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Short Communication

# Cell Immune Response in Mice Skin Stimulated with Different Adjuvants by Intradermal Route

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## Abstract

Adjuvants act in the innate immunity and, when combined to vaccine antigens, can produce a more intense response, improving the antigen presentation, directing the immune system, excellent for new vaccine formulations. This study evaluated the use of the intradermal route and the immune response triggered by a single dose of the adjuvants Aluminum Hydroxide ($Al(OH)_3$), Montanide Pet Gel A (MPGA), Glucopyranosyl Lipid A Stable Emulsion (GLA-SE), and Resiquimod (R-848) in the mice skin. As control mice received sterile saline. MPGA and GLA-SE led to cell recruitment when compared with control group, with intense presence of neutrophils in first 12 hours, replaced by macrophages after 168 hours. R-848 and $Al(OH)_3$ showed similar cell recruitment profiles. Regarding cytokine production, groups that received MPGA and GLA-SE produced high levels of IL-6, TNF-α, and IFN-γ. R-848 and $Al(OH)_3$ groups displayed similar profile of cytokine production only at the first hour. Our results suggest that the intradermal route is efficient inducing immune system activation and GLA-SE was promising adjuvants for a type 1 immune response vaccine.

**Keywords:** Adjuvants; Aluminum hydroxide; Cytokines; Glucopyranosyl Lipid A Stable Emulsion; Immune response; Inflammatory cell infiltrates; Montanide Pet Gel A; Resiquimod

**Abbreviations:** $Al(OH)_3$: Aluminum Hydroxide; Alum: Aluminum Hydroxide; APCs: Antigen Presentation Cells; CEUA: Ethical Committee on the Use of Animals; DCs: Dendritic Cells; DTH: Delayed-type hypersensitivity; GLA-SE: Glucopyranosyl Lipid A Stable Emulsion; HE: Hematoxylin-eosin; MF59: Water-in-oil Squalene-based Emulsion; MPGA: Montanide Pet Gel A; PBMC: Peripheral Blood Mononuclear Cells; R-837: Imiquimod; R-848: Resiquimod; SD: Standard Deviation; TLR4: Toll-Like Receptor 4

## Introduction

Adjuvants are compounds employed in vaccine formulation to improve the effect of co-administered antigens, inducing a stronger, faster, and longer-lasting immune response than generated by the antigen only [1]. Different adjuvants act on the immune system in distinct ways, directly related to their mechanism of action, which induces a diversity of responses in the immune system [2]. Understanding the mechanism of action and cell recruitment kinetics of new adjuvants is essential to the development and improvement of vaccines. Although adjuvants have been employed since the 1920s, only a few are licensed for human use. Aluminum hydroxide (Alum) was the first adjuvant licensed for human use, in 1930 [3]. MF59 (water-in-oil squalene-based emulsion), Imiquimod (R-837), and Resiquimod (R-848) [4], have also been licensed for human use, mostly in Europe. However, as many adjuvants have been discovered empirically, their mechanisms of action and cell recruitment kinetics remain unclear [5].

Glucopyranosyl Lipid A Stable Emulsion (GLA-SE), Montanide Gel, and R-848 are developed adjuvants that have shown great promise in vaccine tests. GLA-SE, used in a vaccine against the Influenza virus, stimulated an increase of humoral and cellular immune response with induction mainly of TNF-α and IFN-γ cytokines [6]. Montanide ISA 720 was evaluated associated with *Leishmania donovani* antigen, inducing a strong Delayed-Type Hypersensitivity (DTH) response, with the production of TNF-α and IFN-γ [7]. R-848 was also able to induce a type 1 immune response, with activation of myeloid and plasmacytoid dendritic cells, lymphocytes, and macrophages [8,9]. However, no data have been published on the time course of the recruitment of innate immune components by these adjuvants. Therefore, we aimed to evaluate the effect of the Montanide Pet Gel A (MPGA), Glucopyranosyl Lipid A Stable Emulsion (GLA-SE), and Resiquimod (R-848) adjuvants in the skin of mice inoculated intradermal route. The time course evaluated spanned from 1 to 336h, fully characterizing the immune response profile stimulated by the adjuvants and allowing a detailed comparison with the classic type II adjuvant, Aluminum hydroxide ($Al(OH)_3$).

