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Immunological Characterization of novel Adjuvants for Vaccines against Pathogenic *Escherichia coli* strains

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Summary

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of humans and animals as part of the commensal microbiota. However pathogenic *E. coli* strains have been isolated and these are usually classified as intestinal or extraintestinal pathogenic *E. coli* (InP*Ec* or ExP*Ec*) according to the disease location and the clinical outcomes.

The extent of multidrug-resistance to last-line antibiotics in both InP*Ec* and ExP*Ec*, together with the recent rising incidence of hypervirulent strains and the emergence of new sequence types among *E. coli* pathotypes are becoming a growing concern since diseases caused by these pathogens are associated with significant human suffering and high costs for the healthcare system. A broad-spectrum *E. coli* vaccine could be a promising alternative to prevent the spread of such diseases while offering the potential for covering against several pathovars at once. A critical goal of new generation vaccines against *E. coli* is to increase the breadth, quality and efficiency of protection and immune response and this could be achieved using adjuvants.

Vaccine adjuvants enhance T and B cell responses by engaging components of the innate immune system. Either acting as delivery systems or as immune-potentiators, these compounds enhance antigen uptake by APCs triggering their maturation or activation, thus promoting immune-modulatory cytokines production that, in turn, elicits local inflammation and cellular recruitment. Between the APCs, macrophages can acquire distinct functional phenotypes, with different functions and transcriptional profiles: classical or M1-activated macrophages, induced by microbial products in the presence or absence of IFN-γ, are microbicidal, tumoricidal and pro-inflammatory; in contrast, alternative or M2 activation by IL-4 and/or IL-13 gives rise to anti-inflammatory and immuno-tolerant cells. These subtypes are thought to represent extremes of a *continuum* of activation states; However, different polarization phenotypes have been shown to differently affect the adaptive immune response with M1 macrophages promoting Th1 and Th17 responses and M2 cells being related to Th2 differentiation.

GSK has identified a series of new adjuvants, called SMIPs (Wu *et al.*, 2014), that trigger members of the TLRs family expressed on a variety of APCs. The aim of the work described in this thesis was to characterize the *in vitro* and *in vivo* effect of GSK TLR2 and TLR-7 agonist adjuvants, named SMIP.2-7, SMIP.7-10 and SMIP.7-11, on macrophage polarization at early time-points and also to verify if the obtained activation state correlates coherently with the adaptive immune response observed after a complete immunization protocol.

On *in vitro* experiments with murine macrophage cell line RAW 264.7, SMIP.2-7, SMIP.7-10 and SMIP.7-11 stimulated a very clear M1 phenotype which was confirmed also on *ex vivo* SMIP-stimulated murine peritoneal macrophages. However, when the SMIPs (TLR7 agonists only) were intraperitoneally injected in mice, either in their soluble forms or formulated with Alum, a mixed polarization phenotype was elicited, with apparent up-

regulation of M2 marker genes as well as pro-inflammatory M1-typical citokines. M1 and M2 cells are thought to have antagonistic roles in the immune response with M1 macrophages being involved in the inflammatory response and M2 macrophages serving to limit excessive Nitric Oxide production and support healing. The ability of these vaccine adjuvants to elicit the expression of both phenotypes' markers at different time-points could play an important positive role in the immune response to a SMIP-containing vaccine, creating an inflammatory-responsive environment within few hours from injection that could favor other immune cells recruitment and vaccine antigen uptake and presentation while other cells would be involved in restoring the system to healing-homeostatic conditions at later time-points.

Vaccine adjuvants can induce immunologic memory to vaccine-antigens through local activation of the innate immune system; since muscle constitutes the preferred site of injection of most human vaccines, the kinetics of cell recruitment into adjuvants (Alum-SMIP.7-10, Alum and SMIP.7-10)- or PBS-treated mice was determined directly in quadriceps muscles at early time-points post-injection. At 24h, Alum-SMIP.7-10 showed to induce recruitment of CD11b+ myeloid cells, in particular monocytes and macrophages, into the injected quadriceps muscles as compared to PBS treated muscle; also B and T cells showed a slight, but still significant, increase in the Alum-SMIP.7-10-treated muscles as compared to the PBS and the SMIP.7-10 alone-injected animals. After two days from injection, also a strong eosinophils influx was observed in the Alum-SMIP.7-10-treated muscles, together with a new wave of monocytes infiltration and a further massive increase in neutrophils and macrophages numbers as compared to the PBS and SMIP.7-10-administered groups; the latter could be presumably addressed to monocyte–macrophage differentiation rather than to a new recruitment of these cells. Monocytes, macrophages and neutrophils resulted the most abundant populations of the Alum-SMIP.7-10 treated muscles up to three days post-injection, meaning that these innate cells are likely involved in antigen "capture" and presentation in the presence of this adjuvant.

Once assessed which are the immune cells involved in the first response to an Alum-SMIP.7-10-containing vaccine, the ability of this adjuvant to increase the immunogenicity of a candidate *E. coli* vaccine antigen was evaluated. *E. coli* considerable antigenic diversity and virulence factor redundancy has undoubtedly hampered the release of a broadly protective vaccine against pathogenic strains. Using the Reverse Vaccinology approach, nine antigens were identified as protective against a mouse sepsis model and among these, the Secreted Surface-associated Lipoprotein of *E. coli* (*SslE*) was the most promising candidate. Functionally, *SslE* is a zinc-metallo-peptidase involved in mucins degradation; such mucinase activity plays an essential role in *E. coli* colonization and virulence. *SslE* already showed to be protective against other ExP*Ec* models as well as against InP*Ec*s strains. Vaccines based on purified antigens normally require multiple doses to achieve protective antibody levels and high cost, which makes their use in the developing world problematic. Alum-SMIP.7-10 showed to strongly increase immunogenicity of *SslE* antigen already after a single dose, inducing a significant increase in antigen-specific IgG titres.

Alum-SMIP.7-10-*SslE* vaccine was also the sole formulation in which specific anti-*SslE* IgG2a and IgG2b subclasses were detectable, indicating that this adjuvant is able to enhance isotype switching. In addition to triggering a humoral response, *SslE*-Alum-SMIP.7-10 formulation was able to induce the higher antigen-specific T cells frequencies than Alum-*SslE* or *SslE* alone. After a complete three-doses immunization schedule, antigen-specific T cells in the Alum-SMIP.7-10 group were predominantly Th1 and, to a lower extent, Th17 polarized, although a good portion of *SslE*-specific CD4+ cells resulted activated, but yet in a Th0 phenotype.

To finally assess the role of macrophages in *SslE* antigen-presentation and the effect of SMIPs on this process, SMIPs-pretreated and *in vitro SslE*-loaded Bone Marrow Derived Macrophages (BMDMs) were used as "Trojan Horse" for the antigen into naïve recipient mice. Adoptivelly-transferred cells successfully presented the antigen, being able to greatly induce *SslE* immunogenicity by promoting a both a sistemic and humoral response even after a sole immunization. SMIPs pretreatment stimulated up-regulation of co-stimulatory molecules that are important for APC-induced T cell activation and overall increased antigen-specific CD4+ T cell expansion above the level reached by *SslE*-loaded but SMIP-untreated macrophages.

Overall, the findings of this thesis emphasize that SMIP adjuvants affect macrophages functionality, but also other cells at the injection site, leading to a rapid selective cellular recruitment that have a strong impact on the stimulation and the success of the following adaptive immune response. In the perspective of the urgent need for development of a broadly protective vaccine against pathogenic *E. coli* strains, Alum-SMIP.7-10 adjuvant can greatly increase the immunogenicity of the candidate antigen *SslE* and would deserve further investigation. However, *SslE* does not cover all known pathogenic strains: a recent study has identified antigen *YncE*, present in >99% of all *E. coli* genomes available, as potential vaccine candidate, showing already protection against a bacteremia model of infection and being recognized by antibodies present in the sera of convalescent urosepsis patients. The idea of a multi-component broad-spectrum vaccine including candidates such as *SslE* and *YncE* formulated with Alum-SMIP.7-10 would need attentive consideration in future *E. coli* vaccine research strategies.

Riassunto

Escherichia coli (*E. coli*) è un batterio gram-negativo, a forma di bastoncello, che si trova comunemente nell'intestino inferiore dell'uomo e degli animali come parte del microbiota commensale. Tuttavia ceppi patogeni di *E. coli* sono stati isolati e di solito sono classificati come *E. coli* o intestinale o extraintestinale (InP*Ec* o ExP*Ec*) in base alla posizione della malattia e ai risultati clinici.

L'entità della resistenza multiresistente agli antibiotici di ultima linea sia in Incec che in ExPEc, insieme alla recente crescente incidenza di ceppi ipervirulenti e all'emergenza di nuovi tipi di sequenze tra i patotipi di *E. coli* stanno diventando una preoccupazione crescente poiché le malattie causate da questi patogeni sono associato a significative sofferenze umane e costi elevati per il sistema sanitario. Un vaccino di *E. coli* ad ampio spettro potrebbe essere un'alternativa promettente per prevenire la diffusione di tali malattie offrendo al tempo stesso anche la possibilità di coprire più pathovar con una sola formulazione. Un vaccino di nuova generazione contro *E. coli* deve essere sicuramente in grado di aumentare dell'ampiezza, la qualità e l'efficienza della protezione e della risposta immunitaria; questo obiettivo può essere raggiunto aggiungendo ad esso i composti adiuvanti più appropriati. Gli adiuvanti vaccinali migliorano le risposte delle cellule T e B coinvolgendo componenti del sistema immunitario innato. Questi composti, agendo come sistemi di trasporto o come immunostimolanti, aumentano il riconoscimento dell'antigene da parte delle cosiddette *Antigen Presenting Cells* (APCs), innescando la loro maturazione o attivazione, promuovendo così la produzione di citochine immuno-modulatorie che, a loro volta, provocano localmente infiammazione e infiltrazione di altre cellule immunitarie. I macrofagi sono le APCs più plastiche, potendo acquisire fenotipi di attivazione distinti che si traducono in diverse funzioni e profili trascrizionali; i macrofagi attivati nella via "classica" o M1, indotti da prodotti microbici in presenza o assenza di IFN-γ, hanno attività battericida, tumoricida e pro-infiammatoria; al contrario, l'attivazione alternativa o M2 di IL-4 e/o IL-13 dà luogo a cellule anti-infiammatorie e immuno-tolleranti. Si pensa che questi sottotipi rappresentino gli estremi di un *continuum* di stati di attivazione. Tuttavia, Fenotipi di polarizzazione differenti stimolano anche diversi effetti sulla risposta immunitaria adattativa, con i macrofagi M1 che promuovono risposte Th1 e Th17 e cellule M2, invece correlate alla differenziazione di tipo Th2.

GSK ha identificato una serie di nuovi adiuvanti, denominati SMIP (Wu *et al.*, 2014), che attivano membri della famiglia dei recettori TLR, espressi su diverse APC. Lo scopo del lavoro descritto in questa tesi è stato quello di caratterizzare *in vitro* e *in vivo* l'effetto di questi adiuvanti, in particolare un agonista del TLR2 e due agonisti del TLR7, chiamati SMIP.2-7, SMIP.7-10 e SMIP.7-11, su polarizzazione dei macrofagi durante le prime ore successive alla stimolazione, verificando poi, , se questo stato di attivazione induceva una risposta immunitaria adattativa coerente con quanto riportato in letteratura al termine di un protocollo di immunizzazione completo.

Negli esperimenti *in vitro* con la linea cellulare RAW 264.7 di macrofagi murine, SMIP.2-7, SMIP.7-10 e SMIP.7-11 hanno indotto un fenotipo M1 molto netto, che è stato confermato anche su negli studi *ex vivo* con macrofagi murini peritoneali. Tuttavia, quando gli SMIP sono stati iniettati per via intraperitoneale nei topi, sia nelle loro forme solubili o in formulazione con Alum, le cellule mostravano un fenotipo di polarizzazione misto, con apparente aumento nell'espressione sia di geni di tipo M2 che di altre citochine tipicamente prodotte de cellule M1, seppur a tempi diversi di stimolazione. Le cellule M1 e M2 svolgono ruoli antagonisti nella risposta immunitaria, con i macrofagi M1 coinvolti nella risposta infiammatoria e gli M2 in grado di limitare la produzione eccessiva di ossido nitrico e di sostenere la riparazione tissutale. La capacità di questi adiuvanti di indurre l'espressione di marcatori di entrambi i fenotipi in diversi momenti potrebbe giocare un ruolo positivo nella risposta immunitaria a un vaccino contenente SMIP creando, entro poche ore dall'iniezione, un'infiammazione a livello locale in grado di favorire il reclutamento di altre cellule immunitarie e aumentando quindi le probabilità di riconoscimento, cattura e presentazione dell'antigene; le funzioni M2 potrebbero essere utili in momenti successivi nella riparazione del danno tissutale e nel ripristino delle condizioni omeostatiche del sistema dopo l'iniezione del vaccino. Poiché il muscolo costituisce il sito preferenziale di somministrazione della maggior parte dei vaccini umani, in questo lavoro di tesi, la cinetica del reclutamento cellulare è stata determinata a 3 diversi time-point post-iniezione direttamente nei quadricipiti di topi trattati con diversi adiuvanti (Alum-SMIP.7-10, Alum e SMIP.7-10 solubile) o con PBS, come controllo negativo. A 24 ore dalla somministrazione, Alum-SMIP.7-10 ha indotto un maggiore reclutamento di cellule mieloidi CD11b+, in particolare monociti e macrofagi, rispetto al muscolo trattato con PBS; allo stesso modo, linfociti B e T hanno mostrato un lieve, ma significativo aumento nei muscoli trattati con Alum-SMIP.7-10 rispetto a quelli iniettati con PBS o SMIP.7-10 nella sua forma solubile. A 48 ore dall'iniezione, sono comparsi anche gli eosinofili nei muscoli che avevano ricevuto Alum-SMIP.7-10, insieme ad una nuova ondata di infiltrazione di monociti e un ulteriore aumento di neutrofili e macrofagi; presumibilmente, la presenza massiccia di questi ultimi potrebbe essere dovuta alla differenziazione postuma dei monociti infiltrati a macrofagi nel sito di iniezione. Tre giorni dopo l'iniezione monociti, macrofagi e neutrofili erano ancora le popolazioni più abbondanti dei muscoli trattati con Alum-SMIP.7-10. Questo porta ad ipotizzare che queste cellule dell'immunità innata siano quelle effettivamente coinvolte nella cattura dell'antigene e nella sua presentazione nelle prime ore dopo la vaccinazione.
Una volta caratterizzate le cellule immunitarie coinvolte nella prima risposta ad Alum-SMIP.7-10, è stata valutata la capacità di questo adiuvante di aumentare l'immunogenicità dell'antigene candidato per il vaccino contro *E. coli*. La considerevole diversità antigenica di questo batterio e la ridondanza dei suoi fattori di virulenza hanno finora ostacolato il rilascio di un vaccino ad ampio spettro di protezione contro i suoi ceppi patogeni. Usando la *Reverse Vaccinology*, nove antigeni sono stati individuati come protettivi contro un modello di sepsi nel modello murino; tra questi, la lipoproteina di superficie di *E. coli* (*SslE*) si è rivelato il candidato più promettente. Funzionalmente, *SslE* è una zinco-metallo-

peptidasi coinvolta nella degradazione delle mucine; tale attività mucinasica gioca un ruolo essenziale nella colonizzazione di *E. coli* e nella sua virulenza. Questo antigene si è già dimostrata protettivo contro altri modelli ExP*Ec* e contro alcuni ceppi InP*Ec*. Tuttavia, i vaccini basati su antigeni purificati normalmente richiedono dosi multiple per raggiungere livelli anticorpali adeguati alla protezione e di conseguenza costi elevati; questo rende problematico il loro utilizzo nei paesi più poveri ed in via di sviluppo. In questi studi Alum-SMIP.7-10 si è rivelato in grado aumentare fortemente l'immunogenicità dell'antigene *SslE*, inducendo un aumento significativo dei titoli IgG specifici per l'antigene già dopo una singola dose. Il vaccino Alum-SMIP.7-10-*SslE* è stato anche l'unica formulazione in cui sono state rilevabili sottoclassi anti-*SslE* IgG2a e IgG2b, grazie alla capacità di questo adiuvante di stimolare il cambio di isotipo delle immunoglobuline. Oltre a scatenare una risposta umorale, la formulazione *SslE*-Alum-SMIP.7-10 è stata anche quella con le più alte frequenze di cellule T antigene-specifico. Al termine di un programma di immunizzazione completo a tre dosi, le cellule T antigene-specifiche nel gruppo trattato con Alum-SMIP.7-10 erano prevalentemente di tipo Th1 e, in misura minore, Th17, sebbene una buona porzione di cellule CD4 + specifiche per *SslE* risultava attivata, ma ancora con fenotipo Th0.

Infine, allo scopo di valutare il ruolo effettivo dei macrofagi nella presentazione dell'antigene *SslE* e l'effetto degli SMIP in questo processo, macrofagi primari derivati dal midollo osseo (BMDM) pretrattati *in vitro* con *SslE* + SMIP sono stati usati come "Cavallo di Troia" per l'antigene ed iniettati in topi riceventi naïve per l'antigene. Le cellule trasferite hanno presentato efficacemente l'antigene, riuscendo a promuovere una risposta sia sistemica che umorale contro *SslE* anche dopo una sola immunizzazione. Il pretrattamento con gli SMIP ha avuto il suo ruolo in questo, stimolando nei BMDM un'aumentata espressione delle molecole co-stimolatorie, importanti per l'attivazione delle cellule T, e l'espansione complessiva delle cellule CD4+ specifiche per l'antigene al di sopra del livello raggiunto dai macrofagi non trattati con *SslE* in assenza di SMIP.

Nel complesso, i risultati di questa tesi sottolineano che gli adiuvanti SMIP influenzano sì la funzionalità dei macrofagi, ma anche altre cellule nel sito di iniezione, portando ad un reclutamento cellulare selettivo rapido che ha un forte impatto sulla stimolazione ed il successo della conseguente risposta immunitaria adattiva. Nella prospettiva di sviluppare al più presto un vaccino ad ampio spettro contro i ceppi patogeni di *E. coli*, l'adiuvante Alum-SMIP.7-10 si è rivelato grado di aumentare notevolmente l'immunogenicità dell'antigene candidato *SslE* e meriterebbe ulteriori indagini. Tuttavia, la proteina *SslE* non è presente in tutti i ceppi patogeni noti. Uno studio recente ha identificato l'antigene *YncE* come potenziale candidato vaccinale alternativo; presente in più del 99% di tutti i genomi di *E. coli* disponibili, *YncE* è già risultato protettivo contro un modello di batteriemia e viene riconosciuto dagli anticorpi presenti nei sieri dei pazienti convalescenti affetti da urosepsi. L'idea di un vaccino multicomponente ad ampio spettro composto dai due antigeni candidati *SslE* e *YncE* e Alum-SMIP.7-10 come adiuvante richiederebbe un'attenta

considerazione nella ricerca futura di una strategia vaccinale efficace contro i diversi ceppi patogeni di *E. coli*.

Chapter I
Introduction

The introduction of vaccines into medical practice at the beginning of the twentieth century has had an extraordinary impact on human health, and represents an unparalleled success story. Vaccines are widely considered to be the most safe and effective medical intervention available. In conjunction with the introduction of antibiotics and modern hygiene practices, vaccines are one of the most efficient strategies for infectious diseases prevention, having also and enormously contributed to a steady decline in the mortality and morbidity of this kind of illnesses. Moreover, thanks to them a life-threatening disease such as smallpox has been completely eradicated, while others such as polio have almost disappeared [Rappuoli *et al.*, 2014]; according to the World Health Organization, vaccination saves 5 lives every minute and will save over 25 million lives from 2011 to 2020 [Ozawa *et al.*, 2017].

Traditional vaccine approaches rely on live, attenuated variants of the targeted pathogen; usually, their administration results in mild and asymptomatic infection, but generates long-lived immunity similar to that observed in individuals who recover from natural infection. For many pathogens, however, whole cell-killed and attenuated vaccines have only limited efficiency or present safety issues. To protect against these organisms, subunit vaccines are used: these include non-living antigens such as inactivated toxins or pathogens, as well as synthetic peptides and recombinant protein subunits.

Despite being safer, these highly purified antigens are often poorly immunogenic and require co-administration with adjuvants, to help stimulate protective immunity based on antibodies and effector T cell functions. The term adjuvant comes from the Latin "*adjuvare*", which means to help or aid [Cox and Coulter, 1997]. Adjuvants, in fact, can be defined as substances that are added to a vaccine to increase the immunogenicity of the final formulation, by stimulating, reinforcing, prolonging, or modulating the adaptive immune response directed towards vaccine antigens.

The *Escherichia coli* vaccine challenge

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of humans and animals as part of the commensal microbiota. *E. coli* genomes consist of conserved regions - the so-called core genome - and more flexible and interspersed regions [Dobrindt *et al.* 2010]; the latter frequently contain foreign DNA such as bacteriophages, genomic and pathogenicity islands [PAIs], which can define new phenotypic characteristics for the host [Dobrindt, 2005; Schmidt, 2010]. Plasmids contribute also to the horizontal gene transfer and the creation of novel combinations of virulence factors leading to new phenotypes and thus the emergence of new pathogens [Kaper, Nataro and Mobley 2004; Nataro *et al.* 2006].

This huge genomic plasticity has led to numerous gene exchange events, resulting in the evolution of new pathogenic strains that cause new types of disease [Creuzburg *et al.* 2011a, b] of public health importance in humans and animals. Based on a combination of clinical, epidemiological and molecular criteria, pathogenic *E. coli* isolates are typically classified as either Extraintestinal Pathogenic *E. coli* (ExP*Ec*) or Intestinal Pathogenic *E. coli* [or InP*Ec*] strains [Monteiro *et al.*, 2018] (**Figure 1A**).

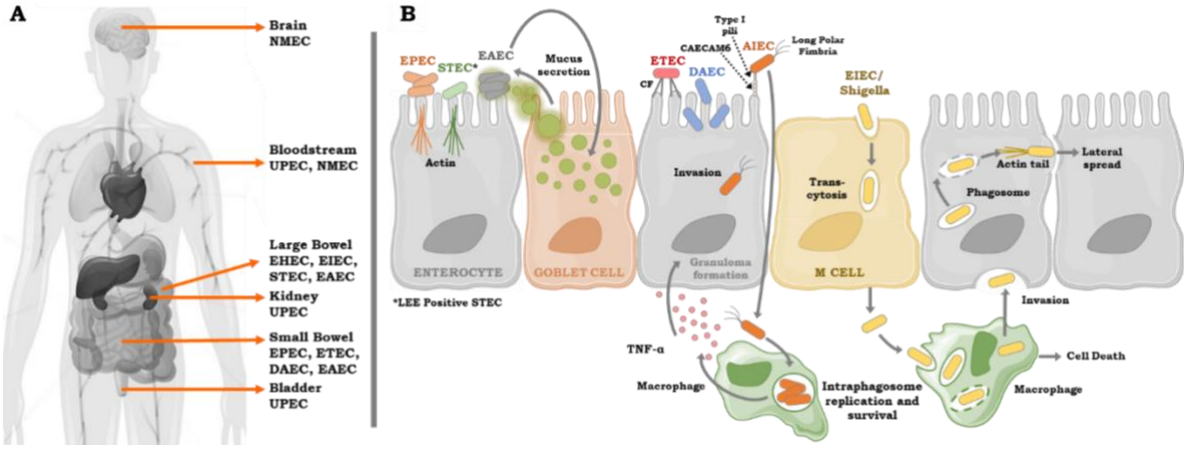


Figure 1. *E. coli* pathogenesis and mechanisms of disease

(A) Sites of pathogenic *E. coli* colonization: pathogenic *E. coli* colonize various sites in the human body. Enteropathogenic *E. coli* (EP*Ec*), enterotoxigenic *E. coli* (ET*Ec*) and diffusely adherent *E. coli* (DA*Ec*) colonize the small bowel and cause diarrhea, whereas enterohemorrhagic *E. coli* (EH*Ec*) and enteroinvasive *E. coli* (EI*Ec*) cause disease in the large bowel; enteroaggregative *E. coli* (EA*Ec*) can colonize both the small and large bowels. Uropathogenic *E. coli* (UP*Ec*) enter the urinary tract and travel to the bladder to cause cystitis and, if left untreated, can ascend further into the kidneys to cause pyelonephritis. Septicemia can occur with both UP*Ec* and neonatal meningitis *E. coli* (NM*Ec*), and NM*Ec* can cross the blood– brain barrier into the central nervous system, causing meningitis. (B) Adherence patterns of enteric *E. coli.* Pathogenic *E. coli* requires adherence to the host epithelium. Enteropathogenic *E. coli* (EP*Ec*) (represented in light orange) and LEE-positive Shiga toxin-producing *E. coli* (ST*Ec*) (represented in light green) are extracellular pathogens that attach to the intestinal epithelium and efface microvilli, forming characteristic A/E lesions. Due to the presence of bundle-forming pili, EP*Ec* is capable of forming microcolonies, resulting in a localized adherence (LA) pattern. Enterotoxigenic *E. coli* (ET*Ec*) (represented in red) uses colonization factors (CFs) for attachment to host intestinal cells. Enteroaggregative *E. coli* (EA*Ec*) (represented in grey) forms biofilms on the intestinal mucosa, and bacteria adhere to each other as well as to the cell surface to form an aggregative adherence pattern (AA) known as "stacked brick." Diffusely adherent *E. coli* (DA*Ec*) (represented in blue) is dispersed over the surfaces of intestinal cells, resulting in a diffuse adherence (DA) pattern. Adherent invasive *E. coli* (AI*Ec*) (represented in orange) colonizes the intestinal mucosae of patients with Crohn's disease and is capable of invading epithelial cells as well as replicating within macrophages. AI*Ec* uses type I pili to adhere to intestinal cells and long polar fimbriae that contribute to invasion. Enteroinvasive *E. coli* (EI*Ec*)/Shigella (represented in yellow) are intracellular pathogens that penetrate the intestinal epithelium through M cells to gain access to the submucosa. EI*Ec*/Shigella escape submucosal macrophages by induction of macrophage cell death followed by basolateral invasion of colonocytes and lateral spread.

InP*Ec*s are responsible for diarrheal diseases of various severity along the gastrointestinal (GI) tract (**Figure 1B**). According to their virulence factors, mechanisms of infection, interaction with the enterocyte, tissue tropism and symptoms [Kaper *et al.*, 2004; Croxen and Finlay, 2010], they can be further subdivided into six subcategories: Entero-Pathogenic *E. coli* (EP*Ec*), Entero-Hemorrhagic *E. coli* (EH*Ec*), Entero-Invasive *E. coli* (EI*Ec*), Entero-Aggregative *E. coli* (EA*Ec*), Diffusely Adherent *E. coli* (DA*Ec*) and Entero-Toxigenic *E. coli* (ET*Ec*). A recently published study by the World Health Organization (WHO) on the global burden of foodborne diseases, estimated that, in 2010, ET*Ec* was the second leading cause of diarrheal diseases worldwide [Havelaar *et al.*, 2015]. Moreover, EP*Ec* was the second leading cause of deaths from diarrheal diseases in the world. Combined, EP*Ec*, ET*Ec* and ST*Ec* were responsible for over 324 million cases of diarrheal diseases in 2010, with more than one third of it affecting children under 5 years of age. Conversely, EH*Ec* strains, mainly affecting developed countries, not only causes diarrheal disease, but is also responsible for clinical complications like hemorrhagic colitis and Hemolytic Uremic Syndrome (HUS), which is an increasing problem in Latin American countries [Kaper *et al.*, 2004; Pianciola *et al.*, 2016; Torres, 2017a]. Among ExP*Ec*s, uropathogenic *E. coli* (UP*Ec*) is the main etiological agent of urinary tract infections (UTIs), accounting for 75% of all uncomplicated UTIs [Flores-Mireles *et al.*, 2015]. Recurrent UTIs are a common problem for young women, causing significant morbidity and care-associated cost [Mobley and Alteri, 2015; Terlizzi *et al.*, 2017]. Another ExP*Ec*, the Neonatal Meningitis-associated *E. coli* (NM*Ec*), is one of the leading causes of early- and late-onset neonatal sepsis [Bonacorsi and Bingen, 2005; Simonsen *et al.*, 2014] (**Figure 1A**).

Antibiotic resistance to commonly used antibiotics such as fluoroquinolones, tetracycline and cephalosporins is now widespread in both InP*Ec*s and ExP*Ec*s [Croxen *et al.*, 2013; Flores-Mireles *et al.*, 2015; Terlizzi *et al.*, 2017; Amezquita *et al.*, 2017; Torres *et al.*, 2017]. The extent of multidrug-resistance, including the last-line antibiotics such as carbapenems, tigecyclin and colistin [Mediavilla *et al.*, 2016; Pournaras *et al.*, 2016; Pitout and De Vinney, 2017] and the recent rising incidence of hypervirulent strains and new sequence types among *E. coli* pathotypes, is becoming a growing concern, with significant public health and economic impacts, especially in the developing world, where treatment options are limited. Among non-antibiotic therapies, a broad-spectrum *E. coli* vaccine could be a promising alternative to prevent the spread of such diseases, while offering the potential for covering against several InP*Ec*s and ExP*Ec*s at once.

SslE mucinase as a good vaccine candidate

The recent rising incidence of hypervirulent multi-drug resistant strains among the pathogenic *E. coli* strains is a major problem for modern society since diseases caused by these pathogens are associated with significant human suffering and high costs for the

healthcare system. In the last years, conventional vaccinology methods based on whole-cell, single antigen or polysaccharide-based approaches have been unsuccessful in providing a highly immunogenic, safe and cross-protective vaccine against pathogenic strains. Among the many challenges, *E. coli* considerable antigenic diversity and virulence factor redundancy has undoubtedly hampered to find common antigens for different pathotypes.

The Reverse Vaccinology [Donati and Rappuoli, 2013] approach was used to *in silico* screen the genome of *E. coli* in order to search and rapidly identify potential vaccine candidates that would be secreted or present on the cell surface of multiple *E. coli* pathogenic strains. The use of this subtractive strategy on the multiple available sequences of *E. coli* genomes have enabled the identification of nine promising conserved antigens that could be the basis for the development of safe and broadly protective vaccines against pathogenic *E. coli* [Moriel *et al.*, 2016]. Among these, *SslE* (Secreted surface-associated lipoprotein of *E. coli*), also known as ECOK1_3385 or YghJ, was shown to be protective in a murine sepsis model with NM*Ec* [Moriel *et al.*, 2010].

The *SslE*-encoding gene is widely distributed in the *E. coli* phylogeny, with a higher presence in intestinal and extraintestinal pathogenic isolates (between 70% and 83%) compared to commensal isolates (59%) [Moriel *et al.*, 2010]. Functionally, *SslE* is a 160 kDa mucin-binding protein able to degrade intestinal mucins including Muc2, Muc3 and bovine submaxillary mucin [Nesta *et al.*, 2014; Luo *et al.*, 2014]. In order to colonize or invade intestinal epithelium, *E. coli* must penetrate the mucus barrier and then either attach to the apical surface of epithelial cells or release toxins to disrupt epithelial integrity [Henderson *et al.*, 1999]. The mucus layer, largely composed of mucins, contains various digestive enzymes and antimicrobial peptides as well as immunoglobulins; the inner layer is densely packed, firmly attached to the epithelium, and devoid of bacteria, whereas the outer layer is movable and has an expanded volume that favors bacterial colonization [Dharmani *et al.*, 2009; McGuckin *et al.*, 2011]. Notably, bacterial pathogens have evolved mechanisms to circumvent this mucus hurdle and directly access the epithelial surface [Kim *et al.*, 2010; Sperandio *et al.*, 2013]. The recent description of *SslE* as a novel *E. coli* mucinase [Nesta *et al.*, 2014; Luo *et al.*, 2014], has opened new outlooks on the mechanisms used by this pathogen to adapt to the intestine: several assays demonstrated that *SslE* mucinase activity helps *E. coli* penetration through the mucus layer, while favoring colonization and allowing bacteria to better reach the epithelial layer [Luo *et al.*, 2014; Valeri *et al.*, 2015]. The evidence that *SslE* expressing bacteria have an enhanced access to the apical epithelial surface was corroborated by an increased pro-inflammatory response, measured as IL-8 release during *E. coli* infection of intestinal mucosa cells [Valeri *et al.*, 2015]. *SslE* has also been associated with biofilm formation in EP*Ec* [Baldi *et al.*, 2012] and with significant tissue damage and hemorrhage in mouse ilea [Tapader *et al.*, 2017], further promoting *SslE* as a virulence factor. Analysis of human sera of convalescent patients from urosepsis [Moriel *et al.*, 2016] or ET*Ec* infections [Roy *et al.*, 2010; Luo *et al.*, 2015] revealed *SslE*-specific antibodies, confirming the immunogenicity of this antigen.

Indeed, *SslE* already showed to be protective against other ExP*Ec* models [Moriel *et al.*, 2010; Nesta *et al.*, 2014] as well as against InP*Ec*s. However, vaccines based on purified antigens normally require multiple doses to achieve protective antibody levels and high cost, which makes their use in the developing world problematic. In addition, problems persist in the elderly or immunocompromised individuals, in which immunogenicity is relatively poor. Thus, the further challenges to for an effective *E. coli* vaccine are to identify vaccine formulations and strategies to elicit stronger primary antibody responses in order to achieve serum levels of protective antibodies and vaccine efficiency.

Immune response to vaccines

The simple but brilliant idea behind vaccines is to mimic the natural infection but limiting its toxicity and reactogenicity, thus providing protection in case of encounter with the real infectious agent. The way by which vaccines provide this protection is through activation of the immune system.

The immune system is composed of an innate arm that reacts very quickly upon recognition of a pathogen, and an adaptive arm that takes longer to be activated but induces long term memory protection and relies on the innate component to be initiated.

The cells and receptors of the innate immune system are critical for the rapid recognition of the infectious agent and initiating a pro-inflammatory response. Activation of the innate immune system is triggered by Pathogen-Associated Molecular Patterns (PAMPs) expressed by the microorganisms. PAMPs are conserved structural microbial components that are absent in multicellular organisms and that are recognized by Pattern Recognition Receptors (PRRs), expressed by cells of vertebrate organisms [Medzhitov and Janeway, 1998]. Phagocytic cells, and in particular macrophages and dendritic cells (DCs), are the most important Antigen Presenting Cells (APCs) that express PRRs and sense the PAMPs in the extracellular and cytoplasmic compartment [Geginat *et al.*, 2015]. Upon recognition, they produce pro-inflammatory cytokines and innate protective molecules that kill the pathogens [Pennock *et al.*, 2013]. The innate immune system therefore represents a first line of protection, being able of mounting a defense within minutes of pathogen invasion. Moreover, the inflammation generated by innate immune cells (neutrophils, macrophages, monocytes, natural killer (NK) cells, DCs (**Figure 1**) informs and directs the expansion and differentiation of adaptive immune cells.

