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Dynamics of APC recruitment at the site of injection following injection of vaccine adjuvants

Susan van Aalst, Irene Stephanie Ludwig, Peter Johannes Sylvester van Kooten, Ruurd van der Zee, Willem van Eden, Femke Broere *

Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

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ABSTRACT

Vaccines often contain adjuvants to strengthen the response to the vaccine antigen. However, their modes of action at the site of injection (SOI) are poorly understood. Therefore, we assessed the local effects of adjuvant on the innate immune system in mice. We investigated the safe, widely used adjuvants MF59 and aluminum hydroxide (alum), as well as trehalose-6,6′-dibehenate (TDB), Complete Freund's Adjuvant (CFA) and the Toll-Like-Receptor-ligands lipopolysaccharide (LPS) and Pam3CysSerLys4 (Pam_3CSK_4). We assessed muscle immune cell infiltration after adjuvant injection and observed 16 h post immunization (hpi) an increased influx with CFA, MF59 and TDB, but not with alum, LPS or Pam_3CSK_4. An elevated influx with the latter three became visible only 72 hpi. Contribution of granulocytes, macrophages and dendritic cells to the influx differed per adjuvant and in time. Adjuvants generally induced a local pro-inflammatory micro-milieu that was transient except for CFA and TDB. The gene expression of CXCL-1, CCL-2 and CCL-5, involved in recruitment of immune cells, varied per adjuvant and corresponded grossly with the observed influx of granulocytes and monocytes/macrophages. Muscles injected with CFA or MF59 (when co-injected with peptide) resulted in APC *ex vivo* capable to induce proliferation of peptide-specific T-cells. By adding *in vitro* an excess of peptide to the APC/T cell co-cultures, we observed an adjuvant-enhanced co-stimulation or antigen presentation by APC after CFA- but not MF59-injection. After TDB-injection this effect was observed only at 72 hpi, but not 24 hpi. Thus the cellular influx profile and the local cytokine and chemokine micro-milieu in the muscle were strongly influenced by the type of adjuvant. Additionally, the capacity of muscle APC to load and present antigen was affected by the adjuvant. These findings may assist the development of novel adjuvanted vaccines in a more rational manner.

1. Introduction

Live attenuated and inactivated whole cell vaccines play an important role in the prevention of many life-threatening infectious diseases but several diseases are still in need of effective vaccines [1]. New developments in vaccine design shift towards safe, though sometimes less immunogenic subunit and synthetic antigens (reviewed in [2,3]). Therefore, the majority of current vaccines require adjuvants to increase immunogenicity. Adjuvants can be categorized into mineral salts, oil-in-water or water-in-oil emulsions, microbial products or combinations thereof [4]. To date, only a limited number of adjuvants is used such as alum (mineral salt), MF59 and AS03 (emulsions) and AS04 (alum with microbial product) [1,5].

Most adjuvants available were developed empirically and their mode of action is only partly elucidated. Earlier work shows that adjuvants exert their effect on the innate immune system [6–12]. The incomplete knowledge on adjuvants' mode of action warrants more research into their effect on innate immune responses at the site of injection (SOI).

In general, successful induction of immunologic memory for vaccine-antigens starts with local activation of the innate immune system. After intramuscular (i.m.) injection, muscle-resident

Abbreviations: APC, antigen presenting cell; CFA, Complete Freunds' Adjuvant; CFSE, 5,6-carboxy-succinimidyl-fluoresceine-ester; CL, contralateral; DC, dendritic cell; DDA, dimethyldioctadecylammonium bromide; dLN, draining lymph node; hPG, human proteoglycan; hpi, hours post immunization; i.m., intramuscular; IL, interleukin; LPS, lipopolysaccharide; MPL, monophosphoryl Lipid A; PAM, Pam_3CSK_4; SOI, site of injection; TDB, trehalose-6,6-dibehenate; TDM, trehalose 6,6′-dimycolate; TNF-α, tumor necrosis factor alpha.

* Corresponding author.
E-mail addresses: s.vanaalst@uu.nl (S. van Aalst), i.s.ludwig@uu.nl (I.S. Ludwig), p.j.s.vankooten@uu.nl (P.J.S. van Kooten), r.vanderzee@uu.nl (R. van der Zee), w.vaneden@uu.nl (W. van Eden), f.broere@uu.nl (F. Broere).

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(immune) cells are activated and produce pro-inflammatory cytokines and chemokines, resulting in recruitment of innate immune cells. Subsequently, antigen presenting cells (APC) and phagocytes take up antigen and transport it to the draining lymph node (dLN) to activate the adaptive response [13].

The squalene-based adjuvants MF59 and AS03 and aluminum-containing adjuvants are thought to ensure effective antigen uptake and presentation by APC [11,14–16] without the need of a depot [16–18], and to create a local immunostimulatory environment [7,9,16].

Complete Freund's Adjuvant (CFA), paraffin oil with heat-killed mycobacteria, is widely used in animal models. The local effect of CFA and the new adjuvant formulation CAF01 – containing trehalose-6,6′-dibehenate (TDB), the synthetic, detoxified form of CFA-derived glycolipid trehalose-6,6′-dimycolate (TDM) – is surprisingly understudied [19–21]. CFA has a depot function which ensures prolonged local antigen availability [21], and contains many immunomodulatory molecules (like TDM) that can induce APC maturation and create a pro-inflammatory environment, resulting in a chemokine-driven influx of innate cells [21,22].

A direct comparison of the local effects of MF59, TDB, CFA and alum in the muscle has not yet been made. Therefore, we compared their effects (and those of TLR-agonists Pam_3CSK_4 and LPS) on the local innate response. We studied the recruitment of immune cells in number, their phenotype and function in the muscle at the SOI early in the immune response (16–72 hpi). More knowledge of the effects of adjuvants on the initiation of the innate immune response can be used for the rational development of adjuvanted vaccines.

