Veterinary Immunology and Immunopathology 160 (2014) 177–183

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Identification of potential biomarkers of disease progression in bovine tuberculosis

Federico Carlos Blanco [a], Fabiana Bigi [a], Marcelo Abel Soria [b,*]

[a] Instituto de Biotecnología, CICVyA-INTA, N. Repetto y De los Reseros, 1686 Hurlingham, Buenos Aires, Argentina
[b] Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, INBA Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form 18 April 2014
Accepted 25 April 2014

Keywords:
Bovine tuberculosis
Biomarker
Discriminant analysis
IL-17

ABSTRACT

Bovine tuberculosis (bTB) remains an important animal and zoonotic disease in many countries. The diagnosis of bTB is based on tuberculin skin test and IFN-γ release assays (IGRA). Positive animals are separated from the herd and sacrificed. The cost of this procedure is difficult to afford for developing countries with high prevalence of bTB; therefore, the improvement of diagnostic methods and the identification of animals in different stages of the disease will be helpful to control the infection. To identify biomarkers that can discriminate between tuberculin positive cattle with and without tuberculosis lesions (ML+ and ML−, respectively), we assessed a group of immunological parameters with three different classification methods: lineal discriminant analysis (LDA), quadratic discriminant analysis (QDA) and K nearest neighbors (k-nn). For this purpose, we used data from 30 experimentally infected cattle. All the classifiers (LDA, QDA and k-nn) selected IL-2 and IL-17 as the most discriminatory variables. The best classification method was LDA using IL-17 and IL-2 as predictors. The addition of IL-10 to LDA improves the performance of the classifier to discriminate ML-individuals (93.3% vs. 86.7%). Thus, the expression of IL-17, IL-2 and, in some cases, IL-10 would serve as an additional tool to study disease progression in herds with a history of bTB.

1. Introduction

Bovine tuberculosis (bTB) is an important animal and zoonotic disease that causes significant financial loss and is a public health hazard. *Mycobacterium bovis*, the causative agent of bTB, is closely related to *Mycobacterium tuberculosis* and both species are included in the *M. tuberculosis* complex. In humans and cattle the disease is primarily an infection of the respiratory system with similar characteristics. The pathogen is transmitted from cattle to humans via aerosol or ingestion of contaminated dairy products. The presence of bTB hinders the development of the dairy and meat industry and is an impediment to international trade. Therefore, the control and eventually the eradication of this disease are essential.

In many of the developed countries, the control of bTB is based on "test and slaughter" programs. Field and/or laboratory diagnostic tests are used to identify potentially infected herds for quarantine, which may be followed by additional diagnostic testing and slaughter of all cattle that show positive test reactions. The economical limitations of developing countries with high prevalence of bTB turn this test and slaughter policy in a non-viable option. In this particular situation, the use of complementary strategies such

* Corresponding author at: Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina. Tel.: +54 11 4524 8000.
E-mail address: soria@agro.uba.ar (M.A. Soria).

http://dx.doi.org/10.1016/j.vetimm.2014.04.008
0165-2427/

as vaccination and diagnostic improvements will be useful for controlling the infection. Blood- or serum-based methods with the potential to differentiate animals in different stages of the disease could help to isolate spreader-animals and limit the dissemination of the pathogen. Biomarkers of disease could improve the ante-mortem diagnosis, which is currently based only on the tuberculin skin test (the official diagnostic test) and IFN-γ determination, as an alternative/complementary method. IFN-γ release assay (IGRA) in whole blood upon specific antigen stimulation has been a useful tool for the detection of infected cattle (Wood and Jones, 2001; Gormley et al., 2006). However, this method is not suitable to differentiate animals with active disease from those infected but with no visible lesions at *post-mortem* examination (Lim et al., 2012; Vordermeier et al., 2002).

To identify biomarkers that can discriminate between tuberculin-positive cattle with and without tuberculosis lesions, we assess in this study three different classification methods: lineal discriminant analysis (LDA), quadratic discriminant analysis (QDA) and K nearest neighbors (k-nn). For this purpose, we used a database of immunological parameters determined in a group of experimentally infected cattle.

