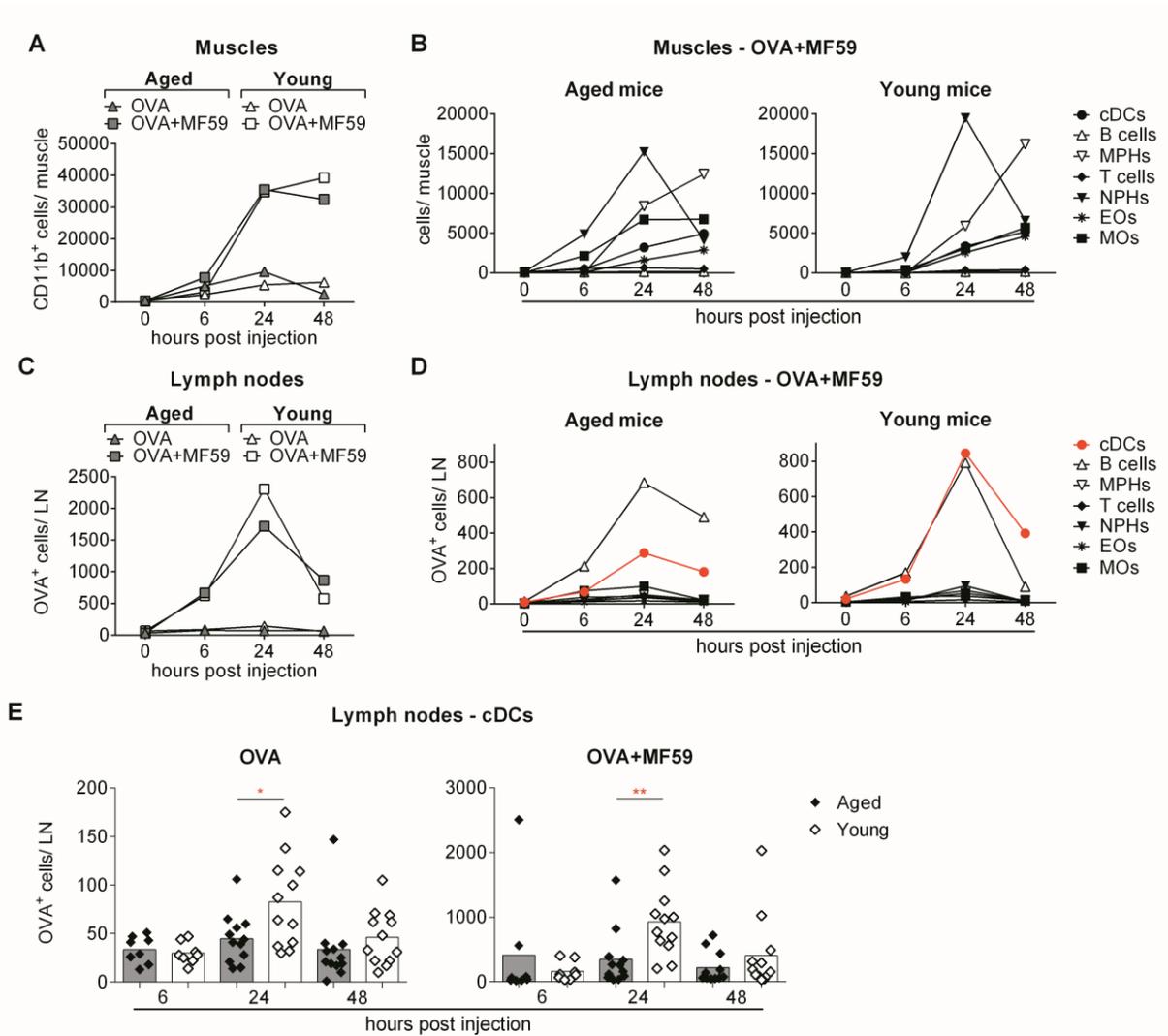
Figure 3 - Cell recruitment into the muscle and antigen uptake in dLNs induced by MF59 in aged and young mice.
(A) CD11b+ cells recruited into the muscle of aged and young mice in response to different treatments. Eight mice per group were injected into both legs with 50 µl of MF59 (1:1, vol:vol, in PBS) or buffer control either in presence or absence of the model antigen OVA (10 µg/ mouse). Cell composition of muscle-derived cells was analyzed by FACS at three time points (6, 24 and 48 hours) post-injection. Values show the means of the 8 treated mice. (B) Comparison of the cell composition in aged and young mice injected with OVA+MF59 as assessed by FACS. (C-D) Total (C) and single cell type (D) antigen-positive cells in draining inguinal LNs. Groups of 8 mice were injected i.m. into both legs with 50 µl of OVA-AF647 (10 µg/ mouse) either unadjuvanted or together with MF59 (1:1, vol:vol, in PBS). LNs were analyzed by FACS at three time points (6, 24 and 48 hours) post-injection to identify specific cells types and antigen-content. Values represent the mean of 8 LNs for each group. (E) Statistical analysis of antigen-positive cDCs into the draining LNs of aged and young mice. Mann-Whitney test: *p < 0.05, **p < 0.01

MF59 contributes to enhanced T follicular helper cell (T$_{FH}$) and Germinal Center (GC) responses but does not overcome limited GC response in aged mice.

Recent evidence demonstrated that enhanced T$_{FH}$ cells and GC responses induced by MF59 are important for its adjuvanticity [347, 358, 359]. To compare T$_{FH}$ and GC B cells responses in aged and young mice, they were immunized with TIV alone or TIV+MF59. Single cells derived from dLNs were analyzed by multicolor FACS 10 days later. MF59 adjuvantation significantly increased CXCR5^{high} PD-1^{high} CD4$^+$ T$_{FH}$ cell responses in both aged and young mice. In aged mice, T$_{FH}$ cell expansion was at least as strong as observed in young mice (no significant difference) (Fig. 4A). In addition, we analyzed GL7$^+$ CD19$^+$ GC B cells and observed a significantly higher number of GC B cells in presence of MF59 in both age groups (Fig. 4B). Despite the enhanced effect of MF59 adjuvantation, the increased number of GC B cells observed in aged mice could not reach the same extent observed in young mice (Fig. 4B).

During GC reaction activated B cells undergo class switching down-regulating the surface molecules IgM and IgD. Switched cells differentiate to Plasma Cells and Memory B Cells. We analyzed the effect of MF59 adjuvantation in GC B cell class switching characterizing this B cell population in two different phenotypes: IgD$^+$ IgM$^{+/-}$ and IgM$^-$ IgD$^-$ GC B cells. We observed that MF59 highly increased the percentage of switched IgM$^-$ IgD$^-$ GC B cells compared to TIV alone in both aged and young mice (Fig. 4C). It is worth noting that aged mice showed an intrinsic limited switched phenotype and that aged mice immunized in presence of MF59 reached similar percentage composition showed by young mice immunized in absence of MF59. This profile reflects nicely what was found for vaccine-induced antibody titers (Fig. 1A+B).

Altogether, our results demonstrated that MF59 adjuvantation of influenza vaccines induces enhanced numbers of T$_{FH}$ and GC B cells and enhanced class switching of GC B cells in both aged and young mice, but intrinsic defects observed in GC B cells of aged mice cannot be completely overcome by MF59.

To further define the relative contribution of the enhancement of T$_{FH}$ cells to MF59-induced GC responses, we analyzed the correlations between T$_{FH}$ and GC B cell numbers in individual mice 10 days after immunization with TIV and TIV+MF59. In young mice, we observed a strong positive correlation between T$_{FH}$ and GC B cells numbers (Fig. 4D, lower panels), indicating that the increase of the number of T$_{FH}$ cells induced by MF59 directly translates to increased GC responses. A similar correlation, but significantly less strong, was observed in young mice immunized with TIV alone. No significant correlations were observed in aged mice immunized both in presence and in absence of MF59, indicating an intrinsic defect in GC responses (Fig. 4D, upper panels). Importantly, despite their impaired immune responses, MF59 could significantly enhanced T$_{FH}$ and GC B cells numbers also in aged mice compared to

un-adjuvanted vaccine, which, even if not directly correlated, could plausibly lead to enhanced GC reaction resulting in higher antibody responses.

Figure 4 (A-C) - MF59 elicits enhanced T follicular helper (T$_{FH}$) cell and Germinal Center (GC) responses in aged and young mice.

dLNs from aged and young mice immunized with TIV+MF59 (or TIV alone as control) were analyzed by FACS 10 days post treatment to determine the number of CXCR5$^+$PD1$^+$ T$_{FH}$ cells (A) and GL7$^+$ GC B cells (B). Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (C) Effect of MF59 adjuvantation in GC B cells class switching; pie charts represent the percentage of two different phenotypes (IgD$^+$IgM$^{+/-}$ and IgM$^-$IgD$^-$) within each GC B cell population isolated from aged and young mice in response to different treatments.
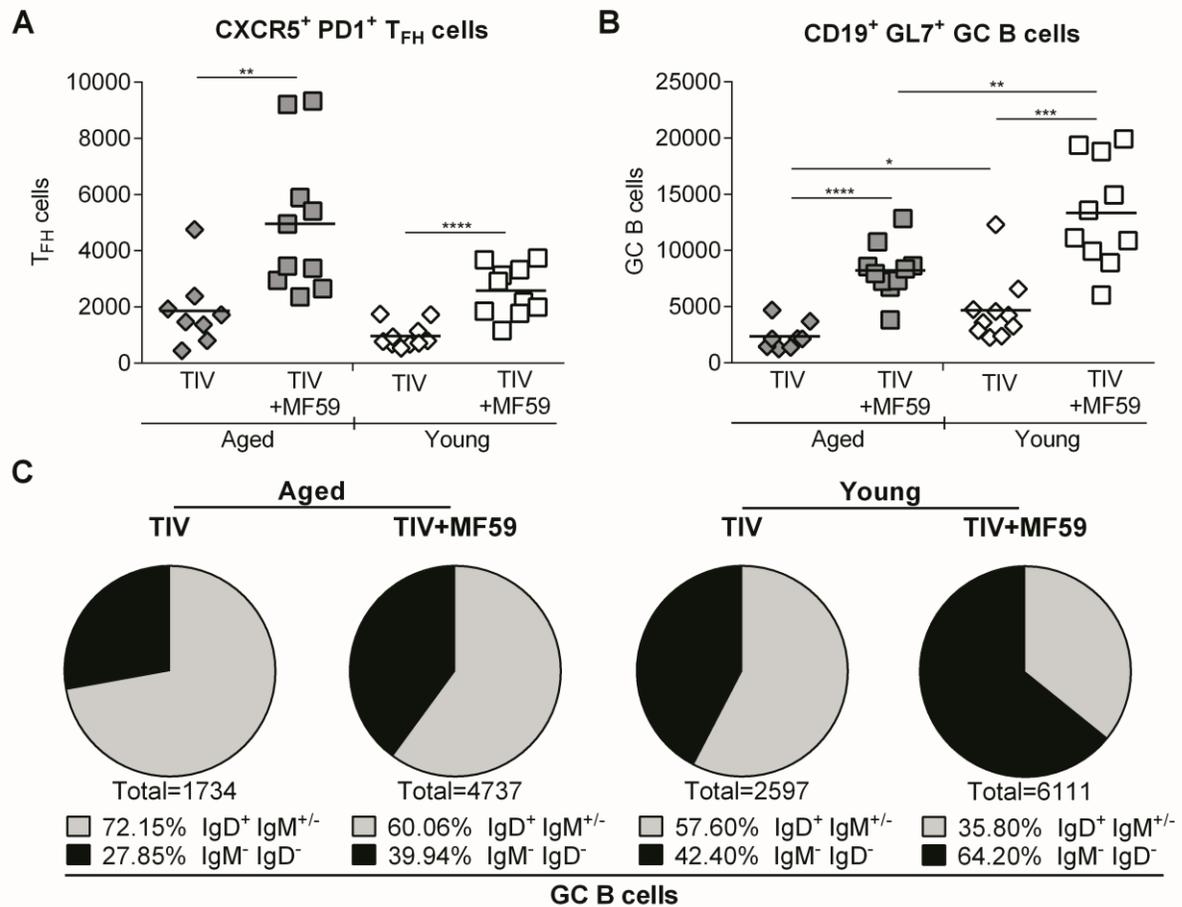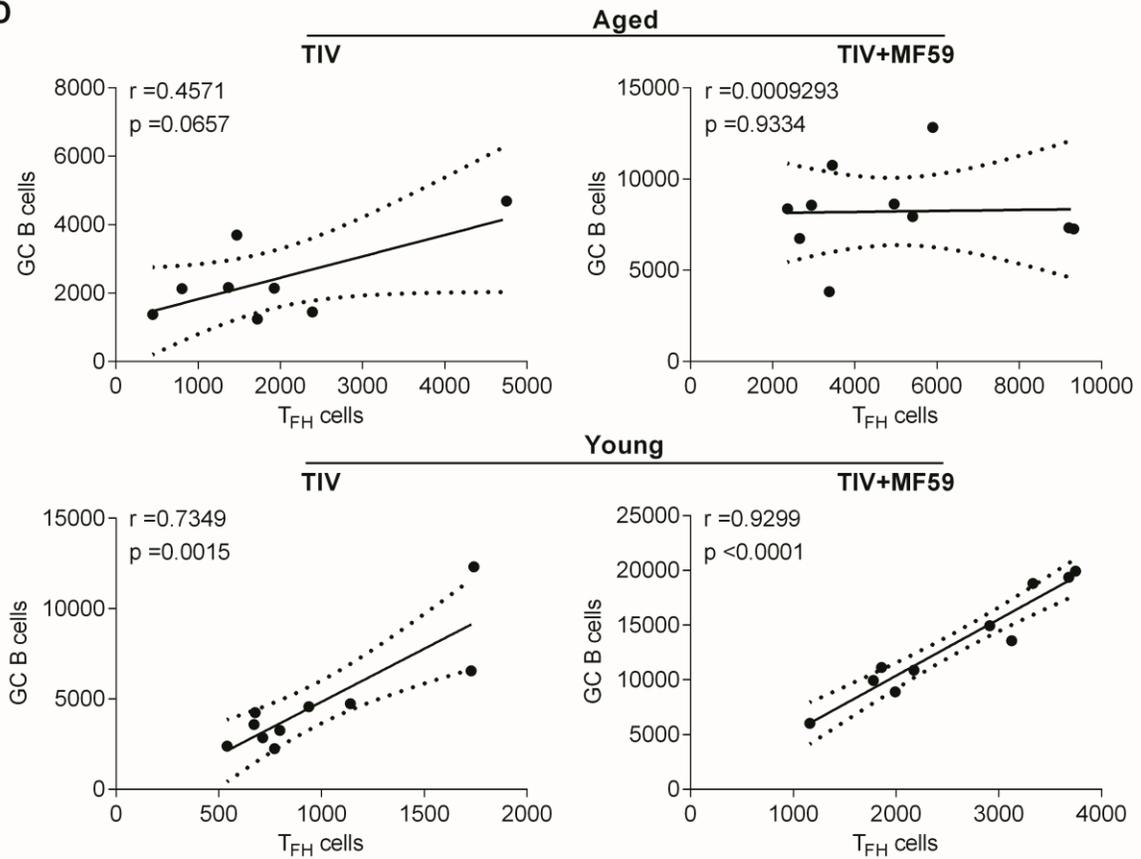
Figure 4 D - MF59 elicits enhanced T follicular helper (T$_{FH}$) cell and Germinal Center (GC) responses in aged and young mice.

