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Research paper

Differential responses of macrophages from bovines naturally resistant or susceptible to *Mycobacterium bovis* after classical and alternative activation

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ABSTRACT

It is known that macrophages from naturally resistant animals possess a strong immune response against bovine tuberculosis to control mycobacterial infections. In the present study, the macrophage phagocytic activity, intracellular bacterial survival, and cytokine gene expression induced by classical and alternative activators against *Mycobacterium bovis* in naturally resistant or susceptible bovines, were evaluated. Animals were classified as naturally resistant or susceptible based on the capacity of their macrophages to allow *M. bovis* (BCG) growth. Peripheral blood macrophages from naturally resistant and susceptible animals were activated by classical and alternative stimuli and challenged with either non-pathogenic *M. bovis* BCG strain or pathogenic 9926 strain. Naturally resistant animals showed the highest phagocytosis index and microbial control after classical and alternative stimuli, being this response higher against the strain 9926 than the non-virulent strain. In addition, the response of macrophages activated by the classical pathway was higher than that under the alternative activation against both types of strains. Furthermore, classical pathway-activated macrophages derived from naturally resistant animals expressed higher levels of the pro-inflammatory markers iNOS, IL-1β, TNF-α, MIP-1 and MIP-3, and the anti-inflammatory markers ARGII and TGF-b, particularly to BCG. The results of this study showed that macrophages from naturally resistant animals produced stronger pro-inflammatory responses than those from susceptible ones to signals provided by classical pathway activators. Its role in innate immunity against *M. bovis* is yet to be determined.

1. Introduction

Bovine tuberculosis is an infectious transmissible disease, mainly caused by *Mycobacterium bovis*, which is characterized by the development of lesions called granulomas (Neill et al., 2001; Aldwell et al., 2001). Its main route of infection in cattle is the airway; most tuberculous lesions are found in tissues related to the respiratory system (Neill et al., 1988). However, the digestive tract can be also an entry port of this bacterium, but the infectious dose required is higher compared with the air way route, in which the infection can be established by a single bacterium (Cassidy et al., 1999a,b).

The transmission of the disease in a herd depends on a number factors associated with environmental conditions faced by the bacteria, the resistance or susceptibility of the host and the virulence of the bacterium. Humid and warm environments, protected from sunlight, benefit bacteria survival for long periods and maintain their infective

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capacity (Neill et al., 1991). However, macrophages can vary their behavior against bacteria by the influence of several factors, including the natural resistance to disease, thus providing the host with a greater possibility to deal with the infection (Bellamy, 2003).

Qureshi et al. (1995) demonstrated that when macrophages derived from naturally resistant cattle were challenged with live *Brucella abortus*, they possessed a greater capacity than those derived from susceptible cattle to control the intracellular growth of *B. abortus*, BCG and *Salmonella dublin*. These authors suggested the use of their approach for the identification of livestock naturally resistant or susceptible to intracellular bacteria, related to a distinct production of pro-inflammatory cytokines and nitric oxide (NO) by macrophages (Harmon et al., 1989).

Macrophage classical activation studies have been developed by Mackaness (1964), who demonstrated that infection of mice with BCG or *Listeria monocytogenes* increased microbicidal activity of macrophages in a stimulus/activator-dependent, but no antigen-specific manner (Gordon, 2003). Some cytokines involved and modulating the classical activation of macrophages to mycobacteria are tumor necrosis factor-alpha (TNF-α), interleukin-1 and interleukin-6, whereas IFN-γ production is stimulated by mycobacteria-activated lymphocytes; these cytokines are typically involved in the immunomodulatory effects and mediate many of the clinical manifestations of tuberculosis (Mackaness, 1964). In addition Gordon (2003) and Mantovani et al. (2004), defined as alternating activation of macrophages that induced by IL-4 and IL-13, which are usually produced by a Th2 type response, inhibiting Th1 response by antagonizing IFN-γ; this phenomenon is particularly associated with allergic processes, and humoral and cellular response to extracellular parasites and pathogens. This pathway is also characterized by the presence of non-opsonic receptors, low levels of the enzyme nitric oxide synthase (iNOS, NOS2) and a marked decreased production of pro-inflammatory cytokines such as IL-1, TNF-α and IL-6. It is also known that IL-4 and other Th2 cytokines increase the macrophage metabolism of L-arginine by the enzyme arginase, which produces ornithine and urea (Mantovani et al., 2004; Adams and Templeton, 1998).

