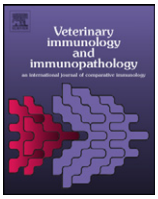
Veterinary Immunology and Immunopathology 135 (2010) 218–225

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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



Research paper

Expression profiling reveals differences in immuno-inflammatory gene expression between the two disease forms of sheep paratuberculosis

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ARTICLE INFO

Article history:
Received 22 May 2009
Received in revised form 26 October 2009
Accepted 30 November 2009

Keywords:
Mycobacterium avium subsp. *paratuberculosis*
MAP
Sheep
Chemokines
Genomics
Pathology

ABSTRACT

Paratuberculosis is a chronic enteropathy of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP); infection of sheep results in two disease forms – paucibacillary (tuberculoid) and multibacillary (lepromatous) associated with the differential polarization of the immune response. In addition the majority of MAP-infected animals show no pathology and remain asymptomatic. Microarray and real-time RT-qPCR analyses were used to compare gene expression in ileum from sheep with the two disease forms and asymptomatic sheep, to further understand the molecular basis of the pathologies. Microarrays identified 36 genes with fold-change of >1.5 and $P \leq 0.05$ in at least one comparison; eight candidates were chosen for RT-qPCR validation. Sequence analysis of two candidates, CXCR4 and IGFBP6, identified three SNPs in each; five were found in all three forms of disease and showed no significant relationship to pathological type. The IGFBP6 G^{3743} A SNP was not detected in asymptomatic sheep. The data show that the two forms of disease are associated with distinct molecular profiles highlighted by the differential expression of chemokine and chemokine receptor transcripts, the protein products of which might be implicated in the different cell infiltrates of the pathologies. The cells within the lesions also show evidence of abnormal activation; they express high levels of cytokine transcripts but have reduced expression levels of transcripts for T cell receptor associated molecules.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a facultative intracellular bacterium that primarily infects macrophages and is the causative agent of paratuberculosis (Johne's disease), a common enteropathy of ruminants. In cattle an extended preclinical phase is dominated by a type 1 immune response that is followed by progression to fatal granulomatous disease, which is linked to a switch to a type 2 response and the production of non-protective IgG1 antibodies (Koets et al., 2002; Kurade et al., 2004). In sheep both type 1 and type 2 responses give rise to disease states (Clarke, 1997) and there seems to be no progression from the paucibacillary (type 1 or tuberculoid) to the multibacillary (type 2 or lepromatous) disease; both are fatal. As with homologous pathologies in tuberculosis and leprosy, the cytokines that dominate paucibacillary paratuberculosis are IFNγ and IL-12, while IL-10 dominates multibacillary disease (Smeed et al., 2007). Only a minority of infected (IS900+) sheep progress to disease and the majority remain asymptomatic with no pathology (Koets et al., 2002; Smeed et al., 2007; de Silva et al., 2009).

We hypothesize that the polarization of the immune response is critical to the clinical outcome of paratuberculosis infection. Intestinal tissue damage that results from a type 1 response involves lymphocyte and eosinophil

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doi:10.1016/j.vetimm.2009.11.015

infiltration and is fundamentally different to that caused by a type 2 response, which leads to epithelioid macrophage infiltration and dissemination of infection (Smeed et al., 2007). The related disease leprosy also has this dichotomy of immunology and pathology (Meisner et al., 2001). Furthermore there is a clear relationship between host genetics and disease type (Fortin et al., 2007); many genes have been implicated but one that has received much attention is solute carrier family 11 (SLC11A1, NRAMP-1 or Bcg), which is linked to both tuberculosis susceptibility (Vidal et al., 1993) and discrimination of the two forms of human leprosy (Meisner et al., 2001). Resistance to MAP infection in mice is also partly linked to this gene, with animals carrying the Bcg^r allele (e.g. C3H) being refractory to infection (Veazey et al., 1995).

The principal cell populations associated with mycobacterial infection are cells of the macrophage lineage, a major function of which is the activation of T cells and initiation of the immune response. Antigen-presenting cell subsets play a crucial role in the polarization of responses through the differential expression of IL-10 and IL-12 (Pulendran et al., 1999). They also show differential expression of pattern recognition receptors (PRRs) (Nalubamba et al., 2008), and differential PRR activation can tailor the immune response (Kapsenberg, 2003). Both TLR2 and CARD15 (NOD2) have been shown to be up-regulated in clinical paratuberculosis (Nalubamba et al., 2008) and involved in both recognition (Ferwerda et al., 2007) and response (Weiss et al., 2001) to MAP. Indeed, there is growing evidence for linkage of TLR2 and/or CARD15 mutations and susceptibility to human mycobacterial diseases (Bochud et al., 2003; Ben-Ali et al., 2004) and bovine MAP infection (Mucha et al., 2009).

