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

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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.

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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-fluoresceine-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