Sede Amministrativa: Università degli Studi di Padova
Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE E BIOTECNOLOGIE
INDIRIZZO DI BIOLOGIA CELLULARE
CICLO XXVI°

The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination

Direttore della Scuola: Ch.mo Prof. Giuseppe Zanotti
Coordinatore d'indirizzo: Ch.mo Prof. Paolo Bernardi
Supervisore: Ch.mo Prof. Cesare Montecucco
Dr. Anja Seubert

Dottorando: Maria Vono

Gennaio 2014

INDEX

SOMMARIO

I vaccini rappresentano senza dubbio l'arma più efficace per combattere e tenere sotto controllo le infezioni [1]. In aggiunta agli antigeni del patogeno, i vaccini contengono adiuvanti utilizzati per potenziare le risposte immunitarie specifiche verso determinati antigeni. Nonostante la loro efficacia e il loro largo uso, il meccanismo di azione di molti adiuvanti è ancora scarsamente caratterizzato [2]. Pertanto, far luce sui meccanismi d'azione degli adiuvanti vaccinali è fondamentale per sviluppare prodotti nuovi, più efficienti e sicuri, e poter così sfruttare appieno il potenziale della vaccinologia [3]. Dopo la vaccinazione, è stato osservato al sito di iniezione il rilascio locale di molecole endogene con la capacità di segnalare "danno" al sistema immunitario, note come allarmine. Per esempio, un rilascio locale di acido urico e DNA è stato osservato nel modello murino dopo vaccinazione con alum, il più diffuso tra gli adiuvanti approvati per uso sull'uomo. Tuttavia, finora non è mai stato esplorato un potenziale ruolo dell'ATP durante la vaccinazione. L'ATP, tra le sue tante funzioni, quando rilasciato nell'ambiente extracellulare in concentrazioni opportune può fungere da allarmina e, come tale è un forte modulatore delle risposte immunitarie [4-6]. Pertanto, in questo lavoro abbiamo indagato se un rilascio di ATP è coinvolto nel meccanismo d'azione di quattro comuni adiuvanti vaccinali: idrossido di alluminio (alum), calcio fosfato (CaPi), adiuvante incompleto di Freund (IFA) e MF59. Sono stati condotti esperimenti *ex vivo* su muscoli murini isolati (tibiale anteriore e quadricipite) e *in vivo* in topi immunizzati intramuscolo con l'adiuvante da testare e il sistema reporter luciferina-luciferasi in grado di segnalare il livello di ATP al sito d'iniezione. Abbiamo osservato che l'iniezione intramuscolare è sempre associata a un debole e transitorio rilascio di ATP. Il rilascio basale di ATP è notevolmente potenziato dall'iniezione di MF59 ma non dagli altri adiuvanti testati.

Pertanto, abbiamo esplorato se e come il rapido e transitorio rilascio di ATP indotto da MF59 al sito d'iniezione potesse contribuire al suo meccanismo d'azione.

Il forte potere adiuvante di MF59 [7, 8] è stato attribuito alla sua capacità di istituire un ambiente immunocompetente al sito di iniezione nel muscolo, caratterizzato da un rapido e transitorio afflusso di un gran numero di cellule immunitarie che captano e assorbono l'antigene e lo trasportano ai linfonodi drenanti [9-11]. Abbiamo qui

dimostrato, che la co-iniezione di apirasi, un enzima in grado di idrolizzare l'ATP, riduce fortemente l'afflusso di cellule immunitarie indotto da MF59 ma non quello indotto da alum o IFA.

Questi risultati indicano che l'abilità di MF59 di indurre un forte afflusso di cellule immunitarie al sito di iniezione è in parte dovuta alla sua intrinseca capacità di rilasciare ATP. Inoltre, abbiamo osservato che la co-iniezione di apirasi e MF59 riduce il numero di cellule antigene-positive che dal muscolo raggiungono i linfonodi drenanti. Tale riduzione si è rivelata tipo cellulare-specifica, infatti il trattamento con apirasi impatta negativamente il numero di cellule B antigene-positive indotto da MF59 nei linfonodi drenanti, suggerendo che le cellule B potrebbero essere un elemento chiave nei "pathways" mediati da ATP durante la vaccinazione.

Efficienti risposte immunitarie di tipo innato si traducono spesso in forti risposte adattative [12]. Pertanto, abbiamo analizzato un eventuale ruolo dell'ATP rilasciato da MF59 sull'attivazione delle cellule T e la produzione di titoli anticorpali antigene-specifici. Di conseguenza, gruppi di topi sono stati immunizzati con un vaccino influenzale trivalente, iniettato come tale o adiuvato con MF59 con o senza apirasi. L'apirasi ha fortemente ridotto la proliferazione delle cellule T vaccino-specifiche e i relativi titoli anticorpali. Questi dati dimostrano che un locale e transitorio rilascio di ATP a livello del sito d'iniezione è necessario per lo sviluppo di risposte immunitarie innate e adattative indotte da MF59 e associano per la prima volta un rilascio extracellulare di ATP a un potenziamento delle risposte immunitarie indotte dalla vaccinazione.

SUMMARY

Vaccines are the most effective agents to control infections [1]. In addition to the pathogen antigens, vaccines contain adjuvants that are used to enhance the specific immune responses. Despite their effectiveness and their wide use, the mechanism of action of many adjuvants is poorly characterized [2]. Therefore, adjuvant research is crucial to better understand how they work and to exploit their full potential in vaccinology [3]. Release of endogenous danger signals has been linked to adjuvanticity, however the role of extracellular ATP during vaccination has never been explored. Extracellular ATP can work as "danger signal" and, as such is a strong modulator of immune responses [4-6]. Here, we tested whether ATP release is involved in the immune boosting effect of four common adjuvants: aluminium hydroxide, calcium phosphate (CaPi), incomplete Freund's adjuvant (IFA) and the squalene-based oil in water emulsion MF59.

Experiments were performed *ex vivo* in excised mice muscles (*tibialis anterior* and *quadriceps*) and *in vivo* in live mice injected with the reporter system luciferase-luciferin that reports on ATP changes. We found that intramuscular injection in general is always associated to a weak transient release of ATP. In contrast, a greatly enhanced ATP release was found upon injection of MF59 but not by all other adjuvants tested.

Therefore, we wanted to dissect whether and how ATP release would contribute to the activity of MF59. The strong adjuvanticity of MF59 [7-8] has been ascribed to its capability to induce an immunocompetent environment in the muscle, characterized by a rapid and transient influx of a large number of immune cells participating in antigen uptake and transport to draining lymph nodes [9-11]. We found that the local injection of apyrase, an ATP-hydrolyzing enzyme, reduced the immune cells recruitment induced by MF59 but not by alum or IFA. These findings indicated that the ability of MF59 to induce migration of different immune cells into the injected muscle is partly due to induced ATP release. Moreover, co-injection of apyrase and MF59 at the muscle injection site reduces the number of antigen positive cells in the draining lymph nodes in a cell type-specific manner. Indeed, co-injection of apyrase negatively impacts the number of antigen positive B cells induced by MF59, suggesting that B cells could be a key component in ATP-mediated signaling during

vaccination. Strong innate immune responses lead to enhanced adaptive immune responses [12]. Accordingly, we compared the impact of MF59-induced ATP release on T cells responses and antibody titers. Groups of mice were immunized with an experimental trivalent influenza vaccine (TIV) either as plain antigens or together with MF59 with or without apyrase. Apyrase strongly inhibited influenza specific T cell responses, total IgG and hemagglutination inhibition titers in response to an MF59-adjuvanted trivalent influenza vaccine. These data demonstrate that a transient ATP release is required for innate and adaptive immune responses induced by MF59 and link for the first time extracellular ATP to an enhanced response to vaccination.

1. INTRODUCTION

1.1 Brief overview on the immune system

Invasion of a host by pathogenic agents triggers a complex set of immune responses through interactions between a diverse array of pathogen-borne virulence factors and the immune surveillance mechanisms of the host [13]. The mammalian immune system comprises an innate and adaptive component. The innate immune system is the first line of defense against pathogens and is mediated by leukocytes including macrophages and dendritic cells (DCs) and by the complement system. It acts immediately using a limited number of germ-line-encoded pattern-recognition receptors (PRRs) that recognize invariant pathogen-associated molecular patterns (PAMPs). PRRs function as sensors that alert the immune system of an imminent danger. The best known members of the PRRs family are the Toll-like receptors (TLRs) [14]. TLRs detect foreign organisms in the extracellular space, in the phagosome or endosome lumen. The cytoplasm instead is surveyed by two different families of PRRs: the retinoic acid-inducible gene (RIG)-like helicases (RLHs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [14]. RLHs are mainly involved in antiviral immunity. NLRs comprise the NOD proteins and the inflammasome scaffold proteins (IPAF, NAIP and NALPs). There is evidence that NLRs mediate immunity against intracellular pathogens and are involved in the pathogenesis of autoinflammatory diseases [15-17].

In contrast, the adaptive immune system, which is long lasting and has immunological memory, is based on a myriad of receptors, which can be soluble, such as the immunoglobulins, or cell-bound as T- or B-cell receptors. Because of this large repertoire of recognition molecules, the immune system discriminates among an almost countless number of antigens. Induction of adaptive immunity not only depends on direct antigen recognition by the antigen receptors but also relies on essential signals that are delivered by the innate immune system [18]. Accordingly, the responses of the innate immune system to pathogens help to initiate adaptive immune responses by different mechanisms. Microbial infection is detected by PRRs and their signaling leads to the activation of phagocytic cells like macrophages and

DCs. These cells besides phagocytosing and eliminating pathogens can also act as so-called Antigen-Presenting Cells (APCs). APCs like DCs, digest the phagocytosed pathogen to peptides, which are subsequently re-exposed on the APCs surface on MHCII molecules. Such, APCs transport pathogen-derived antigens to local lymphoid organs where they are presented to naïve T cells. During the encounter of an APC with an antigen-specific T cell, the latter will get activated and start to proliferate and differentiate. These effector T cells then either leave the lymphoid organs to affect cell-mediated immunity in sites of infection in the tissues or remain in the lymphoid organs to participate in humoral immunity by activating B cells [19]. Protective immunity against reinfection is one of the most important consequences of adaptive immunity operating through the clonal selection of lymphocytes. Protective immunity depends not only on pre-formed antibodies and effector T cells, but most importantly on the establishment of a population of lymphocytes that mediated long lived immunological memory. The capacity of these cells to respond rapidly to restimulation with the same antigen can be transferred to naïve recipients by primed B and T cells [19].

1.2 Regulation of the immune response: the danger theory and endogenous alarmins

Besides the aforementioned immune activation by PRRs, the innate and adaptive immune system can also be activated by endogenous signals that originate from stressed, injured, or necrotic cells, indicating danger to the host. The danger theory was first postulated in 1994 as part of a model of immunity that suggests that the immune system responds to substances that cause damage, rather than to those that are simply foreign [20]. Endogenous danger signals released from necrotic or stressed cells which trigger the inflammatory response after trauma have been termed alarmins or danger-associated molecular patterns (DAMPs) [21]. Oppenheim and Yang have recently grouped several endogenous signals into the category of "alarmins" on the basis of three properties: I) they are rapidly released in response to infection or tissue injury; II) they have chemotactic and activating effects on APCs, particularly DCs; and III) they have potent immunoenhancing activity *in vivo* [22,

23]. Alarmins not only may generate tissue inflammation after injury, but they also could act synergistically with microbial non-self antigens to enhance the inflammatory reaction. Well-known alarmins include heat shock proteins, uric acid, hyaluronan, monosodium urate, thioredoxin, extracellular nucleotides, mitochondrial DNA, high- mobility group box protein 1 (HMGB1), interleukins (such as IL- 1 and IL-33) and reactive oxygen species (ROS) (Table 1) [23, 24]. Moreover, host defense antimicrobial peptides such as B-defensins and cathelicidins [24].

DAMPs	Receptors	Outcome of receptor ligation
Extracellular nucleotides (ATP, ADP, adenosine)	P1, P2X and P2Y receptors (ATP, ADP); A1, A2A, A2B and A3 receptors (adenosine)	DC maturation, chemotaxis, secretion of cytokines (IL-1β, IL-18), inflammation
Extracellular heat shock proteins	CD14, CD91, scavenger receptors, TLR4, TLR2, CD40	DC maturation, cytokine induction, DC migration to lymphnodes
Extracellular HMGB1	RAGE, TLR2, TLR4	Chemotaxis, cytokine induction, DC activation, neutrophil recruitment, inflammation, activation of immune cells
Uric acid crystals	CD14, TRL2, TRL4	DC activation, cytokine induction, neutrophil recruitment, gout induction
Oxidative stress	Intracellular redox-sensitive proteins	Cell death, release of endogenous DAMPs, inflammation
Laminin	Integrins	Neutrophils recruitment, chemotaxis
S100 proteins or calgranulins	RAGE	Neutrophil recruitment, chemotaxis, cytokine secretion, apoptosis
Hyaluronan	TLR2, TLR4, CD44	DC maturation, cytokine production, adjuvant activity

Table 1. Example of well characterized alarmins. (Source: modified from Saïd-Sadier N & Ojcius DM, *Biomed. J.*, 2012 [24])

In recent years, it has become clear that PRRs are not exclusively selective for PAMPs but can also be activated by DAMPs such as monosodium urate crystals and extracellular ATP. The most likely explanation for the presence of a dual control in the activation of innate immunity is that the body is continuously exposed to foreign

microorganisms, which are not necessarily harmful, such us commensal bacteria, and against which it would be even counterproductive to initiate an immune response. To avoid an inappropriate and potentially harmful reaction, a two-step mechanism of control has evolved: detection of a foreign microorganism is not sufficient to trigger inflammation, unless the body has unequivocal proof that this microorganism is also dangerous. The proof is provided by the detection of host cell damage, as assessed by the release of host-derived danger signals [17].

1.2.1 Endogenous danger signals: extracellular ATP

ATP is an important signaling molecule belonging to the purine family. It is produced by cellular respiration and constitutes an indispensable factor for the proper function of a wide variety of enzymes and structural proteins. Extracellular release of ATP takes place in healthy tissue under different conditions, and is involved in a variety of cellular responses such as neurotransmission, vasodilatation, muscle contraction, and cell growth [25, 26]. Thus, in healthy tissues, release of ATP is tightly controlled and its extracellular concentration is kept low by ubiquitous ecto-ATP/ADPases [27].

Intracellular nucleotides like ATP and UTP, normally stored in the cytosol, are released from a variety of cells under conditions of hypoxia, ischemia, inflammation or even mechanical stress and can activate DCs by themselves [28]**.** During tissue injury, trauma or cellular stress, the extracellular levels of these nucleotides can become elevated and are sensed as a potential threat to surrounding tissues.

ATP mediates its effects through ligation of distinct cell-surface purinergic P2 receptors (P2Rs). P2Rs are divided into two families called P2Y (G protein-coupled receptors) and P2X (ligand-gated cation channels), which have distinct effector functions, pharmacological profiles and tissue distribution. Immune and inflammatory cells express P2Y and P2X receptors and their expression is modulated during development and by inflammatory cytokines [29]. Immune cells maintain a steady concentration of ATP in their pericellular environment via a complex mechanism of ATP release and hydrolysis, which is dependent on the activity of ecto-ATPases and ecto-nucleotidases.

Extracellular ATP can accumulate in the pericellular space to concentrations

sufficiently high to activate P2Rs and thus modulate key responses such as chemotaxis, cytokine release, recruitment and activation of neutrophils, macrophages and dendritic cells.

ATP can also stimulate monocyte and microglial cell migration, and has a profound impact on ROS and nitric oxide production [29, 30].

Extracellular ATP is also a well-known activator of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome. The ATP-dependent IL-1β release via NALP3 [31, 32] has been linked to the P2X7 receptor [33]. Moreover, it was shown that pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated P2X7 receptor [34]. Extracellular ATP triggers K+ efflux, inducing gradual recruitment of the pannexin-1 pore and allowing extracellular NLRP3 agonists to access the cytosol [34, 35]. Therefore, ATP is emerging as an important local regulatory factor of the immune responses and P2 receptors are identified as crucial molecular components in the chain of events that leads to inflammatory cell activation. Moreover, ATP is an important cofactor, an enhancer of the inflammatory reaction in response to mitochondrial danger molecules and to microbial-associated molecular patterns. Thus, agents that modulate the extracellular ATP concentration (e.g. soluble ATPases or inhibitors of plasma membrane ecto-ATPases) such as many agonists and antagonists of P2Rs might turn out to be potent modulators of the local inflammatory response and immunological events.

1.3 Immune response to infection and the concept of vaccination

Infectious diseases have been a huge issue for mankind and continue to be a significant medical problem today [36]. Vaccination remains the most effective method of preventing infectious diseases and represents the most relevant contribution of microbiology and immunology to human health.

During the normal course of infection innate immune responses to pathogens, among their many effects, lead to a rapid production of inflammatory cytokines and activation of APCs, such as macrophages and dendritic cells. These responses also contribute to the development of specific adaptive immune responses with production of antibodies and effector T cells that eliminate the pathogen from the

body. After pathogen elimination, many of the effector T and B cells die and antibody levels gradually decline, because the antigens that induced the response have been eliminated. However, memory T and B cells remain, and maintain an enhanced ability to mount faster and more efficiently secondary responses against the same pathogen [19]. Vaccination mimics natural infection and effectively induces pathogen-specific adaptive immunity. The ability to generate antigen-specific memory, which protects against repeated infections with the same pathogens is a fundamental property of the adaptive immune system and determines the success of vaccination [37, 38].

The concept of vaccination originated several hundred years ago from the historical observation that individuals who survived an infectious disease rarely got the same disease a second time [39, 40]. The first evidence of vaccination occurred in the 16th century in China or in India with the practice of variolation, when smallpox pustules from an infected patient were inoculated into healthy individuals to prevent smallpox [41]. Later, in the 18th century, Edward Jenner used cowpox-infected materials to immunize against smallpox and introduced the term "vaccine" [37, 42]. More than 80 years later, Louis Pasteur developed methods for attenuation of bacteria [38] and Salmon and Smith developed methods for inactivation of microorganisms. Together, these advances led to a new era of vaccinology. Then, after World War II the technology of cells grown *in vitro* for virus cultivation was demonstrated by Enders, Weller, and Robbins and then built upon by many other researchers [37]. Virus culture enabled the development of methods for attenuating viral vaccines and allowed the development of several vaccines, including inactivated polio, live polio, measles, mumps, rubella, adenovirus, and varicella [37].

By the latter part of the 20th century, most of the vaccines that could be developed by direct mimicry of natural infection with live attenuated or killed/inactivated pathogens had been developed. Later, the advent of new technologies, such as protein conjugation to capsular polysaccharides and methods to engineer recombinant DNA, led to the development of vaccines for prevention of bacterial pneumonia and meningitis, hepatitis B, and the human papilloma virus vaccine for example. The success of vaccines demonstrates the potential of this approach in reducing the global burden of infectious diseases or even in eradicating them, as in the case of smallpox [43]. However, there are still several diseases that cause considerable global morbidity and mortality for which protective vaccines do not

exist [44]. Some examples are human immunodeficiency virus, mycobacterium tuberculosis, plasmodium falciparum, hepatitis C virus, respiratory syncytial and dengue virus [45-53]. Therefore a better understanding of the vaccination process is needed to develop novel vaccine based on molecular targets that further improve vaccine efficacy.

1.4 Vaccines

The first effective vaccine was developed by Jenner more than 200 years ago and today there are vaccines available against different viral and bacterial pathogens. It is easy to define the properties of an optimal vaccine (Table 2), but few vaccines approach the ideal [54].

Properties of an ideal vaccine
Should give life-long immunity
Should be broadly protective against all variants of an organism
Should prevent disease transmission
Should induce effective immunity rapidly
Should be effective in all vaccinated subjects, including infants and the elderly
Should transmit maternal protection to the fetus
Requires few immunizations to induce protection, ideally one
Would not need to be administered by injection
Should be cheap, stable (no requirement for cold chain), and safe

Table 2. Properties of an ideal vaccine. (Source: modified from Beverley P.C. *Br. Med. Bull.*, 2002 [54])

The most broadly used vaccines are based on live attenuated or inactivated whole organisms. In this case, all of the antigens from the pathogen are present in the vaccine and all are potential targets for an immune response. Furthermore, various microbial components, such as immunostimulatory CpG DNA motifs, double-

stranded RNA, and some glycolipids are known to stimulate the innate immune system. The presence of these PAMPs can provide the vaccine with a built-in "adjuvant". The word adjuvant – originating from the latin word "adiuvare" meaning "to help" - refers to different substances or compounds able to enhance immune responses towards co-administered antigens. Yet, several of these vaccines despite being highly efficient raised concern for their poor tolerability, often caused by pathogen intrinsic toxins or a combination of several PAMPs.

No doubt, vaccines are a powerful tool of preventive medicine. In recent years, the progress in vaccine development has been great and the number of lives saved has been impressive. However, these successes arrived but not without failures. A tragic example, known as "the Cutter incident" occurred in the 1950s with a polio vaccine. The polio virus was identified in 1908 by Karl Landsteiner. At its peak in the 1940s and 1950s, polio paralyzed or killed over half a million people worldwide every year [55]. In April 1955 more than 200.000 children in the USA received a polio vaccine in which the process of inactivating the live virus was defective. Within days there were reports of paralysis and the vaccination programme against polio was abandoned. Subsequent investigations revealed that the vaccine, manufactured by the California-based family firm of Cutter Laboratories, had caused 40.000 cases of polio, killing many children or leaving them with varying degrees of paralysis [56].

Over the years, the bio-technological advancements have given us laboratory powers to better understand disease etiology and produce newer and improved vaccine products. The results have been amazing and including the total eradication of smallpox and the prevention of many other dangerous infectious diseases.

Anyway, it is important to remember that the development of a vaccine, from basic research, through vaccine preparation and pre-clinical and clinical testing, to actual application and disease control, is technically challenging, hard-working, very expensive and take up much time. For example, early vaccination against smallpox started in the late 1700s while vaccine became widely available by the early 1900s. Anyway, it took decades longer to totally eliminate transmission in North America and Europe and years more to eliminate it from the rest of the world.