2. Material and methods

2.1. Mice

BALB/c mice (female; 8–10 weeks) were obtained from Charles River Laboratories and human proteoglycan (hPG) specific TCR-5/4E8-transgenic mice [23] were bred at the Central Animal Laboratory of Utrecht University, The Netherlands. Mice were kept under standard conditions and received water and food *ad libitum*. Experiments were approved by the Utrecht University Animal Experiments Committee.

2.2. Adjuvant injections

Mice were immunized with an i.m. injection in the quadriceps with 50 μl adjuvant in PBS. We chose to take the untreated CL muscle of a PBS-injected animal as control to be able to monitor (potential) effects induced by the injection as such. MF59-adjuvant® (Novartis Vaccines) and CFA (Difco, 1 mg/ml) were mixed with equal volumes of PBS. Aluminum hydroxide (alum; Alhydrogel, Invivogen) and LPS (*E. coli* 0127:B8 L4516, Sigma) were prepared as 0.5 mg/ml solutions and PAM_3CSK_4 (VacciGrade™, Invivogen) as 0.2 mg/ml in PBS. TDB (VacciGrade™, Invivogen) was prepared as a suspension of 1 mg/ml in PBS containing 2% DMSO and sonicated before use. Control mice were injected with PBS or PBS supplemented with 2% DMSO as vehicle control.

The doses MF59 and alum received by the mice were equal to 1/10th of the human adult dose of Fluad® and Engerix B® containing MF59 and alum respectively. This dose is considered a good representation of the human dose. Antigens are generally emulsified in CFA in the 1:1 (V:V) ratio [22,24], therefore we chose to mix CFA with PBS in this ratio before administration. Dosage of the TLR-ligands LPS and Pam_3CSK_4 and Mincle agonist TDB were the most commonly used dosages in literature; LPS [25], Pam_3CSK_4 [26] and TDB (often in DDA formulation) [19,27]. For the co-culture experiments, adjuvants were supplemented with 100 μg hPG peptide. This peptide (^{70}ATEGRVRVNSAYQDK^{84}; Genscript) is recognized by the TCR-5/4E8-Tg T-cells [23].

2.3. Single cell suspensions muscle

Quadriceps muscle of both injected and CL muscle were harvested, tendons were removed and muscles were digested for 30 min at 37 °C in 3 mL IMDM containing 0.05% type II collagenase solution (Worthington Biochemicals), 10 μg/ml DNAseI (Boehringer Mannheim) and 0.5% BSA (Sigma). The enzymatic reaction was stopped with excess medium and cells were washed and filtered through a 70 μm nylon mesh (BD-Biosciences).

2.4. Co-culture

TCR-5/4E8-Tg $CD4^+$ T-cells were isolated from naïve TCR-5/4E8-Tg mice and labeled with 0.5 μM 5,6-carboxy-succinimidyl-fluores ceine-ester (CFSE) as described [28]. Single cell suspensions from muscle, harvested 24 or 72 hpi, were labeled with CellTrace Violet (Invitrogen) for 10 min with 5 μM CellTrace Violet in PBS according to manufacturer's instructions.

CFSE-labeled TCR-5/4E8-Tg $CD4^+$ T-cells were co-cultured for 72 h at 37 °C, 5% CO_2 with CellTrace-labeled cells from muscle (1/4th muscle/2×10^5 $CD4^+$ T-cells) with or without addition of peptide (10 μg/ml). Cells were cultured in IMDM containing FBS (Lonza, 5%), β-mercaptoethanol (Gibco; 5×10^{-5} M), penicillin (Gibco; 100 units/ml) and streptomycin (Gibco; 100 μg/ml). 1/4th muscle corresponds with CD11b + cell numbers at 24 h/72 hpi of ~5,000/5000 (PBS), ~5,000/33,000 (alum), ~40,000/50,000 (MF59), ~45,000/125,000 (CFA), ~200,000/170,000 (TDB), ~14,000/33,000 (LPS), 25,000/50,000 (PAM).

2.5. Cytokine production

Supernatants of the muscle cell-$CD4^+$ T cell co-cultures were collected after 72 h for multiplex cytokine assays respectively IFN-γ, IL-5 and IL-17, using the Magpix (Luminex XMAP) system according the manufacturer's instructions. Briefly, supernatant (or a dilution hereof) together with magnetic capture beads for the respective cytokines were added to polystyrene, black, 96 flat bottom plates, (Greiner bio-one, 655096). Subsequently, biotin-conjugated detection antibodies and Streptavidin-PE (BD Bioscience) incubation. The antibody pairs (coat:detect) used were AN-18:XMG1.2, TRFK5:TRFK4 and TC11-18H10:TC11-8H4.1 for IFN-γ, IL-5 and IL-17 respectively. The concentrations of cytokines in the tested samples were calculated using standard curves and the MFI data was analyzed using a 5-parameter logistic method (xPONENT software, Luminex, Austin, USA).

2.6. Flow cytometry analysis

Cells were stained with the monoclonal antibodies F4/80-FITC (BM8; Biolegend), GR1-PE (RB6-8C5; Immunotools), I-A/I-E-HorizonV450 (M5/114.15.2), CD11b-PerCPCy5.5 (M1/70), and CD11c-APC (N418) (eBioscience). Subsequently, cells were measured on a FACSCanto II Flow cytometer (BDBiosciences). Analysis was performed with FlowJo v7.6.5 (Tree Star). Proliferation of the cells after co-culture was gated as follows: single cells in the lymphocyte gate were gated on $CD4^+$ cells. Subsequently, to exclude potentially interfering cells from muscle we excluded cells that were positive for Cell trace Violet. In this Cell trace $Violet^-$ $CD4^+$ cell population, we determined proliferation as%divided $CFSE^+CD4^+$ cells.

2.7. Quantitative real time PCR

Single cell suspensions of muscle were resuspended in TRIzol® Reagent (Ambion) for total mRNA extraction. The acid-guanidi

nium-thiocyanate-phenol-chloroform extraction protocol was performed according to manufacturer's instructions. Transcription of mRNA to cDNA was performed with the iScript™ cDNa Synthesis Kit (Bio-Rad) according to manufacturer's instructions, using a T100™ Thermal Cycler (Bio-Rad).