Correlations at day 10 post immunization between T$_{FH}$ and GC B cells in individual mice immunized with TIV with or without MF59.

The MF59-induced ATP signaling pathway is active in aged mice contributing to its adjuvanticity.

We have recently demonstrated that MF59 plays a unique capacity to greatly increase ATP release from injected muscles and this is essential for its adjuvant effect [4]. To test if this MF59 prerogative was conserved and still active in aged mice, we used the well-established reporter system luciferase-luciferin to monitor MF59-stimulated ATP release from injected muscles in aged mice and compared it to the one observed in young mice. In aged mice, we found that MF59 injection induced a fast and prominent ATP signal that was significantly higher than ATP release caused by needle injury of PBS injection in the contralateral muscle (Fig. 5A and 5B). No significant differences were found when comparing ATP signals from aged muscles to signals from young muscles.

Our previous study has also demonstrated that MF59 co-injection with apyrase, an enzyme that rapidly hydrolyzes ATP to AMP, could quench the induced extracellular ATP signal and, as a consequence, partially abolish the induction of innate and adaptive immune response by MF59 [4]. Accordingly, we compared the effect of apyrase inhibition on MF59-induced ATP release in aged and young mice assessing CD4$^+$ Th and antibody responses. Groups of mice were immunized with TIV, either as plain antigens or together with MF59 in presence or absence of apyrase. We found that in aged mice as well as in young mice, the enhanced T cell response induced by MF59-adjuvanted vaccine was completely abolished by co-injection of apyrase (Fig. 5C). In addition, young and aged mice immunized with TIV in presence of MF59+apyrase showed significantly decreased HI antibody titers compared to mice receiving TIV+MF59 (Fig.5D). Importantly, whereas in young mice apyrase co-injection did not bring HI antibody titers to the level observed in mice immunized with unadjuvanted vaccine, in aged mice apyrase co-injection completely reduced HI titers to the same level observed in aged mice immunized with TIV alone (Fig. 5D). This last finding suggests first that in young mice MF59 activates more than one pathway since inhibition by apyrase of ATP-dependent pathway does not completely abrogate the whole adjuvant effect, and second that in aged mice ATP release is the only pathway still functioning induced by MF59, thereby contributing to its efficient adjuvant effect.
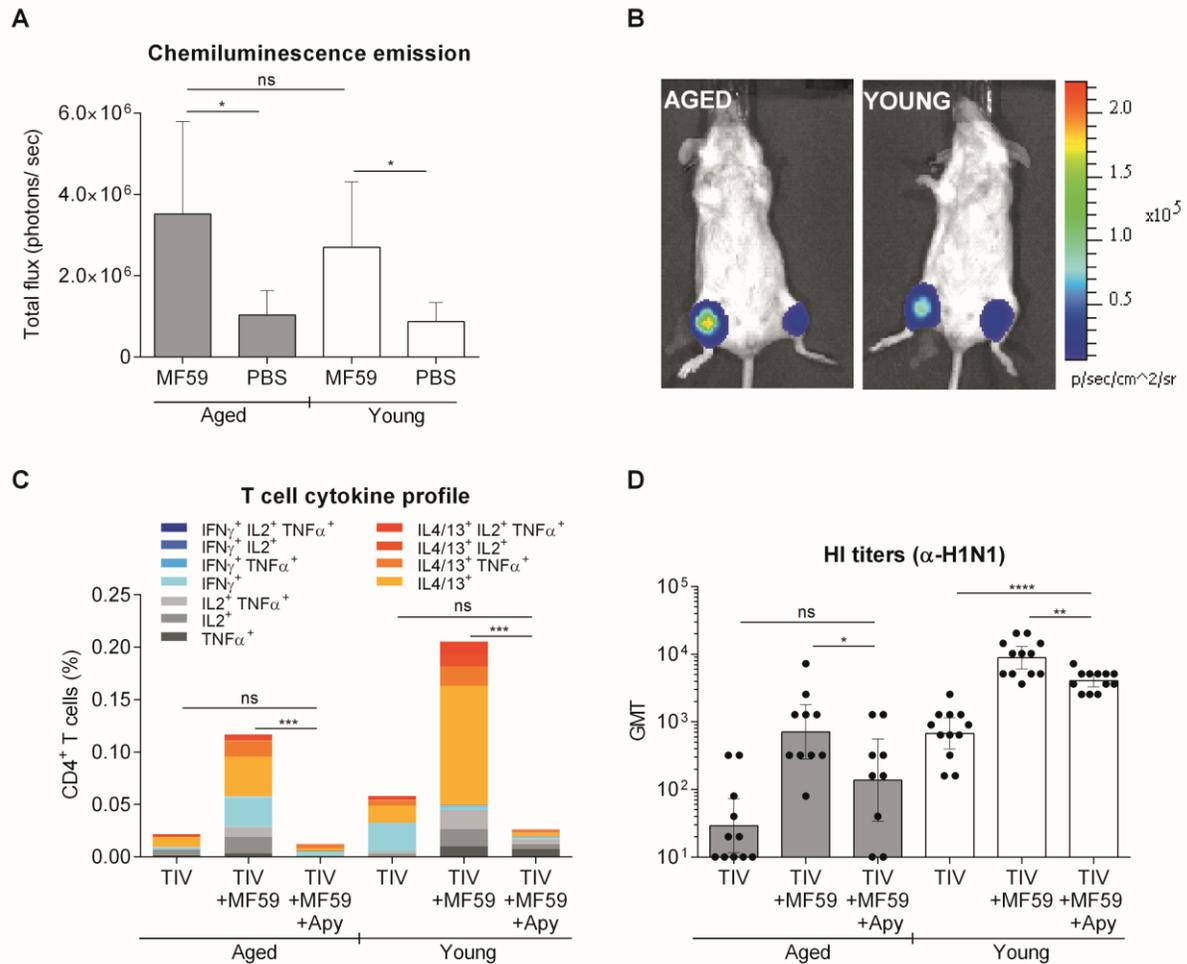
Figure 5 - MF59 similarly induces ATP release in muscle of young and aged mice and this contributes to its adjuvanticity.

(A-B) ATP release in aged and young mouse muscles induced by injection of MF59. (A) Quantitative analysis of chemiluminescence emission over time (number of photons/sec in the region of interest) obtained after intramuscular injection of MF59 or PBS together with the mixture luciferase-luciferin that reports on ATP changes. Data show mean values ± SD from at least four independent experiments. (B) Representative images taken 3 min after intramuscular injection of MF59 (right hind limb) or PBS (left hind limb). (C-D) Co-injection of apyrase abrogates the adjuvant effect of MF59 on vaccine-specific Th cells and HI antibody response. (C) Mice were immunized twice (4 weeks apart) with TIV and MF59 in presence or absence of apyrase (10 U/ leg). Spleens were taken 2 weeks after the second immunization and vaccine-specific CD4$^+$ Th cells were re-activated by *in vitro* stimulation. Their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. The bars show cumulative numbers of vaccine-specific cytokine expressing cells, while the individual color code indicates the type of cytokines expressed by the respective cells. (D) Mice were vaccinated as before. Serum samples were drawn two weeks after the second immunization and TIV-specific HI titers towards H1N1/ California were measured; values represent means of Log2 titers of 8 mice per group ± s.d. Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant

DISCUSSION

Although the potent adjuvant effect of MF59 in increasing the efficacy of influenza vaccine in the elderly (>65 years) population is well established, the molecular mechanisms triggered by this well-characterized oil-in-water emulsion have not been yet examined in this population. In the current study, we compared aged mice (> 18 months) and young mice in most of the relevant assays for MF59 activity and found that in aged mice the release of endogenous ATP at the site of injection induced by MF59 is well conserved and plays a crucial contribution to its adjuvant activity. The effect of apyrase in reduction of MF59-boosted HI titers was even more evident in aged mice compared to young mice, indicating that in this population, ATP might be the most important contributor involved in MF59 adjuvanticity.

Likewise downstream pathways such as induction of cytokines in the injection site resulting in infiltration of a multitude of different immune cells and efficient antigen uptake were not significantly different in aged and young mice.

Instead we could demonstrate that numbers of antigen-loaded cDCs in dLNs of immunized mice were significantly impaired in aged mice. Since this was the case both in plain vaccinated mice and in mice receiving MF59-adjuvanted formulations, we conclude that most likely intrinsic impairment of efficient cDC activation and/ or migration are the cause. DCs are pivotal for initiation and regulation of innate and adaptive immune responses and constitute the key cell type to present antigen to naïve T cells.

Similar findings of DC impairment due to aging were published by a group assessing influenza infection in young and aged mice [160, 360]. The authors could show that NLRP3 inflammasome is impaired in elderly mice. Accordingly IL-1β secretion in response to influenza infection is affected. IL-1β is one of the key inflammatory cytokines and important for immune activation events like DC activation and function [360, 361]. That impaired DC activation in aged mice was key for an efficient response against influenza infection could further be demonstrated by Stout-Delgado and colleagues, by adoptive transfer experiments of DCs from young to aged mice and vice versa [160].

Whether solely IL-1β- or additional other cytokine-impairments during aging impact on proper DC function is unknown. Fundamental changes in the cytokine network are caused by and during aging. In fact chronic inflammation during senescence was termed "inflammaging" [362, 363]. This should cause also considerable imbalances in the response towards adjuvant injection since cytokines and chemokines are thought to play a fundamental role especially for MF59 adjuvanticity [310, 314-316]. In fact it was speculated that MF59 acted primarily via the induction of a "chemokine-driven immune amplification

loop" [314]. This principle was also termed "reverse targeting" [364], nicely illustrating that instead of targeting antigens to immune cells, MF59 acts by recruiting immune cells towards the injected adjuvant and co-injected antigens. Altered cytokine- and chemokine-networks due to aging should impact also MF59-induced responses. As it happens we found that serum cytokines were reduced in aged mice.

A multitude of additional MF59-induced cytokines can be identified assessing muscle-derived mRNA by microarray [297, 315, 351]. Such analyses have proven to be a powerful tool to assess adjuvant induced transcriptional changes in the injection site. We are currently performing microarray studies to further dissect changes caused by aging. Comparing untreated muscles of aged and young mice as well as MF59-injected muscles should allow us to draw conclusions on eventual imbalances in basal level cytokines and adjuvant-stimulated upregulation and secretion.

Future studies will have to show whether all observed immune deficiencies – cytokine imbalances, cDCs activation or migration and GC B cells and isotype switch – are all caused by inflammaging or whether differential factors contribute. Yet, some important immune activation pathways like the "alarmin" ATP release are fully intact also in aged mice. Since extracellular ATP contributes considerably to MF59-induced cytokine release, "reverse targeting" and efficient induction of Th cells [4] this might explain the proven success of this vaccine adjuvant especially in elderly populations.

ACKNOWLEDGEMENTS

Supplementary Figure 1 - (A-C) Hemagglutination inhibition (HI) titers towards H3N2/ Perth (A) and B/ Brisbane (B) and PR8 (C); values represent GMT of 12 mice per group with 95% CI. (D) Kinetic of viral loads in lung supernatants of young mice following influenza infection; the curve represents GMT as $TCID_{50}$/ mouse with 95% CI. Mann-Whitney test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns = not significant
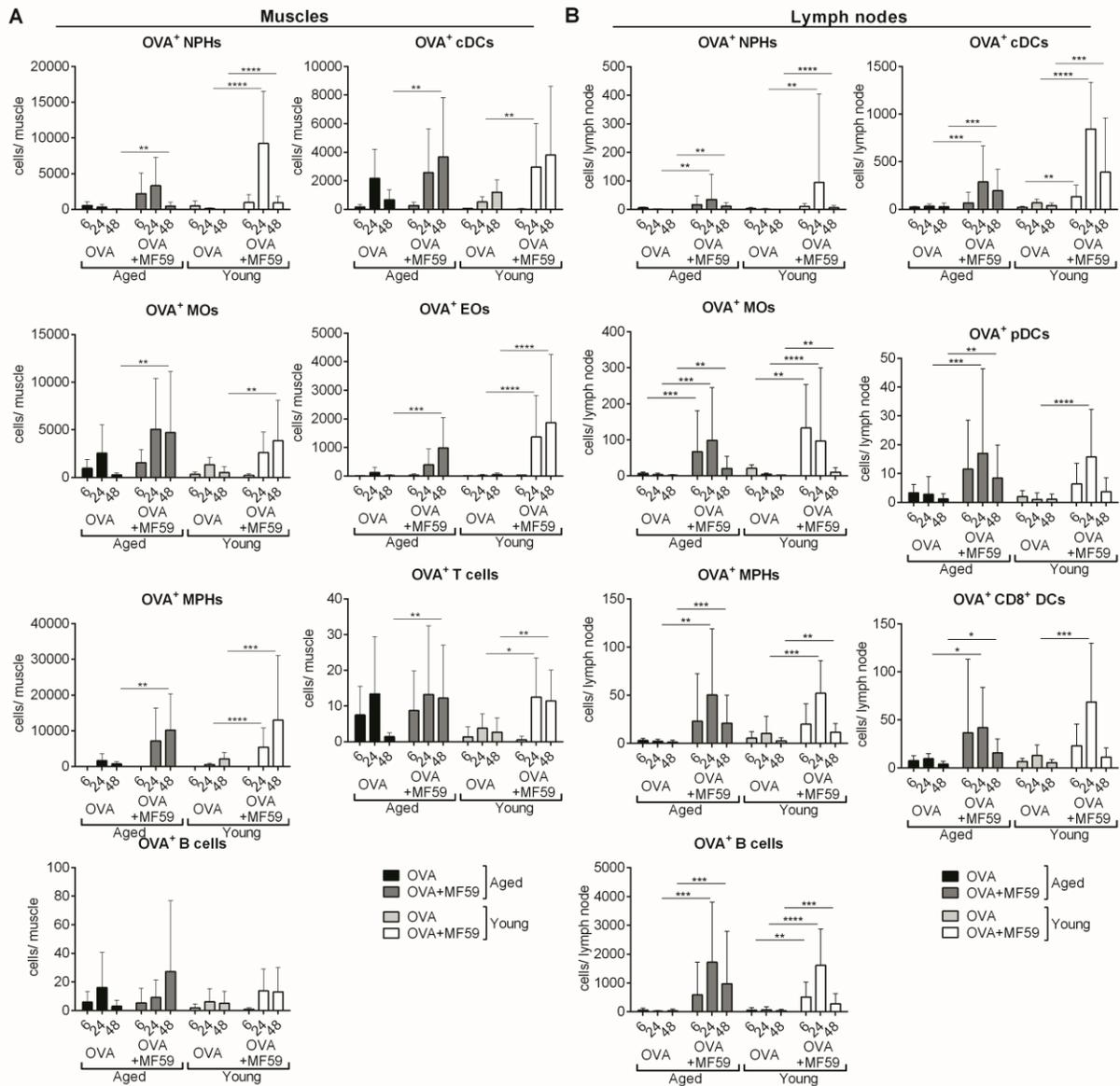
Supplementary Figure 2 - Antigen-positive cell recruitment into the muscle and into the dLNs in response to MF59 injection in aged and young mice.