Recently, more defined vaccines based on partially purified preparations from the organism or recombinant subunit proteins have been developed. These types of vaccines usually have a better safety profile. However, their development requires knowledge of the best target antigens to induce a protective immune response.

Importantly, the better safety profile comes at the cost that the highly purified antigens, by themselves, are often not sufficiently immunogenic. Therefore, adjuvants usually need to be added to these types of vaccines. Moreover, subunit vaccines do not have an inherent ability to be delivered to appropriate sites for optimal immune stimulation, unlike live attenuated organisms, for example, where the natural invasiveness of the pathogen provides effective delivery. Thus, in designing new effective and safe vaccines, several key elements are required. First, an antigen against which an immune response is targeted. Second, an adjuvant for efficient stimulation of the innate immune system and finally, delivery systems to target the vaccine to appropriate cells of the immune system to ensure optimal stimulation [39].

The advent of the genomic era led to many changes in vaccinology. The continuing advances in genome sequencing technologies and bioinformatics enabled researchers to explore a microorganism's genome for antigen discovery. Microbial genomes contain all possible antigens and these are used as the starting point to capture information for vaccine development. This process, known as 'Reverse Vaccinology', was first proposed in 2000 based on the identification of novel meningococcal vaccine candidates from the genome sequence of a Neisseria meningitidis serogroup B strain [57]. Nowadays, the Structural Vaccinology (SV) approach is coming on stream. SV is the evolution of Reverse Vaccinology: a genome-based approach combined with structural biology, with the idea that protective determinants can be used to selectively engineer the antigens that can be re-designed and simplified for inclusion in vaccine combinations. The rational structure-based antigen optimization has many goals. Among these are the facilitation of industrial-scale production of the antigens combination, a greater immunogenicity and safety profile. Structural vaccinology is particularly powerful in case of antigenic variation between closely related strains and species and can lead to the development of improved vaccines against several pathogens and potentially help resolve challenges in manufacturing or efficacy [58]. Anyway, despite the increase in our knowledge of the immune system and host-pathogen interactions and despite the advent of new technologies, we still lack effective vaccines for many diseases.

Sometimes the vaccine design may not have been optimal and the vaccine used may not have induced the correct type of immune response. For example, antibodies are not protective against some intracellular pathogens, and T cell responses may be

more effective. Sometimes polarized specific immune responses mediated by CD4+ T helper (Th) lymphocytes, based on their profile of cytokine production (type 1 or Th1 and type 2 or Th2) are needed. Therefore, the recent emphasis on T cell-stimulating rather than conventional antibody-inducing vaccines may be really helpful in such cases.

Adjuvants have been identified as crucial factors in order to not only activate the immune system, but to steer the responses in the desired direction. Accordingly, the choice of adjuvant can affect the nature of antibodies and T cells produced, so a careful choice of adjuvant is needed to induce the desired and most appropriate immune responses. Moreover, adjuvants and other components may then be added to improve the characteristics of the vaccine, such as stability, potency, protective efficacy, and the number of doses required. These include delivery systems (e.g. emulsions, liposomes, and polymers), and compounds able to direct vaccines to specific cells of the immune system [59].

1.5 Vaccines adjuvants

Vaccine adjuvants are used to enhance immune responses towards co-administered antigens, thereby improving vaccine potency, immunological memory or cross-protection [2, 3]. Different classes of compounds display adjuvant activity in pre-clinical models; among them, bacterial products, mineral salts, emulsions, microparticles, nucleic acids, small molecules, saponins and liposomes [60, 61]. The idea that some materials could improve immune responses was recognized many years ago with the work of Ramon and Glenny, who used tapioca and aluminium hydroxide to enhance the immune responses of horses or guinea pigs to diphtheria and tetanus toxoids [62, 63]. Nowadays, experimental adjuvants range from simple molecules such as calcium phosphate (CaPi) to very complex mixtures like Incomplete Freund's adjuvant (IFA) made of a water-in-oil emulsion or Complete Freund's adjuvant (CFA) that additionally includes killed Mycobacteria [64]. IFA is a water-in-oil emulsion, which can be mixed with antigens and pattern molecules. IFA lacks the bacterial antigens that are present in the complete form and which are notorious for the induction of discomforting side effects. Although IFA-antigen

emulsions elicit long lasting IgG responses, they also stimulate the activation of T-cytotoxic and T-helper lymphocytes [65, 66]. In spite of such desired properties, IFA is not approved for routine immunotherapy in humans, but is merely used in investigational clinical trials. For human vaccines, adjuvants of highly defined properties that combine efficacy with complete safety are needed and to date only very few compounds are licensed. These include aluminum salts, calcium phosphate, oil in water emulsions, virosomes and the TLR4 agonist, monophosphoryl lipid A (MPL). Calcium phosphate has previously been shown to be an effective adjuvant in man for a number of vaccines, including diphtheria and tetanus toxoids [67], but was gradually replaced with alum in 1960s. In addition, there are a large number of adjuvants currently in development, aimed at boosting CD4+ helper T cell, CD8+ cytotoxic T cell, and humoral immune responses.

Some of the safest and most efficient adjuvants licensed for human use – like aluminium hydroxide and the oil-in-water squalene-based emulsion MF59 - have been empirically identified and their mechanism of action is still not fully understood [60, 68, 69]. A better understanding of the molecular mechanisms of adjuvanticity is needed to develop novel and improved adjuvants based on a more rational design. The identification of new molecular targets that further improve vaccine efficacy is very important, particularly for primary diseases like tuberculosis, malaria, AIDS and other diseases that are waiting for new-generation adjuvants that could overcome the current vaccine failures [1].

Adjuvants can act in several not exclusive ways to enhance the immune responses and to generate effective immunological memory. Many of their effects seem to be on antigen-presenting cells such as dendritic cells. Indeed, adjuvants can affect the migration, maturation, antigen presentation, and expression of costimulatory molecules by DCs, and these events in turn improve the responses of T and B lymphocytes to antigen [3]. This activation of DCs can occur either directly via signaling of PRRs like TLRs. On the other hand, DCs activation can occur via a complex cross-talk with other cell types, both immune and non-immune cells. Accordingly, adjuvants are often subdivided in TLR-dependent and TLR-independent adjuvants (Table 3) [70].

Adjuvants	Formulations
TLR-indipendent adjuvants	
Alum ✔	Mineral salts
MF59 ✔	Squalene Oil-in-water emulsion
AS03 ✔	Squalene Oil-in-water emulsion
AF03 ✔	Squaline Oil-in-water emulsion
Virosomes ✔	Liposomes
ISCOMs ✔	Particulate comprising cholesterol and phospholipids
ISCOMATRIX ✔	Particulate comprising cholesterol, phospholipid and saponin
Montanides (ISA 720 and 51) ✔	Water-in-oil emulsions
LT/LTK63 ✔	Bacterial toxins
QS-saponin-based synthetic adjuvants (QS21) ✔	Aqueous
Muramyl tripetide phosphatidyl-ethanolamine (MTP-PtdEtn) ✔	Oil-in-water emulsion
IL-1 family cytokines (i.e., IL-1α, IL-1β, IL-18, and IL-33) ✔	Pro-inflammatory cytokines
TLR-dipendent adjuvants	
MPL ✔	TLR4 agonist
AS04 ✔	Alum + MPL
CpG ✔	TLR9 agonist
Imiquimod ✔	TLR7 agonist
Flagellin ✔	TLR5 agonist
AS01 or AS02 ✔	Combination of liposomes, MPL and QS21
AS15 ✔	Combination of liposomes, MPL, QS21 and CpG

Table 3. TLR-dependent and TLR-independent adjuvants in testing or use in human. Adjuvants marked with a green tick are components licensed for human vaccines, while those with a red tick have been developed or tested in clinical trials, but are not yet approved. (Source: modified and updated from De Gregorio *et al.*, *Front Immunol.* 2013 [70]).

1.5.1 TLR-dependent adjuvants

Many of the vaccines currently used for human use contain whole attenuated or killed microorganisms and therefore PAMPs are present. PAMPs can engage PRRs, which act as microbial sensors expressed by DCs and other leukocytes. Currently, many PAMPs by themselves are used as natural vaccine adjuvants, among these for example CpG oligonucleotides and MPL. The mechanisms of action of TLR-dependent adjuvants are well characterized. Indeed, TLR signaling has many effects on antigen presentation to DCs, increase in co-stimulatory molecules and cytokine levels, leading to the upregulation of cytokines, MHC class II, and co-stimulatory molecules and to the migration of DCs to the T cell area of lymph nodes. A variety of immunostimulatory compounds, including proteins, lipids, carbohydrates, and nucleic acids, have been shown to be TLR ligands and are currently being used experimentally or in clinical trials as vaccine adjuvant.

1.5.2 TLR-independent adjuvants

In contrast, most conventional adjuvants such as alum, emulsions such as MF59, or incomplete Freund's adjuvant elicit efficient immune responses to vaccine in the absence of TLRs. Their mechanism of action is only poorly understood despite their broad use [68-70]. Interestingly, the most conventional have a common feature: they are particulate compounds known to modulate antigen persistency or antigen uptake by DCs and therefore have been generally defined as antigen delivery systems. But it is likely that antigen-delivery is not their only mechanism. Probably these compounds activate the immune system through pattern recognition receptors and signaling machineries different from TLRs. Recently, other intracellular innate receptors that sense a variety of immunomodulatory compounds, such as NLRs, RIG-like receptors and intracellular DNA receptors, have been demonstrated to activate the innate immune responses, and possibly the adaptive immune responses, in a TLR-independent manner [71, 72]. It is very important to understand how these innate sensors or their downstream signaling pathway(s) mediate the adjuvant-induced innate and adaptive immune responses in order to develop potent and safe vaccine [73]. Among TLR-indipendent adjuvants, aluminum salts are the most

widely used adjuvants in human vaccination. Indeed, although insoluble aluminium salt based adjuvants have been used extensively since the early 20th century, only recently some significant insights have been gained as to how alum actually works [68]**.** The aluminum-containing adjuvants that are licensed for human use are aluminum hydroxide Al(OH)x, commonly known as alum, and aluminum phosphate (Al(OH)x(PO_4)y in which some hydroxyl groups of Al(OH)x are replaced by a phosphate group, and whose precise composition depends on the method of preparation [74]. Over the last years it has been shown that three potential mechanisms are often cited to explain how aluminum-containing adjuvants increase humoral immunity: I) the formation of a depot by which the antigen is slowly released to enhance the antibody production; II) the induction of inflammation and the subsequent recruitment and activation of antigen presenting cells that capture the antigen and III) the conversion of soluble antigen into a particulate form so that it is better phagocytosed by antigen presenting cells.

Injection of alum in mice i.p. or i.m. results in a rapid inflammatory response and release of many inflammatory cytokines [75, 76] that attract cells of the innate immune system at the injection site. Particularly, IL-1β levels increase few hours after injection [77, 78]. IL-1β production is dependent on the activation of myeloid differentiation primary response protein 88 (MyD88) and the assembly of the NLRP3 inflammasome, and subsequent activation of caspase-1 [78]. However, the role of the inflammasome and IL-1β in alum's activity is controversial [79, 80]. In addition, there is the local release of endogenous uric acid and DNA [81- 83], probably by local tissue damage at the injection site. In particular, it was shown that recruited monocytes take up antigen, process it and re-expose antigen peptides on MHCII molecules on their way to the draining lymph nodes while differentiating into DCs. In the nodes, mature DCs then activate antigen-specific T cells that differentiate into T helper cells. Particularly in the mouse, this response is Th2 based. In the spleen, and possibly in the draining nodes, there is also recruitment of Ly6$^+$ monocytes, IL-4$^+$ eosinophils that stimulate also B cell responses. In humans, it also appears that alum-formulated vaccines mainly act at the level of the monocyte, inducing phenotypic and functional maturation [9, 84, 85]. Together, these experiments demonstrated that monocyte-derived DCs are necessary to mediate the adjuvant effects of alum.

After aluminum-containing adjuvants, oil-in-water emulsions, such as MF59, are the

most common adjuvants licensed for human use. Since MF59 turned out to become a particular focus of this work we dedicated a separate chapter to this attractive adjuvant formulation. MF59 composition and informations about its mode of action are described below in much greater detail.

1.5.3 Combination adjuvants

It has been proposed that particulate adjuvants (delivery systems) should be combined to TLR agonists to optimize vaccines based on poorly immunogenic proteins. Combinations of TLR agonists like CpG, with delivery systems such as mineral salts, emulsions, and microparticles are superior to the individual adjuvant components in inducing humoral and cellular responses [70, 86]. An example is the Adjuvant System 04 (AS04) developed by GlaxoSmithKline that combines the TLR4 agonist, MPL, and aluminum salt [87].

1.5.4 The TLR-independent adjuvant MF59: mechanism of action

MF59 is an oil-in-water emulsion consisting of squalene (4.3% v/v), and two non-ionic surfactants tween 80 (0.5% v/v) and span 85 (0.5% v/v), emulsified in citrate buffer (10 mM) (Fig. 1). MF59 is a well-established, safe and potent emulsion-based vaccine adjuvant that has been licensed since 1997 in more than 20 countries, for use in an influenza vaccine for the elderly (Fluad®). The overall safety profile of MF59 has been established clinically through an extensive pharmacovigilance evaluation [88]**.** MF59 consists of small (~160 nm in diameter) squalene oil droplets stabilized by the addition of tween 80 and span85, which are widely used as emulsifiers in foods, cosmetics and pharmaceuticals [89]. Squalene is a biosynthetic precursor to cholesterol and steroid hormones. Squalene is synthesized in the liver in humans and circulates in the bloodstream, but is most abundant in the skin, where it is the main component of sebaceous secretions. Moreover, significant quantities of squalene are naturally present in adipose tissue, muscles and lymph nodes. Hence, squalene is

both biodegradable and biocompatible and is also a normal component of the human diet. This was also an important reason for the selection of squalene during the development of MF59. An oil that has no inherent biological activity, that is a normal component in the human body at the site of administration, and that can be easily metabolized and excreted, is of great advantage for safety and tolerability. Shark livers provide the natural source of squalene for MF59 while tween 80 and span 85 are sourced from plants [9, 61].

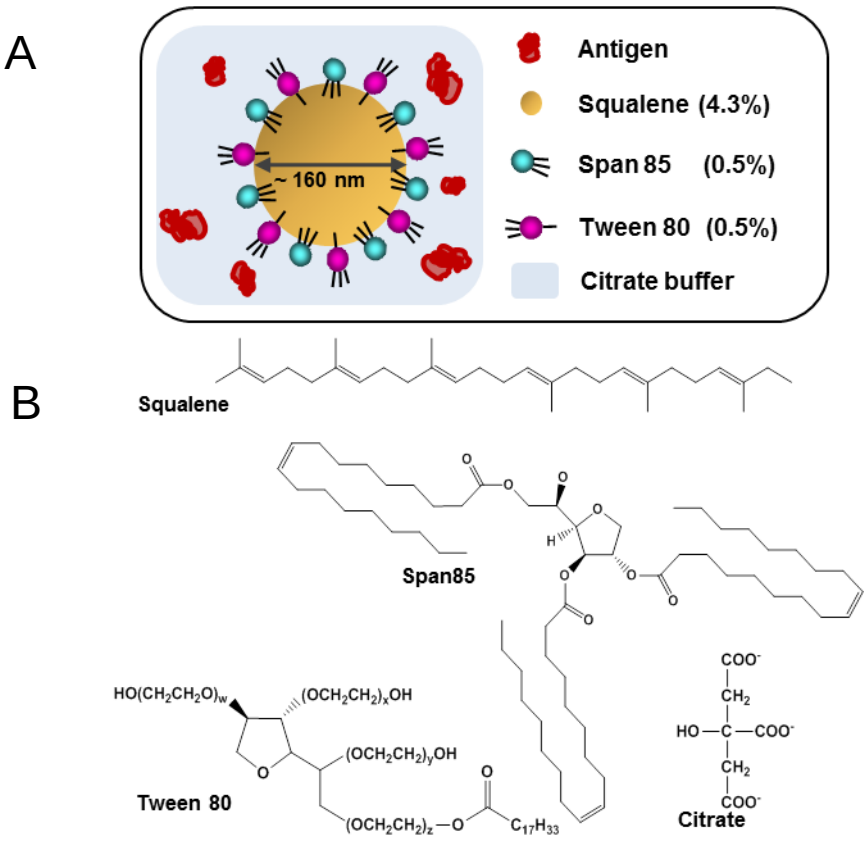

Fig. 1. Composition of MF59 emulsion. ***A***, Sketch of MF59 emulsion and ***B***, chemical structures of its individual components (Source: Calabrò *et al*., *Vaccine* 2013 [89]).

Over the years, the mechanism of action of MF59 has been extensively evaluated (88-92). Briefly, normal tissue-resident cells like macrophages and muscle cells are initially activated by injection of MF59 and are able to induce a local immunocompetent environment at the muscle injection site. A mixture of cytokines, chemokines and other factors is produced by these cells. The released chemoattractants act on immune cells and result in their migration from the blood stream into the muscle. The recruited target cells, including monocytes and granulocytes, also produce cytokine and chemokines upon contact with MF59, thereby further amplifying the chemokine gradient. This mechanism results in signal amplification and to a significant influx of phagocytic cells into the injection site. The overall higher number of cells available locally increases the chance of interaction between an APC and the antigen. Higher numbers of antigen-positive APCs lead to a more efficient transport of Ag to the lymph nodes, enhance the probability of APCs encounter with antigen-specific T cells and thus result in better T cell priming (Fig. 2) [9].

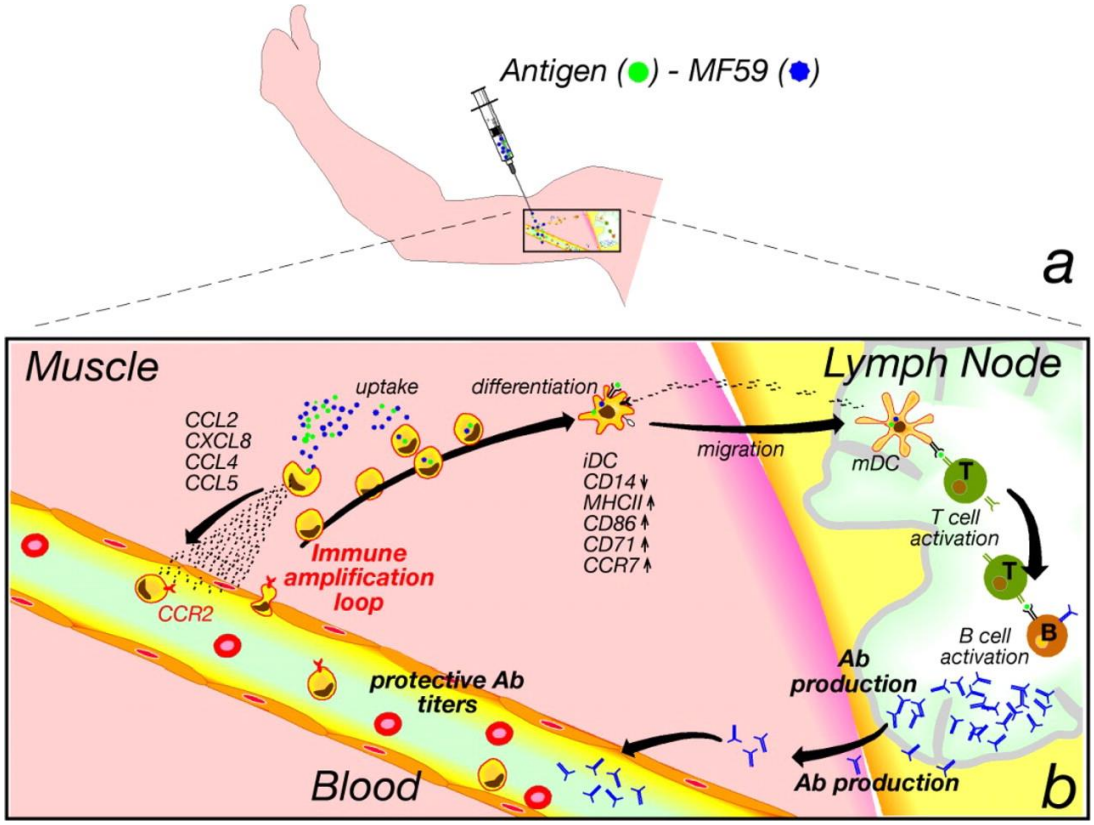

Fig. 2. **Model for the mechanism of action of MF59 at the muscle injection site**. MF59 creates atransient and local immunocompetent environment in the muscle following injection. Target cells are activated and a large panel of genes is differentially regulated in response to the adjuvant. Many of

these genes code for chemokines and cytokines. An increase in chemokines levels lead to the influx of many immune cells from the blood stream into the muscle. Recruited cells can also respond to the adjuvant by chemokine secretion thereby creating an immune amplification loop. All recruited cells can take up the adjuvant and antigen and transport them to the draining lymph nodes. Thus, MF59 leads to a much higher number of antigen presenting cells in the lymph nodes where they can interact with Ag-specific T cells to start adaptive immune responses. Strong innate immune responses leads to enhanced adaptive immune responses which translates to higher numbers of Ag-specific effector and memory T cells, such as antibody-titers and finally to greater vaccine potency (Source: Seubert A. *et al., J. Immunol.*, 2008 [9])

To understand which cells are a primary target of MF59, different human immune cells were cultured *in vitro* alone or in presence of the adjuvant. Monocytes, macrophages and granulocytes were found to be specifically activated by MF59. It was demonstrated that they all responded to the adjuvant in a similar manner, by producing the following chemokines: MCP-1 (also known as CCL-2), IL-8 (CXCL-8), MIP-1α (CCL-3) and MIP-1β (CCL-4). *In vivo*, these chemokines attract mainly monocytes and granulocytes, the same cell types that released chemokines in response to MF59 exposure *in vitro* [9]. Accordingly it was postulated that a key component of the mechanism of MF59 was chemokine-driven immune cell recruitment and chemokine-release that would create a positive feedback loop, strongly enhancing the numbers of immune cells at the injection site. These cells could then further participate in antigen uptake and transport to the draining lymph nodes.