In an immature stage, APCs - in particular DCs and macrophages - specialize in uptake of antigens thanks to their PRRs equipment. Pathogen invasion is normally accompanied by the replication of the pathogen followed by tissue damage; the combination of these two components serves to activate numerous PRRs present in the local tissue as well as tissue-localized innate immune cells such as macrophages and DCs. PRRs stimulation results in activation of multiple signaling pathways and the subsequent increase in the expression of a plethora of effector molecules, including Major Histocompatibility Complex (MHC), co-stimulatory molecules and pro-inflammatory chemokines and cytokines. The resulting

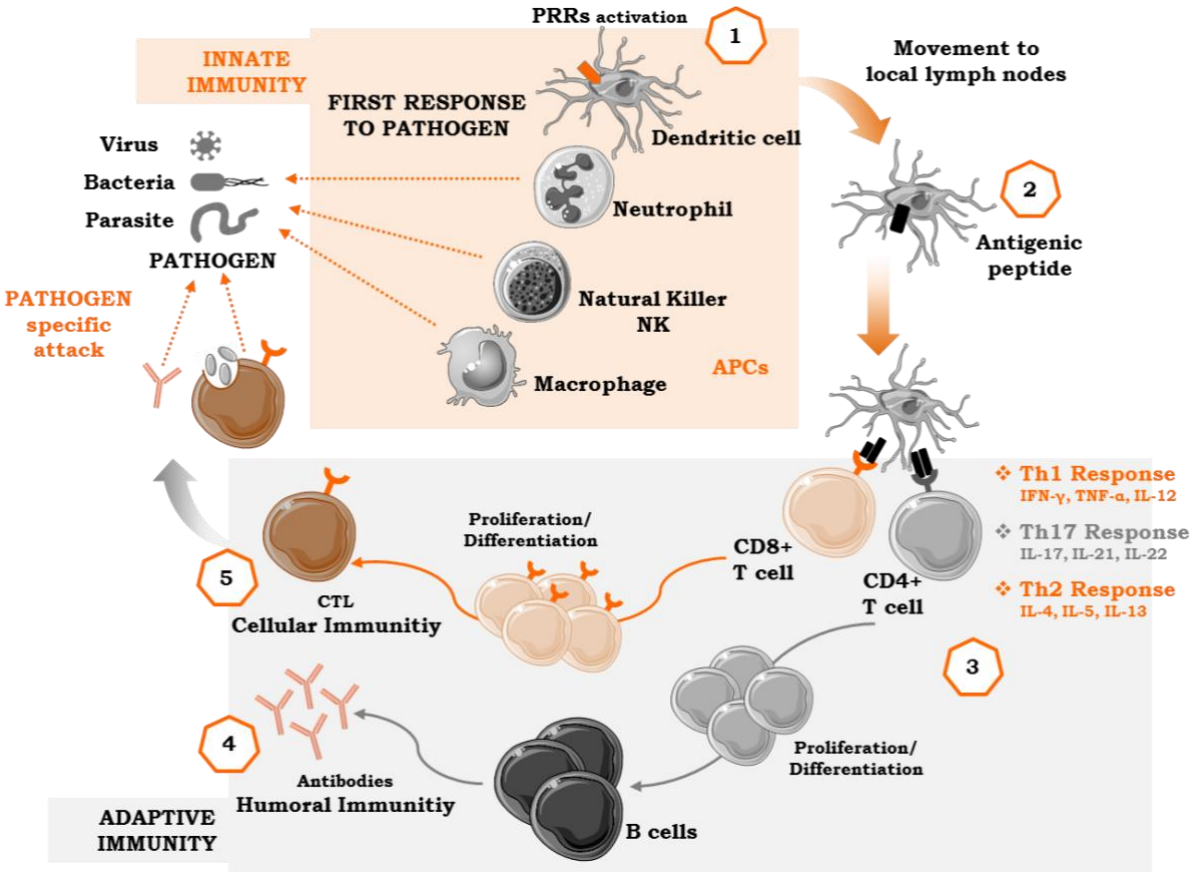
production of inflammatory chemokines and cytokines serves to draw in a host of innate cells (macrophages and neutrophils), which can provide some local support for the immediate containment of the infection. This inflammatory process further induces the activation of local APCs, such as DCs, to not only take up cellular/pathogen debris but to also increase their expression of the chemokine receptor CCR7, which induces their migration into the T cell zones of the local SLO [Pennock *et al.*, 2013]. During migration, antigen acquired within the inflamed tissue is processed and presented in both class I and class II MHCs on the cell surface for presentation to naïve T cells (**Figure 1**). In conjunction with antigen processing and presentation, the DC further matures in its expression of the various costimulatory surface molecules and cytokines. This maturation process is characterized by the loss of endocytic and phagocytic capacities and an increase in the surface expression of co-stimulatory molecules, such as CD80, CD86 and CD40. Effective antigen presentation is mediated by a sequence of signals, the first being the antigen itself; in particular, naïve lymphocytes recognize peptides or epitopes presented by APCs through classical MHC I and non-classical MHC II molecules [Morrow *et al.*, 2012]. The combination of increased surface antigen/MHC and costimulatory molecules facilitates the effective stimulation of antigen-specific CD4 and CD8 T cells, whose presence has increased due to local inflammation, increasing T cell trafficking in, and restricting trafficking out, of the SLO.

Depending on the nature of the maturation stimulus, the subset of APCs and the local environment in which antigen is recognized, innate cells are able to prime naïve T cells and then induce clonal expansion and differentiation into T helper 1 (Th1), T helper 2 (Th2), or T helper 17 (Th17) cells, all of which are distinguishable on the basis of their receptors and subsequent cytokine production profile [Jin *et al.*, 2012; Netea *et al.*, 2005; Murphy *et al.*, 2002]. The resulting stimulation of antigen-specific CD4 T cells results in their migration to the boundary between the T cell zones and the B cell follicle [Vinuesa *et al.*, 2011]. There they have the opportunity to interact with antigen-specific B cells that have responded to pathogen antigens that have either been dragged in by migrating DCs or have drained through the lymphatics [Pennock *et al.*, 2013]. Effective communication between the CD4 T cell and B cell results in their migration into and formation of a secondary follicle, where B cells undergo somatic hyper mutation and class-switch recombination to form higher-affinity pathogen-specific antibodies (**Figure 2**).

Thus, it is the nature of the danger signal at the initial site of infection that provides the immune system with the necessary information about the nature of the antigen and instructs the type of immune response needed to control the infection. Indeed, responding to the inflammatory stimuli, cells of the adaptive arm of the immune response (B cells, αβ T cells, and γδ T cells) are stimulated to expand in number (proliferate) and to differentiate into cells with a range of functions appropriate for the immunological challenge.

Figure 2. Innate and adaptive immune response to pathogen/vaccine actions.

(1) PRRs activation on APC (2) Antigen processing and presentation to T cells; (3) Enhancement of Th response; (4) Stimulation of humoral immune response. (5) Enhancement of cellular cytotoxic immune response.



Upon elimination of the invading pathogen, the majority of adaptive cells die and leave behind an array of memory cell subsets; these memory cells offer a diversity of migratory properties and functions, collectively mediating a rapid, specific and protective immune response upon subsequent encounter (**Figure 2**). Regardless of their specific phenotype, the pool of memory cells differs from naïve cells in three important parameters.

First, despite the 90–95% die from antigen-specific T cells, the frequency of cells that remain is still 10–100 fold higher than the precursor frequency of naïve cells that were present before the pathogen encounter, strongly enhancing patrolling and surveillance of the host. Second, memory cells have the capacity to access both Secondary Lymphoid Organs (SLOs) and peripheral tissues, even under conditions of homeostasis. Contrary to the primary response, where the trafficking patterns of the naïve T cells forces them to wait

in the draining SLO until antigen and/or migrating APCs present them with antigen days after the initial infection, the presence of memory cells *in situ* enables their immediate response to a pathogen reencounter, contributing to the dramatically shorter response time of the secondary response [Pennock *et al.*, 2013]. An interesting aspect of the immediate response by memory cells is that their production of effector chemokines and cytokines serve to draw in many innate immune cells as effectors.

Thus, while the quality and quantity of innate immune activation facilitates the primary adaptive response, it is the adaptive response that facilitates the recruitment and activation of innate cells during the secondary response. Third, memory cells are more sensitive to antigen stimulation through their TCR and are less dependent on costimulatory molecules to enable their productive response. As a result, memory cells are able to respond to the presentation of minimal amounts of antigen during the early hours after the initial reinfection. There are two major advantages from an adaptive response to the host: primarily, it allows the host to form an immune response that is specifically tailored to the invading pathogen; moreover, it forms a pool of memory cells from these specific effectors that can last for many years, capable of protecting the host against reinfection by their rapid response. This combination of specificity and memory are the mechanistic underpinnings for the clinical success of vaccination [Pennock *et al.*, 2013]. Historically, the first vaccines developed, many of which are still in use today, were made by killed or attenuated microorganism [De Gregorio and Rappuoli, 2014]. These vaccines are very efficacious since they are recognized by the immune system as live pathogens. As such, they contain both the PAMPs recognized by the PRRs expressed by the innate immune system as well as the antigenic components that are recognized by the specific T and B cells. Therefore, they activate both arms of the immune system and are able to generate a protective immune memory. On the other hand, they present in some cases high reactogenicity which may result in several side effects. With the increased need to vaccinate an increasingly younger and healthier population and the demand to use safer and better tolerated vaccines, scientific research led to the development of subunit vaccines that contain only recombinant or purified antigens from the microorganism. These vaccines are generally well tolerated but lack the PAMPs that are required for the activation of the innate immune system and often are not able to generate a potent and durable protective immune response. Therefore, one of the objectives for the development of the new generation vaccines is to activate the innate immune system with the use of adjuvants. Adjuvants are systems able to potentiate the specific immune response induced by the antigen with which they are co-administered. They have for the most part been developed empirically, without a clear understanding of their cellular and molecular mechanisms of action. The majority of today's vaccines contain adjuvants that were added for the purpose of enhancing the magnitude, type, onset, and duration of the acquired immune response. However, recent data suggest that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system, rather than by direct effects on the lymphocytes themselves [McCartney *et al.*, 2009; McKee *et al.*, 2007, 2010; O'Hagan and De Gregorio,

2009]. As previously explained, innate and acquired immunity are intimately linked through APCs, in particular macrophages and DCs. The innate immune system has a critical role as a first line of defense for primary responses against microbes to occur, because it confers an immediate nonspecific mechanism of protection through Patterns Recognition Receptors (PRRs). In fact, innate immune cells have a variety of PRRs that recognize conserved microbial PAMPs, as well as signals released by dying cells, termed Damage-Associated Molecular Patterns (DAMPs).

PRRs can be found in all the membranes of the cells in the innate immune system and they are not specific for any given pathogen or antigen. Although there are several hundred varieties, all the genes of the PRRs are encoded in the germ-line to ensure limited variability in their molecular structures [Clem, 2011]. Since the outset of the study of innate immunity, the interactions between PAMPs and PRRs have been extensively analyzed; Toll-like Receptors (TLRs), Nucleotide binding Oligomerization Domain-like Receptors (NLRs), C-type Lectin Receptors (CLRs), and Retinoic Acid-Inducible gene-1-like Receptors (RLRs) are the main groups of PRRs that are widely expressed on immune cells (**Figure 4**). LPS (endotoxin), peptidoglycan (cell walls), lipoproteins (bacterial capsules), hypo-methylated DNA (CpG found in bacteria and parasites), double-stranded DNA (viruses), and flagellin (bacterial flagella) naturally target PRRs [Jang *et al.*, 2015]; these antigens are produced by microbial cells and not by human cells. Recognition of PAMPs by PRRs leads to complement activation, opsonization, cytokine release, and phagocyte activation during infection or in presence of danger signals, but also directly modulate adaptive immune responses, which is specific to the invading agent. This property has been efficaciously used in heterologous pathogens or attenuated pathogen-based vaccines for years.

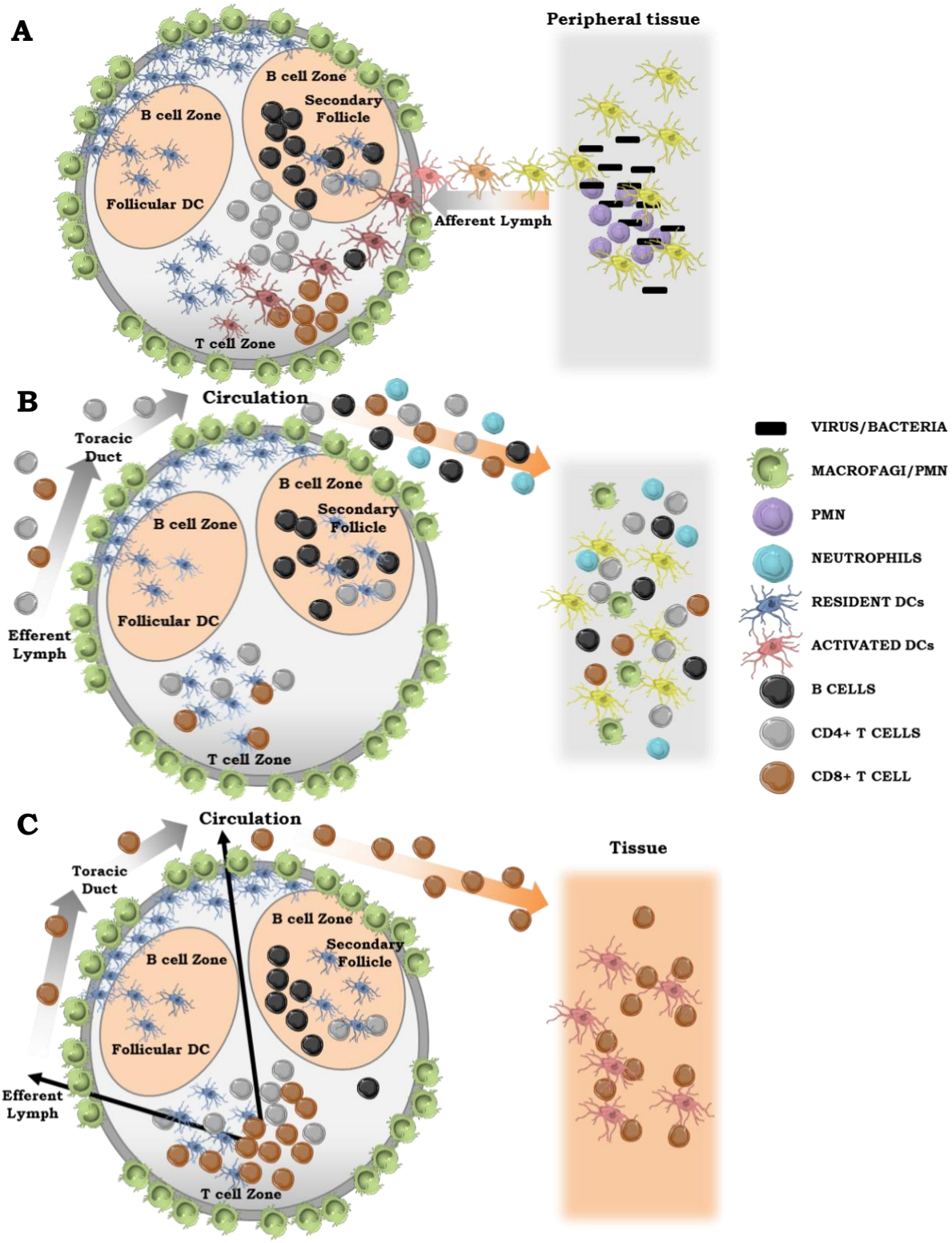


Figure 3. The anatomy of a T cell response.

(A) Microbe invasion and proliferation at the site of infection leads to the initial recruitment of phagocytes and containment of the infection. Tissue-resident APCs acquire antigen and migrate into the proximal draining lymph nodes after being activated by the local inflammatory processes. During transit, APCs process and present antigens in the context of class I and class II MHC and upregulate various cell surface molecules and cytokines important in providing the necessary co-stimulation to T cells within the SLO. (B) Antigen-stimulated CD4 T cells collaborate with B cells to promote

antibody production, class switching, and memory B cell formation. CD4 and CD8 T cells clonally expand and migrate out of the SLO and into the infection site, where their effector functions facilitate the elimination of the pathogen. Also, memory B cells and neutrophils move to the tissue (C) Most T cells die off, leaving a memory pool with precursor frequency, antigen sensitivity and trafficking capacity optimized for initiating rapid secondary responses *in situ*.

Benefits of adjuvants to vaccine formulations

The key objective of vaccination is the induction of an effective pathogen-specific immune response that leads to protection against infection and/or disease caused by that pathogen, and that may ultimately result in its eradication. The concept that the immune response to antigens can be improved by the addition of certain compounds into the vaccine formulation was demonstrated about one hundred years ago, when Aluminum salts were introduced in vaccine formulations and were referred to as "adjuvants". Since then, Aluminum salts have been widely used in vaccines to aid in antigen presentation and delivery, acting as adjuvants in order to generate effective immune responses.

Over the past years, many efforts have been made to investigate how and why adjuvants work; a recent, greater understanding of innate and adaptive immunity and their close interaction at the molecular level in the host response to a pathogen has enabled vaccine researchers to use adjuvants to their full advantage. Indeed, besides being traditionally used to increase the magnitude of an adaptive response to a vaccine, adjuvants are becoming increasingly important to allow vaccine formulations to selectively stimulate immunological pathways to obtain the desired type of antigen-specific immune response (humoral and/or cell-mediated) [Kenney and Cross, 2010; Pulendran *et al.*, 2010].

Clinically, adjuvants are used to increase the response to a vaccine in the general population (increasing mean antibody titers and/or the fraction of subjects that become protectively immunized), but they are particularly crucial for immunization in populations with reduced responsiveness because of age [infants and elderly], disease, or therapeutic interventions [Podda, 2001; Beran, 2008], or when the efficacy of a vaccine is limited. In addition, adjuvants can also increase immune response durability by inducing immune memory and persistence: these improved antibody responses can be correlated with a robust germinal center formation and an expansion of antigen-specific follicular helper T cells (TFH), a subtype of T cells that provide help to B cells for antibody production and class switching [Linterman and Hill, 2016].

Adjuvants are included in vaccines to induce enhanced immune responses to vaccine antigens. In particular, recent advances have shown that these compounds are able to (i) increase the biological half-life of vaccines, (ii) enhance antigen uptake by APCs, (iii) activate/maturate APCs, (iv) induce the production of immune-modulatory cytokines, (v) activate inflammasomes, and (vi) stimulate local inflammation and cellular recruitment.

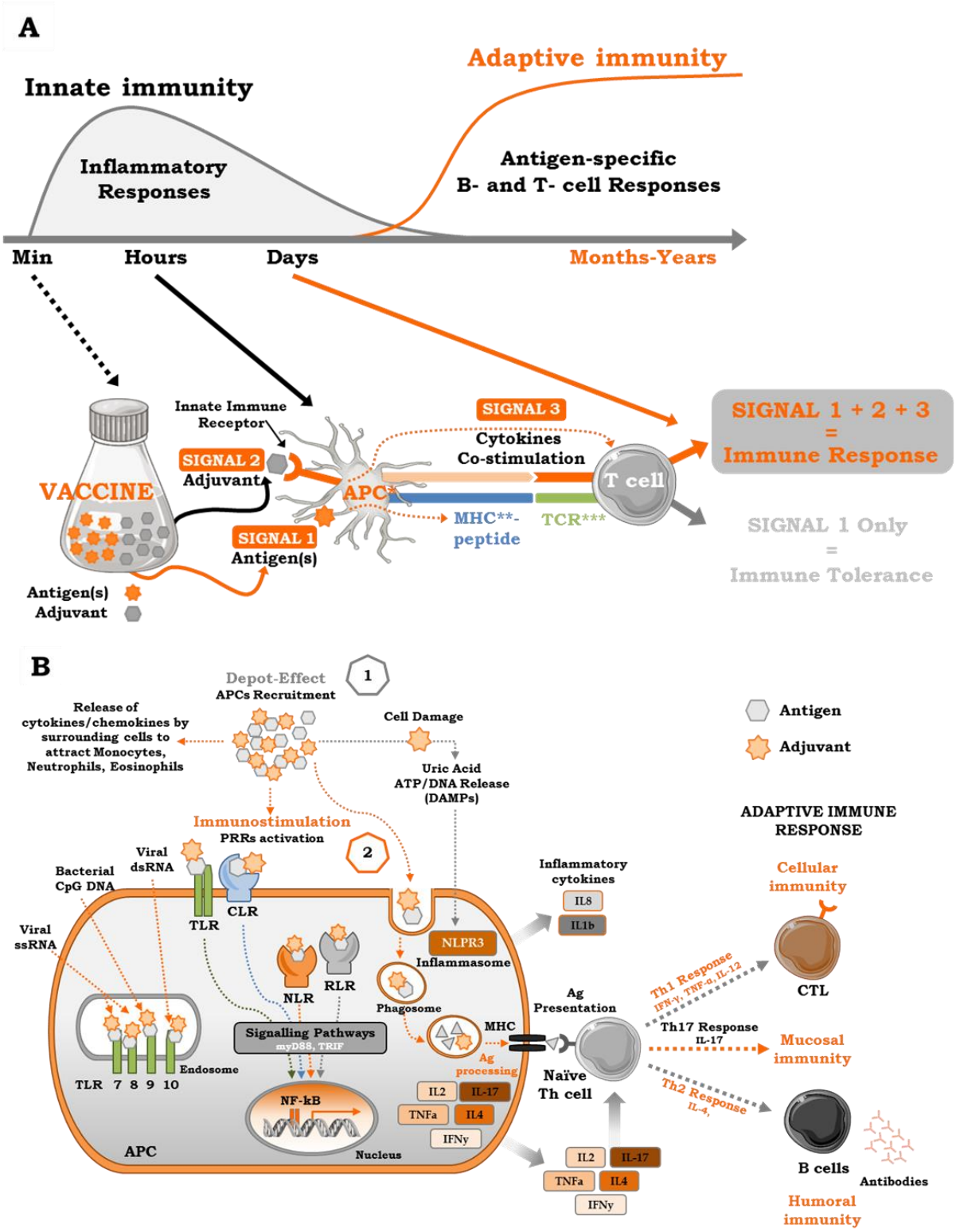


Figure 4. Adjuvanted antigen activation of APC

(A) Vaccine adjuvants can activate the innate immune system by acting like Pathogen-associated Molecular Patterns and thus can enhance or restore the ability of the immune system to identify vaccine antigen as a pathogen with subsequent activation/maturation of APC and activation of the adaptive immune system. (B) (1) Delivery system localizes antigen and immune potentiator at the site of injection, favoring its encounter with the APC. (2) Immune potentiator directly activates APC through PRRs, which bind PAMPs and DAMPS. This activation leads to increased co-stimulatory

capacity of APCs, up-regulation of MHC molecules and secretion of innate protective molecules and pro-inflammatory cytokines that recruit immune cells. Antigen processing step provides the specific pathogen epitopes to be presented by APCs to naïve CD4 T cells through MHC class II molecules. CD4 T cells undergo clonal expansion and differentiation toward specific T helper phenotypes (Th1, Th2, Th17) and provide help to antigen-specific B cells to undergo class switching and conversion to plasma cells that secrete antigen specific antibodies. Activated APCs can also act directly on CD8 T cells that recognize MHC class I-peptide complexes, with CD4 Th cells providing support to license them to become effector cytotoxic T lymphocytes (CTLs) (Mechanism Not shown).

A very diverse range of compounds and materials can achieve an adjuvant effect, and different criteria may be used to group adjuvants in order to allow a rational comparison. The increasing understanding of the mechanisms behind the functioning of the immune system has allowed classifying adjuvants into two major classes: (I) the immune potentiators that activate the innate immune system and potentiate the antigen-specific response (immunogenicity) and (II) the delivery systems, that vehicle the immune potentiators and antigen to the right cells and organs of the immune system [Singh and O'Hagan, 2002; Pashine *et al.*, 2005] (**Figure 4**) Delivery systems, such as nanoparticles, liposomes, virosomes or recombinant vectors, are particulate materials that are able to function as carriers to which antigens can be associated, to stabilize the antigens and to allow them to be present for a longer time at the site of injection ("depot-effect"; **Figure 4**); moreover, since their dimensions are consistent with those of pathogens, they can be taken up by phagocytosis into APCs, creating local pro-inflammatory response that in turn recruit other innate immune cells to the site of injection, further enhancing antigen delivery and uptake. By contrast, immune-potentiators exert direct stimulatory effects on immune cells and also initiate the immune response through activation of innate immunity (**Figure 4**). Although immune-potentiators are a very broad class of materials, typically they are purified components of bacterial cells or viruses.
Consequently, they are recognized as 'danger signals' by receptors present on immune cells, particularly APC. Once these receptors are engaged, cells respond accordingly through activation of the innate immune response. Independently of the mechanism of action, these compounds facilitate the use of smaller doses of antigen [Banzhoff *et al.*, 2009; Boyle *et al.*, 2007; Schwarz *et al.*, 2009], permitting comparable responses with substantially lower amounts of antigen. This is important when large-scale vaccination is urgent and production facilities limiting, as in the emergence of a pandemic influenza strain; moreover, they reduce the number of doses required to induce protection immunity, an ability that is essential in countries where multiple injections raise compliance issues and/or significant logistic challenges [Banzhoff *et al.*, 2009; Halperin *et al.*, 2006; Schwarz *et al.*, 2009].

Mode of action of empirically derived adjuvants

The development of new adjuvants is still necessary to improve immunogenicity of existing vaccines, to develop new vaccines against diseases that are difficult to target (*e.g.* HIV, Malaria and Tuberculosis) or to respond quickly to new emerging infectious diseases. For many years, the only two allowed adjuvants in the clinic were Aluminum salts (generically referred to as Alum) and the oil in water emulsions MF59 [Singh *et al.*, 2006; Mbow *et al.*, 2010]. Alum has been used in the clinic for almost a century, while MF59 was licensed for adjuvanted Flu in the elderly in 1997 and, together with another emulsion, AS03, was licensed in Europe in the 2009 for the vaccine against the H1N1 pandemic Flu [Buonsanti and D'Oro, 2016]. Despite their extensive use, the mechanism of action of Alum and MF59 started to be investigated only in recent years [De Gregorio *et al.*, 2013]. They act both as immune potentiators and delivery systems; as delivery systems, they create an antigen depot and increase antigen uptake at the site of injection [Dupuis *et al.* 1998; Morefield *et al.*, 2005]. As adjuvants, they induce a local pro-inflammatory reaction that contributes to increase immunogenicity [Goto *et al.*, 1997; Mosca *et al.*, 2008; Seubert *et al.*, 2008]. However, their mechanism of action differs: Alum seems to activate NALP-3 component of the inflammasome complex [Eisenbarth *et al.*, 2008; Kool *et al.*, 2008; Hornung *et al.*, 2008; Li *et al.*, 2008], while MF59 requires functional MyD88 adaptor protein and induces the release of ATP at the site of injection [Seubert *et al.*, 2011; Vono *et al.*, 2013]. Moreover, it has been demonstrated that MF59 localizes in the subcapsular and medullary macrophage compartments of mouse draining LNs in which it promotes accumulation of the vaccine antigen and thus facilitates the deposition of the immune complexes-trapped antigen onto activated follicular DCs [Cantisani *et al.*, 2015].

PRRs agonists as vaccine immune potentiators

Alum salts and emulsions were developed empirically and have been used for many years without knowing their mechanism of action. However, the field of adjuvant discovery underwent a drastic change recently, when the molecular mechanisms of immune activation become to be elucidated and PRRs were discovered as key molecules that regulate the innate immune signaling. Among them, TLRs and NLRs are the most studied PRRs that can control and modulate the cellular immune response [Iwasaki and Medzhitov, 2004; Philpott *et al.*, 2014] (Table 1).

Control of innate and adaptive immunity by TLR signaling

Over the past few decades, there has been significant progress in understanding the molecular mechanisms involved in antigen recognition and in the induction of immune response. The stimuli involved in the activation and maturation of APCs can act either independently or synergistically to promote cytokine secretion as well as up-regulation of PRRs expression. Integration of the host response to several PAMPs or DAMPs allows for a

highly tailored immune response [Bianchi, 2007; Medzhitov *et al.*, 1997]. Most pathogens contain several PAMPs that are recognized by the host cell PRRs that act in concert to elicit protective immune responses. Therefore, it is logical to design vaccines with multiple PAMPs/DAMPs to stimulate complementary and/or redundant PRR signaling pathways to mimic what occurs in nature. Vaccine components should instruct APCs at the site of vaccination as to the type of immune response required to establish effective immunity and immunologic memory to combat subsequent, natural infection.

TLRs are the best characterized PRRs; thirteen TLRs are present in mammalian species and each appears to have a specific and distinct function in innate immunity.

As other innate immune receptors, TLRs are germ-line encoded PRRs that recognize a broad range of PAMPs associated with bacteria, viruses, parasites, and fungi to initiate innate immune responses and to instruct adaptive immunity [Akira *et al.*, 2006; Iwasaki and Medzhitov, 2004; Takeda *et al.*, 2003].

Among the first group, TLR2 recognizes lipoproteins of the cell wall of gram-positive bacteria, TLR4 recognizes lipopolysaccharide (LPS) of gram-negative bacteria and TLR5 is stimulated by the flagellin protein from a variety of bacteria. Among the latter category, TLR3 and TLR7/8 sense double-strand and single strand RNA (ds-RNA, ss-RNA), respectively, while TLR9 recognizes viral and bacterial unmethylated-CpG-containing DNA. The ligand-binding domain consists primarily of a repeating pattern of a Leucine-Rich Repeat (LRR) motif, which provides an adaptable structural matrix for interactions with a variety of distinct ligands [Bell *et al.*, 2003; Kobe *et al.*, 2001].

Signaling through all TLRs, with the exception of TLR3, involves an intracellular cascade that includes the Myeloid Differentiation primary response gene 88 (MyD88), IL-1 Receptor Activated Kinase (IRAK), TIR-Associated-Protein (TIRAP), Toll Receptor-Associated Activator of Interferon (TRIF), Toll Receptor-Associated Molecule (TRAM), and Tumor Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF-6), leading to activation of NF-kB [Takeda *et al.*, 2003]. NF-kB activation results in the induction of pro-inflammatory cytokines, such as IL-1β and TNF-α, and induction of MHC molecules and co-stimulatory signals that provide the link from pathogen recognition by the innate immune system to activation of the adaptive immune system [Van Duin *et al.*, 2006]. In the case of the endosomal receptors TLR7/8 and TLR9, MyD88 activates IRF7 and the secretion of type I Interferons (IFN). TLR3 is the unique TLR that uses directly the adaptor protein TRIF with subsequent activation of IRF3 and IRF7 [Kawai *et al.*, 2010].

Some of the specific mechanisms by which activation of the TLR system promotes adaptive immune responses include:

- Antigen internalization and maturation of DCs [Schjetne *et al.*, 2003];
- Influencing migration of DCs [Means *et al.*, 2003];
- Promoting Th1 responses [Roman *et al.,* 1997];
- Cross-priming and -presentation [Heit *et al.*, 2004; Schulz *et al.*, 2005];
- Reversal of tolerance [Pasare and Medzhitov 2003; Yang *et al.*, 2004; Serra *et al.*, 2003; Peng *et al.*, 2005];

❖ Up-regulation of MHC and co-stimulatory molecules [Cella *et al.*, 1997; Hertz *et al.*, 2001].

Many natural molecules that were used experimentally as potential adjuvants, such as LPS, dsRNA, CpG-rich bacterial DNA were shown to bind TLRs. In addition, recent studies have shown that commonly used prophylactic vaccines BSG, Influvac®, Typhim Vi® and yellow fever vaccine, induce activation of DCs through TLR signaling [Querec *et al.*, 2006; Pulendran, 2009; Schreibelt *et al.*, 2010]. Several TLR agonists have been studied as potential vaccine adjuvant and some of them have been tested in clinical trials. The first molecule targeting a TLR to have been licensed as adjuvant is Monophosphoryl Lipid A (MPL-A) that target TLR4 [Casella and Mitchell, 2008]. In combination with Alum, it makes the new adjuvant AS04, approved for HBV and HPV vaccines [Didierlaurent *et al.*, 2009]. MPL-A is a natural molecule but other synthetic analogs have already been tested in clinical trials in combination with Alum or formulated with emulsions [Persing *et al.*, 2002; Dupont *et al.*, 2006; Santini-Oliveira *et al.*, 2015]. TLR5 agonist bacterial flagellin has adjuvant properties when mixed with antigens, however current application is focused on the generation of fusion proteins of recombinant vaccine antigens and flagellin [Huleat *et al.*, 2007; McDonald *et al.*, 2007; Mizel *et al.*, 2009]; immunization with flagellin-containing vaccines induces a mixed Th1 and Th2 cell immune response and also leads to enhanced secretion of antigen-specific IgG and local IgA responses [Liu *et al.*, 2010; Hong *et al.*, 2012]. TLR7 or TLR8 agonists are not approved as vaccine adjuvant components, however the synthetic TLR7 ligand imidazoquinoline Imiquimod (R-837) has been licensed in a topical cream formulation for dermatologic diseases [Meyer *et al.*, 2008] and was tested in clinical trials as adjuvant in anti-cancer and infectious diseases [Toussi and Massari, 2014]. However, the most advanced TLR ligand tested as adjuvants are two synthetic CpG containing DNA oligonucletides (CpG7909 and ISS1018) alone or formulated with Alum and nanoemulsion, that were tested with HBV, malaria, influenza, antrax and cancer vaccines [Steinhagen *et al.*, 2011; Shirota *et al.*, 2014]. CpG7909 was tested in phase I/II clinical trials with a 7-valent pneumococcal conjugate or HBV vaccine in HIV patients which are usually hypo-responsive to these vaccinations. These adjuvanted vaccines induced an increased number of high responders and achieved an extended long-term protection compared to the non-adjuvanted vaccines [Cooper *et al.* 2008; Sogaard *et al.*, 2010].

