Assessment of potential adjuvanticity of recombinant *Leishmania infantum* eukaryotic initiation factor in a murine model.

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ABSTRACT

One important goal in a successful vaccine formulation is to identify adjuvants that promote the appropriate immune responses and are safe for use in humans. The majority of adjuvants elicit humoral responses instead of the desired strong cellular responses, while the most potent possess high toxicity. Therefore, the development of novel adjuvants remains an urgent need. The *Leishmania* eukaryotic initiation factor (LeIF) antigen is considered as a natural Th1-type adjuvant that has the ability to induce cytokine secretion by immune cells of healthy individuals.

In this study, we evaluated the adjuvant properties of the recombinant *Leishmania infantum* eIF (rLieIF) by co-administering it along with OVA antigen in the peritoneal cavity of BALB/c mice. The positive control group, received alum (a widely used adjuvant in humans) plus OVA. At 6 and 24h post-immunization, the peritoneal exudate cells (PEC) were harvested and the phenotype of recruited immune cells was determined. Moreover, we evaluated their capacity to produce NO upon *in vitro* stimulation with rLieIF, and/or IFN-γ, and/or LPS, as wells as their uric acid levels. Moreover, at 2h post-immunization, we identified the relative gene expression of the IL-1β that is associated in alum's mechanism of action.

Mice that received OVA-rLieIF exhibited significant recruitment of neutrophils and monocytes in the peritoneal cavity, at 24h post-immunization. Furthermore, PEC derived from OVA-rLieIF-immunized mice produced significant amounts of NO when stimulated with rLieIF or rLieIF-IFN-γ. Moreover, rLieIF induced a 50-fold augmentation of IL-1β gene expression, while it did not affect the levels of uric acid.

In conclusion, this study brings additional knowledge on the adjuvant activity of LieIF that involves the IL-1β gene expression and suggests that LieIF may play a dual role in vaccination strategies.

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INTRODUCTION

The term “adjuvant” comprises all the substances that have the ability to act as immunopotentiators, influencing both the amount and the quality of the adaptive immune response (1). Adjuvants are used in vaccine formulations in order to strengthen the poor immunogenicity of antigens, to induce particular immune responses and to reduce the number of vaccinations (2). Therefore, the designing of a potent adjuvant is particularly important to a successful vaccine development.

Vaccine adjuvants are represented by different classes of compounds (e.g. microbial products, emulsions, microparticles and liposomes) which exert their function by diverse and unclear mechanisms of action, such as by “depot formation”, “Ag targeting” and “inflammation” (3). The range of approved adjuvants for human vaccines is very limited and is mainly comprised of two compounds, aluminum phosphate and the oil-in-water emulsion MF59 (4).

The *Leishmania* eukaryotic initiation factor (LeIF) antigen is considered as a natural Th1-type adjuvant that has the ability to induce cytokine secretion by immune cells of healthy individuals (5). In the present study we evaluated the potency of *Leishmania infantum* eukaryotic initiation factor (LieIF) to act as an adjuvant by co-administering it along with OVA antigen in the peritoneal cavity of BALB/c mice. Furthermore, we investigated the mechanism(s) of LieIF-mediating adjuvant function.

METHODS AND MATERIALS

Immunization protocol

Four groups of female BALB/c mice (G1, G2, G3 and G4), obtained from the breeding unit of Hellenic Pasteur Institute, were immunized in the right lower quadrant of peritoneum with: Imject Alum suspension (Thermo Scientific) (10 mg) containing 10 μg of OVA (OVA – Alum, (G1)), 10 μg of OVA in combination with 10 μg of recombinant LieIF (OVA-rLieIF, (G2)), 10 μg of OVA (OVA, (G3)) and PBS (G4).

Relative gene expression

2h post-immunization (p.i.), we determined the relative gene expression of the IL-1β, by real-time PCR.

Response of innate immune system cells

6 and 24 h after injection, the peritoneal exudate cells (PEC) were harvested and labeled with anti-CD11b-FITC together with anti-Ly6C-PE and anti-Ly6G-PE (BD Pharmingen) monoclonal antibodies. Cells were analyzed in FACSCalibur cytometer and data were analyzed with FlowJo V.10.0.8 software.

In vitro assays

At 24h p.i., the produced NO upon *in vitro* stimulation of PEC with rLieIF, and/or IFN-γ, and/or LPS, was measured with Griess reaction.

At 6 and 24h p.i., the levels of uric acid were determined according to the manufacturer’s instructions, using an auto-analyzer.

