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Cry1Ac protoxin from *Bacillus thuringiensis* promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF-α cytokines

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ABSTRACT

Bacillus thuringiensis Cry1Ac protoxin (pCry1Ac) is a promising mucosal adjuvant, but its action mechanism is unknown. We examined *in vivo* whether pCry1Ac promotes the activation of macrophages in the peritoneum, spleen and mesenteric lymph nodes or in the lungs and bronchoalveolar lavage after intraperitoneal or intranasal pCry1Ac administration, respectively, in BALB/c mice. pCry1Ac upregulated the costimulatory molecules CD80 and CD86 in these macrophages, but with distinct kinetics. *In vitro* stimulation of resident macrophages with pCry1Ac upregulated CD80 and CD86 and enhanced the production of the pro-inflammatory cytokines TNF-α, IL-6 and MCP-1. To investigate whether the pCry1Ac-induced activation was mediated through MAPK pathways, we pretreated RAW 264.7 cells with signaling inhibitors of MEK, JNK and p38 MAPKs (PD98059, SP600125 and SB203580, respectively). pCry1Ac-induced upregulation of CD86 and CD80 was partially inhibited by the MEK inhibitor. While LPS-induced upregulation mechanisms of CD80 and CD86 appear to be different; as these were particularly inhibited by MEK and JNK inhibitors, respectively. pCry1Ac-induced IL-6 and MCP-1 production was especially inhibited with the p38 MAPK inhibitor, whereas TNF-α was only slightly inhibited upon treatment with JNK and p38 MAPK inhibitors. Therefore macrophage stimulation with pCry1Ac induced the upregulation of CD80 and CD86, and the production of IL-6, TNF-α and MCP-1, possibly, through the MEK and p38 MAPK pathways. It also promoted the nuclear translocation of NF-κB p50 and p65, the upregulation of MHC-II, and the activation of T CD4+ cells. These results suggest that pCry1Ac induced macrophage activation through mechanisms which differ partially from the LPS-induced.

1. Introduction

The use of mucosal adjuvants represents a feasible strategy to improve vaccines because most pathogens enter the body *via* these routes. To date, cholera toxin (CT) and the heat-labile toxin (LT) from *Escherichia coli* are the most powerful mucosal adjuvants known, but they are too toxic for clinical use [1,2]. Although non-toxic CT and LT mutants have been reported, these mutants provide only partial adjuvant activity [3]; thus, safe mucosal adjuvants are still required.

Cry proteins from *Bacillus thuringiensis* are considered safe for vertebrates, as they are used as biopesticides [4–6], and are massively and inexpensively produced [7]. Interestingly, we have previously shown that recombinant soluble Cry1Ac protoxin (pCry1Ac) is a potent systemic and mucosal immunogen and adjuvant in mice [8–10], and appears to be safe as no mice have died by the administration of pCry1Ac, even after 3 intraperitoneal (i.p.) doses of 100 μg [8].

We have also demonstrated that pCry1Ac can act as an effective and protective adjuvant in two murine parasitic infection models. The intranasal coadministration of pCry1Ac with amoebic lysates conferred complete protective immunity against a lethal challenge with *Naegleria fowleri* [11]. Likewise, pre-treatment with pCry1Ac alone increased protection against *Plasmodium* [12] and *Naegleria* infection in mice. These findings suggest that the adjuvant effects induced by pCry1Ac may occur in part due to its ability to activate innate immune mechanisms. However, this hypothesis remains to be tested.

The immunostimulatory action of most adjuvants of microbial origin, such as lipopolysaccharides (LPS), involves the upregulation of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells (APCs) and the induction of cytokines [13,14]. APC activation occurs following innate immune recognition, and the binding of these costimulatory molecules to the T cell costimulatory receptors CD28 and CTLA-4 is essential for the activation and regulation of T cell

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immunity [15–17]. LPS is not used as a component in vaccine preparations due to its high toxicity, but it is a valuable, widely used experimental tool, and its mechanism of action on APCs is one of the best described [18–20]. In contrast, the mechanism of action through which the pCry1Ac adjuvant exerts its immunomodulatory effects on APCs, such as macrophages, is unknown. Because pCry1Ac administered by either the i.p. [21] or intranasal route induces strong systemic and mucosal immune responses as well as adjuvant effects [10,11], in this study, we selected these administration routes to test the *in vivo* effect of pCry1Ac on the activation of macrophages. We isolated macrophages at different time points following *in vivo* stimulation from tissues that we expected to be activated based on their location near the site of antigen administration. For comparison, as a positive control, we used mice that were injected with a low, non-lethal dose of LPS [22].

We determined whether pCry1Ac promoted B7.1 (CD80) and B7.2 (CD86) upregulation in macrophages harvested from the peritoneum, spleen and mesenteric lymph nodes (in mice injected *via* the i.p. route) or from the lungs and BAL (in mice injected *via* the intranasal route). These parameters were also measured *in vitro* in resident macrophages obtained from the same tissues and stimulated with pCry1Ac. In addition, we assessed whether the *in vitro* stimulation with pCry1Ac enhanced proinflammatory cytokine production.

Accumulated evidence indicates that nuclear factor kappa B (NF-κB) is a major transcription factor that plays a pivotal role in immune and inflammatory responses by regulating genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as COX-2 and iNOS [23,24]. Furthermore, the activation of NF-κB can be triggered by mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun NH2-terminal kinase (JNK) [25].