Table 1. Pattern Recognition Receptors

TLR	Localization	Ligands	Signal Adaptor	Production
TLR1	Cell surface	Bacterial lipoproteins from *Mycobacteria*, *Neisseria*	MyD88	IC
TLR2	Cell surface	Triacylated lipoproteins, Zymosan yeast particles,	MyD88	IC

		Peptidoglycans, Lipoproteins, Glycolipids, LPS		
TLR6	Cell surface	Yeast zymosan, lipotechoic acid, lipopeptides from mycoplasma	MyD88	IC
TLR3	Endosomes	Viral dsRNA, (poly (I:C)	TRIF	IC, type1 IFN
TLR4	Cell surface/ endosomes	Lipopolysaccharides (LPS) Paclitaxel	TRIF MyD88	IC, type1 IFN
TLR5	Cell surface	Bacterial Flagellin	MyD88	IC
TLR7	Endosomes	ssRNA Imidazoquinolines (R848, R-837)	MyD88	IC, type1 IFN
TLR8	Endosomes	ssRNA, Imidazoquinolines (R848)	MyD88	IC, type1 IFN
TLR9	Endosomes	CpG DNA CpG oligonucleotides	MyD88	IC, type1 IFN
TLR10	Endosomes	Profilin-like proteins	MyD88	IC
NOD1	Cytoplasm	Diaminopimelate-containing muramyl tripeptide mostly found in Gram-negative bacterial peptidoglycan	RIPK2	IL-1β
NOD2	Cytoplasm	Muramyl dipeptide from gram-positive and Gram-negative bacterial peptidoglycan	RIPK2	IL-1αβ
NLRP3	Cytoplasm	ATP, viral RNA, Muramyl dipeptide, Imidazoquinoline, Uric acid cristals, Silica, Aluminum Salts, Chitosan, QuilA	Inflammasome	IL-1αβ, IL-18
MMR (Macrophage Mannose Receptor)	Cell surface	Mannose, Fucose	CRD domanis	Antigen uptake
CLRs (Dectin-1, Mincle, DC-SIGN)	Cell surface	β-Glucan, virus	Src, Syk	T cell interaction
RLRs (RIG-1, MDA5)	Cytoplasm	Viral double strand RNA	IRF	IC, type1 IFN
STING	Cytoplasm	DNA	TBK1-IRF3	IFN-β

NOD-like Receptors

NLRs family is composed of 22 cytoplasmic receptors each of one containing a central nucleotide-binding domain (NOD) for agonist binding plus an N-terminal effector domain that activates a specific signaling cascade. NLRP3, also known as NALP3, is the most known member of the NLRPs subfamily of NLRs, which is characterized by an N-terminal

pyrin domain. In association with the ASC adapter and Caspase-1, NALP3 compose the NLRP3 inflammasome that catalyzes the cleavage of pro-IL-1β and pro-IL-18 to the respective mature cytokines. It has been shown that NAPL3 may detect a wide range of PAMPs and DAMPs such as ATP, uric acid, silica, chitosan and Aluminum salts and each of these stimuli activates its own signaling cascade, resulting in the release of a common downstream signaling event which then activates NALP3 inflammasome [Benko *et al.*, 2008; Geddes *et al.*, 2009]. NOD1 and NOD2 initiate the NF-kB-dependent and MAPK-dependent gene transcription; both receptors sense the peptidoglycan from the bacterial cell wall, although they recognize different structure of this PAMP. NOD1 detects DiAminoPimelic acid (DAP)-containing muropeptide, which is typically present in Gram-negative bacteria [Girardin *et al.*, 2003], whereas NOD2 senses Muramyl DiPeptide (MDP) moieties, ubiquitously present in both Gram-negative and Gram-positive bacteria [Girardin *et al.*, 2003]. The NOD-driven activation of innate immunity is indeed crucial to trigger an adaptive immune response to bacterial infection and this has opened up the opportunity to use NOD-ligands as vaccine adjuvants [Geddes *et al.*, 2009; Hancock *et al.*, 2012]. The ability to trigger NLRs has been shown for old and extensively used vaccine adjuvants, such as the Aluminum salts that induce the activation of NOD1/2 and NLRP3, respectively.

Combination of immune potentiators and Delivery systems

Many of the new immune potentiators tested in preclinical and/or clinical setting proved to be very potent in activating the immune system, but their potency often goes along with high toxicity. Delivery systems may limit their toxic effect by localizing the immune potentiators and the vaccine antigens at the injection site: in this way, the adjuvant only acts on the immune cells that will infiltrate the injection site, enhancing the antigen-specific immune response [O'Hagan and Valiante, 2003], while reducing the risk of a systemic inflammation. The combination of immune potentiators and delivery systems can improve vaccines in different ways:

- Enhance the immune response to intrinsically poorly immunogenic antigens;
- Increase immunogenicity of antigens that are not effective in the elderly or infants;
- Induce a more prolonged protection;
- Elicit a different type of immune response;
- Shift the immune response towards a specific T cell phenotype (Th1, Th2 or Th17);
- Generate antibodies with higher affinity or avidity;
- Reduce the dose of antigen in the vaccine
- Reduce the number of doses required to achieve full protection [Black *et al.*, 2015].

The majority of the TLR agonists currently under investigation as adjuvants are tested in combination with first generation adjuvants (Aluminum salts, oil in water emulsions and liposomes) that behave essentially as particulate carriers [O'Hagan and Fox, 2015].
However, several studies are also exploring the use of multiple TLR agonists combinations associated to different delivery systems [Coffman *et al.*, 2010; Mutwiri *et al.*, 2011]. AS01

is a multicomponent adjuvant, made by liposomes containing both MPL and the saponin QS21, which is part of two novel vaccines, one based on a RTS,S recombinant antigen that demonstrated efficacy in preventing malaria in Phase III trials, and a subunit vaccine against herpes zoster which was shown to be very efficacious in both old and immunocompromised subjects [Agnandji *et al.*, 2011; Cunningham, 2016]. Other groups showed that the combination of TLR4 and TLR7 ligands co-delivered with antigens through synthetic nanoparticles have a synergistic effect on immunogenicity compared to each single TLR ligand [Kasturi *et al.*, 2011]. Also a combination of NOD1 and NOD2 receptor ligands when encapsulated into biodegradable Poly Lactic Acid (PLA) nanoparticles, showed to be more efficacious compared to free ligands at increasing the immune response to the gag p24 HIV-1 antigen delivered in separate PLA particles [Pavot *et al.*, 2013].

Small Molecules Immune-Potentiators as new adjuvants

Natural TLR ligands and their synthetic analogues have been extensively tested for this purpose in preclinical studies and some of them are advanced in the clinic [Maisonneuve *et al.*, 2014]. However, recently the discovery and development of new molecules as vaccine adjuvants has often relied on synthetic analogues more than on natural compounds. New rational approaches are now being adopted to facilitate progresses to clinical development of new adjuvants; these rely on the use of High-Throughput Screening (HTS) methodologies and medicinal chemistry activities. This strategy, borrowed from the drug discovery field, has two requisites: the availability of chemical libraries containing small molecules able to trigger an innate immune response and an *in vitro* assay that is predictive for their potential adjuvant activity. The compounds that display immunological activity (*hits*) are prioritized based on potency, stability, specificity and chemical-physical parameters and optimized with an iterative process of chemical modifications based on structural activity relationship (SAR). Secondary screening can also be performed to evaluate mechanism of action, toxicity or other parameters of the lead compounds. Afterwards, these molecules can be tested in relevant animal models to investigate their efficiency and benchmarked against known adjuvants [Pashine *et al.*, 2005]. This approach can lead to the discovery of new adjuvants, proceeding on two fronts: while targeted medicinal chemistry efforts can be helpful to optimize known chemical structures based on the knowledge of their molecular mechanism of action, the use of random screening for immunological active compounds, independently of their structure, can lead to the identification of completely novel chemical structure [Pashine *et al.*, 2005]. The latter approach is used for the discovery of new adjuvants without the bias about the mechanism of action of the potential targets and ligands. The only criteria that need to be satisfied are the capacity to activate some *in vitro* biological system that allows to predict an immunological function. Such assays can be for example the release of secreted cytokines and chemokines by cell lines or primary immune cells, which are generally detected by ELISAs or other immune techniques, or the activation of transcription factors which can be detected by gene reporter assays using colorimetric or

luminescent read-outs. For example, in the case of adjuvants targeting the different TLRs, a cell line expressing a specific TLR and a gene reporter system can be used in an HTS approach to test a library of natural or synthetic analogues with the aim to identify a new class of agonist for that receptor or to optimize a lead with improved activity [Buonsanti and D'Oro, 2016].

Design of adjuvants targeting Toll-Like Receptors

Activation of the innate immune system has the potential to induce qualitative different adaptive immune responses by providing different signals during priming of naïve CD4 T cells, driving their response toward distinct specific T helper profiles (Th1, Th2, Th17). Those signals are strongly influenced by TLRs signaling. For example, while TLR2 or TLR5 engagement lead to a balanced Th1/Th2 mixed response, TLR4 engagement drives T cell priming more to a Th1-like phenotype, and nucleic-acid sensing TLRs are particularly potent to induce CD8 T cell responses [Coffman *et al.*, 2010]. Therefore, a rational design approach could make possible to develop immune potentiators that selectively activate the desired immune outcome.

TLR7 has been the first receptor of the innate immune system which was shown to be activated by small drug-like synthetic molecules, leading to the identification of the compounds in the imidazoquinoline family as Small-Molecule Immune Potentiators (SMIPs) triggering TLR7 and/or TLR8. The TLR7 agonists Imiquimod and Resiquimod have been investigated for their adjuvant activity in many preclinical models [Vasilakos *et al.*, 2013]. In all these studies emerges that, although they are able to increase immunogenicity and to induce a strong immunological memory, they also induce systemic inflammation and an unacceptable reactogenicity. The hypothesis is that the undesired effects are due to the high biodistribution of these small molecules that results in a generalized inflammation. Indeed, these small molecules do not behave as good adjuvants when administered simply mixed with the antigen, whereas improve their effect when they are co-delivered with the antigen and retained at the injection site.

Many companies are involved in the identification of small molecules TLR7 agonists as new adjuvants. GSK Vaccines was also interested in the identification of new SMIPs targeting TLR7 but with limited biodistribution, in order to increase vaccine efficacy with minimal side effects. This strategy was based on the hypothesis that a poorly soluble compound would be locally retained with the co-delivered antigen at the muscle injection site, were its activity as immune stimulator would be beneficial, minimizing its systemic adsorption, thus potentially reducing the release of inflammatory mediators, which only results in undesired reactogenicity. Indeed, safety and tolerability are the primary attributes of each vaccine formulation and need to guide also the design and optimization of the adjuvant component. The initial medicinal chemistry effort was focused on the identification of a novel class of selective TLR7 agonists, belonging to the Benzonaphthyridines (BZN) chemical group. Then a systematic modification of the BZN chemical structure, combined

with a panel of *in vitro* screening assays, led to a rational design of TLR7-agonists that were less soluble than classical small molecules like Resiquimod: after *in vivo* muscle administration to mice, these lipophilic BZN were extensively retained at the injection site and induced a limited systemic inflammatory response. Thus, these new lipophilic BZNs were able to increase immunogenicity of the co-administered antigens much more than Resiquimod: these results supported the hypothesis that the systemic innate immune activation induced by highly soluble small molecules, such as Imiquimod and Resiquimod, results in a "waste inflammation" that was not functional to their adjuvant efficacy [Buonsanti and D'Oro, 2016]. However, these newly designed SMIPs presented an easy predictable issue: being highly lipophilic, these compounds were poorly soluble and therefore difficult to formulate reproducibly at the scale required for manufacturing of a licensed vaccine and at the physiologic buffer conditions necessary for their use in humans. In addition, it was shown that they were retained at the injection site for more than two weeks and this excessive long persistence was likely not necessary for the adjuvant effect. These drawbacks were eliminated by engineering SMIP-based adjuvants with a combination of medicinal chemistry and vaccine formulation science. A new screening was performed to identify BZN analogs that were selective TLR7 agonists but more soluble in aqueous solutions at physiologic pH.

Soluble TLR7-SMIPs were then designed for adsorption to Aluminum Hydroxide (Alum) inserting a PolyEthylene Glycol (PEG) linker and a terminal phosphonate group which allow their adsorption to Alum via ligand exchange. Therefore, the second generation SMIPs were selected based on their solubility at neutral pH and a stable adsorption to Alum. When these compounds were not adsorbed to Alum they exhibited a reactogenic profile similar to Resiquimod *in vivo* and, like this molecule, they were extremely poor of the lipophilic SMIPs and were even more effective adjuvants than the first generation [Wu *et al.*, 2014]. SMIP-7.10, one of these phosphonate BZN compounds, bound to Alum and defined as Alum-TLR7, was selected for further development as vaccine adjuvant in glycocongiugate vaccines [Buonsanti *et al.*, 2016]. The discovery of Alum-TLR7 represented the first example of a new adjuvant based on a synthetic small-molecule and obtained by a rational design.

The criteria for the screening of the SMIPs were to deemphasize potency while focusing on safety and tolerability. With this aim in mind, the selected molecules displayed adequate but not maximum potency in the *in vitro* immunoassays, while still possessing the physicochemical characteristics that allowed for optimal Alum adsorption and therefore less systemic exposure. This approach represents a new general strategy that can be applied to engineer many small molecules for use as vaccine adjuvants.

Macrophage polarization

In the healthy organism, the innate immune system provides the first line of defense against external or internal danger signals, by initiating a protective inflammatory response

that develops during time through different phases, from initiation and full inflammation, to resolution and re-establishment of tissue integrity. The first phase of an inflammatory response is aimed at destroying pathogens and is followed by a phase in which dead and dying cells, damaged extracellular matrix material and cellular debris are removed, to end up with a recovery phase in which the tissue is repaired and restored to a healthy fully functional condition. In fact, if the defense against harmful threats is a priority for avoiding tissue damage, maintaining homeostasis (*i.e.*, maintaining tissue morphology and tissue function) is the ultimate goal of a tissue in multicellular organisms [Matzinger *et al.*, 2007]. Monocytes/macrophages play major roles in development, scavenging, inflammation and anti-pathogen defenses, both by the direct elimination of foreign agents and in organizing each different phase of the inflammatory process [Van Furth and Cohn, 1968]. When not properly regulated or in excess, inflammation may contribute to many and different pathological conditions, from autoimmune and chronic inflammatory diseases to atherosclerosis and cancer (Italiani and Boraschi, 2014 and references therein). Moreover, they have a central role in tissue development, by shaping the tissue architecture and in surveillance and monitoring of tissue changes, by acting as sentinel and effector cells. Especially, they are important in maintaining homeostasis, by clearing apoptotic or senescent cells and by repairing and remodeling structural and functional integrity of the tissue soon after damage.

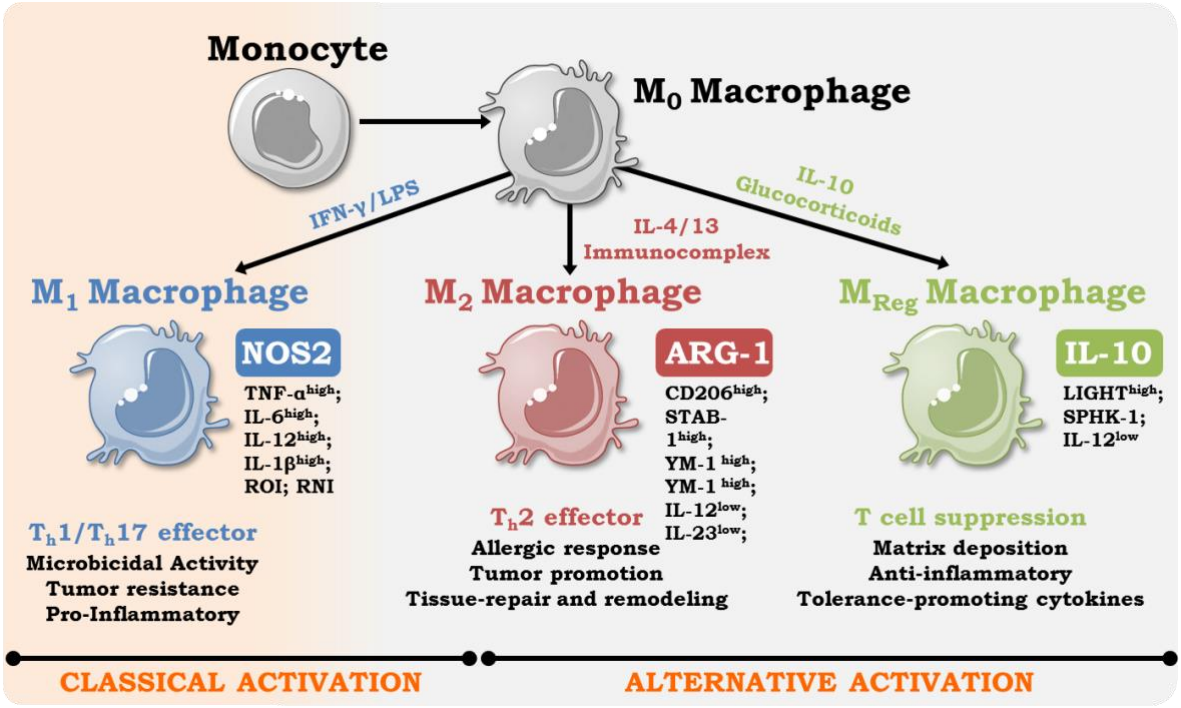
In 1893, Elie Metchnikoff first described macrophages ("the big eaters" in Greek) as tissue resident cells able to eat and kill infectious agents. Macrophages are heterogeneous and versatile cells found essentially in all tissues of adult mammals, where they can represent up to 10–15% of the total cell number in quiescent conditions; this number can increase further in response to inflammatory stimuli. The specialization of macrophages in specific microenvironments explains their heterogeneity. These populations, that acquire different names according to their tissue localization (*e.g.* histiocytes in the connective tissue, Kupffer cells in the liver, osteoclasts in the bone, alveolar macrophages in the lungs, microglial cells in the central nervous system, Langerhans cells in the skin and others), have such highly different transcriptional profiles that they could be considered as many different and unique classes of macrophages [Gautier *et al.*, 2012]. Apart from tissue-specific functions, all tissue macrophages share a series of common functions encompassing clearance of cell debris, immune surveillance, wound healing, defense against pathogens and the initiation and resolution of inflammation.

Different tissues define different phenotypes of both resident macrophages and inflammatory cells macrophages recruited from the reservoirs of blood (*i.e.* also meaning monocytes-derived macrophages), spleen and bone marrow [Geissman *et al.*, 2010], phenotypes that are necessary for the tissue-specific needs of defending, maintaining and regaining homeostasis [Lech *et al.*, 2012]. In facts, macrophages own the capacity to polarize into different functional phenotypes in response to the tissue micro-environmental changes that occur during the different phases of an inflammatory response, enabling them to steer adaptive immune response in different direction. This highlights the central role of

macrophages in immune defense, overturning the long-held notion that macrophages need to be activated by T cells.

In vivo, macrophages can adopt a variety of functional phenotypes depending on subtle and continuous changes in the tissue micro-environment. The so-called process of macrophage polarization occurs through different activation programs by which macrophages carry out their defensive functions. In this way, macrophages become able to respond with appropriate functions in distinct contexts, modifying their metabolic functions from a heal/growth promoting setting to a killing/inhibitory capacity [Mills, 2000 and 2012]. According to these major functional differences, macrophages are normally distinguished as M1 or M2-activated. M1 and M2 macrophages can also have distinct features in terms of chemokine production profiles [Frankenberger *et al.*, 2012] and iron and glucose metabolism [Barnes *et al.*, 2005]; however, the main distinction between the two phenotypes is that in M2 macrophages the arginine metabolism is shifted to ornithine and polyamines, while in M1 cells it is shifted to NO and citrulline [Lech *et al.*, 2012]. M2-produced ornithine can promote cell proliferation and repair through polyamine and collagen synthesis, fibrosis and other tissue remodeling functions [Pesce *et al.*, 2009], while M1-produced NO is an important effector molecule with microbicidal activity and cell proliferation inhibitory capacity [Macmicking *et al.*, 1997].

Differently, Mosser and Edwards [2008] have suggested a macrophage classification that takes into account the three functions of these cells in maintaining homeostasis: host defense, wound healing, and immune regulation. Classifying macrophages according to



these functions provides three basic macrophage populations: classically activated macrophages (also known as M1), wound-healing macrophages (M2), and regulatory macrophages (MReg) [Fleming *et al.*, 2011; Manjili *et al.*, 2014] (**Figure 5**).

Figure 5. Schematic representation of Macrophage Polarization

Microbial products, Interferon γ (IFN-γ), Granulocyte-Macrophages Colony Stimulating Factor (GM-CSF) or various TLR ligands activate macrophages toward an M1 functional program (Classically Activated Macrophages), with secretion of large amounts of pro-inflammatory cytokines such as IL-1β, TNF-α, Type 1 IFN, IL-6, IL-12, IL-18 and IL-23. M1 macrophages are irreplaceable during acute infectious diseases since they provide host protection against intracellular bacteria or viruses by production of toxic effector molecules such as Nitric Oxide (NO) or Reactive Oxygen Intermediates (ROI) [Verreck *et al.*, 2005]. Typical characteristics of M1 cells include also enhanced antigen presentation via higher expression of the major histocompatibility complex class II (MHC II) and co-stimulatory molecules CD80 and CD86, participating as inducers and effector cells in polarized Th1 responses.

In contrast, M2 activation phenotype (Alternatively Activated Macrophages) is induced by fungal cells, immune complexes, helminthic infections, complement components, apoptotic cells, Macrophage Colony Stimulating Factor (M-CSF), IL-4, IL-13, IL-10 and Tumor Grow Factor β (TGF-β). This activation leads to the secretion of high amounts of IL-10 and low levels of IL-12. M2 phenotype responses occur in wound healing-type circumstances and/or in absence of infections. Such responses can also be further amplified by IL-4, IL-10, or IL-13. These "resting" macrophages are also characterized by the up-regulation of Dectin-1, DC-SIGN, Mannose Receptors, Scavenger Receptor A, Scavenger Receptor B-1, CD163, CCR2, CXCR1 and CXCR2 [Martinez *et al.*, 2009]. Instead of generating NO or ROI, M2 macrophages produce ornithine and polyamines through the Arginase pathway [Gordon and Martinez, 2010; Mantovani *et al.*, 2002], to promote growth functions and production of the extracellular matrix. Actually, the M2 terminology encompasses a continuum of functionally diverse states rather than a unique activation state. Accordingly, M2 macrophages can be further divided specifically into M2a, M2b, M2c and M2d subsets, based on the different stimuli of induction for each type and their distinct gene expression profiles [Mantovani *et al.* 2004; Rőszer 2015]. In general, these macrophages take part in polarized Th2 responses, allergy, parasites clearance, tissue remodeling, angiogenesis and tumor promotion [Sica and Mantovani, 2012].

Finally, Regulatory Macrophages, or M-Reg, represent one of the basic macrophage population and they are mainly involved in immune regulation: physiological role of M-Reg is to dampen the immune response and limit immunopathology. Unlike classically activated macrophages, M-Reg produce high levels of anti-inflammatory IL-10 cytokine and turn off IL-12 synthesis. Unlike M2 Macrophages, M-Reg do not stimulate Arginase activity and they do not contribute to the production of the extracellular matrix. M-Reg can arise following innate or adaptive immune responses; in particular, this polarization state is induced after Fc-γR ligation by IgG complexes in occurrence of PAMPs (*e.g.* lipopolysaccharide or lipoteichoic acid) recognition via TLRs, or following stress responses (signal initiated by glucocorticoid hormones). Phenotypically, M-Reg express the

Sphingosine Kinase-1 (SPHK-1) and LIGHT (TNF superfamily 14) markers, that might be used to identify M-Reg in mouse.

Progress has been made in defining the molecular mechanism underlying macrophage polarization, including signaling pathways, miRNA, epigenetic modification, post-transcriptional regulators and transcriptional factors [Lawrence and Natoli, 2011; Biswas *et al.*, 2012; Smale, 2010]. The heterogeneity of macrophage functions can be considered as a consequence of interaction with different immunological pathways (*e.g.*, interaction with different growth and survival factors, interaction with lymphoid and myeloid cytokines, interaction with pathogens, resolution), rather than attributing them to distinct macrophages subsets [Gordon and Martinez, 2010]. The M1/M2 classification is useful to understand the plasticity of macrophages but it may be taken as a simplified conceptual framework describing a continuum of diverse functional states, of which M1 and M2 activation states are not ontogenically defined subsets but represent the extremes of the functional continuum [Wonk *et al.*, 2012].

Macrophages and T cell Response

The description of macrophages polarization is leading immunologists to take a step back and revise their concept on how the immune system works [Mills, 2012 and 2014]. The M1 and M2 definition was formulated by mirroring the Th1/Th2 polarization concept, suggesting that Th1/Th2 cells do instruct M1/M2 polarization. However, it is now well-known that the reverse is true, that is that macrophages are initiating and directing T-cell polarization.

The capacity of taking up and presenting antigen (*i.e.*, the linking function between innate and adaptive immunity) is one of the most important features of tissue macrophages [Ley *et al.*, 2014], as they are described as professional APCs. Some monocytes that enter the tissue during inflammation do not differentiate into macrophages and are able to take up antigen in the tissue and carry it to lymph nodes where they can present it to naïve T-cells [Jakubzick *et al.*, 2013]; however, tissue macrophages are also able to present antigen, despite the fact that they do not recirculate to lymph nodes after antigen uptake. Tissue macrophages are highly phagocytic and can take up microorganisms and other matter in the tissue and this is their major function both in homeostasis and during inflammation. Antigen presentation may occur also in non-lymphoid organs, inducing antigen-specific local activation and expansion of primed T-cells, but not of naïve T-cells [Italiani and Boraschi, 2014 and references therein]. The hypothesis proposed by Ley is that initial priming of naïve T-cells occurs in the lymph node (to which antigen-loaded tissue monocytes recirculate), but that the full activation and effector functions of T-cells occur in the tissue where the inflammatory reaction is taking place, upon the productive interaction and formation of immunological synapse between primed T-cells and the antigen-presenting tissue macrophages. The differentiation of antigen-specific CD4 T cells into various Th cells occurs by induced expression of cytokines produced by DCs, as well

as other cells within the local environment, which can be immune cells and/or structural cells like stromal or epithelial cells. The combination of cytokines and mediators sensed by the T cells guide their polarization into TH cells.

- Th1 cells generally support cell-mediated immune responses and promote protective immunity against intracellular pathogens, especially those capable of infecting dendritic cells and macrophages. Their secretions of IFN-γ, as well as TNF, induce activation of macrophages and upregulation of iNOS enzyme. The subsequent generation of NO by macrophages has direct effects on pathogen replication [Henard and Vazquez-Torres, 2011]. IFN-γ is also a major inducer in the activation of CTLs which are specially equipped to handle intracellular infections. Finally, Th1 cells induce preferential production of IgG2 antibodies, a subclass involved in virus neutralization.
- Th2 cells participate in the clearance of parasitic helminths, and respond to IL-4 secreted by innate tissue resident cells. IL-4 and IL-13, two signature cytokines of Th2 cells, drive macrophage-mediated killing of helminths [Allen and Maizels 2011]. Additionally, IL-13 acts on goblet cells, favoring production of MUC5A to expel parasites from intestines. TH2 cells mediate potentiation of allergic responses and asthma by directing B cell secretions of IgG1.
- Th17 cells are essential in the control of extracellular bacterial and fungal infections. They were shown to control infection in models of *Citrobacter rodentium* [Mangan *et al.* 2006], *Salmonella Typhimurium* [Blaschitz and Raffatellu, 2010], *Klebsellia pneumoniae* [Aujla *et al.* 2008] and *Candida albicans* [Saijo *et al.* 2010]. Th17 cells have been shown to develop in mice upon infection with the enterohemorragic *Escherichia coli* strain O157:H7 [Atarashi *et al.* 2015]. IL-17A and IL-17F, that are Th17 cells signature cytokines [Ishigame *et al.* 2009], promote the recruitment of neutrophils by inducing secretion of chemo-attractants (IL-8, CXCL1, CXCL2) by target cells. Neutrophils are essential players in innate and adaptive immunity [Leliefeld *et al.* 2015], actively participating in the clearance of many pathogens by phagocytosis, degranulation of antimicrobial compounds, activation of oxidative burst and release of DNA traps filled with antimicrobial proteins [Kaufmann *et al.* 2016]. In addition, Th17 cells are also an important source of the cytokine IL-22, a pleiotropic cytokine; the combined secretion of IL-17 and IL-22 by these T cells increase the secretion of antimicrobial peptides S1008A and S1009A by skin keratinocytes [Liang *et al.* 2006].
- Treg cells are responsible for maintaining self-tolerance and immune homeostasis, and thus hold a key role in keeping inflammation responses from being deleterious. There are two distinct pools of Treg cells [Yuan and Malek 2012; Bollrath and Powrie 2013]: natural Treg (nTreg) cells, which develop and differentiate in the thymus, and induced Treg (iTreg) cells, which derive from conventional CD4 T cells in the periphery. iTreg cells are greatly involved in maintaining tolerance to food antigens and commensal bacteria in the gut. iTreg key cytokines, including IL-10 and TGFβ, negatively regulate effector T cells, thus dampening the inflammatory response [Rubtsov *et al.*, 2008].

Different polarization phenotypes stimulate different effects on adaptive immune response. Most likely, M1 macrophages are the antigen-presenting cells (APC) that induce activation/polarization of effector Th1 and Th17 cells upon production of IL-12 and IL-23. Likewise, M2 macrophages, which produce TGF-β and express the αVβ8 Integrin, are likely involved in the polarization of Th2 and Treg cells [Gordon and Taylor, 2005; Mantovani *et al.*, 2011; Arango Duque and Descoteaux, 2014; Ley, 2014].

Aim and scope of the thesis

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of humans and animals as part of the commensal microbiota. However, pathogenic *E. coli* strains have been isolated and these are usually classified as intestinal or extraintestinal pathogenic *E. coli* (InP*Ec* or ExP*Ec*) according to the disease location and the clinical outcomes.

Antibiotic resistance to commonly used antibiotics such as fluoroquinolones, tetracycline and cephalosporins is now widespread in both InP*Ec*s and ExP*Ec*s [Croxen *et al.*, 2013; Flores-Mireles *et al.*, 2015; Terlizzi *et al.*, 2017; Amezquita *et al.*, 2017; Torres *et al.*, 2017]. The extent of multidrug-resistance to last-line antibiotics such as carbapenems, tigecyclin and colistin [Mediavilla *et al.*, 2016; Pournaras *et al.*, 2016; Pitout and De Vinney, 2017], the recent rising incidence of hypervirulent strains and new sequence types among *E. coli* pathotypes are becoming a growing concern due to the significant economic impacts to the public health. A broad-spectrum *E. coli* vaccine could be a promising alternative to prevent the spread of such diseases while offering the potential for covering against several InP*Ec*s and ExP*Ec*s at once.

A critical goal of new generation vaccines against *E. coli* is to increase the breadth, quality and efficiency of protection and immune response using adjuvants. Adjuvants are substances or mixture of substances that are able to stimulate the immune system to develop a robust and durable adaptive response against the selected vaccine antigen and, in addition, to ensure the proper *in situ* antigen release, with no adverse systemic reactions. Only a few adjuvants have been so far approved for human use, such as Aluminum salts, the oil-in water-emulsion MF59 and the LPS derivative MPL-A. These adjuvants work by boosting B cell responses, thus increasing the antibody production against the specific antigen and increasing the number of specific memory B cells [De Gregorio *et al.*, 2009].

GSK has identified a series of new adjuvants, called SMIPs [Wu *et al.*, 2014] able to trigger members of the TLRs family expressed on a variety of APC. APCs are cells of the innate immune system, including dendritic cells and macrophages, thus inducing the key molecular events that ultimately lead to innate immune responses and the development of antigen-specific adaptive immunity. Macrophages are the most functionally diverse (plastic) cells of the hematopoietic system and their main function is to respond to pathogens and modulate the adaptive immune response through antigen processing and presentation. These cells undergo specific differentiation into distinct functional phenotypes depending on the local tissue environment: the M1 phenotype is characterized by the production of high levels of pro-inflammatory cytokines, an ability to mediate resistance to pathogens, strong microbicidal properties, high production of reactive nitrogen and oxygen intermediates, and promotion of Th1 responses. In contrast, M2 Macrophages are characterized by their involvement in parasite control, tissue remodeling, immune regulation, tumor promotion and efficient phagocytic activity, mainly promoting Th2 oriented immune responses [Arango Duque, Descoteaux 2014].

Different TLRs play a central role in macrophage activation and can induce different polarization phenotypes. The aim of the work described in this thesis was to characterize the *in vitro* and *in vivo* effect of GSK TLR agonist adjuvants on macrophage polarization at early time-points, then assessing the effective role of these APC on antigen presentation upon vaccine injection and verifying if the obtained activation state coherently correlates with the adaptive immune response observed after a complete immunization protocol.
The identification of a portfolio of TLR agonists capable of eliciting optimal antibody production, along with a cellular-mediated response to *E. coli* subunit vaccines, would be a dramatic improvement in the design of a broadly protective vaccine against pathogenic *E. coli*.

Chapter II
Materials and Methods

Adjuvants

The adjuvants SMIP.2-7, SMIP.7-10 and SMIP.7-11 were all provided by GSK Vaccines. SMIP.7-10 and SMIP.7-11 were used in both *in vitro* experiments and animal studies at different doses, soluble or pre-adsorbed to Aluminum Hydroxide (Alum). SMIP.2-7 was use on *in vitro* experiments. Alum was also used alone, as benchmark adjuvant.

Animal studies

Groups of 5–10 female 8-week-old BALB/C (Charles River) mice were used for *in vivo* experiments. For macrophage polarization studies, animals were intra-peritoneally injected at day 0 with a total volume of 100µl of either Alum (200µg), or different amounts of TLR7 agonist (SMIP.7-10 or SMIP.7-11), in their soluble forms or pre-adsorbed to constant 200µg dose of Alum. Non-injected naïve mice were used as control group. For innate cells recruitment experiment and for immunization studies, animals were intramuscularly injected, while in the adoptive transfer, recipient mice were intravenously perfused.
All animal studies were approved by GSK Animal Welfare Body and carried out in accordance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 26/2014) and with the GSK Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (authorization number 689/2015-PR). All mice were housed under specific pathogen-free (SPF) conditions at the GSK Vaccines Animal Resource Center, which is an AAALAC (Association of Assessment and Accreditation of Laboratory Animal Care) accredited facility.

Single cells suspension preparation from mouse spleens and quadriceps

Quadriceps muscles were harvested and placed into tissue culture dishes containing RPMI (Gibco) on ice. The muscles were cut into small pieces and digested with Liberase DL and DNAse I (Roche) in RPMI for 2h at 37°C under constant agitation. The cell suspension was centrifuged, resuspended in DMEM (Gibco) and filtered through a 70µm nylon mash (Becton Dickinson) before cell-counting and staining with fluorescently labeled antibodies and FACS analysis.

Cell culture and preparation

The murine macrophage cell line RAW 264.7 was cultured in complete Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS, 2mM L-Glutamine, 100 U/ml Penicillin, 100µg/ml Streptomycin, 50mg/ml Gentamicin, 25mM HEPES, and 10mM Sodium Pyruvate (all from Gibco, Thermo Fisher, Carlsbad, CA, USA). Cells were maintained at 37°C in a 5% CO_2 incubator.

Peritoneal cells from wild-type BALB/c mice were collected by intra-peritoneal injection of 5ml of ice cold PBS, immediately after euthanasia. Lavages were centrifuged 10 minutes at 300g, supernatants were removed and pellets were resuspended in complete DMEM. Total cells were counted and plated according to the number of live cells; after 2h of incubation at 37°C and 5% CO_2, macrophages were purified by washing cells thoroughly three times with PBS to remove non-adherent cells. Alternatively, peritoneal macrophages were isolated from total peritoneal lavages of naïve BALB/c mice by depletion of magnetically labeled non-target cells using the Macrophage Isolation Kit Peritoneum (Miltenyi Biotech).

Bone Marrow Derived Macrophages (98% purity) were obtained from *in vitro* differentiation of bone marrow cells. Bone marrow was isolated from the femurs of Balb/c mice of mice and cultured overnight in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100µg/ml streptomycin and 2mM glutamine. Non-adherent cells were collected by centrifugation and cultured at 4×10^6 cells/ml in the presence of 20 ng/ml MCSF to generate bone-marrow derived macrophages; after 3 days in culture, fresh media was added; at day 6 days, the adherent cells were detached using ice-cold Phosphate Buffered Saline (PBS; Gibco, Thermo Fisher, Carlsbad, CA, USA) + EDTA 10mM, counted, centrifuged and resuspended in Endotoxin-Free PBS for injection (Sigma)

Analysis of mRNA levels

Total RNA was isolated from RAW 264.7 and peritoneal macrophages using TRIzol-Chloroform (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. After DNase I treatment (RQ1 RNase-Free DNase, Promega), according to manufacturer's protocol, concentration and purity of RNAs was measured using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and 2µg from each sample were reverse-transcribed using Oligo-dT primers and 5 U/µl of Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), in a total volume of 20µl. cDNAs were then amplified with the LightCycler480 Sybr Green kit (Roche) and the primers listed in the table below. Data were analyzed using the comparative cycle threshold method and normalized using the gene *Actb* that encodes β-actin as housekeeping gene. All Results were expressed as relative mRNA expression compared to untreated macrophages.

