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Characterization of tuberculous granulomas in different stages of progression and associated tertiary lymphoid tissue in goats experimentally infected with *Mycobacterium avium* subsp. *hominissuis*

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ABSTRACT

Oral infection of goats with *Mycobacterium avium* subsp. *hominissuis* (MAH) resulted in a large variety of granulomas in organized gut-associated lymphatic tissues and intestinal lymph nodes. To characterize the cellular composition of granulomas, $CD4^+$, $CD8^+$, γδ, B lymphocytes and plasma, $CD25^+$, $CD68^+$, MHC-II^+, $Ki67^+$ and endothelial cells were labeled in consecutive frozen sections by immunohistochemistry and acid fast bacilli (AFB) by Kinyoun stain. Granulomas with extensive necrosis, little mineralization and variable numbers of AFB surrounded by many $CD4^+$ T cells, but only few epitheloid macrophages were observed in severely sick goats at 2–3 mpi. They were interpreted as exuberant immune reaction. Organized granulomas with very few AFB were seen in clinically healthy goats at 13 mpi. The necrotic cores were surrounded by a zone of granulomatous infiltrate with many epitheloid macrophages and few lymphocytes. This zone was initially wide and highly vascularized and became progressively smaller. It was enclosed by an increasing layer of connective tissue. All organized granulomas were surrounded by compartimentalized tertiary lymphoid tissue. The granulomas in experimental infection of goats with MAH reflect the heterogeneity of lesions seen in mycobacterial infections of humans and ruminants and are therefore valuable for comparative research.

1. Introduction

A hallmark of *Mycobacterium* (M.) *tuberculosis* complex (MTC) infection is the presence of structured granulomas with central necrotic cores with variable mineralization surrounded by epitheloid macrophages, multinucleated giant cells (MGC), other inflammatory cells and connective tissue [1–4]. These granulomas are sites of host-pathogen interactions that on the one hand may limit mycobacterial growth and tissue pathology, but on the other hand can be exploited by the mycobacteria for survival [4,5]. They are highly dynamic structures with an enormous morphological heterogeneity within an individual and even within one organ [5,6]. Thus, they are important read out parameters for the stage of infection and the reaction to anti-tuberculous treatments and animal models that develop the spectrum of granulomas seen in human tuberculosis (TB) are highly desirable.

The currently used small animal models in experimental TB research are often not capable to reproduce the complete spectrum of granuloma heterogeneity [5–7]. Most mouse strains develop poorly organized granulomas. Granulomas with necrosis, but without the important feature of calcification are described in C3HeB/FeJ mice [6,8]. The guinea pig model overcomes these shortcomings [9,10]. However, both guinea pigs and mice develop only progressive fatal disease without a phase of latency [1,11]. Granulomas with morphology comparable to human TB have been described in infected macaques; however, ethical issues, costs and availability of animals are limitations of this model [6,11]. The zebrafish model revealed new aspects of the interaction between *M. marinum* and the innate immune response due to the unique opportunity of live imaging during granuloma formation, but zebrafish embryos are not able to mount an adaptive immune response [4,12].

Cattle have been used in TB research for a long time, whereas goats have increasingly gained acceptance in recent years. Both are naturally susceptible to *M. bovis* and *M. caprae*, and may undergo a phase of latency [11,13]. Granulomas with a wide spectrum of

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heterogeneity can be observed [2,11,14]. They have been classified as: stage 1 – clusters of epitheloid macrophages, stage 2 – granulomas with minimal necrosis and variably thin fibrous capsule, stage 3 – granulomas with central caseous and mineralized necrosis and complete fibrous capsule and stage 4 – granulomas with extensive multicentric caseous and mineralized necrosis and marked peripheral fibrosis [2].

After experimental oral infection of goats with *M. avium* subsp. *hominissuis* (MAH), an opportunistic, non-tuberculous mycobacterium, goats developed either severe fatal disease with marked ulcerative and granulomatous lesions predominantly in organized gut-associated lymphoid tissue (oGALT) and extensive caseonecrotic granulomas in intestinal lymph nodes (ILN) at 2–3 month after inoculation (mpi) or a wide variety of morphologically different granulomas in oGALT and ILN at 13 mpi. The objectives of this investigation were to characterize the morphology of granulomas seen in the goats infected with MAH and to compare them with those seen in other granuloma models and in human TB. Thus cellular composition and presence of MAH were examined in the different granulomas. A special focus was on the layer of lymphocytes in the periphery of granulomas resembling tertiary lymphoid tissue (3rd LT) described recently in human TB [3,15], since it has been suggested that the interaction between mycobacteria and 3rd LT determines the development of latency versus progression in TB [15,16].