RESULTS

- The intraperitoneal injection of rLieIF-OVA resulted in a significant recruitment of neutrophils and monocytes in the peritoneal cavity, compared to the negative control group (G4), at 24h p.i. (Chart 1).
- At 2h p.i., we determined the relative change in IL-1β gene expression in peritoneal exudate cells and we observed that rLieIF combined with OVA; induced a 50-fold increase in gene expression compared to OVA alone (Chart 2).
- Furthermore, we observed that rLieIF did not affect the levels of uric acid (Chart 3).
- At last, PEC derived from OVA-rLieIF-immunized mice produced significant amounts of NO when stimulated *in vitro* with rLieIF or rLieIF+IFN-γ (Chart 4).

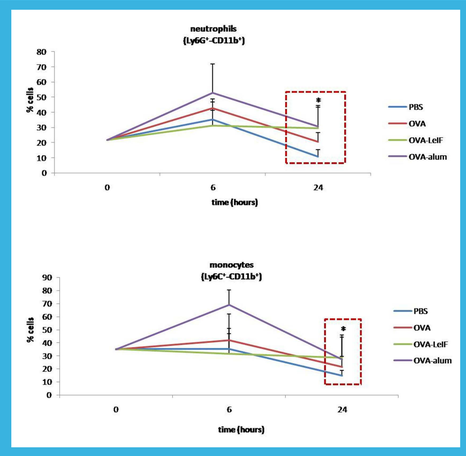

Chart 1. Influx of cells of innate immunity in the peritoneal cavity induced by rLieIF.

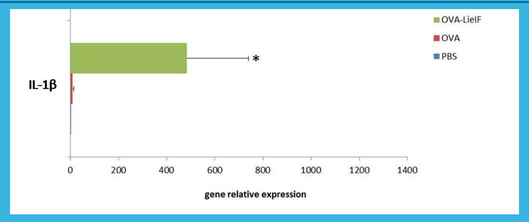

Chart 2. Relative expression of IL-1β gene in the cells of the peritoneal cavity.

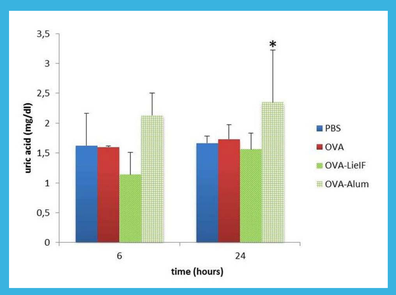

Chart 3. Determination of uric acid levels.

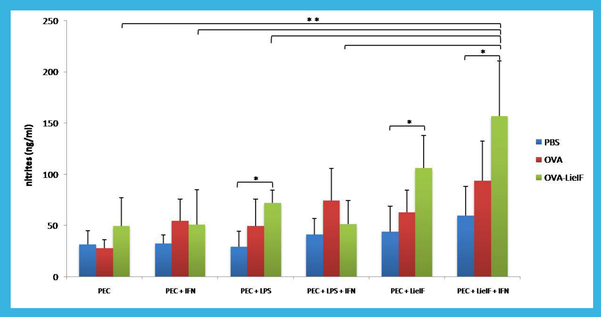

Chart 4. NO production *in vitro* from peritoneal exudate cells of immunized mice.

DISCUSSION

Alum remains the most widely used adjuvant for nearly 90 years both in veterinary and human vaccines (6). The selection of next generation adjuvants should be developed with better understood mechanisms of action in order to realize all the potential benefits that adjuvants offer.

Recombinant LieIF resulted in the attraction of innate cells at the injection site, like alum (7). Nevertheless, its effect was weaker than alum’s corresponding effect, as we did not observe significant attraction of innate immune cells as early as at 6h p.i.

Alum causes potent recruitment of neutrophils at the injection site that is accompanied by the production of IL-1β cytokine which provides a necessary and sufficient signal to support T cell response to antigens (8). We noticed that rLieIF also induced the expression of IL-1β gene, at 2h p.i.

Moreover, it has been shown that alum’s activity is dependent on the production of uric acid, as its neutralization does not lead to the recruitment of monocytes. More specifically, uric acid acts as a “danger signal” for the activation of influx of immune cells. We observed that rLieIF did not increase the levels of uric acid, suggesting the induction of a different cell attracting mechanism.

Finally, *in vitro* stimulation of PEC derived from rLieIF-OVA immunized mice; with rLieIF +/- IFN-γ led to increased NO production compared with the levels from cells derived from negative control group receiving the same stimulation. Moreover, PEC derived from rLieIF-OVA immunized mice induced significant production of NO upon stimulation with rLieIF +/- IFN-γ compared to all the other stimuli (LPS, IFN-γ).

CONCLUSIONS

Leishmania derived eukaryotic initiation factor (LeIF) is exploited not only as vaccine antigen, but also as adjuvant in vaccine development against infectious diseases.

Our study brings a deeper understanding of the mechanisms that mediate the adjuvant properties of LieIF, which is prerequisite for the successful design of more sophisticated vaccines.

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