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Immunomodulatory effects of azithromycin on the establishment of lipopolysaccharide tolerance in mice ☆

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ABSTRACT

Recent reports suggest that azithromycin can shift macrophage polarization towards the alternatively activated M2 phenotype. In order to investigate its immunomodulatory activity *in vivo*, the influence of azithromycin on survival and cytokine production was assessed in the LPS tolerance model which is characterized by an M2 skewed response. For induction of tolerance, mice received an intraplantar injection of 30 μg LPS, 24 h prior to intravenous challenge with 350 μg LPS. Azithromycin (100 mg/kg) was administered orally, 2 h before LPS application. Influence of treatment on survival and cytokine concentration in serum was monitored. Azithromycin alone, instead of LPS, could not induce an LPS tolerant state. However, when administered before LPS priming it significantly increased survival, which was enhanced by concomitant azithromycin before LPS challenge. Azithromycin had no effect on survival when administered only prior to the LPS challenge. Tolerance induction by LPS priming was associated, upon LPS challenge, with decreased serum concentrations of pro-inflammatory cytokines, TNFα, IL-12p40 and CCL5, and increased serum concentrations of the anti-inflammatory cytokines, IL-10 and IL-1ra. Azithromycin treatment, prior to LPS priming, further reduced serum TNFα and CCL5, yielding the greatest inhibition when the macrolide was also given prior to LPS challenge. Serum concentrations of the anti-inflammatory cytokines, IL-10 and IL-1ra, were unchanged following azithromycin treatment. In summary, we have confirmed the immunomodulatory activity of azithromycin, as reflected in its ability to augment tolerance induction to LPS, promoting increased survival and reduced pro-inflammatory cytokine production, without affecting overt inflammation to LPS or anti-inflammatory cytokine production.

1. Introduction

Inflammation is a pathophysiological state caused by infection and/or injury. However, the amplitude and duration of the inflammatory response is tightly regulated since uncontrolled inflammation can lead to extensive tissue damage and even death. One of the protective mechanisms is development of refractoriness to repeated stimulation with the same danger signal. In the case of endotoxin (bacterial lipopolysaccharide, LPS) the phenomenon of tolerance induction has been observed *in vitro* and *in vivo* [1]. The molecular basis of endotoxin tolerance is not yet fully understood. Interestingly, endotoxin tolerant monocytes/macrophages resemble in many characteristics alternatively (M2) activated macrophages [2,3]. Differential cytokine production is a key feature of polarized macrophages. In M2 macrophages, inflammatory cytokines (*e.g.* TNFα, IL-12) are downregulated and anti-inflammatory cytokines (*e.g.* IL-10, IL-1ra) upregulated [4].

Macrolide antibiotics are natural or semisynthetic polyketides that consist of a macrocyclic lactone ring containing 14, 15 or 16 atoms to which one or more sugars are attached. Erythromycin, a secondary metabolite isolated from *Saccharopolyspora erythraea*, was the first macrolide to be introduced to clinical use over 50 years ago. Subsequently, several semisynthetic derivatives of erythromycin, including azithromycin (9-deoxy-9a-aza-9a-methyl-9a-homoerythromycin A), were designed to broaden the antimicrobial spectrum, reduce gastrointestinal side effects and increase acid stability and bioavailability [5]. Nowadays, macrolides are widely used in the treatment of respiratory and urogenital tract, skin and soft tissue infections.

In addition to their efficacy in the treatment of bacterial infections, many studies over the last 20 years have demonstrated that certain macrolides are effective in the treatment of various chronic inflammatory disorders, particularly of the respiratory tract. Significant improvements in clinical symptoms and survival have been reported in numerous clinical studies in patients with diffuse panbronchiolitis, cystic fibrosis and bronchiolitis obliterans syndrome, following the introduction of erythromycin, clarithromycin or azithromycin into therapy [6]. The

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clinical efficacy of macrolides in these diseases is not directly related to their antimicrobial activity [7].

Macrolides such as azithromycin and clarithromycin seem to modulate the inflammatory response, rather than just suppressing it, as do standard anti-inflammatory or immunosuppressive drugs [8]. Modulatory effects of clarithromycin on LPS-stimulated IL-8 production and ERK1/2 activation have been reported in human bronchial epithelial cells *in vitro*. An initial decrease in IL-8 production and ERK1/2 phosphorylation in response to the macrolide was followed by an increased response to LPS activation, which with longer incubation times was normalized to baseline levels [9]. In addition, administration of roxithromycin and erythromycin to healthy animals is associated with immuno-enhancing properties [10–12], while in various animal models of inflammation anti-inflammatory effects were observed [13]. Similarly, a 3-day standard dosing regimen of azithromycin in healthy volunteers caused initial stimulation of neutrophil degranulation and the oxidative burst to particulate stimuli, followed by a delayed inhibition of neutrophil function and of circulating chemokine concentrations, in association with an increase in numbers of circulating apoptotic cells and sustained levels of the drug in circulating neutrophils [14].

Recent reports suggest that macrolides like azithromycin and clarithromycin inhibit neutrophil-dominated pulmonary inflammation through modulatory effects on mononuclear phagocytes. Azithromycin, for instance, has been shown to restore the deficient phagocytosis of apoptotic cells (efferocytosis) *in vitro* by alveolar macrophages from COPD patients [15]. An improved phagocytic ability of macrophages with increased mannose receptor (CD206) expression has also been observed following 12 weeks treatment of COPD patients with azithromycin [16]. Pro-resolution and pro-homeostatic properties of azithromycin have recently been shown in an animal model of peritonitis as well [17]. Several groups have reported that azithromycin is able to alter macrophage phenotype from pro-inflammatory M1 towards alternatively activated M2 cells *in vitro* [18–21].