Eight mice per group were injected i.m. into both legs with 50 µl of OVA-AF647 (10 µg/ mouse) either unadjuvanted or together with MF59 (1:1, vol:vol, in PBS). Cell composition of mucle-derived (A) or dLNs-derived (B) OVA- positive cells was analyzed by FACS at three different time points (6h, 24h and 48h post injection). Values show the means of 8 mice per group. Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

Positive contribution of adjuvanted influenza vaccines on the resolution of viral-bacterial co-infections.

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Keywords: influenza, bacterial superinfection, vaccine adjuvants

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ABSTRACT

Background

Most pre-clinical studies assess vaccine effectiveness in specific-pathogen-free animals in controlled environments. This is unrealistic given that humans are continuously exposed to different commensals and pathogens in sequential and mixed infections. Accordingly, complications from secondary bacterial infection are a leading cause of influenza-associated morbidity and mortality. New vaccination strategies are needed to control infections on simultaneous fronts.

Methods

We compared different anti-influenza vaccines for their protective potential in a viral-bacterial co-infection model. Mice were immunized with H1N1/A/California/7/2009 subunit vaccines, formulated with different adjuvants inducing either Th1- [MF59+CpG], Th2- [MF59] or Th17- [LTK63] prone immune responses, and were sequentially challenged with mouse-adapted influenza virus H1N1/A/Puerto Rico/8/1934 and *Staphylococcus aureus* USA300, a clonotype emerging as a leading contributor in post-influenza pneumonia in humans.

Results

Unadjuvanted vaccine controlled viral infection, yet mice had considerable morbidity from viral disease and bacterial superinfection. In contrast, all adjuvanted vaccines efficiently protected mice both from viral disease and bacterial co-infection but with different efficiency: Th1 > Th1/2 = Th17.

Conclusions

Our studies should help to better understand how differential immunity to influenza skews immune responses towards co-infecting bacteria, and moreover to discover novel modes to prevent bacterial super-infections in the lungs of people suffering from influenza.

INTRODUCTION

Seasonal and pandemic influenza is still one of the major causes for mortality and morbidity worldwide [1, 365]. Symptoms can vary from mild disease to death depending on strain virulence, host immune status and environmental factors. Often, complications are caused by superinfection of otherwise harmless respiratory bacteria that get the upper hand once the immune system is debilitated by influenza infection [2, 366]. Notably, bacterial infections maybe a primary cause of mortality associated with influenza infection in the absence of pre-existing comorbidity [187, 367]. This phenomenon was most dramatically observed during the 1918 Spanish Flu that killed more people than the First World War. In fact, already at the time, it was stated that: "if influenza condemns, additional infection executes" [32, 368].

The multifactorial interplay between host status, viral and bacterial factors has been assessed in mouse models [183, 193, 203]. Though it is well established that influenza infection enhances susceptibility to secondary bacterial infections by enhancing bacterial adhesion on epithelial cells [200, 205] and impairing host responses [214, 217, 218, 369, 370], it is less clear to what extent an ongoing immune response towards influenza impacts on the type of immune response against co-colonizing bacteria. Would different flavours of immune responses, such as individual T helper (Th) profiles, specific cytokine environments and immune cell compositions, have a beneficial or detrimental impact on bacterial disease?

All these factors can be modulated by vaccination against influenza with different adjuvants able to skew Th-profiles. Accordingly, we evaluated adjuvants MF59 (Th1/2), MF59+CpG (Th1) [6] or LTK63 (Th17) [7] for their capacity to modulate disease resolution of mixed viral-bacterial infections.

The development of novel vaccines that protect not only from vaccine-matched influenza infection, but induce broader-spectrum memory responses with adequate Th profiles to exert positive bystander effects on eventual bacterial co-infections could help control infections on simultaneous fronts.

MATERIAL AND METHODS

Vaccines

Purified, egg-derived H1N1/A/California/7/2009 (H1N1/ Cal) influenza subunit antigen was concentrated through the KrosFlo research II Complete TFF System (Spectrum Laboratories) as described elsewhere [371].

Oil-in-water emulsion adjuvant MF59 was prepared as described before [316]. CpG 1826 oligonucleotide (5'-TCC ATG ACG TTC CTG ACG TT-3') with all phosphothioate backbones was purchased by Primm. LTK63, the non-toxic mutant of LT, was produced in our laboratories as reported earlier [372].

Mice that were pre-exposed (p.e.) to a sublethal dose (2.5 10^{-2} tissue-infectious-dose-50 ($TCID_{50}$)) of mouse-adapted influenza H1N1/A/Puerto Rico/8/1934 (PR8) virus were used as positive controls in challenge studies.

Mice and Immunizations

Groups of 6 to 8-weeks-old female pathogen-free BALB/c mice (Charles River) were used in agreement with institutional and European guidelines. All animal experiments were approved by the Italian Health Ministry and carried out in accordance with Italian legislation (Legislative Decree 116/92). Animals were immunized three times sublingually (s.l.) with 30 µl (H1N1 (10 µg) + LTK63 (5 µg)), or twice intramuscularly (i.m.) with 50 µl in each quadriceps muscle (1 µg H1N1 administered either as plain vaccine, or together with MF59 (1:1, vol:vol), or with MF59 (1:1, vol:vol) + CpG (10 µg)) four weeks apart (days 1 (only s.l.), 28 and 56).

Virus challenge

Mice were anesthetized and challenged intranasally (i.n.) with 30 µl (15 µl/ nostril) of 2.1 $TCID_{50}$ of PR8 virus three weeks after the final vaccination. After viral challenge, mice were monitored for 16 days for weight loss and euthanized if humane endpoints (= 25% weight loss) were reached.

Serum and bronchoalveolar lavage (BAL) sample collection

Serum samples were collected two weeks after each immunization or at different time-points post infection (p.i.).

BAL samples were collected at different time-points p.i. by injecting 1.5 ml PBS and 0.1% BSA intratracheally.

Determination of vaccine- and Infection-specific antibodies by ELISA, microneutralization (MN) and hemagglutination inhibition assay (HI) as well as CD4$^+$ T cell responses

Vaccine- and Infection-induced immune responses were determined as described in Supplementary Materials or elsewhere [7, 373].

Determination of cytokine concentration in lung homogenates

At indicated time-points p.i., six mice per group were sacrificed and lungs harvested. Organs were homogenized in a buffer containing tissue protein extraction reagent (T-PER, Pierce) and complete protease inhibitor cocktail (Roche) using a gentleMACS^{TM} dissociator (Miltenyi Biotec) according to manufacturer's instruction. Protein concentrations in lung homogenates were determined using BCA Protein Assay kit (Pierce). Cytokine analyses were performed using the Luminex Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay (Biorad) according to the manufacturer's protocol.

Cell recruitment into lungs

Lung cell composition was determined by FACS as described in Supplementary Materials or elsewhere [373].

Bacterial challenge

Staphylococcus aureus (*S. aureus*) Lac (USA300) Sm^R (streptomycin resistant) bacteria were cultured at 37°C and 250 rpm in tryptic soy broth supplemented with streptomycin (500 μg/ ml) until an optic density of A_{600} = 2, corresponding to a concentration of 10^9 CFU/ ml. Bacteria were harvested by centrifugation, washed and suspended in PBS for mouse infection. Animals were anesthetized and challenged i.n. with 10^7 CFU of *S. aureus* in a volume of 30 μl (15 μl/ nostril) at day 6 after influenza infection. Infected mice were monitored daily for 10 days as described for single virus challenge.

Determination of viral titers and bacterial CFU in lungs

Viral titers in homogenized lung samples were determined by TCID$_{50}$ assay performed on MDCK cells by ten-fold serial dilutions of supernatant samples as described [352], yet using Ultra-MDCK-Medium (Lonza) supplemented with 1% PSG (Life Technologies) and 1 ug/ ml TPCK-treated trypsin (Sigma-Aldrich). Bacterial CFU were measured via plating of serial dilutions of lung homogenates on tryptic soy agar plates supplemented with streptomycin (500 μg/ ml).

Statistical analysis

All statistics were performed using GraphPad Prism software (version 6.0) using Mantel-Cox test for survival or Mann-Whitney test for the other read-outs. p values < 0.05 were considered significant.

RESULTS

Immune profiles induced by different adjuvants and administration routes

Influenza infection is controlled by protective neutralizing antibodies and by cytotoxic CD8^{+} T cells [26]. Yet, CD4^{+} T helper (Th) cells have been also implicated in protection via various pathways [102]. Accordingly, we characterized the individual immune profiles induced by the different vaccine formulations. Figure 1 shows that immunization with plain influenza antigens (Ag) induced moderate vaccine-specific antibody titers. In contrast, Ag formulated with MF59 with or without CpG induced about one log higher total IgG (Fig. 1A) and functional, hemagglutination-inhibition (HI) titers (Fig. 1B). Also sublingual administration of influenza Ag formulated with LTK63 induced measurable total and functional antibody titers. Yet, these were significantly lower than the MF59±CpG-induced ones, and were similar to not-adjuvanted vaccine. As expected, each vaccine adjuvant induced a different Th profile in the spleen (Fig 1C). All adjuvants enhanced Th responses as compared to plain vaccine antigens. Yet, the individual profiles of intracellular cytokines were dramatically different: MF59 led to a mixed Th1/Th2 profile, MF59+CpG to more Th1-prone responses and LTK63 to Th17 cells.
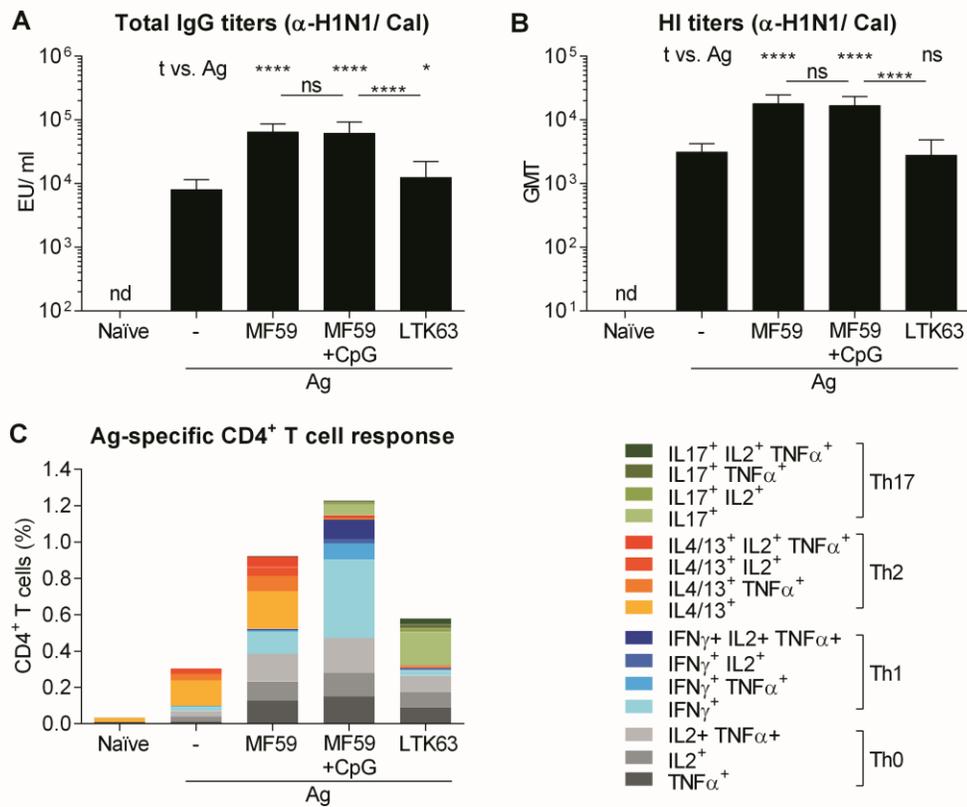
Figure 1 - Profiles of different vaccine-induced adaptive immune responses.
Mice (15/ group) were immunized twice intramuscularly (i.m.) or three time sublingually (s.l.) 4 wk apart with Ag (antigen: H1N1/A/California/7/2009 subunit, H1N1/ Cal) alone or combined with adjuvants, as indicated: MF59 (1:1 vol:vol), MF59 (1:1 vol:vol) + CpG (10 μg), LTK63 (5 μg) and Ag (0.1 μg for i.m and 10 μg for s.l. immunization). (A–B) Serum samples were obtained 2 wk after the last immunization, and Ag-specific antibody titers were measured. (A) Total IgG antibody titers towards H1N1/ Cal. Values represent geometric mean titers (GMT) as ELISA Units/ ml (EU/ ml) with 95% CI. (B) Hemagglutination inhibition (HI) GMT titers towards H1N1/ Cal with 95% CI. Mann-Whitney test (t): *$p < 0.05$, ****$p < 0.0001$, ns = not significant, nd = not detected. (C) Spleens from 3 mice per group were taken 3 wk after last immunization and Ag-specific CD4^{+} Th cells were reactivated by *in vitro* stimulation. Their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. Cumulative data from two independent experiments are shown as numbers of vaccine-specific cytokine expressing cells and the individual colour code indicates the type of cytokines expressed by the respective cells reported as mean CD4^{+} T cells (%).