## Material and Methods

### Mice and Adjuvants stimulation

This study was approved by the Ethical Committee on the Use of Animals (CEUA) of the Federal University of Ouro Preto (protocol 2011/26). Male outbred Swiss mice (4 to 8-week-old) were divided into five groups (n=5 mice/group/time): (1) Control group received sterile saline (NaCl 0.9%, pH 7.2-7.4); (2) $Al(OH)_3$ group received a 180 µg of Aluminum hydroxide (Sigma-Aldrich Co St. Louis, MO, USA); (3) MPGA group received 50 µL of Montanide Pet Gel A (Seppic, Paris, France); (4) GLA-SE group received a 50 µg of Glucopyranosyl Lipid A Stable Emulsion (Alexis Biochemicals, San Diego, USA); and (5) R-848 group received a 25 µg of Resiquimod (Sigma Chemical CO, St Louis, USA). Mice received a single 50 µL dose of each adjuvant by intradermal route at the dorsum previously shaved, and were evaluated at several time points (1, 12, 24, 48, 96, 168, and 336h). At each time point the animals were anesthetized intraperitoneally with sodium thiopental 2.5% (Tiopentax - Cristalia®, São Paulo, Brazil, 30 mg/Kg). Mice received a lethal dose of sodium thiopental 2.5% and necropsied to collect the skin from the inoculation site.

### Mice Skin Collection and Preparation

Skin from adjuvant inoculated mice were collected and divided into two parts. One of these was preserved in 10% buffered formalin for morphometric analysis. Posteriorly, it was dehydrated, embedded in paraffin, cut by microtome into 5 µm sections, mounted on slides and stained with Hematoxylin-Eosin (HE) for histological procedures [10]. The rest of the fragment was stored at -80°C until use.

### Morphometric analysis

The dermis/epidermis inflammatory cell infiltrates were measured analyzing 20 images of randomly selected fields (total area $1.5x10^6$ µm², 40x objective). Leica QWin software (Leica Microsystems, Wetzlar, Germany) was used for analysis of images by counting the cell nuclei present in tissue sections. The skin appendages such as hair follicles and sebaceous glands were excluded from the morphometric analysis as a standard procedure.

### Immune Cellular Characterization

The slides with skin sections were used for immune cellular characterization in previously quantified inflammatory cell infiltrates. The cell profile was attained by differential leukocyte count of main inflammatory cells (neutrophils, macrophages, eosinophils, and lymphocytes) [10].

### Cytokines Measurement

Briefly, skin samples from the inoculation site were weighed and received 700 μL of inhibitor protease (Sigma Chemical Co, St Louis, USA) and were homogenized in a Tissuelyser (Quiagen, USA). The homogenate samples were centrifuged at 10,000 x g for 10 minutes at 4 ºC and the supernatants were collected for cytokine measurement. The cytokines IFN-γ, TNF-α, IL-6, IL-10, and IL-4 were measured by Cytometric Bead Array (BD Biosciences) [10]. The events were acquired on the FACSCalibur cytometer (BD Biosciences). The results were obtained using the FCAP Array™ v1.0.2 software (BD Biosciences, San Jose, CA, USA) and expressed by picograms per milliliter (pg/mL) of total protein.

### Statistical Analysis

Statistical analyzes were performed using the GraphPad Prism 8.0 software (Prism Software, Irvine, CA, USA). The data were plotted as absolute values ± Standard Deviation (SD) from duplicate sets of experiments. The Kolmogorov-Smirnoff test was used to confirm the normality of the data. Analysis of variance (one-way ANOVA) was performed, followed by the Turkey multiple comparison test to determine the significant differences between groups concerning inflammatory cell infiltrates and differential leukocyte count. The statistical significance was set at $p\text{-value} < 0.05$.

## Results

### Adjuvants display Different Cell Recruitment kinetics in Mice Skin after Intradermal Inoculum

The analysis of cell recruitment in the mice skin showed that all adjuvants' groups increased recruitment of cells compared with the C group, however with distinct kinetics. In addition, the adjuvants (MPGA, GLA-SE, and R-848), demonstrated an increase in cells recruited at the site of inoculation when compared with classic type II adjuvant $Al(OH)_3$. MPGA and GLA-SE groups showed a similar response, albeit with an earlier and more durable cell recruitment in the former. Furthermore, GLA-SE was able to induce a significant increase in the number of cells at most of the time points evaluated (12, 24, 48, 96, and 168h), whereas MPGA showed a delay in the increase of cell recruitment (Figure 1a). Representative photomicrographs of histological sections of the inflammatory cell infiltrate in the mice skin at the inoculation site were shown in Figure 1b. Generally, the inflammatory cell infiltrates were observed in the muscle layer and deep dermis, causing enlargement of the entire dermis.

