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Mycobacteria-induced anaemia revisited: A molecular approach reveals the involvement of NRAMP1 and lipocalin-2, but not of hepcidin

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ABSTRACT

Anaemia is a frequent complication of chronic infectious diseases but the exact mechanisms by which it develops remain to be clarified. In the present work, we used a mouse model of mycobacterial infection to study molecular alterations of iron metabolism induced by infection. We show that four weeks after infection with *Mycobacterium avium* BALB/c mice exhibited a moderate anaemia, which was not accompanied by an increase on hepatic hepcidin mRNA expression. Instead, infected mice presented increased mRNA expression of ferroportin (*Slc40a1*), ceruloplasmin (*Cp*), hemopexin (*Hpx*), heme-oxygenase-1 (*Hmox1*) and lipocalin-2 (*Lcn2*). Both the anaemia and the mRNA expression changes of iron-related genes were largely absent in C.D2 mice which bear a functional allele of the *Nramp1* gene. Data presented in this work suggest that anaemia due to a chronic mycobacterial infection may develop in the absence of elevated hepcidin expression, is influenced by *Nramp1* and may involve lipocalin-2.

Introduction

Chronic infectious diseases are frequently accompanied by anaemia. It is widely accepted that the anaemia results from the efforts of the host to decrease iron availability for invading microorganisms. Iron retention inside cells of the reticulo-endothelial system has been postulated to be at the basis of this "Iron Withholding System" (Weinberg 1992). Macrophages are responsible for the recycling of large amounts of iron, following ingestion and destruction of senescent red blood cells. The iron that is derived from the degradation of haemoglobin is usually released to the circulation in order to be re-utilized in the bone marrow for incorporation into newly forming erythrocytes (Knutson and Wessling-Resnick 2003).

In states of inflammation, the iron recycling process is blocked. Experiments in cells of the mononuclear phagocyte lineage suggest that this may involve LPS-mediated ferroportin-1 down-regulation through a Toll-like receptor 4 (TLR4)-dependent pathway (Yang et al. 2002). In addition, the hepatic iron-regulated peptide hormone hepcidin is highly induced during inflammation. Hepcidin is a key regulator of iron recycling and absorption (Andrews 2004; Ganz 2006; Hentze et al. 2004; Weiss 2009) which exerts its effect by binding to ferroportin to promote its internalization and degradation (Nemeth et al. 2004). Hepcidin is secreted into the urine of human subjects following injection of LPS, with a peak around 6 h, preceded by an increase in serum IL-6 levels, and followed by a decrease in serum iron (Kemna et al. 2005). In mice, it has also been shown that the injection of microbial products, such as LPS or complete Freund's adjuvant (which contains mycobacterial components) lead to the induction of hepcidin mRNA expression in the liver, with maximum values between 1.5 and 16 h post-treatment (Constante et al. 2006; Frazer et al. 2004; Pigeon et al. 2001; Roy et al. 2004). When synthetic hepcidin is injected intraperitoneally into mice, a sharp decrease in serum iron is observed, starting 1 h post-treatment and lasting around 48 h (Rivera et al. 2005). Hepcidin has thus been considered a fundamental player in the development of the anaemia of inflammation and chronic infection. However, all the studies described above are limited to relatively short time frames, more accurately reflecting the course of an acute infection or inflammation than a chronic situation.

Mycobacteria are intracellular pathogens of macrophages, causing insidious long-lasting infections, both in humans and other

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hosts. Human mycobacterial infections are frequently accompanied by anaemia (Hasibi et al. 2008; Maartens et al. 1990; Muzaffar et al. 2008). More than twenty years ago, Marchal and Milon have shown that mice infected with *Mycobacterium bovis* BCG (BCG) developed anaemia, which was most severe at 4 weeks post-infection. These authors have shown that BCG-induced anaemia was T-cell dependent and resulted from decreased erythropoiesis (Marchal and Milon 1981, 1986). At the time of these studies, hepcidin, ferroportin, and several other proteins involved in iron metabolism had not been described. Interestingly, these authors observed that the degree of anaemia induced by mycobacterial infection depended on the mouse strain, being more severe in C57BL/6 than in C3H/He mice (Marchal and Milon 1986). These two mouse strains differ, among many other genes, in the *Nramp1* allele. *Nramp1* (Natural Resistance-Associated Macrophage Protein 1, also known as Solute Carrier Family 11, Member a1 or *Slc11a1*) was discovered as a crucial mouse gene determining resistance or susceptibility to several intracellular pathogens, present in a locus previously known as *Bcg*, *Lsh* or *Ity* (Vidal et al. 1993). Its high homology with *Nramp2/DMT1/DCT1/Slc11a2*, a known iron transporter, as well as several functional studies, indicate that NRAMP1 is a proton-coupled divalent cation transporter, capable of transporting Fe^{2+}, as well as Mn^{2+} and Zn^{2+} (Courville et al. 2006). Unlike NRAMP2, however, NRAMP1 is present exclusively in cells of the myeloid lineage (namely neutrophils, macrophages and dendritic cells), in the membranes of lysosomal vesicles (Courville et al. 2006). NRAMP1 may contribute to the host defence against infection by a multitude of mechanisms, ranging from element starvation to alterations in phagosome maturation, the production of reactive oxygen and nitrogen intermediates or the modulation of the release of inflammatory mediators (Blackwell et al. 2003; Cellier et al. 2007). A role for NRAMP1 in normal iron metabolism has recently been shown. *In vitro*, *Nramp1* expression was increased in macrophages undergoing erythro-phagocytosis or heme ingestion and the expression of a functional NRAMP1 protein increased the release of iron from these sources (Soe-Lin et al. 2008). *In vivo*, *Nramp1*-deficient mice exhibited decreased capacity to recycle the iron resulting from erythrophagocytosis, showing augmented iron retention in the liver and spleen (Soe-Lin et al. 2009).

