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Interleukin-13 reduces hyperalgesia and the level of interleukin-1β in BALB/c mice infected with *Leishmania major* with an up-regulation of interleukin-6

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ABSTRACT

The anti-inflammatory cytokines interleukin-10 (IL-10) and interleukin-13 (IL-13) were shown to reduce hyperalgesia in some models such as rats exposed to UV rays. In addition, IL-10 was also shown to reduce hyperalgesia in high dose of *Leishmania major*-induced inflammation in BALB/c mice accompanied by a significant decrease in the levels of interleukin-1β (IL-1β) in the paws of infected mice, while no effect on the levels of IL-6 was observed. In this study, we injected BALB/c mice with a high dose of *L. major* and treated them with IL-13 (15 ng/animal) for twelve days (excluding the weekends) and hyperalgesia was assessed using thermal pain tests. Furthermore, the levels of IL-1β and IL-6 were also assessed at different post-infection days. Our results show that IL-6 and more importantly IL-1β don't play a direct role in the *L. major*-induced hyperalgesia and that IL-13 induces this hyperalgesia through the down-regulation of IL-1β and another proinflammatory cytokine (most probably TNF-α). Furthermore, our data show that IL-13 leads to the upregulation of the level IL-6 which initially seems to have no direct role in the induced hyperalgesia. Therefore, we suggest that the *L. major*-induced hyperalgesia is mainly mediated by the cytokine cascade leading to the production of sympathetic amines.

1. Introduction

Leishmania major (*L. major*) is an obligate intracellular parasitic protozoan which infects mononuclear phagocytes (Alexander et al., 1999; Wyler, 1982) of resistant hosts such as humans, rats and CBA mice with a stable cell-mediated immune response leading to self-recovery. On the contrary, BALB/c mice is a susceptible host to *L. major* which often mounts an antibody response as it succumbs to progressive infection (Bretscher et al., 1992). Activation of either branch of the immune system in turn depends on other factors such as genetic makeup of the host (Gorczynski and MacRae, 1982; Launois et al., 1997a), the infecting dose of parasites (lower dose induces cell-mediated rather than humoral immunity) (Bretscher et al., 1992) and the cytokine milieu (Gumy et al., 2004) especially during early stages of the infection (Aguilar-Torrentera and Carlier, 2001; Gumy et al., 2004). In BALB/c mice, the early production of IL-12 is accompanied by concomitant production of Th2 cytokines such as TGF-β, IL-4/IL-13 and IL-10 (Noben-Trauth et al., 2003; Sacks and Noben-Trauth, 2002) that in turn inhibit IL-12 function (Solbach and Laskay, 2000) and natural killer (NK) cells which are crucial for Th1 cell activation to clear the intracellular parasite (Awasthi et al., 2004; Scharton-Kersten and Scott, 1995). On the other hand, *L. major* infection in resistant hosts results in Th1 cells activation with the production of IL-2, Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α) (Stout and Bottomly, 1989). In particular, IFN-γ activates macrophages to express iNOS2 and nitric oxide formation which kills the intracellular amastigotes (Awasthi et al., 2004). Although IL-4 is thought to play a crucial role in determining the course of infection (Launois et al., 1995) since it acts within a critical time window to render CD4+ cells unresponsive to IL-12 and become Th2 cells (Launois et al., 1997b), other studies have debated such hypothesis (Scott et al., 1996) stating that mutant BALB/c mice deficient in IL-4 only possess partial (Noben-Trauth et al., 1999) or even complete lack (Mohrs et al., 2000) of impaired Th2 polarization in response to *L. major*. IL-13 is also postulated to determine susceptibility to infection as overexpression of IL-13 gene in normally resistant C57BL/6 mice leads to susceptibility and deletion of IL-13 gene in normally susceptible BLAB/c mice leads to resistance (Matthews et al., 2000). A contrary hypothesis is recently put forward to describe a promoting role of IL-13 and IL-4 in Th1 response against *Leishmania* (Alexander and McFarlane, 2008).