### Differential Leukocyte Count Profile in Mice Skin after Intradermal Inoculum with Adjuvants

The adjuvants MPGA and GLA-SE displayed similar cell recruitment profiles with an increase of neutrophils at 12 hours, 40% and 67% respectively. At 168 hours, these percentages decreased to 2% and 8%, respectively. We observed a neutrophils replacement by macrophages, with advancement of kinetics, at 168 hours in MPGA (23%) and GLA-SE (28%) groups. In contrast, the R-848 group had a similar cell recruitment profile with $Al(OH)_3$ group (Figure 1c).

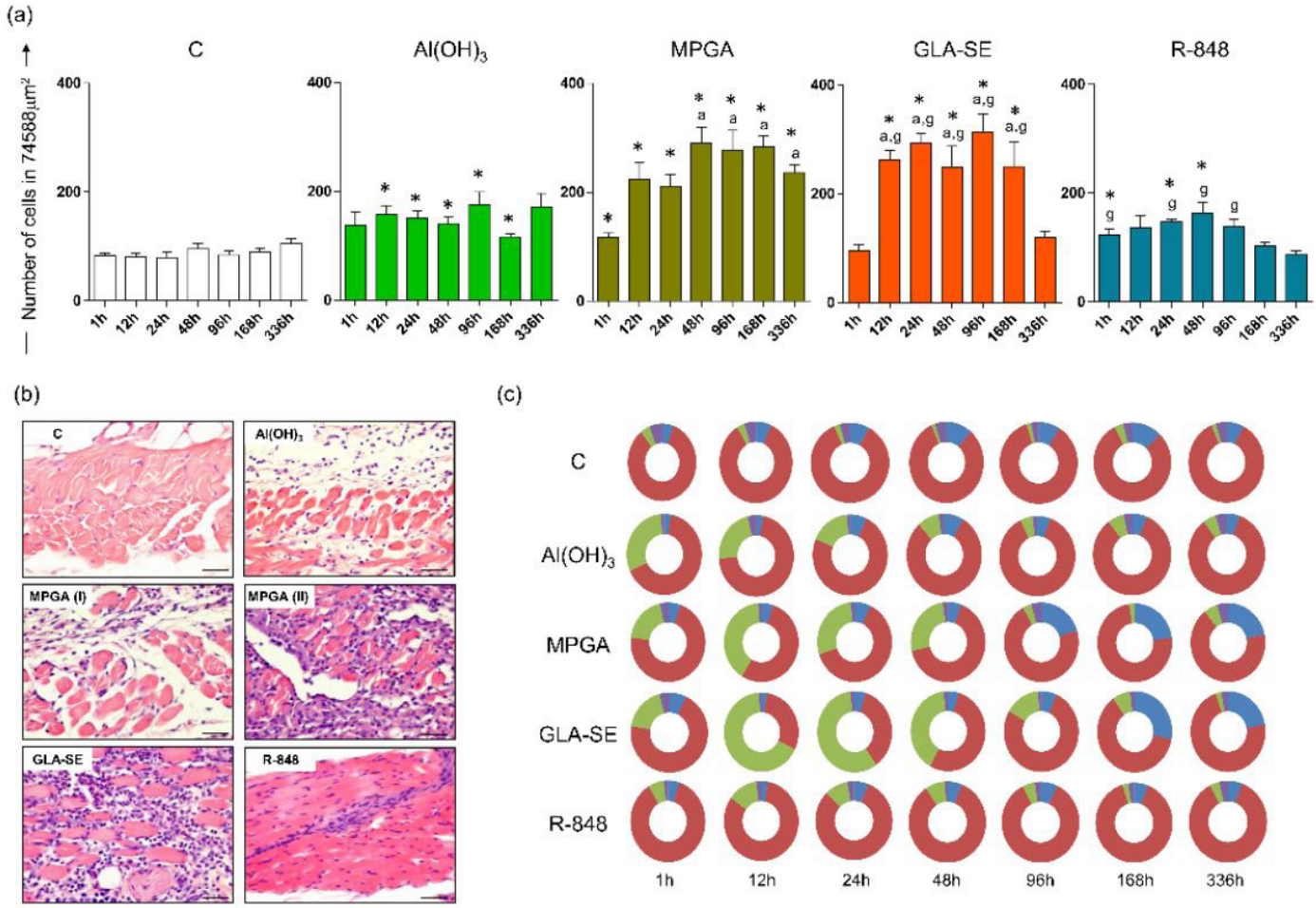


**Figure 1:** Adjuvants promote different cell recruitment kinetics in mice skin after intradermal inoculum. Mice received a single dose of sterile saline (C) or the adjuvants, Aluminum Hydroxide ($Al(OH)_3$) ; Montanide Pet Gel A (MPGA); Glucopyranosyl Lipid A Stable Emulsion (GLA-SE); and Resiquimod (R-848), and evaluated at several time points (1, 12, 24, 48, 96, 168, and 336h) (n=5 mice/group/time). **(a)** Graphs represent the longitudinal evaluation of the inflammatory cell infiltrates in mice skin after intradermal inoculum with adjuvants. Results are expressed as absolute values ± SD. The letters "a" and "g" indicate the significant difference between the times of 1 hour and 336 hours, respectively, in the longitudinal analysis and (*) indicates significant difference from the control group (p-value<0.05). **(b)** Representative photomicrographs of histological sections of the inflammatory cell infiltrate in the mice skin at the inoculation site. MPGA (I) at 1h and MPGA (II) 48h until 336h; GLA-SE at times of 12 to 168 hours and R-848 over the time. Slides shown at 40× magnification. Hematoxylin-eosin. Bar = 50 µm. **(c)** Differential leukocyte count profile after intradermal inoculum with adjuvants. The different immune cellular types of neutrophils, macrophages, lymphocytes, and eosinophils were represented by the colors green, blue, red, and purple, respectively.

### Cytokine Profile Produced in Mice Skin after Intradermal Inoculum with Adjuvants

MPGA and GLA-SE groups showed increased levels of IL-6 at 12 hours, and increased levels of TNF-α and IFN-γ at 24 hours. R-848 and $Al(OH)_3$ groups displayed a similar cytokine profile inducing higher levels of IL-6, TNF-α, and IL-10 at 1 hour, although $Al(OH)_3$ also induced IL-4 cytokine (Figure 2).