It was found that monocytes could undergo phenotypical changes upon incubation with MF59. The cells adopted a more DC-like phenotype, so it was further hypothesized that the MF59 adjuvant induces a monocyte-to-DC differentiation. As DCs are considered to be the key antigen presenting cell type which prime naïve T cells and initiate adaptive immunity, an increase in numbers of this important cell type could translate into an overall higher frequency of immunity initiating cells at the injection site, and within the local lymph node. Importantly, the results generated *in vitro* with human cells were consistent with the available data in mice [90], which showed mainly monocyte/macrophage recruitment into the injection site, with a minor fraction of DCs, probably derived from differentiation rather than direct recruitment.

Moreover, gene expression profiles induced in the mouse muscle following injection of several adjuvants revealed that MF59 leads to upregulation of genes involved in immune activation within the muscle after local administration [11]. MF59 was found to be a much broader activator of transcriptional changes than other commonly used adjuvants such as alum or CpG oligonucleotides [11]. Many of the upregulated genes coded for cyto- or chemokines, cytokine receptors, adhesion molecules involved in leukocyte migration, and antigen-presentation related genes. It was also shown by confocal microscopy that MF59 triggered a more rapid influx of CD11b+ cells from blood compared with other adjuvants. Moreover, muscle cells were identified as additional potential target cells of MF59. Indeed, muscle cells are responsive to the adjuvant by upregulation of the early innate activation markers JunB and pentraxin3 (Ptx3) [11].

Despite these encouraging results it is still unclear, which signaling pathways and immune receptors are triggered by MF59. It has been suggested for several adjuvants, such as alum, that their activity depends on the activation of the NLRP3-inflammasome, which is required for the correct processing of a number of pro-inflammatory cytokines, including IL-1β. However, MF59 was shown to work in a NLRP3-independent manner by two independent groups [91, 92]. Instead it was demonstrated that the adaptor molecule MyD88 is crucial for MF59 adjuvanticity [91] as well as the apoptosis-associated speck-like protein containing CARD (ASC) [92]. Further studies will be necessary to dissect all of the steps of the MF59-induced signaling cascades.

1.5.5 Danger signals in the mechanism of action of TLRs-independent adjuvants

The danger theory proposed by Matzinger could explain how particulate adjuvants trigger innate and adaptive immunity [20]. Alarmins lost from cells subsequent to trauma or infection may serve as local endogenous adjuvants.

Endogenous danger signals have been linked to adjuvanticity, for example recent reports have shown that alum induces tissue damage at the injection site and promote the release of endogenous DAMPs. In particular, endogenous uric acid and DNA

release are involved in the adjuvanticity of alum [81-83]. Moreover, alum-driven DNA release has been implicated in the priming of naïve T cell after the first dose of vaccine and has only a partial effect on antibody responses, suggesting that additional mechanisms are involved in alum adjuvanticity [83]. However the role of extracellular ATP during vaccination has never been explored. As reported above, extracellular ATP has many immunomodulatory properties and there are evidences of its ability to enhance immunity acting on P2Rs. Monocyte-derived dendritic cells have been shown to express a number of P2Rs of both the P2X and the P2Y series [93, 94]. Exposure of monocyte-derived dendritic cells to ATP results in up-regulation of CD54 and MHC class II molecules, induces secretion of IL-12, and augments stimulatory capacity for allogeneic T cells [94]. And in combination with TNF-α, ATP has been shown to increase the expression of co-stimulatory molecules such as CD80, CD83, and CD86 in monocyte-derived dendritic cells [95].

Furthermore, the ability of dendritic cells derived from murine fetal skin to present antigens was reduced by oxidized ATP, an irreversible inhibitor of P2XRs, and clones of dendritic cells selected for lack of $P2X_7Rs$ also had decreased Ag-presenting ability, suggesting a role for this receptor in Ag presentation [93].

Exogenous administration of a purinergic agonist, ATPγS, can enhance cutaneous immunity by augmenting the Ag uptake by APCs. These data demonstrate that ATP could work as an endogenous adjuvant and that the release of ATP during infection or trauma to the skin may result in enhanced cutaneous immunity suggesting that agonists of P2Rs represent a potentially useful class of therapeutic adjuvants [96]. Moreover, Denkinger and collegues found that suramin, a small molecular weight naphthylurea agonist of P2X/P2Y receptors, has adjuvant properties *in vivo*. Their results suggest that suramin promotes its adjuvant effects via activation of APCs, probably through modulation of P2Y or P2X receptors [97]. Therefore as ATP triggers a myriad of immunological events we started a project to investigate a possible link among the release of ATP at the vaccine injection site and adjuvanticity.

1.6 Aim of the project

Vaccine adjuvants are also known as “the dirty little secret of immunologists”, a line coined by Charlie Janeway. Indeed, despite their effectiveness and their wide use, the mechanism of action of many adjuvants is still poorly characterized [2]. Therefore, a better understanding of adjuvanticity is needed to develop novel adjuvants based on molecular targets that further improve vaccine efficacy. This is particularly important for primary diseases for which protective vaccines do not exist [3]. An examination of the chemical nature of four major vaccine adjuvants – alum, CaPi, IFA and MF59 – suggested that they could interact with the phospholipid bilayer of cell membranes, via hydrogen bonding or ionic interactions with the head groups of phospholipids/ glycolipids and/or via hydrophobic interactions with the hydrocarbon chains of lipids. As vaccines are frequently administered by intramuscular injection, we posited that: I) a high local concentration of adjuvant is generated in a confined portion of the muscle, and that II) the first cell membrane they get in contact with is the sarcolemma. Since we found that the muscle injection of membrane interacting snake phospholipases A2 (PLA2) myotoxins induces the release of ATP, which is contained in large amounts inside muscle fibers [98, 99], we decided to evaluate the possibility that other putative membrane interacting agents, such as the major adjuvants mentioned above, might similarly induce ATP release. This possibility would be particularly relevant in the context of adjuvanticity as ATP is a "danger signal" acting on a variety of purinergic P2 receptors and, as such, is a strong modulator of immune responses [4-6]. Therefore, we tested the possibility that extracellular ATP is involved in boosting the immune response by the aforementioned four common adjuvants.

2. *RESULTS*

2.1 Adjuvant-induced ATP release from injected mouse muscles

In order to test our hypothesis, we monitored the adjuvant-stimulated ATP release in mice using the reporter system luciferase-luciferin. In the presence of ATP, luciferase catalyzes oxidation of luciferin with emission of photons that can be recorded by an appropriate imaging apparatus.

Recent work showed that cells engineered to stably express luciferase on their plasma membrane (PmeLUC- cells) are suitable to detect changes in extracellular ATP concentration *in vivo* [100]. However, in preliminary experiments, we found that i.m. injected luciferase adsorbs onto muscle fibers *in vivo* and efficiently reports on ATP changes within the muscle. Furthermore, administration of soluble luciferase causes a smaller perturbation of tissue homeostasis than injection of the PmeLUC- cell suspension. This read-out is so sensitive that even the low ATP release due to needle injury can be detected at the injection site. Testing the different adjuvants, we found that only MF59-injection induces a fast and prominent ATP signal that is significantly higher than ATP release caused by PBS injection in the contralateral muscle (Fig. 3, *A* and *E*). In contrast, CaPi- or IFA-inoculation do not increase ATP release over buffer control (Fig. 3, *B*, *C*, *F*, *G*) and alum even appears to decrease the signal (Fig. 1, *D* and *H*). Yet, alum readily binds many proteins. Since we observed that ATP binds to alum *in vitro* the luminescence reduction could be a consequence of ATP and/ or luciferin-luciferase adsorption onto the adjuvant surface. Quantitative photoemission evaluation revealed that MF59-injection increased extracellular ATP levels about three fold compared to those triggered by PBS- (CaPi-, IFA- or alum-) containing mixtures (Fig. 3, *E* to *H*). Extracellular ATP increases within 2-3 min following MF59 injection, and then declines over the following 5-6 min. Signal decline is most likely due to ATP dilution in tissue fluids and to the ATP-hydrolyzing activity of ecto-ATPases, which are present on the surface of sarcolemma and stromal cells [101]. To test this latter possibility and to have an independent evaluation of ATP release, we injected *ex vivo tibialis anterior* and *quadriceps* muscles with MF59 or buffer control and measured ATP release into the

medium (Fig. 3, *I* and *J*). The assay confirms prior findings, yet ATP release lasts longer, possibly owing to the different diffusion kinetics of the fluids within the peri-muscle milieu in the two different set-ups. Furthermore, in the *ex vivo* assay, ATP freely diffuses into the bathing solution, and therefore partially escapes hydrolysis, indirectly supporting the explanation that in the *in vivo* experiments ATP is immediately exposed to the degrading activity of ecto-ATPases.

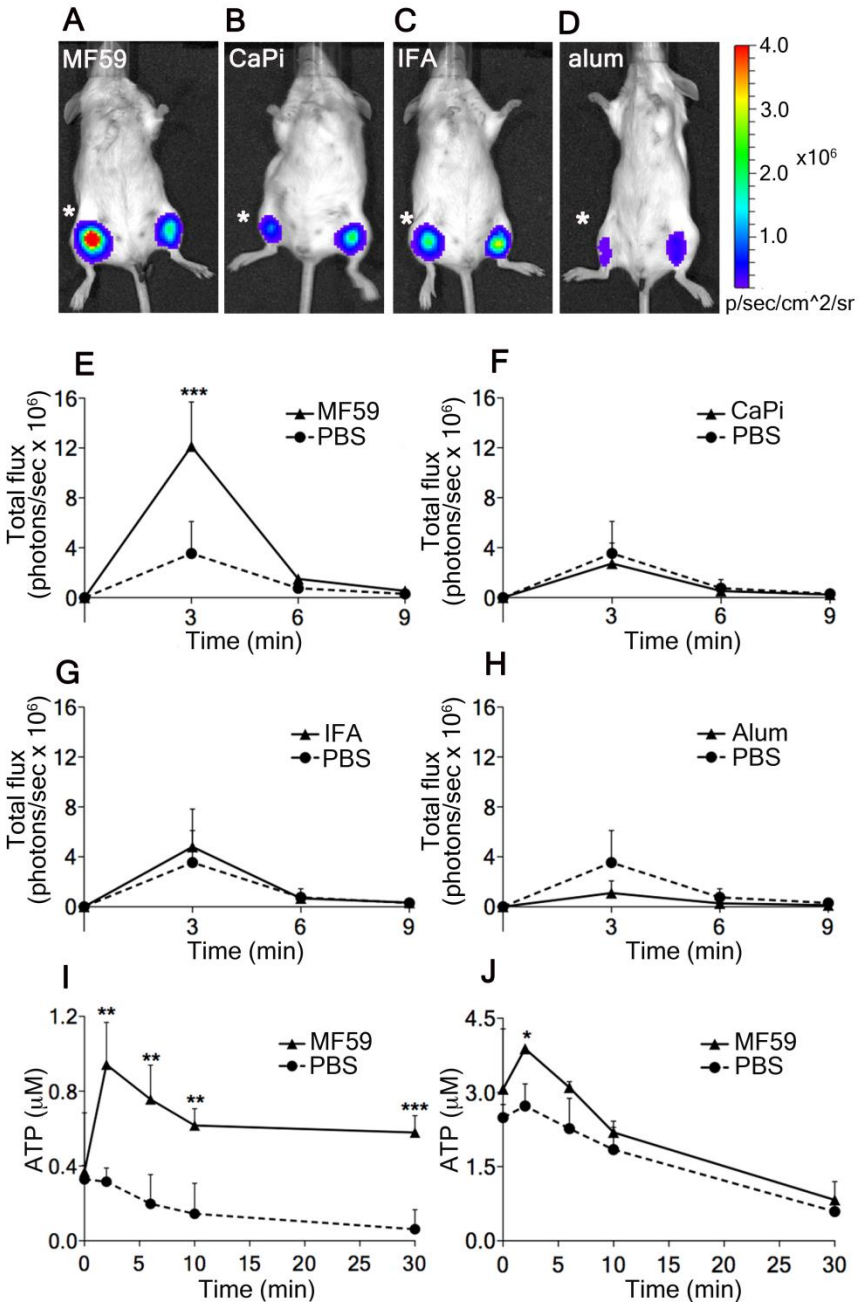

Fig. 3. Adjuvant-induced ATP release in mouse muscles. (***A-D***)**:** representative images taken 3 min after intramuscular injection of adjuvants (right hind limb; asterisk) or PBS (left hind limb) in Balb/c

mice together with the mixture luciferase-luciferin that reports on ATP changes. (***E-H***) corresponding quantitative analyses of chemiluminescence emission over time (number of photons/sec in the region of interest). (***A*** and ***E***) MF59 (40% v/v), (***B*** and ***F***) CaPi (50 μg), (***C*** and ***G***) IFA (40% v/v), (***D*** and ***H***) alum (100 μg); (***I*** and ***J)*** ATP release from *ex vivo* mouse muscles injected with MF59. Mouse *tibialis anterior* (***I***) and *quadriceps* (***J)*** muscles were exposed and injected with MF59 (40% v/v), continuous lines; the dotted line refers to the injection of the same volume of PBS. Muscles were rapidly removed and suspended in oxygenated buffer at 37°C. ATP released into buffer was quantified at the given time points using the luciferin-luciferase assay and a known ATP standard. Data show mean values + SD from at least four independent experiments. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$.

MF59 consist of small squalene oil droplets stabilized by the addition of two non-ionic surfactants, tween 80 and span 85, emulsified in citrate buffer. An examination of the chemical nature of MF59 nanoparticles, suggested that they could potentially interact with the phospholipid bilayer of cell membranes. In particular the two surfactants, located on the particle surface, are putative membrane active agents. We tested which of the individual ingredients of MF59 would be responsible for its ability to release ATP. Not surprisingly, ATP release is caused by tween 80 and span 85 but not by squalene oil alone (Fig. 4).

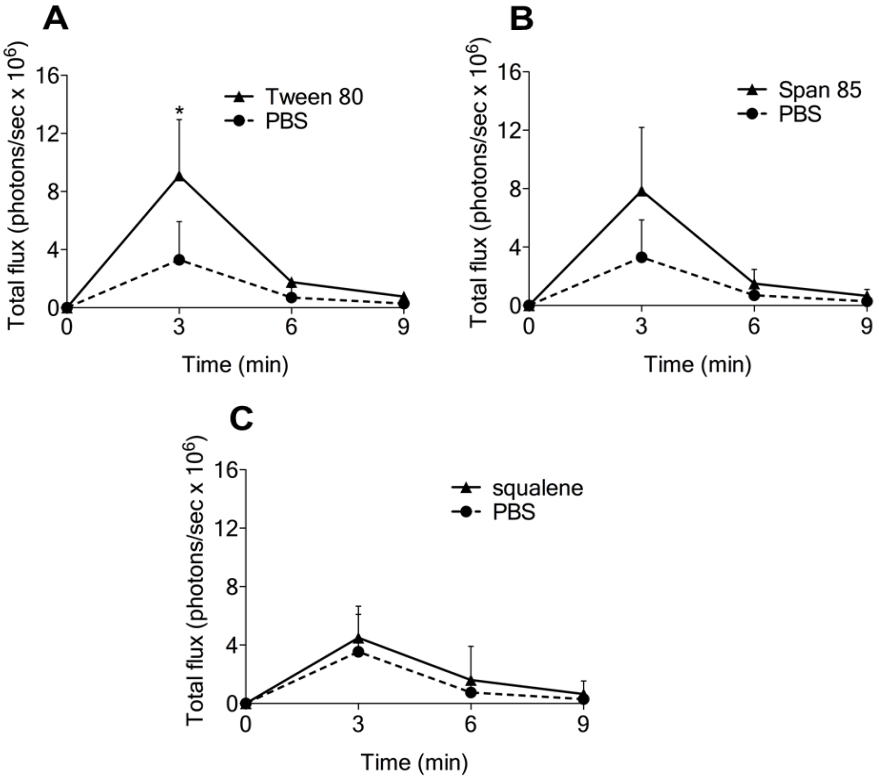

Fig. 4. Tween 80 and span 85 are responsible for the MF59-induced ATP release. ***A-C,*** quantitative analysis of light emission after imaging of BALB/c mice (5 mice/group) i.m. injected with (***A***) tween 80 (0.5% v/v), (***B***) span 85 (0.5% v/v) or (***C***) squalene (4.3% v/v) (left leg) and PBS (right leg). Chemiluminescence is reported as the number of photons per sec in the region of interest; Data show mean values + SD from at least four independent experiments. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$.

Taken together, our results clearly document that MF59 displays a unique capacity to greatly increase ATP release from injected muscles. To assess whether this ATP release would be essential for the adjuvant effect of MF59, we quenched extracellular ATP by co-injection of MF59 with apyrase, an enzyme that rapidly hydrolyzes ATP to AMP [102]. Figure 5 (panels *A* and *B*) shows that apyrase completely abolishes the MF59-induced ATP signal. On the basis of this result, we could proceed to determine whether co-injection of apyrase would alter induction of innate and adaptive immune responses by MF59.

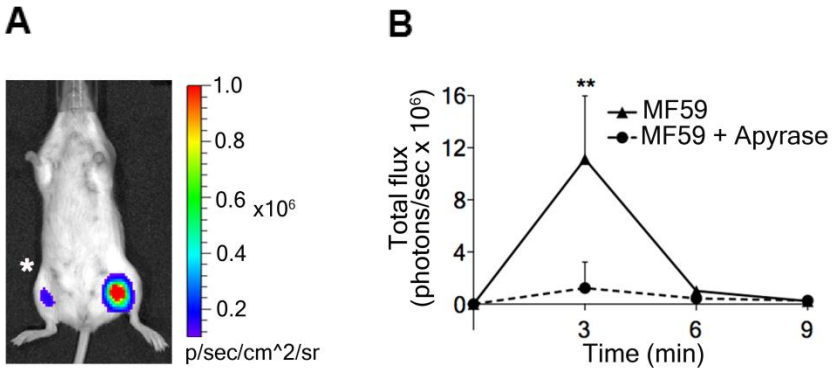

Fig. 5. MF59-induced ATP release in mouse muscles and the effect of apyrase (***A)*** representative image taken 3 min after intramuscular injection of MF59 (40% v/v) + apyrase (10U; asterisk) or MF59 alone (contralateral muscle) and (***B***) the corresponding quantitative analysis over time. Data show mean values + SD from at least four independent experiments. Unpaired, two-tailed Student's T-test (T): ** $P< 0.01$.

2.2 Immune cell recruitment induced by MF59 injection is inhibited by apyrase

Over the years, ATP has emerged as an important activator and modulator of immune responses, among several other danger molecules that are released from cells by a variety of pathogens of differing physical, chemical and biological nature [5, 6, 103]. In particular, it was shown that ATP released by stressed or dying cells promotes recruitment and activation of phagocytes. Therefore, we investigated whether and how ATP release could contribute to the activity of MF59.

The strong adjuvant-effect of MF59 [7, 8] has been ascribed to its capability to induce an immunocompetent environment in the muscle, characterized by a rapid and transient influx of a large number of CD11b+ immune cells participating in antigen uptake and transport to draining lymph nodes [9-11]. To clarify the role of ATP in MF59-induced cell recruitment, mice were injected i.m. with MF59 in the presence or absence of apyrase. After 24 hours, muscles were harvested and their content of neutrophils, monocytes, DCs and macrophages was determined by flow cytometry (the gating strategy is shown in Fig. 6). Co-injection of apyrase clearly lowered MF59-induced cell recruitment (Fig. 7), indicating that ATP release is in part responsible for cell influx. Yet, on the other hand, ATP by itself does not have an appreciable effect, which is true for both degradation-sensitive ATP and degradation-resistant ATPγS. However, this is not surprising, as the injection of a single ATP bolus does not reproduce the graded concentration of extracellular ATP that appears to be necessary to support chemotaxis [6, 104]. In addition, MF59 might induce the release of additional danger- or damage-associated signals and chemotactic factors that may synergize with ATP, as shown for example for mesenchymal stem cell responses to CXCL12 and ATP [105, 106]. In favor of this hypothesis is also the finding that injection of alum and IFA also resulted in a significant recruitment of CD11b+ cells in the injected muscle. Yet, for all adjuvants, cell influx was significantly lower as compared to MF59 (Fig. 7*E*) and reached levels similar to mice treated with MF59 and apyrase. At the same time co-injection of apyrase did not have any effect on alum- and IFA-mediated cell recruitment, consistently with the previous finding that they are not good ATP inducers in the muscle (Fig. 3*G* and *H*).