Table 2. Real time qPCR Primers sequences

Murine Target Gene	Primer Sequence 5'-3'
m-Actin FW	CCAGAGCAAGAGAGGTATCC
m-Actin RV	CTGTGGTGGTGAAGCTGTAG
m-ARG-1 FW	CACCTCCTCTGCTGTCTTCC
m-ARG-1 RV	AAGAAAAGGCCGATTCACCT
m-ARG-2 FW	ACAGGGTTGCTGTCAGCTCT
m-ARG-2 RV	TGATCCAGACAGCCATTTCA
m-IL-10 FW	CTGGGTGAGAAGCTGAAGAC
m-IL-10 RV	ATCACTCTTCACCTGCTCCA
m-IL-12 FW	GAAAGACCCTGACCATCACT
m-IL-12 RV	CCTTCTCTGCAGACAGAGAC
m-IL-1β FW	ACCTGCTGGTGTGTGACGTTCC
m-IL-1β FW	GGGTCCGACAGCACGAGGCT
m-IL-6 FW	AGTTGCCTTCTTGGGACTGA
m-IL-6 RV	ACAGGTCTGTTGGGAGTGGT
m-LIGHT FW	CTGCATCAACGTCTTGGAGA
m-LIGHT RV	GATACGTCAAGCCCCTCAAG
m-NOS2 FW	TCAGAGCCACAGTCCTCTTT
m-NOS2 RV	TCCATGCAGACAACCTTGGT
m-SPHK-1 FW	TCCTGGAGGAGGCAGAGATA
m-SPHK-1 RV	GCTACACAGGGGTTTCTGGA
m-STAB-1 FW	TATGTGCCGACCAGGTATGA
m-STAB-1 RV	CTGCTCTTAACCGCAGGAAC
m-TNF-α FW	GTAGCCCACGTCGTAGCAA
m-TNF-α RV	GGTGAGGAGCACGTAGTCG
m-YM-1 FW	ACTTTGATGGCCTCAACCTG
m-YM-1 RV	AATGATTCCTGCTCCTGTGG

Flow cytometry staining and analysis

Murine Peritoneal cells were counted and seeded in a 96-well plate (approximately 10^6cells/well). After two washes with sterile PBS, cells were stained with 100µl of 1:300 Live/Dead Aqua (Invitrogen), incubated for 20 minutes in the dark, and finally washed again with PBS. To saturate Fc receptors, 25µl of 1:50 Fc-block (Mouse Fc Block, BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) were added to the cells for further 20 minutes at 4°C. At the end of this incubation, cells were stained with 25µl of a pre-titrated monoclonal antibody mix diluted in PBS for further 25 minutes at 4°C, in the dark. The antibodies used to characterize macrophage polarization phenotypes were Anti-Mouse F4/80 eFluor 450 (Clone BM8, eBioscience, San Diego, CA, USA), Anti-Mouse CD86 PE/Cy5 (Clone GL-1, Biolegend, San Diego, CA, USA), Anti-Mouse CD80 PE/Dazzle 594 (Clone 16-10A1, Biolegend, San Diego, CA, US), Anti-Mouse MHCII FITC (Clone 2G9, BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA), Anti-Mouse CD36 Alexa Fluor 647 (Clone HM36, Biolegend, San Diego, CA, USA), Anti-Mouse CD369 (Dectin-1) PE (Clone RH1, Biolegend, San Diego, CA, USA), Anti-Mouse CD206 Alexa Fluor 700 (Clone C068C2, Biolegend, San Diego, CA, USA), and Anti-Mouse CD68 Brilliant Violet 605 (Clone FA-11, Biolegend, San Diego, CA, USA). After staining, cells were washed with PBS, and fixed with Cytofix (BD Bioscience, Becton Dickinson, Franklin Lakes, NJ, USA) for 20 minutes at 4°C. Finally, after two additional washes, cells were maintained in PBS until reading. The Flow Cytometer used was LSRII (BD Bioscience, Becton Dickinson, Franklin Lakes, NJ, USA).

NF-κB Luciferase Reporter Assay

TLR-specific activation assays were performed using Human Embryonic Kidney 293 (HEK293) cells expressing Luciferase under control of the NF-κB promoter and stably transfected with mouse TLR7 (HEK-Blue mTLR7). HEK-Blue mTLR7 transfected cells were maintained in DMEM complemented with 4.5 g/liter glucose and HEPES (Invitrogen), 10% FBS, 1% Penicillin-Streptomycin solution (Invitrogen) and specific antibiotics (Puromycin, 5µg/ml; Blasticidin, 10µg/ml). For the NF-κB luciferase assay, 25,000 cells/well were seeded in 90µl of complete DMEM without antibiotics in 96-well µClear® luciferase plates (PBI International) and incubated for 24 h at 37°C. The day after, cells were stimulated either with SMIP.7-10 and SMIP-7.11 at 100nM or with the commercial TLR7 agonist R848, at the same concentration. All stimuli were tested in duplicate. After incubation for 6h at 37 °C, supernatants were aspired from each well, and cells were lysed for 20 minutes at room temperature using 20µl/well of 1:5 diluted passive lysis buffer (Promega). Produced luciferase was detected using 100µl/well luciferase assay reagent (Promega) and emitted light was immediately quantified using a Tecan Spark Luminometer (TECAN). NF-κB activation of cells stimulated with GMMA is expressed as fold-increase of emitted light over the average result of PBS-stimulated control cells.

Mesoscale multiplex analysis

Mesoscale 7-spot (MSD Technology) analysis for cytokines IL-6, KC-Gro, IL-1β, TNF-α, IL-10, IL-12, and IFN-γ was performed with 25µl of supernatants from Pmacs according to the manufacturer's instructions. Concentrations of the different cytokines in the samples were determined in comparison with the preloaded standard in the plates.

ELISA assays

For all ELISA assays, 96-well Nunc-Immuno MicroWell MaxiSorp flat bottom plates (Thermofisher Scientific) were used. Plates were coated with 100µl of antigen *SslE* at 1µg/ml in PBS pH 7.4 overnight at 37°C. Plates were blocked for 1 hour at 37°C + 5% CO2 with PBS + 0.05% Tween 20 + 3% Bovine Serum Albumine (BSA, Sigma-Aldrich). Plates were then incubated with diluted serum or fecal pellet supernatants (in series of 2X dilutions) in PBS + 1% BSA for 2 hours at 37°C and 5% CO2. For each assay, 100µl of goat anti-mouse anti IgG (H+L), anti-IgG, anti-IgG2a and anti-IgG2b secondary antibody coupled to Alkaline Phosphatase (all from Southern Biotech, Birmingham, AL, USA) was added at the appropriate dilution in PBS + 1% BSA and incubated for 2 hours at 37°C and 5% CO_2: all antibodies from. Between each of these steps, plates were washed three times with PBS-0.05% Tween20. Plates were then incubated with 100µl of Alkaline Phosphatase Liquid substrate (Sigma-Aldrich) for 30 min at room temperature and read on a SpectraMax microplate reader (Molecular Devices) at 405nM.

In vitro re-stimulation of antigen-specific CD4 T cells and intracellular cytokines staining

Single cell suspensions were obtained from spleens of each mouse by homogenization through a 70µm nylon cell-strainer (Becton-Dickinson, BD). Red blood cells were lysed and cells cultured in RPMI 1640 (Gibco), supplemented with 10% Fetal Bovine Serum (FBS; Hyclone), 50µM β-mercaptoethanol (Sigma), 100 U/ml Penicillin, 100µg/ml Streptomycin, and 2mM Glutamine (Invitrogen Life Technologies). For T-cell cytokine responses, cells were plated at 2 x 10^6 cells/well in 96-well U-bottom plates in the presence of anti-CD28 Ab (1µg/ml) (BD) and *SslE* (10µg/ml or 50µg/ml), or with anti-CD28 alone (unstimulated), or with anti-CD28 plus anti-CD3 (0.1 µg/ml) (BD). After overnight stimulation, 5 µg/ml Brefeldin A (Sigma Aldrich) were added for an additional 4 hours. Cells were then stained with Live/Dead Near InfraRed viability marker (Molecular Probes-Life Technologies), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and then incubated with anti-CD16/CD32 Fc block (BD Biosciences). The staining included the following mAbs: PerCP-Cy5.5-conjugated anti-CD3 (BD), V500-conjugated anti-CD4 (BD), PE-TexasRed-conjugated anti-CD8 (Invitrogen), PE-conjugated anti-IFNγ (BD), Alexa700-conjugated

anti-TNF-α (BD), Alexa488-conjugated anti-IL-4 and anti-IL-13 (eBioscience), APC-conjugated anti-IL-2 (BD), PE-Cy7-conjugated anti-IL-17 (eBioscience) and V450-conjugated anti–CD44 (BD). Cells were acquired on a LSRII (BD) and analyzed using FlowJo software (TriStar). CD44 and CD4 double-positive T cells were gated on IL-2, TNF-α, IFN-γ, IL-17, IL-4 and IL-13: frequencies of *SslE*-specific T cells were calculated after subtracting the background measured in the corresponding negative control for each cytokine.

Statistical analysis

GraphPad Prism 7 software was used to perform the statistical analysis of the data. Mann-Whitney two-tailed test was used to test for statistical significance of differences between two experimental groups. Significance was calculated as following: * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; ns, not significant ($P\geq0.05$).

Chapter III
Results

Both SMIPs shift Macrophage polarization towards the M1 pro-inflammatory phenotype *in vitro*

Several factors may determine vaccine effectiveness; the choice of the right vaccine antigen that is able to induce a protective immune response is crucial, but its formulation with suitable adjuvants compounds is equally important. Indeed, adjuvants are able to stimulate the immune system to develop a robust and durable adaptive response against the selected vaccine antigen and, in addition, they ensure the proper *in situ* antigen release, with no adverse systemic reactions, inducing the key molecular events that ultimately lead to innate immune responses and the development of antigen-specific acquired immunity. Among the innate immune cells, macrophages are the most functionally diverse and plastic cells of the hematopoietic system and they are part of the first-line of response to vaccine administration, being able to stimulate and modulate adaptive immune response through antigen processing and presentation.

Recently, GSK has developed a new type of adjuvants, called SMIPs (Small Molecules Immuno-Potentiators) that act as TLRs agonist, triggering different members of the TLRs family expressed on APCs, including dendritic cells and macrophages. It is widely described that different TLRs play a central role in macrophage activation and can induce different polarization phenotypes.

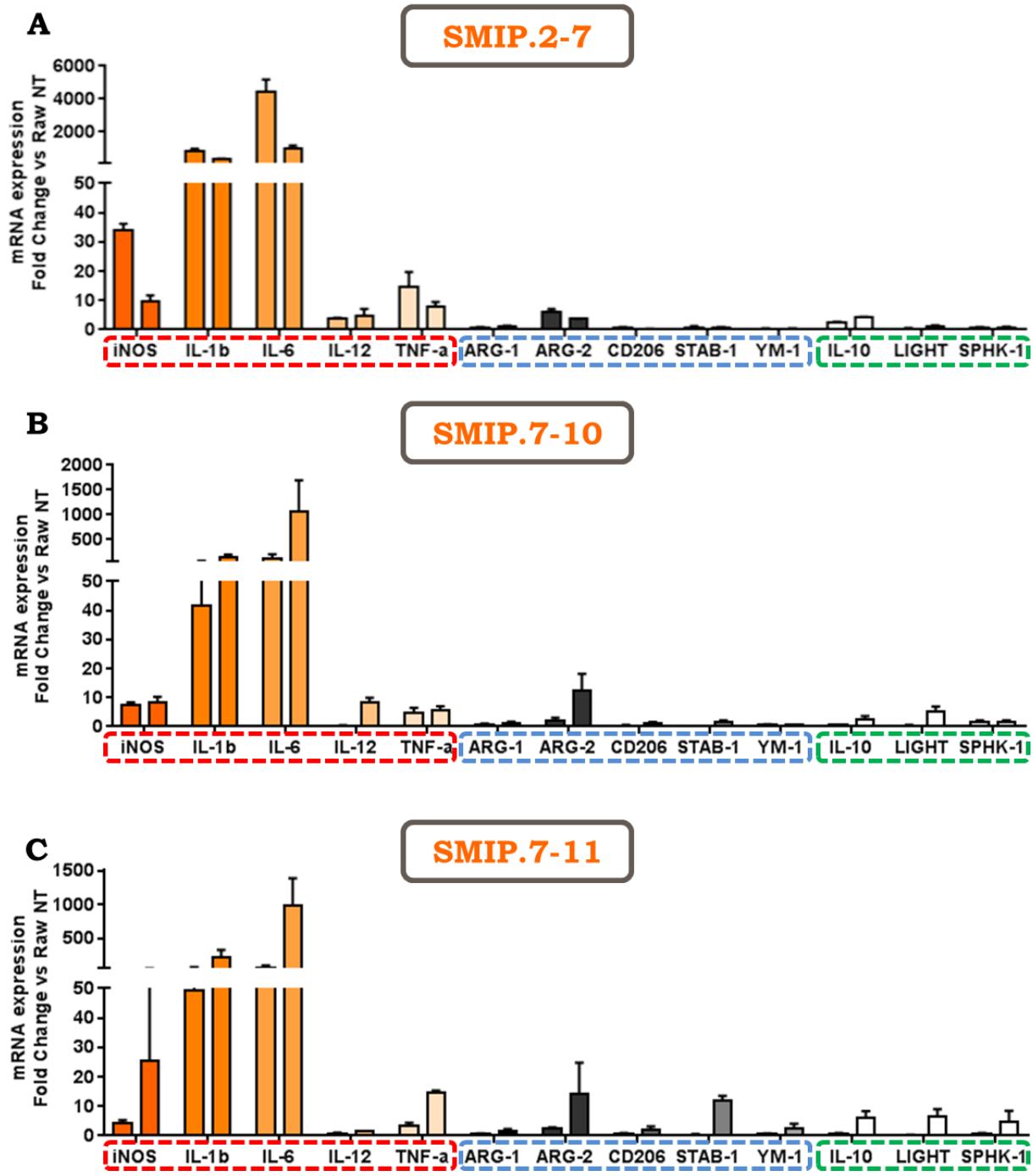
From the screening of the entire SMIP library that was generated, three TLRs agonists were selected to be developed as possible immuno-potentiators based on their efficacy, specificity and chemical/physical properties. Therefore, we tested them as potential adjuvants to be included in an effective *E. coli* vaccine formulation. As a first experiment, the contribution of these SMIPs, named SMIP.2-7 (TLR2 agonist), SMIP.7-10 and SMIP.7-11 (both TLR7 agonists) on macrophage polarization was studied in cell-based *in vitro* assays using the murine macrophage cell line RAW 264.7. Cells were seeded in 12-well plates (5 x 10^5 per well) and stimulated with TLR2 and TLR7 agonists at 100nM final concentration, for 6 and 24 hours; total RNA was isolated from each well and mRNA expression of many polarization markers was analyzed (**Figure 6**), in order to evaluate the kind of activation phenotype induced by the SMIPs compounds.

The genes encoding the signature of M1 pro-inflammatory markers, such as Inducible Nitric Oxide Synthase (*iNOS* or known as *nos-2*), *il-12*, *il-6*, *tnf-a* and *il-1β* were significantly up-regulated, compared to the non-treated (NT) RAW 264.7 cells (**Figure 6A, 6B, 6C**) with all adjuvants. Differently, M2 (*arg-1*, *arg-2*, *cd206*, *stab-1* and *ym-1*) and M-Reg (*il-10*, *light* and *sphk-1*) phenotypes signature markers did not show any significant variation, with only *arg-2* M2 and *il-10* M-Reg markers showing a slight increase (about 4-5 fold) above the non-treated control with both TLR2 and TLR7 agonists. Among the three TLRs agonist,

SMIP.2-7 was able to stimulate even stimulating a greater inflammatory response, as suggested by the stronger induction of *il-1β* and *il-6* cytokines expression.

To further confirm the results of the gene expression analysis, a 7-spot multiplex cytokine analysis was performed on SMIP-treated Raw 264.7 cells supernatants after 6h or 24h stimulation (**Figure 6C**, **6D**). The Mouse Pro-Inflammatory 7-Plex Tissue Culture Kit by Mesoscale (MSD Technology) provides assay-specific components for the simultaneous quantitative determination of mouse IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, KC-Gro (homologue of human pro-inflammatory cytokine IL-8), and TNF-α in cell culture supernatants. Although with a lower level of up-regulation as compared to their respective genes expression, all the pro-inflammatory cytokines included in the assay kit showed to be highly released in cell supernatants upon treatment with the three SMIPs.

Overall, these results suggested that RAW 264.7 murine macrophages polarized predominantly towards a M1 phenotype upon stimulation with both TLR2 and TLR7 agonists *in vitro*.


A
SMIP.2-7
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Fold Change vs Raw NT
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4000
2000
50
40
30
20
10
0
iNOS
IL-1b
IL-6
IL-12
TNF-a
ARG-1
ARG-2
CD206
STAB-1
YM-1
IL-10
LIGHT
SPHK-1
B
SMIP.7-10
mRNA expression
Fold Change vs Raw NT
2000
1500
1000
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50
40
30
20
10
0
iNOS
IL-1b
IL-6
IL-12
TNF-a
ARG-1
ARG-2
CD206
STAB-1
YM-1
IL-10
LIGHT
SPHK-1
C
SMIP.7-11
mRNA expression
Fold Change vs Raw NT
1500
1000
500
50
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iNOS
IL-1b
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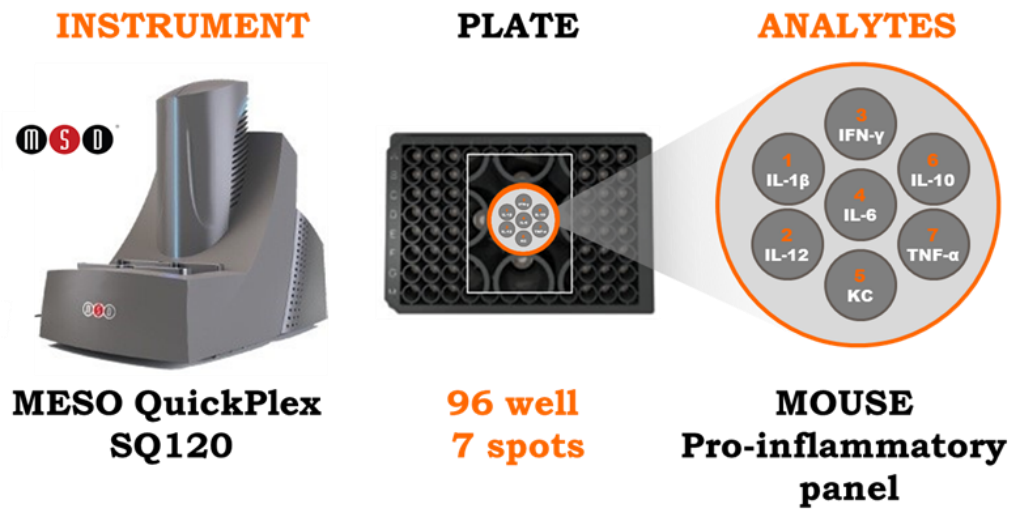
D


INSTRUMENT
PLATE
ANALYTES
3 IFN-γ
1 IL-1β
6 IL-10
4 IL-6
2 IL-12
7 TNF-α
5 KC
MESO QuickPlex SQ120
96 well 7 spots
MOUSE Pro-inflammatory panel

E

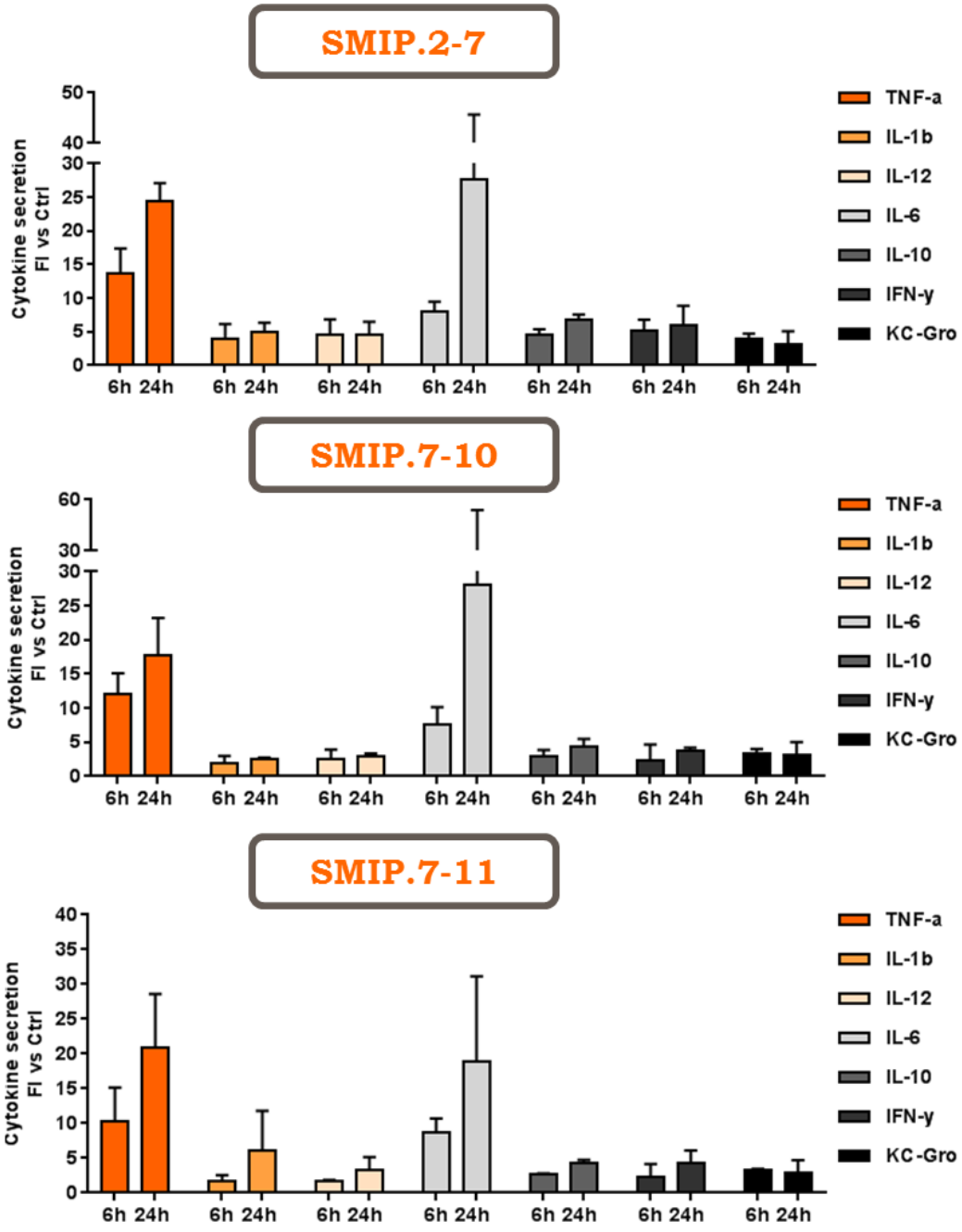

SMIP.2-7
SMIP.7-10
SMIP.7-11
Cytokine secretion FI vs Ctrl
6h 24h
TNF-a
IL-1b
IL-12
IL-6
IL-10
IFN-γ
KC-Gro

Figure 6. Macrophage polarization in response to SMIPs *in vitro*

RAW 264.7 cells were stimulated for 6h or 24h with the three TLRs agonists SMIP.2-7(A), SMIP.7-10 (B) and SMIP.7-11 (C). The mRNA levels of the genes encoding markers of the M1 (Red box: *iNOS*, *il-1β*, *il-6*, *il-12*, *tnf-α*), M2 (Blue box: *arg-1*, *arg-2*, *cd206*, *stab-1*, *ym-1*) and MReg (Green box: *il-10*, *light, sphk-1*) populations were analyzed by Real Time qPCR (left bar of each gene for 6h time-point; right bar of each gene for 24h time-point). Values are expressed as Fold-Change to non-stimulated RAW 264.7 (Raw NT). Data shown represent the mean with standard deviation (SD) of ten *in vitro* independent experiments. (D, E) Mesoscale 7-spot (MSD Technology) analysis for cytokines IL-6, KC-Gro, IL-1β, TNF-α, IL-10, IL-12, and IFN-γ was performed with 25µl of supernatants from SMIPs-treated or untreated Raw 264.7 cells, according to the manufacturer's instructions. Concentrations of the different cytokines in the samples were determined in comparison with the preloaded standard in the plates. Values are expressed in Fold-Change to non-stimulated Raw 264.7 cells; data shown are the mean with SD of 3 independent experiments.

SMIP.2-7 and SMIP.7-11 shift macrophage polarization towards M1 pro-inflammatory phenotype at early time-points also on *ex-vivo* stimulated peritoneal macrophages.

Starting from these preliminary data, the aim of the next set of experiments was to confirm the *in vitro* obtained results on primary macrophages.

Macrophages begin their lives as monocytes, which are produced in the bone marrow and circulate throughout the bloodstream; they can leave the bloodstream and enter other tissues and organs in the body where they undergo further differentiation and become "resident" macrophages. Resident macrophages can be found in many tissues including connective tissues, liver, lung, lymph nodes, and skin. The peritoneal cavity provides an easily accessible site for harvesting moderate numbers of resident macrophages. Moreover, peritoneal macrophages (PMacs) are relative quiescent in terms of immunological and secretory functions, as they are finally differentiated but naïve to any type of polarization. Thus, to evaluate the contribution of SMIPs also on primary murine macrophages, PMacs were harvested from Balb/c mice by standard lavage of peritoneal exudates with 5 ml ice-cold PBS and cultured in dishes. PMacs non-adhere *in situ* but they become adherent on tissue-culture treated plates: therefore, after washing the dishes for three times with cold PBS, they can be separated from other cells present in the peritoneal cavity. PMacs were then detached from the dish, counted, plated in 6-well-plates and stimulated with SMIP.2-7 and SMIP.7-11 at 100nM final concentration for 6 and 24 hours; total RNA was isolated and mRNA expression of polarization markers was analyzed (**Figure 7A, 7B**). SMIP.7-10 was not tested on PMacs due to the similar behavior of the two TLR7 agonists observed in the *in vitro* assay.

In agreement with the observations of the *in vitro* model, SMIPs were able to induce a predominant M1 phenotype of activation at the earlier time-point, with induction of *Nos2* and inflammatory cytokines up-regulation. However, at the later time-point, gene expression analysis showed an increase in few M2 (*arg-1*) and MReg markers (*il-10*, *light*); Of note, is the re-stimulation of *arg*-1 expression after 24h incubation of PMacs with both TLR agonists.

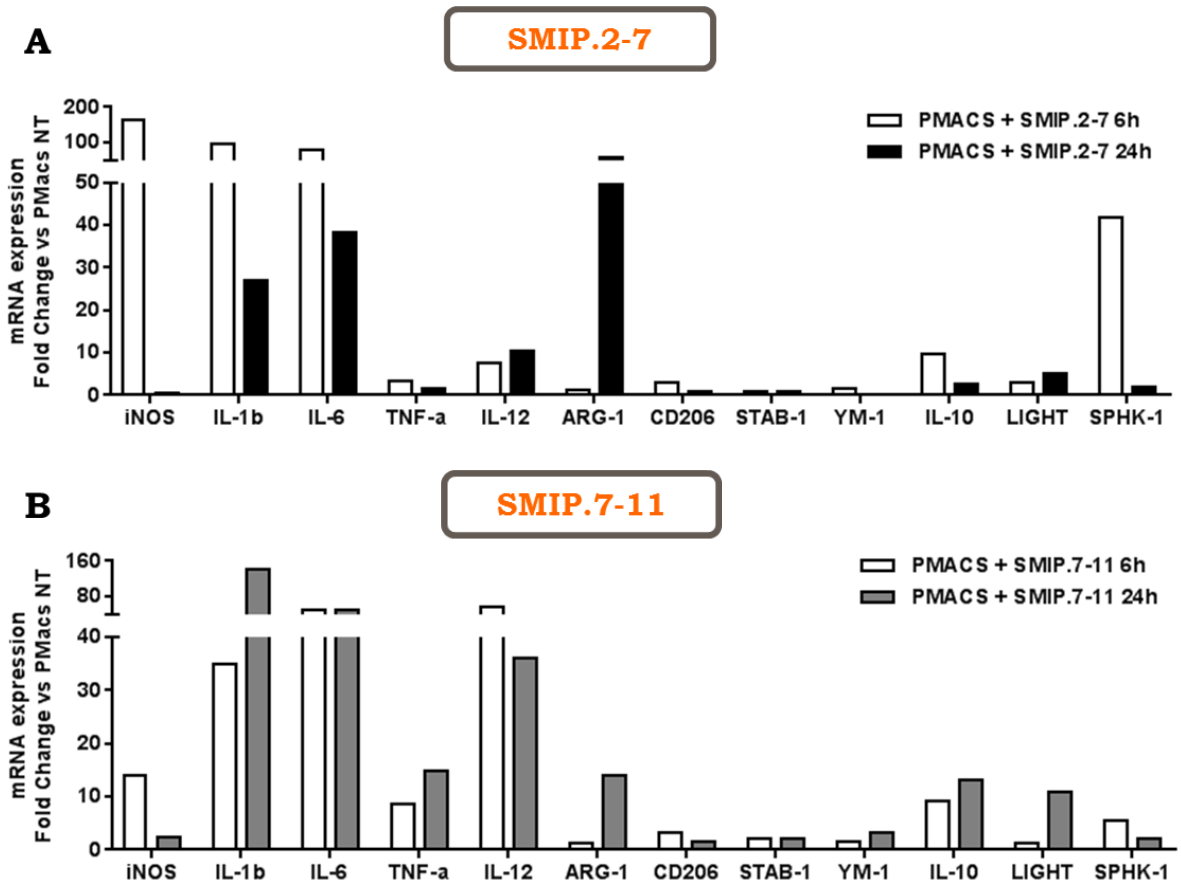


Figure 7. Macrophage polarization in response to SMIPs *ex vivo*

PMacs were obtained by peritoneal lavage of Balb/c mice and stimulated for 6h or 24h with SMIP.2-7 (A) or SMIP.7-11 (B). The mRNA levels of the genes encoding markers of the M1, M2, and MReg populations were analyzed by Real Time qPCR.

The adjuvant effect of SMIP.7-10 and SMIP.7-11 involves NF-κB activation

After all the *in vitro* evidences accumulated and due to the similar macrophagic activation profile induced by the TLR2 and the TLR7 agonists tested, and considering that the latter have been more characterized as potential vaccine adjuvants [Buonsanti *et al.*, 2016], it was decided to pursue all the studies only on TLR7 agonists.

TLR7 recognizes small synthetic molecules such as Loxoribine and R848 (Imidazoquinoline compound) and its signaling upon activation involves Nuclear Factor Kappa B (NF-κB) signal transduction pathways.

To evaluate if the adjuvant effect of SMIPs is actually mediated by triggering of TLR7 we took advantage of the commercially available HEK-Blue mTLR7 cells, that are precisely designed for studying the stimulation of mouse TLR7 (mTLR7) by monitoring the activation of NF-κB; these cells are obtained by co-transfection into HEK293 cells of the mTLR7 gene and an optimized Firefly Luciferase (*Luc*) reporter gene. The latter is driven by four copies of NF-κB response element located upstream of the minimal TATA promoter: after activation by pro-inflammatory cytokines or stimulants of lymphokine receptors, endogenous NF-κB transcription factors bind to the DNA response elements, inducing transcription of the luciferase reporter gene. To assess that the SMIPs were actually able to bind TLR7 and correctly stimulating the canonical MyD88 signal transduction cascade that lead to NF-κB activation, HEK-Blue mTLR7 cells were treated with the same concentration (100nM) of the two GSK adjuvants or the commercial R848 TLR7 ligand for 6h and then the Firefly Luciferase Activity (as to say NF-κB activity) was measured using TECAN Spark Luminescence Plate-Reader (**Figure 8**). Luciferase activity of the cells stimulated with the SMIPs was compared with the signal intensity of the non-treated or the R848 treated HEK-Blue mTLR7 cells. These results clearly show that either SMIP.7-10 or SMIP.7-11 do activate TLR7 signaling pathway with similar potent NF-κB level of induction; moreover, they both give a greater signal than the commercial TLR7 ligand, R848.

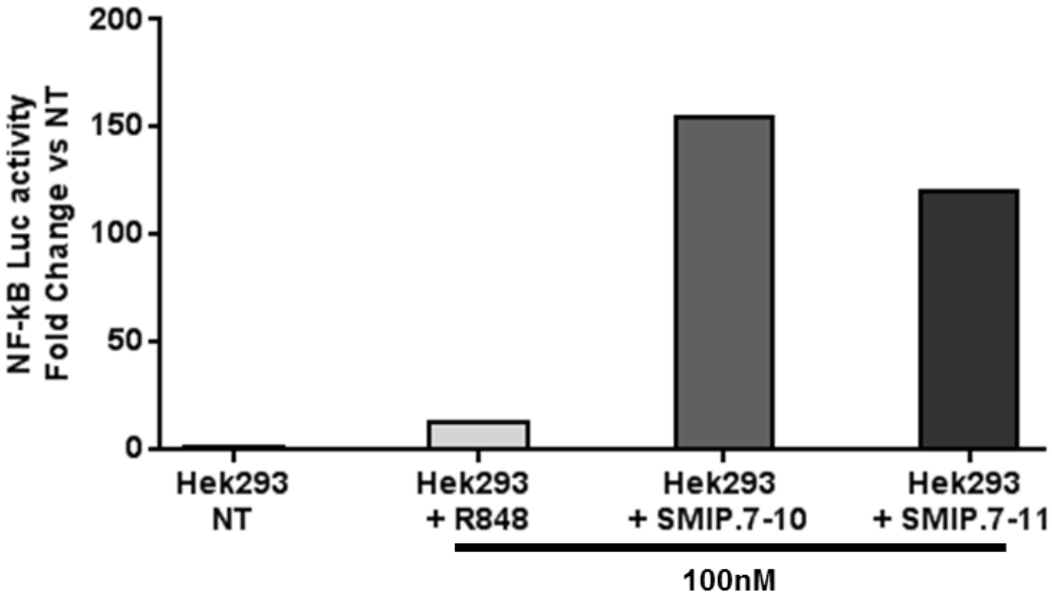


Figure 8. NF-κB level of activation upon stimulation with SMIPs or R848

Luciferase activity assay was performed using HEK-Blue mTLR7 cells, to evaluate NF-κB level of activation upon stimulation with different TLR7 agonists. Values are expressed as fold change to the untreated cells.