Potential of vaccine formulations to protect from a lethal, heterologous influenza challenge

Next, we assessed the potential of the individual vaccine formulations to protect from a lethal influenza challenge. Three weeks after the last immunization, mice were challenged with mouse-adapted H1N1/A/PR8/1934 (PR8) influenza virus and monitored for 16 days for body weight and viral titers. In naïve mice excessive weight loss (Fig. 2A) and mortality (Fig. 2B) were indicative of ongoing disease for up to nine days. During this period virus replicated rapidly for three days, then immune responses succeeded in controlling the infection (Suppl. Fig. 1). In contrast, weight loss in vaccinated mice was much milder (Fig. 2A), survival rate was greatly increased (Fig. 2B), and viral replication in lungs was contained significantly earlier (Fig. 2C). Interestingly, all adjuvanted formulations were significantly better as compared to antigen alone. This effect was especially evident at day 6 post infection (p.i.). Only mice that received influenza Ag with MF59+CpG controlled the infection early on (Fig. 2C) and showed less than 10% weight loss (Fig. 2A). This is little surprising since this formulation induced a Th1 profile (Fig. 1C) that should be the most suitable immune response towards a viral infection [27].
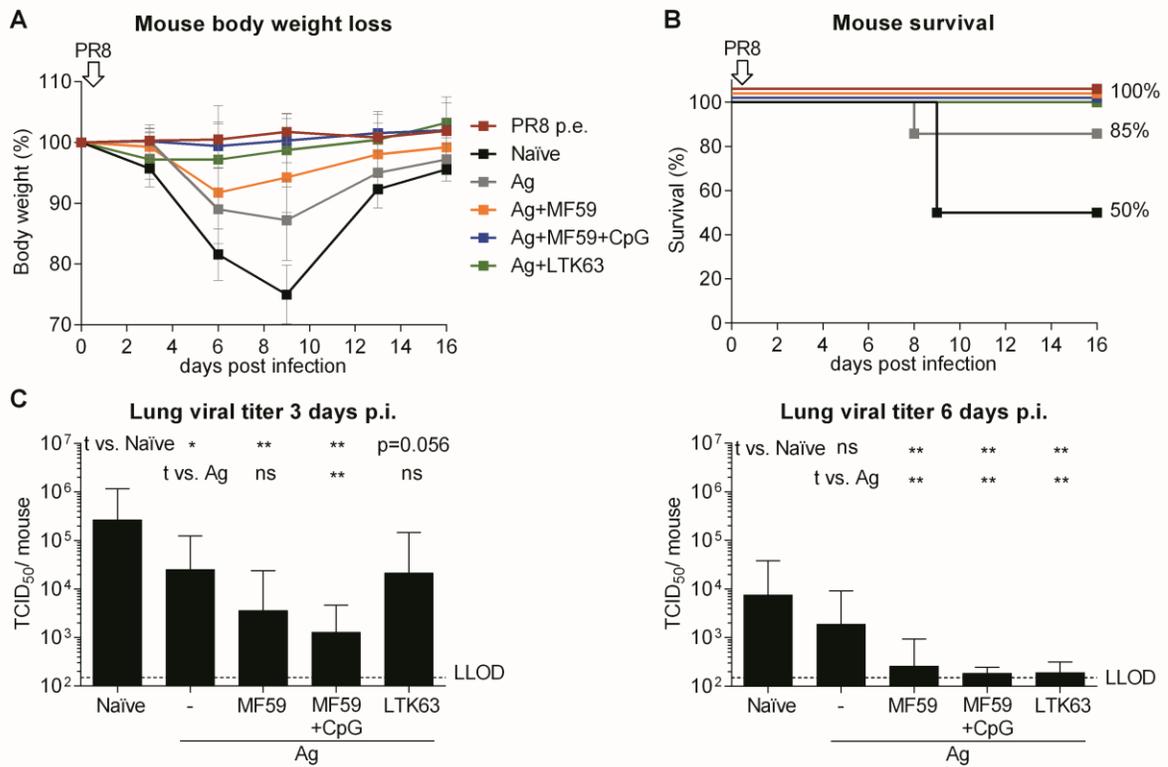
Figure 2 - Protective efficacy of different vaccine formulations after heterologous influenza challenge
(A-B) Mice (10/ group) were immunized as described in Fig.1. Three wk after the last immunization, mice were challenged intranasally (i.n.) with 2.1 TCID50 of influenza virus PR8. Mice that were previously pre-exposed (p.e.) to a sublethal dose influenza challenge were used as positive control. Animals (6/ group) were monitored for body weight loss for 16 days post influenza infection (p.i.) and euthanized if humane endpoints were reached. The empty arrows indicate the day of influenza (PR8) challenge. (A) Body weight loss of influenza infected mice. Curves represent mean percentage (%) of mouse weights at indicated days p.i. compared to initial body weights ± SD. (B) Survival curve of respective mice. Final survival rate of each group of mice is reported at the end of individual curve. (C) Mice were immunized and infected with influenza as reported before. Lungs were taken three and six days p.i. and viral titers were assessed. Cumulative data from two independent experiments are shown, each with 3 mice/ group and time-point. Values represent GMT as TCID50/ mouse with 95% CI. Mann-Whitney test (t): *p < 0.05, **p < 0.01, ns = not significant. LLOD = lower limit of detection.

To further characterize immune responses after challenge, we sacrificed mice at different time-points p.i. and assessed cell composition of dissociated lung tissue by FACS analysis. Single cells were stained with a large panel of cell type-specific antibodies in order to identify most of the infiltrating cell types (gating strategy in Suppl. Fig. 2).

As shown in Figure 3A+B and Supplementary Figures S3+S4, naïve mice and those received plain antigens had a high infiltrate of a variety of immune cells in their lungs. Cell influx peaked between days 6 and 9 p.i., and especially inflammatory cells like neutrophils, monocytes and monocyte-derived macrophages were strongly enhanced (Fig. 3B and Suppl. Fig. 3+4). In contrast, all mice that received adjuvanted vaccine formulations, independently from the type of adjuvant, had a significantly reduced infiltrate as compared to naïve or plain-vaccinated mice. This was also evident for the overall infiltrate as well as for individual inflammatory cell types (Fig. 3A+B and Suppl. Fig. 3+4).
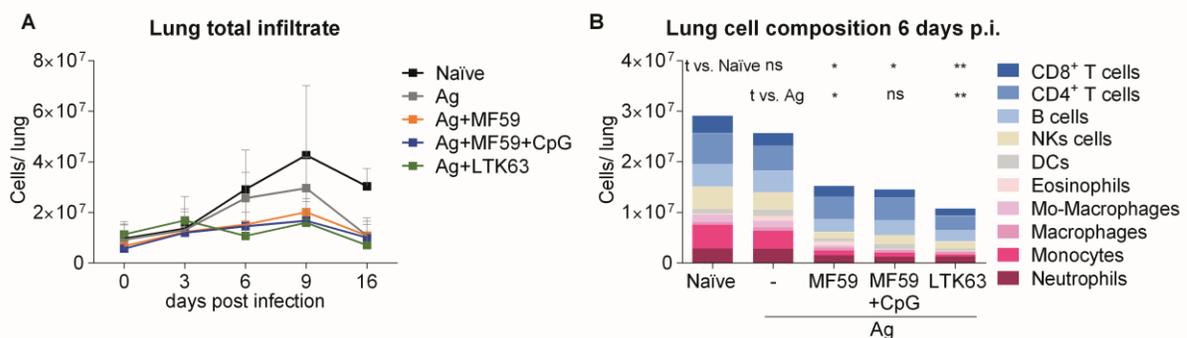
Figure 3. Lung immune cell recruitment during influenza challenge.
Mice were immunized and infected with PR8 virus as before. Lungs were taken at indicated time-points p.i. and total number of cells infiltrated was determined. Immune cell composition was assessed by cell surface marker staining and FACS analysis. Cumulative data from two independent experiments are shown, each with 3 mice/ group and time-point. (A) The curve represents mean of total cell counts/ lung + SD. (B) The bars show cumulative numbers of cell types at 6 days p.i. normalized to total cell count in the lungs. The individual colour code indicates mean numbers of respective cell types. Mann-Whitney test (t): *p < 0.05, **p < 0.01, ns = not significant.

Interestingly, mice that received s.l. immunization with LTK63 showed almost no weight loss and very little inflammatory lung infiltrate, even though vaccine-induced total and functional HI antibodies were significantly lower than those of the other adjuvanted groups (Fig. 1 A+B). HI titers are a correlate of protection for influenza infection during homologous challenge [28, 29]. Yet, antigenically drifted viruses may appear during the annual influenza season and this usually leads to reduced efficacy of recommended vaccines [30, 31]. In our model, mice were immunized with antigens from H1N1/A/California/7/2009 virus but challenged with the closely related but heterologous mouse-adapted PR8.

In order to dissect the mechanism(s) of protection, we tested serum of naïve and vaccinated mice for the capability to cross-react with (Fig. 4A) and eventually block PR8 viral infection in vitro in a micro-neutralization (MN) test (Fig. 4B). Cross-protective MN titers were not detected in any of the groups during the first six days p.i., though cross-reactive antibodies were measured. Neutralizing antibodies were detected in all groups from day 9 onwards likely due to the onset of adaptive immunity against virus-infection. We further tested the presence of cross-reactive IgA in BAL samples at different time-points during the infection (Fig. 4C). Only mice that received influenza antigen with LTK63 via the mucosal route had detectable IgA before the infection that cross-reacted with the challenge virus PR8.
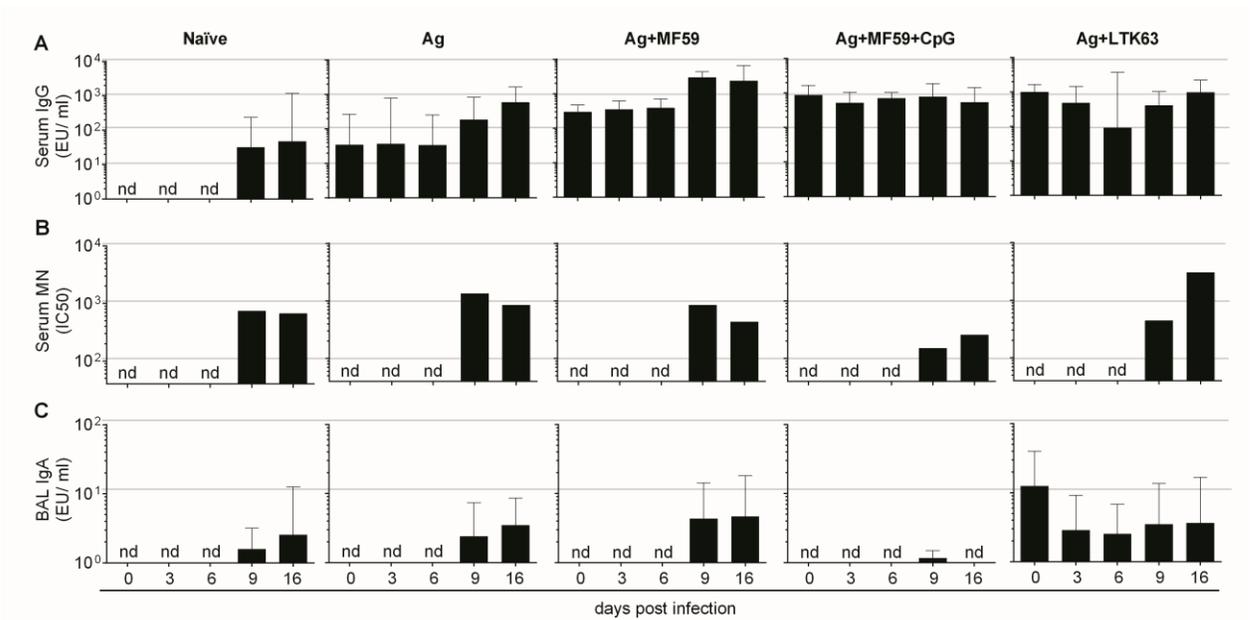
Figure 4 - PR8-specific serological and mucosal humoral response.

Mice were immunized and infected with influenza PR8 virus as reported before. Cumulative data from two independent experiments are shown, each with 3 mice/ group and time-point. Serum and (bronchoalveolar lavages) BAL samples were obtained at the indicated days p.i., and PR8-specific antibody titers were measured. (A) Total IgG antibody titers in serum. Values represent GMT as EU/ ml with 95% CI. (B) PR8 virus micro-neutralization (MN) antibody titers in serum. Values represent titers of pooled samples that correspond to a 50% inhibition of infection (IC50). (C) PR8-specific IgA titers in BALs. Values represent GMT as EU/ ml with 95% CI. nd = not detected

Polarized T helper cells are recruited into the lungs upon influenza infection

Next, we tested the quantity and quality of vaccine-specific Th cells that are recruited to the site of infection (Fig. 5). Single cell suspensions of dissociated lungs were prepared at different time-points p.i., and Ag-specific CD4$^+$ Th cells were identified as cytokine-secreting cells by intracellular staining and FACS analysis after *in vitro* stimulation (gating strategy in Suppl. Fig. 5). Few vaccine-specific Th cells were found prior to virus challenge (Fig. 5A). Only mice receiving LTK63-adjuvanted vaccine had measurable Th responses with a characteristic Th17 profile.

Numbers of Th cells in lung infiltrates increased during the course of infection in all treatment groups, peaking between days 6-9 p.i. As expected, the characteristic Th profiles induced by the different vaccine formulations were also maintained in Th cells infiltrating infected lungs: a Th1/Th2 profile for MF59, Th1-prone responses for MF59+CpG and Th17 responses for LTK63. This was further confirmed measuring the respective indicator cytokines IFNγ (Th1), IL-5 (Th2) and IL-17 (Th17) in lung homogenates via multiplex-bead ELISA (Fig. 5B). Whether vaccine-induced Th cells contributed to viral clearance via secretion of immune-activating cytokines and/ or via direct effector functions still remains to be determined.