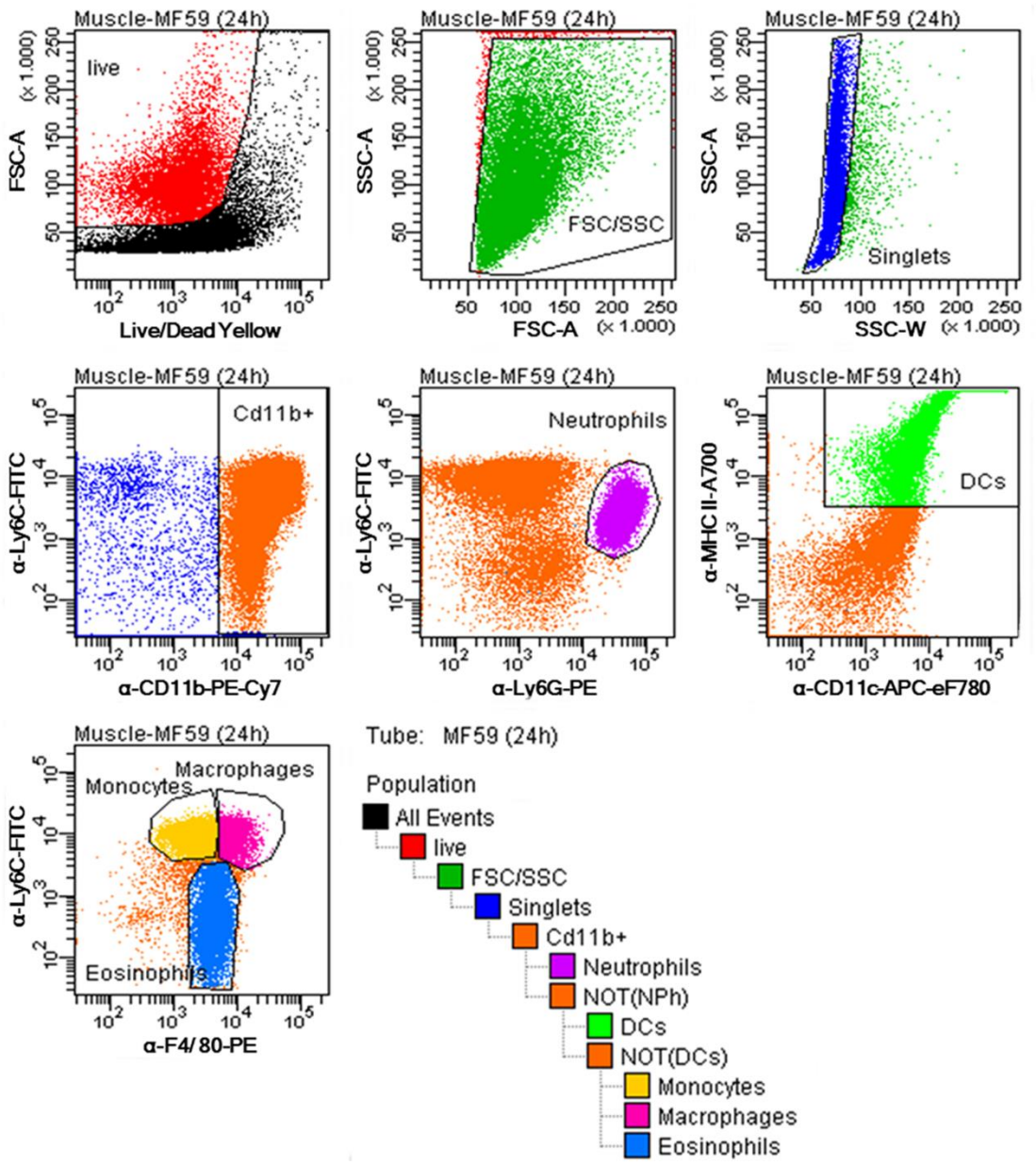

Fig. 6. Gating strategy of muscle derived cells. Muscle single cell suspensions were prepared and analyzed by FACS applying the depicted gating strategy.

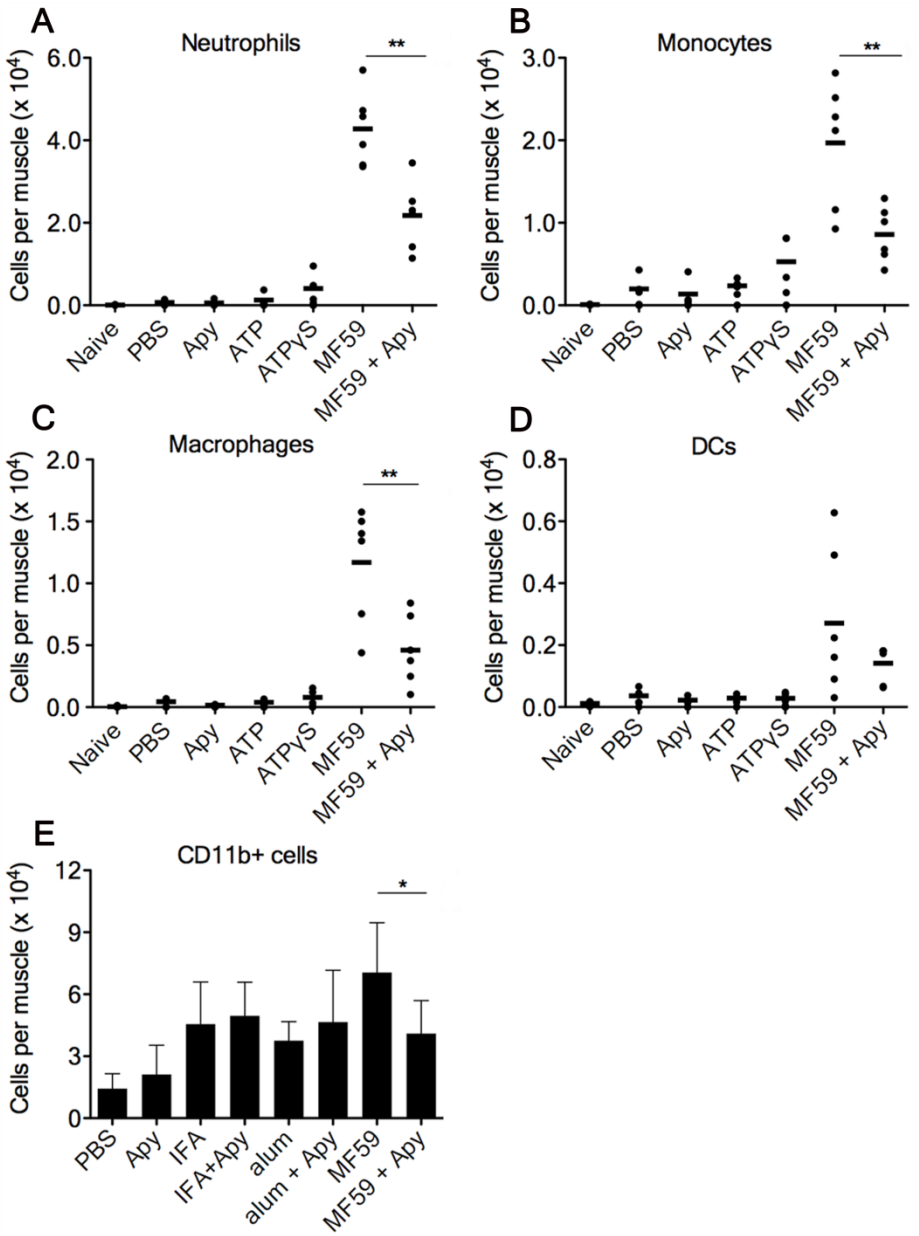

Fig. 7. Co-injection of apyrase reduces immune cell recruitment induced by MF59, but not by IFA or alum. (***A-D***) Groups of mice were injected i.m. with the indicated compounds, at the following doses: MF59 (40% v/v), ATP or ATP-γS (5mM), apyrase (Apy) (10U/ leg) or PBS. Single cell suspensions of treated muscles were analyzed by FACS 24 h post-injection. Dots show numbers of the respective cell type per individual muscle (N ≥4 per group), while black bars indicate arithmetic means. (***A***) Neutrophils, (***B***) monocytes, (***C***) macrophages, and (***D***) dendritic cells (DCs). (***E***) Groups of mice were injected i.m. with the indicated compounds, at the following doses: MF59 (20% v/v), IFA (40% v/v), alum (100 μg), apyrase (10U/ leg) or PBS, all in presence of Ovalbumin (10 μg/mouse). Numbers of CD11b+ cells are reported, data show mean values + SD from 8-12 muscles/group. The injection of all tested adjuvants results in a significant recruitment of CD11b+

cells in muscle when compared to PBS-inoculation. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$.

An important function of adjuvants is to enhance antigen-specific T cell priming and the production of antigen-specific, high affinity antibodies by B cells. These processes take place in the draining lymph nodes (LNs) and require the presence of an antigen. Therefore, we marked the cell types that may serve as major antigen vehicles and investigated whether a local ATP release at the muscle injection site could modulate antigen uptake and transport to draining lymph nodes. We used Ovalbumin-AF647 (OVA) as antigen. After intramuscular injection of different adjuvants and labeled OVA, we analyzed the antigen-positive cells in the draining inguinal lymph nodes of treated muscle 24 h post injection (Fig. 8). We found that only MF59 strongly enhances the number of OVA-positive cells in draining inguinal lymph nodes while alum, IFA and ATPγS did not when compared to OVA injection. At this timepoint we detected a significant number of OVA-positive B cells and different DC subsets in the draining LNs of mice immunized with MF59 (+/- apyrase). A lower number of OVA-positive monocytes, macrophages and neutrophils are also present.

Surprisingly, co-injection of apyrase and MF59 reduces the number of antigen positive cells and particularly the number of OVA+ B cells induced by MF59 (Fig. 8 *A* and *B*). It is known that free antigen that arrives in draining lymph nodes is sequestered by subcapsular sinus macrophages. Antigen-non specific B cells can then transport the antigen on their surface deeper into the node and transfer it to follicular DCs. The reduction of OVA-positive B cells observed after co-injection of MF59 and apyrase could have different reasons: ATP could either directly regulate the carrier activity of antigen-non specific B cells or exert effects up-stream on other cell types, such as sub-capsular macrophages. Recently, it was reported that ATP and products of ATP hydrolysis can modulate B cells functions with different effects on naïve and activated B cells such as T cell-B cell interactions [107]. Therefore we are wondering if B cells could be a key component in ATP-mediated signaling during vaccination.

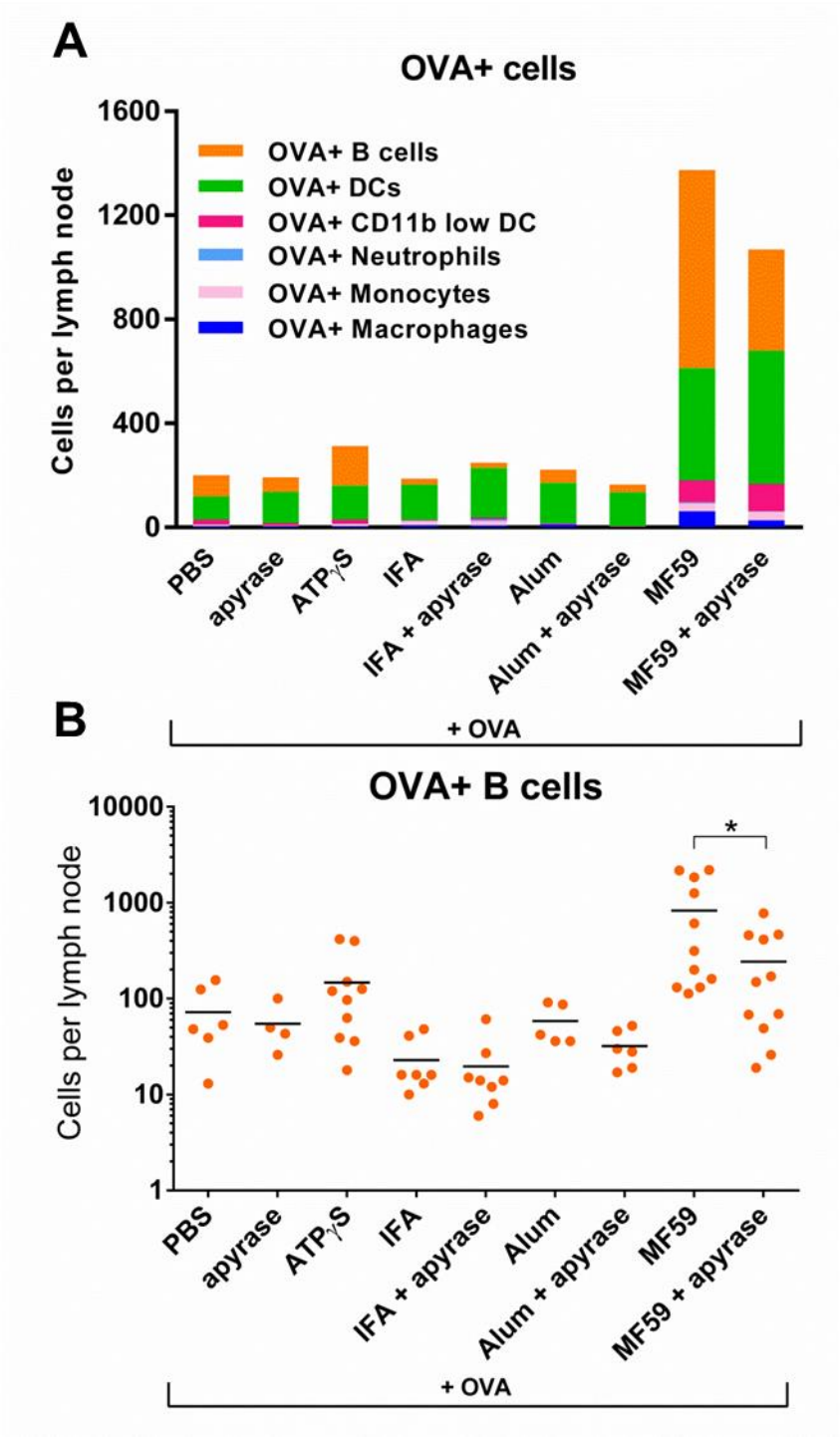

Fig. 8. Antigen-positive cells in draining inguinal lymph nodes. (***A-B***) Groups of mice were injected i.m. with the indicated compounds, at the following doses: MF59 (20% v/v), IFA (40% v/v), alum (100 μg), apyrase (10U/ leg) or PBS, all in presence of Ovalbumin (OVA-AF647; 10 μg/mouse). LNs that drain the treated muscle were analyzed by FACS 24 h p.i. to identify specific cells types and Ag-content. (***A***) Values represent the mean of at least 4 LNs for each group. (***B***) Dots show numbers of OVA+ B cells per individual lymph node (N ≥4 per group), while black bars indicate arithmetic means. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$.

2.3 ATP release contributes to adjuvanticity of MF59

A strong recruitment of immune cells at the injection site leads to enhanced antigen uptake and transport to draining lymph nodes, which translates into overall strongly enhanced adaptive immune responses [9, 10]. Accordingly, we assessed the impact of MF59-induced ATP release on $CD4^+$ T helper responses and antibody titers. Groups of mice were immunized with an experimental trivalent influenza vaccine (TIV) either as plain antigens or together with MF59 in presence or absence of apyrase. Control experiments showed that the injection of TIV antigens alone does not induce ATP release (Fig. 9).

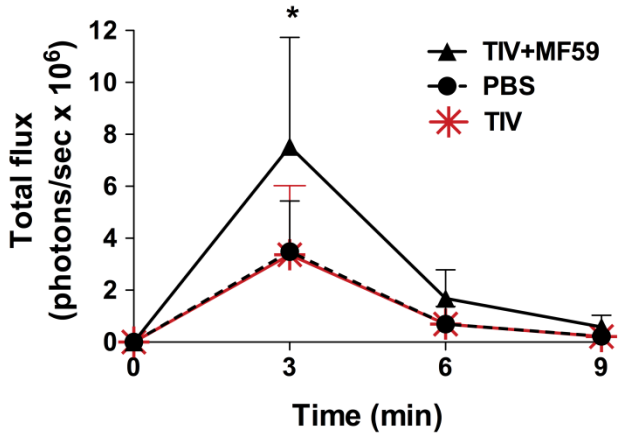

Fig. 9. The injection of a trivalent influenza vaccine (TIV) alone does not induce ATP release. Quantitative analyses of chemiluminescence emission over time (number of photons/sec in the region of interest). For each mouse, one leg was injected intramuscularly with a mixture composed of the reporter and TIV antigens (0.1 µg each antigen) alone while the contralateral leg was injected with the reporter solution plus PBS or TIV antigens adjuvanted with MF59 (20% v/v). Data show mean values + SD from 8-11 mice per experimental condition in independent experiments. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$.

We found that MF59-induced ATP release strongly contributes to adjuvanticity, both for T-helper cell responses and vaccine-specific antibody titers. Vaccine-specific T-helper cells were reactivated by *in vitro* stimulation of splenocytes from immunized

mice and assessed by FACS for intracellular cytokine expression; gating strategy and representative FACS blots are shown in Fig. 10.

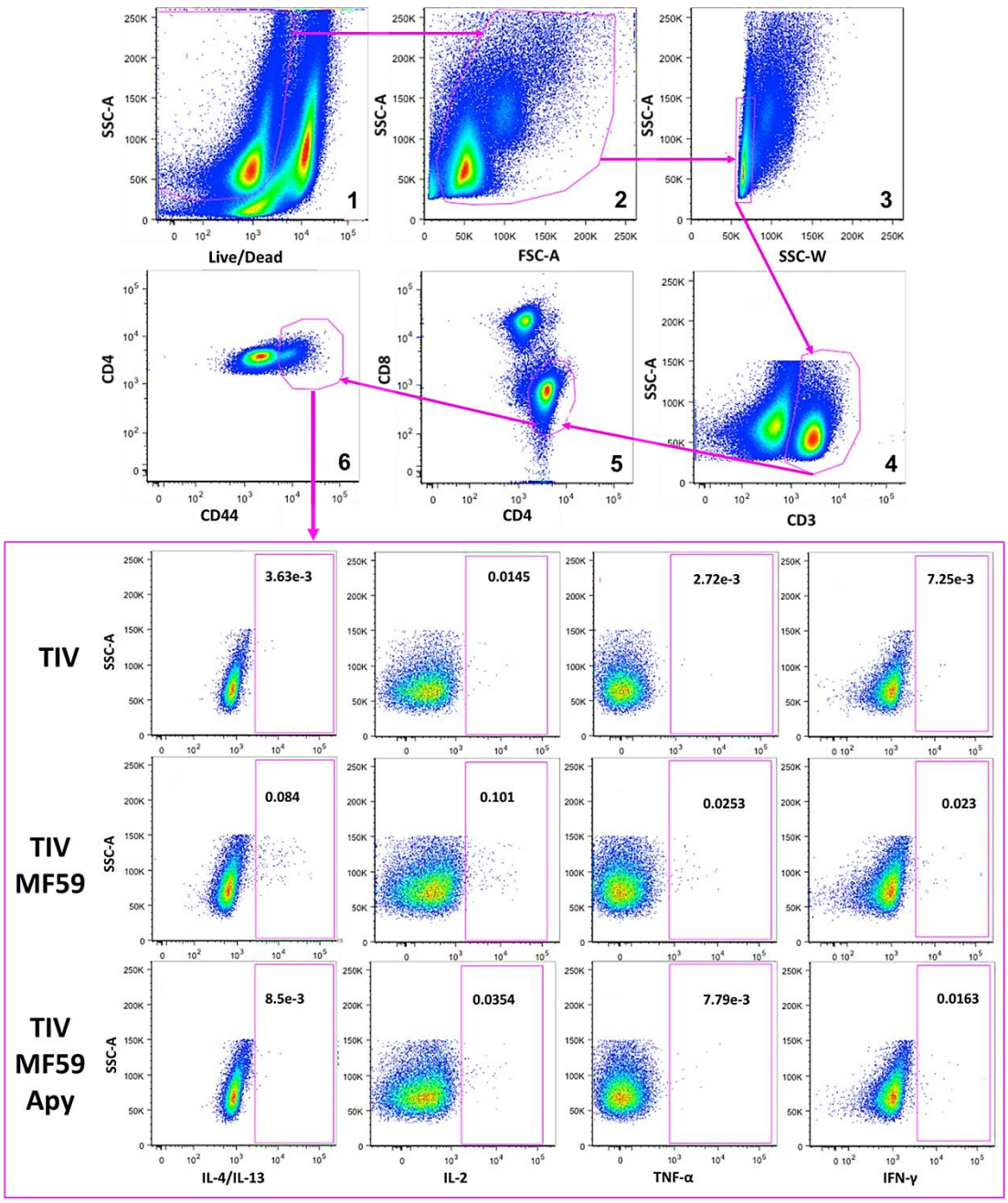

Fig. 10. Gating strategy of spleen derived cells. Spleens from 4 mice/group were taken 2 weeks after the first immunization and vaccine-specific T helper cells were re-activated by *in vitro* stimulation. CD4+/ CD44+ T helper cells were identified by the depicted gating strategy. Their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. Cytokine producing cells were identified (numbers inside the gate refers to cytokine-positive cells per

total CD4+ T cells) and multiple cytokine-expressing cells were calculated by Boolean gating. Shown are representative FACS blots from mice immunized with TIV, TIV+MF59 or TIV+MF59+apyrase, respectively.

MF59-adjuvanted vaccine induced high T cell responses already after the first immunization, but this effect was completely abolished by co-injection of apyrase (Fig. 11*A*). Similar results were observed after the booster dose (Fig. 11*B*). MF59 induced a mixed Th1/ Th2 profile, but apyrase-mediated abrogation of ATP signaling had more impact on Th2 (IL4/13+) responses as compared to Th1 (IFNγ+). This is even more evident after the booster vaccination.

MF59 significantly enhanced antibody titers against all three vaccine antigens as compared to TIV alone already after the first vaccination (Fig. 11*C*) with a mixed IgG1/ IgG2 profile (Fig. 12). The prominent adjuvant effect of MF59 not only leads to a large increase in total antibody titers, but also increased functional hemagglutination inhibition (HI) titers, that are considered a correlate of protection for influenza vaccinations (7, 8) (Fig. 11*E*). Apyrase-co-injection modestly reduced the antibody responses induced by the first injection of MF59-TIV. However the adjuvant effect of MF59 was still significant (Fig. 11*C* and *E*). Only after the booster immunization, the apyrase-mediated reduction of total IgG, IgG1 and HI titers became highly significant (Fig. 11*D*, *F* and Fig. 12).