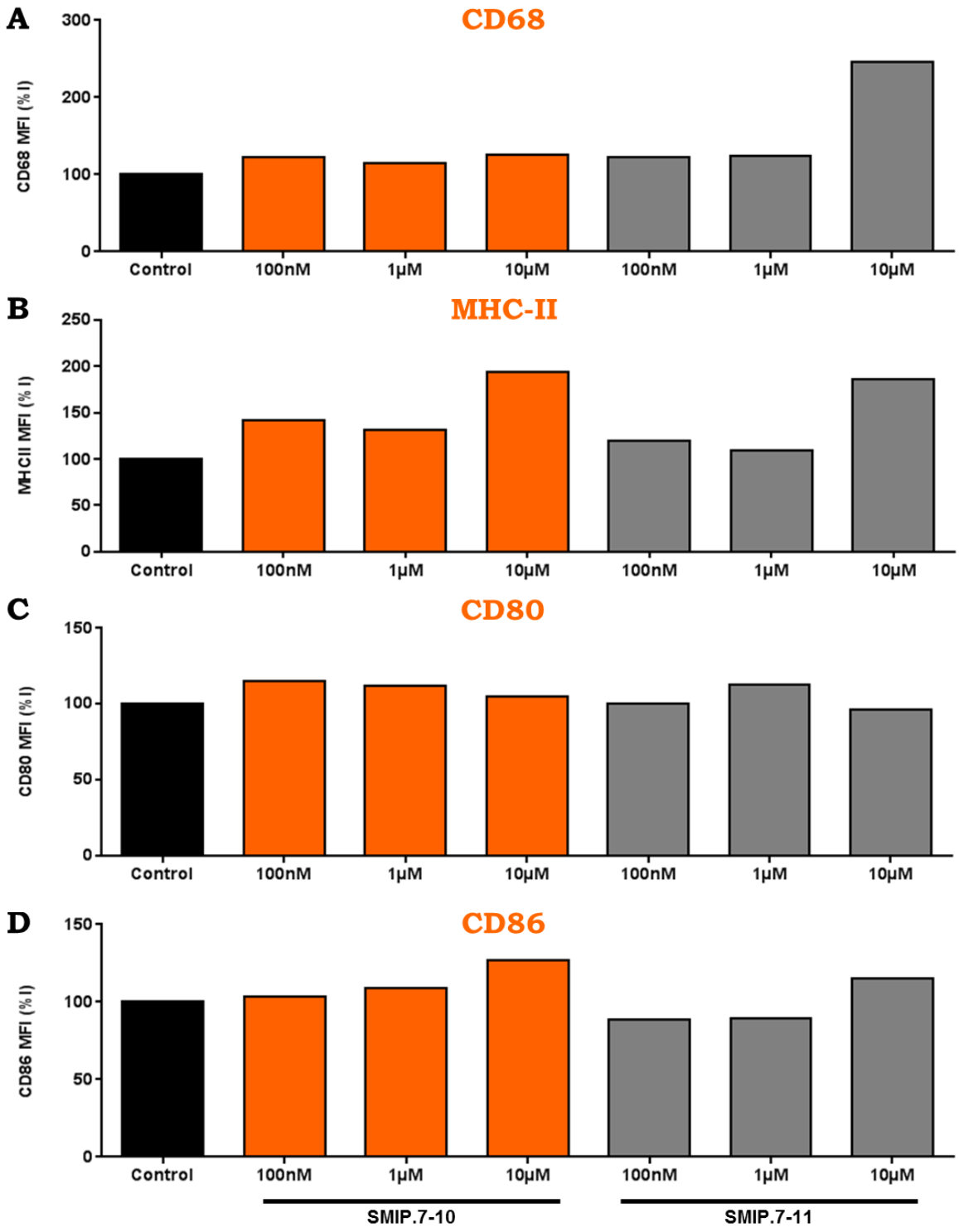
SMIP.7-10 and SMIP.7-11 modulate also polarization surface markers expression on macrophages

Due to the similar macrophagic activation profile induced by the TLR2 and the TLR7 agonists tested, and considering that the latter has been more characterized as potential adjuvant [Buonsanti *et al.*, 2016], it was decided to pursue our studies only on TLR7 agonists. Phenotypical distinction between polarized macrophages involves also differential expression of cell surface receptors; among these, CD80, CD86, and MHC-II that serve as T cells co-stimulatory molecules, are associated with M1 activation, whereas the Mannose Receptor-1 (Mrc-1, also known as CD206), Dectin-1 and CD36 fit the M2 signature [Martinez *et al.*, 2008].

Given the contrasting properties of pro-inflammatory M1 and anti-inflammatory M2 macrophages and in order to deepen the apparent mixed phenotype that emerged from gene expression analysis of PMacs *ex vivo* experiments, flow cytometry analysis for cell-surface polarization markers was also performed on these cells.

In order to have a purified population, murine peritoneal macrophages were immune-magnetically negatively sorted from peritoneal lavages and stimulated *ex vivo* (**Figure 9**).

Interestingly, a 24h incubation of PMacs with different doses of SMIP.7-10 and SMIP.7-11 resulted only in the down-regulation of CD36, CD206 and Dectin-1 M2 markers (**Figure 9E, 9F, 9G**), as compared to the untreated cells. By contrast, expression of co-stimulatory MHC-II, CD80 and CD86 molecules (M1 markers) (**Figure 9B, 9C, 9D**), showed a modest increase over control, with major effect of the highest dose of both adjuvants, especially for CD86 expression. Similarly, the other M1 marker CD68 (**Figure 9A**) was not modulated by SMIPs, with only a mild up-regulation on peritoneal macrophages treated with the 10μM dose of SMIP.7-11. These results coherently correlate with the gene-expression analysis obtained from *in vitro* stimulated RAW 264.7 cell line that showed an M1-oriented activation upon stimulation with GSK adjuvants. This kind of polarization, in combination with the increased surface expression of MHC-II and co-stimulatory molecules, would facilitate the effective stimulation of antigen-specific CD4 and CD8 T cells in response to a SMIP-containing vaccine.


A
CD68
CD68 MFI (% I)
300
200
100
0
Control
100nM
1μM
10μM
100nM
1μM
10μM
B
MHC-II
MHCII MFI (% I)
250
200
150
100
50
0
C
CD80
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100
50
0
D
CD86
CD86 MFI (% I)
SMIP.7-10
SMIP.7-11

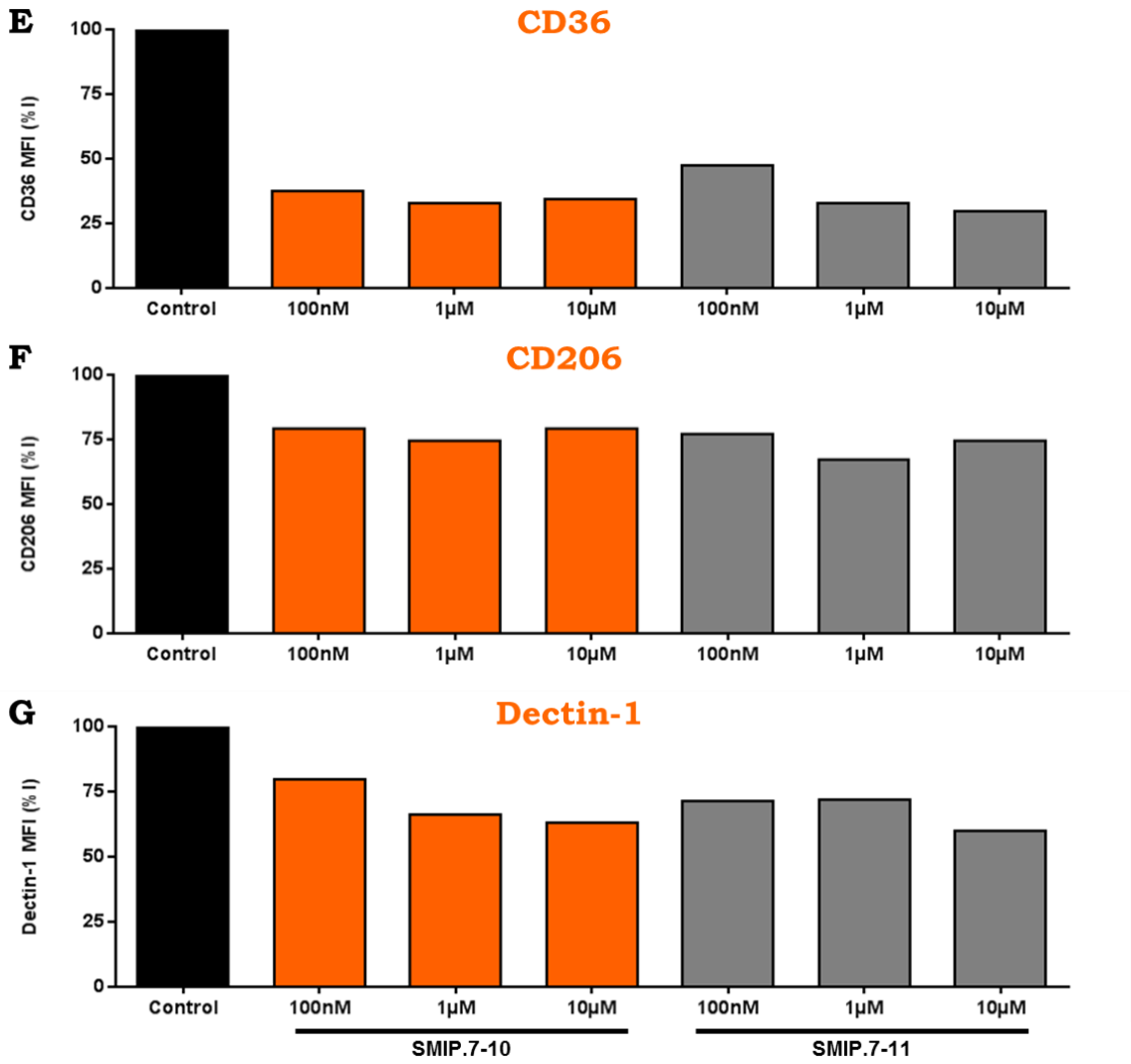


Figure 9. FACS analysis for PMacs stimulated with SMIP.7-10 and SMIP.7-11. PMacs were stimulated for 24h with TLR7 agonists. Surface expression of (A) CD68, (B) MHCII, (C) CD86, (D) CD80, (E) CD36, (F) CD206 and (G) Dectin-1 was measured by flow cytometry on F4/80+, live macrophages. Values are expressed as Percentage of Increase (%I) in Mean Fluorescent Intensity (MFI) compared to the non-treated PMacs.

Both TLR7-agonists shift Macrophage polarization towards a mixed activation phenotype *in vivo*.

Assays to characterize the effect of vaccine formulations on macrophages can provide valuable insights into their mechanism of action. However, *in vitro* models have the obvious limitation that macrophages in isolation are not receiving signals from the extracellular matrix components, as well as from other cell types, which may influence their responses to pathogens. Dendritic cells and resident macrophages are the first phagocytes to enter into action upon vaccine injection, followed by the recruitment of neutrophils from the bloodstream. Therefore, after the first evaluation of the contribution of the two GSK TLR7 agonists on macrophage polarization in cell-based *in vitro* and *ex-vivo* assays, it was necessary to verify the coherence of results on animal models, evaluating the best dose, formulation and time-point to detect macrophage recruitment and to define their activation phenotype *in vivo*.

To this purpose, SMIP.7-10 and SMIP.7-11 adjuvants were injected in mice at two doses, either in their soluble form or formulated with Alum(OH) (**Figure 10**). This new condition was introduced because, in the perspective of a human *E. coli* SMIP-containing vaccine, SMIPs could not be injected as soluble compounds due to the excessive reactogenicity that these molecules would have at a systemic level; moreover, Wu *et al.* (2014) and Buonsanti *et al.* (2016) already proved that SMIP.7-10 adsorbed to Alum is able to increase functional antibodies against glycoconjugate-based or protein based-vaccines, leading to a great increment of breadth of coverage when compared to Alum-adjuvanted vaccine alone, avoiding any sign of toxicity neither systemically nor at the site of injection.

The expression/regulation of genes encoding mediators of the innate immune response, cytokines and cell-surface activation markers, at 24h and 48h post-intraperitoneal injection was measured using different techniques, such as RT-qPCR (**Figure 11A-G**) and FACS staining (**Figure 13A-G**). Moreover, the number of total cells (macrophages and other cells) in the peritoneal cavity was evaluated to understand if different doses and formulation of adjuvant injections could induce cell recruitment (**Figure 12**). Balb/C female mice were injected intraperitoneally on day 0 with only Alum (200 µg dose) or Alum-SMIP.7-10 or -SMIP.7-11, consisting of a constant 200 µg dose of Alum and different doses of the TLR7 agonist, as described in material and methods.

Alum (**Figure 11A**), which is known to activate a Th2 response, indeed, promoted an M2 activation of macrophages, with significant up-regulation of the main anti-inflammatory phenotype markers ARG-1, ARG-2, CD206, YM-1 and IL-10 at the FACS analysis; in line with literature, also TNF-α and IL-1β showed an important increase, meaning that also a pro-inflammatory activation, probably via the inflammasome, is occurring at the same time; neverthless iNOS was not induced.

A similar mixed polarization was also elicited from the two SMIPs in their soluble forms, which induced few M2 marker genes (ARG-1, ARG-2, IL-10), togheter with a high

expression of TNF-α, IL-6 and IL-1β. In the same way, injections with different doses of 10μg or 50μg SMIPs adsorbed to Alum resulted in a mixed activation phenotype, with genes TNF-α ARG-1, ARG-2 and YM-1 highly up-regulated by all the formulations; iNOS was induced only by Alum-SMIP.7-10 at the lower dose tested (10μg), whereas M-Reg genes, LIGHT (*Tnfsf14*) and SPHK-1, appeared up-regulated only in the animal group that was injected with Alum-SMIP.7-11 formulation (**Figure 11**). Interestingly, Alum addition to SMIPs seems to block the IL-6 pro-inflammatory cytokine expression, that was observed with both SMIPs administered alone; this would be consistent with what previously shown by Wu *et al.* (2014), in which it was demostrated that Alum adsorption to SMIP molecules could enhance the immunogenicity of these adjuvants while at the same time reducing their systemic reactogenicity, actually measured as IL-6 serum levels. Similarly excessive IL-10 secretion in response to SMIPs is attenuated by Alum addition.

Many reports describe the so-called depot-effect of particulate adjuvants, such as Alum: they are described as delivery system adjuvant, being able to increase the persistence of the antigen and consequently the recruitment of innate immune cells at the site of injection, thus augmenting the chances for the vaccine to be up-taken by APCs. In agreement with this knowledge, all the Alum-SMIP formulations were able to recruit a higher number of cells at the site of injection than the Alum alone, in a dose-independent manner, at the 24h time-point. However, this effect lasted until the later time-point only for Alum-SMIP.7-10 (**Figure 12**) formulation.

For the FACS analysis of cell-surface markers of polarization (**Figure 13A-G**), the results also showed a mixed type of macrophage activation, with markers of both M1 (CD68, MHCII, CD86 and CD80; **Figure 13A-D**) and M2 (CD36, CD206; **Figure 13E-F**) appearing up-regulated in all the groups treated with SMIPs + Alum formulations at 24h, compared to the untreated control group. On the contrary, Dectin-1 M2 marker (**Figure 13G**) resulted more expressed in the group treated with the SMIPs alone, as compared to the control group.

Overall, these *in vivo* results suggested that SMIPs intraperitoneal injection, either in soluble form or adsorbed to Alum, elicit general activation of many distinct polarization markers; differently from what observed in the *in vitro* studies, in which both SMIPS induced a clear M1-shifted polarization, *in vivo* data are controversial and the "phenotypization" is not straightforward. However, going into details, among the two TLR7 agonists, there were some relevant differences: indeed, Alum-SMIP.7-10 induced more IL-12 and causes less CD206-expressing cells (M2) or cells expressing MReg marker than SMIP.7-11 (**Figure 11**). Also, SMIP.7-10 showed to recruit higher numbers of properly activated cells at the site of injection, inducing mostly M1 cells (MHC-II+, CD80/86+) and less M2 and MReg cells.

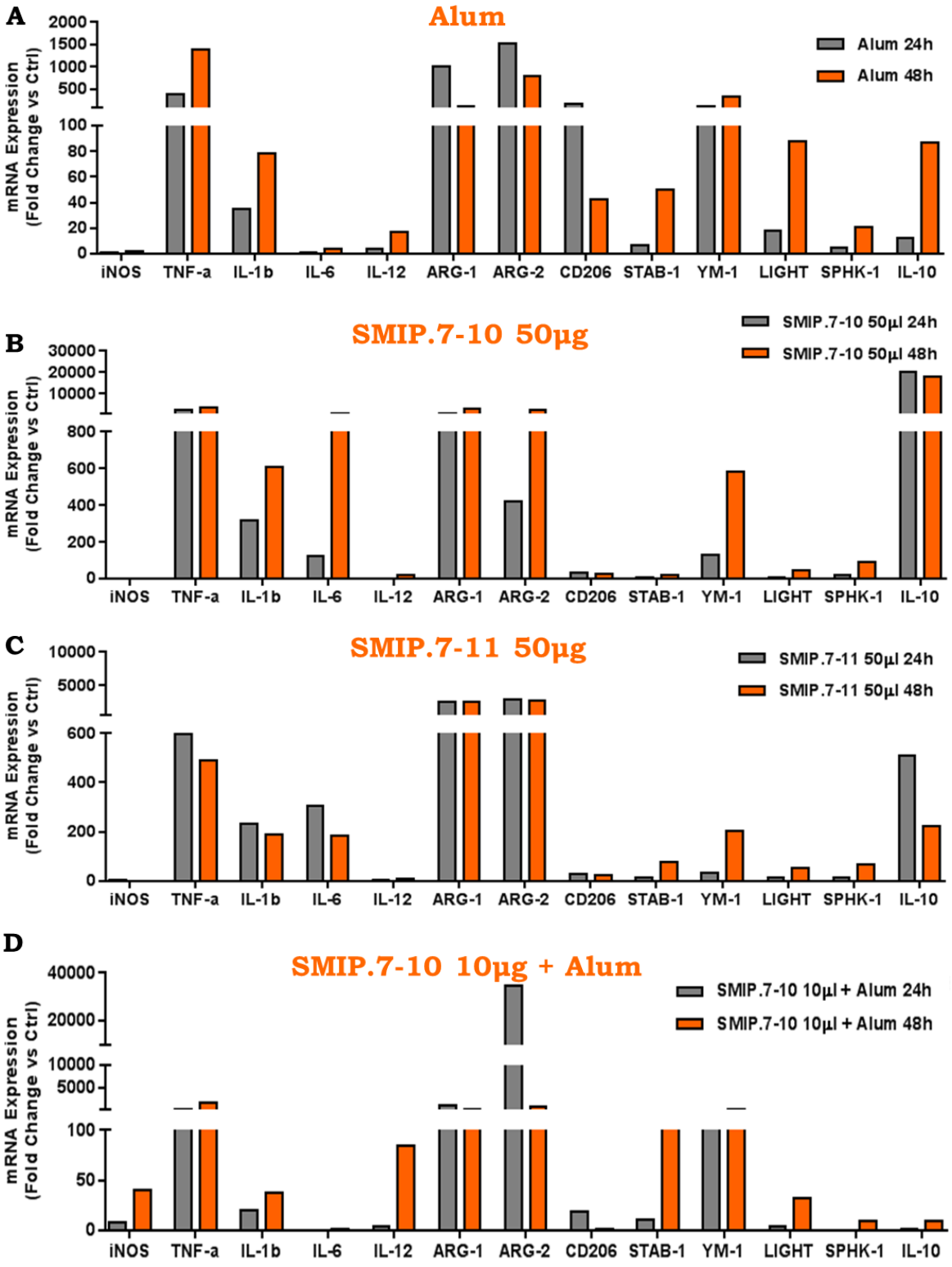
For all these reasons, being the aim of this thesis to find an adjuvant that would drive preferentially Th1/Th17 responses against *E. coli* antigen – normally associated with M1 cells – SMIP.7-10 was chosen for further analysis.

Experimental Groups	SMIP dose (µg)	Alum (OH) (200µg)	Volume IP
Non treated	/	/	100µl
Alum	/	Yes	100µl
SMIP.7-10	50	No	100µl
SMIP.7-11	50	No	100µl
SMIP.7-10	10	Yes	100µl
SMIP.7-10	50	Yes	100µl
SMIP.7-11	10	Yes	100µl
SMIP.7-11	50	Yes	100µl

Balb/c mice

Figure 10. Animal study scheme plan.

Balb/C mice (8 or 10/group) were injected at day 0 with SMIP alone or formulated with Alum at the indicated doses and peritoneal lavages were collected at 24h and 48h post-injection.


A
Alum
Alum 24h
Alum 48h
mRNA Expression (Fold Change vs Ctrl)
2000
1500
1000
500
100
80
60
40
20
0
iNOS TNF-a IL-1b IL-6 IL-12 ARG-1 ARG-2 CD206 STAB-1 YM-1 LIGHT SPHK-1 IL-10
B
SMIP.7-10 50µg
SMIP.7-10 50µl 24h
SMIP.7-10 50µl 48h
mRNA Expression (Fold Change vs Ctrl)
30000
20000
10000
800
600
400
200
0
iNOS TNF-a IL-1b IL-6 IL-12 ARG-1 ARG-2 CD206 STAB-1 YM-1 LIGHT SPHK-1 IL-10
C
SMIP.7-11 50µg
SMIP.7-11 50µl 24h
SMIP.7-11 50µl 48h
mRNA Expression (Fold Change vs Ctrl)
10000
5000
600
400
200
0
iNOS TNF-a IL-1b IL-6 IL-12 ARG-1 ARG-2 CD206 STAB-1 YM-1 LIGHT SPHK-1 IL-10
D
SMIP.7-10 10µg + Alum
SMIP.7-10 10µl + Alum 24h
SMIP.7-10 10µl + Alum 48h
mRNA Expression (Fold Change vs Ctrl)
40000
20000
10000
5000
100
50
0
iNOS TNF-a IL-1b IL-6 IL-12 ARG-1 ARG-2 CD206 STAB-1 YM-1 LIGHT SPHK-1 IL-10

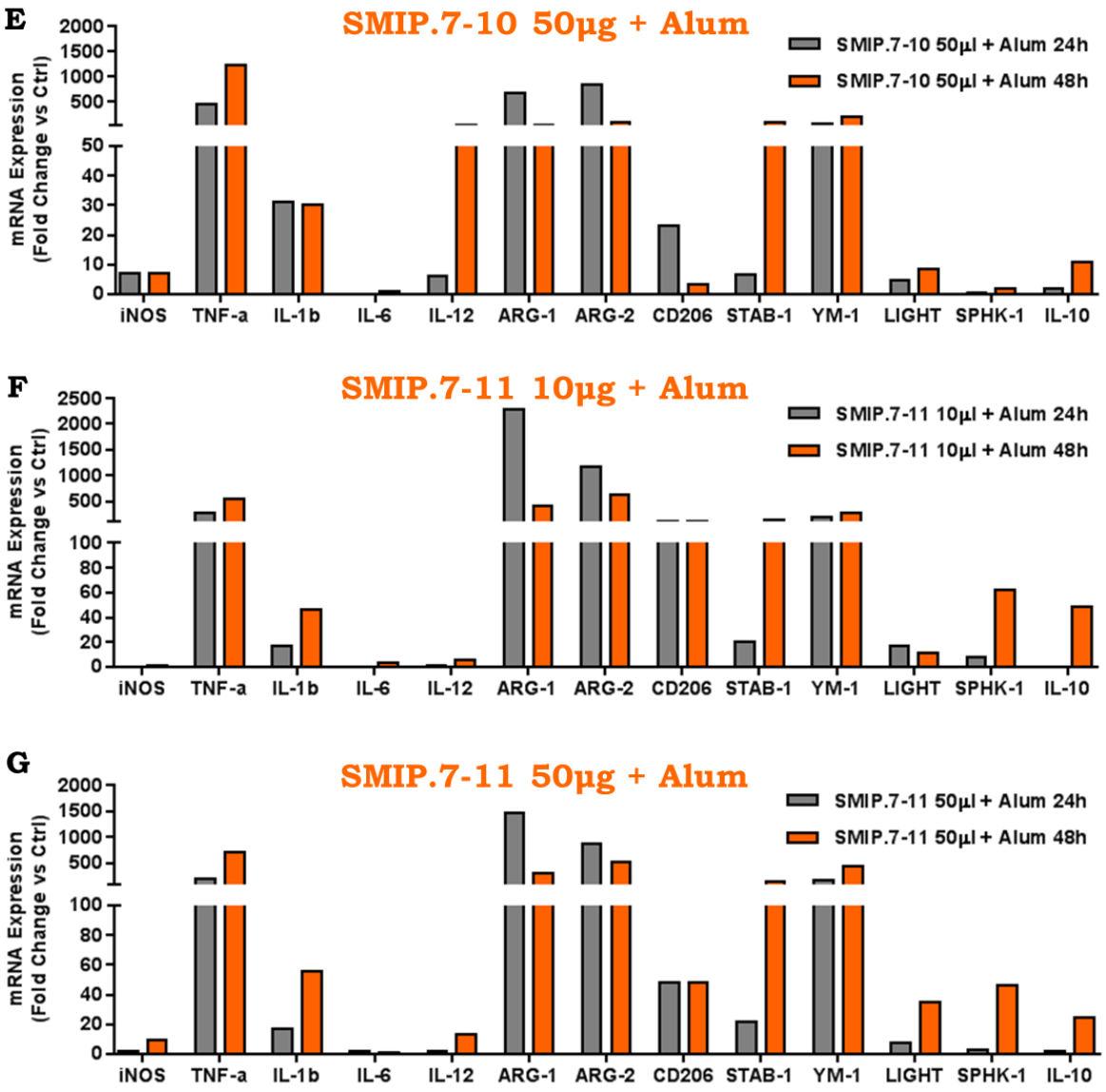


Figure 11. Effect of Alum, SMIPs or Alum-SMIPs on macrophage polarization *in vivo*.
Balb/C mice (8 or 10/group) were injected at day 0 with SMIP alone or formulated with Alum, at the indicated doses. At 24h and 48h post-injection, peritoneal lavages were collected and cells and mouse RNAs were isolated to analyze gene expression by Real time qPCR. Mean values for each group (pool) are shown in the graph as fold change to the untreated mice and reported to Actin, as housekeeping gene.

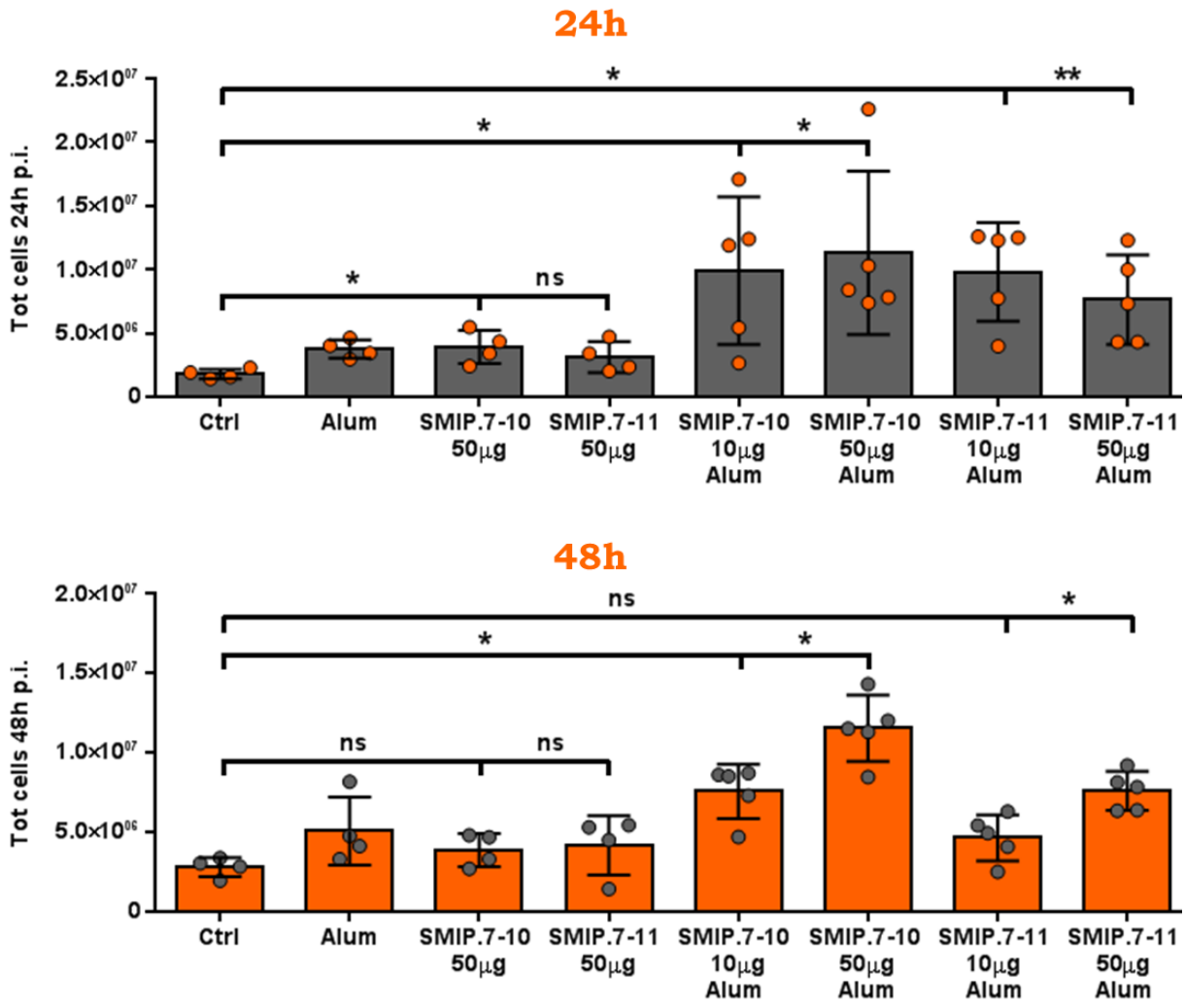
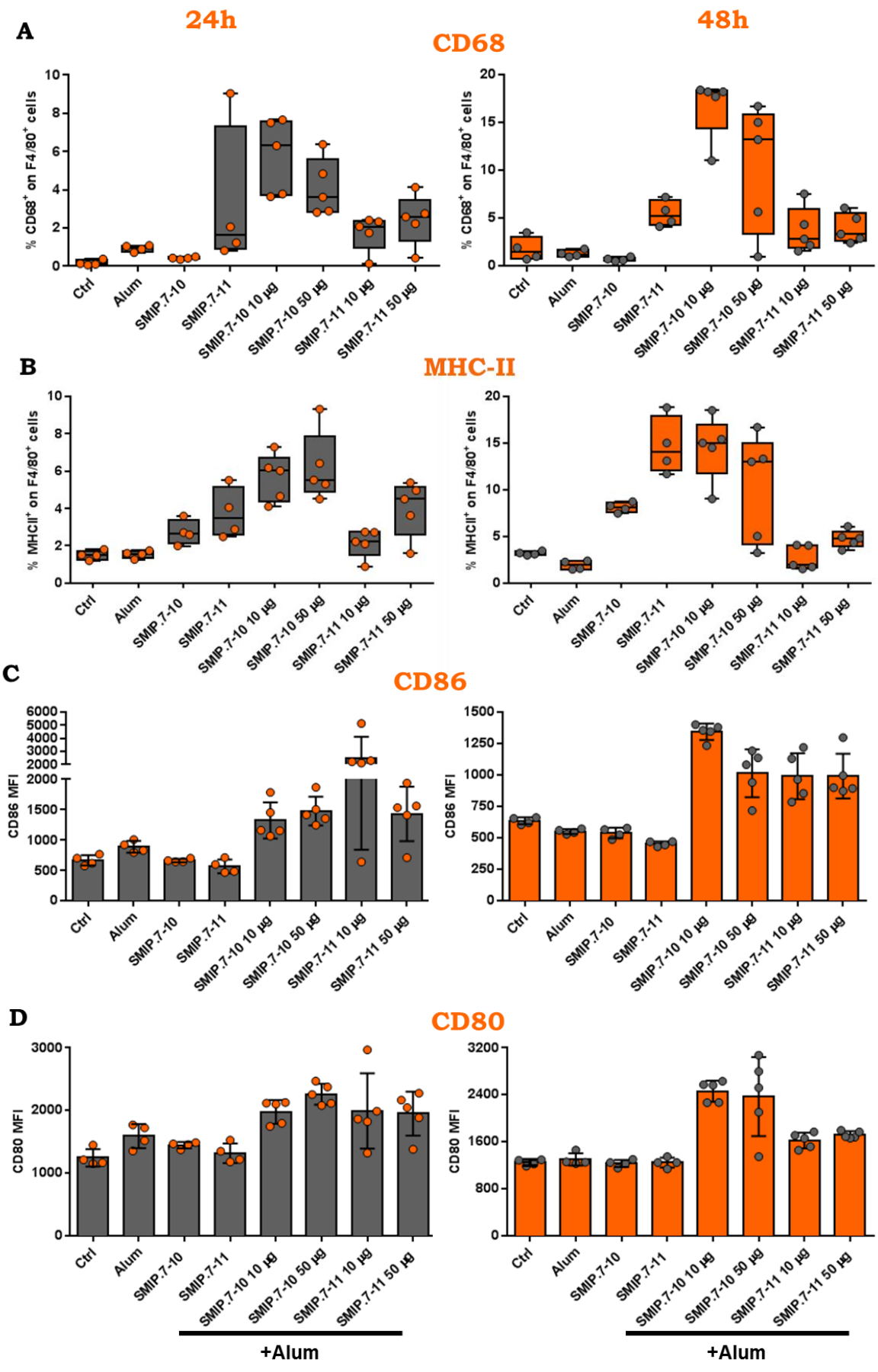


Figure 12. Effect of Alum, SMIPs or Alum-SMIPs on cell recruitment upon IP injection.

Balb/C mice (8 or 10/group) were injected at day 0 with SMIP alone or adsorbed to Alum, at the indicated doses. After collection, single mouse peritoneal washes were counted with Nucleocounter instrument. Each dot represents one animal. Statistical significance was determined using a Mann-Whitney test for the difference in Cell recruitment at 24h and 48h post-injection among untreated (Ctrl) and SMIP ± Alum treated animals (**P < 0.01, *P < 0.05, ns = non-significant).