Quantitative RT-PCR (3 min at 95 °C, followed by 40 cycles of 20 s. at 95 °C and 45 s. at 59.5 °C) was performed in a MyiQ iCycler (Bio-Rad) in 25 µl using iQ™ SYBR® Green Supermix (Bio-Rad) with 0.25 µM final primer concentrations (Table 1). All primers were designed and tested in house, except for Cxcl-1 [29]. Relative amounts of mRNA were determined using the Pfaffl-method [30] with HPRT and GAPDH as reference genes.

2.8. Statistical analysis

One-way Anova (two-tailed) with post hoc Tukey's Test was performed using Prism v6.05 (Graphpad). When indicated, data was log-transformed prior to analysis. Differences were considered significant at $p < 0.05$.

3. Results

3.1. CD11b$^+$ cellular influx after intramuscular injection with adjuvant

To study cells involved in the response to adjuvants we determined the recruitment of innate cells to the SOI in adjuvant-injected muscle up to 72 hpi. Most of the recruited cells were found to be CD11b$^+$ (Supplementary Fig. 4a). Injection with TDB, CFA and MF59 induced a significant CD11b$^+$ cell influx early post immunization ($p < 0.001$; Fig. 1a). CFA injection induced a continuous increase reaching 20 times more CD11b$^+$ cells at 72 hpi than PBS, whereas TDB led to a CD11b + cell peak at 24 hpi and remained high with 38 times more CD11b + cells at 72 hpi than PBS. After the relatively high CD11b + influx at 16 hpi for MF59-injection, a moderate increase was observed, but much lower than for CFA or TDB and at 72 hpi similar for Pam_3CSK_4. In contrast, injection with alum or TLR-ligand LPS and showed CD11b$^+$ cell numbers similar to PBS for the first 24 hpi, before they increased at 72 hpi (4, and 8-fold, respectively). Injection with PBS or 2% DMSO in PBS induced low influxes that were slightly higher than in the CL ($20 \times 10^3 \pm 13 \times 10^3$ CD11b$^+$ cells (SOI) versus $5 \times 10^3 \pm 3.5 \times 10^3$ (CL); Fig. 1b). None of the adjuvants caused a CD11b$^+$ cell influx in the CL.

Table 1
Primers used in RT-qPCR. Forward and reverse primers (indicated in the 5′ to 3′ direction) used in the RT-qPCR performed on immune cells from muscle. Shown are the primer pairs for the genes HPRT, GAPDH, IL-1β, TNF-α, CCL-2, CCL-5, CXCL-1.

Primer	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
HPRT	CTG GTG AAA AGG ACC TCT CG	TGA AGT ACT CAT TAT AGT CAA GGG CA
GAPDH	CAA CTC ACT CAA GAT TGT CAG CAA	GGC ATG GAC TGT GGT CAT GA
IL-1β	CAA CCA ACA AGT GAT ATT CTC CAT G	GAT CCA CAC TCT CCA GCT GCA
TNF-α	CCC TCA CAC TCA GAT CAT CTT CT	GCT ACG ACG TGG GCT ACA G
CXCL-1	CTG CAC CCA AAC CGA AGT C	AGC TTC AGG GTC AAG GCA AG
CCL-5	AGC AGC AAG TGC TCC AAT CT	AAG CGA TGA CAG GGA AGC TA
CCL-2	TTA AAA ACC TGG ATC GGA ACC AA	GCA TTA GCT TCA GAT TTA CGG GT

3.2. Granulocytes, macrophages and dendritic cells in cellular influx

To characterize cells in the influx, we stained for granulocytes (CD11b$^+$GR1$^+$), macrophages (CD11b$^+$F4/80$^+$) and dendritic cells (DC; CD11c$^+$MCHII$^+$).

At 16 hpi CFA, TDB and MF59, but not LPS, Pam_3CSK_4 or alum, resulted in increased numbers of granulocytes, mainly neutrophils (CD11b$^+$Ly6G$^+$; data not shown), and macrophages in the muscle (Fig. 2a, b; $p < 0.001$). With CFA, granulocytes and macrophages peaked at 72 hpi, whereas with TDB influx peaked at 24 hpi. MF59, LPS and Pam_3CSK_4 induced maximal granulocyte numbers at 24 hpi. However, granulocyte numbers at 24 hpi differed per adjuvant as did their subsequent decline, with a rapid decline in MF59 and LPS injected muscle, but not in TDB or Pam_3CSK_4 injected muscle. From 16 hpi onwards, a continuous increase of macrophages was observed for MF59 and PAM_3CSK_4 but not LPS. Alum resulted in minor increases in granulocyte and macrophage numbers only at 72 hpi ($p < 0.0003$).

CFA, TDB, MF59 and, at a later stage, also alum resulted in higher DC numbers compared to PBS (Fig. 2c; 24 hpi). TDB resulted in an early increase of DC numbers at 16 hpi that peaked at 24 hpi and declined again at 72 hpi, whereas MF59 and CFA induced an increase over time, with cell numbers similar to TDB at 72 hpi. Alum resulted in 8×10^3 DC 72 hpi, two times more than PBS. In contrast, the numbers of DC in the influx induced by LPS (significant) and PAM_3CSK_4 (trend) were lower than after PBS (Fig. 2c).