2. Materials and methods

2.1. Cattle infections, sample collection and necropsy

Thirty Holstein-Fresian calves (3–6 months old) from three independent experimental infections were inoculated intratracheally with 10^6 to 10^7 colony forming units (CFU) of *M. bovis* NCTC 10772 or *M. bovis* 04-303, as described previously (Blanco et al., 2011). All experiments conformed to local and national guidelines on the use of experimental animals and category III infectious organisms. The thirty animals used in this study were negative for IFN-γ by ELISA assay (Bovigam, Zurich, Switzerland) and tuberculin skin test at the beginning of the experiments but positive by both assays at the end of the experiments (data not shown). Blood samples were taken at the beginning of the experiment, for evaluation of preimmune status, and at several times after infection. For this particular study, all the analyzed samples corresponded to 60 days post infection (dpi). Heparinized blood (10 mL) from each animal was used for Bovigam and peripheral blood mononuclear cells (PBMC) isolation by gradient centrifugation over Histopaque 1077 (Sigma–Aldrich, St. Louis, USA) following the manufacturer's protocol. PBMCs were incubated at 37 °C in RPMI complete medium supplemented with 10% of bovine fetal serum (Internegocios, Mercedes, Argentina) and 20 μg/mL final concentration of purified protein derivative from *M. bovis* (PPDB) (Biocor, Zurich, Switzerland). The incubations were performed on 12-well tissue culture plates for 16 h for RNA extraction and 48 h for flow cytometry determinations. At the end of each experiment, the calves were euthanized and thin slices of lungs and lymph nodes of the head and pulmonary region were analyzed looking for granuloma formations. The animals were classified in two groups ($n = 15$) based on the presence or absence of macroscopic lesions. Representative images of granulomas are shown in supplementary Fig. 1.

Supplementary Figure 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.04.008.

2.2. Interferon gamma release assay

Heparinized blood samples were dispensed in 200 μl aliquots into individual wells of a 96-well plate. The wells contained whole blood plus 20 μg/ml *M. bovis* PPD (Prionics, Zurich, Switzerland) or PBS. The blood cultures were incubated for 18 h and plasma was harvested and stored at −80 °C. Interferon gamma concentrations in stimulated plasma were determined using a commercial ELISA based kit (Bovigam; Prionics, Zurich, Switzerland). Absorbance of standards and test samples were read at 450 nm. The optical density (OD) for the PBS controls, which was usually approximately 0.1 OD units, was used to normalize individual readouts and to calculate optical density indexes (ODIs), where the results obtained by antigen stimulation were divided by the results for the PBS-stimulated cultures.

2.3. Flow cytometry

For flow cytometry determinations, 2×10^6 PBMCs were incubated with PPDB. To evaluate the expression of CD4 (MCA1653A647, IgG2a), CD8 (MCA837PE, IgG2a) and CD25 (MCA2430F and MCA2430PE) surface markers, cells were stained with fluorescent conjugated monoclonal antibodies (AdDSerotec, Oxford, UK). The stained cells were analyzed in a FACSCalibur cytometer (BD, Franklin Lakes, NJ, USA) using Cell Quest software. Analysis gates were set on lymphocytes according to forward and side scatter. IL-2R expression was analyzed in CD4+ and CD8+ populations. Percentages of IL-2R-expressing cells were calculated as the ratio of CD4+ or CD8+ cells expressing CD25 and total CD4+ or CD8+ cells. Representative dot-plots are included in supplementary Fig. 2.

Supplementary Figure 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.04.008.

2.4. Expression of cytokines in peripheral blood mononuclear cells stimulated with purified protein derivative from M. bovis

Cytokine mRNA expression analyses were performed in peripheral mononuclear blood cells. Briefly, PBMCs were isolated from heparinized blood by gradient centrifugation over histopaque 1077 (Sigma–Aldrich, St. Louis, USA), following the manufacturer's protocol, and subsequently incubated at 37 °C with PPDB for 16 hs (Blanco et al., 2009b). Immediately after incubation, cells were resuspended in RNAeasy RLT stabilization buffer and total RNA was extracted using the rest of the commercial kit and following the manufacturer's protocol (RNAeasy, QIAGEN, Hilden, Germany). RNA quality and quantity as well as cDNA synthesis were assayed as described previously (Blanco et al., 2009b).