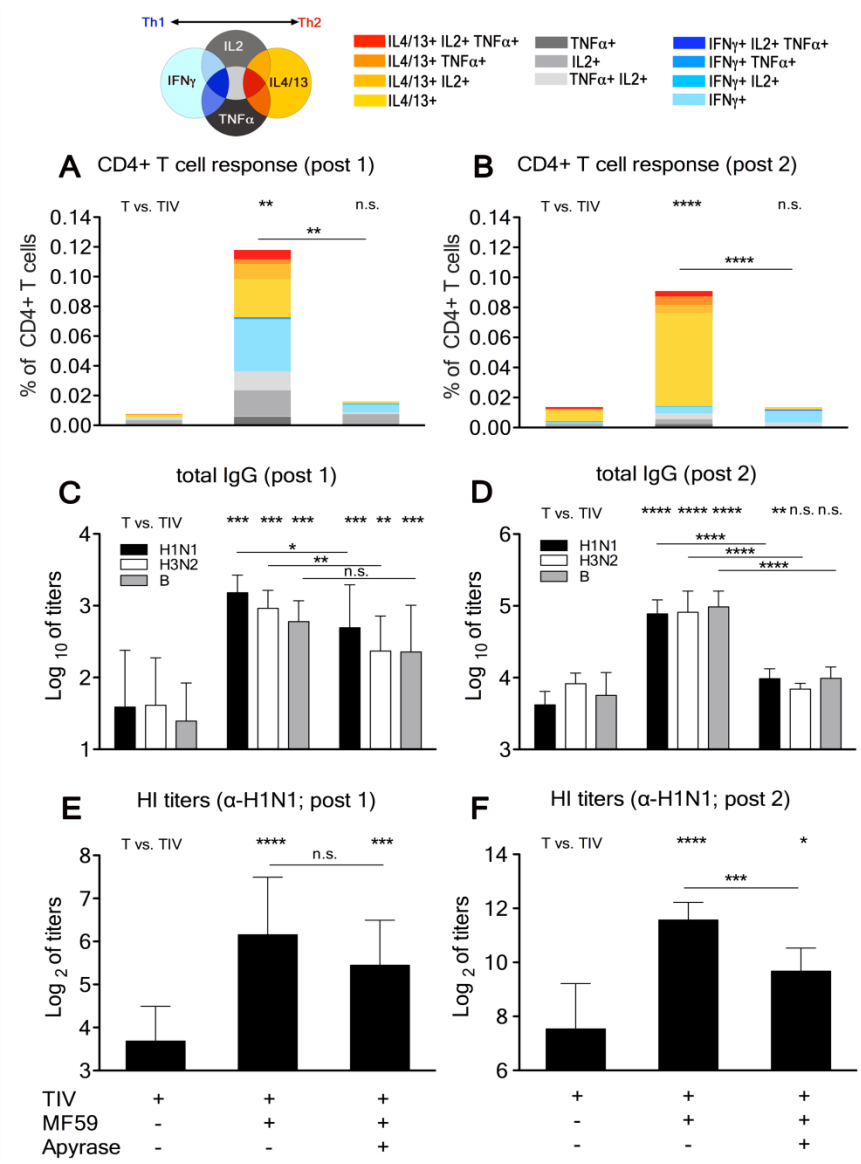

Fig. 11. Co-injection of apyrase inhibits adjuvanticity of MF59 to a trivalent influenza vaccine. (***A***-***F***) 12 mice/group were immunized twice (4 weeks apart) with a trivalent influenza vaccine (TIV) and adjuvants as indicated: MF59 (40% v/v), apyrase (10 U/ leg) and TIV (0.1 µg each antigen). (***A***, ***D***) Spleens from 4 mice/group were taken 2 weeks after each immunization and vaccine-specific $CD4^+$ T helper 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 after the first (***A***) and second (***D***) immunization, while the individual color code indicates the type of cytokines expressed by the respective cells as indicated in the figure legend. (***C*** -***F***) Serum samples were drawn two weeks after each immunization and vaccine-specific antibody titers were measured. Total IgG antibody titers towards H1N1/California, H3N2/Perth, and B/Brisbane after the first (***C***) and second (***D***) immunization. Values represent mean logarithmic titers (log 10) of 8-12 mice/group + SD. Hemagglutination inhibition titers towards H1N1/California after the first (***E***) and second (***F***) immunization; values represent means of Log2

titers of 8-12 mice/group + SD. Unpaired, two-tailed Student's T-test (T): **P*< 0.05,** *P*< 0.01, *** *P*<0.001,**** *P*<0.0001**.**

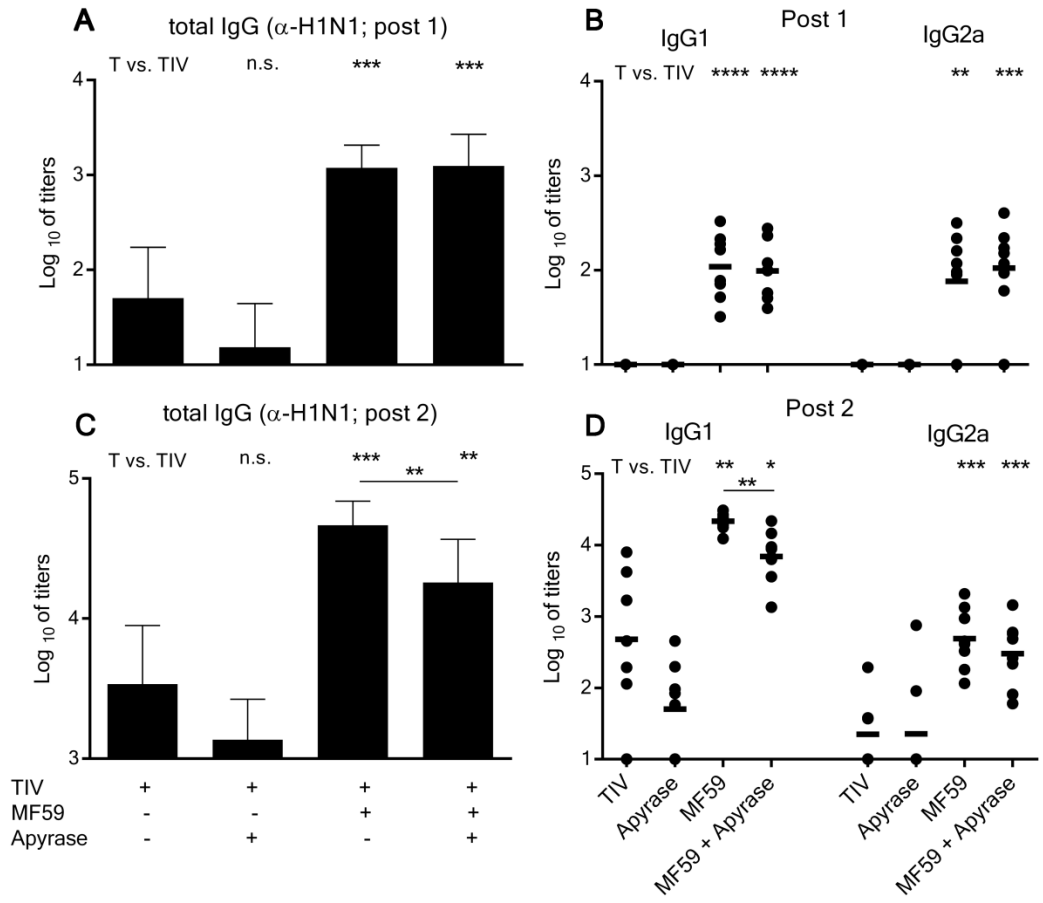

Fig. 12. Apyrase reduces vaccine-specific IgG1 levels induced by MF59. (***A-D***) Mice were immunized twice (4 weeks apart) using the different formulations indicated in the abscissae. The following doses were used: MF59 (20% v/v), apyrase (10 U/ leg) and a trivalent influence vaccine (TIV) (0.1 μg of each antigen). Serum samples were drawn two weeks after both immunizations and the H1N1-specific total IgG, IgG1 and IgG2a levels in sera were measured by ELISA after the first (***A*** and ***B***) and second (***C*** and ***D***) immunization. Values represent mean logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): * *P*< 0.05, ** *P*< 0.01, *** *P*<0.001, **** *P*<0.0001.

Similar results were observed using other antigens like ovalbumin (Fig. 13).

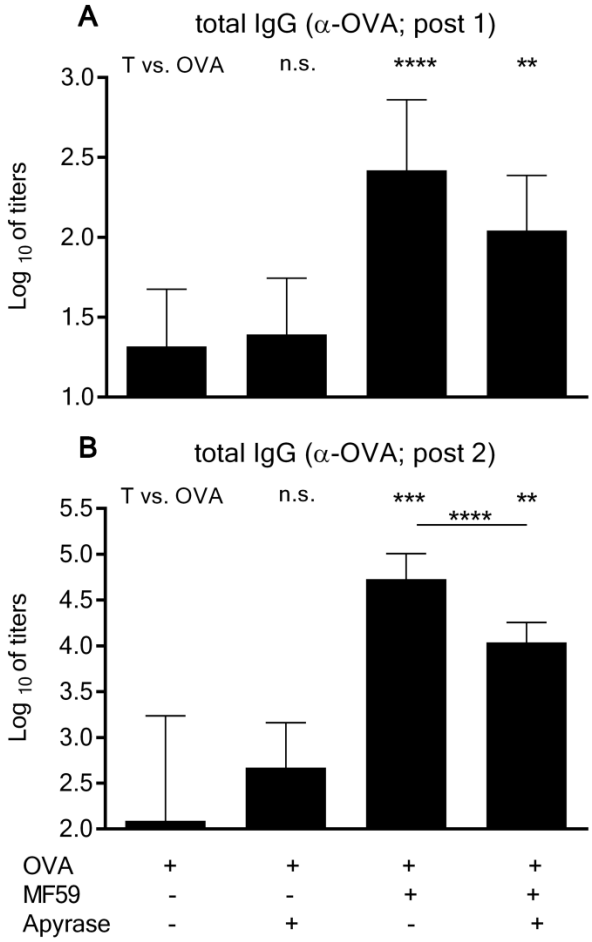

Fig. 13. Co-injection of apyrase reduces ovalbumin (OVA)-specific antibody titers enhanced by MF59. (***A*** and ***B***) Mice were immunized twice (4 weeks apart) with endograde OVA and the indicated compounds. The following doses were used: MF59 (40% v/v), apyrase (10 U/ leg) and OVA (10 μg/mouse). Serum samples were drawn two weeks after each immunization and OVA-specific antibody titers were measured by ELISA after the first (***A***) and second (***B***) immunization. Values represent mean logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): ** $P< 0.01$, *** $P<0.001$, **** $P<0.0001$.

We wondered whether the partial effect of apyrase after the first immunization with TIV-MF59 was due to the use of a high dose of adjuvant that cannot be blocked entirely. Therefore we titrated down MF59 from 40% (standard dose used here) to as

little as 2.5%. At all conditions tested, a significant reduction of antibody titers by apyrase was detected after the booster vaccination but not after the first dose (Fig. 14).

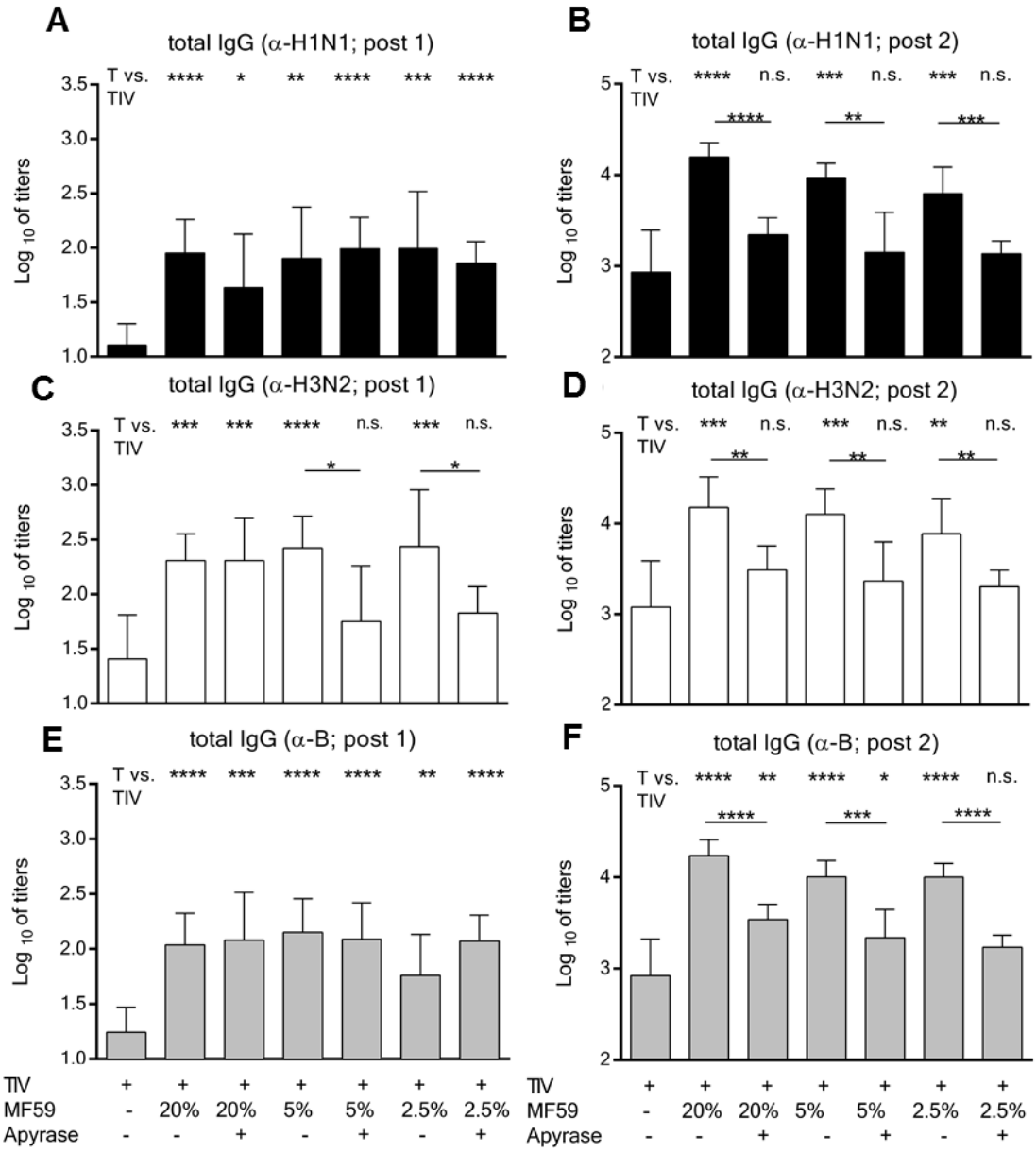

Fig. 14. Effect of apyrase on antibody responses induced by decreasing doses of MF59. (***A***-***F***) Mice were vaccinated with a trivalent influenza vaccine (TIV) and different doses of MF59 (20% v/v, 5% v/v and 2.5% v/v) with or without apyrase (10 U/leg). Serum samples were drawn two weeks after each immunization and the total IgG antibody titers towards (***A*** and ***B***) H1N1/California, (***C*** and ***D***) H3N2/Perth, and (***E*** and ***F***) B/Brisbane were measured by ELISA after the prime (post 1) and the booster vaccination (post 2). Values are the mean of logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$, **** $P<0.0001$.

In summary, our data demonstrate that ATP is required for CD4+ T cell responses induced by MF59 and for secondary antibody responses, but not for primary antibody responses.

In order to better dissect the effect of apyrase on MF59 adjuvanticity we performed an additional experiment using TIV. One group of mice received two doses of MF59+apyrase as before. In the other groups apyrase was added to MF59 only during prime or only during boost (Fig. 15). The group receiving MF59 plus apyrase twice had severely reduced Th cell responses after the first and the second vaccination as previously shown. Apyrase co-injection only during prime had a very significant effect on CD4 T cells responses after the first dose but only modestly reduced CD4 T cell frequencies after the second dose. Apyrase added only during boost did not have any significant effect on CD4 T cell frequencies (Fig. 15*B*). Antibody titers were significantly reduced in mice that received MF59+apyrase either during both immunizations or during the prime (Fig. 15*C*) while apyrase-addition during the booster dose had no significant effect.

Interestingly we did not detect any difference in antibody titers in the group of mice that received apyrase twice compared to the mice that received apyrase only during the prime. From this experiment, we concluded that the effect of apyrase on antibody responses induced by MF59 most likely resulted from an inhibition of T cell priming during the first vaccination.

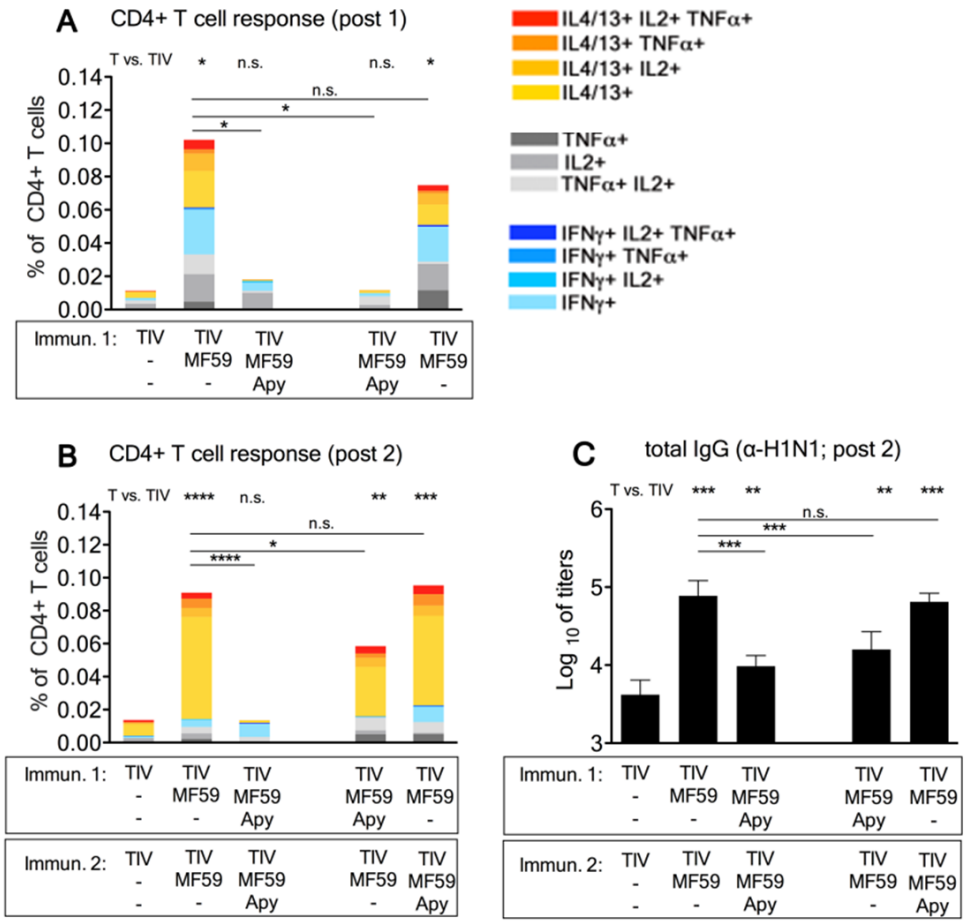

Fig. 15. ATP release induced by MF59 is essential during the first vaccination. Mice were immunized as before with a trivalent influenza vaccine (TIV) and adjuvants as indicated. One group of mice received two doses of MF59+apyrase while in the other groups apyrase (Apy) was added to MF59 only during prime or only during boost. (***A***, ***B)*** Spleens from 4 mice/group were taken 2 weeks after each immunization and vaccine-specific $CD4^+$ T helper cells were re-activated by *in vitro* stimulation as before. The bars show cumulative numbers of vaccine-specific cytokine expressing cells after the first (***A***) and second (***B)*** immunization, while the individual color code indicates the type of cytokines expressed by the respective cells. (***C)*** serum samples were drawn two weeks after the second immunization and total IgG antibody titers towards H1N1/California were measured by ELISA. Values represent mean logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$, **** $P<0.0001$.

To test this hypothesis, we performed adoptive transfer experiments of MF59-primed immune cells. We wanted to confirm whether primed T helper cells lead to the

greatly enhanced antibody titers at boost or whether other primed immune cell types or immune serum contributed to the effect.

Initially, we evaluated the possibility to transfer adaptive responses induced by vaccination to a naïve recipient injecting MF59-primed immune cells. Therefore, mice were vaccinated with a TIV plus MF59. Mice were then sacrificed 10 days after the prime; spleens and lymph nodes were collected and processed to obtain single immune cells suspensions. Cells were then resuspended in PBS and injected i.v. into naïve recipient mice. One group of recipient mice received only PBS as negative control. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks after the immunization sera were collected. Fig. 16 shows the total IgG antibody titers towards H1N1/California; mice that received MF59-primed cells both from spleens and LNs show higher total IgG titers when compared to mice that received PBS. Therefore, the priming effect induced by the first immunization with TIV plus MF59 can be transferred to naïve recipient mice injecting primed-immune cells.

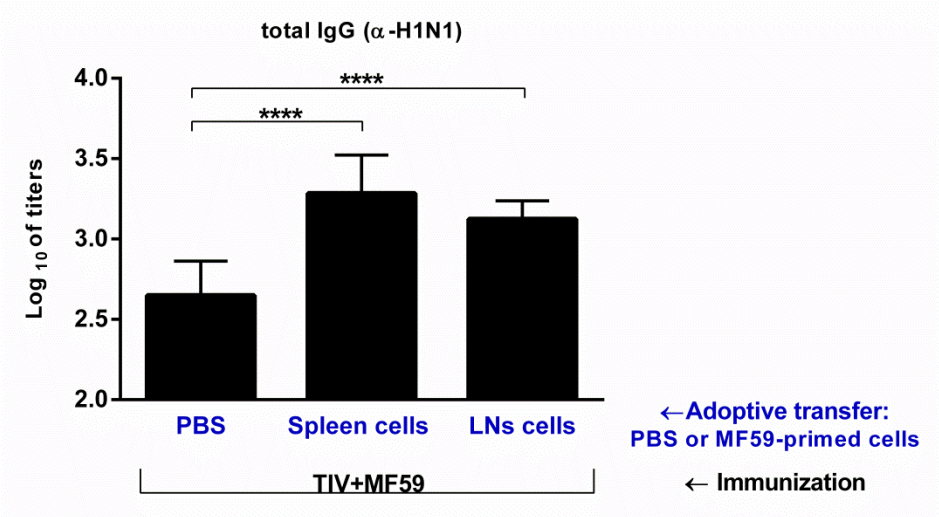

Fig. 16 MF59-primed immune cells can transfer adaptive responses induced by vaccination to a naïve recipient. Groups of 12 mice were vaccinated with a TIV plus MF59 and then 10 days after prime were sacrificed. Spleens and lymphnodes were collected and processed to obtain single immune cells suspensions then injected i.v. in naïve recipient mice. One group of recipient mice received only PBS as negative control. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks later serum samples were drawn and total IgG antibody titers towards H1N1/California were measured by ELISA. Values represent mean logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): **** $P<0.0001$.