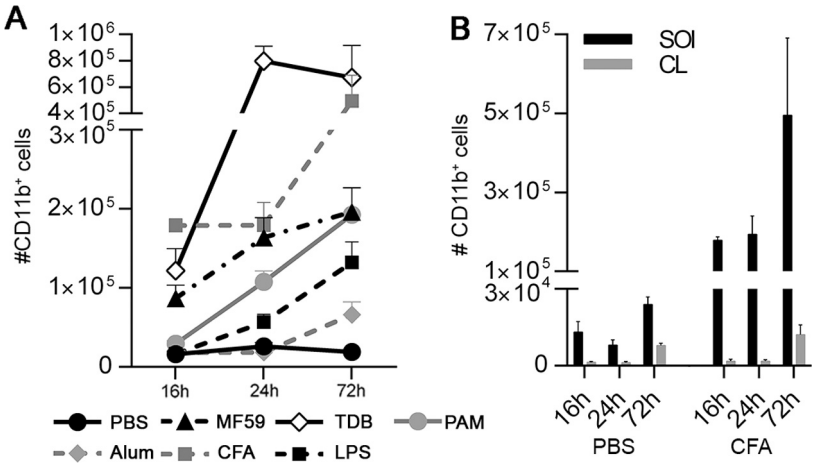

Fig. 1. Differential CD11b$^+$ cell influx after i.m. adjuvant injection. Flow cytometric analysis of CD11b$^+$ cell numbers in muscle tissue after adjuvant injection. Mice were i.m. (M. quadriceps) injected with alum, MF59, CFA, TDB, LPS or Pam_3CSK_4. Mock injected animals received PBS. Influx after adjuvant injection is shown at time points 16, 24 and 72 hpi. (A) Absolute numbers of CD11b$^+$ cells in the injected muscle for all adjuvant and (B) s in the treated (SOI) or untreated (CL) muscle for PBS and CFA are depicted. Shown are means ± SEM of 3–13 animals per data point. Significant differences between groups are indicated in Supplementary Fig. 1.

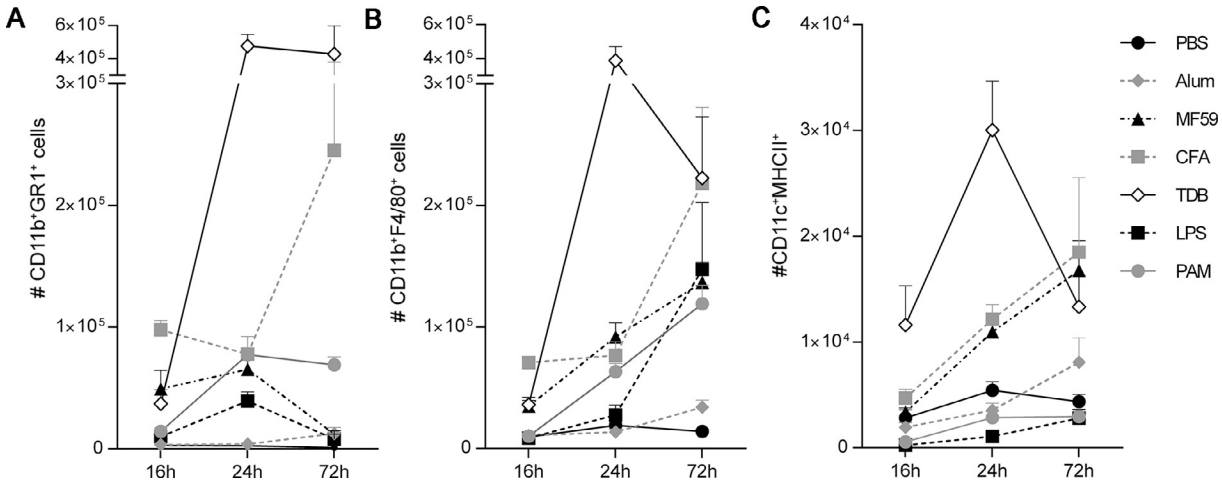

Fig. 2. Cell influx profiles of granulocytes, macrophages and DC after intramuscular adjuvant injection. Flow cytometric analysis of (A) granulocytes ($CD11b^+GR1^+$), (B) macrophages ($CD11b^+F4/80^+$) and (C) DC ($CD11c^+MCHII^+$) in muscle tissue after adjuvant injection. Mice were i.m. (m. quadriceps) injected with alum, MF59, CFA, TDB or with one of the TLR-ligands (LPS, Pam_3CSK_4). Mock injected animals received PBS. Influx is shown at time points 16, 24 and 72 hpi. (A-C) Absolute numbers of cells in the adjuvant-treated and PBS muscle are depicted over time. Shown are means ± SEM of 3–13 animals per data point. Significant differences between groups are indicated in Supplementary Fig. 2. The change in cellular composition over time per individual adjuvant is indicated in Supplementary Fig. 5.

All DC found in the muscle were > 90% positive for the CD11b + marker (Supplementary Fig. 4b–d).

Summarizing, most adjuvants induced a fast, but mostly transient, influx of granulocytes to the SOI, followed by recruitment of macrophages and DC. However, the cell numbers and kinetics of the influx differed per treatment.

3.3. Intramuscular adjuvant-induced cytokine and chemokine profiles

After intramuscular injection we analyzed the local gene expression of pro-inflammatory cytokines and chemokines (Fig. 3). All adjuvants except alum induced early upregulation of the genes interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) compared to PBS, which was most pronounced for CFA and MF59. Only TDB and CFA induced a prolonged increased expression of IL-1β and TNF-α at 72 hpi.

At 16 hpi gene expression of CXCL-1 was upregulated in all cases, except for alum, and most obvious for CFA. The effect was transient since levels returned to PBS values at 72 hpi for all adjuvants. The effect of adjuvant on CCL-5 was more diverse. At 16 hpi CCL-5 was upregulated after CFA, TDB, LPS and Pam_3CSK_4. CFA and TDB, but not the TLR-ligands, induced increased expression up to 72 hpi. MF59 had no effect on local CCL-5 gene expression, whereas alum induced higher levels only at 72 hpi. The gene expression of CCL-2 at 16 hpi was upregulated after MF59 and TDB and tended to be higher, though not significantly, after CFA, LPS and Pam_3CSK_4. CCL-2 gene upregulation after TDB was short, whereas its expression after CFA, MF59, LPS and Pam_3CSK_4 was high up to 72 hpi.