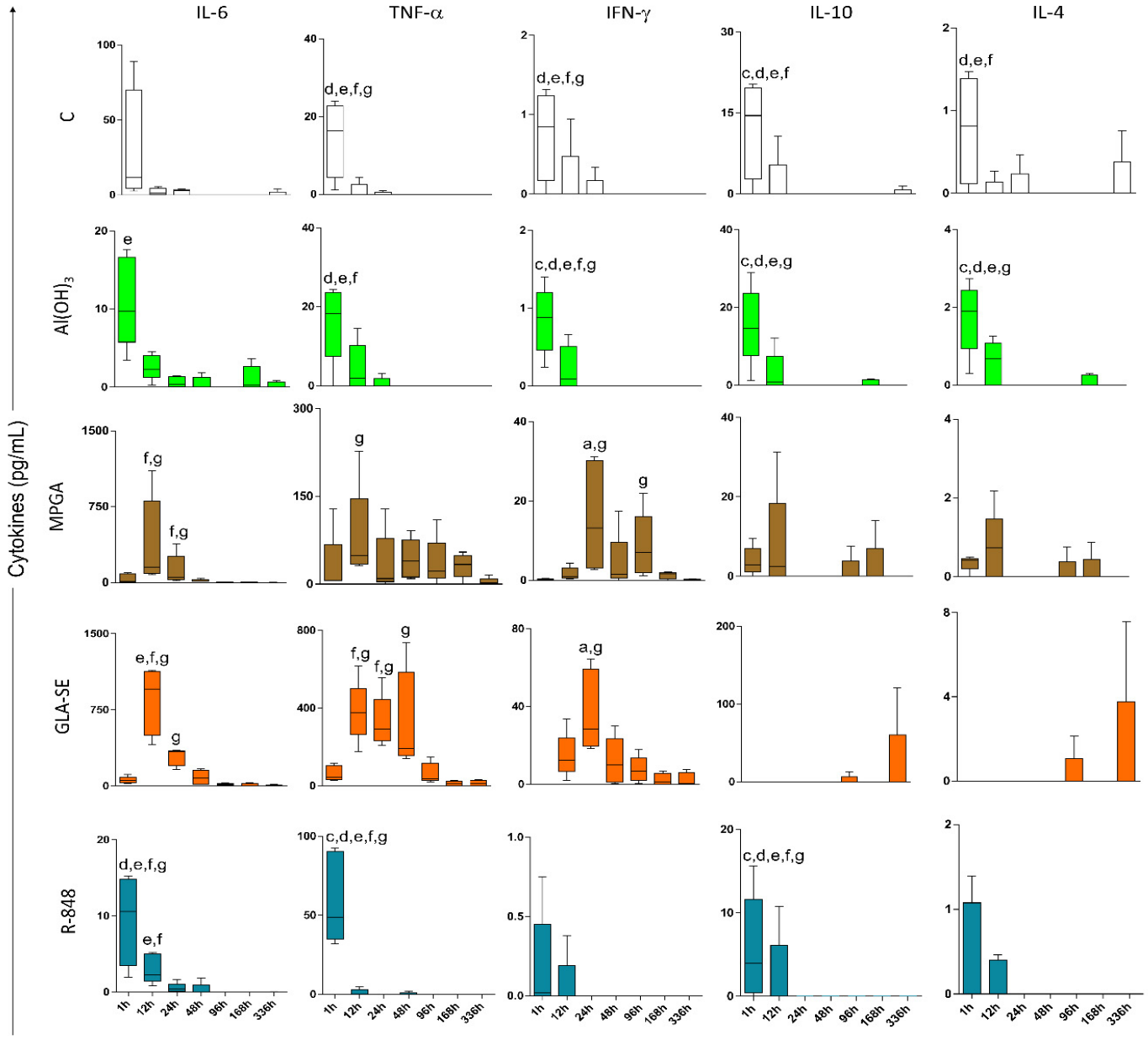


**Figure 2:** Cytokine profile produced in mice skin after intradermal inoculum with adjuvants. Mice received a single dose of sterile saline (C) or the adjuvants, Aluminum hydroxide ($Al(OH)_3$) ; Montanide Pet Gel A (MPGA); Glucopyranosyl Lipid A Stable Emulsion (GLA-SE); and Resiquimod (R-848), and evaluated at several time points (1, 12, 24, 48, 96, 168, and 336h) (n=5 mice/group/time). Longitudinal evaluation ofIL-6, TNF-α, IFN-γ, IL-10, and IL-4 cytokines production. Data are expressed as absolute values ± SD. The letters "a", "b", "c", "d", "e", "f" and "g" indicate the significant difference (p-value < 0.05) between the times 1h, 12h, 24h, 48h, 96h, 168h and, 336h respectively.

## Discussion

The present study investigated the inflammatory cell infiltrates, cell recruitment and cytokine production, overtime after intradermal inoculum of GLA-SE, MPGA, and R-848 adjuvants. GLA-SE and MPGA adjuvants promoted a strong induction of pro-inflammatory cytokines production, resulting in intense and long-lasting cell recruitment. The IFN-γ and TNF-α production after stimulation by these compounds (GLA-SE and MPGA) were more intense and more prolonged than observed for the other cytokines. On the other hand, R-848 and $Al(OH)_3$ demonstrated a similar pattern of cell recruitment, however only $Al(OH)_3$ adjuvant displayed IL-4 production as expected. GLA-SE, a synthetic compound derived from LPS, is a Toll-like receptor 4 (TLR4) agonist, known to induce strong cellular activation with promotion of type 1 immune response [6,11]. Here, we show that this response starts as early as 1 hour after inoculation, as indicated by IL-6 production, with strong peaks of TNF-α detected at 12 hours and high production of IFN-γ at 24 hours. These results contrast with those of Lambert et al. (2012) [12], who reported a delayed presence of IL-6 and no difference in TNF-α production after subcutaneous injection of GLA-SE. Moreover, our time course experiment showed that cytokine production persists for 48 hours, decaying after that to normal levels. The early detection of IL-6 in the skin allows faster antigen recognition by APCs, and the persistence of TNF-α and IFN-γ might explain the intense and long-lasting cell recruitment in the dermis demonstrated. This prolonged immune response is an important property for vaccine development as it allows better antigen recognition and posterior specific T-cell presentation.