Here, we characterized the development of anaemia in mice chronically infected with *Mycobacterium avium*. Using two strains of mice expressing different alleles of *Nramp1* (Potter et al. 1983), we identified hepatic iron-related genes whose expression is significantly affected by *M. avium* infection.

Materials and methods

Mice

The BALB/c (NRAMP1-S) and C.D2 (NRAMP1-R) congenic mouse strains (Potter et al. 1983) were bred and housed at the Instituto de Biologia Molecular e Celular (IBMC) animal facility. The animals were kept inside individually ventilated cages, bearing high efficiency particulate air (HEPA) filters and were fed sterilized food and water *ad libitum*. For the experimental treatments, 8–12 weeks old females were used. All animal experiments were carried out in compliance with the animal ethics guidelines of the institute, and the national and European regulations for the care and handling of laboratory animals.

Bacteria

M. avium strain 2447, forming smooth transparent (SmT) colonies, was isolated from an AIDS patient and was a gift from Dr. F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Mycobacteria were grown to mid-log phase in Middlebrook 7H9 medium (Difco, Sparks, MD) containing 0.05% Tween 80 (Sigma, St. Louis, MO) at 37 °C. Bacteria were harvested by centrifugation, suspended in a small volume of saline containing 0.05% Tween 80, briefly sonicated to disrupt bacterial clumps, diluted and stored in aliquots at −70 °C until use.

Experimental iron overload

To induce iron overload, mice were injected intraperitoneally with 10 mg of iron, as iron–dextran (Sigma). Control mice received an equivalent amount of dextran (Sigma) by the same route. Animals were infected two weeks after the iron–dextran treatment.

Iron overload was confirmed by quantifying liver and spleen non-heme iron by the bathophenanthroline method as previously described (Rodrigues et al. 2006).

Experimental infection and quantification of bacterial load in the organs

Mice were infected intravenously, through a lateral tail vein, with 10^6 CFU of *M. avium*. Control animals received the same volume of saline. At the time points of interest, mice were sacrificed and blood and tissues were harvested for the different analysis. For bacterial quantification, the livers and spleens were aseptically collected and homogenized in a 0.05% Tween 80 solution in distilled water. Serial dilutions were plated into Middlebrook 7H10 agar medium and the plates were incubated at 37 °C for 1 week, when the colonies were counted.

Histological analysis

Samples of liver and spleen were fixed in buffered formaldehyde and incorporated in paraffin blocks. Sections were stained by haematoxylin-eosin for general histological characterization. Ferric iron was detected by Perls' blue staining. A Leica DMLB microscope (Leica Cambridge Lda., Cambridge, UK) equipped with a color CCD (charge-coupled device) camera was used to examine the liver and spleen sections.

Immuno-histochemistry for Lipocalin-2 was performed as previously described (Marques et al. 2008). Briefly, liver sections (10 μm) were stained with anti-mouse lipocalin-2/neutrophil gelatinase-associated lipocalin (R&D Systems, Minneapolis, MN, USA) at 1:500 dilution as primary antibody. Biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) was used at 1:200 following standard procedures. Slides were then subjected to Ziehl-Nielsen coloration following standard techniques. Samples were analyzed using an optical microscope (BX61; Olympus).

Quantification of tissue ferritin

Tissue samples were homogenized in a lysis buffer (50 mM Hepes (Gibco), 1% IGEPAL C-630 (Sigma), 1% cocktail of proteases inhibitors P840 (Sigma)) and cleared by centrifugation (10,000 rpm for 10 min). Ferritin concentrations in the lysates were determined by an enzyme-linked immunosorbant assay (ELISA) using polyclonal antibodies (Abs) raised against mouse recombinant H and L ferritin subunits, using the corresponding recombinant homopolymers for calibration The specificity and the absence of cross-reactivity of the Abs were previously described (Ferreira et al. 2001; Santambrogio et al. 2000). The microtiter plates were coated with polyclonal Ab specific for mouse H or L ferritin. For revelation, the same Ab labeled with biotin was used, followed by incubation with streptavidin (Sigma) and o-phenylenediamine dihydrochloride (Sigma) as chromogenic substrate. Protein content of tissue lysates was measured by the BCA protein assay kit (Pierce). The results were expressed as ng of ferritin per mg of protein.