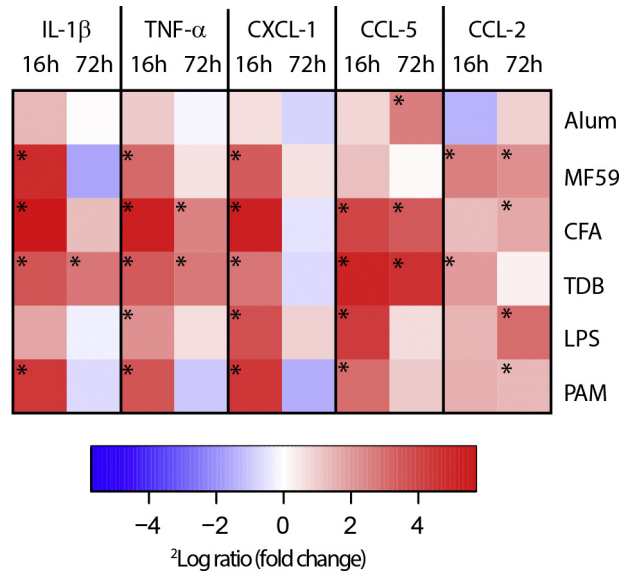

Fig. 3. Relative gene expression of IL-1β, TNF-α, CXCL-1, CCL-5, CCL-2 in the muscle after intramuscular adjuvant injection. Quantitative RT-PCR for gene expression of cytokines IL1-β and TNF-α and chemokines CXLC1, CCL5, CCL2 in muscle tissue 16 and 72 h post adjuvant injection. Mice were i.m. (m. quadriceps) injected with alum, MF59, CFA, TDB or with one of the TLR-ligands (LPS, Pam_3CSK_4). Mock injected animals received PBS. The relative gene expression was determined with the Pfaffl method and as calibrators, Hprt and Gapdh were used. Data is expressed as fold change relative to PBS and is 2-log transformed. Shown are means ± SEM of 3–13 animals per data point. Significance was determined using one-way Anova (two-tailed) with Tukey's correction. Asterisks indicate significant differences in expression compared to PBS ($^*P < 0.05$).

3.4. Antigen presentation and activation of muscle-APC

Muscle derived cells of mice injected 24 h or 72 h prior to harvest with hPG peptide and adjuvant, were co-cultured for 3 days with TCR-5/4E8-Tg $CD4^+$ T-cells. The cultures were *in vitro* supplemented with cognate peptide to assess the activation status (co-stimulatory ability), and hence the capability to activate cognate T-cells, of (recruited) muscle APC.

In the presence of *in vitro* added peptide, stimulation of TCR-5/4E8-Tg $CD4^+$ T-cells with control APC harvested 24 hpi and 72 hpi from the PBS-injected muscle induced considerable proliferation (Fig. 4a). At 24 hpi, this proliferation was further enhanced with APC derived from muscle injected with peptide plus CFA (17% net proliferation), but not alum, MF59, TDB, LPS or Pam_3CSK_4 (Fig. 4b). At 72 hpi, besides CFA, also TDB showed an increased proliferation of TCR-5/4E8-Tg $CD4^+$ T-cells (net proliferation of 23% and 31% respectively, compared to 5% for PBS; Fig. 4c). This was not the case for APC in muscle exposed to alum, MF59, LPS or Pam_3CSK_4, which induced proliferations similar to PBS.

To address T-cell differentiation as result of the adjuvants we determined the cytokine production in the co-culture's supernatants (Fig. 4d). All TCR-5/4E8-Tg $CD4^+$ T-cells in culture produced