To identify the role of the different immune cell types and antibodies on the priming effect, we separately transferred either I) serum (100 µl), II) whole CD3+ T cells or III) B cells from TIV plus MF59 immunized mice (12 mice/group) to naïve recipient mice (8 mice/group). $CD3^+$ T cell and B cell suspensions were prepared by magnetic separation using the MACS cell separation systems (Miltenyi Biotec) and $CD3^+$ T or B cell isolation kit (Miltenyi Biotec). The adoptive transfer was performed as described above. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks after immunization sera were collected. Fig. 17 shows the total IgG antibody titers towards H1N1/California. Mice that received MF59-primed CD3+ cells – but not primed B cells or immune serum - show higher total IgG titers when compared to mice that received PBS. Therefore, the priming effect induced by the first immunization with TIV plus MF59 can be transferred to naïve recipient mice injecting primed T cells. Previously, we speculated that a local ATP release induced by MF59 is essential during prime, to induce T cell responses that are important at the boost for a beneficial effect on antibody titers. Our results from adoptive transfer experiments clearly demonstrate the beneficial effect of primed T cell on the development of secondary responses after vaccination with a TIV-adjuvanted with MF59.

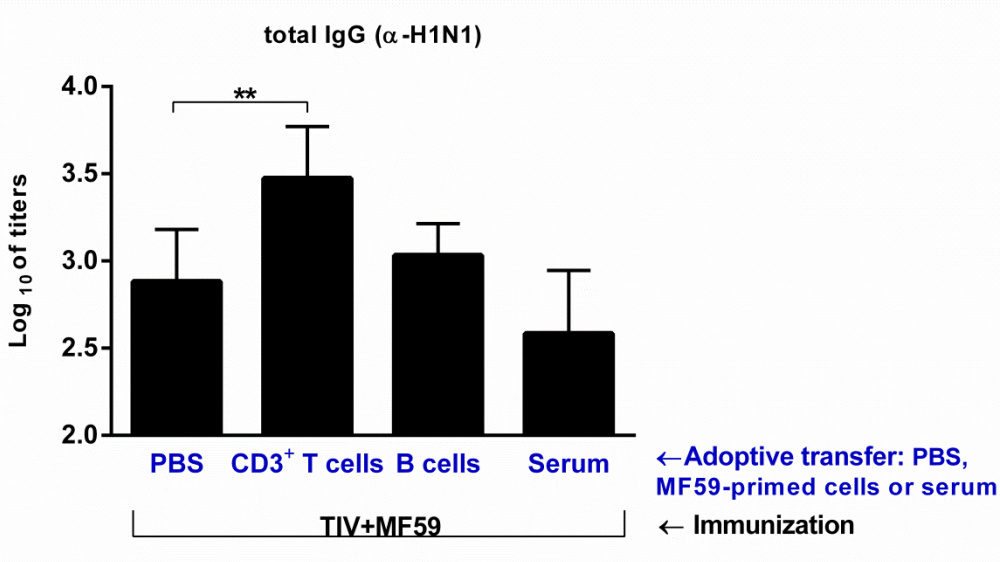

Fig. 17. MF59-primed $CD3^+$ T cells are responsible for the priming effect during vaccination with a TIV plus MF59. To identify the role of different immune cell types and antibody on priming

effect we separately transferred serum (100 µl), whole CD3+ cells and whole B cells from TIV plus MF59 immunized mice (12 mice/group) to naïve recipient mice (8 mice/group). The adoptive transfer experiment was performed as described above. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks later serum samples were drawn and total IgG antibody titers towards H1N1/California were measured by ELISA. Values represent mean logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): ** $P<0.01$.

From our experiments we concluded that adjuvant-induced ATP contributes to enhanced recruitment of immune cells into the injection site, to enhanced numbers of antigen-positive cells in draining lymph nodes – with a special focus of antigen-positive B cells – and to subsequent T cell priming. Primed CD4+ T cells then exert their prominent helper effect during the booster immunization.

After having dissected the chain of immune events induced by MF59 and the contribution of ATP to each individual response, we asked whether the effect of apyrase was specific for MF59 or whether it could be extended to other adjuvants (Fig. 18). We found that apyrase did not significantly reduce antibody responses generated by TIV antigens alone or in combination with IFA and alum, which do not stimulate ATP release in the muscle (Fig. 3, *C* and *D*). This is true both for primary antibody responses after the first dose (Fig. 18*A*) and secondary responses (Fig. 18*B* and Fig. 19*A*) as well as for functional HI titers (Fig. 19*B*). The analysis of T cell responses revealed that inhibition of antigen specific Th cells by apyrase is predominantly observed in the presence of MF59. Addition of apyrase to TIV antigens alone or formulated with IFA resulted in a non-significant reduction of vaccine-specific CD4 T cells. However, apyrase significantly reduced CD4 T cells induced by TIV formulated in alum (Fig. 18*C*). Apyrase mediated hydrolysis of the baseline level of extracellular ATP induced by injury might be responsible for the observed reduction of specific CD4 T cells in the groups that did not receive MF59 as adjuvant.

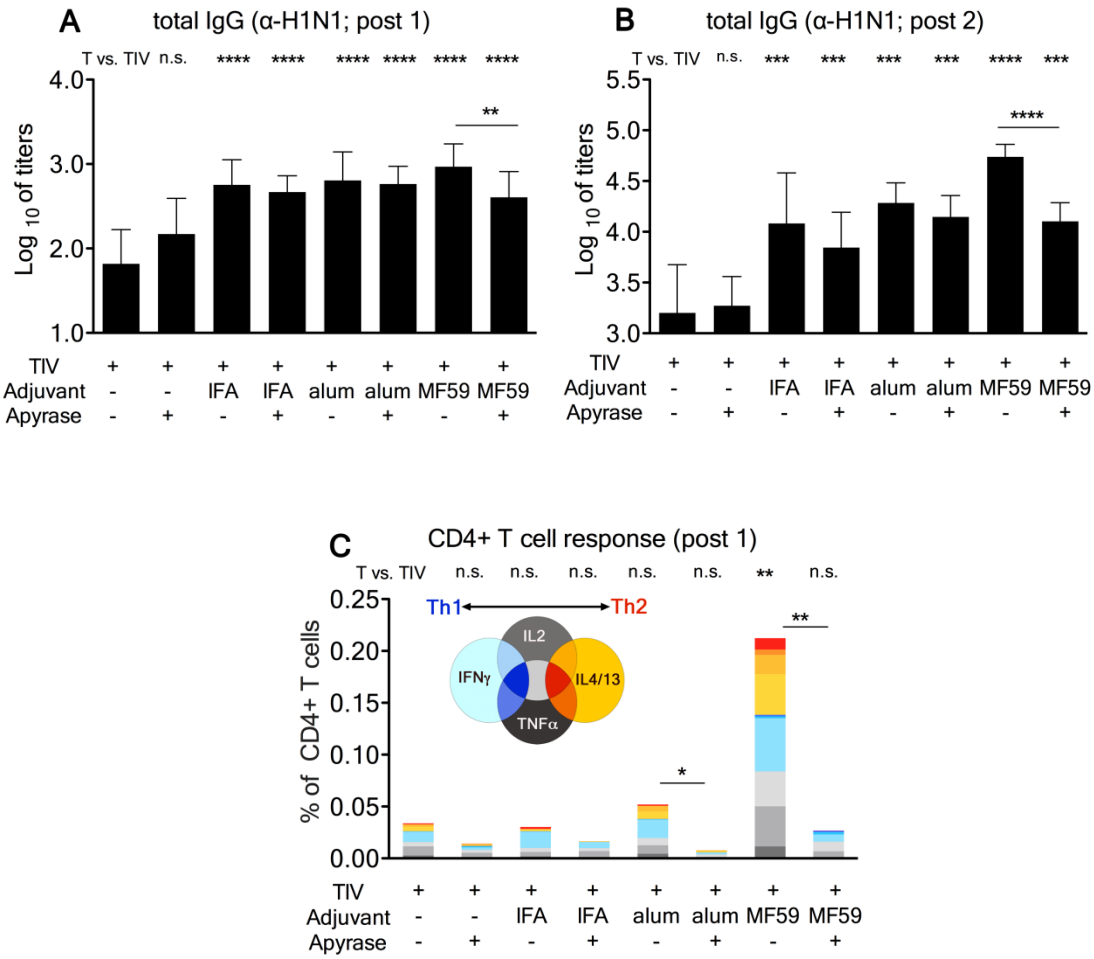

Fig. 18. Apyrase inhibits antibody responses induced by TIV adjuvanted with MF59, but not with alum and IFA. (***A-C***) 12 mice/group were immunized as before with a trivalent influenza vaccine (TIV) and adjuvants as indicated. The following doses were used: MF59 (40% v/v), IFA (40% v/v), alum (100 µg), apyrase (10 U/ leg) and TIV (0.1 µg each antigen). (***A***, ***B***) Serum samples were drawn two weeks after the first (***A***) or second (***B***) immunization and total IgG antibody titers towards H1N1/California were measured by ELISA. Values represent mean logarithmic titers (log 10) of 8-12 mice mice/group + SD. (***C***) Spleens from 4 mice/group were taken 2 weeks after the first immunization and vaccine-specific $CD4^+$ T helper 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 as indicated in the figure legend. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$, **** $P<0.0001$.

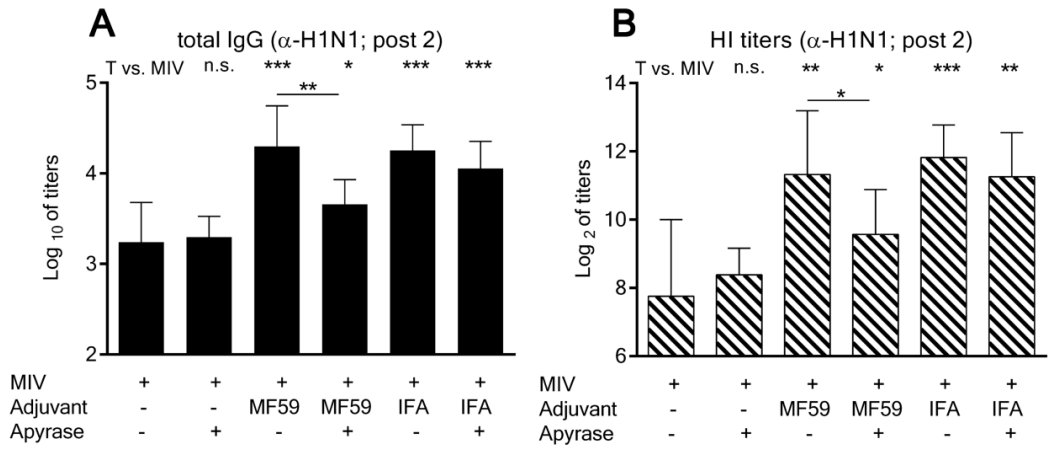

Fig. 19. Co-injection of apyrase abrogates adjuvanticity of MF59, but not of IFA. Mice were vaccinated with a monovalent influenza vaccine (MIV) and different adjuvants (MF59 20% v/v or IFA 40% v/v) with or without apyrase (10 U/leg). (***A***) Total IgG antibody titers towards H1N1/California. Values represent the mean logarithmic titers (log 10) of 8 mice/group + SD. (***B***) Hemagglutination inhibition titers towards H1N1/California; values represent means of Log2 titers of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): * $P< 0.05$, ** $P< 0.01$, *** $P<0.001$.

2.4 ATP by itself has not adjuvant effect

Our results clearly show that ATP release contributes to adjuvanticity of MF59. We questioned whether ATP by itself could induce a measurable adjuvant effect. Therefore we immunized mice with TIV and different concentrations of ATP or the hydrolysis resistant ATP-γS (Fig. 20, *A* to *C*). As expected, MF59 increased total IgG titers in response to all three influenza antigens, whilst ATP co-administration did not boost antibody responses at any concentration tested. The efficient immune modulation by ATP might depend on timely graded and local concentrations of ATP and/or on synergies with other alarmins released by MF59 injection. Probably the injection of ATP does not mimic the localized tissue release of ATP and of other factors induced by MF59 in the muscle.

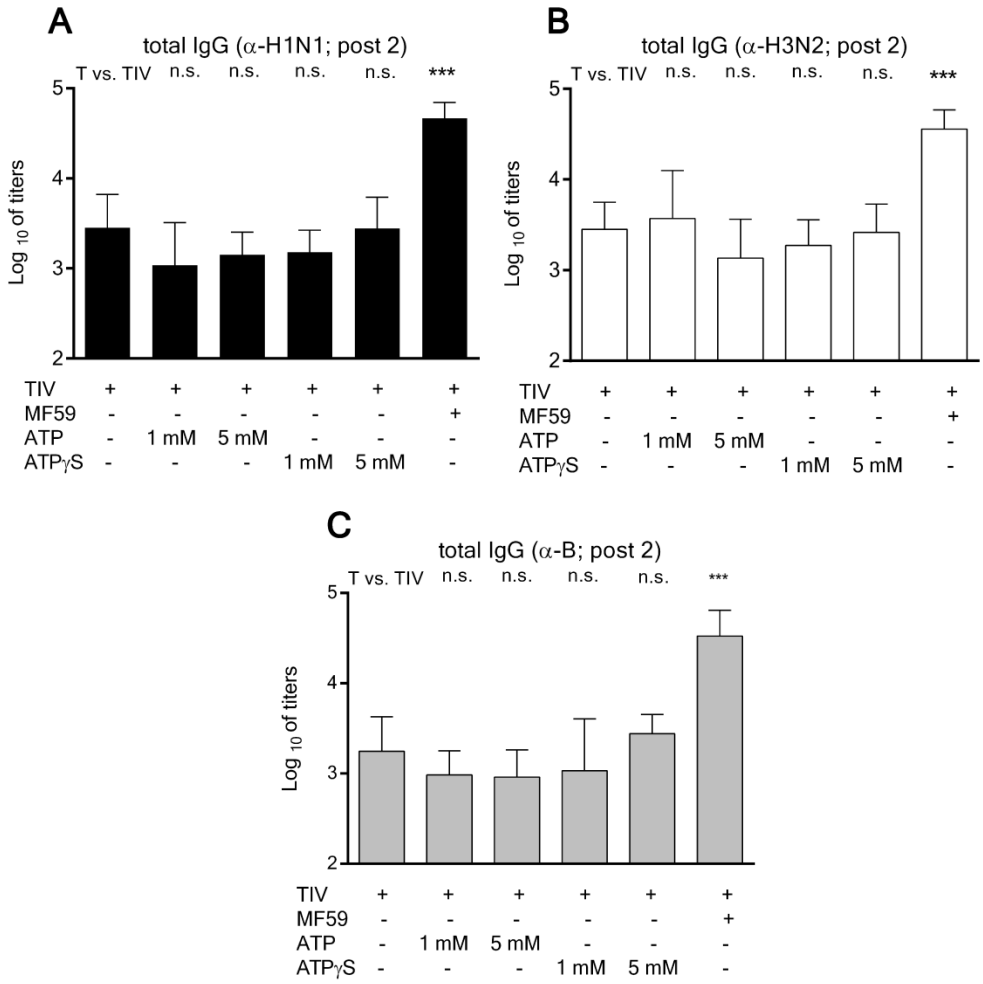

Fig. 20. Injection of ATP or ATP-γS does not have any adjuvant effect. Groups of mice were immunized twice with a 4-week interval using the different formulations indicated in the abscissae. The following doses were used: MF59 (20% v/v), ATP or ATP-γS (1mM and 5mM) and a trivalent influence vaccine (TIV) (0.1 μg of each antigen). Serum samples were drawn two weeks after the second immunization and the total IgG antibody titers towards (***A***) H1N1/California, (***B***) H3N2/Perth, and (***C***) B/Brisbane were measured by ELISA. Values are the mean of logarithmic titers (log 10) of 8 mice/group + SD. Unpaired, two-tailed Student's T-test (T): *** $P<0.001$.

2.5 Muscle cells could be the main target of MF59

Previous studies have indicated that MF59 injection induces activation of muscle fibers, as revealed by JunB translocation and Pentraxin 3 expression [11], suggesting that skeletal muscle fibers, with their high content of ATP, are a major target of MF59. To further investigate this possibility we assessed effects of MF59 on murine skeletal muscle C2C12 myotubes in culture. We found that MF59 induces a very rapid release of ATP from myotubes within minutes, in agreement with our data *in vivo* and *ex vivo* (Fig. 21 *A*). eATP targets P2 receptors. An important consideration is that the purinergic receptors of the P2X family are cation-selective channels [108]. ATP binding to a P2X receptor opens the channel and allows the transmembrane passage of K^+, Na^+, and Ca^{2+} along their concentration gradients [4,17]. As purinergic receptors are present in murine muscle cells [109, 110] this led us to consider the possibility that ATP released by muscle cells may bind to neighboring cells with a consequent alteration of the permeability of sarcolemma to ions. As ions flux/exchange across membranes modulate immune responses, we investigated these phenomena. Here, we show that MF59-treated myotubes rapidly release potassium. It would be interesting to evaluate a possible role of ATP in MF59-induced potassium efflux (Fig. 21 *B*). A very fast K^+ and ATP release from myotubes suggests that MF59 induces a rapid alteration of the permeability of sarcolemma. Recently, it was found that a cytosolic K+ efflux is sufficient to activate the NLRP3 inflammasome by bacterial toxins and particulate matter [111]. As MF59 works independently from NLRP3 inflammasome activation [91, 92] we propose that other events triggered by ATP and ions exchanges are required for its adjuvanticity.

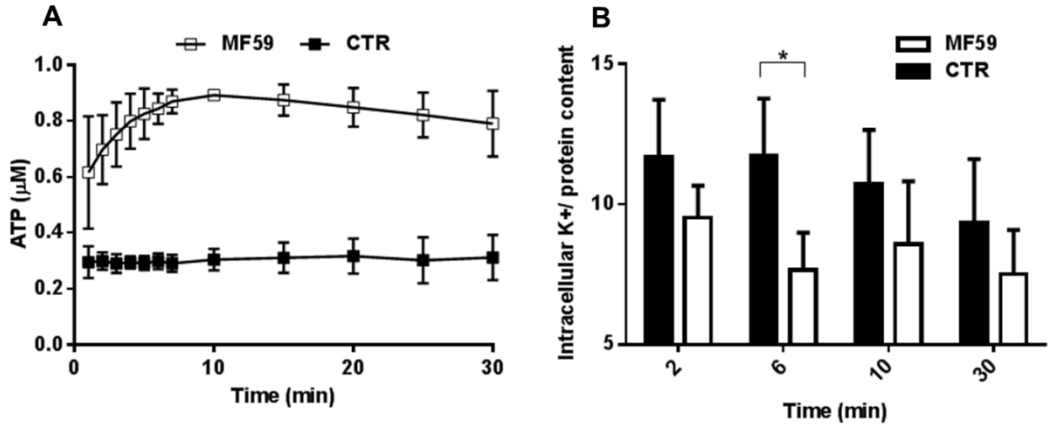

Fig. 21. MF59 induces release of ATP and K+ from murine C2C12 muscle cells. (***A***, ***B***) Cells were incubated with MF59 or PBS diluted in DMEM to a final dose of (50% v/v). At the indicated timepoints cells were rapidly washed twice with a choline buffer and then lysed in 120 μL of the same buffer containing 0.5% (w/v) of Triton X100. (***A***) Time course of ATP release in medium. Points are the average of values obtained in three different experiments ± SD. (***B***) The amount of intracellular K+ is reported, K+ levels are expressed as ppm (parts per million**)** normalized to the corresponding protein content (evaluated by BCA assay); values are the mean of 3 independent experiments + SD.

3. DISCUSSION

Taken together, our results provide strong evidence that ATP released from injected muscle is a crucial contributor to the adjuvant activity of MF59. The immunological step in which ATP is required is naïve T cell priming following the administration of the first dose of vaccine. Efficient T cell priming is required for an optimal antibody response after the boost. Our results from adoptive transfer experiments clearly demonstrate the beneficial effect of primed T cell on the development of secondary responses after vaccination with a TIV-adjuvanted with MF59. The effect of apyrase was more evident for CD4+ T cell responses in the Th2 compartment. Accordingly, we observed a strong reduction of IgG1 antibodies after the booster dose of MF59-TIV vaccine.

The data available at this stage do not address directly the question concerning which cells release ATP around the injection site. However, our preliminary results on C2C12 myotubes strongly suggest that skeletal muscle fibers, with their high content of ATP, are a major target of MF59 in agreement with previous studies that have indicated that MF59 injection induces activation of muscle fibers [11]. At variance from MF59, neither alum nor CaPi and IFA were found to increase ATP release from muscles over injection-induced background level. However, this negative result leaves open the possibility that other "danger signaling" molecule(s) could be involved in their adjuvant activities. Indeed, recent reports have implicated endogenous uric acid and DNA release in the adjuvanticity of alum [81- 83]. Similarly to the role of ATP for MF59, alum-driven DNA release has been implicated in the priming of naïve T cell after the first dose of vaccine and has only a partial effect on antibody responses, suggesting that additional mechanisms are involved in alum adjuvanticity [78, 79]. Even in the case of MF59, the results presented here indicate that ATP is a crucial contributor, but not the sole factor involved in MF59 adjuvanticity.

Exogenous ATP is active on an array of purinergic receptors that can modulate intracellular signaling. In particular, ATP is known to activate the NLRP3 inflammasome complex via the P2X7 receptor, inducing the maturation and

release of pro-inflammatory cytokines such as IL-1β [17, 32]. Interestingly, NLRP3 has also been linked to alum mechanism of action [78- 80] and therefore P2X7 may represent an obvious link between ATP and adjuvanticity. However, two independent studies have shown that the mechanism of MF59 is independent from NLRP3 inflammasome activation [91, 92] and therefore we propose that other events triggered by purinergic receptors are required for adjuvanticity. We have reported that MF59 adjuvanticity depends on signaling pathways that involve the protein MyD88 [91]. MyD88 is a common adaptor of most Toll like receptors (TLRs) and of IL-1 family cytokine receptors such as IL-1R, IL-18R or the IL-33R ST2. As MF59 does not trigger TLR signaling *in vitro*, it is more likely that MyD88 signaling downstream of IL-1 family receptors could be crucial for MF59 activity. Interestingly, it was shown that ATP-induced signaling via purinergic receptors does not only lead to inflammasome-dependent IL-1 release, but can also induce the inflammasome-independent release of IL-33 [112]. Recently it was shown that P2X7 receptor positively regulates MyD88-dependent NFκB activation and inflammatory signaling pathways [113]. It would be interesting to explore whether the strong adjuvant activity of MF59 is compromised in P2X7 KO mice. Additionally, it would be interesting to ascertain whether apyrase treatment in MyD88 KO mice might lead to total abrogation of all hallmarks of MF59 action, including generation of an immunocompetent environment, cell recruitment, antigen uptake, translocation and activation of adaptive immunity.