Macrophages have also reported to differentially respond to Th1 and Th2 cytokines stimuli; for instance, classically (Th1) activated macrophages produce NO earlier compared with Th2 cytokines-activated macrophages, in which NO is not produced and the metabolism of L-arginine is increased, which suggests that the cytokines immune influence, either Th1 or Th2, follows different pathways (Adler et al., 1995; López et al., 2003).

The ability to efficiently control an infection is a neglected aspect in the selection of domestic species, as the urgent need to provide more volume to feed the human population has focused attention on productive qualities, without considering the natural resistance of individuals to certain diseases. Therefore, it is necessary to generate a greater understanding of the mechanisms of natural resistance to certain pathogens of importance to veterinary and human health, such as *M. bovis*.

2. Materials and methods

2.1. Bacteria

Non-pathogenic *M. bovis* BCG Danesa strain was a gift from Dr. Iris Estrada of the Escuela Nacional de Ciencias Biológicas at Instituto Politécnico Nacional, whereas *M. bovis* 9926 is a field strain isolated from a 8-year old female bovine, from Acatic, Jalisco, México, which presented tuberculosis lesions at postmortem examination. Bacteria were grown in Middlebrook 7H9 broth (Becton Dickinson, Cockeysville, MD) with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson) at 37 °C. The bacterial suspension was centrifuged at 2500 × *g* for 10 min, and the pellet was suspended in RPMI 1640 plus 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 20 mM sodium bicarbonate (CRPMI) (Gibco BRL, Life Technologies, Grand Island, NY). Bacteria suspension was then passed twice through a 27-gauge needle in order to disrupt the clumps. One-mililiter aliquots were stored at −80 °C for at least 1 day, and the inoculum was titrated by plating serial dilutions on Middlebrook 7H11 medium plus 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson).

2.2. Cell preparation and culture

Peripheral blood was obtained from 4- to 5-year old healthy adult female cattle from a disease-free herd (not vaccinated, exposed, or challenged) housed at Campus de Ciencias Agropecuarias of the Universidad Autonoma de Nuevo Leon (UANL), Mexico. Macrophages were obtained from peripheral blood mononuclear cells (PBMC) by the method of Campbell and Adams (1992), with slight modifications. In brief, blood was collected from the jugular vein into 60-ml syringes containing acid-citrate-dextrose solution and was centrifuged at 1000 × *g* for 30 min. Buffy coats were diluted in 30 ml of citrated PBS, layered onto 15 ml of Percoll (1.077 g/ml, Pharmacia, Uppsala, Sweden), and centrifuged at 1200 × *g* for 25 min. PBMC were then removed from the interface between the plasma and Percoll solution, pooled, diluted in 50 ml of citrated PBS, and centrifuged at 500 × *g* for 15 min. Next, cell pellets were washed three times with citrated PBS at 500 × *g* for 10 min, suspended in CRPMI containing 4% autologous serum to facilitate adherence, placed at 5×10^6 PBMC in 50-ml Teflon flasks, and cultured 4 h at 37 °C under 5% CO_2. Non-adherent cells were then removed by three washes with pre-warmed PBS, and adherent monocytes were cultured, as described above, in CRPMI plus 12% autologous serum for 10–12 days until they differentiated to macrophages, as reported by others (Macedo et al., 2013; Campbell and Adams, 1992).

2.3. Nitrite determination

Macrophages were incubated for 22 h in triplicates, in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY), in a total volume of 7 ml of CRPMI plus 15% heat inactivated fetal calf serum (FCS) with 50 ng/ml *Escherichia coli* O26:B6 LPS (Sigma–Aldrich, St. Louis, MO) plus 100 ng/ml rbIFN-γ to prompt the classical