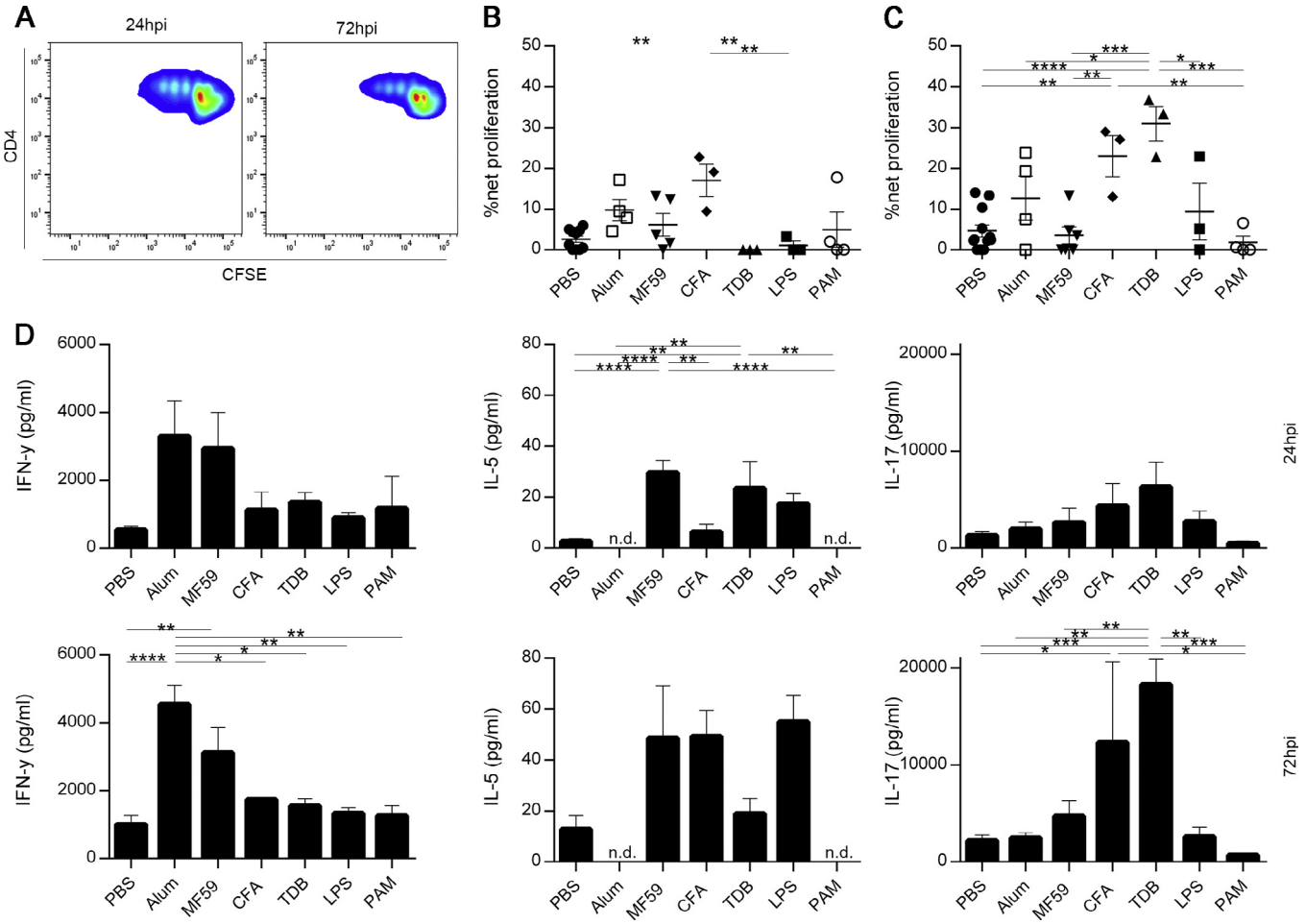

Fig. 4. Co-culture of adjuvant-plus-antigen-treated muscle cells with CFSE-labeled TCR-5/4E8-Tg CD4$^+$ T-cells combined with *in vitro* added peptide. Mice were i.m. (m. quadriceps) injected with 100 μg hPG peptide plus alum, MF59, CFA, TDB or with one of the TLR-ligands (LPS, Pam_3CSK_4). Mock injected animals received hPG peptide in PBS. Muscles were harvested 24 hpi and 72 hpi, collagenase digested and the muscle-digest (1/4th muscle) was co-cultured with CFSE-labeled TCR-5/4E8-Tg CD4$^+$ T-cells for 72 h in the presence of hPG-peptide. CFSE dilution was determined by flow cytometry. (A) Representative plots of CFSE dilution of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture with APC from muscle injected with PBS with *in vitro* added hPG peptide at 24 hpi (left) and 72 hpi (right). (B, C) Net proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture with APC from muscle 24 hpi (B) and 72 hpi (C). (D) Cytokine concentrations (pg/ml) for IFN-γ, IL-5 and IL-17 in supernatant after 72 h of co-culture of T cells together with muscle-cells isolated from muscle 24 h (top) or 72 h (bottom) post immunization and in the presence of 10 μg/ml hPG peptide. We determined as background the average proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells induced by APC from the CL of PBS-injected animals. Thus, net proliferation was determined as (% proliferation induced by adjuvant-affected APC at SOI) – (% proliferation induced by APC from the CL of PBS). The average background proliferation was ~70% and ~60% at 24 hpi and 72 hpi, respectively. Shown are means ± SEM of 3–14 animals per group. Each symbol represents an individual animal. Significance was determined using one-way Anova (two-tailed) with Tukey's correction. Asterisks indicate significant differences in expression ($^*P < 0.05$, $^{**}P < 0.01$).n.d.: not detected.

to some extent IFN-γ and IL-17. IFN-γ production was significantly higher for MF59 and alum and IL-17 production was significantly higher for T-cells in culture with APC derived from TDB and CFA injected animals at 72 hpi. IL-5 production was, although low, higher for MF59 and TDB.

3.5. Antigen capture and presentation by muscle-APC

To investigate whether the observed differences in T-cell proliferation were caused by the activation status and presentation of the APC alone, or also by (*in vivo*) antigen capture in the muscle, we performed the co-culture experiments also directly *ex vivo*, without *in vitro* added cognate peptide.

Muscle APC isolated 24 h after i.m. injection with peptide in combination with CFA or MF59-adjuvant, but not alum, LPS or Pam_3CSK_4, were much more efficient to induce proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture than APC from animals that received hPG peptide in PBS (Fig. 5a, top). On average, CFA and MF59 resulted in 21% and 17% net proliferation compared to 1% for PBS (Fig. 5b). Similarly, APC retrieved 72 hpi from CFA and MF59-injected muscle induced *ex vivo* proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells without added peptide (Fig. 5a, bottom). An average net proliferation of 30% and 20% was found for CFA and MF59, respectively, compared to 1.6% for PBS (Fig. 5c). APC in muscle exposed to alum, LPS or Pam_3CSK_4 induced proliferations similar to PBS at this time point. Within the TDB-treated group we observed large variation at both time points, with net proliferations of 10% and 7% at 24 hpi and 72 hpi respectively, not significantly higher than PBS.

To address T-cell differentiation as result of the adjuvants we determined the cytokine production in the co-culture's supernatants (Fig. 5d). We observed a T_H1/T_H2 mixed profile for MF59 as shown by IFN-γ and IL-5 production, whereas CFA and TDB induce a more T_H1/T_H17 profile for their obvious induction of IL-17 production, besides IFN-γ. LPS, Pam_3CSK_4 and alum induce only minimal cytokine production, corresponding with their low induction of proliferation.