L. major-induced hyperalgesia has been reported in BALB/c mice; when a high dose of parasite (4×10^6 promastigotes) is injected per

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hind paw, a sustained hyperalgesia associated with an up-regulation of interleukin-1β (IL-1β) and Nerve Growth Factor (NGF) occurs (Kanaan et al., 2000). Conversely, when a low dose of parasite is used, (4×10^3 promastigotes), hyperalgesia is transient with a rise in IL-1β and IL-6 (Karam et al., 2006). Th2 cytokines such as IL-4, IL-10 and IL-13 are known to produce analgesia, an action mediated through the inhibition of proinflammatory cytokines like TNF-α, IL-1β and PGE2 (Cunha et al., 1999; Lorenzetti et al., 2001; Poole et al., 1995; Saade et al., 2000; Vale et al., 2003). In the context of *L. major*-induced hyperalgesia, the picture is mixed: while IL-10 has been shown to reduce hyperalgesia in BALB/c mice with a down-regulation of IL-1β but not IL-6 (Karam et al., 2007), IL-13 increases hyperalgesia with an upregulation of IL-1β in rats without rendering them more susceptible to infection (Haber et al., 2008).

Till date, there is no data on the effects of IL-13 upon *L. major*-induced hyperalgesia nor the levels of the proinflammatory cytokines in BALB/c mice. Our study addresses this research question with a focus on the TNF-α, IL-1β, IL-6 and PGE2.

In inflammatory type of hyperalgesia, prostaglandins and sympathetic amines are the end-effectors which directly sensitize nociceptors causing hyperalgesia (Khasar et al., 1999; Lorenzetti and Ferreira, 1985; Safieh-Garabedian et al., 2002; Tonussi and Ferreira, 1994), and their production is preceded by the release of a cascade of cytokines and chemokines (Cunha et al., 2005). It is widely accepted that TNF-α, mainly produced by macrophages, initiates the cascade by inducing the release of IL-1β and IL-6 and which in turn stimulate the cyclooxygenase pathway leading to the production of prostaglandins. On the other hand, TNF-α can induces IL-8 production, which in its turn leads to the production of sympathomimetic mediators in another pathway (Cunha et al., 1992). During inflammation, IL-1β is mainly produced by monocytes, macrophages and glial cells which stimulate cyclooxygenase (COX)-2 and subsequent release of prostaglandins (Newton et al., 1997) causing hyperalgesia. Antibodies to IL-1β only partially neutralize LPS-induced inflammatory hyperalgesia signifying the roles of other mediators (Cunha et al., 1992). Direct administration of IL-1β, either peripherally or centrally, induces hypernociception in rats (Oka et al., 1993). For *L. major*-induced hyperalgesia, IL-1β was found to be upregulated with hyperalgesia (Kanaan et al., 2000; Karam et al., 2006; Loram et al., 2007).

Although IL-6 is a key player among other cytokines recognized for mediating local hyperalgesia in inflammation (Loram et al., 2007), a more central role has been suggested in the context of chronic and neuropathic pain (Oka et al., 2007; Sommer and Kress, 2004) where an inhibitory and anti-nociceptive effects have been attributed to it (Flatters et al., 2003; Naka et al., 2002). Our previous study did demonstrate a transient rise of local IL-6 levels with *L. major* infection at both high and low doses, and its exact role in hyperalgesia remains unclear except that it may mediate via IL-1β production (Karam et al., 2006). Here, we chose to study the effect of IL-13 on the levels of IL-1β and IL-6 in the paws of BALB/c mice infected with *L. major* in correlation with the observed hyperalgesia.

2. Aim of the study

In this study we investigate the effect of IL-13 on *L. major*-induced hyperalgesia and the levels of the local proinflammatory cytokines IL-1β and IL-6 in BALB/c mice to unravel the role of those cytokines in this model of inflammatory hyperalgesia.