Altogether, this data suggest that the different vaccine formulations may induce protective immunity through the differential contribution of cytokine-secreting Th cells, functional antibodies in blood and/ or at mucosal sites, and eventually other mechanisms.
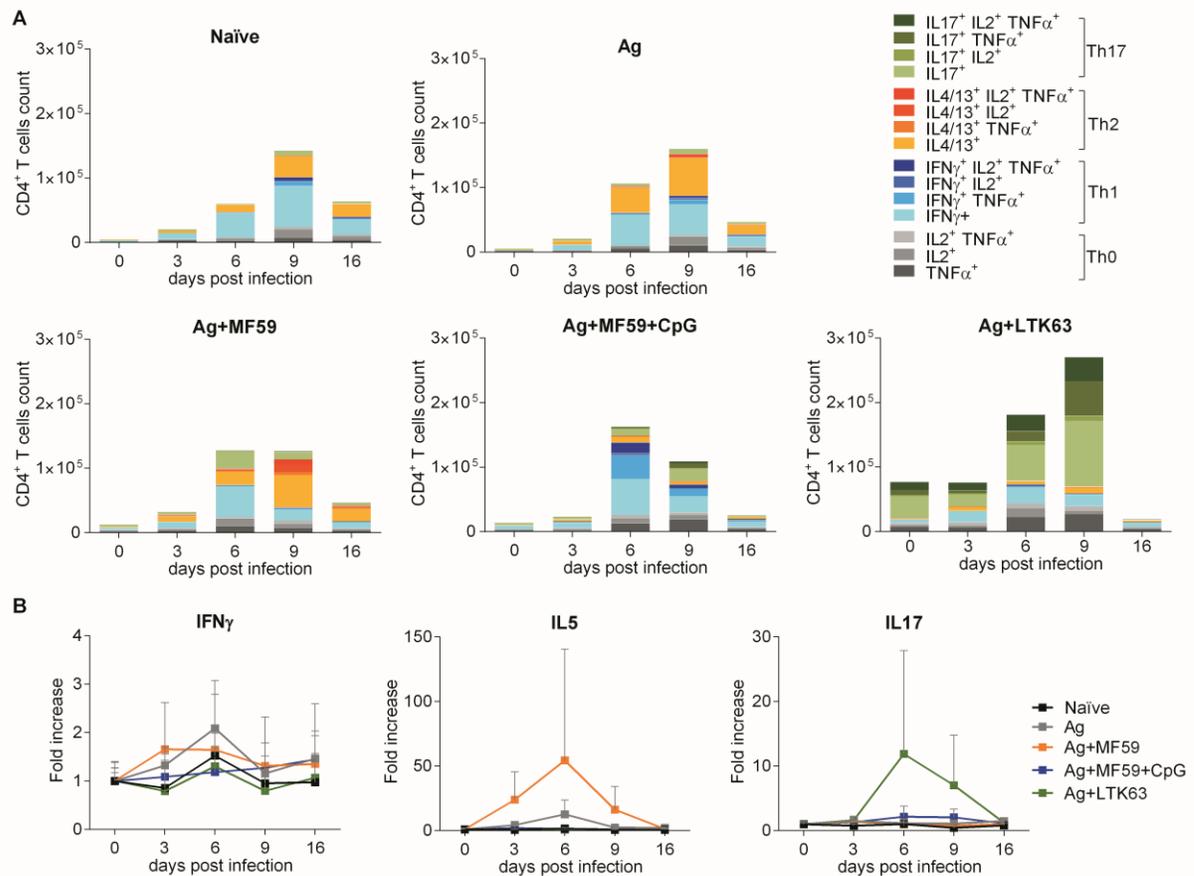
Figure 5 - Each vaccine formulation induces characteristic Th-polarized immune responses in the lungs.
Mice were immunized and challenged with PR8 virus as before. Lungs were taken at indicated day p.i and Ag-specific CD4$^+$ Th cell response (A) or total cytokine concentration (B) were evaluated. Cumulative data from two independent experiments are shown, each with 3 mice/ group and time-point. (A) Ag-specific CD4$^+$ Th cells were stimulated *in vitro* and their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. The bars show cumulative numbers of Ag-specific cytokine expressing cells normalized to total cell counts in the lungs. The individual colour code indicates the type of cytokines expressed by the respective cells. (B) Cytokine concentration in lung homogenates. The curves represent mean cytokine concentration as fold increase compared to pre-infection concentration + SD.

Bystander effects of adjuvanted influenza vaccines on viral-bacterial co-infection

Finally, we characterized the performance of the different vaccine formulations in a viral-bacterial co-infection model that more realistically reflects the conditions encountered in real life.

Mice immunized with different influenza vaccines were challenged with PR8 like before, and were subsequently challenged on day 6 p.i. with 10^7 CFU of *Staphylococcus aureus*. Day 6 post-influenza infection was chosen for various reasons: (i) the overall timeframe is in line with actual influenza infection and bacterial superinfection [193], (ii) mice of the three different adjuvant groups had similar inflammatory lung infiltrates (Fig. 3B), similar low viral titers (Fig. 2C), and similar quantity of Ag-specific Th cells in infected lungs (Fig. 5A). Yet, the quality of Th profiles (Fig. 5A) and the respective cytokine environment in lungs (Fig. 5B) were fundamentally different and peaked on day 6 p.i.

Since it is believed that different Th profiles have differential effectiveness against different pathogens (Th1 for virus and other intracellular pathogens, Th2 for extracellular bacteria and parasites, and Th17 for intracellular bacteria), we were intrigued to know whether the respective cytokine environment would have a positive or negative impact on super-infecting bacteria.

Mice infected just with *S. aureus* had only minor weight loss and no mortality during the following days without any statistical difference between the different treatment groups (Suppl. Fig. 6A+B). In contrast, co-infected mice had enhanced morbidity and mortality (Fig. 6A+B) as compared to single influenza infection (Fig. 2A+B). In particular, naïve mice and those vaccinated with plain antigens showed a dramatic weight loss and less than 30% survival after influenza-*S. aureus* co-infection. In contrast, mice receiving adjuvanted influenza vaccines were mostly protected from severe weight loss and showed 65-100% survival rates. As expected, Th1-prone mice (Ag+MF59+CpG), that were better protected during single influenza infection, better counteracted also the secondary bacterial challenge as compared to the other adjuvanted formulations.

On the day of co-infection, naïve and plain vaccinated mice had similar body weight loss (Fig. 6A) and similar viral titers in lungs (Fig. 6C). Yet, while plain vaccinated mice continued to control viral load with or without bacterial co-infection and had lower viral titers in the lungs on day 7 p.i. than on the previous day, naïve mice showed the classical rebound of viral titers observed during bacterial superinfections [2, 9]. And, similarly to exacerbation of viral disease by co-infecting bacteria, also bacterial loads were greatly enhanced by viral pre-infection (Fig. 6D). While comparable bacterial counts were obtained from lungs of all groups of mice during single bacterial infection (Fig. 6D, solid bars), bacterial loads were significantly enhanced in naïve and plain vaccinated mice in the co-infection model (Fig. 6D, striped bars).

In contrast, all mice receiving adjuvanted vaccines controlled viral loads to low levels by the day of the co-infection (Fig. 6C). Importantly, bacterial counts in lungs were also efficiently controlled as compared to naïve mice and those receiving not-adjuvanted vaccine (Fig. 6D). As expected from previous results, the overall outcome was best for mice that received vaccines with MF59+CpG via systemic vaccination. Mice vaccinated i.m. with MF59- or s.l. with LTK63-adjuvanted vaccine had slightly greater weight loss (Fig. 6A), lower survival rate (Fig. 6B) and some *S. aureus* overgrowth after co-infection (Fig. 6D).

Altogether our results indicate that - while all adjuvanted influenza vaccines clearly contribute to resolution of viral-bacterial co-infections - Th1 inducers would be the most appropriate. Future studies will have to show whether differences are due to differentially induced Th profiles and cytokine environment at the site of infection or whether other mechanisms like induction of differential antibody isotypes or ADCC are involved.
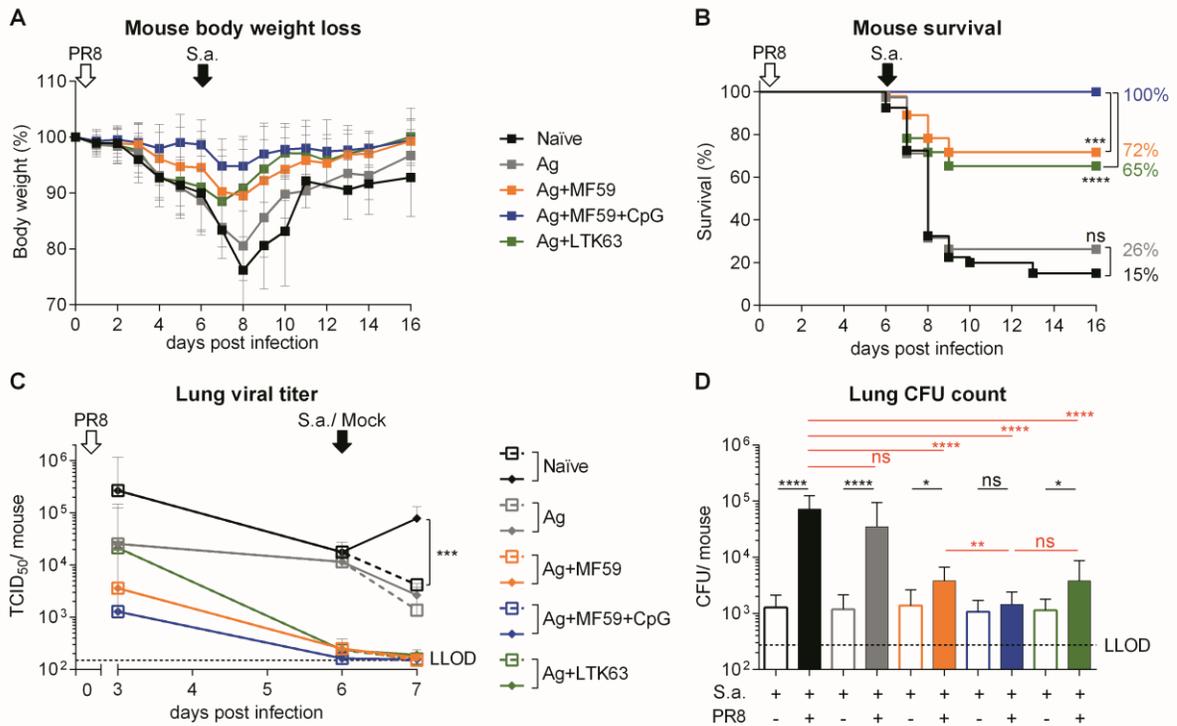
Figure 6 - Bystander effects of adjuvanted influenza vaccines during viral-bacterial co-infection

At least twelve mice per group and experiment were immunized and challenged with PR8 virus as before. Six days p.i. mice were co-infected i.n. with 10^7 CFU of *S. aureus* Lac USA300. Vaccinated mice that were infected with virus or bacteria only were used as controls. Cumulative data from five independent experiments are shown. The empty arrows indicate the day of influenza (PR8) challenge, while the solid arrows indicate *S. aureus* (S.a.) infection. (A-B) All animals were monitored for body weight loss for 16 days post influenza infection and euthanized if humane endpoints were reached. (A) Body weight loss of influenza-*S. aureus* co-infected mice. Curves represent mean percentage (%) of mouse weights at indicated days p.i. compared to initial body weights ± SD. (B) Survival curve of the respective mice. Final survival rate of each group of mice is reported at the end of respective curve. (C) Lungs from 3-6 mice per group and experiment were taken at indicated days p.i. and viral titers were assessed. The curves represent GMT as $TCID_{50}$/ mouse with 95% CI. Starting from 6 days p.i. dashed lines represent mice infected only with influenza while solid lines are used for influenza-*S. aureus* co-infected (co-inf) mice. (D) Lungs from 3-6 mice per group and experiment were taken 24 h after *S. aureus* infection and CFU counts were assessed. Values represent geometric mean counts as CFU/ mouse with 95% CI. Mantel-Cox (B) and Mann-Whitney (C-D) tests: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant. LLOD = lower limit of detection.

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DISCUSSION

The upper respiratory tract is exposed to numerous pathogens simultaneously, and viral-bacterial co-infection in the lung is a common clinical manifestation. Only recently, the complex interplay of different commensals and pathogens with the host immune system and its impact on immune-maturation or -suppression is getting full attention [2, 374, 375].

Complications from secondary bacterial infection are a leading cause of morbidity and mortality associated with influenza virus infection. The situation is becoming more challenging due to extensive and sometimes inappropriate use of antibiotics that leads to the emergence of antibiotic-resistant bacteria. Hence, infectious diseases remain an important unmet medical need and new concepts of vaccination strategies or treatments to control infections on simultaneous fronts are needed.

Accordingly, we assessed the impact of different influenza vaccination strategies in a mouse model of viral-bacterial co-infection.

Influenza infection is controlled mostly by protective neutralizing antibodies and by antiviral effector T cells [26, 102]. Upon vaccination or infection, subjects develop innate and adaptive immune responses clearing the virus. Strain-specific antibody and memory responses are induced which are directed against major viral epitopes (hemagglutinin and neuraminidase), and exert selective pressure on circulating influenza strains. Virus mutations drive antigenic drifts and shifts to achieve immune evasion and necessitate updating of seasonal influenza vaccines regularly in order to match strains. The selective pressure against many pathogens is higher on B cell epitopes (direct immune targets via antibody binding to matching epitope) than on T cell epitopes (indirect immune targets via immune activation) [376].

Conventional influenza vaccination strategies aim to induce high functional antibody titers that are considered a correlate of vaccine efficacy [259, 377]. While this is certainly true against homologous virus strains, the situation could be different when infections are caused by circulating strains that do not match with the antigens contained in the seasonal vaccine.

During vaccine development, little attention is usually paid to the concomitant induction of CD4$^+$ T cell responses and other potential mechanisms of protection. In our model, HI titers alone were not predictive of vaccine efficacy, since plain vaccinated mice and those receiving antigens together with the mucosal adjuvant LTK63 had similar vaccine-specific HI titers, but strikingly differential morbidity and mortality after challenge with a heterologous influenza strain or in mixed viral-bacterial co-infection. Though HI titers of LTK63-immunized animals were about one log lower than titers from the other

adjuvanted groups, they were similarly protected from weight loss, inflammatory infiltrate, and viral loads, while plain vaccinated mice performed considerably worse in all settings. An explanation might be the induction of higher CD4$^+$ Th cell responses by LTK63, the altered Th profile, the additional induction of mucosal IgA antibodies or other factors. Most likely the different factors contribute to varying content to protection.

Successful vaccination with influenza vaccines via the sublingual route could path the development of novel needle-free vaccines [7]. Such vaccines would have the advantage of being self-administrable, avoid needle-phobia and pain and could therefore enhance vaccine uptake considerably.

Most importantly, mice receiving LTK63-adjuvanted vaccine sublingually were equally protected from bacterial overgrowth in mixed infections as MF59-adjuvated vaccinated mice. MF59-adjuvanted vaccines were specifically developed for elderly populations and are currently considered – together with similar adjuvanted influenza vaccines – as the gold standard of influenza vaccines for protection of immune compromised subjects, of elderly and infant populations [3, 286, 310]. Notably aged people (>65 years) are also the ones most susceptible to influenza-induced bacterial superinfections [158]. It would be interesting to apply our influenza+*S. aureus* co-infection model to aged mice and eventually identify the proper anti-influenza vaccination strategy specific for this target population to counteract the two pathogens together.