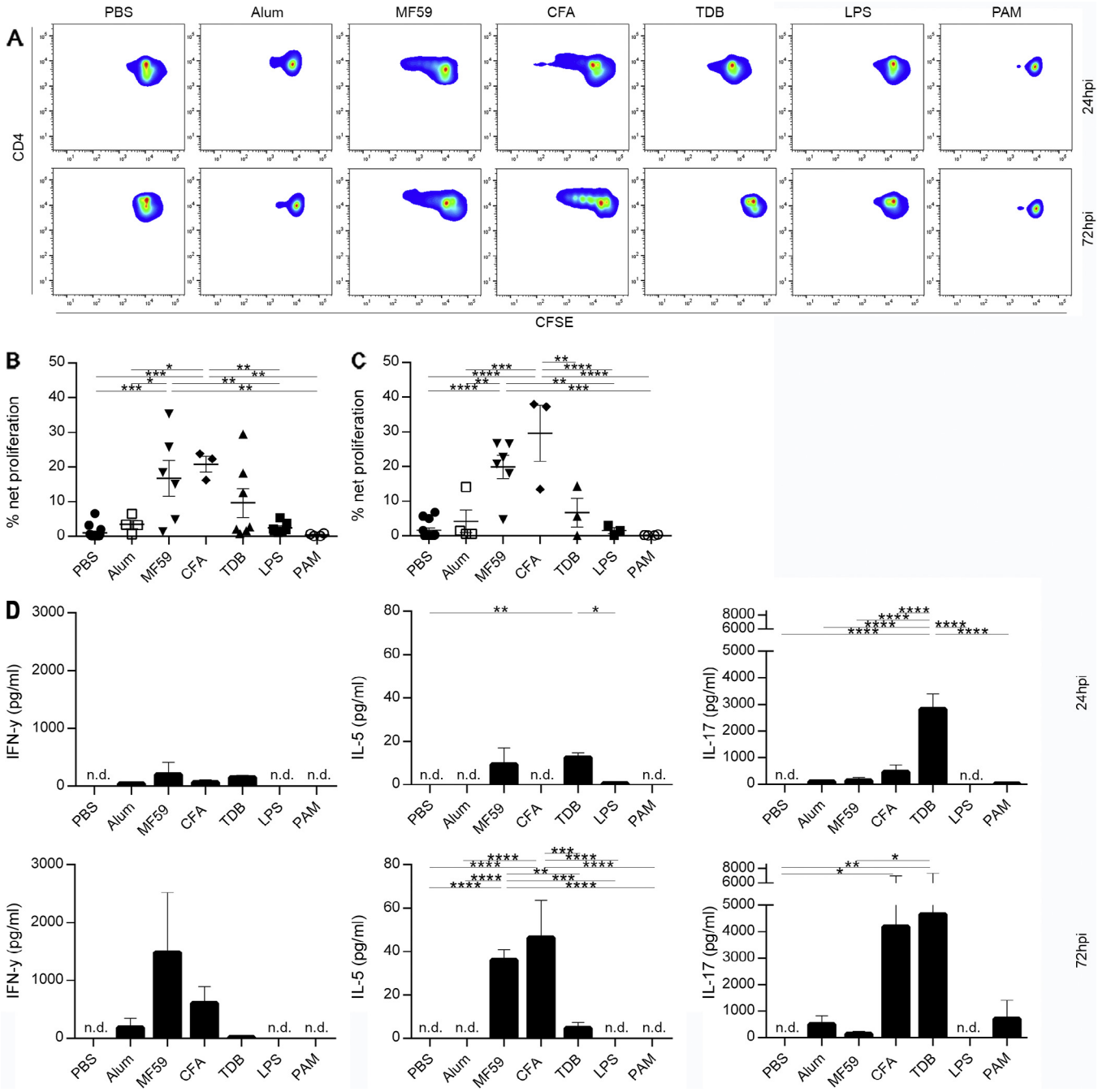

Fig. 5. Co-culture of cells from adjuvant-plus-antigen-injected muscle with CFSE-labeled TCR-5/4E8-Tg CD4$^+$ T-cells. Mice were i.m. (m. quadriceps) injected with 100 μg hPG peptide plus alum, MF59, CFA, TDB or one of the TLR-ligands (LPS, Pam_3CSK_4). Mock injected animals received hPG peptide in PBS. Muscles were harvested 24 hpi and 72 hpi, collagenase digested and cells from the muscle-digest (1/4th muscle) were co-cultured with CFSE-labeled TCR-5/4E8-Tg CD4$^+$ T-cells for 72 h. CFSE dilution was determined by flow cytometry. (A) Representative plots of CFSE dilution of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture with APC from muscle 24 hpi (top) and 72 hpi (bottom). (B, C) Net proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture with APC from muscle 24 hpi (B) and 72 hpi (C). (D) Cytokine concentrations (pg/ml) for IFN-γ, IL-5 and IL-17 in supernatant after 72 h of co-culture of T cells together with muscle-cells isolated from muscle 24 h (top) or 72 h (bottom) post immunization. Net proliferation was determined as described in Fig. 4. The average background proliferation of TCR-5/4E8-Tg CD4$^+$ T-cells in co-culture with APC from CL of PBS was ~20% at 24 hpi and ~30% at 72 hpi. Shown are means ± SEM of 3–12 animals per group. Each symbol represents an individual animal. Significance was determined using one-way Anova (two-tailed) with Tukey's correction. Asterisks indicate significant differences in expression ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). n.d.: not detected.

4. Discussion

The cellular (and molecular) mechanisms of action of many adjuvants are not fully clear. Here, we present a direct comparison of local early responses of the innate immune system after intramuscular injection of several adjuvants.

In the first 72 hpi, local (recruitment) effects of vaccines are largely mediated by the adjuvant and not the antigen [7]. Therefore, we analyzed local cellular recruitment after adjuvant treatment only. The influx observed after all treatments, though very variable in numbers, consisted largely of myeloid cells expressing the integrin marker CD11b$^+$, essential for adhesion and migration across the endothelium or epithelium to infiltrate tissue [31]. Within our set of adjuvants we could distinguish a group of adjuvants that induced an early and high influx of CD11b$^+$ cells (CFA, TDB and MF59) and a group (PAM_3CSK_4, LPS and alum) that induced a more

modest influx from 24 hpi onwards (Fig. 1). This in contrast to studies showing a myeloid ($CD11b^+$) cell influx 24 hpi with alum [7,32]. This difference is likely related to the dose used in our study, which was 3–6 times lower.