2. Material and methods

2.1. Tissue samples

The experimental infection of goat kids was carried out in strict accordance with European and National Law for the Care and Use of Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (Permit Number: 04-002/12). All experiments were performed in containment of biosafety level 2 under supervision of the authorized institutional Agent for Animal Protection. During the entire study, every effort was made to minimize suffering. A total of 24 goat kids of the breed "Thüringer Wald Ziege" (20 males, 4 females) were used in this study as described recently (Schinköthe et al., submitted). In brief, 18 goat kids received MAH 10 times every 2–4 days beginning at the age of 10–21 days resulting in a total dose of 2.13×10^{10} cfu MAH. The inoculum was mixed with milk replacer and fed by bottle. Between 2–3 mpi, 2 animals died spontaneously and 7 had to be euthanized for animal welfare reasons because of severe clinical symptoms of elevated body temperature, weight loss and apathy. The remaining 9 goats developed mild transient increase in body temperature and depression around 2 mpi, recovered and remained healthy until necropsied at 13 mpi.

Ulcerative lesions primarily restricted to the oGALT and granulomas in draining ILN were seen in goats necropsied at 2–3 mpi and numerous granulomas of varying size at the same sites in goats necropsied at 13 mpi. After macroscopic documentation of lesions, multiple sites of altered oGALT and ILN were collected for histology, immunohistochemistry (IHC) and bacterial cultivation. To investigate the cellular composition, lesions and/or granulomas were collected from at least one jejunal Peyerís patch (JPP), ileal Peyerís patch (IPP) and ILN of 5 of the 9 goats necropsied at 2–3 mpi and from 1 to 2 JPP and ILN as well as 1–2 different sites of IPP in 9 out of the 9 goats necropsied at 13 mpi. This resulted in a total of 6 JPP, 5 IPP, 5 ILN from goats at 2–3 mpi and 13 JPP, 11 sites of IPP and 12 ILN from goats at 13 mpi.

These tissue samples were snap frozen in 2-methyl-butane at −70 °C immediately after euthanasia. Consecutive frozen sections of 5 μm thickness were collected on glass slides coated with chrome alum gelatin.

2.2. Staining of frozen tissue sections

Frozen sections were stained with hematoxylin and eosin (HE) for classification of granulomas based on a previously described scheme (Table 1, Schinköthe et al., submitted). The following 6 stages were differentiated: stage 1 (initial), stage 2 (solid), stage 3 (monocentric necrosis and mineralization), stage 4 (multicentric necrosis and mineralization), stage 5 (fibrotic organization) and stage 6 (extensive caseous necrosis). Azan staining was used to detect collagen fibers and the Kinyoun stain to detect acid fast bacilli (AFB) (TB stain kit K, BD Diagnostics, US). Numbers of AFB were semi-quantitatively assessed at ×1000 magnification in a reference area (RA) of 2359 μm^2 on representative sections (Table 2).

2.3. Immunohistochemistry (IHC) for cell surface markers and proliferation

The cell surface markers CD4, CD8, TcR1-N24, CD79α, CD25, CD68, and MHC-II were labeled in frozen sections. A monoclonal antibody against Ki-67 was used for differentiation of proliferating cells and a polyclonal rabbit antiserum to human van Willebrand Factor (vWF) for detection of endothelial cells. The monoclonal antibodies used and cell types detected are summarized in Table 3.

Frozen sections were air dried and fixed for 10 min (min) in acetone at room temperature except for sections used for the detection of CD25 which were directly transferred in 1:1 acetone-methanol at −20 °C for 10 min and for the detection of Ki-67 which were fixed in neutral buffered formalin for 10 min at room temperature. Endogenous peroxidase was blocked by incubation with 0.06% phenylhydrazine in PBS at 37 °C for 40 min. Ki-67 antigen was retrieved by heating in citrate buffer in a microwave oven for 10 min. Peroxidase-conjugated sheep anti-mouse IgG (NA931 V, GE Healthcare Europe, Freiburg, Germany) was used as second anti-

Table 1
Characterization of granulomas in oGALT and lymph nodes of goats infected with *M. avium* subsp. *hominissuis*.

Granuloma stage	Cellular composition	Necrosis and mineralization	Fibrosis
1 (Initial)	epitheloid macrophage cluster, ±MGC, interspersed and encircling lymphocytes, single neutrophils	no necrosis	–
2 (Solid)	epitheloid macrophages, ±MGC, interspersed with lymphocytes and neutrophils	minimal necrosis and/or mineralization	–
3 (Monocentric necrosis and mineralization)	±epitheloid macrophages, ±MGC, lymphocytes and neutrophils surround necrosis and/or mineralization	monocentric caseous necrosis and/or mineralization	variable, –/+++
4 (Multicentric necrosis and mineralization)	±epitheloid macrophages, ±MGC, lymphocytes and neutrophils surround necrosis and/or mineralization	multicentric caseous necrosis and/or mineralization	variable, –/+++
5 (Fibrotic organization)	minimal cellular infiltrate	fibrosis > necrosis and mineralization	+++
6 (Extensive caseous necrosis)	many lymphocytes, interspersed epitheloid macrophages and neutrophils,	irregular necrosis with minor mineralization	–

oGALT, organized gut-associated lymphoid tissue; MGC, multinucleated giant cells; ±, missing or present at variable number; –, not present; +++, severe.