We showed that all adjuvants are efficient inducers of Th responses. CD4$^+$ T cells have been implied in prediction of vaccine effectiveness and memory Th cells are believed to contribute to protection against influenza challenge via various pathways [102, 349]. As indicated by their name, Th cells provide crucial help to B and CD8$^+$ T cells. They are important for the fast induction of protective antibodies and provide stimulatory signals that determine differential isotype switch. In BALB/c mice Th1 cells induce preferentially a switch towards IgG2a/b, Th2 cells IgG1 and Th17 cells additionally IgA. But besides their helper function, Th cells might play also more direct roles via immune activation due to their release of effector cytokines at the site of infection coordinating an antiviral state in infected tissues or by acting as cytotoxic CD4$^+$ T cells.

Th1 cells are usually considered to be the most important in the defence against virus and intracellular bacterial pathogens; Th2 cells were implied in protection from parasites and extracellular bacteria; and Th17 cells are important players at mucosal sites, against fungal pathogens and intracellular bacteria. It could be expected that the most efficient immune response against an influenza-bacterium mixed infection would be constituted by a mixed Th1/ Th17 response.

In line with expectations, Th1-inducing (MF59+CpG) vaccine formulation performed even better than the other adjuvanted vaccines. Mice that experienced more Th1-polarized immunity were better protected from prior influenza disease and consequently they efficiently counteracted secondary bacterial infection.

Future experiments will have to show, whether these differences translate into measurable greater vaccine effectiveness. Nevertheless, our work contributes with a new approach to the quest of the development of better influenza vaccines in order to avoid regular updating of seasonal influenza vaccines to match circulating seasonal and eventual pandemic strains. Universal flu vaccines that induce broadly cross-protective antibodies are certainly a promising strategy [378]. But also the contribution of adequate vaccine adjuvants for the induction of the most adequate immune profiles should be key.

The development of novel vaccines that protect not only from vaccine-matched influenza infection, but induce broader-spectrum memory responses with adequate Th profiles to exert positive bystander effects on eventual bacterial co-infections could help to control infections on simultaneous fronts.

ACKNOWLEDGMENTS

SUPPLEMENTARY FIGURES
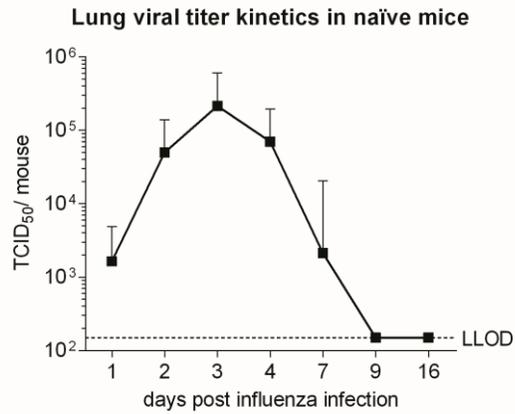
Supplementary Figure 1 - Lung viral titer kinetics in naïve mice.

Forty-two mice were challenged i.n. with 2.1 $TCID_{50}$ of influenza PR8 virus. Lungs from 6 mice were taken at indicated days p.i. and viral titers were assessed. The curve represents GMT as $TCID_{50}$/ mouse with 95% CI. LLOD = lower limit of detection.
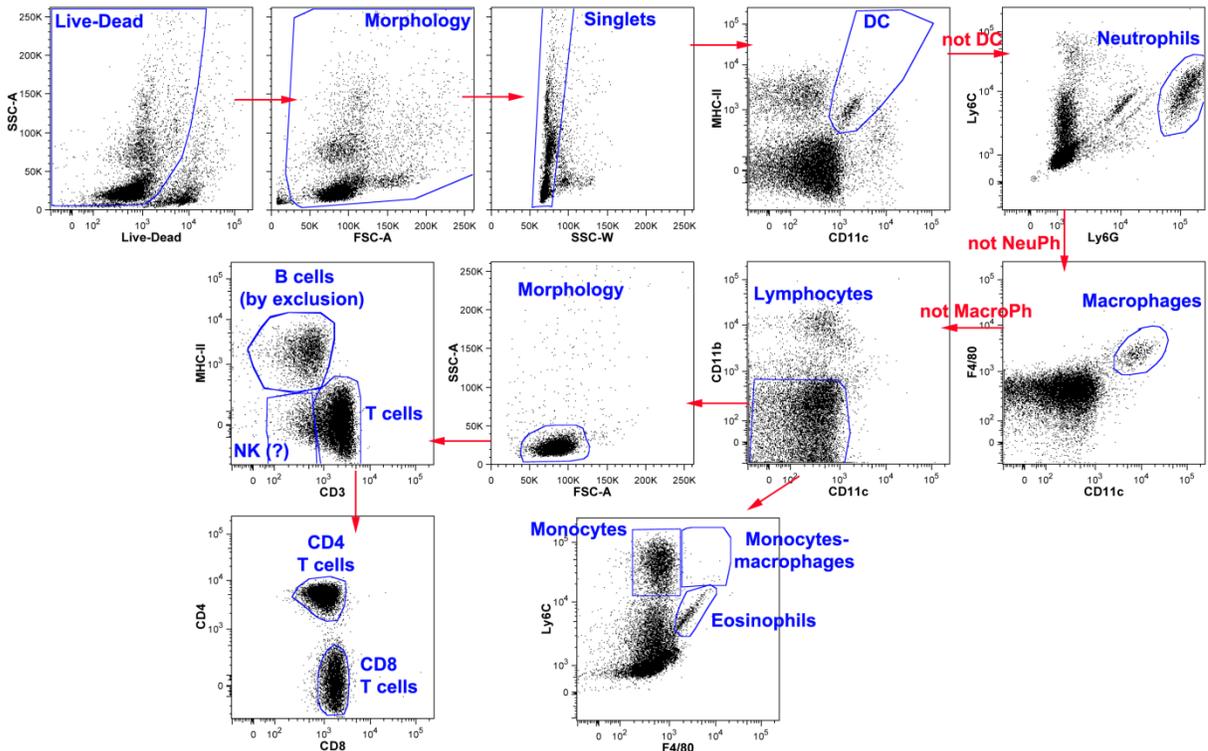
Supplementary Figure 2 - Gating strategy: lung immune cell recruitment.

Gating strategy of lung-derived cells. Lung single cell suspensions were prepared and analyzed by FACS, applying the depicted gating strategy.
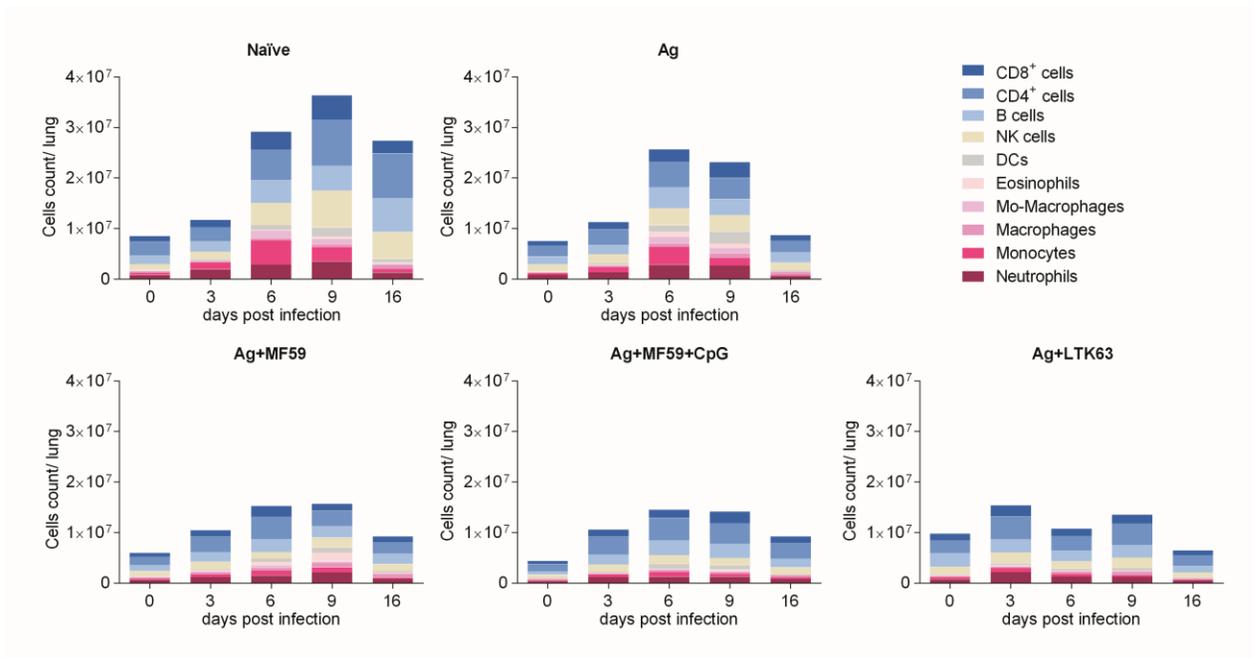
Supplementary Figure 3 - Lung immune cell recruitment kinetics.

Mice were immunized with Ag and adjuvants as indicated and challenged with influenza virus PR8. Lungs from 3 mice per group were taken at indicated days p.i and immune cell composition was determined by cell surface markers staining and FACS analysis. Cumulative data from two independent experiments are shown as numbers of cell types normalized to total cell counts in the lungs and the individual colour code indicates mean numbers of specific cell type.
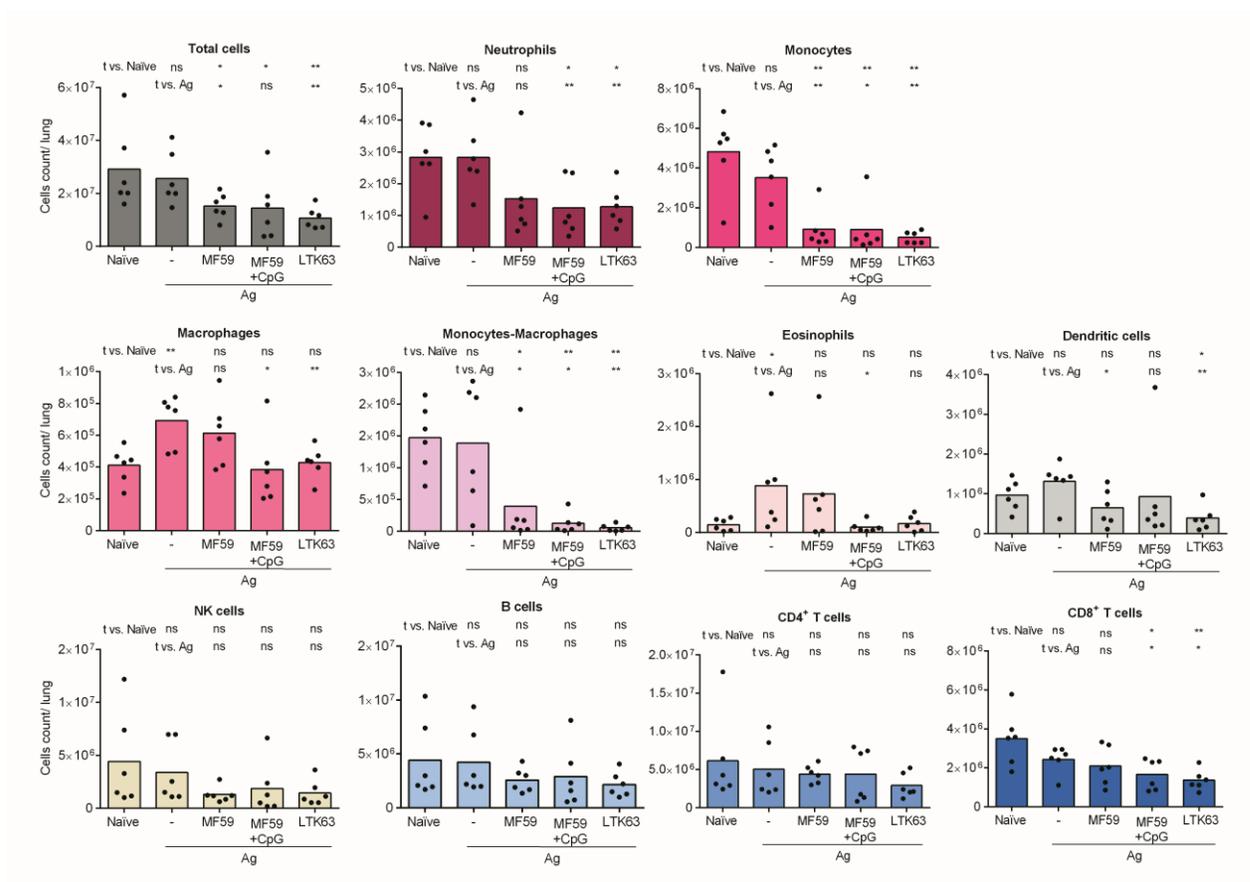
Supplementary Figure 4 - Lung immune cell recruitment: single cell types at 6 days p.i.
Mice were immunized and challenged PR8 virus as before. Single-cell suspensions of lungs were analyzed by FACS 6 days p.i. Cumulative data from two independent experiments are shown, each with 3 mice/ group and time-point. Dots show numbers of the respective cell type per individual lung (n=6 per group), whereas bars indicate means. Mann-Whitney test (t): *p < 0.05, **p < 0.01, ns = not significant.
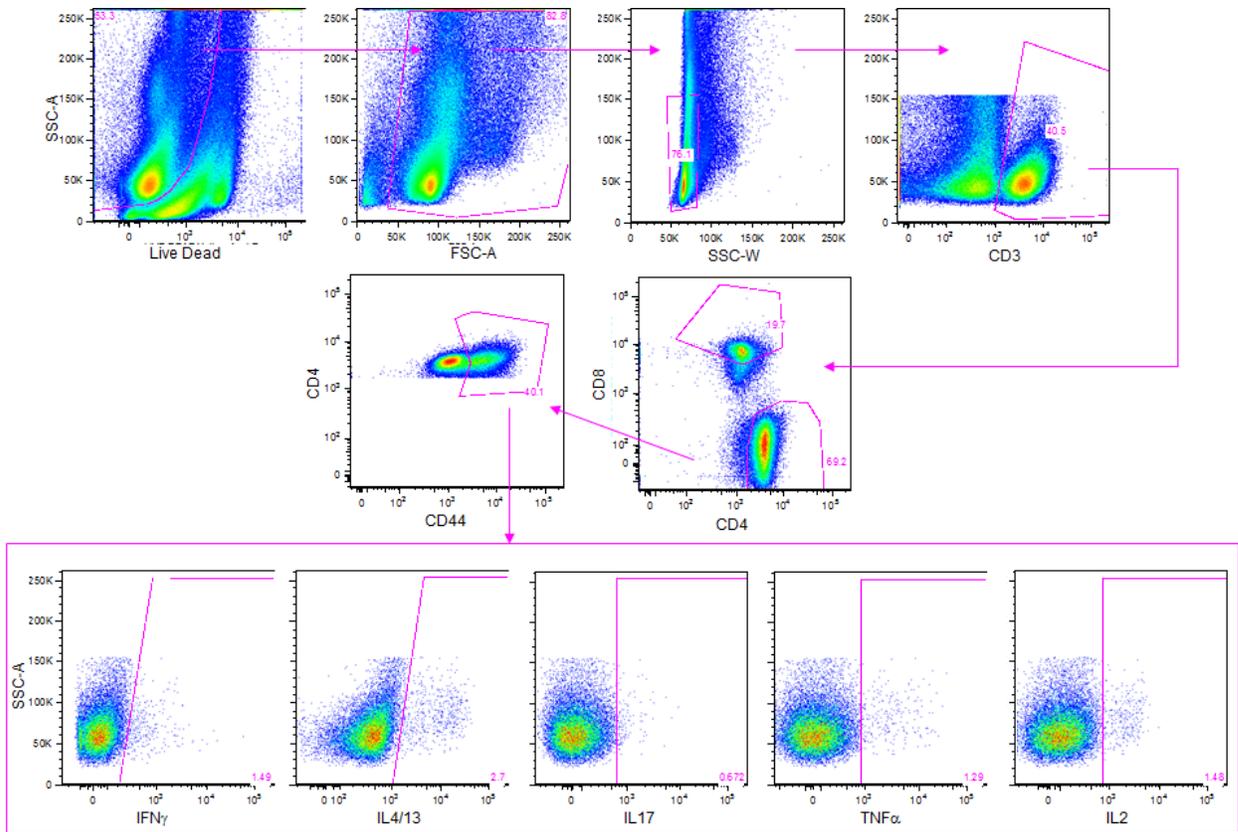
Supplementary Figure 5 - Gating strategy: lung Th cell response.