Indeed, the three main MAPKs, ERK1/2, JNK and p38, have been shown to be absolutely required for LPS-induced inflammation [20,26,27].

Therefore, to investigate the mechanism of action of pCry1Ac in comparison with that of LPS, we determined whether pretreatment with MAPK inhibitors suppressed the pCry1Ac-induced upregulation of CD80 and CD86 and the production of proinflammatory cytokines in RAW 264.7 macrophages.

Finally to reinforce our findings indicating that pCry1Ac activates macrophages we evaluated the expression of MHC-II, CD40, the translocation of NFkB and performed *in vitro* stimulation assays evaluating the activation and proliferation of T cells CD4. The outcomes indicate that Cry1Ac is able to activate macrophages.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), L-Glutamine, 20 mM HEPES, 5 mM 2-β-mercaptoethanol, 1% essential amino acids were obtained from (GIBCO BRL, NY 14072. Fc γ II/III, fluorochrome-labeled monoclonal antibodies CD86, CD80, F4/80, CD11b, CD11c, MHC-II, CD40, CD3, CD4, CD8, CD25, CD69 and CBA mouse Inflammation Kit were purchased from BD Biosciences, (San José CA, USA). Actin, histone 4 or p50 NF-kB antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibody was from Thermo scientific Pierce (Rockford, IL. 61105 USA). Polymixin B resin was from (Bio-Rad, Hercules, CA, USA). Anti p65 NFkB antibody was purchased from Biosciences. Enhanced chemiluminescence was from (Amersham Life Science, Buckinghamshire, UK). Roswell Park Memorial Institute 1640 (RPMI-1640) medium, LPS (*E. coli*, serotype 0111:B4), E-toxate, penicillin, streptomycin, PD98059, SP600125, SB203580, Nonidet P-40, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, Carboxyfluorescein succinimidyl ester (CFSE), dithiothreitol, Tween 20 and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Recombinant pCry1Ac

Recombinant pCry1Ac was purified from IPTG-induced *E. coli* JM103 (Pos900) cultures as described previously [11]. Endotoxin levels in the purified pCry1Ac preparations were tested using the E-toxate, kit and were found to be below 0.1 EU/ml. These preparations were further treated with an excess of a polymixin B resin to remove any possible endotoxin remnants.

2.3. Animals

The BALB/c female mice used in this study were 6 to 8 weeks old. These mice were housed in filter-top cages and were provided with sterile food and water *ad libitum* at the FES Iztacala, Universidad Nacional Autónoma de México animal house facility in accordance with the institutional and national official guideline, NOM-062-ZOO-199, for the use and care of laboratory animals.

2.4. Parenteral and nasal administration of pCry1Ac

The *in vivo* and *in vitro* effects induced by pCry1Ac were compared with those induced by LPS because LPS is a well-known macrophage activator [18,20,22].

To determine the *in vivo* effect of the administration of pCry1Ac (5 μg) or a non-lethal dose of LPS (1 mg/kg), in BALB/c mice, the animals received one antigen dose by either the i.p. or i.n. route [21,28] and were sacrificed at 24, 48, 72 and 96 h after the stimulus. The dose of 5 μg of pCry1Ac was elected because had been tested in previous a study as a dose able to increase survival to plasmodium in mice [12] and also it is a dose able significant antibody responses by i.n. and i.p. routes. Macrophages were then collected and stained to determine the expression of costimulatory molecules.

2.5. Macrophage isolation

Untreated mice or mice treated i.p. or i.n. with pCry1Ac or LPS were sacrificed under ether anesthesia at 24, 48, 72 and 96 h after stimulation. Immediately, blood from the heart was extracted and centrifuged at 500 *g* at 4 °C for 15 min. The serum was removed and aliquoted into two tubes and snap-frozen at −70 °C until use. After blood collection, the mice were sacrificed, and the pulmonary vasculature was perfused with 5 ml of PBS *via* the left ventricle. Peritoneal exudate cells (PECs) were collected by peritoneal lavage with 5 ml of ice-cold phosphate-buffered saline (PBS) containing 5% FCS. The peritoneal cavity was gently agitated for 3 min before retrieval of peritoneal fluid. Spleen and MLNs were excised and disaggregated through a sterile nylon mesh filter using 8 ml of RPMI-1640 medium supplemented with 5% fetal calf serum (FCS). To collect bronchoalveolar lavage (BAL) samples, the trachea and lungs were excised completely and washed thoroughly with cold RPMI-1640 medium to eliminate blood. Then, 1 ml of cold medium was used to flush out the trachea to collect transudate from the lungs, and the transudate was placed in a sterile 5 ml Petri dish. To collect lung macrophages, the lungs were excised, and the cells were disaggregated in the same manner as was used for the spleen and MLNs.

The cell suspensions obtained from the different tissues (spleen, MLNs, lungs) or lavages (peritoneal and BAL) of individual mice were washed by centrifugation with RPMI-1640 medium and then plated in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, 20 mM HEPES, 5 mM 2-β-mercaptoethanol, 1% essential amino acids, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), (supplemented RPMI-1640 medium). The cells were cultured for 2 h at 37 °C and 5% CO_2 in 12-well culture plates to allow macrophages to adhere [29]. The non-adherent cells were removed and discarded by gentle washing twice with warm RPMI-1640 medium. The adherent cells were recovered either by washing with ice-cold PBS containing 0.5 mM EDTA (PBS-EDTA) or with the aid of a policeman. The adherent