Fast recruitment of neutrophilic granulocytes to the muscle together with a reduction in numbers at 72 hpi, was observed for most adjuvants except for CFA and CFA-related TDB, where numbers remained high (Fig. 2a). Neutrophils are essential in the first line of defence against pathogens, but prolonged activation of these cells can result in sustained inflammation and tissue injury (reviewed in [33]). In the present study, CFA-treatment, known to induce severe inflammation [21], attracted significant numbers of neutrophils to the SOI for a longer period of time, whereas the safe and widely used MF59 showed early recruitment of neutrophils followed by a swift clearance in accordance with other reports [7,26].

Similar to other studies [7,32], adjuvant injection induced next to the neutrophil influx, a second influx of other innate cells. CFA, TDB and MF59 successfully recruited macrophages and DC to the SOI (Fig. 2b, c). PAM_3CSK_4, and LPS, although later, ensured influx of predominantly macrophages (Fig. 2b, c). Since these TLR ligands have a high solubility and thus easy systemic distribution, this might have impacted cell recruitment. Previously, it was shown that the LPS-derivative monophosphoryl Lipid A in AS04, a combination-adjuvant with alum, increased DC and inflammatory monocytes 24 hpi in dLN [6]. It is possible that a similar effect was induced in our study, but since we focused on the effects of adjuvant at the injection site, effects in LN were outside our scope.

We found that immune cells are recruited to the muscle, but numbers and cell-types differ per adjuvant. This is most likely induced by distinct cytokines and chemokines in a local adjuvant-specific micro-milieu. Adjuvant generally induced a local pro-inflammatory micro-milieu with a variable expression of IL-1β and TNF-α mRNA in the muscle (Fig. 3). Their upregulation was transient for MF59, LPS and PAM_3CSK4 as noticed earlier for MF59 [9]. In contrast, both CFA and TDB retained a pro-inflammatory profile, consistent with the strong local reactogenicity of CFA [21] and the potent induction of pro-inflammatory cytokines by TDB *in vitro* [34]. All adjuvants, except alum, induced expression of the neutrophil-attracting chemokine CXCL-1 similar to that of IL-1β and TNF-α, but with a more rapid decrease (Fig. 3). This matches the fast, but temporary influx of neutrophils (Fig. 2a). CCL-2 is involved in the attraction of inflammatory monocytes, monocyte-derived macrophages and DC [35]. Upregulation of this gene coincided grossly with an increased influx of monocytes (Supplementary Fig. 3) and macrophages (Fig. 2b). Only TDB showed an obvious influx of monocytes and macrophages without upregulation of CCL-2 at 72 hpi, potentially due to redundant chemokines, e.g. CCL-7 [36].

Essential in vaccine responses is the uptake of antigen by APC and subsequent activation of T-cells. We tested the effect of adjuvant on the antigen presenting capacities of APC attracted to the SOI. Especially CFA and MF59 attracted high numbers of APC (Fig. 2b, c) and, when injected together with a T-cell epitope, this resulted in APC capable to induce *ex vivo* proliferation of T-cells (Fig. 5). To test whether the adjuvant enhanced co-stimulation and/or antigen presentation to antigen-specific T-cells, peptide was added *in vitro* to the co-culture. This enhancing effect was found with the depot-forming CFA but not with MF59 (Fig. 4). The latter suggests that MF59 does not induce more maturation of APC than PBS at the injection site. We confirmed this by *in vivo* expression of CD86 on muscle-DC after MF59 injection, which was equal to PBS (data not shown), consistent with an *in* vitro study that showed that MF59 is mainly involved in the differentiation of monocytes to DC, and not DC maturation [11,37]. Apparently the main effect of MF59 is to recruit APC (Fig. 2) or to enhance antigen uptake *in vivo* (Fig. 5) as described earlier [7,14]. The high response already induced by PBS APC after peptide addition (Fig. 4a) emphasizes that uptake of antigen is the limiting factor in the experiments of Fig. 5.

Remarkably, TDB recruited similar numbers of APC as MF59 or CFA (Fig. 2b, c), but when injected with peptide, did not result in cognate T-cell activating APC (Fig. 5). Potentially, peptide-loaded APC might have migrated already to the dLN, or the APC recruited by TDB did not take up antigen adequately, possibly caused by the large numbers of neutrophils present at the SOI after TDB (Fig. 2a). Yang and colleagues showed that during the first 24 h after immunization neutrophils may inhibit antigen capture and presentation by DC and macrophages [38]. This might explain why 24 h after TDB-injection the muscle-APC supplemented with peptide induced equally high cognate T-cell proliferation as PBS-muscle derived APC, but much higher at 72 hpi (Fig. 4). In the *in vivo* situation, the neutrophils may have inhibited the initial antigen uptake of APC and so their presentation to T-cells. In contrast, the combination adjuvant of TDB in DDA, CAF01, successfully induced cellular T-cell responses in a tuberculosis vaccine [20]. Possibly this is caused by the antigen depot forming capacity of DDA liposomes and/or the DDA enhanced uptake and presentation of antigen at the SOI [39,40].

To address T-cell differentiation as a result of adjuvant, we determined the presence of T_H1, T_H2 and T_H17 cytokine expression in the co-culture's supernatants. CFA and TDB induced a predominantly Th1/Th17 profile, whereas MF59 induced a more Th1/Th2 profile (Fig. 5d). These data correspond with the profiles described in literature for the respective adjuvants [1,27]. The other adjuvants induced no or very limited proliferation and thus the cytokines were below detection limit.

5. Conclusion

In this study we showed that vaccine adjuvants directly and specifically affect the local micro-milieu, differentially attract immune cells and determine the antigen-presenting capacity of APC at the SOI, in this way influencing the immune response to an adjuvanted vaccine. Such understanding of local effects of adjuvants on the innate immune system may help rational vaccine development.

Conflict of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.02.005.

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