3. Materials and methods

Adult female BALB/c mice (20–30 g) were used in all the experiments performed in this study. The animals were housed under optimal conditions of light and temperature (12 hour light and 12 hour dark cycles; 22 ± 3 °C) and received solid food and water ad libitum. During the period of the experiments, the mice were kept, in groups of five, in clear plastic cages with solid floors covered with 3–6 cm of saw dust. All experimental procedures were carried out with strict adherence to the ethical guidelines for the study of experimental pain in conscious animals (Zimmerman, 1983).

L. major (MHOM/SU/73/5 ASKH) parasites (provided by the London School of Hygiene and Tropical Medicine) were grown at 22 1 °C to 25 ± 1 °C in a standard biphasic medium and subcultured weekly. The solid medium (underlay) was made of bactoagar supplemented with 10% rabbit blood, while the liquid medium (overlay) consisted of Lock's solution (4 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl and 0.2 g glucose in 500 ml distilled water) supplemented with 10% heat-inactivated fetal calf serum (at 56 °C for 30 min), 100 IU/ml of penicillin and 100 IU/ml of streptomycin (Sigma). The overlay solution containing the promastigote forms of the parasite were centrifuged for 10 min at 2500 rpm and the parasites were re-suspended in 1 ml Lock's medium. The parasite count was determined in a trypan blue solution using a hemocytometer and readjusted to have 2.5×10^6 parasites per 50 μl Lock's medium.

The reported results were based on two sets of experiments each involving different groups of mice (n = 5 in each group) which were used either for the behavioral pain tests or the determination of the levels of cytokines in the paws of the mice.

3.1. Behavioral measurements

Five groups of mice were subjected to different pain tests three days before any treatment, then one group was treated intraperitoneally (i.p.) with IL-13 (15 ng/animal) for 12 days (excluding the weekend), the second group received intraplantar injection (i.pl.) with a high dose of *L. major* at the left hind paw (2.5×10^6 promastigotes/50 μl Lock's medium), and the third group received an i.pl. injection of high dose of *L. major* plus i.p. IL-13 (15 ng/animal) for 12 days (excluding the weekend). The two remaining groups acted as controls, one with no treatment and the other with i.pl. injection of Lock's medium (50 μl /left hind paw). All groups were subjected to pain tests at least three times weekly following the injections for a period of 30 days.

The hot plate (HP) and the tail flick (TF) tests were used to assess the thermal pain thresholds. For the HP test, each animal was placed on a heated pad with the temperature being maintained at T = 51 °C (±0.5 °C). The latency of paw licking or jumping was taken as an index of pain threshold (Kanaan et al., 1996). For the TF test, the tails of the mice were immersed into a beaker of distilled water, with a temperature of T = 50 °C (±0.5 °C). The time interval between the immersion of the tail and the tail flicking reaction was recorded. Each animal was tested three times with a three minute interval between each consecutive measurement and the average was recorded.

3.2. Immunoassay

Five sets of mice, each containing five groups (n = 5), were used in this part of the study. The first set was injected with IL-13 i.p. (15 ng/animal) for 12 days (excluding weekend). The other two sets were injected with a high dose of i.pl. *L. major*, but one set was also treated with i.p. IL-13 (15 ng/animal) for 12 days (excluding weekend). In addition two sets were used as control (one consists of naive mice and the other of mice injected with *L. major*-free Lock's medium).

The mice were sacrificed one group per time interval at days 2, 4, 6, 13 and 21 post-injection under deep anesthesia (pentobarbital 50 mg/kg, i.p.). The skin of both right and left hind paws of the mice, was removed, weighed and stored at −80 °C before processing. The removed paws were individually homogenized for 1 min at a speed of 20,000 rpm in 1.2 ml of homogenization buffer which consists of 2.3376 g of NaCl, 0.5 g of bovine serum albumin (BSA), 50 μl of Tween 20 and 2 tablets protease inhibitor in 100 ml of phosphate buffered saline (PBS). Each 100 ml of PBS contains: 0.818 g NaCl, 0.02 g KCl,