A
24h
48h
CD68
% CD68+ on F4/80+ cells
B
MHC-II
% MHCII+ on F4/80+ cells
C
CD86
CD86 MFI
D
CD80
CD80 MFI
Ctrl
Alum
SMIP.7-10
SMIP.7-11
SMIP.7-10 10 µg
SMIP.7-10 50 µg
SMIP.7-11 10 µg
SMIP.7-11 50 µg
+Alum

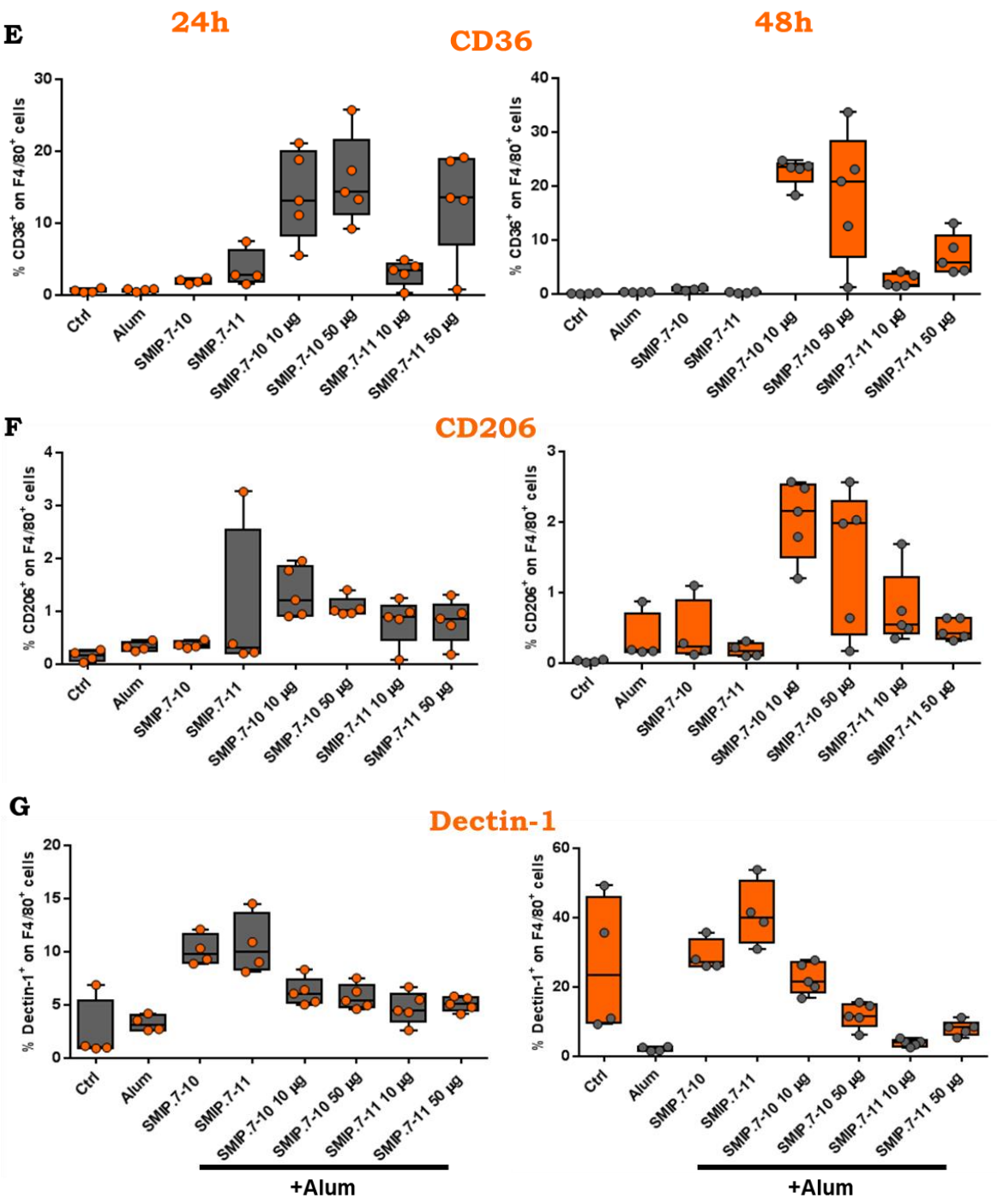


Figure 13. Effect of Alum, SMIPs or Alum-SMIPs on macrophage cell-surface expression *in vivo*. Balb/C mice (8 or 10/group) were injected at day 0 either with SMIPs alone or formulated with Alum at the indicated doses. At 24h and 48h post-injection, peritoneal lavages were collected and cells were stained to analyze cell-surface markers expression by FACS (A-G). Values for each group of mice are reported in the graph as either % of positive cells or as Mean Fluorescence Intensity (MFI), always gated on the total F4/80+ cells.

Alum-SMIP.7-10 induces high monocytes and macrophages infiltration at the site of injection up to 72 hours post-injection

Earlier works showed that adjuvants induce immunologic memory for vaccine-antigens through local activation of the innate immune system. Indeed, more knowledge of the effects of adjuvants on the initiation of the innate immune response directly at the site of injection could be used for the rational development of adjuvanted vaccines. Many adjuvant studies have assessed cell recruitment into the peritoneum as injection site due to the easy access and handling of peritoneal lavage-fluid. Yet, the muscle constitutes the site of injection of most human vaccines. Skeletal muscle contains few immune cells and sufficient delivery of vaccine antigen therefore likely requires inflammation, including recruitment of such cells to the site of vaccine administration. Mast cells, macrophages and dendritic cells have been described to be present in the resting muscle and could play a role during the creation of an immunostimulatory environment together with other cell types like endothelial cells [Calabrò *et al.*, 2011].

Cell recruitment into the muscle following immune activation, inflammation or damage is less studied and limited data are available regarding recruitment or activation of single innate cell types.

In the first 72 hours post-injection, local effects of vaccines are largely mediated by the adjuvant and not the antigen [Calabrò *et al.*, 2011], therefore, local cellular recruitment upon adjuvant treatment only was analyzed. In this experiment, Alum and SMIP.7-10 alone were used as benchmarks for comparing how Alum-SMIP.7-10, also known as Alum-TRL7 [Buonsanti *et al.*, 2016], induces innate recruitment and activation at the site of injection; intramuscularly PBS-injected animals were used as negative control. The kinetics of cell recruitment into the adjuvant- or PBS-treated animals was determined in quadriceps muscles at 24h, 48h and 72h post-injection. In order to identify the composition of immune cells recruited into the muscle at each time-point, multicolor FACS analysis was performed (**Figure 14A**) on the single cell suspensions obtained by mechanical plus enzymatic digestion of the muscle tissue (**Figure 14B**). Figure 12A shows the marker combination used to distinguish the major infiltrating cell types at high resolution; in particular, this analysis allowed us to identify Neutrophils (CD3-, MHCII int, Ly6G+, Ly6C low, SSC low), Eosinophils (CD3-, MHCII-, CD11b+, Ly6G-, F4/80int, Ly6C- SSC high), Monocytes (CD3-, MHCII int, Ly6G int, CD11b+, Ly6C+, SSC low), Macrophages (CD3-, Ly6G-, CD11c-, Ly6C+, CD11b+, F4/80+) and finally T cells (CD3+, MHCII-, SSC low) and B cells (CD3-, F4/80-, MHCII+, SSC low).

Adjuvants administration effect was first evaluated after 24 hours (**Figure 15A**), which is a time point that had already shown strong local innate immune activity in mice [Calabrò *et al.*, 2011].

Alum-SMIP.7-10 as well as Alum alone, although at lower level, were able to induce the recruitment of CD11b+ myeloid cells, in particular monocytes (5- to 7-fold above PBS) and

macrophages (4- to 6-fold more than PBS), into the injected quadriceps muscles (**Figure 15A-1**). In contrast, the unformulated SMIP.7-10-treated muscles, probably due to its soluble nature and its poor capacity to retain at the site of injection, revealed similar cell number and composition as the muscle of the PBS-injected control mouse (**Figure 15A-1**). This evidence confirmed that the cellular influx was specific to adjuvant injection, thus excluding muscle damage due to liquid injection as a sufficient cause for cell recruitment. At this early time-point, also a significant increase (Mann-Whitney T-test: $p < 0, 05$) of about 6-fold more neutrophils was observed in Alum and Alum-SMIP.7-10-treated groups as compared to the PBS and the SMIP.7-10-injected muscles (**Figure 15A-1, -2, -3**). At 24h post-injection, also T cells (around 2-fold) and B cells (3-fold) showed a slight, but still significant (Mann-Whitney test: $p < 0, 05$), increase in the Alum-SMIP.7-10-treated muscles as compared to the PBS and the SMIP-injected animals. At this first time-point of observation, the amount of eosinophils of all the adjuvants-treated groups was similar to the numbers observed in the PBS control group.

Differently, at the later time point analyzed, which is 48h post-injection (**Figura 15B-1**), a strong eosinophils influx was observed mainly in the Alum-SMIP.7-10-treated muscles, together with a new wave of monocytes infiltration and a further massive increase in neutrophils (around 40-fold more than PBS-administered animals) and macrophages numbers (20-fold above PBS and SMIP.7-10 groups); the latter could be presumably addressed to monocyte–macrophage differentiation rather than to a new recruitment of these cells. To a higher extent than the earlier time-point, a 10-fold increase in T cells recruitment was found in the Alum-SMIP.7-10 treated muscles, whereas the amount of B increased approximately 2-3-fold as compared to the PBS-injected group (**Figure 15B-1, -2, -3**). At the last and latest time-point analyzed, which is 72h (**Figure 15C-1**), monocytes and macrophages continued to be the most abundant populations of the Alum-SMIP.7-10 treated muscles. Moreover, after three days post-injection, both Alum and Alum-SMIP.7-10 were still able to induce neutrophils infiltration, increasing the amount of this cell population up to 70-fold above the PBS group; however, a stronger neutrophils recruitment was observed in response to Alum-SMIP.7-10 (**Figure 15C-1, -2, -3**).

Once assessed the quality, the quantity and the kinetics of the innate immune cells influx at the site of injection upon adjuvant, and thus vaccine injection, the question to be answered was whether recruitment is actually the key event in vaccine and/or adjuvant-mediated immune enhancement. At whatever time-point analyzed, most if not all cells that infiltrated the adjuvant injection site were CD11b positive, with a great part of them being infiltrating monocytes differentiating to macrophages. Recruitment of these cells likely play a critical role for immune enhancement of the administered vaccine since one of macrophages main function is to modulate the adaptive immune response through antigen processing and presentation; moreover, macrophage differentiation into distinct functional phenotypes, namely M1 or M2 polarization, can drive the activation and differentiation of naïve T cells to acquire different effector functions.

Since Alum-SMIP.7-10, as well as SMIP.7-10 alone, was shown to induce a mixed polarization phenotype on macrophages when injected intraperitoneally, another objective of this study was to evaluate macrophage polarization upon adjuvant intramuscular injection at early time-points. In order to characterize macrophage polarization into the muscle at each time-point, additional polarization surface markers were added to the multicolor FACS analysis that was performed for innate cells characterization; in particular, on total macrophage population (CD11b+, F4/80+), CD86, CD80, MHCII markers were used to distinguish M1 polarized macrophages, whereas CD206 and CD36 were used as M2 markers (**Figure 13D**). Yet, the dataset has to be considered counter-intuitive and does not allow any conclusion on polarization status of infiltrating immune cells in the injection site. While *in vitro* exposure to SMIP.7-10 induced a more M1-prone phenotype, in this dataset the molecules involved in antigen presentation like MHCII and co-stimulatory molecules CD80/CD86 (M1 polarization markers) seem to be down-regulated in Alum-SMIP.7-10 injected muscles as compared to PBS control. This becomes more evident in later time-points.

A

Balb/c mice

Groups	Formulation
Group 1	PBS
Group 2	Alum
Group 3	Alum-SMIP.7-10
Group 4	SMIP.7-10

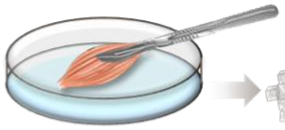
On mouse quadriceps
24h-48h-72h

FACS Staining

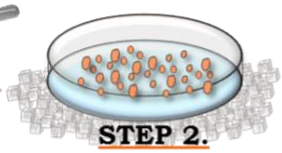
- Neutrophils: CD3-, MHCII int, Ly6G+, Ly6C low, SSC low
- Eosinophils: CD3-, MHCII-, CD11b+, Ly6G-, F4/80 int, Ly6C-
- Monocytes: CD3-, MHCII int, Ly6G int, CD11b+, Ly6C+, SSC low
- T cells: CD3+, MHCII-, SSC low
- B cells: CD3-, F4/80-, MHCII+, SSC low
- Mϕ: CD3-, Ly6G-, CD11c-, CD11b+, F4/80+
  - M1 markers : CD86, CD80, MHCII
  - M2 markers : CD206, CD36

B

Balb/c mice



Mouse Quadriceps



STEP 2.

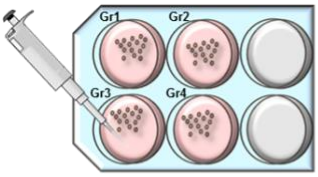
Single cell suspension preparation

STEP 1.

Muscles were cut into 2-4mm little pieces with a lancet in Petri dishes on ice

Enzimatic dissociation

RPMI +
DNase +
Liberase DL



STEP 3.

- Liberase inactivation with RPMI + FBS 10% + PSG
- Cells were pooled during filtering through 70µm cell strainer
- Cells suspensions were washed at 1500 rpm for 7'
- and resuspended in 1 ml of complete media for counting

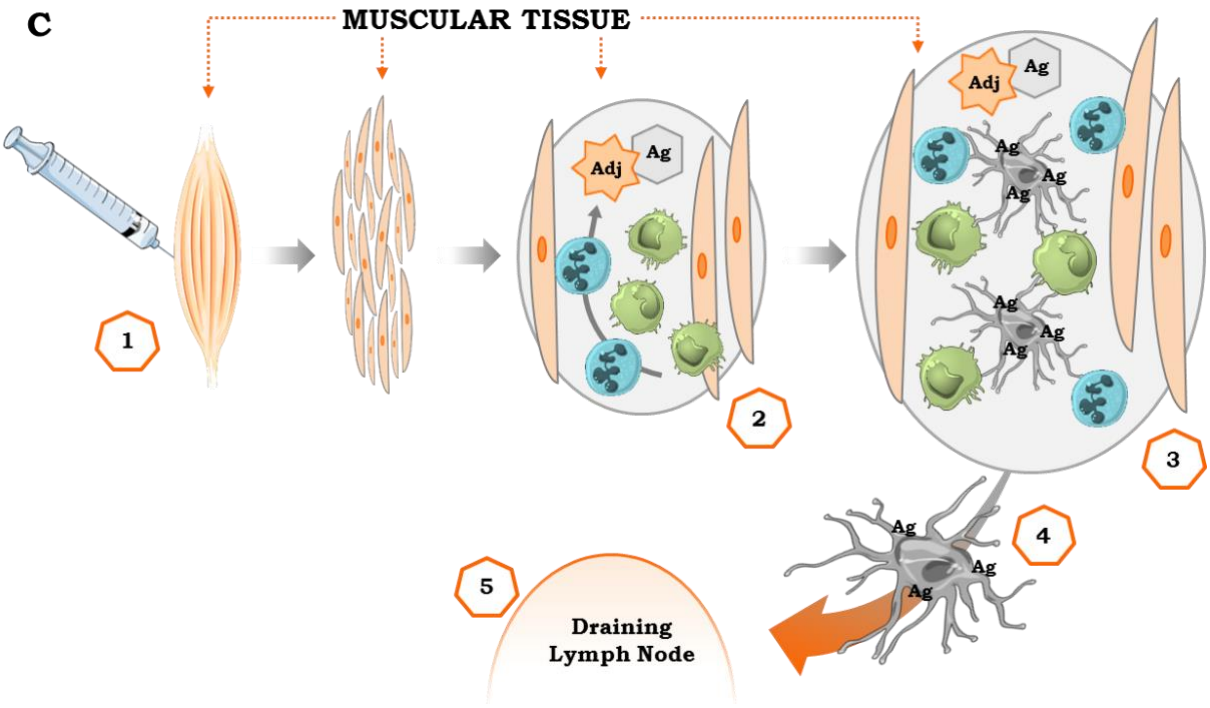
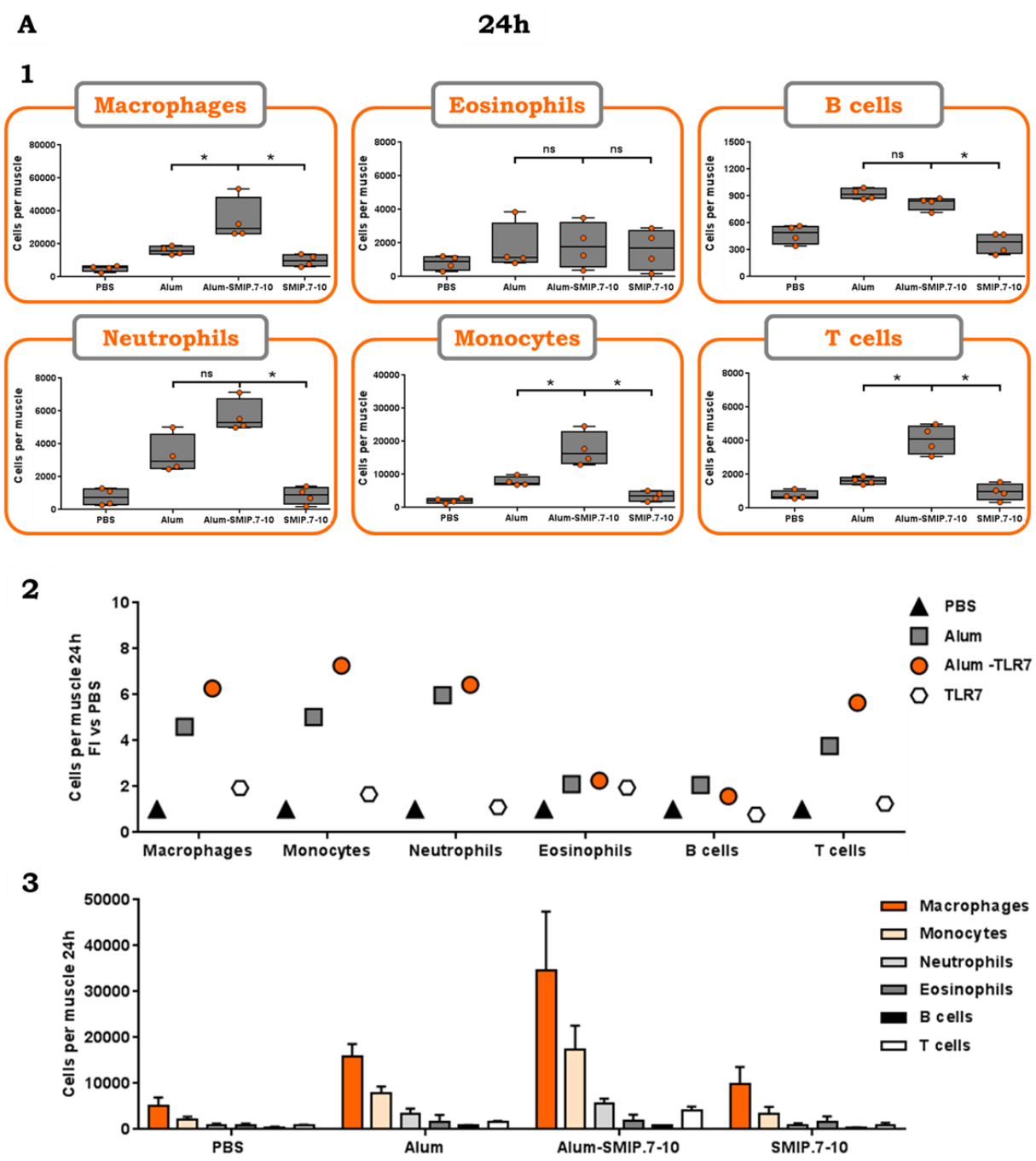


Figure 14. Experimental protocol, gating strategy for innate cells characterization and initiation of a vaccine response at the site of injection

(A) Multicolor FACS analysis (16 colors) to distinguish the innate immune cells recruitment and macrophage polarization at the site of adjuvants injection. (B) Protocol for muscles dissociation; muscles were firsly cutted in small pieces with a lancet and then incubated for 2 hours in Liberase and DNAse containing RPMI, with manual pipetting every 15 minutes to help tissue-disruption. (C) Following injection (1), the pathogen-associated patterns contained in vaccine antigens attract APCs and neutrophils that patrol throughout the body (2). Elicitation of sufficient "danger signals" by the vaccine antigens (Ag)/adjuvants (Adj) activates monocytes and dendritic cells (3); the activation changes their surface receptors and induces their migration along lymphatic vessels (4), to the draining lymph nodes (5) where the activation of T and B lymphocytes will take place.


A
24h
1
Macrophages
Eosinophils
B cells
Neutrophils
Monocytes
T cells
Cells per muscle
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10
ns
*
2
Cells per muscle 24h
FI vs PBS
PBS
Alum
Alum -TLR7
TLR7
Macrophages
Monocytes
Neutrophils
Eosinophils
B cells
T cells
3
Cells per muscle 24h
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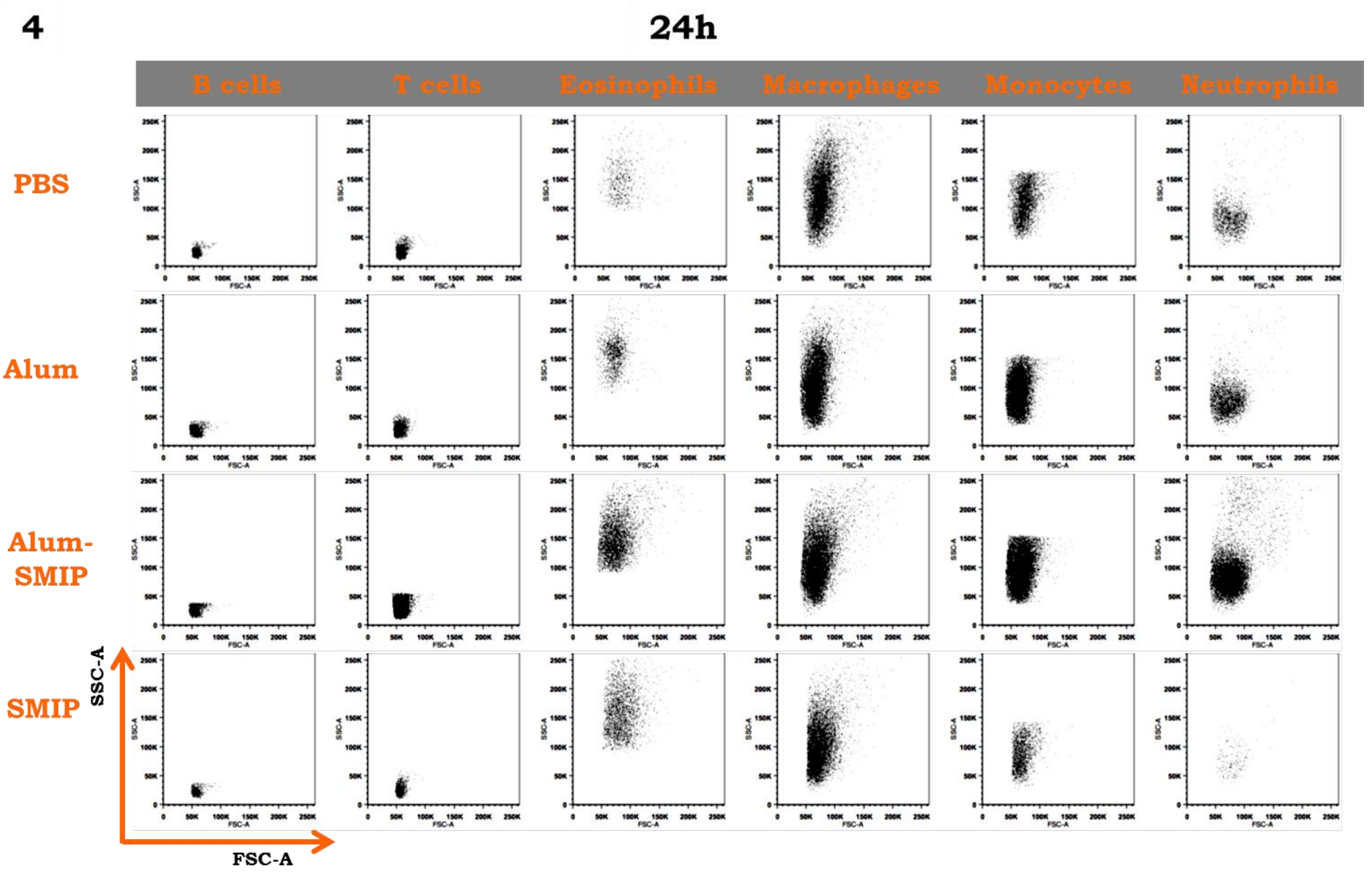
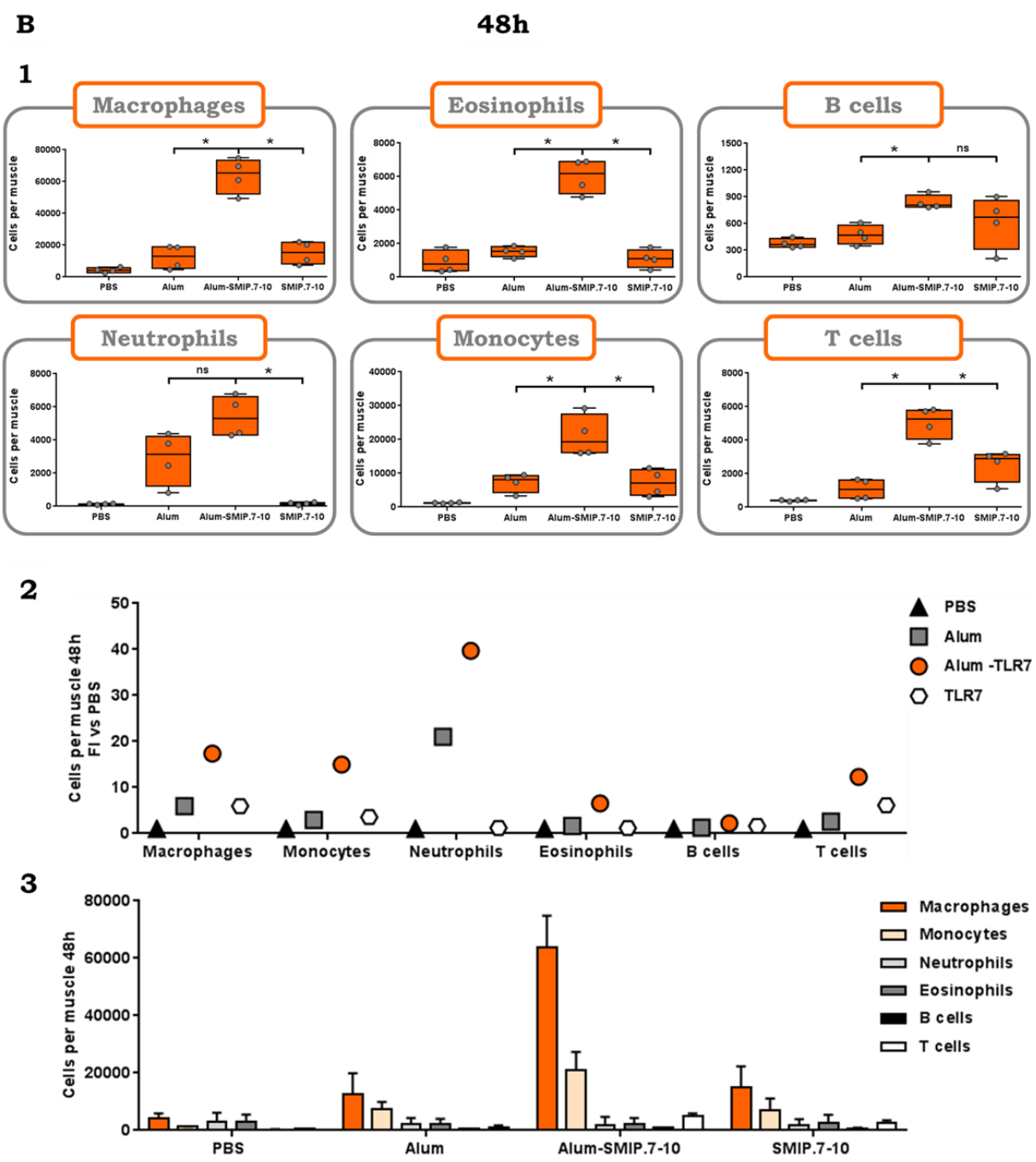
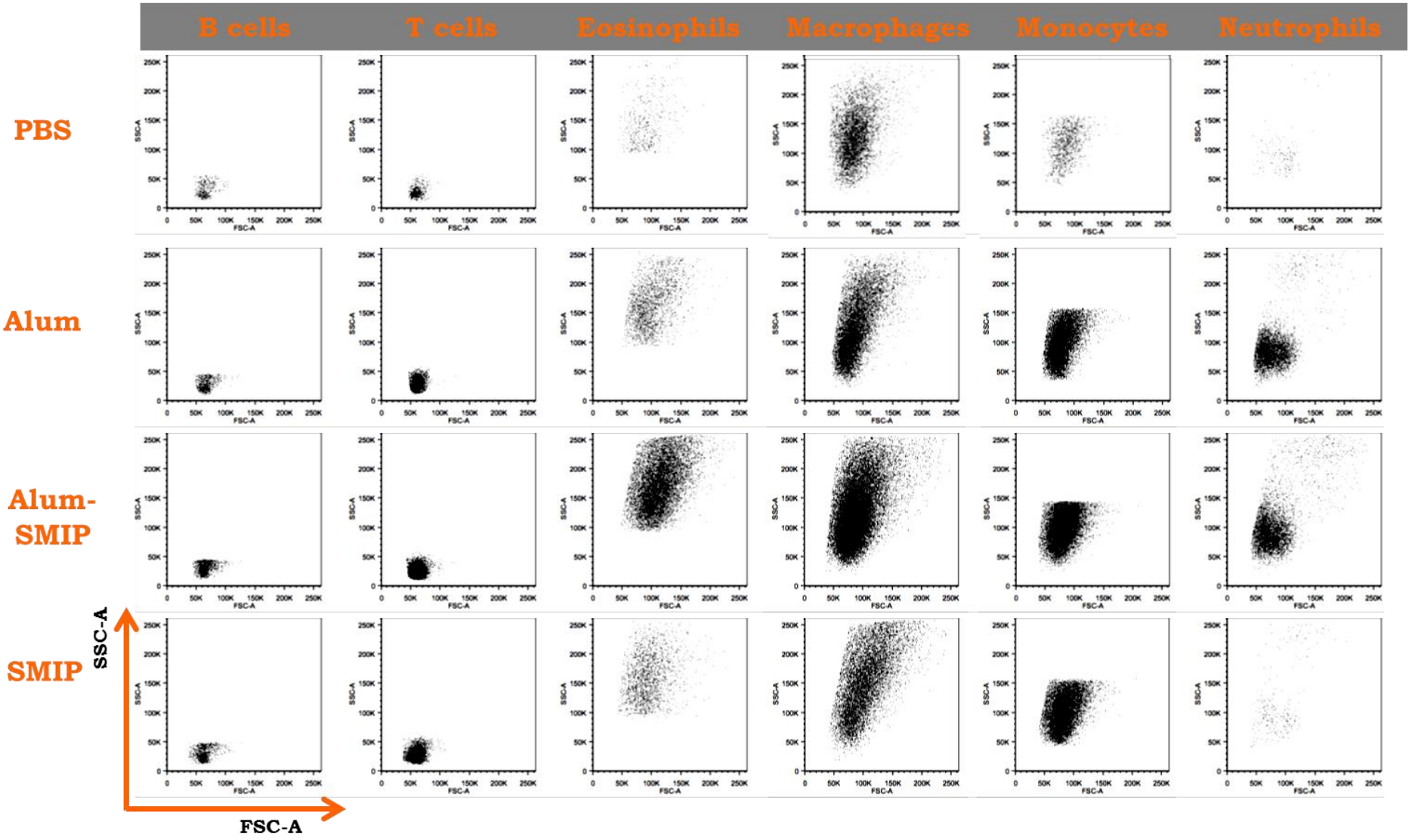
Neutrophils
Eosinophils
B cells
T cells
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10

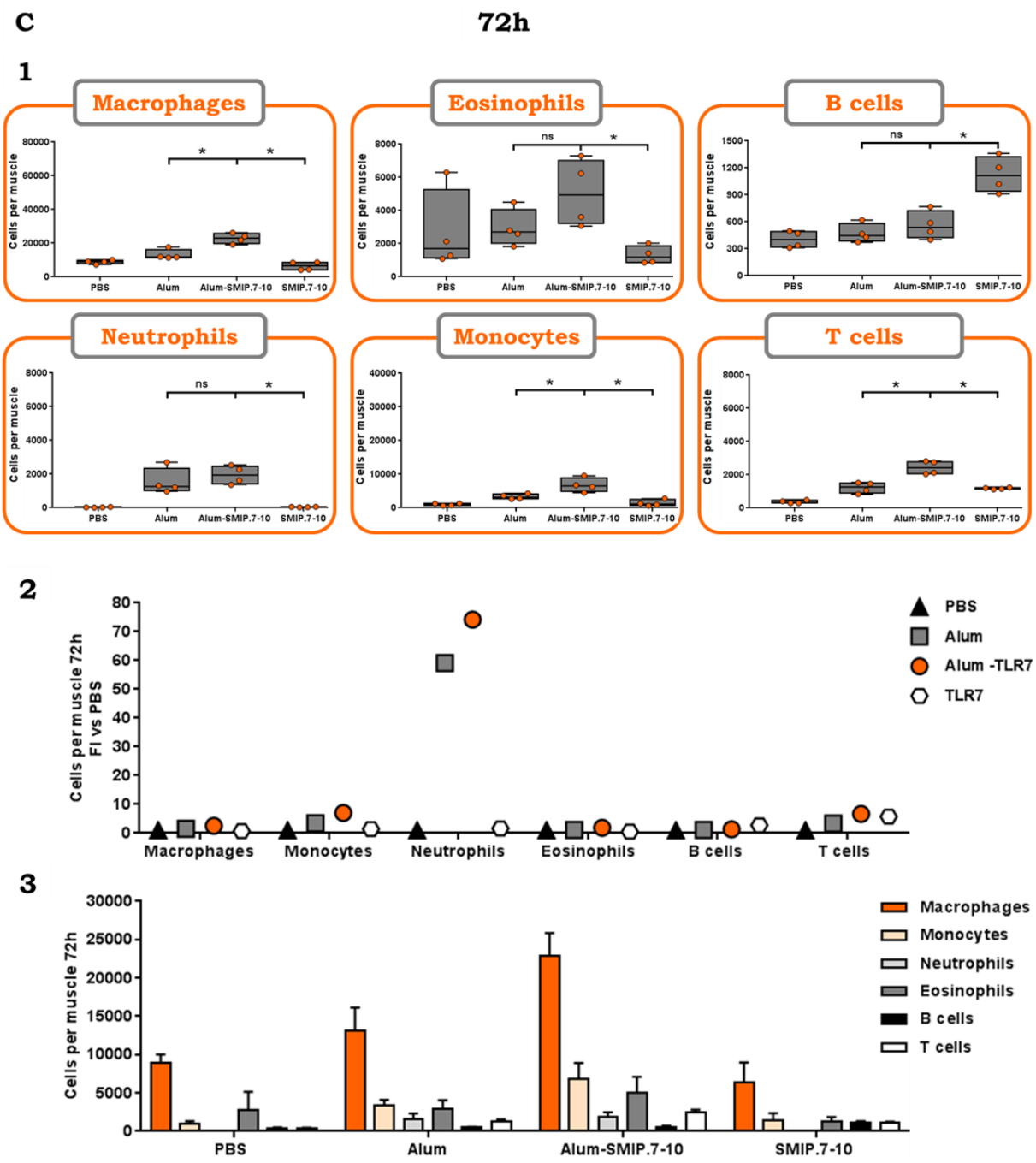

4
24h
B cells
T cells
Eosinophils
Macrophages
Monocytes
Neutrophils
PBS
Alum
Alum-SMIP
SMIP
SSC-A
FSC-A