Furthermore, the specific reduction of OVA-positive B cells observed in the draining LNs after co-injection of MF59 and apyrase remains to be elucidated; different reasons could explain this phenomenon: ATP could either directly regulate the carrier activity of antigen-non specific B cells or exert effects up-stream on other cell types, such as sub-capsular macrophages. Confocal fluorescence microscopy of the draining lymph node at different times after injection could help us to elucidate how ATP-mediated pathways take part on the aforementioned immunological events.

Moreover, it would be fascinating to shed light on the molecular pathways modulated by ATP after vaccination with MF59, for example by performing

microarray analysis of the whole muscle injected with MF59 with or without apyrase.

This work supports the model that adjuvanticity involves induction of host molecules acting as danger signals and propose a new model for the mechanism of action of MF59 (Fig. 22). The latter activate the immune system to the advantage of a more sustained and protective immune reaction to vaccines.

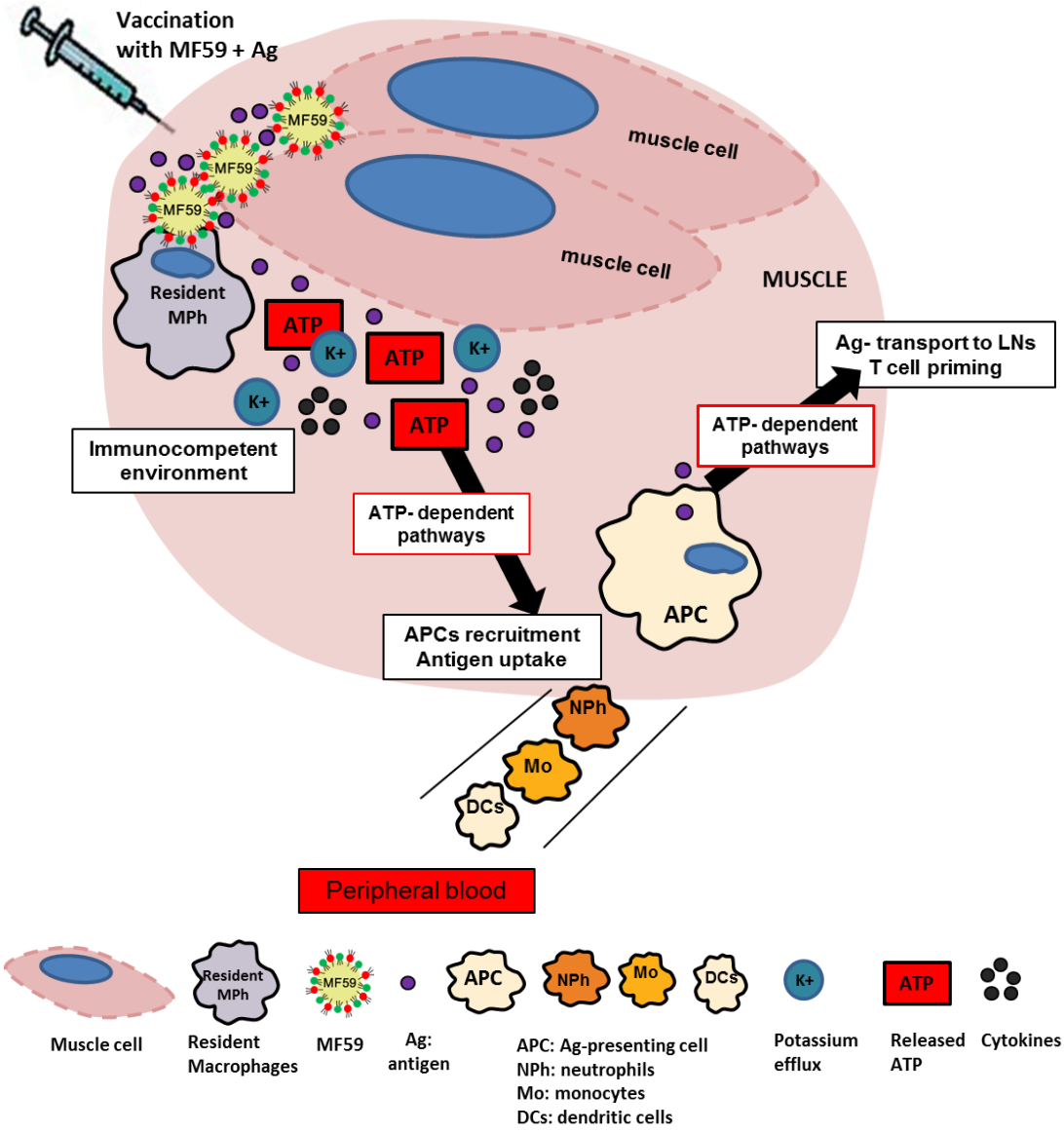

Fig. 22. New hypothetic model for the mechanism of action of MF59. Vaccination with MF59 induces a local release of endogenous danger signals, such as ATP and K+ and the production of cytokines and chemokines. These agents working together create a transient and local

immunocompetent environment at the muscle injection site. An increase in chemokines [9, 10] and ATP levels in the extracellular compartment leads to the influx of many immune cells from the peripheral blood into the muscle. Recruited cells can also produce chemokine creating an immune amplification loop. All recruited cells can take up the antigen and transport it to the draining lymph nodes to induce adaptive immune responses. We found that the MF59-induced ATP release is required for APCs recruitment, Ag uptake and transport to draining LNs. At last**,** but not least we found that ATP-mediated pathways are required for naïve T cell priming following the administration of a first dose of a trivalent influenza vaccine. These strong innate immune responses induced by MF59 translates into a higher numbers of Ag-specific effector T cells, such as antibody-titers and finally to greater vaccine potency. Our data demonstrate for the first time that a transient ATP release is required for innate and adaptive immune responses induced by MF59 although ATP-mediated pathways remain to be explored.

These findings lead to a large area of investigation to identify compounds that induce the most appropriate and advantageous danger signals boosting immune response to vaccines. We could show that ATP can contribute to a broad panel of immune events ranging from innate immunity to adaptive humoral and cellular responses, representing an attractive target to improve vaccine responses.

4. *MATERIALS & METHODS*

4.1 Mice

Pathogen-free BALB/c mice (purchased from Charles River) aged 6-8 weeks were used in this study in agreement with institutional and European guidelines. All experimental procedures involving animals were carried out in accordance with the Italian Animal Welfare Act and were approved by the local authority veterinary service at the University of Padova, Ferrara and at the Animal Ethical Committee of Novartis in Siena.

4.2 Adjuvants

MF59, a Novartis proprietary oil-in-water emulsion consisting of 4.3% (v/v) squalene, 0.5% (v/v) tween 80, 0.5% (v/v) span 85 in citrate buffer (10 mM), was prepared by homogenization at 12,000 psi with a Microfluidizer (model 110Y; Microfluidics, Newton, MA). The emulsion was sterilized and stored at 4 °C. The mean particle size of the emulsion droplets determined with a Mastersizer X (Malvern Instruments) was 194 ± 76 nm. Aluminium hydroxide was from Novartis, calcium phosphate from Brenntag Biosector, and Incomplete Freund's Adjuvant from Difco Laboratories. All tested adjuvants were diluted in PBS.

4.3 *In vivo* bioluminescence imaging

In vivo bioluminescent imaging was performed with an ultra low-noise, high sensitivity cooled CCD camera mounted on a light-tight imaging chamber (IVIS Lumina System, Caliper, Perkin Elmer). Tracking, monitoring and quantification of signals were controlled by the acquisition and analysis software Living Image. Mice were anesthetized with a continuous flux of isoflurane, positioned in the instrument chamber and injected with a 50 µl syringe fitted with a 29 gauge needle (Hamilton). For each mouse, one leg was injected i.m. with a mixture

composed of the reporter (luciferase-luciferin mix, Promega) and of the adjuvant to be tested: MF59 (40% v/v), alum (100 µg), CaPi (50 µg), and IFA (40% v/v), in a total volume of 20 µl. The individual components of MF59 (squalene, Span 85 and Tween 80) were formulated in PBS at the same dose as within the MF59 emulsion. The contralateral leg was injected with the reporter solution plus the PBS used for adjuvant dilution. One mouse per each experimental run was monitored immediately after injections; luminescent images were obtained with constant exposure time periods of 3 minutes for a total of 9 minutes; regions of interest were defined manually around the injection site to determine the total photon flux as number of photons/second. Experiments with apyrase were performed in the same conditions: for each mouse, one leg was injected with a mixture composed of the luciferase-luciferin mix plus MF59 (40% v/v) and apyrase (10U, Sigma) while the contralateral leg was injected with the reporter solution plus MF59 (40% v/v) alone. For all injections the final volume was 20 µl.

4.4 Muscle isolation and *ex vivo* ATP measurement

Mouse hind limb muscles (*tibialis anterior*, or *quadriceps*) were injected with 25 µl of MF59 (40% v/v) diluted in PBS in one leg or with the same volume of PBS in the contralateral one and then rapidly isolated from mice and immediately transferred to vials containing 1 mL oxygenated (95% O_2, 5% CO_2) physiological buffer (139 mM NaCl, 12 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KH_2PO_4, and 11 mM glucose, pH 7.4) at 37 °C. The ATP released in the buffer was determined with the ATPlite^{TM} luciferase assay (Perkin-Elmer). Briefly, the supernatant of control and treated muscles were collected at the indicated timepoint in a white 96-well plate, and mammalian lysis solution was added. The plate was shaken for 5 min at 700 rpm at RT. ATP substrate solution was added and a 5-min shake was performed in the dark. After 10 min, the luminescence was measured by Fluoroskan Ascent FL (Thermo Electron Corporation). ATP concentration was calculated from a calibration curve obtained using ATP standard solutions.

4.5 Cell recruitment into muscle, Ag-uptake and transport to draining lymph nodes

Groups of mice were injected with 25 µl/ muscle of either MF59 (40% or 20% v/v), alum (100 µg), and IFA (40% v/v), all with or without apyrase (10U/ muscle), with ATP-γS or ATP (5mM, Sigma), or PBS buffer control. Experiments were performed injecting either adjuvants alone or in presence of the model antigen ovalbumin (OVA-AF647; Molecular Probes, Invitrogen). 24 h post-injection, mice were sacrificed and quadriceps muscles were harvested and processed to obtain single cell suspensions. Briefly, muscles were placed into tissue culture dishes containing HBSS (Gibco) on ice. The muscles were cut into small pieces and digested with 0.05% type II collagenase (Worthington Biochemicals) in HBSS for 30 min at 37 °C under agitation. The cell suspension was centrifuged, resuspended in DMEM (Gibco) and filtered through a 70 µm nylon mash (Becton Dickinson). Cells were stained with Live/Dead Fixable Yellow (Invitrogen) and combinations of the following antibodies: α-Ly6C-FITC, α-CD11b-PE-Cy7, α-Ly6G-PE, α-CD3-PerCPCy5.5 (all from BD Pharmingen) and α-I-A/I-E-AlexaFluor700, α-F4/80-PacificBlue, α-CD11c-APC-eFluor780 (all from eBioscience). The stained cells were analyzed using a FACS LSR II Special Order System (BD Biosciences) using BD DIVA software (BD Biosciences). For Ag-uptake studies and transport to draining lymph nodes mice were injected i.m. as above with MF59 (40% v/v), alum (100 µg), IFA (40% v/v), all with or without apyrase (10U/ muscle), ATP-γS (5mM, Sigma), or PBS buffer control, all in presence of fluorescently labeled ovalbumin (OVA-AF647). Then, 24 h p.i. lymph nodes were collected, cut into small fragments and digested with collagenase D (0.8 mg, Roche) and DNase I (0.25 mg, Roche) in HBSS (Gibco) medium for 30 min at 37 °C under continuous agitation. Digested fragments were filtered through a 70 µm nylon mash, stained with fluorescently labeled antibodies as described above for muscle cell suspensions and analyzed by FACS.

4.6 Vaccine formulation and immunization

Experimental trivalent influenza vaccine 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 in immunogenicity experiments. The vaccine contains purified subunit antigens and is standardized for HA content by single-radial-immunodiffusion. For adjuvanticity experiments, groups of eight to twelve animals were immunized two times on days 0 and 28 in the quadriceps muscles of both hind legs with 25 µl vaccine/leg (50 µl total per mouse). Doses were 0.3 µg (0.1 µg each antigen) of either influenza soluble trivalent egg-derived antigen alone; antigens mixed with research grade MF59 (40% v/v), alum (100 µg) or IFA (40% v/v), all with or without apyrase (10 U/ muscle), apyrase alone, ATP-□S or ATP (1 mM or 5 mM). Serum samples of individual mice were collected 2 weeks after each immunization and evaluated for total immunoglobulin G (IgG) antibody titers by ELISA and hemagglutination inhibition (HI) titers by the hemagglutination inhibition assay. All formulations were optimized for pH and osmolality to physiological conditions. For some experiments, mice were immunized as described before with 10 µg/mouse Endograde ovalbumin (Hyglos).

4.7 ELISA

Serum samples were obtained two weeks after the first and second immunization and Ag-specific total IgG, IgG1 or IgG2a measured by ELISA. For flu specific ELISA, MaxiSorp plates (Nunc) were coated with 2 µg /ml of each antigen (H1N1 California, H3N2 Perth, B Brisbane) in PBS overnight at 4 ° C. For OVA-specific ELISA, we used MaxiSorp plates (Nunc), coated with 50 µg/ml of OVA (Hyglos) in coating buffer (Na_2CO_3/$NaHCO_3$, pH 9.6) overnight at 4°C. Plates were washed three times with 0.05% Tween in PBS and then blocked with FC Blocking (Candor Bioscience) for 2 h at RT, then washed as before and incubated for 1 h at RT with individual mouse sera. Plates were washed and incubated for 2 h at RT with secondary anti-mouse total IgG (Sigma). Plates were washed and P-

nitrophenyl phosphate disodium (Sigma) added. After 30 minutes the reaction was blocked with EDTA (3%, pH 8). Color reaction was measured with SpectraMax (Molecular Devices) reader by determining OD at 405 nm. The titers were normalized with respect to the reference serum assayed in parallel.

4.8 Determination of antibodies by Hemagglutination Inhibition assay

The Hemagglutination Inhibition (HI) assay was carried out on individual sera taken 2 weeks after the second immunization. Sera were pretreated with DENKA receptor destroying enzyme (RDE, Biogenetics) in a 1:3 volume ratio for 18 h at 37°C. Samples were inactivated at 56°C for 30 min and stored at 4°C until use. Before use the virus antigen was titrated using a 50 µl serial tenfold dilutions of the stock vial. The virus working dilution (i.e. dilution factor to reach 4 Hemagglutinating Units (HAU) in a 25 µl volume) was calculated by dividing by 8 the previously determined stock titer, and was checked daily before the assay by the 4 units test. Twofold serial dilutions of 25 µl pretreated sera and positive control sera were treated with 25 µl of virus working dilution. After gentle shake, samples were incubated at room temperature for 60 min. 50 µl of turkey red blood cells suspension were dispensed in each well and plates were again incubated RT for 60 min. The test endpoint was determined by visual inspection for an agglutination reaction: a red dot formation indicated a positive reaction (inhibition), whereas a diffuse patch of cells a negative reaction (hemagglutination). The titer was defined as the highest serum dilution at which hemagglutination was inhibited, and the antibody concentration corresponds to the reciprocal value of the titer.

4.9 *In vitro* re-stimulation of antigen-specific $CD4^+$ T cells

Four mice per group were sacrificed 2 weeks after each immunization and spleens were collected to assess the frequency and phenotype of Ag-specific $CD4^+/CD44^+$ T cells induced by vaccination. Spleens were processed to obtain single cell

suspensions, red blood cells lysed and the splenocytes cultured in RPMI (Gibco) containing 10% FCS (HyClone), beta-mercaptoethanol and antibiotics. 2 x 10^6 splenocytes/well were stimulated in the presence of α-CD28 antibody (1 μg/ml) (Becton-Dickinson) and trivalent influenza antigens (1 μg/ml each Ag; total 3 μg/ml), or with α-CD28 alone (negative control, <0.1% total cytokine-positive cells), or with α-CD28 plus α-CD3 (0.1 μg/ml) as positive control (Becton-Dickinson). After overnight stimulation, Brefeldin A (2.5 μg/ml)(Sigma–Aldrich) was added for additional 4 h to inhibit cytokine secretion. Cells were washed and stained with Live/Dead Fixable Yellow Dead Cell Stain Kit (Invitrogen). Cells were fixed, permeabilized, and stained with the following mAbs: α-CD8-PETexasRed (Invitrogen); α-CD3-PerCP-Cy5.5; α-CD44-v450; α-CD4-V500; α-TNF-α- AlexaFluor700; α-IFNγ-PE; and α-IL-2-APC (all Becton-Dickinson) α-IL-4-FITC; α-IL-13-FITC (e-Bioscience). Cells were then acquired using a FACS LSR II Special Order System (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). For each individual mouse, frequencies of influenza-specific T cells were calculated by subtracting the background measured in the corresponding negative control for each cytokine.

4.10 Adoptive transfer

Groups of 12 mice were vaccinated with a trivalent influenza vaccine (TIV) plus MF59 and then 10 days after prime were sacrificed. Spleens and lymph nodes were collected and processed to obtain single immune cells suspensions then injected i.v. in naive recipient mice. One group of recipient mice received only PBS as negative control. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks later serum samples were drawn to evaluate vaccine-specific total IgG antibody titers. We verified that MF59-primed immune cells could transfer adaptive responses induced by vaccination to a naive recipient mice. Therefore, to identify the role of different immune cell types and antibodies on priming effect we separately transferred serum (100 μl), whole CD3+ cells (T cells) and whole B cell from TIV plus MF59 immunized mice (12/group) to naive recipient mice (8/ group). Spleens and lymph nodes were processed to obtain single immune cells suspensions. $CD3^+$ cells and B cells suspensions were

prepared by magnetic separation using an autoMACS (Miltenyi Biotec) and $CD3^+$ T or B cell isolation kit (Miltenyi Biotec). Cells or sera were resuspended in PBS and injected i.v. in naive mice. One group of recipient mice received only PBS as negative control. The day after transfer, recipient mice were immunized with TIV plus MF59 and two weeks after immunization sera were collected to evaluate vaccine specific antibody titers.

4.11 C2C12 cell cultures

Murine skeletal muscle C2C12 cells were obtained from the American Type Culture Collection (CRL-1772; ATCC), and were maintained at subconfluent levels in DMEM (Gibco) supplemented with 10% FBS (EuroClone). To induce differentiation (5–6 d), cells were grown to 80% confluence and then the medium was replaced with DMEM supplemented with 2% equine serum (Gibco) and changed every 24–48 h.

4.12 Potassium measurement

Cell were incubated with MF59 or PBS diluted in DMEM to a final dose of (50% v/v). At the indicated timepoint cells were rapidly washed twice with a choline buffer (129 mM CholineCl, 1.5 mM CaCl2, 0.8 mM MgCl2, 5 mM H3PO4, 5 mM citric acid, and 5.6 mM glucose, pH 7.4) and then dissolved in 120 μL of the same buffer containing 0.5% w/v of Triton X100. Samples were diluted in bidistilled water and the K+ content was measured by flame photometry with a Perkin-Elmer Analyst atomic absorption photometer. K+ levels for each sample are normalized to the corresponding protein content evaluated by BCA assay.

4.13 Statistical analysis

All statistics were performed using GraphPad Prism software. The unpaired two-sample Student's T-test was used. *P* values less than 0.05 were considered significant.

5. BIBLIOGRAPHY

1. Rappuoli, R. & Aderem, A. A 2020 vision for vaccines against HIV, tuberculosis and malaria. *Nature* **473,** 463–469 (2011).
2. McKee, A. S., MacLeod, M. K. L., Kappler, J. W. & Marrack, P. Immune mechanisms of protection: can adjuvants rise to the challenge? *BMC Biol.* **8**, 37 (2010).
3. McKee, A. S., Munks, M. W. & Marrack, P. How do adjuvants work? Important considerations for new generation adjuvants. *Immunity* **27**, 687–90 (2007).
4. Burnstock, G. A. B. Physiology and pathophysiology of purinergic neurotransmission. *Physiol. Rev.* ***87***, 659–797 (2007).
5. Di Virgilio, F. Purinergic mechanism in the immune system: A signal of danger for dendritic cells. *Purinergic Signal.* **1**, 205–9 (2005).
6. Junger, W. G. Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* **11,** 201–12 (2011).
7. Wack, A. *et al.* Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine* **26**, 552–61 (2008).
8. Caproni, E. *et al.* MF59 and Pam3CSK4 boost adaptive responses to influenza subunit vaccine through an IFN type I-independent mechanism of action. *J. Immunol.* **188,** 3088–98 (2012).
9. Seubert, A., Monaci, E., Pizza, M., O'Hagan, D. T. & Wack, A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J. Immunol.* **180,** 5402–5412 (2008).
10. Calabro, S. *et al.* Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine* **29,** 1812–23 (2011).
11. Mosca, F., Molecular and cellular signatures of human vaccine adjuvants. *Proc Natl Acad Sci U S A.* **105,** 10501-6 (2008).