MPGA and GLA-SE displayed a similar pattern of cytokine production. Furthermore, MPGA persisted for 336 hours at the inoculation site, probably due to its gel-based composition, consistent with the results by Vialle et al. (2010) [13] obtained with intramuscular injection in guinea pigs. This gel-based depot system might explain the persistence of IFN-γ levels for 96 hours. Regarding inflammatory process, GLA-SE and MPGA adjuvants promoted the earliest (12h and 48h) and most intense cell recruitment among all adjuvants tested, around 3-fold more than the control group. Moreover, cell persistence was very long, lasting until 168 hours for GLA-SE and 336 hours for MPGA at levels 2.7-fold and 2.2-fold higher than control and 2.15-fold and 1.4-fold higher than ($Al(OH)_3$), respectively. TLR4 agonists, such as GLA-SE, activate the immune system via TRIF and MyD88 pathways, which are related to the induction of chemokines/cytokines involved in cell recruitment and presentation pathways [12]. The persistence of MPGA in the inoculation site and long-lasting inflammation demonstrate that this gel-based composition presents a strong ability to induce the immune system to engage in intense cell recruitment. Vialle and colleagues [13] demonstrated that in association with OVA, MPGA induced sustained antigen release overtime, with recruitment of the innate immune system by a pro-inflammatory profile in the injected muscle and improved phagocytosis of the antigen increasing the activity of Antigen Presentation Cells (APCs). $Al(OH)_3$ adjuvant presents the same depot system mechanism, promoting around 1.7-fold more cell recruitment than the control group, however it is not able to induce strong inflammation as GLA-SE and MPGA.

Additionally, R-848 did not present remarkable cell recruitment to the dermis. This pattern was observed in $Al(OH)_3$ and R-848 groups may be explained by the fact that these adjuvants induced the production of IL-10, an important immunoregulatory cytokine [14]. The persistence and intensity of the responses induced by GLA-SE and MPGA make these adjuvants particularly promising in vaccine formulations, once may provide a more constant and longer antigen presentation than achieved with currently approved adjuvants. The inflammatory process profile generated by the adjuvants varied over time. MPGA and GLA-SE adjuvants promoted high neutrophil recruitment at 12 hours that was persistent until 24 hours after inoculation. The presence of this cell at the inoculation site acts as the first line of defense against pathogens, releasing cytokines that induce the recruitment of other cell types such as macrophages [15]. At later time points, the cell profiles changed to a reduced number of neutrophils and an increased number of macrophages. Macrophages together with dendritic cells (DCs) are among the main cells responsible for phagocytosis and antigen presentation to T cells [16]. The quick time recruitment of neutrophils and long-lasting macrophages creates a good microenvironment for antigen presentation, confirming the potential of these adjuvants for vaccine development. In contrast, R-848 did not show a significant cell profile variation during the time course. Differently, Kwissa et al. (2012) [17] demonstrated that intramuscular immunization of nonhuman primates with R-848 induced neutrophil increase on PBMC, although was not observed the cell migration from blood to the muscle.

Our results demonstrate the potential of adjuvants in stimulating the cellular immune response, which is directly associated with the dose inoculated and the route of administration [18]. Most existing vaccines use subcutaneous or intramuscular routes. The advantage of the intradermal route is an easier and faster access route, minimally invasive, and allows lower antigen dose, related to reducing the costs of a vaccine [19]. Here, we demonstrate that the intradermal route is as efficient as other routes in inducing immune system activation. Moreover, a complete-time course study evaluating a profile of innate immune responses completely elucidates the behavior of response triggered by different adjuvants, thus allowing the choice of a better adjuvant for each vaccine.

## Conclusion

In this sense, based on our results we conclude that GLA-SE adjuvant is the most promising to compose vaccine candidates against intracellular pathogens due to its capacity to promote important pro-inflammatory cytokines production with high and long-lasting cell recruitment, which creates a perfect microenvironment for cell activation and posterior antigen presentation.

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