B
48h
1
Macrophages
Eosinophils
B cells
Neutrophils
Monocytes
T cells
Cells per muscle
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10
ns
2
Cells per muscle 48h
FI vs PBS
PBS
Alum
Alum -TLR7
TLR7
Macrophages
Monocytes
Neutrophils
Eosinophils
B cells
T cells
3
Cells per muscle 48h
Macrophages
Monocytes
Neutrophils
Eosinophils
B cells
T cells
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10

4

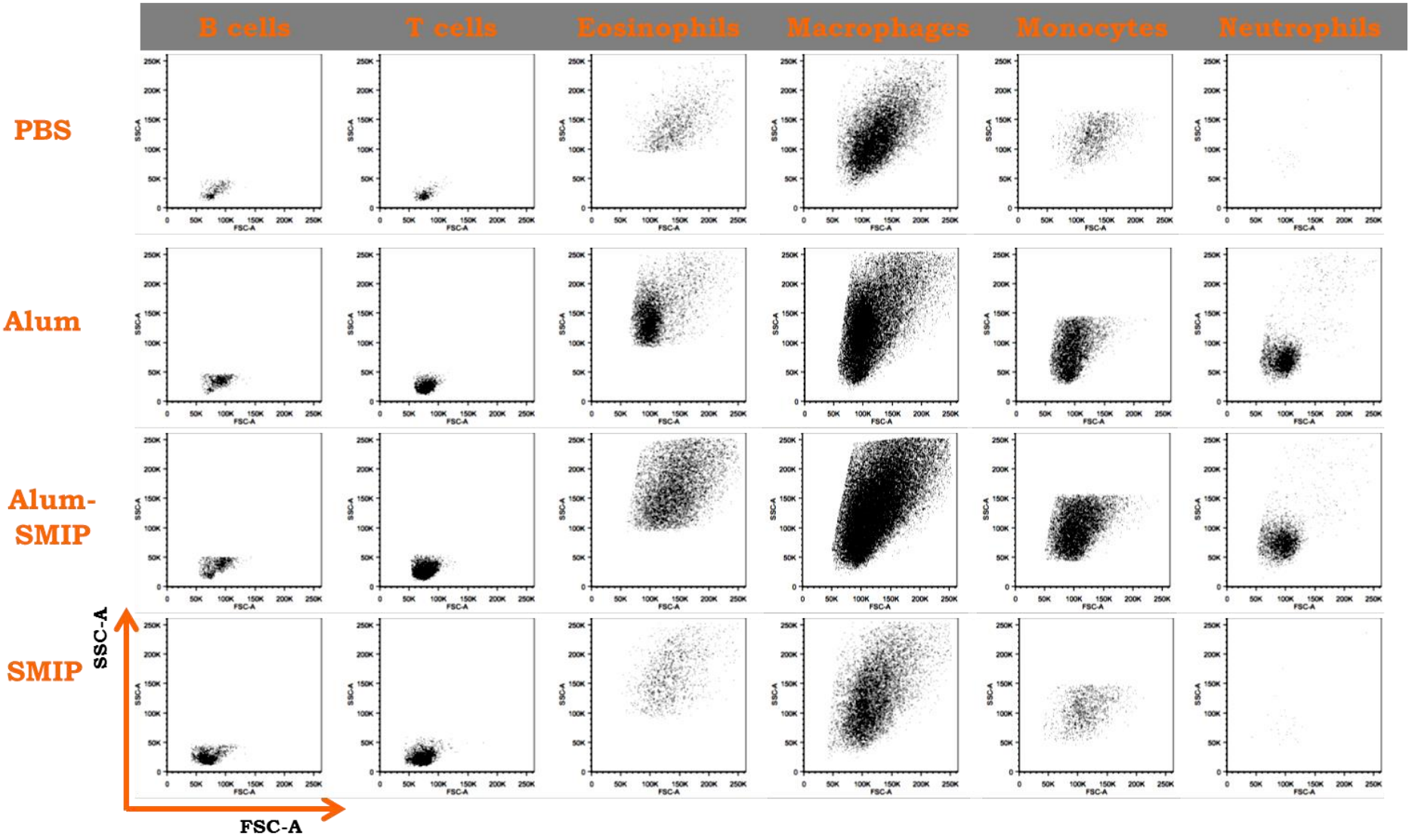
48h


B cells
T cells
Eosinophils
Macrophages
Monocytes
Neutrophils
PBS
Alum
Alum-SMIP
SMIP
SSC-A
FSC-A


C
72h
1
Macrophages
Eosinophils
B cells
Neutrophils
Monocytes
T cells
Cells per muscle
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10
ns
*
2
Cells per muscle 72h
FI vs PBS
PBS
Alum
Alum -TLR7
TLR7
Macrophages
Monocytes
Neutrophils
Eosinophils
B cells
T cells
3
Cells per muscle 72h
Macrophages
Monocytes
Neutrophils
Eosinophils
B cells
T cells
PBS
Alum
Alum-SMIP.7-10
SMIP.7-10

4

72h


B cells
T cells
Eosinophils
Macrophages
Monocytes
Neutrophils
PBS
Alum
Alum-SMIP
SMIP
SSC-A
FSC-A

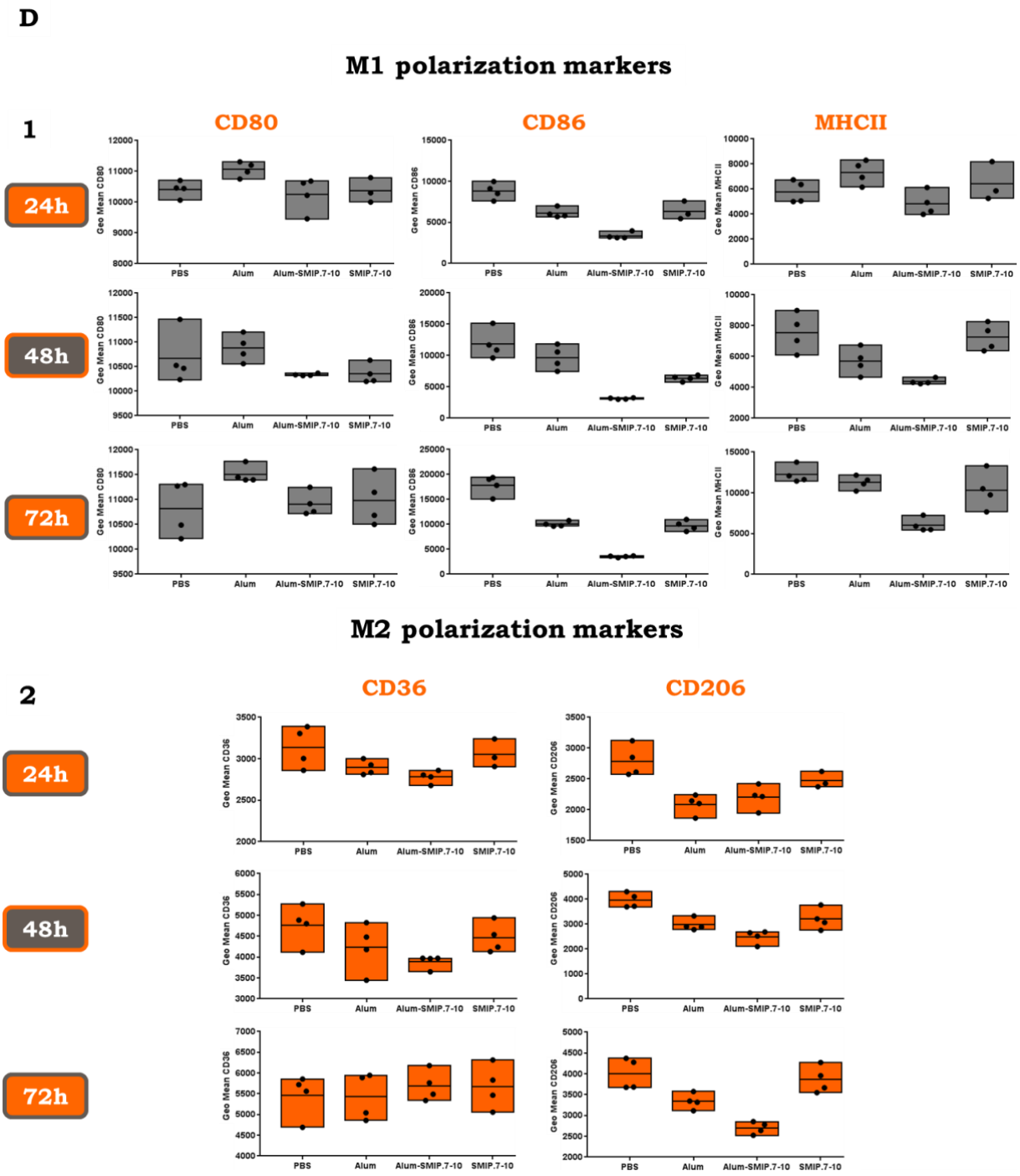


Figure 15. Kinetics of cell recruitment into the muscle in response to Alum-SMIP.7-10-injection. Twelve mice per group (four animals per time-point) were injected into both legs with Alum-SMIP.7-10 and SMIP.7-10 alone or Alum, as benchmark adjuvants. The cell composition of adjuvants treated and PBS-injected muscles of all mice was assessed at (A-1) 24h; (B-1) 48h; (C-1) 72h post-injection by FACS. (A/B/C-2) Fold-increase vs PBS control group for all the innate cells population at the three time-points. (A/B/C-3) Prevalence of the single cell populations in all the experimental groups. (D) Polarization phenotypes of adjuvants-recruited macrophages was evaluated by FACS. Mann-Whitney test was applied (*$P < 0.05$; ns = non statistically significant)

Alum-SMIP.7-10 strongly increases *SslE Escherichia coli* antigen immunogenicity via parenteral route, stimulating a strong systemic immune response

Having assessed which are the immune cells involved in the first response to an Alum-SMIP.7-10-containing vaccine, also the ability of this adjuvant to increase the immunogenicity of a candidate *E. coli* vaccine antigen was evaluated.

In this experiment (see study design and protocol in **Figure 16**), BALB/c mice were immunized three times with Alum-SMIP.7-10-*SslE*, Alum-*SslE* and *SslE* alone 2 or 3 weeks apart, at day 1 ("Prime" injection) and day 21/35 ("Boost" injections) and the immune response profile of the antigen was characterized at the systemic level. Antibody titres were assessed following each immunization: compared to Alum-formulated *SslE* ($P < 0,05$) or to *SslE* alone ($P < 0,0001$), Alum-SMIP.7-10 overall enhanced the magnitudue and the quality of antibody response, showing to strongly increase immunogenicity of *SslE* antigen already after one immunization; in fact, a significant increase in antigen-specific IgG titres was seen as early as after the Prime immunization with *SslE* antigen adjuvanted with Alum-SMIP.7-10 (**Figure 17A**). An even increased anti-*SslE* IgG response with the Alum-SMIP.7-10 adjuvanted formulation was observed two weeks post-second dose (**Figure 17B**). Sera obtained after the second immunization were further analyzed for immunoglobulin subclasses IgG1, IgG2a and IgG2b: among all the groups, *SslE* formulated with Alum-SMIP.7-10 induced the highest anti-*SslE* IgG1 titers; moreover, it was the only condition in which specific anti-*SslE* IgG2a and IgG2b subclasses were detectable (**Figure 17B**), indicating that only Alum-SMIP.7-10 is able to enhance isotype switching. IgG2a and IgG2b antibodies have different complement fixing activity and this is important for bacterial killing.

This result brought to speculate that formulating *SslE* with Alum-SMIP.7-10 would shift the immune response towards a Th1 phenotype, as indicated by the increase of the IgG2a + IgG2b/IgG1 *ratio* in the antigen specific antibodies (**Figure 17C**).

This hypothesis was confirmed by the results of the cytokine expression profile of *SslE*-specific-CD4 Th cells. To look at the cellular immune response to antigen *SslE*, splenocytes were isolated at day 35 and 49 (14 days post-second and third immunization, 5 mice/group and 10 mice/group, respecitvely) and re-stimulated with antigen *SslE* overnight (with the last 4 hours with Brefeldin A, 5μg/ml). Cells were then prepared for flow cytometry analysis by intracellular staining with CD3, CD8, CD4, CD44, TNFα, IFN-γ, IL-2, IL-4, IL-14 and IL-17 murine monoclonal antibodies, in order to identify Th cells (CD4 and CD44 positive cells) and in particular Th1 (IFN-γ, TNF-α and IL-2 positive cells), Th2 (IL-4 and IL-13 positive cells) and Th17 (IL-17A positive cells) populations. After the second immunization (post-II) (**Figure 18A**), a greater expansion of antigen-specific T cells was observed in both adjuvant-*SslE* groups as compared to the antigen alone. However, the Alum and Alum-SMIP.7-10 groups differed in terms of Th response polarization: indeed, altough both

adjuvants were able to stimulate similar frequencies of antigen specific CD4 T cells, Alum-induced Th cells were not yet polarized, being mostly Th0 and only few of them Th1-oriented. Instead, at the same time-point, over the half of antigen-specific T cells in the Alum-SMIP.7-10 group were polarized toward the Th1 phenotype (**Figure 18A, C**). At the post-III time-point (**Figure 18B**), the situation changed dramatically; in facts, the third immunization pushed over one-third of the Alum-*SslE*-induced T cells across a Th2-oriented functional activation. On the contrary, also at this later time-point, the addition of a TLR7 agonist to *SslE* vaccine antigen determined a Th1 switch in the type of Th cell response, altough a good portion of antigen specific CD4 cells resulted yet in a Th0 unpolarized state. Moreover, after three immunizations, some Alum-SMIP.7-10 induced-antigen specific CD4 T cells differentiate towards a Th17 phenotype.

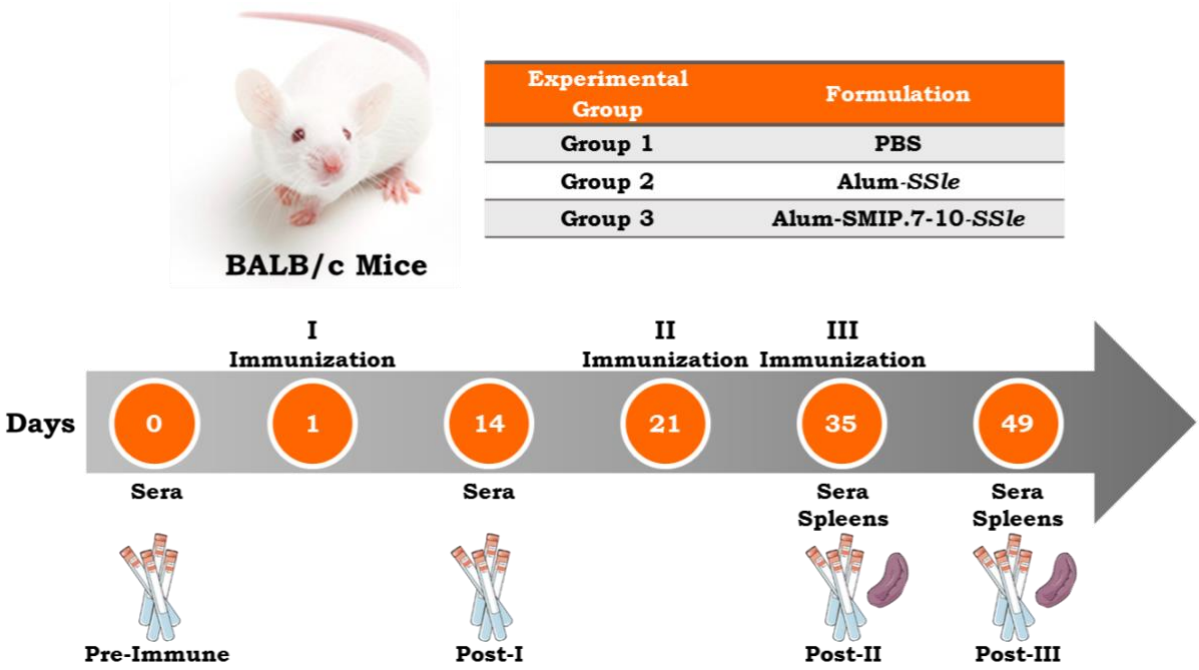
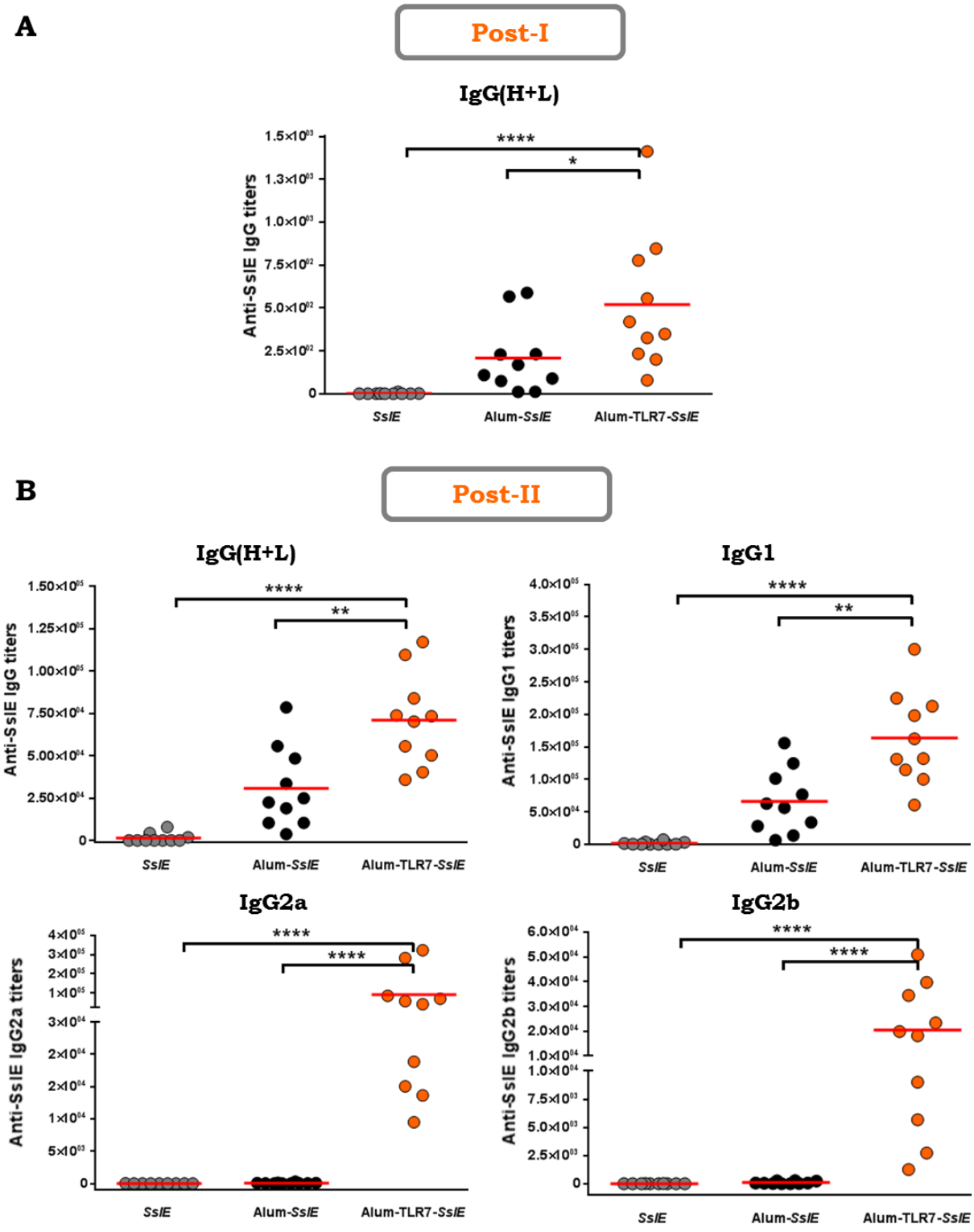


Figure 16. Study design for the *SslE* immunization experiment.

Balb/C mice (15/group) were immunized two (5 mice/group) or three times (10 mice/group) at day 1, 21 and 35 with *SslE* vaccine antigen, alone or formulated either with Alum or Alum-SMIP.7-10. Sampling included sera and spleens and occurred always 14 days after each immunization.


A
Post-I
IgG(H+L)
Anti-SsIE IgG titers
****
*
SsIE
Alum-SsIE
Alum-TLR7-SsIE
B
Post-II
IgG(H+L)
IgG1
IgG2a
IgG2b
Anti-SsIE IgG titers
Anti-SsIE IgG1 titers
Anti-SsIE IgG2a titers
Anti-SsIE IgG2b titers
****
**
****

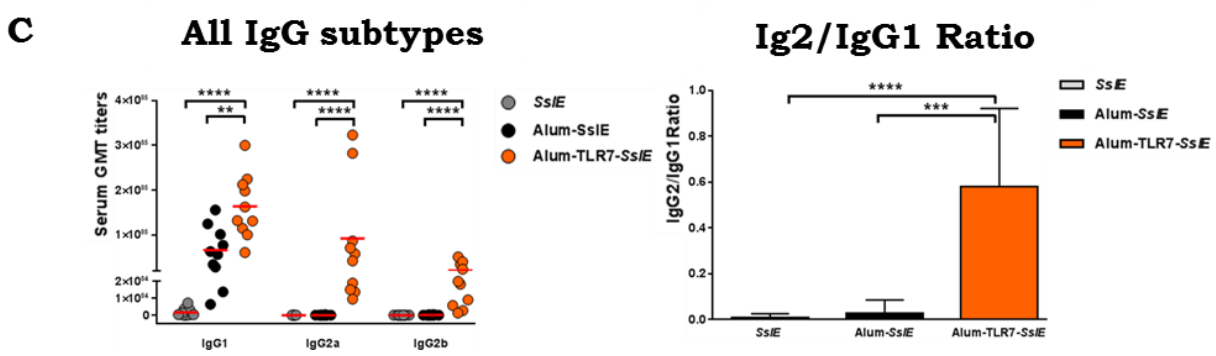


Figure 17. Effect of Alum-SMIP.7-10 on humoral the immune response to *SslE* vaccine.
14 days after the first (A) and the second (B) immunization, total IgG specific for the *SslE* antigen were measured by ELISA. For the post-second dose time-point (B), all the IgG subclasses were also evaluated.Values for each mouse in all groups and the geometric mean (bar) with 95% CI titers are reported in the graph. (C) IgG2a+IgG2b/IgG1 ratio.
Mann-Whitney test was applied: ****P < 0.0001, ***P < 0.001, *P < 0.05.

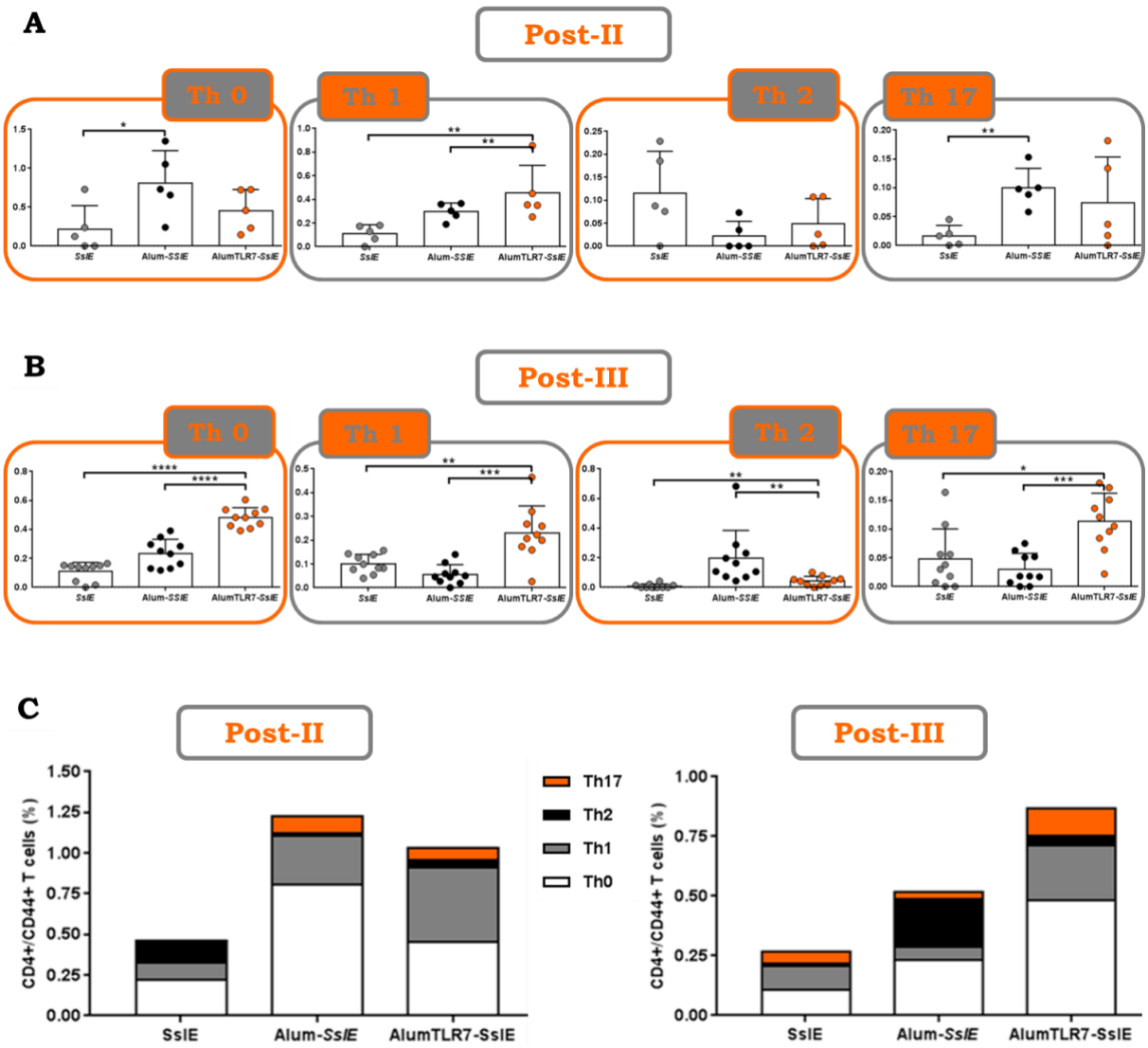


Figure 18. Cytokine quantification of murine splenocytes after immunizations.
Splenocytes from immunized mice were harvested and purified at day 35 (A) (post-II, 5 mice/group) and 49 (B) (post-III, 10 mice/group). Splenocytes were seeded at 10^6 cells/well, cultured with recall antigen *SslE* (10µg/ml final concentration) and incubated overnight at 37°C (last 4 hours with Brefeldin-A). Cells were then stained with Live/Dead and fluorescent antibodies for flow cytometry acquisition. Percentages of Ag-specific cytokine-secreting CD4 T cells were analyzed by intracellular staining. (C) Bars show the average of 5/10 mice per group of CD4CD44+ T cells producing IL-17 with/without IL-2 and TNF-α (Th17); IL-2 and/or TNF-α (Th0); IFN-γ with/without IL-2 and TNF-α (Th1); IL-4 and IL-13 with/without IL-2 and TNF-α (Th2).
Mann-Whitney test was applied: ****$P < 0.0001$, ***$P < 0.001$, *$P < 0.05$.

Macrophages are directly involved in *SslE* antigen presentation

In the previous study, BALB/c mice were intramuscularly immunized twice with *SslE E. coli* antigen alone or in combination with Alum or Alum-SMIP.7-10 (Alum formulated with SMIP.7-10) vaccine adjuvants and the immune response profile of the antigen have been characterized at the systemic level. Alum-SMIP.7-10 inclusion in vaccine formulation was actually able to significantly increase overall *SslE* immunogenicity by inducing higher antigen specific antibody titer, as compared to the immunization with the antigen alone or adjuvanted with Alum, even after the first step of immunization.

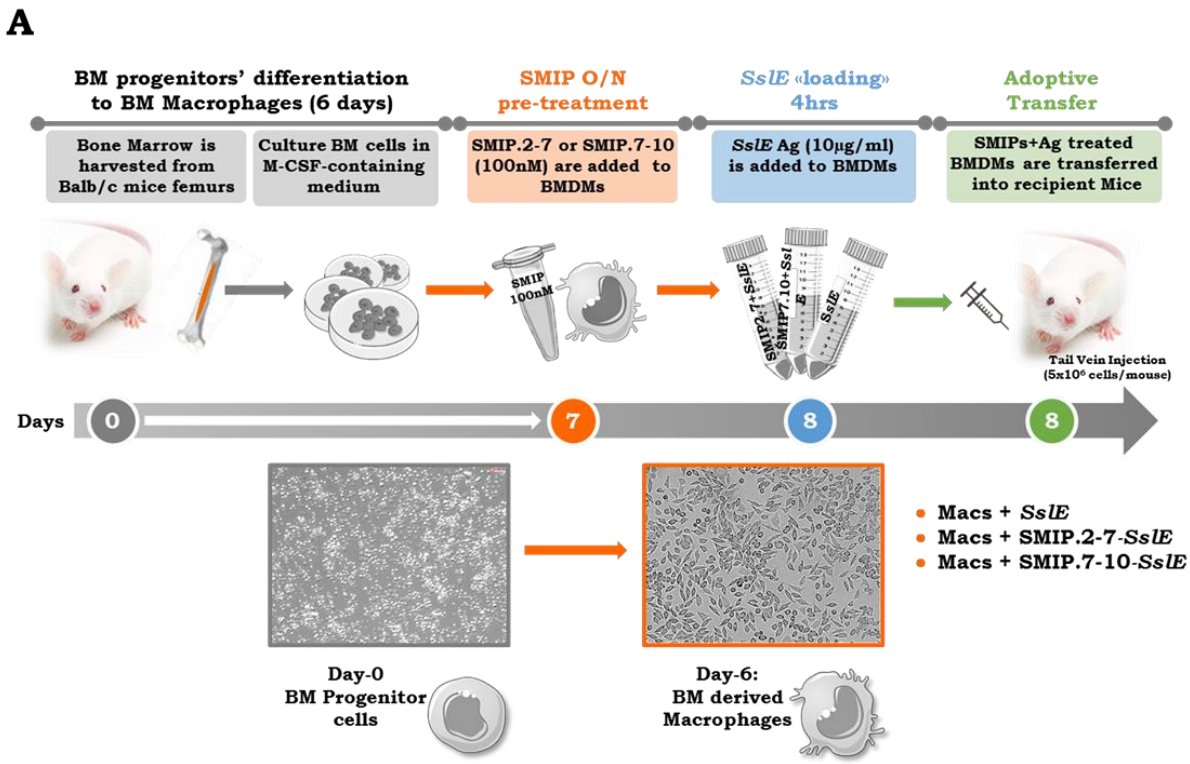
To understand if macrophages do have a role in *SslE* antigen-presentation and if SMIPs TLR agonists can enhance this process, it was evaluated whether adoptive transfer of *in vitro* antigen-stimulated Bone Marrow Derived Macrophages (BMDMs) was actually able to raise *SslE* immunogenicity by promoting an humoral response, measured as anti-*SslE* IgG ELISA titers in animal sera at two weeks post-injection (day 14).

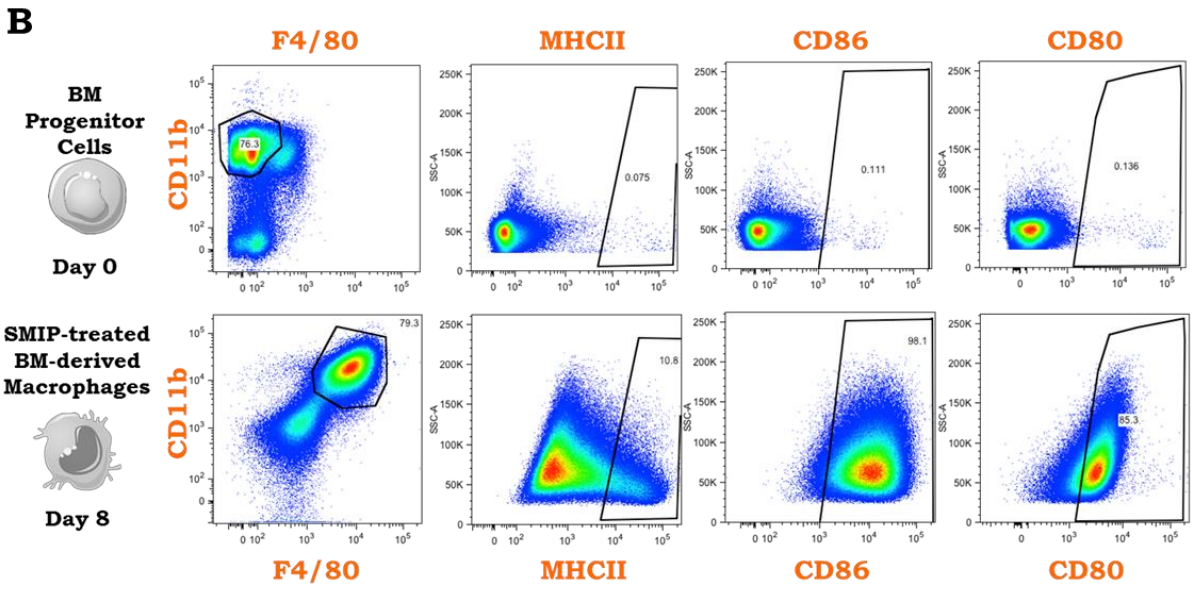
BMDMs are primary macrophage cells, derived from bone marrow cells *in vitro* in the presence of growth factors. Macrophage-Colony Stimulating Factor (M-CSF) is a lineage-specific growth factor that is responsible for the proliferation and differentiation of committed myeloid progenitors into cells of the macrophage/monocyte lineage. Bone marrow was isolated from femurs of BALB/c mice and bone marrow precursor cells were grown in DMEM with 10% FBS in non-tissue culture Petri dishes in the presence of M-CSF. Under these conditions, the bone marrow monocyte/macrophage progenitors proliferated and differentiated into a homogenous population of mature BMDMs. The efficiency of the differentiation has been assessed using FACS analysis of CD11b and F4/80 surface antigens expression (**Figure 19A-B**). At this time point, they have been overnight incubated with SMIP.2-7 or SMIP.7-10 and then stimulated for 4h with *SslE*. At the end of stimulation, cells have been washed, harvested and counted to be adoptively transferred intravenously into naïve recipient Balb/c mice (5 x 10^6 cell/recipient mouse). BMDMs that received the antigen but not the adjuvant were used as control. Overall, *in vitro* Ag-"loaded" macrophages were able to successfully present *SslE* antigen to the immune cells of the recipient mice and to induce an antigen specific immune response already after one immunization. Indeed, all the experimental groups showed an increase in antigen-specific IgG titres. In addition, compared to the control group that receive only the antigen, SMIP-pretreatment of macrophages did have a role in the enhancement of *SslE* presentation (**Figure 20A**). This was probably due to the up-regulation of co-stimulatory molecules MHC-II, CD80 and CD86 that was observed by FACS analysis in overnight SMIPs-stimulated BMDMs (**Figure 19B**). Likewise, SMIP-pretreated macrophages showed a significant up-regulation of the main M1 markers, as well as IL-10 MReg immunomodulatory cytokine, as verified by gene expression analysis on BMDMs prior to antigen addition via Real-time qPCR (**Figure 19C**); this pro-inflammatory activation could have led to enhanced immune cells recruitment and activation upon injection into singenic mice, hence favoring antigen presentation and induction of a specific immune response.