Gating strategy of lung Th cell responses. Lung single cell suspensions were prepared, Ag-specific cells were stimulated *in vitro* and analyzed by FACS, applying the depicted gating strategy.
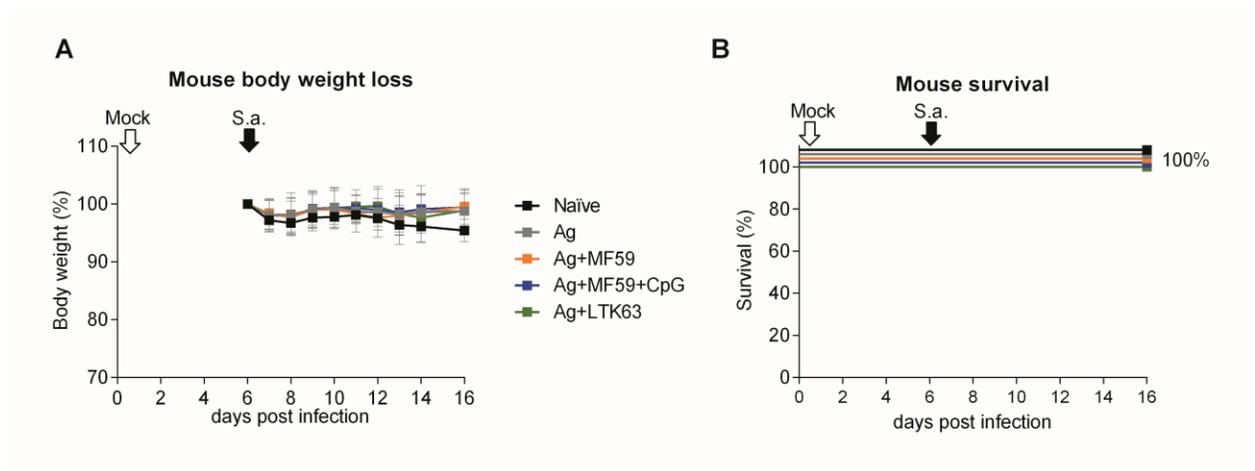
Supplementary Figure 6 - Mouse weight loss and survival during single *S. aureus* infection.

At least twelve mice per group and experiment were immunized as reported before and infected i.n. with 10^7 CFU of *S. aureus* Lac USA300 at the corresponding time of infection for viral/ bacterial co-infected mice. All animals were monitored for body weight loss for 10 days after bacterial infection and euthanized if humane endpoints were reached. The empty arrows indicate the day of Mock infection, while the solid arrows indicate *S. aureus* (S.a.) infection. Cumulative data from five independent experiments are shown. (A) Body weight loss of *S. aureus* infected mice. Curves represent mean percentage (%) of mouse weights at indicated days p.i. compared to initial body weights ± SD. (B) Survival curve of respective mice.

SUPPLEMENTARY MATERIAL AND METHODS

Determination of antigen-specific antibody titers by ELISA

Titration of H1N1/ Cal or PR8-specific IgG was performed on individual sera while titration of specific IgA was performed on individual BAL. Polysorp plates (Nunc) were coated overnight at 4°C with 2.6 µg/ml of H1N1 antigen in PBS and blocked with SmartBlock (Candor) for 1 h at 37°C. Plates were washed three times with PBS 0.05% Tween 20 and, for IgG titration, incubated for 1 h at 37°C with individual mouse sera serially diluted 2-fold in PBS 1% BSA 0.01% Tween 20. These plates were washed and incubated for 1.5 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). For IgA titration, blocked plates were incubated for 20 min at 37°C with individual mouse BAL serially diluted 2-fold (starting dilution 1:10) in PBS 1% BSA 0.05% Tween 20 and alkaline phosphatase-conjugated goat anti-mouse IgA. All plates were washed and P-nitrophenyl phosphate disodium was added. The reaction was stopped adding a solution of 3% EDTA pH 8, and absorbance measured with SpectraMax (Molecular Devices) reader at 405 nm.

Antibody titers are the dilutions that gave an optical density (OD) higher than mean + 5 SD of the average OD obtained with pre-immune sera. The titers were normalized by using a reference serum assayed in parallel and reported as ELISA Units/ml (EU/ml).

Determination of antibodies by hemagglutination inhibition assay (HI)

To remove non-specific inhibitors, serum samples were pre-treated with DENKA receptor-destroying enzyme (Biogenetics) for 18 h at 37°C according to the manufacturer's instructions and then inactivated at 56°C for 30 min. HI assay was then performed on individual sera. Briefly, 25 µl of two-fold serially diluted samples were incubated with 25 µl of strain-specific influenza antigen (Whole virus, containing four hemagglutinating units) for 1 h at room temperature. 50 µl turkey red blood cells suspension was dispensed in each well and plates were again incubated at room temperature for another 1 h. Reactions were followed through visual inspection: a red dot formation indicated a positive reaction (inhibition) and a diffuse patch of cells a negative reaction (hemagglutination). All sera were run in duplicate. The titer was defined as the serum dilution at which the last complete agglutination inhibition occurred. The antibody concentration corresponds to the reciprocal value of the titer.

Neutralization assay

Neutralization activities in serum samples from immunized mice were detected using a microneutralization assay (MN). Pooled sera were inactivated at 56°C for 30 min and then serially

diluted 3-fold in minimal essential medium (MEM, Gibco) with Penicillin, Streptomycin and Glutamine (PSG, Life Technologies), Trypsin 1X 1:250 and incubated for 1 h at 37°C with 300 TCID50 of mouse adapted influenza A/Puerto Rico/8/1934 (H1N1) virus. The first dilution tested was 1:80. All samples were then transferred on MDCK cells, plated in a 96-well plate (20,000 cells/ well). The final volume was 200 µl/ well. Each sample was incubated for 18 h at 37°C in 5% CO2. Cells were then washed with PBS, fixed with Fixation Buffer (BD Cytofix) and permeabilized with a solution of PBS 0.1% BSA 0.1% Tween 20. The expression of viral proteins was detected by ELISA with a monoclonal antibody against matrix and nucleoprotein (α-M/NP-FITC) conjugate with fluorescein isothiocyanate (Oxoid) followed by an anti-FITC polyclonal antibody conjugated with horse-radish peroxidase (HRP) (Roche). O-phenylenediamine dihydrochloride (Sigma) was used as substrate and the absorbance recorded at 450 nm using SpectraMax (Molecular Devices) reader. Inhibition of infection of 50% was determined by a 4-parameters fitting curve (SoftMaxPro) and the corresponding titers represented as the reciprocal of the dilution. All sera were run in duplicate.

In vitro stimulation of antigen-specific CD4+ T cells

Mice were challenged i.n. with PR8 virus three weeks after the last immunization as described before. At the indicated time points, three mice per group were sacrificed and spleens (only day 0) and lungs were collected to assess frequency and phenotype of Ag-specific CD4+ T cells induced by vaccination and infection. Single-cell suspensions were obtained, red blood cells lysed by RBC lysis buffer (Biolegend) and the cells were cultured in RPMI (Gibco) containing 10% FCS (HyClone), beta-mercaptoethanol and antibiotics (PSG 1%). Splenocytes were stimulated in the presence of anti-CD28 antibody (1 µg/ ml) (BD Biosciences) and antigen H1N1 (0.3 µg/mL), or with anti-CD28 alone (negative control, <0.1% total cytokine-positive cells), or with anti-CD28 plus anti-CD3 (0.1 µg/ ml) (BD Biosciences; positive control). After overnight stimulation at 37°C in 5% CO2, Brefeldin A (2.5 µg/ml; Sigma–Aldrich) was added for additional 4 h. Lung derived cells were stimulated for 4 h at 37°C in 5% CO2 in the presence of anti-CD28 and 2 µg/ ml of PepMix HA Influenza-A (H1N1) California (JPT) in medium with Brefeldin A. Same negative and positive controls described before were used. Cells were washed and stained with LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen). Cells were fixed, permeabilized, and stained with the following antibodies: anti-CD4-V500, anti-CD3-PerCp-Cy5.5, anti-CD44-Pacific Blue, anti-TNF⬚-AlexaFluor700, anti-IFN⬚-PE and anti-IL2-APC (all BD Biosciences); anti-CD8-PE-Taxas Red (Invitrogen); anti-IL4-FITC, anti-IL13-FITC and anti-IL17-PE-Cy7 (all e-Biosciences). Cells were acquired on a LSR-II SORP (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Cell recruitment into the lungs

Mice were challenged i.n. with PR8 virus three weeks after the last immunization as described before. At the indicated time points, three mice per group were sacrificed and lungs were harvested and processed using the gentleMACSTM dissociator (Miltenyi Biotec). To facilitate tissue disruption, cells were enzymatically digested with 2 mg/ ml type D collagenase (Roche) and 40 μg/ ml DNase I (Roche) in HBSS medium (Gibco) for 30 min at 37°C in 5% CO_2. An aliquot of cell suspension was taken and temporally stored at 4°C for bacterial titration. The cell suspension was then centrifuged and an aliquot of supernatant was taken and stored at -80°C for viral titer analysis. Cells in the pellet were suspended in PBS 10 mM EDTA and filtered through a 70 μm nylon Cell Strainer (BD Biosciences). Red blood cells were lysed by RBC lysis buffer (Biolegend). White blood cells were washed with PBS and stained with LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen). Cells were fixed and stained with the following antibodies: anti-Ly6C-FITC, anti-CD11b-PE-Cy7, anti-Ly6G-PE, anti-CD11c-APC, anti-CD3-PerCp-Cy5.5, anti-CD8-V500 (all BD Pharmingen) and anti-MHCII-AlexaFluor700, anti-F4/80-eFluor450, anti-CD4-APC-Cy7 (all eBiosciences). Cells were acquired on a LSR-II SORP and analyzed by BD FACSDiva software (BD Biosciences).

DISCUSSION

Influenza is still an important source of disease in humans and secondary bacterial infections (SBI) associated with cases of influenza are a leading cause of severe morbidity and mortality, especially among high-risk groups such as the elderly and young children. Vaccines, antiviral and antibiotic therapies are now readily available to separately control and prevent influenza or bacterial infections. Yet, pathogen resistances to antibiotic and antiviral treatments are increasing worldwide. And influenza virus is continuously subjected to antigenic drift and therefore heterologous viral strains may appear and spread during annual influenza season reducing anti-influenza vaccination success [275]. Taking into account that humans are usually colonized by a multitude of commensal or pathogenic organisms at the same time, novel approaches of interventions, such as adjuvanted influenza vaccines with a broader spectrum or even more general "respiratory disease vaccines", are needed. With the current work we set to contribute knowledge on important parameters determining success or failure of such novel interventions like immune status of the host, host-pathogen interactions and even more complex viral-bacterial co-infections.

Influenza A H1N1 and H3N2 and influenza B are most circulating viral strains up to now and trivalent influenza vaccines (TIV) are produced every year to fight against the seasonal epidemics induced by these viruses. Current vaccines consist mainly in live-attenuated virus, in inactivated split or subunit vaccines. Traditional vaccines aim at the induction of hemagglutinin (HA)-specific neutralizing antibodies which are not cross-protective [70, 260]. Moreover these vaccines exhibit a lower immunogenicity and efficacy in those age groups with a greater need to be vaccinated against influenza, i.e. young children and the elderly [266, 379, 380]. A possible strategy to improve vaccines effectiveness is to formulate split or subunit TIV with appropriate adjuvants. These compounds are able to enhance the immune response elicited by an antigen and generally exert their effect by improving antigen delivery or targeting specific immune pathways to improve the immunogenicity of vaccines [276-278]. Currently licensed adjuvants for influenza vaccine usage are the squalene oil-in-water emulsion systems MF59 (Novartis) and AS03 (GlaxoSmithKline) [3, 286, 310, 346]. AS03 has been used in conjunction with monovalent preparations of inactivated 2009 pandemic H1N1 and pre-pandemic H5N1 virus vaccines [292, 293], while MF59 has been licensed for use with seasonal vaccines in the elderly in some countries, as well as for pre-pandemic and pandemic vaccines [3]. Importantly, MF59-adjuvanted vaccines are proven to induce a stronger immune response in the elderly (>65 years old) [290, 299, 300] and have double the efficacy of

unadjuvanted formulations in young children [288, 381]. Besides boosting antibody titres, MF59 adjuvant has also been shown to broaden the immune recognition repertoire in H5 [280, 281, 303, 304] and seasonal TIV vaccines [305-307], generating cross-reactive antibodies as well as stimulating both humoral and cellular immunities. The latter capacity is especially interesting, since it has been demonstrated in humans and animal models that natural influenza infection confers protection not only against homologous but also heterologous virus strains through CD4$^+$ and CD8$^+$ T cell mediated immunity [382-386]. Therefore the induction of cross-reactive T cell response may be a promising approach for the development of more broadly protective vaccines.