To begin to unravel the complex interactions between MAP and the immune system that gives rise to bovine paratuberculosis, Coussens and colleagues have used bovine leukocyte microarrays to examine transcript changes in peripheral blood mononuclear cells (PBMC) (Coussens et al., 2002) and macrophages (Murphy et al., 2006). In addition they have examined cattle ileal tissue (Aho et al., 2003) in order to understand the mechanisms that lead to intestinal tissue damage and have identified a number of genes involved in the immunology and pathogenesis of bovine paratuberculosis (including insulin-like growth factor binding protein-6 – IGFBP6). Our own studies (Smeed et al., 2007; Nalubamba et al., 2008) have focussed on ovine paratuberculosis because of the defined pathologies in sheep and exploit an oligonucleotide microarray of ∼600 immuno-inflammatory genes (Watkins et al., 2008) to gain insight into the role of these genes in the development of the different pathologies. The defined nature of the disease phenotype in sheep facilitates the identification of genetic markers associated with pathological type, and might also inform studies in cattle.

Competitive hybridization of cDNA isolated from the ileum (the site of paratuberculosis lesions) between the three infected groups was performed to identify changes to transcript expression associated with each disease group and develop molecular signatures of each pathological form. Quantitative real-time RT-PCR (RT-qPCR) on selected candidate genes was used to validate the arrays and the data were analysed to identify and compare physiological pathways associated with the contrasting pathologies. Data sets gathered from such experiments provide the potential to follow pathways of immune reactivity as well as assessing disease states that can then be related to specific gene expression signatures. In addition, as the epidemiology of these mycobacterial diseases suggests a genetic susceptibility (Ben-Ali et al., 2004) we investigated this link, by a preliminary single nucleotide polymorphism (SNP) analysis, between polymorphisms in two of the candidates and pathological form.

2. Materials and methods

2.1. Experimental animals and tissues

MAP-infected animals presented with clinical Johne's disease (diagnosed by a veterinary surgeon: prolonged weight loss, inappetance and depression, and occasional diarrhoea) were out bred, female sheep (Table S1) of a variety of breeds and ages. All sheep were euthanized and diagnosis was confirmed by histopathology, Ziehl Neelsen (ZN) staining and IS900 real-time quantitative PCR with a sensitivity of five genome equivalents (Eishi et al., 2002); sheep of similar ages from the same flocks (Nalubamba et al., 2008), with no signs of clinical Johne's disease and positive for IS900 DNA were considered asymptomatic (Smeed et al., 2007). IS900 is a specific marker for the precise identification of MAP (Green et al., 1989). Animal procedures were performed under a valid Animals (Scientific Procedures) Act 1986 Project Licence.

2.2. RNA isolation

Terminal ileum sections (∼0.5 g) were placed in five volumes of RNA*later* (Qiagen, Crawley, UK), incubated overnight at 4 °C and stored at −80 °C. Tissues were homogenized in guanidine isothiocyanate lysis buffer (RNeasy, Qiagen) and RNA was isolated using the RNeasy Maxi kit, eluted in 0.8 ml of RNase-free water, precipitated with ethanol and resuspended in 0.3 ml of RNase-free water. Samples were DNase I digested (Qiagen) and genomic DNA contamination assessed by GAPDH PCR and a no reverse transcriptase control. RNA quality was assessed using a RNA 6000 Nano LabChip on the Agilent 2100 bioanalyzer and quantified using a NanoDrop ND-1000 spectrophotometer; all samples had an RNA integrity number >7; $n = 6$ for the microarray experiments.

2.3. Preparation of labelled cDNA and microarray hybridization

cDNA generation and indirect labelling was performed using the Fairplay III Microarray Labelling Kit (Stratagene, Cambridge, UK) incorporating Cy3 and Cy5 dyes (GE Healthcare, Amersham, UK); the DyeEx spin 2.0 kit (Qiagen) removed unincorporated dye. Pre-hybridization and hybridization was carried out using the Pronto!™ Kit (Corning, Loughborough, UK). This analysis utilized the ruminant immuno-inflammatory gene universal array (Watkins et al., 2008); 4824 spots from 596 genes.