Among the SMIPs, TLR2 agonist SMIP.2-7 was able to induce even a more potent specific humoral response than TLR7 agonist SMIP.7-10 (**Figure 20A**).

In order to evaluate if *SslE*-loaded macrophages were able to induce a T cell antigen-specific immune response, spleens were harvested at day 14 and reduced to single-cell suspension. Splenocytes were then *in vitro* re-stimulated with *SslE* overnight before the addition of 5μg/ml of Brefeldin A for 4 hours at 37°C + 5% CO_2. Cells were then prepared for Flow cytometry analysis by intracellular staining with CD3, CD8, CD4, CD44, TNF-α, IFN-γ, IL-2, IL-4, IL-13 and IL-17 murine monoclonal antibodies in order to evaluate the induction and eventually the polarization of the T cell response. Normally post-I is a very early time-point to observe a systemic response, but in this experiment adoptively trasferred mice (**Figure 20B**) showed already a great activation of antigen-specific T cells; however, most of them are still in a non polarized Th0 state and this could mean that the response has been successfully triggered by *SslE*-loaded macrophages but it still in an immature phase (**Figure 20B, upper panel**).

In fact, differentiation of naïve CD4 T cells (Th0) into Th1 or Th2 cells determines whether antigen will raise a cellular or a humoral immune response; the maturation pathway chosen by the Th0 cell is often decisive for the outcome of disease and depends among others on the co-stimulatory attributes of the APC and the nature and abundance of cytokines provided by the APC and the microenvironment. The presence of the SMIPs induced significantly higher T cells frequencies than the control. Analysis of the accompanying cytokine secretion revealed that, although the two SMIPs stimulated similar levels of *SslE*-specific T cells activation, SMIP.2-7 and SMIP.7-10 differently stimulated the flavour of the Th response, with TLR2 agonist inducing a Th2-oriented T cell activation, whereas SMIP.7-10 inducing a Th1 polarization (**Figure 20B, lower panel, 20C**). Noteworthy, the level of Th17 induction was similar in both SMIPs-preincubated groups, and significantly higher than the control.


A
BM progenitors' differentiation to BM Macrophages (6 days)
SMIP O/N pre-treatment
SslE «loading» 4hrs
Adoptive Transfer
Bone Marrow is harvested from Balb/c mice femurs
Culture BM cells in M-CSF-containing medium
SMIP.2-7 or SMIP.7-10 (100nM) are added to BMDMs
SslE Ag (10µg/ml) is added to BMDMs
SMIPs+Ag treated BMDMs are transferred into recipient Mice
SMIP 100nM
SMIP2-7+SslE
SMIP7.10+SslE
SslE
Tail Vein Injection (5x10⁶ cells/mouse)
Days
0
7
8
8
Macs + SslE
Macs + SMIP.2-7-SslE
Macs + SMIP.7-10-SslE
Day-0 BM Progenitor cells
Day-6: BM derived Macrophages


B
F4/80
MHCII
CD86
CD80
BM Progenitor Cells
Day 0
SMIP-treated BM-derived Macrophages
Day 8
CD11b
SSC-A
76.3
0.075
0.111
0.136
79.3
10.8
98.1
85.3

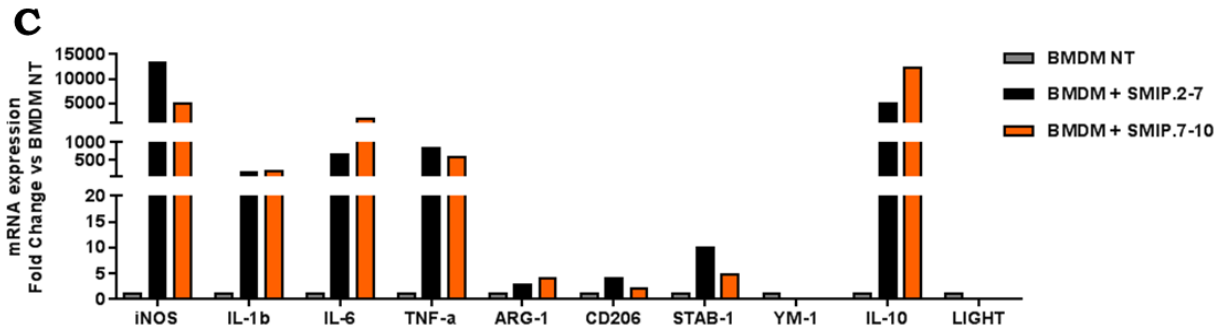
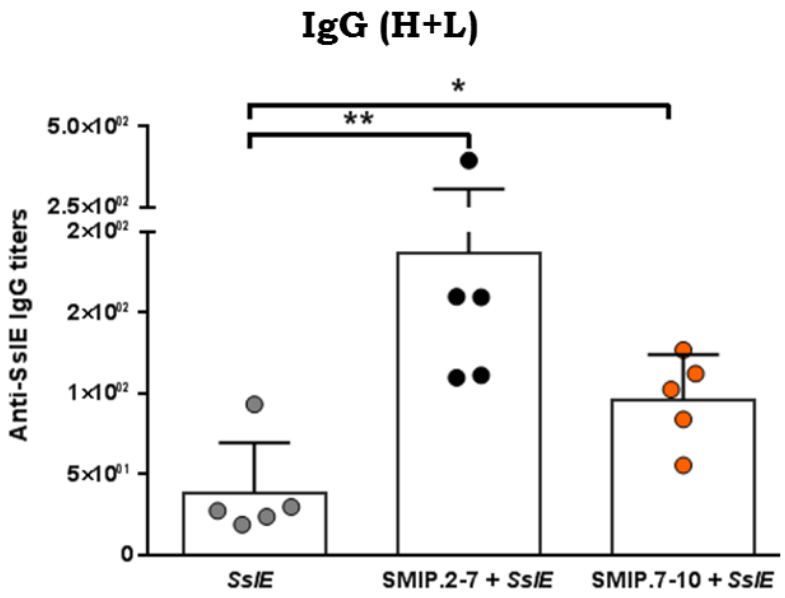


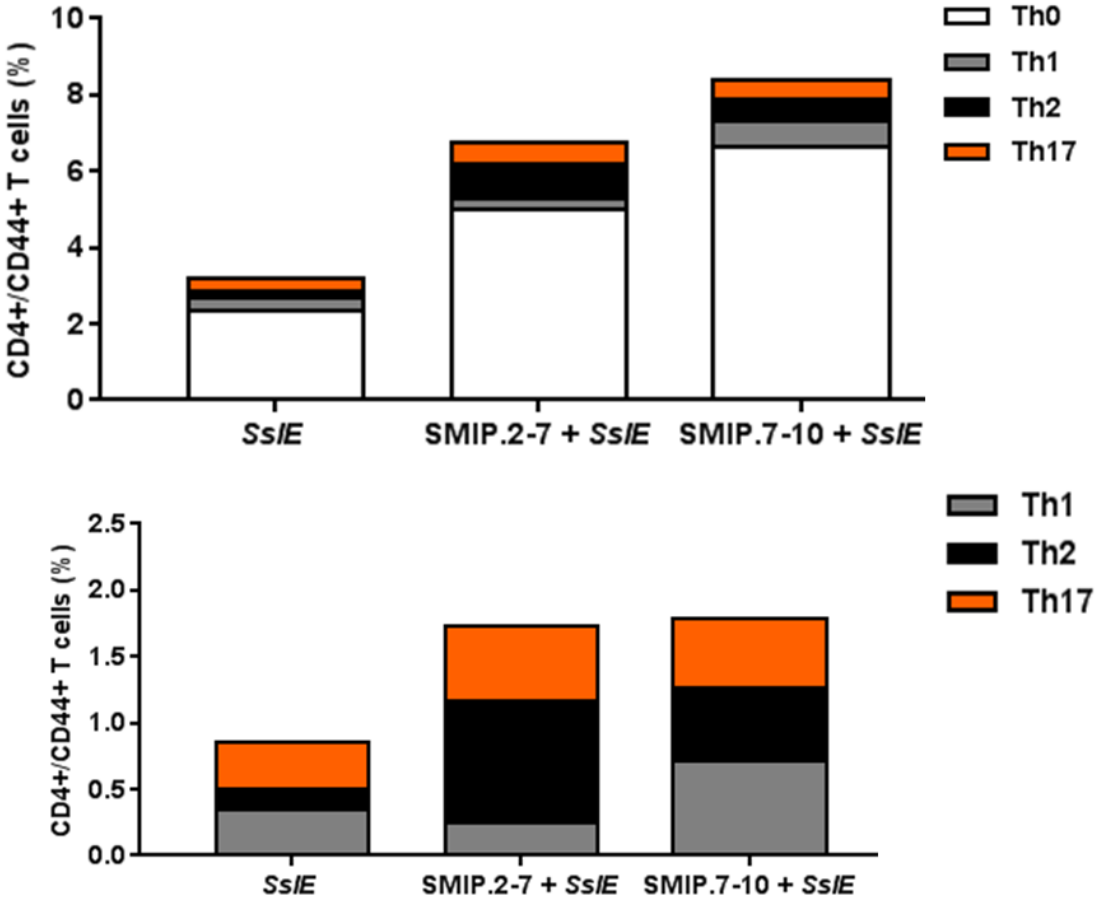
Figure 19. BMDMs *in vitro* differentiation protocol and flow cytometry characterization before adoptive transfer. (A) Bone marrow preparation for macrophage differentiation. At day 7 BMDMs were overnight incubated with SMIPs and stimulated with the *SslE* antigen for 4h before being adoptively transferred into recipient mice. (B) Surface expression of CD11b and F4/80 (murine macrophage-specific marker) was analyzed by flow cytometry at day 0 (BM progenitor cells) and at day 8 (after BMDMs *in vitro* differentiation and SMIP stimulation, prior to incubation with *SslE*) in order to test effective macrophage differentiation. SMIP-treated BMDMs showed very high expression of M1 surface markers MHCII, CD86 and CD80, that are also co-stimulatory molecules involved in the antigen presentation process, while this is not observed in BM progenitor cells. (C) Real-time qPCR for gene expression analysis of M1 (*iNOS*, *il-1β*, *il-6*, *tnf-α*), M2 (*arg-1*, *cd206*, *stab-1*, *ym-1*) and MReg (*il-10*, *light*) polarization markers on SMIP-treated BMDMs revealed an M1-predominant altough mixed phenotype of activation. Results are expressed ad fold-change above untreated (NT) BMDMs.

A

Post-I


IgG (H+L)
*
**
5.0×10^02
2.5×10^02
2×10^02
2×10^02
1×10^02
5×10^01
0
Anti-SslE IgG titers
SslE
SMIP.2-7 + SslE
SMIP.7-10 + SslE

B


10
8
6
4
2
0
CD4+/CD44+ T cells (%)
Th0
Th1
Th2
Th17
SslE
SMIP.2-7 + SslE
SMIP.7-10 + SslE
2.5
2.0
1.5
1.0
0.5
0.0
CD4+/CD44+ T cells (%)
Th1
Th2
Th17
SslE
SMIP.2-7 + SslE
SMIP.7-10 + SslE

C

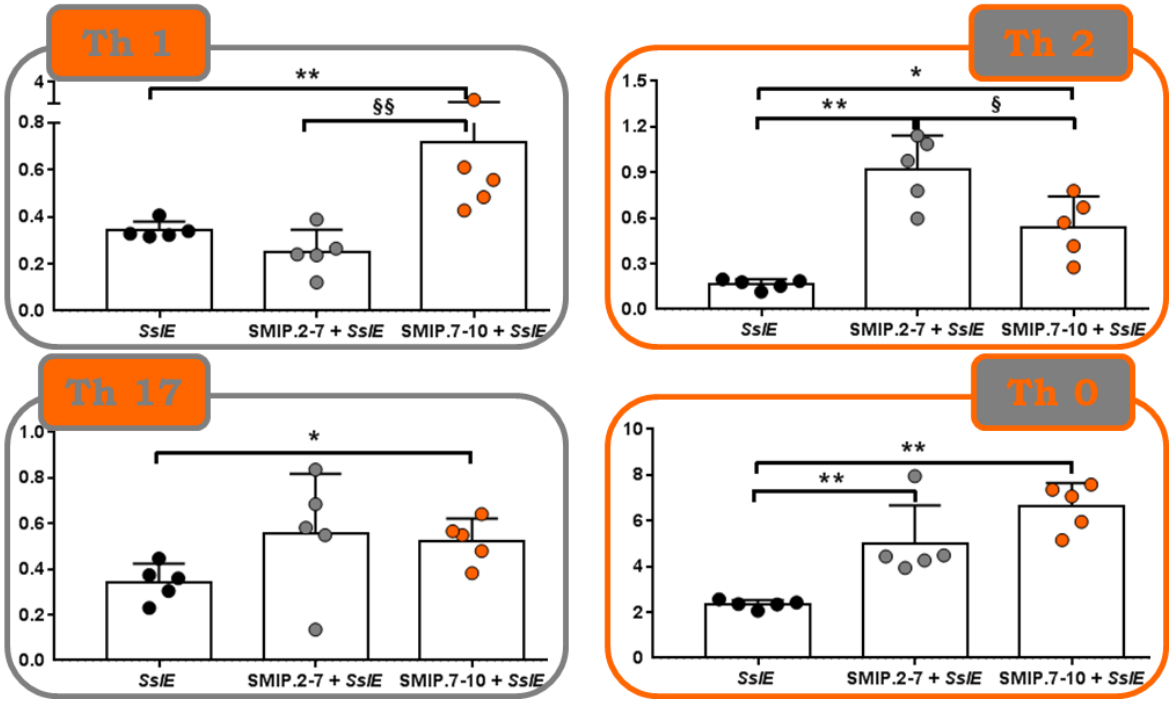


Figure 20. Effect of SMIP.2-7 and SMIP.7-10 on humoral and cellular immune response to *SslE* antigen.

(A) 14 days after the first immunization, total IgG specific for the *SslE* antigen were measured by ELISA. (B) Evaluation of the antigen-specific T cell reponse. Upper panel show all the Th populations on the CD4/CD44+ positive T cell; lower panel show only the frequencies of Th1, Th2 and Th17 populations. (C) Frequencies of the single Th populations. Bars show the average of 5 mice per group of CD4CD44+ T cells producing IL-17 with/without IL-2 and TNF-α (Th17); IL-2 and/or TNF-α (Th0); IFN-γ with/without IL-2 and TNF-α (Th1); IL-4 and IL-13 with/without IL-2 and TNF-α (Th2). Mann-Whitney test was applied: ****P < 0.0001, ***P < 0.001, *P < 0.05.

Chapter IV
Discussion

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of humans and animals as part of the commensal flora. Normally, *E. coli* colonizes the infant gut within hours of birth and establishes itself as the most abundant facultative anaerobe of the human intestinal microflora for the remainder of life, equipped with the abilities to grow in the ever-changing environment in the gut and cope with the mammalian host interaction. Nevertheless, *E. coli* can survive in many different ecological habitats, including abiotic environments and is considered a highly versatile species; known habitats of *E. coli* include soil, water, sediment and food.

However, pathogenic *E. coli* strains also exist and these isolates are typically categorized based on their mechanisms of disease and clinical outcomes. Genome sizes of *E. coli* can differ by a million base pairs between commensals and pathogenic variants and this extra genetic content can contain virulence and fitness genes. Comparative genomics have shown that *E. coli* genomes are split between a shared, conserved set of genes - called the core genome - and a flexible gene pool. The pathogenic ability of *E. coli* is therefore largely afforded by the flexible gene pool through the gain and loss of genetic material at a number of hot spots throughout the genome [Touchon *et al.*, 2009], resulting in Horizontal Gene Transfer (HGT).

The prevention of *E. coli* infections is of pressing concern from both the public health and economic perspectives [Smith *et al.*, 2007]. Although the use of antibiotics is an important key strategy to treat the infections, the recent rising incidence of hypervirulent multi-drug resistant strains among the pathogenic strains is a major problem for modern society since diseases caused by these pathogens are associated with significant human suffering and high costs for the healthcare system. The most effective preventive strategy would be the existence of a vaccine, but to date *E. coli*'s considerable antigenic diversity and virulence factor redundancy, together with the difficulty in predicting vaccine coverage and the lack of a correlate of protection, have led to numerous promising pre-clinical data not being confirmed by human studies [Brumbaugh *et al.*, 2012; Ahmed *et al.*, 2013], thus hampering the release of a broadly protective vaccine against all the pathogenic strains.

Vaccines are certainly one of the discoveries of the past century that most affected human kind, collectively contributing to a reduction of childhood mortality and an increase in life expectancy. The key objective of vaccination is immunity, in other words to elicit an effective pathogen-specific immune response that leads to protection against infection caused by that pathogen and that may ultimately result in its eradication, while avoiding unwanted side effects of infectious diseases. Immunity is characterized by increased titers of specific antibodies and higher frequencies of antigen-specific B and T cells with accelerated and increased functional responses in the case of re-encounter of the same

agent. Biochemical, serological and microbiological methods have traditionally been used to dissect pathogens and identify potential antigens for vaccine development. These approaches were important to provide the basis of vaccinology, but showed to be time-consuming, leading to years or even decades of research. Inactivation and attenuation were the first choice for many years, but the difficulty of cultivating some microorganisms in vitro and the fact that even attenuation may result in detrimental or unwanted immune responses showed that these approaches are impractical in some instances [Purchell *et al.*, 2007]. Even the purification of specific antigens failed in many cases in providing protective vaccine candidates, since the methods usually employed led to the identification of the most abundant, but also most variable and less suitable, vaccine candidates [Rappuoli, 2000].

Although successful for many pathogens, conventional vaccinology still left many diseases uncontrolled. Nonetheless, the sequencing of the first bacterial genome in 1995 led the vaccine development to enter a new era and to open a new chapter in the vaccine development guidebook. Suddenly, all the proteins encoded by a microorganism were available and for the first time after more than two centuries and it was possible to identify vaccine candidates without using the traditional techniques. This new approach, named Reverse Vaccinology, have given full access to all the proteins that a microorganism can encode and, by computer analysis, have allowed to identify potential surface-exposed proteins in a reverse manner, starting from the genome rather than the microorganism. In a subtractive comparative genome analysis, called subtractive reverse vaccinology, genes conserved between pathogenic and nonpathogenic strains of a same or even related species could be discarded, leading to the selection of antigens most strictly related to the pathogenesis and, therefore, avoiding any impact on the commensal flora. This approach could also reduce the number of antigens to be tested and, consequently, the time for the delivery of a vaccine.
In the case of *E. coli*, in spite of the huge genomic diversity, the subtractive reverse vaccinology approach allowed to identify nine potential vaccine targets as protective against a mouse sepsis model of *E. coli*; among these, the Secreted Surface-associated Lipoprotein of *E. coli* (*SslE*) was the most promising candidate [Moriel *et al.*, 2016], being also widely conserved in all *E. coli* pathotypes.

Novel vaccine strategies include the so-called subunit vaccines, which encompass only the part of the pathogen to which immune recognition results in protection. The high purity of these vaccines makes adverse events less likely, but it also makes the vaccines less immunogenic and potentially less effective. For example, they require multiple booster doses to achieve protective antibody levels and high costs, which makes their use in the developing world problematic. Therefore, improving the efficacy of these vaccines, in particular increasing the rapidity by which they induce a protective immune response, would be a great benefit for public health.

To overcome this inadequacy, adjuvants are added to vaccine formulations to boost the immune system in order to increase the immunogenicity of vaccine antigens; in this way, they may significantly reduce the amount of antigen needed or the number of doses required for optimal response [Buonsanti *et al.*, 2016]. The discovery of vaccine adjuvants dates back to 1925 [Christensen, 2016]; over the past years, many efforts have been made to investigate how and why these compounds work. A recent, greater understanding of innate and adaptive immunity and their close interaction at the molecular level in the host response to a pathogen has enabled vaccine researchers to use adjuvants to their full advantage. Recent data suggest that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system rather than by having direct effects on the lymphocytes themselves. These compounds, either acting as delivery systems or as immune-potentiators, are able to enhance antigen uptake by APCs by activating or inducing their maturation, thus promoting immune-modulatory cytokines production that, in turn, elicits local inflammation and cellular recruitment. Among APCs, macrophages can acquire distinct activation phenotypes in response to vaccine adjuvants, implementing different functional programs and transcriptional profiles. Different polarization phenotypes stimulate also different effect on adaptive immune response, with M1 macrophages promoting Th1 and Th17 responses and M2 macrophages being instead related to Th2 responses, tissue remodeling and immune tolerance [Arango Duque and Descoteaux, 2014]. GSK has identified a series of new adjuvants, called SMIPs (Wu *et al.*, 2014), that trigger members of the TLRs family expressed on APCs. The aim of the work described in this thesis was to characterize the *in vitro* and *in vivo* effect of GSK TLRs agonist adjuvants on macrophage polarization at early time-points and also to verify if the obtained activation state correlates coherently with the adaptive immune response observed when formulated with *E. coli SslE* vaccine antigen in complete immunization protocol.

The ideal adjuvant to be included in a protective vaccine against *E. coli* should activate macrophages towards an M1 functional program in order to elicit a systemic immune response (therefore a Th1 response) while also inducing an optimal and synergistic local response at the intestinal mucosa. Firstly, the effect of SMIP.2.7, SMIP.7-10 and SMIP.7-11 on *in vitro* experiments using murine macrophage cell line RAW 264.7 was evaluated. Gene expression analysis by Real Time qPCR of the most significant polarization markers indicated that all SMIPs induced an M1 phenotype. *Ex vivo* FACS analysis on purified murine peritoneal macrophages treated with GSK adjuvants coherently correlated with the gene-expression analysis, showing an M1-oriented activation. After all the *in vitro* evidences accumulated and due to the similar macrophagic activation profile induced by the TLR2 and the TLR7 agonists tested, and considering that the latter have been more characterized as potential vaccine adjuvants [Buonsanti *et al.*, 2016], it was decided to pursue our studies only on TLR7 agonists.

During the process of SMIPs screening and optimization, Wu *et al.* [2014] hypothesized that the key to the successful use of these small molecule immune-potentiators (SMIP) as vaccine adjuvants was keeping these compound localized at the site of injection; localization should optimize triggering of the desired innate immune responses at the site where the vaccine antigens are concentrated facilitating the initiation of the specific adaptive immune response, also reducing the release of the SMIP into the systemic circulation, potentially keeping its serum levels below the concentration that triggers a systemic inflammatory response [O'Hagan *et al.*, 2003; Didierlaurent *et al.*, 2009; Mbow *et al.*, 2011]. To achieve this localization, TLR7 agonist SMIPs were engineered allowing them to be adsorbed to Aluminum Hydroxide (Alum), a very well-known adjuvant with a long history of safe use in humans [Buonsanti *et al.*, 2016]. SMIP7.10 and SMIP.7-11 were intraperitoneally injected in Balb/C mice on day 0, either in their soluble forms or formulated with a constant dose of Alum. In agreement with this rational design approach, also in this experiment Alum-containing SMIP formulations showed to recruit significantly higher numbers of cells at the site of injection compared to their soluble forms and to Alum alone, while at the same time reducing their systemic reactogenicity. In fact, while *il-6* expression was high in mice immunized with the free SMIPs, levels comparable to Alum alone were induced by the same compound adsorbed to Alum [Wu *et al.*, 2014]. Excluding from these major differences, gene expression analysis and FACS staining showed that a mixed polarization phenotype was elicited from both SMIPs either Alum-formulated or in their soluble forms, with up-regulation of M2 marker genes as well as pro-inflammatory M1-typical citokines. As previously said, M1 and M2 cells are thought to have antagonistic roles in the immune response, with M1 macrophages being involved in the inflammatory response and M2 macrophages serving to limit excessive Nitric Oxide production and support healing [Mills, 2012]. The production of Nitric Oxide (NO) by Nitric Oxide Synthase 2 (NOS2) from arginine is a key element of the innate immune response as NO is toxic to many pathogens and important during an inflammatory response. Arginine is the sole amino acid substrate for NO production and notably host macrophages are often divided into two classes, namely M1 and M2, based on the alternative expression of ARG1 and NOS2. The ability of these vaccine adjuvants to elicit the expression of both ARG1 and NOS2 on macrophages at different time-points could play an important beneficial role in the immune response to a putative SMIP-containing vaccine, creating an inflammatory-responsive environment within few hours from injection that could favor vaccine antigen uptake and presentation while being able to restore the system to healing-homeostatic conditions at later time-points. Thus, this SMIPs-induced mixed M1/M2 phenotype could stimulate different immune pathways and therefore induce different types of Th response, likely increasing the breadth of protection of the vaccine.

Earlier works showed that adjuvants induce immunologic memory for vaccine-antigens through local activation of the innate immune system [Coffman *et al.*, 2010]. A deeper knowledge of the specific effects of adjuvants on the initiation of the innate immune

response directly at the site of injection could be very helpful for the identification of efficient adjuvants and to rationally design new and better ones. Even though muscles constitute the most relevant site of injection for most human vaccine, cellular infiltration and activation of single innate cell types following vaccination-induced immune activation, inflammation or damage have been very poorly characterized. In this study, cell recruitment events induced by Alum-SMIP.7-10, Alum and SMIP.7-10 alone into mouse quadriceps were analyzed in detail up-to three days post-injection. As described by O'Hagan *et al.* [2012] and Calabrò *et al.* [2011] for MF59, a key element of the mechanism of action of adjuvants at the administration site is the creation of a transient 'immunocompetent' local environment at the injection site, resulting in the recruitment of crucial immune cells for antigen and adjuvant uptake and transport to the local lymph nodes, where the immune response is triggered. In this study, the kinetics and the prevelence of the single innate cells populations differed among the three adjuvants tested; however, at whatever time-point analyzed, all findings strongly indicated that Alum-SMIP.7-10 target simultaneously a wide variety of immune cells; most, if not all, cells that infiltrated the adjuvant injection site were CD11b positive, with a great part of them being infiltrating monocytes differentiating to macrophages. Recruitment of these cells likely play a critical role for immune enhancement of the administered vaccine since one of macrophages main function is to modulate the adaptive immune response through antigen processing and presentation. Of note, also neutrophils and to a different extent and timing, eosinophils, were highly recruited at the site of injection. Neutrophils and monocytes/macrophages have been shown to work in concert as inducers and effectors of adaptive immunity [Silva, 2010]. Several publications have reported that granulocytes may serve as antigen-vehicles to the lymphatics under certain conditions [Mack *et al.*, 2005; Wang *et al.*, 2007; Sokol *et al.*, 2008], possibly helping to initiate adaptive immune responses in different ways, either by releasing important cytokines that attract monocytes and DCs and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state [Bennouna *et al.*, 2003] and/or by transporting intracellular pathogens to draining lymph nodes [Abadie *et al.*, 2005], while delivering phagocytosed antigens to professional APCs. That said, it is open to speculation whether monocytes/macrophages are the single cell type among the recruited cells that are essential for the mechanism of adjuvants like Alum-SMIP-7.10 or whether the immune response is orchestrated in such a robust and/or redundant manner that massive reduction in one or several populations among the recruited cells would be compensated for by other cells, therefore not strongly impacting on Alum-SMIP-7.10 adjuvanticity.

In any case, the overall higher number of cells available locally increases the likelihood of interaction between an antigen presenting cell and the antigen and therefore leads to a more efficient transport of Ag to the lymph nodes, which results in better T cell priming. Additionally, Alum-SMIP.7-10 may enhance and accelerate the differentiation of cells towards macrophages and alter their phenotype. Unfortunately, the analysis of macrophages polarization in adjuvant-administered muscles did not allow any conclusion

on activation status of infiltrating immune cells at the injection site. Unexpectedly the molecules involved in antigen presentation like MHCII and co-stimulatory molecules CD80/CD86 (M1 polarization marker) seemed to be downregulated in Alum-SMIP.7-10 injected muscles as compared to PBS control; this effect was even more evident at later time-points. However, instead of such an apparent M2-polarization, this finding might be based on the constantly evolving cell composition of the injection site with newly infiltrating cells ("not yet polarized"), cells that already got activated and prepare for migration to draining lymph nodes ("various states of activation and polarization") as well as cells destined for clean-up and tissue remodeling ("M2 cells"). Alternatively, it is not possible to exclude that this contradictory result could be also an artefact of the FACS analysis, related to the on-going monocyte-macrophage differentiation that is happening over the time, hereby diluting the macrophage population (already expressing a certain amount of CD80/86 and MHCII) with the newly recruited monocytes that express naturally much less of these markers and upregulate them during differentiation towards macrophages. This could explain why Alum-SMIP.7-10 group, which had the highest recruitment numbers, here appeared to have the lowest markers expression.

Once assessed which are the immune cells involved in the first response to an Alum-SMIP.7-10-containing vaccine, the ability of this adjuvants to increase the immunogenicity of the candidate *SslE E. coli* vaccine antigen was evaluated. The *sslE* encoding gene is widely distributed in the *E. coli* phylogeny, with a higher presence in intestinal and extraintestinal pathogenic isolates compared to commensal isolates; functionally, *SslE* is a zinc-metallo-peptidase involved in mucins degradation; such mucinase activity plays an essential role in *E. coli* colonization and virulence; *SslE* already showed to be protective against other ExP*Ec* models as well as against InP*Ec*s. Yet, very little is known about the mechanism behind *SslE* protective efficacy.
In this work, Alum-SMIP.7-10 was able to greatly increase overall *E. coli SslE* purified antigen immunogenicity, as compared to the immunization with the antigen alone or adjuvanted with Alum. Moreover, while Alum induced a Th2-biased response, Alum-SMIP.7-10 induced a Th1 prevalent response. Release of Th1 cytokines from T helper cells, in turn, was responsible for IgG2a and IgG2b antibody switching. Among IgG subclasses, IgG2a and IgG2b are generally considered to be the most potent for activating antimicrobial effector responses since their *Fc* regions mediate many effector functions, such as Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC), which ultimately lead to an increase in bactericidal activity; Similarly, Alum-SMIP.7-10 adjuvant was shown to enhance immune responses to glycoconjugate vaccines with significant increase in the frequency of antigen-specific Th1 and decrease in Th2 cells [Buonsanti *et al.*, 2016]. Recently, Vo *et al.* [2017] described an important effect of Alum-SMIP.7-10 on the generation of the B cell memory compartment, showing that this adjuvant is able to induce the recruitment of naïve antigen-specific B cells within the draining LN to help sustaining the germinal center reaction. In the perspective of an

effective *E. coli* potential vaccine, beside the Th1-oriented T cell response, in this work Alum-SMIP.7-10-*SslE* formulation showed also to induce the production of IL-17 from antigen-specific T helper cells, (namely Th17 cells) that contributes in activating antimicrobial activities of neutrophils and macrophages [Isailovic *et al.*, 2015; Mancini *et al.*, 2016].

Finally, in the contest of vaccination and induction of a protective immune response, antigen presentation step is crucial since it allows for the specificity of adaptive immunity. Antigen-presenting cells are vital for effective adaptive immune response, as the functioning of both cytotoxic and helper T cells is dependent on APCs. The latter are normally distinguished between professional and non-professional; macrophages are considered part of the former category. However, the APC involved in activating T cells is usually considered to be a dendritic cell while macrophages contribution in antigen-presentation and T cell priming has been less described. As a conclusive experiment for this thesis, the objective was to understand the effective role of macrophages in *SslE* antigen-presentation and if SMIPs addition could somehow enhance this process. Although inducing similar polarization phenotypes on macrophages *in vitro*, SMIP.2-7 and SMIP.7-10 have distinct molecular targets, namely TLR2 and TLR7, which could affect antigen presentation in different ways. SMIPs-pretreated Bone Marrow Derived Macrophages (BMDMs) and *in vitro SslE*-loaded were used as "Trojan Horse" for the antigen into naïve recipient mice. Adoptively-transferred cells were successfully able to present the antigen, being able to greatly increase *SslE* immunogenicity by promoting both a systemic and humoral response even after one sole immunization. SMIPs pretreatment stimulated up-regulation of co-stimulatory molecules that are important for APC-induced T cell activation and overall increased antigen-specific CD4 T cell expansion above the level reached by *SslE*-loaded and SMIP-untreated macrophages. Moreover, although both SMIP.2-7 and SMIP.7-10 were able to polarize macrophages towards the M1 pro-inflammatory phenotype, the two TLRs agonists stimulated T cells responses with different flavors; with similar and promising *SslE*-specific Th17 cells frequencies, TLR2 agonist promoted a prevalence of Th2-polarized antigen-specific T cells whereas TLR7 elicited a predominant Th1 activation type. CD4 Th1 cells secrete IL-2, IFN-γ and TNF-α, contributing to the elimination of intracellular pathogens directly and indirectly through macrophage activation and support to CD8 T-cell differentiation. By contrast, CD4 Th2 cells mainly secrete IL-4, IL-5, IL-6, IL-10, and IL-13, exerting direct antimicrobial functions against extracellular pathogens (helminths and parasites) and essentially providing support to B lymphocytes. However, although providing protection in different ways towards specific targets, both Th1 and Th2 cells support B-cell activation and differentiation during extra-follicular responses. It could be though very interesting to evaluate which SMIP is able to induce and to what extent, isotype switching, that is usually very important to assess vaccine efficacy. Besides this difference, both SMIPs were able to induce similar and *SslE*-specific Th17 cells frequencies. Of note, newborn and infants normally display an impaired

Th1 response, that is likely responsible for the lower response to vaccines and the increased susceptibility to infections in this age [Levy, 2007]; therefore, although these results are very preliminary, it could be thus conceivable to further investigate on the possibility to formulate *SslE E. coli* antigen either with SMIP.2-7 or SMIP.7-10 (plus Alum) depending to the target population to which the vaccine will be administered.

Chapter V
Concluding Remarks

The ultimate goal of vaccination is to generate protection against disease causing pathogens. Protective immunity against different pathogens requires different immune responses that can be generated by using appropriate vaccine adjuvants. Therefore, a detailed knowledge of the mechanisms of action of adjuvants is very important in the rational design of vaccines. In recent years, considerable advances have been made in understanding the mechanisms of action of various adjuvants, particularly the activation of innate immunity via various mechanisms. The future of vaccine adjuvant research is heading toward developing novel combination adjuvants that consist primarily of PRRs agonists and particulate adjuvants. While combining different adjuvants results in potent formulation that can enhance the quality and quantity of immune response against vaccine antigens, adjuvant combinations may also have more complex mechanisms of action. Safety remains the major concern when it comes to adjuvant approval for human use. Detailed understanding of the mechanisms of action of adjuvants will provide some insight into their safety.

Overall, the findings of this thesis emphasize that adjuvants affect not only APCs, but also the other cells at the injection site, leading to a rapid selective cellular recruitment that have a strong impact on the stimulation and the success of the following adaptive immune response. In the perspective of the urgent need for development of a broadly protective vaccine against pathogenic *E. coli* strains, it was shown that a parenteral immunization using Alum-SMIP.7-10 adjuvant is able greatly increase the immunogenicity of the candidate antigen *SslE*. That said, it is coming to knowledge that although widespread and protective against different *E. coli* pathovars, *SslE* does not cover all known pathogenic strains. A recent study has identified antigen *YncE*, present in >99% of all *E. coli* genomes available, as potential vaccine candidate, showing already protection against a bacteremia model of infection and being recognized by antibodies present in the sera of convalescent urosepsis patients [Moriel *et al.*, 2016]. The idea of a multi-component broad-spectrum vaccine including candidates such as *SslE* and *YncE* formulated with Alum-SMIP.7-10 definitely would need attentive consideration in future *E. coli* vaccine research strategies.

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