The aim of the present study was to investigate the immunomodulatory effects of azithromycin *in vivo*. In the light of recent reports that azithromycin can shift monocyte/macrophage polarization towards the alternatively activated M2 phenotype [18–21], the influence of this macrolide on survival and cytokine production was investigated in the LPS tolerance model which is characterized by an M2 skewed response.

2. Materials and methods

2.1. Animals

Studies were performed on 8 week old male C57bl6 mice weighing 23–24 g (Charles River, Germany). Mice were maintained under standard laboratory conditions (temperature 23–24 °C, relative humidity 60 ± 5%, 15 air changes per hour, artificial lighting with circadian cycle of 12 h). Pelleted food and tap water were provided *ad libitum*. All procedures on animals were approved by the ethics committee of GlaxoSmithKline Research Centre Zagreb Limited, and performed in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Materials

LPS from *Escherichia coli* serotype 0111:B4 was obtained from Sigma Chemical Co. (US). Azithromycin dihydrate was from PLIVA Inc. (Croatia). Antibodies for enzyme-linked immunosorbentassay (ELISA) were purchased from R&D Systems (US). All other reagents, if not indicated otherwise, were from Sigma Chemical Co. (US).

2.3. In vivo experiments

For induction of endotoxin shock, animals were challenged with 350 μg (~15 mg/kg) LPS i.v. For induction of tolerance, mice received an intraplantar injection of 15 or 30 μg (~0.6 or ~1.3 mg/kg) LPS, 24 h prior to intravenous challenge with 350 μg LPS. Azithromycin (100 mg/kg) or only vehicle was administered orally, 2 h before LPS application, either for priming or challenge. Azithromycin was first dissolved in dimethlysulfoxide (DMSO) and then diluted with 0.5% (w/v) methylcellulose [final concentration of DMSO was 5% (v/v)]. The compound was further solubilised by addition of an equimolar quantity of citric acid. The solution obtained was administered in a volume of 10 ml/kg (b.w.). The selected dose was previously demonstrated to increase survival of animals in a mouse model of septic shock [22].

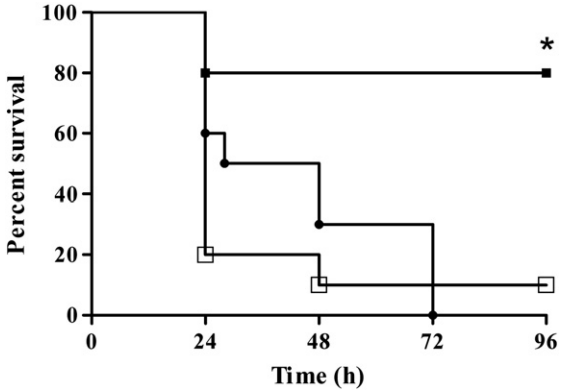

Fig. 1. Induction of LPS tolerance with survival rate of naive (filled circles) and LPS primed (open and filled squares) mice following intravenous challenge with 350 μg LPS. For induction of tolerance, mice received an intraplantar injection of 15 (open squares) or 30 μg (filled squares) LPS, 24 h prior to intravenous challenge with 350 μg LPS. n = 10 animals per group.*$p<0.05$ *vs.* 350 μg LPS i.v. Mantel–Cox test.

Survival of animals was monitored for 4 days. For cytokine determination in serum, animals were anesthetised with Isoflurane (Abbott Laboratories, US) and exsanguinated by puncturing the *a. carotis communis* 0.5 or 4 h after the LPS injection. Serum was collected and stored at −20 °C until use. In survival studies, 10 animals were included per treatment group. Seven animals per treatment group were used in experiments for cytokine determination. All experiments were repeated at least twice.

2.4. ELISA

Cytokine concentration in serum was determined by sandwich ELISA using capture and detection antibodies according to the manufacturer's instructions. Optical density was measured at 450 nm by use of a microplate reader. The concentration of cytokines was determined

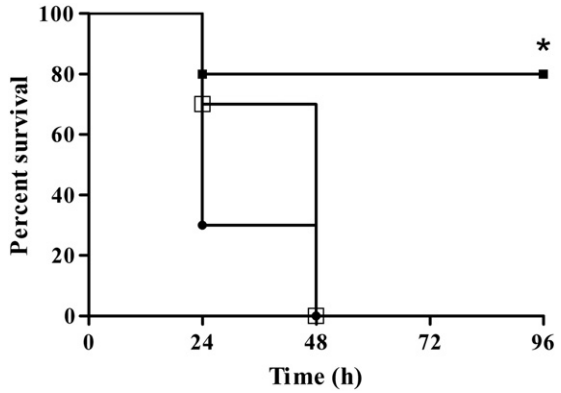

Fig. 2. Azithromycin alone cannot induce a state of tolerance to LPS. Survival rate is shown of naive (filled circles), LPS (filled squares) and azithromycin (100 mg/kg) pretreated (open squares) mice following intravenous challenge with 350 μg LPS. For induction of tolerance, mice received an intraplantar injection of 30 μg LPS or 100 mg/kg azithromycin p.o., 24 h prior to intravenous challenge with 350 μg LPS. n = 10 animals per group.*$p<0.05$ *vs.* 350 μg LPS i.v. Mantel–Cox test.