In this thesis we first of all deeply investigated the mechanism of action of MF59 in aged mice: this pre-clinical model fits better the human target population of MF59-adjuvanted vaccines. We then assessed whether influenza vaccines could be further improved. To that end we evaluated the impact of MF59 and two other experimental adjuvants on subunit anti-influenza vaccines on protection of mice from heterologous influenza challenge and SBI.

In the latter study we showed that the three adjuvanted-immunization strategies used to vaccinate mice (antigens (Ag)+MF59±CpG and Ag+LTK63) were able to differently skew the adaptive immunity towards Th1- or Th2- or Th17-polarized cellular responses and to elicit systemic and/ or mucosal neutralizing antibodies. All these adaptive immune components together with those of innate immunity efficiently counteracted heterologous virus infection. As expected the effectiveness of adjuvanted-vaccines was superior to that of unadjuvanted one and moreover the kinetics of disease evolution and inflammatory responses vary in the differently vaccinated mice. In line with the established correlation between high serological hemagglutination inhibition (HI) titres and vaccine efficacy [70, 260], H1N1/A/California/7/2009 subunit vaccine formulated with MF59 alone or in combination with CpG increased comparable functional antibody titres and consequently mice were well protected from influenza challenge. Yet, virus used in the infection was the heterologous H1N1/A/Puerto Rico/8/1934 (PR8) and the two vaccine formulations conferred slightly different levels of protection. Analysing lung viral titres at early time points, body weight loss, anti-PR8 serum microneutralization (MN) titres and bronchoalveolar lavages (BALs) IgA titres, we observed that Th1-biased mice (Ag+MF59+CpG) had a faster reaction to the infection and were more efficiently protected from disease than those vaccinated with Ag+MF59 (mixed Th1/Th2 response). This should be little surprising since Th1 response is considered the most adapt towards a viral infection [102].

Traditional inactivated vaccines are delivered via systemic routes (usually intramuscularly, i.m.), but mucosal vaccination is becoming an interesting field of research that aim to enhance cross-reactive

immunity and overcome immune defects due to senescence [330, 387, 388]. Live attenuated influenza vaccines (LAIV) are currently used in Russia and since 2003 are also approved by the FDA. They are delivered intranasally (i.n.) and have been shown to induce high level of heterologous protection via long-lasting systemic and mucosal responses [274, 389, 390]. Efficacy of this vaccination strategy has been proven in children 2 to 7 years old [391]. However, because of the inherent risk of immunizing with live viruses, LAIV are not recommended for immunocompromised individuals or people in close contact with these vulnerable populations. Appropriate mucosal subunit vaccines would be the safer option. Gallorini *et al*. have recently shown that sublingual (s.l.) administration of LTK63-adjuvanted influenza vaccine elicits comparable antibody titres to those of i.m. immunization with conventional unadjuvanted influenza vaccine [7]. Furthermore, they found that adjuvanted s.l. vaccination enhances Ag-specific Th17 cells and neutralizing mucosal IgA that are not induced by i.m. immunization. Here we continued the exploration of this interesting vaccination strategy comparing its adjuvanticity and protective efficacy with those of traditional i.m. immunizations formulated as plain antigens or combined with MF59±CpG. As expected, functional HI antibody titres elicited by Ag+LTK63 immunization were comparable to those of unadjuvanted vaccine. Yet, mucosally vaccinated mice were able to efficiently counteract heterologous PR8 challenge similarly to other adjuvanted vaccinated ones; on the contrary unadjuvanted vaccine was not sufficient to control neither lung viral replication nor inflammatory response. Encouraged by these results, we further investigated possible mechanisms of protection elicited by s.l. vaccination. It is known that respiratory tract HA-specific IgA antibodies have functional role in protection against influenza [392]. In our study we showed that only mice immunized with Ag+LTK63 via the mucosal route had detectable levels of IgA in their lungs able to cross-react with the challenge virus PR8 prior the infection. Moreover only these mice showed measurable Ag-specific Th cell in lung (with Th17 profile) before receiving the infection. Starting from these results we can hypothesize that mucosal IgA and/ or Th cell response can account for heterologous protection in s.l. vaccinated mice. It was not within the scope of this study to investigate the specific mechanism of protection of the vaccination strategy. Additional studies would be needed to test our hypothesis and eventually to further validate the efficacy of adjuvanted s.l. vaccination route in conferring protection towards heterologous influenza viruses.

Influenza infection is itself an important medical issue, but it becomes a severe threat for public health if associated with SBI. Post-influenza bacterial pneumonia is a major cause of morbidity and mortality associated with both seasonal and pandemic influenza virus illness. Notably the mechanisms responsible for this viral-bacterial synergy have remained elusive and historically have been attributed to

virus-induced lung tissue damage [200, 393, 394]. However, recent studies on animal models have demonstrated that preceding influenza infection induces a dysfunctional host anti-bacterial innate [212, 214, 218, 219, 221, 370] and adaptive [213, 217, 220] immune response and have identified this mechanism as the major contributor to SBI susceptibility. Vaccination remains fundamental to prevent influenza and bacterial infections especially because antiviral and antimicrobial resistance is increasing [395-397] and many treatments have shown adverse effects on the host [398, 399]. Data from animal models indicate that vaccination against influenza viruses effectively prevent bacterial associated pneumonia [5, 240-242, 400]. An important caution about current influenza vaccines is that partial protection of related strains may not be sufficient to alleviate bacterial complications. In our study we used different vaccine formulations to prime the immune system towards different Th profiles during a heterologous influenza virus infection and we then investigated their impact on SBI caused by *S. aureus* USA300. We found that the weak protection induced by unadjuvanted influenza vaccine was not sufficient to efficiently block bacterial over-growth. Importantly plain vaccinated mice showed a delayed response to heterologous influenza challenge if compared to adjuvanted-vaccinated ones, but they were still able to continue viral clearance even after SBI onset. On the contrary, naïve mice showed a reduced control of viral replication as consequence of bacterial super-infection resulting in a rebound of influenza titers in their lungs the day after *S. aureus* co-infection. Notably, naïve and plain antigens vaccinated mice showed high inflammatory monocytes and neutrophils counts in their lungs at the day of co-infection - i.e. 6 days post-influenza infection (6d p.i.) - which did not help in bacterial clearance but in contrast they seemed to worsen the evolution of SBI. The role of neutrophils in dual influenza-bacterial infection remains controversial: some studies showed that influenza induces a diminished recruitment of neutrophils to the lungs via type I IFN signalling and this correlates with impaired clearance of secondary infecting bacteria [214, 401]; on the contrary other works demonstrated significant neutrophils accumulation in influenza infected lungs which contribute to tissue pathology via neutrophil extracellular traps (NETs) formation and consequent increased susceptibility to SBI at days 6 and 7 p.i. [58, 221, 222]. This might also depend on differential activation status of recruited neutrophils. The flexibility of these innate cells - with responses as different as apoptosis, NETosis, upregulation of MHC-II and antigen presentation - was underappreciated for a long time [402]. It would be interesting to further evaluate the role of neutrophils in our model of mixed influenza-*S. aureus* infection and moreover to phenotypically compare those present in influenza-challenged lungs of naïve and unadjuvanted vaccinated mice.

All adjuvanted immunization strategies applied in our study enhanced Ag-specific Th cell response and mice were significantly better protected than naïve or plain vaccinated animals during heterologous influenza infection and secondary bacterial challenge. Interestingly, mice experiencing Th1-polarized immunity during influenza encounter (Ag+MF59+CpG) were better protected from SBI than mice receiving other adjuvanted vaccine formulations. It would be interesting to deeply investigate if this higher protection is just due to a better control of primary influenza infection or if Th1 polarization of the immunity may have some impact on bacterial pneumonia. It is known that release of IFNγ by Th1 cells activates macrophages and enhances bacterial killing [403, 404]. Similar the release of IL-17 by Th17 cells impacts on recruitment and activation state of neutrophils [404, 405]. The specific vaccine-induced cytokine environment during influenza infection should impact therefor also on co-infecting bacteria.

The essential role of Th responses to control bacterial colonization has also been appreciated in different models of single bacterial infection. Antigen-specific T helper cells confer protection from nasopharyngeal colonization of *Streptococcus pneumoniae* [339] and especially IL-17 secreting Th17 cells have been shown to be important [406]. Similarly changing the flavour of a Th response from Th2 to Th1 by adequate adjuvants has shown to enhance protection from *Bordetella pertussis* colonization in the mouse model [407].

We took this concept one step further and tested the positive bystander effect of vaccination-induced influenza-specific Th cells on a bacterial co-infection.

Our results lead us to suppose that neutralization of influenza virus at early stages after infection is extremely important to further efficiently counteract bacterial super-infections indirectly by not weakening immune responses and/ or altering the cellular state of the respiratory tract. However, if the virus is not rapidly blocked but it has the possibility to replicate at low rate for the first 3 days p.i., the presence of a proper cross-reactive T-cell-based immunity elicited by adjuvanted-vaccination is of great help to then reduce pathogenicity induced by SBI via cytokine-mediated activation of phagocytic cells like macrophages or neutrophils and efficient bacterial killing. Our hypothesis needs to be further confirmed by focusing on mechanism of protection established by various anti-influenza vaccine formulations in our model of mixed infections. Particularly we have to properly test virus-specific Th cell response on the light of new effector functions attributed to CD4^{+} T cells during viral infection [102] and considering recent findings about the frail balance and strict timing of type 1 (IFNγ), type 2 (IL-13) [220] and type 17 (IL-17) [217] cytokines production during single influenza/ bacterial infection and their positive or detrimental impact on SBI.

Importantly we showed that also MF59-adjuvant is still an effective enhancer of immunity induced by vaccination though being more Th2-prone than the other adjuvants tested. In the mouse model MF59 induces a mixed Th1/ Th2 response [6, 317]. Yet, this situation is different in human vaccinations. In humans MF59-adjuvanted vaccines induce Th1 responses [304], probably due to the fact that humans are not naïve to influenza but pre-exposed to prior influenza infections. The positive bystander effect of influenza-vaccination-induced Th cells on SBI should accordingly be more pronounced in humans than in the mouse model. Among adjuvants tested in the second study, only MF59 is currently licensed for human applications and here we highlighted that its added value to influenza vaccines is not solely due to its amplification of immunogenicity but also by conferring broader protection against a heterologous virus infection and a positive impact to counteract SBI. In contrast, unadjuvanted vaccine induced only minor adaptive response and it poorly protected mice from mismatched viral infection and SBI: mice showed excessive lung inflammatory cells infiltration, viral load comparable to that of naïve ones and they were very negatively affected by *S. aureus* secondary challenge.

The mechanism of action of MF59 has been studied a lot in pre-clinical models [4, 297, 311, 313-316, 350], but all investigations have been conducted in young mice (6-8 weeks, comparable to young adults) which do reflect the immune response in elderly. Considering that MF59 is specifically used to vaccinate aged people, we evaluated which immune events induced by this adjuvant were still active in aged mice (>18 months). Similar to young mice, also in older ones MF59 potentiated immunogenicity of TIV enhancing Th cell and antibody responses as well as vaccine efficacy. Since we consider these responses essential for the protection from SBI, we would expect to confirm the added value of adjuvants for influenza vaccines also in elderly mice. Respective studies will be done in the future.

Yet, MF59 did not completely overcome the reduced ability of a senescent immune system to respond to vaccination. Older adults have well-known innate and adaptive immunity defects which reduce the effectiveness of anti-influenza vaccines in this population [341]. Our results demonstrated that also in aged mice there are intrinsic impairments associated with immunosenescence that even the strong adjuvant effect of MF59 could not overcome. Indeed in older mice we obtained lower magnitude of antibody response, higher variability in Th cell response, fewer influxes of conventional dendritic cells (cDC) to dLNs and more limited germinal center (GC) B cells maturation than in younger ones. We have previously shown that transient ATP-release at injection site gives an important contribution to MF59-adjuvanticity [4]. Here we demonstrated that in old mice that this pathway is fully functional and that its activation is extremely crucial because it seemed to be the only one still functioning in the context of immunosenescence. This finding is even more important since we demonstrated that ATP signalling is

essential for efficient priming of Th cells. Having identified an immune activation pathway that is not impacted by aging and that targets immune cells that can play a fundamental role in protection from viral or bacterial infections should find broad application in diverse preventive and therapeutic treatments.

Our data added to the mounting evidence that MF59-adjuvanted influenza vaccines play a fundamental role for the protection especially of the elderly population, while not adjuvanted subunit and split-virus vaccines have shown limited effectiveness [145, 266, 340].

LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent Cell-mediated Cytotoxicity
Ag	Antigen
APC	Antigen Presenting Cell
BALs	Bronchoalveolar Lavages
cDC	conventional Dendritic cell
CFU	Colony Forming Unit
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
dLNs	Drain Lymph Nodes
EMA	European Medicines Agency
FDA	Food and Drug Administration
GC	Germinal Center
GISN	Global Influenza Surveillance Network
H1N1	Hemagglutinin 1 Neuraminidase 1
H1N1/Cal	H1N1/A/California/7/2009
HA	Hemagglutinin
HI	Hemagglutination Inhibition (assay)
i.m.	intramuscularly
i.n.	intranasally
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISG	IFN-stimulated Gene
LAIV	Live Attenuated Influenza Vaccine
M1/ M2	Matrix protein 1/ 2
MHC	Major Histocompatibility Complex
MLD_{50}	50% mouse lethal dose
MN	Microneutralization (assay)

MO	Monocyte
MoA	Mechanism of Action
MPH	Macrophage
MRSA	Methicillin Resistant *Staphylococcus aures*
NA	Neuraminidase
NI	Neuraminidase Inhibition (assay)
NK	Natural Killer cell
NLRP3	Nucleotide Oligomerization Domain (NOD)-like Receptor Family Pyrin Domain 3
NP	Nucleoprotein
NPH	Neutrophil
NS1	Nonstructural protein
OVA	Ovalbumin
p.i.	post infection
PAMP	Pathogen Associated Molecular Pattern
pDC	plasmacytoid Dendritic Cell
PR8	H1N1/A/Puerto Rico/8/ 1934
PRR	Pattern Recognition Receptor
PVL	Panton-Valentine Leukocidin
RIG-I	Retinoic Acid-inducible Gene I
RNA	Ribonucleic Acid
s.l.	sublingually
SBI	Secondary Bacterial Infection
$TCID_{50}$	50% tissue culture infectious dose
T_{FH}	T follicular helper cell
Th	T helper cell
TIV	Trivalent Inactivated Influenza Vaccine
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor α
T_{reg}	Regulatory T cell
WHO	World Health Organization

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