12. Ott, G., Barchfeld, G. L. & Van Nest, G. Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. *Vaccine **13***, 1557–1562 (1995).
13. Kumar, H., Kawai, T. & Akira, S. Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* **30,** 16–34 (2011).
14. Meylan, E., Tschopp, J. & Karin, M. Intracellular pattern recognition receptors in the host response. *Nature* **442,** 39–44 (2006).
15. Ogura, Y., Sutterwala, F. S. & Flavell, R. A. The inflammasome: first line of the immune response to cell stress. *Cell* **126,** 659–662 (2006).
16. Stojanov, S. & Kastner, D. L. Familial autoinflammatory diseases: genetics, pathogenesis and treatment. *Curr. Opin. Rheumatol.* **17,** 586–599 (2005).
17. Di Virgilio, F. Liaisons dangereuses: P2X(7) and the inflammasome. *Trends Pharmacol. Sci.* **28,** 465–472 (2007).
18. Schenten, D. & Medzhitov, R. The control of adaptive immune responses by the innate immune system. *Adv. Immunol.* **109,** 87–124 (2011).
19. Janeway, C. A. Jr, Travers, P., Walport, M., & Shlomchik, M. J. Immunobiology: The Immune System in Health and Disease. 5th edition. Garland Publishing, New York and London (2001).
20. Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12,** 991–1045 (1994).
21. Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* **81**, 1-5 (2007).
22. Harris, H. E. & Raucci, A. Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep*. **7,** 774-8 (2006).
23. Oppenheim, J. J. & Yang, D. Alarmins: chemotactic activators of immune responses. *Curr. Opin. Immunol.* **17**, 359-65 (2005).
24. Saïd-Sadier, N. & Ojcius, D. M. Alarmins, inflammasomes and immunity. *Biomed. J.* **35,** 437–49 (2012).
25. Ralevic, V. & Burnstock, G. Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50,** 413–492 (1998).
26. Gordon, J. L. Extracellular ATP: Effects, sources and fate. *Biochem. J.* **233**, 309–19 (1986).

27. Robson, S. C. *et al.* Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J. Exp. Med.* **185,** 153–163 (1997).
28. Lazarowski, E. R., Homolya, L., Boucher, R. C. & Harden, T. K. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J. Biol. Chem.* **272,** 24348–24354 (1997).
29. Bours, M. J. L., Swennen, E. L. R., Di Virgilio, F., Cronstein, B. N. & Dagnelie, P. C. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol. Ther.* **112,** 358–404 (2006).
30. Bours, M. J. L., Dagnelie, P. C., Giuliani, A. L., Wesselius, A. & Di Virgilio, F. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front. Biosci. (Schol. Ed).* **3,** 1443–1456 (2011).
31. Kahlenberg, J. M. & Dubyak, G. R. Mechanisms of caspase-1 activation by P2X7 receptor-mediated K+ release. *Am. J. Physiol. Cell Physiol.* **286,** C1100–C1108 (2004).
32. Di Virgilio, F. The therapeutic potential of modifying inflammasomes and NOD-like receptors. *Pharmacol. Rev.* **65,** 872–905 (2013).
33. Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440,** 228–232 (2006).
34. Pelegrin, P. & Surprenant, A. Pannexin-1 mediates large pore formation and interleukin-1b release by the ATP-gated P2X7 receptor. *The EMBO Journal* **25**, 5071–5082 (2006).
35. Kanneganti, T. D. *et al.* Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* **26,** 433–443 (2007).
36. O'Hagan, D. T., Singh, M. & Ulmer, J. B. Microparticle-based technologies for vaccines. *Methods* **40,** 10–19 (2006).
37. Plotkin, S.A. History of Vaccine Development. Springer, New York (2011).
38. Siegrist, C. A. Vaccine immunology. In: Plotkin, S. A., Orenstein, W. A., Offit, P. A., eds. Vaccines. 5th edition. Saunders Elsevier, Philadelphia, PA (2008).

39. Pashine, A., Valiante, N. M., Ulmer, J. B. Targeting the innate immune response with improved vaccine adjuvants. *Nat Med*. **11**, S63-8 (2005).
40. Amsen, D., Backer R. A. & Helbig C. Decisions on the Road to Memory. In: Katsikis, P.D., Schoenberger S.P. & Pulendran B. Crossroads Between Innate and Adaptive Immunity IV. Springer (2013).
41. Finley, J.H., Jr. The Complete Writings of Thucydides: The Peloponnesian War. Modern Library, New York (1951).
42. Jenner, E. On the origin of the vaccine inoculation. Shury, London, UK (1801).
43. Breman, J. G. & Arita, I. The confirmation and maintenance of smallpox eradication. *N. Engl. J. Med*. **303**, 1263–1273 (1980).
44. Koff, W. C. *et al*. Accelerating next-generation vaccine development for global disease prevention. *Science* **340** (6136), 1232910 (2013).
45. Houghton, M., & Abrignani, S. Prospects for a vaccine against the hepatitis C virus. *Nature* **436**, 961–966 (2005).
46. Johnston, M. I., & Fauci, A. S. An HIV vaccine—Evolving concepts. *N. Engl. J. Med*. **356**, 2073–2081 (2007).
47. Langhorne, J., Ndungu, F.M., Sponaas, A.M., & Marsh, K. Immunity to malaria: More questions than answers. *Nat. Immunol*. **9**, 725–732 (2008).
48. McMichael, A. J., Borrow, P., Tomaras, G. D., Goonetilleke, N. & Haynes, B. F. The immune response during acute HIV-1 infection: Clues for vaccine development. *Nat. Rev. Immunol.* **10**, 11–23 (2010).
49. Skeiky, Y. A., & Sadoff, J. C. Advances in tuberculosis vaccine strategies. *Nat. Rev. Microbiol*. **4**, 469–476 (2006).
50. Walker, L. M. *et al*. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* **326**, 285–289 (2009).
51. Burton, D. R. Antibodies, viruses and vaccines. *Nat. Rev. Immunol*. 2, 706–713 (2002).
52. Whitehead, S. S., Blaney, J. E., Durbin, A. P. & Murphy, B. R. Prospects for a dengue virus vaccine. *Nat. Rev. Microbiol*. **5**, 518–528 (2007).
53. Sallusto, F., Lanzavecchia, A., Araki, K. & Ahmed, R. From Vaccines to Memory and Back. *Immunity* **33**, 451–463 (2010).

54. Beverley, P. C. Immunology of vaccination. *Br. Med. Bull.* **62,** 15–28 (2002).
55. "What is Polio". Canadian International Immunization Initiative. p. 3. Retrieved 9 May (2007).
56. Offit, P. A. The Cutter Incident: How America's First Polio Vaccine Led to a Growing Vaccine Crisis. Yale University Press, New Haven/London, Pages 240pp (2005).
57. Rappuoli, R. Reverse vaccinology. *Curr. Opin. Microbiol.* 3: 445– 450 (2000).
58. Cozzi, R., Scarselli, M. & Ferlenghi, I. Structural vaccinology: a three-dimensional view for vaccine development. *Curr. Top Med. Chem.* **13**(20):2629-37 (2013).
59. Moyle, P. M. & Toth, I. Modern subunit vaccines: development, components, and research opportunities**.** *Chem. Med. Chem*. **8**(3):360-76 (2013).
60. Tritto, E., Mosca, F. & De Gregorio, E. Mechanism of action of licensed vaccine adjuvants. *Vaccine* **27,** 3331–3334 (2009).
61. O'Hagan, D. T., Ott, G. S., Nest, G. V., Rappuoli, R. & Giudice, G. D. The history of MF59(®) adjuvant: a phoenix that arose from the ashes. *Expert Rev Vaccines*, **12**(1), 13-30 (2013).
62. Ramon, G. Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de sérum antidiphtérique. *Bull. Soc. Centr. Med. Vet*., **101**, 227–234 (1925).
63. Glenny, A., Pope, C., Waddington, H. & Wallace, U. J. The antigenic value of toxoid precipitated by potassium alum. *Pathol. Bacteriol*., **29**, 38–45 (1926).
64. Coffman, R. L., Sher, A. & Seder, R. A. Vaccine adjuvants: putting innate immunity to work. *Immunity* **33**, 492–503 (2010).
65. Speiser, D. E. *et al*. Rapid and strong human CD8þ T cell responses to vaccination with peptide, IFA, and CpG oligonucleotide 7909. *J. Clin. Invest*. 115:739–46 (2005).
66. Moss, R. B., Diveley, J., Jensen, F. & Carlo, D. J. *In vitro* immune function

after vaccination with an inactivated, gp120-depleted HIV-1 antigen with immunostimulatory oligodeoxynucleotides. *Vaccine* 18: 1081–7 (2000).

67. Aggerbeck, H. & Heron I. Adjuvanticity of aluminium hydroxide and calcium phosphate in diphtheria-tetanus vaccines. *Vaccine*, 13 (14): 1360–1365 (1995).

68. Lambrecht, B. N., Kool, M., Willart, M.A. & Hammad, H. Mechanism of action of clinically approved adjuvants. *Curr. Opin. Immunol.* **21**, 23–9 (2009).

69. O'Hagan, D. T., Ott, G. S., De Gregorio, E. & Seubert, A. The mechanism of action of MF59 - An innately attractive adjuvant formulation. *Vaccine* **30**, 4341–8 (2012).

70. De Gregorio, E., Caproni, E. & Ulmer, J. B. Vaccine adjuvants: mode of action. *Front Immunol.* **4**, 214 (2013).

71. Ishii, K. J. & Akira, S. Innate immune recognition of, and regulation by, DNA. *Trends Immunol.* **27,** 525–32 (2006).

72. Creagh, E. M. & O'Neill, L. A. J. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* **27,** 352–357 (2006).

73. Ishii, K. J. & Akira, S. Toll or toll-free adjuvant path toward the optimal vaccine development. *J. Clin. Immunol.* **27,** 363–371 (2007).

74. Hem, S. L., Johnston, C. T. & HogenEsch, H. Imject Alum is not aluminum hydroxide adjuvant or aluminum phosphate adjuvant. *Vaccine* **25**, 4985–4986 (2007).

75. McKee, A. S. *et al.* Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* **183,** 4403–4414 (2009).

76. Kool, M. *et al.* Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* **181,** 3755–3759 (2008).

77. Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S. & Flavell, R. A. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453,** 1122–1126 (2008).

78. Li, H., Nookala, S. & Re, F. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. *J. Immunol.* **178,** 5271–5276 (2007).

79. Li, H., Willingham, S. B., Ting, J. P. Y. & Re, F. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J. Immunol.* **181,** 17–21 (2008).

80. Franchi, L. & Núñez, G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *Eur. J. Immunol.* **38**, 2085–2089 (2008).

81. Kool, M. *et al.* Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* **205**, 869–82 (2008).

82. Marichal, T. *et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nat. Med.* **17**, 996–1002 (2011).

83. McKee, A. S. *et al.* Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E1122-31 (2013).

84. Ulanova, M., Tarkowski, A., Hahn-Zoric, M. & Hanson, L. A. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect. Immun.* **69,** 1151–1159 (2001).

85. Rimaniol, A. C. *et al.* Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. *Vaccine* **22,** 3127–3135 (2004).

86. Goto, N., *et al.* Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties Vaccine, 15: 1364–1371 (1997).

87. Garçon, N. Preclinical development of AS04. *Methods Mol. Biol.* **626,** 15–27 (2010).

88. O'Hagan, D. T. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. *Expert Rev. Vaccines* **6,** 699–710 (2007).

89. Calabrò, S., *et al.* The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. *Vaccine **31***, 3363-9 (2013).

90. Dupuis, M., et al., Dendritic cells internalize vaccine adjuvant after intramuscular injection. *Cell Immunol.* **186**, 18-27 (1998)

91. Seubert, A. *et al.* Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 11169–74 (2011).

92. Ellebedy, A. H. *et al.* Inflammasome-independent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 2927–2932 (2011).

93. Berchtold, S. *et al.* Human monocyte derived dendritic cells express functional P2X and P2Y receptors as well as ecto-nucleotidases. *FEBS Lett.* **458,** 424–428 (1999).

94. Ferrari, D. *et al.* The P2 purinergic receptors of human dendritic cells: identification and coupling to cytokine release. *FASEB* **14,** 2466–76 (2000).

95. Mutini, C. *et al.* Mouse dendritic cells express the P2X7 purinergic receptor: characterization and possible participation in antigen presentation. *J. Immunol.* **163,** 1958–1965 (1999).

96. Granstein, R. D. *et al.* Augmentation of cutaneous immune responses by ATP gamma S: purinergic agonists define a novel class of immunologic adjuvants. *J. Immunol.* **174,** 7725–7731 (2005).

97. Denkinger, M., Shive, C. L., Pantenburg, B. & Forsthuber, T. G. Suramin has adjuvant properties and promotes expansion of antigen-specific Th1 and Th2 cells in vivo. *Int. Immunopharmacol.* **4,** 15–24 (2004).

98. Cintra-Francischinelli, M. *et al.* Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of acceptors. *Cell. Mol. Life Sci.* **66**, 1718–1728 (2009).

99. Cintra-Francischinelli, M. *et al. Bothrops* snake myotoxins induce a large efflux of ATP and potassium with spreading of cell damage and pain. *Proc Natl Acad Sci USA* **107**, 14140–14145 (2010).

100. Pellegatti, P. *et al.* Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One* **3,** e2599 (2008).

101. Kannan, S., Elimban, V., Fandrich, R. R. & Dhalla, N. S. Immunolocalization of the sarcolemmal Ca 2 + / Mg 2 + ecto-ATPase (myoglein) in rat myocardium. *Mol. Cell. Biochem.* **197,** 187–194 (1999).

102. Komoszynski, M. & Wojtczak, A. Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. *Biochim Biophys Acta* **1310,** 233-241 (1996).

103. Pugin, J. How tissue injury alarms the immune system and causes a systemic inflammatory response syndrome. *Ann. Intensive Care* **2**, 27 (2012).

104. Pimorady-Esfahani, A., Grounds, M. D. & McMenamin, P. G. Macrophages and dendritic cells in normal and regenerating murine skeletal muscle. *Muscle nerve* **20**, 158–166 (1997).

105. Rayah, A., Kanellopoulos, J. M. & Di Virgilio, F. P2 receptors and immunity. *Microbes Infect.* **1,** 1–9 (2012).

106. Ferrari, D. *et al.* Purinergic stimulation of human mesenchymal stem cells potentiates their chemotactic response to CXCL12 and increases the homing capacity and production of proinflammatory cytokines. *Exp. Hematol*. **39**, 360–74 (2011).

107. Saze, Z., *et al*. Adenosine production by human B cells and B cell-mediated suppression of activated T cells. *Blood*. **122**, 9-18 (2013).

108. La Sala, A. *et al.* Alerting and tuning the immune response by extracellular nucleotides. *J Leukoc Biol*. **73**, 339-43 (2003).

109. Banachewicz, W., Supłat, D., Krzemiński, P., Pomorski, P. & Barańska, J. P2 nucleotide receptors on C2C12 satellite cells. *Purinergic Signal.* **1**, 249–257 (2005).

110. Deli, T. *et al.* Contribution from P2X and P2Y purinoreceptors to ATP-evoked changes in intracellular calcium concentration on cultured myotubes. *Pflugers Arch.* **453,** 519–529 (2007).

111. Muñoz-Planillo, R. *et al*. K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* **38**, 1142-53 (2013).

112. Kouzaki, H., Iijima, K., Kobayashi, T., O'Grady, S. M. & Kita, H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J. Immunol.* **186**, 4375–4387 (2011).

113. Liu, Y., Xiao, Y., Li, Z. P2X7 receptor positively regulates MyD88-dependent NF-kB activation. *Cytokine* **55**, 229-36 (2011).

6. *APPENDIX*

6.1 Abbreviations

$(Al(OH)x(PO_4)y$, the chemical formula for aluminium phosphate

Ab, antibody

ADP, adenosine 5'-diphosphate

AF, alexa fluor

Ag, antigen

AIDS, acquired immunodeficiency syndrome

Al(OH)x, the chemical formula for aluminium hydroxide

Alum, aluminium hydroxide (in this context)

AMP, adenosine 5'-monophosphate

APCs, Ag-presenting cells

APY, apyrase

AS, Adjuvant sistem (as AS01,AS02, AS03, AS04)

ASC, apoptosis-associated speck-like protein containing CARD

ATP, adenosine triphosphate

ATPγS, adenosine 5′-O-(3-thio)triphosphate

B, influenza strain B/Brisbane/60/2008

BALB/c, a mouse strain

BCA, bicinchoninic acid

C2C12**,** a mouse myoblast cell line

CaPi, calcium phospate

CARD, caspase activation and recruitment domain

CCL, C-C motif chemokine ligand (as CCL-2)

CCR, C-C motif chemokine receptor

CD, Cluster of Differentiation (as CD4, CD8, etc.)

CFA, complete Freund's adjuvant

CpG, cytosine guanine dinucleotide

CTL, Cytotoxic T Lymphocytes

CXCL, CXC chemokine ligand

DAMPs, danger-associated molecular patterns

DCs, dendritic cells

DMEM, Dulbecco's modified Eagle's medium

DNA, deoxyribonucleic acid

DNase, deoxyribonuclease

ds, double-stranded (as dsDNA or RNA)

eATP, extracellular ATP

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbent assay

FACS, fluorescence-activated cell sorter

FBS, fetal bovine serum

FCS, fetal calf serum

FITC, fluorescein isothiocyanate

h, hour (only with numbers)

H1N1, influenza strain H1N1 A/California/7/2009

H3N2, influenza strain H3N2 A/Perth/16/2009

HA, hemagglutinin

HBSS, Hanks' balanced salt solution

HI, hemagglutination inhibition

HIV, human immunodeficiency virus

HMGB1, high- mobility group box protein 1

i.m., intramuscular

i.p., intraperitoneal

i.v., intravenous

IFA, incomplete Freund's adjuvant

IFN, interferon (e.g., IFN-γ)

Ig, immunoglobulin (also IgA, IgD, IgE, IgG, IgM)

IL, interleukin (e.g., IL-2)

IPAF, ICE protease-activating factor

K+, potassium

LNs, lymph nodes

LRR, leucine-rich repeat

mAb, monoclonal Ab

MACS, magnetic-activated cell sorting

MCP, monocyte chemoattractant protein

MHC, major histocompatibility complex (as MHCI, MHCII)

min, minute (only with numbers)

MIP, macrophage-inflammatory protein

MIV, monovalent influenza vaccine

ml, milliliter (only with numbers)

mM, milliMolar (only with numbers)

MPL, monophosphoryl lipid A

MyD88, myeloid differentiating factor 88

N, number in study or group

n.s., not significant

NAIP, NLR family, apoptosis inhibitory protein

NALPs, NACHT-LRR and pyrin domain-containing protein

NLRP3, Nod-like receptor family, pyrin domain-containing 3

NLRs, nucleotide-binding oligomerization domain (NOD)-like receptors

NOD, nucleotide-binding oligomerization domain

OD, optical density

OVA, ovalbumin

P, p-value

p.i., post injection

P2Rs, P2 receptors

P2X, subclasse of P2 receptors (ligand‑gated cation channels) e.g. P2X7

P2Y, subclasse of P2 receptors (G protein‑coupled receptors)

PAMPs, pathogen-associated molecular patterns

PBS, phosphate-buffered saline

PE-Cy7, phycoerythrin-Cyanin 7

PE, phycoerythrin

PerCP, peridinin chlorophyll protein

PerCPCy5.5, PerCP- cyanine 5.5

PLA2, phospholipases A2

Post 1, post first immunization

Post 2, post second immunization

PRRs, pattern-recognition receptors

Ptx3, pentraxin 3

QS, *Quillaja Saponaria* (as QS21)

R, receptor (e.g., IL-2R)

RIG, retinoic acid-inducible gene

RLHs, retinoic acid-inducible gene (RIG)-like helicases like receptors (controllala)

RNA, ribonucleic acid

ROS, reactive oxygen species

RPMI (usually RPMI 1640)

RT, room temperature

s, second (use only with numbers)

SD, standard deviation

SV, structural vaccinology

Th cell, T helper cell (as Th1, Th2)

TIV, trivalent influenza vaccine

TLRs, Toll-like receptors

TNF, tumor necrosis factor (as TNF-α)

U, unit (only with numbers)

UTP, uridine 5'-triphosphate

v/v, volume to volume ratio (%)

w/v, weight to volume ratio (%)

6.2 Publications

1. Vono M, Taccone M, Caccin P, Gallotta M, Donvito G, *et al*. **Adjuvant-induced muscle ATP release potentiates response to vaccination.** *Proc Natl Acad Sci U S A.* 2013; 110(52):21095- 100.

2. Caccin P, Pellegatti P, Fernandez J, Vono M, Cintra-Francischinelli M, *et al*. **Why myotoxin-containing snake venoms possess powerful nucleotidases?** *Biochem Biophys Res Commun.* 2013; 430(4):1289-93. *

3. Brun P, Brun P, Vono M, Venier P, Tarricone E, *et al.* **Disinfection of Ocular Cells and Tissues by Atmospheric-Pressure Cold Plasma.** *PLoS ONE* 2012; 7(3): e33245. *

*Publications obtained by collaborative projects outside my main research topic.

6.3 Acknowledgements

First I want to thank my advisors Professor Cesare Montecucco at the University of Padua and Dr. Anja Seubert at the Novartis Research Center in Siena.

I started the first phase of my research project in the laboratory of Professor Cesare Montecucco at the Department of Biomedical Sciences, University of Padua. It was a great honor and a privilege working with Prof. Cesare Montecucco and his excellent team. Then, I moved to Siena in Novartis in the laboratory of Dr. Anja Seubert at the Department of Immunology, where the joy and enthusiasm she has for research was contagious and motivational for me. I really appreciate all their contribution of ideas, time, and funding to make my Ph.D. experience possible and productive. In both of these places I had the possibility to work in a multicultural, dynamic and stimulating environment and the great opportunity to learn a lot about science and life.

I am glad I had opportunity to work with Professor Francesco Di Virgilio (University of Ferrara), Dr. Ennio De Gregorio and Dr. Rino Rappuoli (Novartis Vaccines & Diagnistics, Siena). They made a significant contribution to the project, with fascinating ideas and suggestions.

The members of both Montecucco and Seubert group have contributed a lot to my PhD with good advices and collaborations. I am especially grateful to Paola Caccin and Irene Zornetta (University of Padua), Marianna Taccone, Marilena Gallotta e Vanessa Zurli (Novartis Vaccines & Diagnistics, Siena) for helping me with experiments and discussions. Thanks to Emiliano Palmieri and Michele Pallaoro (Novartis Vaccines & Diagnistics, Siena) for adjuvant formulations; Thanks to the Novartis Animal and FACS facilities, Samule Calabrò, Daniele Casini, and Alessandra Bonci (Novartis Vaccines & Diagnistics, Siena) for technical assistance. Sylvie Bertholet (Novartis Vaccines & Diagnistics, Siena) for helpful discussions. Giovanna Donvito, Simonetta Falzoni and Marzia Scarletti (University of Ferrara) for help with IVIS technique.

Last but not least, I would like to thank my family for all their support and encouragement.

Thank you.